

BASIC RESEARCH IN MALARIA

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ELVIO H. SADUN

Assistant Editor
ARTHUR P. MOON

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E. H. Sadun

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PROCEEDINGS OF
THE HELMINTHOLOGICAL SOCIETY
OF WASHINGTON

PARTICIPANTS

Doctor Masamichi Aikawa

Institute of Pathology
Case Western Reserve University
Cleveland, Ohio 44106

Doctor Darrell W. Anthony

Entomology Research Division
Agriculture Research Service
USDA, Gainesville, Florida 32001

**Lieutenant Commander Joseph C. Armstrong,
MSC, USN**

Naval Medical Research Institute
National Naval Medical Center
Bethesda, Maryland 20014

Doctor John D. Arnold

Harry S. Truman Laboratory
Kansas City General Hospital
Medical Center
2322 Holmes Street
Kansas City, Missouri 64108

Doctor Gunter F. Bahr

Chief, Biophysics Branch
Armed Forces Institute of Pathology
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Richard Beaudoin

Naval Medical Research Institute
National Naval Medical Center
Bethesda, Maryland 20014

Professor L. J. Bruce-Chwatt

Ross Institute of Tropical Hygiene
London School of Hygiene and Tropical Medicine
Keppel Street (Gower Street)
London, WC1E 7HT, England

Doctor Ernest Bueding

Department of Pathology
Johns Hopkins University
School of Hygiene and Public Health
615 North Wolfe Street
Baltimore, Maryland 21205

Colonel Edward L. Buescher, MC

Director and Commandant
Walter Reed Army Institute of Research
Washington, D. C. 20012

Colonel Francis C. Cadigan, Jr., MC

Commanding Officer
U.S. Army Medical Research Unit
Kuala Lumpur
Department of State
Washington, D. C. 20521

Lieutenant Colonel Craig J. Canfield, MC

Assistant to the Director for Malaria Research
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor George Carter

Department of Pharmacology
West Virginia University Medical Center
Morgantown, West Virginia 26506

Doctor Shao-Chia Chou

Department of Pharmacology
University of Hawaii School of Medicine
3675 Kilauea Avenue
Honolulu, Hawaii 96816

Doctor David F. Clyde

Director, Institute of International Medicine
University of Maryland School of Medicine
Baltimore, Maryland 21201

Professor Sydney Cohen

Department of Chemical Pathology
Guy's Hospital Medical School
London, S.E. 1, England

Lieutenant Colonel Edward J. Colwell, MC

U.S. Army Medical Component, Southeast
Asia Treaty Organization
APO San Francisco 96346

Doctor K. A. Conklin

Department of Pharmacology
University of Hawaii School of Medicine
3675 Kilauea Avenue
Honolulu, Hawaii 96816

Professor Augusto Corradetti

Director, Laboratory of Parasitology
Istituto Superiore di Sanita
Rome, Italy

Lieutenant Colonel Carter L. Diggs, MC

Department of Medical Zoology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Nicholas R. DiLuzio

Department of Physiology
Tulane University School of Medicine
New Orleans, Louisiana 70112

Doctor Donald Fairbairn

Zoology Department
University of Massachusetts
Amherst, Massachusetts 01002

Mr. Earl H. Fife
Chief, Department of Serology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Coy D. Fitch
Department of Internal Medicine
Saint Louis University
School of Medicine
1325 South Grand Boulevard
Saint Louis, Missouri 63104

Professor P. C. C. Garnham
Imperial College Field Station
Ashurst Lodge
Ascot, Berkshire, S15 7DE,
England

Doctor Howard C. Goodman
Chief, Immunology
World Health Organization
1211 Geneva 27, Switzerland

Lieutenant Colonel Anthony P. Hall, MC
Headquarters, Walter Reed Army Institute of
Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor William L. Hanson
Department of Veterinary Pathology and
Parasitology
University of Georgia School of Veterinary
Medicine
Athens, Georgia 30601

Doctor C. A. Homewood
Department of Parasitology
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool L3 5QA, England

Doctor Lee Howard
Office of Health
Agency for International Development
Washington, D. C. 20523

Doctor Robert L. Kaiser
Director, Malaria Program
Center for Disease Control
Atlanta, Georgia 30333

Doctor Maurice E. King
IIT Research Institute
10 West 35th Street
Chicago, Illinois 60616

Doctor Julius P. Kreier
Department of Microbiology
The Ohio State University
College of Biological Sciences
484 West 12th Avenue
Columbus, Ohio 43210

Doctor Tibor Lepes
Director, Division of Malaria Eradication
World Health Organization
1211 Geneva 27, Switzerland

Doctor Seth H. Lourie
Department of Rehabilitation Medicine
Upstate Medical Center
Syracuse, New York 13210

Professor Lucio Luzzatto
Department of Haematology
University College Hospital
Ibadan, Nigeria

Captain Robert O. McAlister, MSC
Department of Medical Zoology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Gerald J. McCormick
Department of Endocrinology and Metabolism
Division of Medicine
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Charles A. M. Meszoely
Department of Biology
Northeastern University
Boston, Massachusetts 02115

Doctor Louis H. Miller
Laboratory of Parasite Diseases
National Institutes of Health
Bethesda, Maryland 20014

Major General William H. Moncrief, Jr., MC
Commanding General
Walter Reed Army Medical Center
Washington, D. C. 20012

Major Walter Noll, MC
US Army Medical Component
Southeast Asia Treaty Organization
APO San Francisco 96346

Doctor Ruth S. Nussenzweig
Department of Preventive Medicine
New York University School of Medicine
550 First Avenue
New York, New York 10016

Professor Wallace Peters
Department of Parasitology
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool L3 5QA, England

Doctor Robin D. Powell
Medical Service
Veterans Administration Hospital
Iowa City, Iowa 52240

Doctor Richard B. Prior
Department of Microbiology
The Ohio State University
484 West 12th Avenue
Columbus, Ohio 43210

Doctor Leo Rane
University of Miami Medical Research Annex
U.S. Army Chemotherapy Research Project
5750 N.W. 32nd Avenue
Miami, Florida 33142

Doctor Karl H. Rieckmann
Rush-Presbyterian-St. Luke's Medical Center
Army Medical Research Project
Box 112, Joliet, Illinois 60434

Doctor Carmen Ines River-Ortiz
Department of Preventive Medicine
New York University Medical Center
550 First Avenue
New York, New York 10016

Doctor Richard N. Rossan
Gorgas Memorial Laboratory
P.O. Box 2016
Balboa Heights, Canal Zone

Doctor Elvio H. Sadun
Chief, Department of Medical Zoology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Emanuele Salvidio
Universita degli Studi di Genova
Istituto Scientifico di Medicina Interna
16132 Genova-Viale Benedetto XV n.6
Italy

Doctor John E. Scanlon
Department of Pathobiology and Comparative
Medicine
School of Public Health
University of Texas
P.O. Box 20186, Astrodome Station
Houston, Texas 77025

Doctor Imogene Schneider
Department of Entomology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Doctor Jerome V. Schnell
Section of Medical Microbiology and Tropical
Medicine
University of Hawaii School of Medicine
3675 Kilauea Avenue
Honolulu, Hawaii 96816

Doctor Thomas M. Seed
Department of Microbiology
The Ohio State University
484 West 12th Avenue
Columbus, Ohio 43210

Major Herbert E. Segal, MC
U.S. Army Medical Component
Southeast Asia Treaty Organization
APO San Francisco 96346

Doctor Alan M. Shefner
IIT Research Institute
10 West 35th Street
Chicago, Illinois 60616

Doctor Irwin W. Sherman
Department of Life Sciences
University of California
Riverside, California 92502

Doctor Wasim A. Siddiqui
Section of Medical Microbiology and Tropical
Medicine
University of Hawaii School of Medicine
3675 Kilauea Avenue
Honolulu, Hawaii 96816

Doctor George L. Spitalny
Department of Preventive Medicine
New York University Medical Center
550 First Avenue
New York, New York 10010

Doctor Daniel J. Stechschulte
Robert B. Brigham Hospital
125 Parker Hill Avenue
Boston, Massachusetts 02120

Doctor Charles R. Sterling
Institute of Pathology
Case Western Reserve University
Cleveland, Ohio 44106

Major Douglas R. Stutz, MSC
Department of Medical Zoology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Brigadier General Richard R. Taylor, MC
Commanding General
U.S. Army Medical Research and Development
Command
Washington, D. C. 20314

Doctor John A. Terzakis
Lenox Hill Hospital
100 East 77th Street
New York, New York 10021

Doctor Paul E. Thompson
Department of Veterinary Pathology and
Parasitology
University of Georgia School of Veterinary
Medicine
Athens, Georgia 30601

Brigadier General W. D. Tigertt, MC
Commanding General
Madigan General Hospital
Tacoma, Washington 98431

Doctor Jack D. Tiner
Department of Laboratories
Beth Israel Medical Center
10 Nathan D. Perlman Place
New York, New York 10003

Doctor William Trager
The Rockefeller University
66th Street and York Avenue
New York, New York 10021

Doctor Jerome P. Vanderberg
Department of Preventive Medicine
New York University Medical Center
550 First Avenue
New York, New York 10016

Doctor Knox Van Dyke
Assistant Professor of Pharmacology
West Virginia University Medical Center
Morgantown, West Virginia 26506

Doctor Ronald A. Ward
Department of Entomology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Mr. Bruce T. Welde
Department of Medical Zoology
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

Captain Rowland N. Wilkinson, MSC
U.S. Army Medical Component
Southeast Asia Treaty Organization
APO San Francisco 96346

Doctor Martin D. Young
Director, Gorgas Memorial Laboratory
P.O. Box 2016
Balboa Heights, Canal Zone

Doctor Rokus A. de Zeeuw
Laboratory for Analytical Chemistry
State University
Antonius Deusing Laan No. 2
Groningen, The Netherlands

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I

INTRODUCTION

Preface: Malaria Chemotherapists— Perched on the Shoulders of Giants

E. H. SADUN

Walter Reed Army Institute of Research, Walter Reed Army Medical Center,
Washington, D. C. 20012

There is a tendency in malaria research, perhaps in all research, to assume that the past is devoid of valuable information. Observations dating from a former period are often considered to be unsophisticated, unskilled or unimportant. Among research scientists there is a fashionable pursuit of newness, and enormous efforts are being made to come up with fresh ideas and new solutions. Modern technology may have thrust upon us the dehumanizing effect of losing our historical perspective. But history is, after all, a review of past experiences which influence present events.

For nearly a decade an extensive malaria research program has been supported by the U.S. Army Medical Research and Development Command to generate knowledge which can be applied to developing more effective methods and agents in the fight against malaria. This current program had from its inception a primary concern with chemotherapy. Aware of man's tendency to neglect experience, Tigertt (1966) called our attention to the words of Santayana (1922): "Progress far from consisting in change depends on retentiveness . . . when experience is not retained, as among savages, infancy is perpetual. Those who cannot remember the past are condemned to repeat it." Tigertt (1966) also admonished, "Malaria can never be regarded with complacency and always must remain high on the military medical research priorities list. If we, and those who follow us, fail to recognize this, we and they deserve to be classified as savages in the sense the word was used by Santayana." These words provided guidance in the succeeding years and prompted me to preface the International Panel Workshop held in 1969 with a series of ruminations entitled "Back to Antiquity" (Sadun, 1969).

Since most of the U.S. Army's malaria program is centered on the development of anti-

malarials, I would like at this time to view retrospectively some of the works of two Italian scientists of the 17th Century, Sebastiano Badi and Francesco Torti, whose contributions to the management of malaria stand out above all others. Their publications are remarkable documents of their logical thinking, their wise counsel and the lasting effect that they exerted on the history of medicine. Although they were great individualists, very different from one another in temperament and in relation to their observations, both were instrumental in dealing a mortal blow to Galenism, the firmly held popular belief that only blood letting or purgatives could remove the exudates of disease. As a result of their work and of the development of modern chemotherapy, Galen's writings which influenced the practice of medicine and remained virtually unchallenged up to the 17th Century, seem now nothing more than a blind belief in magic.

The extraordinary subsequent contributions made by Laveran who discovered the malarial parasite in human blood, by Marchiafava and Golgi who clarified its significance in relation to clinical findings and by Ross and Grassi who described its development in the mosquito, all helped establish the etiology of malaria and create the basis for prophylaxis. Yet, the therapeutic approaches to malaria were not substantially improved for at least two centuries after the works of Badi and Torti.

The 17th Century was a period of intense intellectual and spiritual individualism. This was the age of Shakespeare and Milton, Velasquez and Rembrandt, Descartes and Spinoza. Although the reasons for the development of the experimental method as opposed to the metaphysical Aristotelian and Tolemaic doctrines are complex, it was not accidental that many of the greatest scientific contributions also occurred in that century. It is sufficient to recall names such as Newton, Bacon, Har-

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vey, von Leeuwenhoek, Malpighi, Swammerdam, Redi, Vallesnieri and many other investigators who set the basis for a modern scientific approach.

Perhaps the most striking event in the history of chemotherapy was the introduction, in Europe in 1640, of the bark of a tree, designated as chinchona by Juan de Vega, the physician of Count Chinchon, Spanish Governor of Peru. The story that the Countess Chinchon was cured of malaria by the administration of the bark is widely circulated, but it is probably without foundation. Nevertheless, the name chinchona given by Linnaeus to the "fever tree" has been accepted by botanists for nearly three centuries and is a part of our cultural heritage. After the chinchona bark was introduced to Europe, the relative merits of the drug against fevers was passionately debated. A serious controversy between those who favored this medicament and those who opposed it soon divided practically all of Europe's physicians into two camps.

This controversy was activated and inflamed by a number of factors. Galenism had to be reconciled with the observations that the chinchona bark stopped the intermittent fevers and produced an apparent cure. Moreover, since the bark was imported by the Jesuits who profited greatly by its sale, the anticlerical elements of that period claimed that its value was purely for business, not medicine (Castiglioni, 1936). The experimental evidence for its effectiveness was also greatly hindered by the belief that malarial disease was caused by bad air emanating from swamps. Because of the lack of knowledge of the etiology of intermittent fevers, malaria diagnosis was made purely on clinical grounds, and was thus frequently confused with other fevers against which the chinchona bark had no curative effect. Perhaps the greatest obstacle to the demonstration of this drug's effectiveness, was the lack of standards for testing the purity of the preparations used. It was common practice among unscrupulous merchants to "cut" chinchona bark powder with inert substances to increase their profits. It seems ironic that today many dealers in addictive drugs increase their profits by using a variation of the same swindle, the illicit distribution to their victims of heroin mixed

with the active principle of chinchona, now referred to as quinine.

The facts could not be denied for long. Even one of the most conservative Italian physicians of the period, Bernardino Ramazzini, who insisted that no medicines should be used unless their effectiveness was thoroughly documented and their exact compatibility known, eventually admitted that chinchona was indeed specific against intermittent fevers and concluded that the revolution brought about by this drug in the history of medicine was comparable to the discovery of gunpowder in the history of warfare (Castiglioni, 1936).

Although Sydenham was among the first to recognize the specific virtues of chinchona against quartan malaria and suggested that it eliminated the fevers without need of expelling the humors, the greatest contributions were made by Badi and Torti who studied the effectiveness of this drug on a systematic basis and succeeded in dispelling many meaningless speculations by their precise documentation of data. Badi was among the first to report that this drug was effective against intermittent fevers. Torti demonstrated that it had no analogous effect on fevers of other origins and thus, on the basis of its specificity, used chinchona chemotherapy as an aid in diagnosis by separating malaria from other fevers. Without the knowledge of the etiologic agent and without the aid of a microscope, these two scientists accurately classified various types of intermittent fevers and described their clinical manifestations.

Sebastiano Badi was born in the early part of the 17th Century (the year is unrecorded) in Genoa where he graduated in medicine. After a brief period in Rome as a physician at the Court of Cardinal DeLugo he was appointed Public Health Physician and Physician in Chief of two hospitals in Genoa. He was a strong supporter of the therapeutic value of chinchona and was perhaps the first to use it in Italy against intermittent fevers. In an earlier book (Badi, 1656) he presented an interesting historical background on the origin and use of chinchona. In a subsequent book (Badi, 1963) he recommended for the first time the use of the chinchona bark particularly in the tertian intermittent fevers. He had learned about the beneficial activity of this

bark from Antonio Bolli, a merchant who lived in Peru, who had stated that the Indians were proclaiming its virtues long before the arrival of the Spaniards. This is indeed surprising since malaria, which in the second half of the 15th Century was widespread in Europe, especially in Italy, Spain and Portugal, was carried to the West Indies and the American mainland by the conquering Spaniards (Hoeppli, 1969). Although for a few generations following the arrival of Columbus there were no reports of this disease in America, many areas became malarious after an ever increasing number of presumably infected negro slaves were brought to this continent (Hoeppli, 1969).

Some untoward effect from the use of this medicament had been observed occasionally in Europe. Yet, Bolli insisted that in Peru he had never seen anyone with an intolerance to this drug. Badi, therefore, suspected that the bark which was being sold in Europe as the "powder of the Jesuits" might be heavily adulterated. Later, these suspicions were confirmed when Badi learned from Cardinal De Lugo that such frauds were indeed occurring frequently. He began using chinchona cautiously, comparing the effects when given in the form of a powder, as an extract, as a distillate or as an alcoholic tincture, and later concluded that the preferable method was to give it in powder form together with wine. In a poetic passage Badi tried to explain the reason for using an infusion in wine. "Why, I could not tell. Perhaps because wine absorbed the quality of the powder and the powder acquires the strength from the wine. In fact, the wine is a friend of the heart for the extraordinary sympathy it has for it" (Book 1, Chapter XXIV). However, he warned that the drug should be given only to those people without gastrointestinal problems and who had been previously purged. As an exception to this rule he recommended that the drug should be given in all cases of pernicious malignant intermittent fevers.

Badi was obviously aware that his recommended therapy was a departure from the current Aristotelian views that only God can make miracles. Therefore, in his two interesting books, he repeatedly attempted to justify the effectiveness of this drug by insisting that it was a natural substance and that the evi-

dence indicated that the fevers were eliminated by an occult quality of the bark. He stated that the skeptics may have been right in their learned objections, but regardless of their theories this drug "always provides a cure in every case, without delay" (Book 1, Chapter XIII). Being unable to reconcile his findings with Galenism, he emphasized that this drug "has been discovered not through reasoning or philosophy but through experience" (Book 1, Chapter XIII). This statement is a denial of rationalism, which holds that man can know about things which are beyond experience, and promotes empiricism, i.e. a concerted effort to base all probabilities on verifiable experience, relying on facts to hold in the future as they have in the past. Obviously, this concept is basic to the scientific method, the essential ingredients of which are discovery, proof, reproducibility and logical deduction.

In praising the drug, Badi insisted that the bark was effective against the quotidian, tertian, quartan and even chronic fevers. "It is simply marvelous, the rapidity with which the bark acts. As soon as it is given even in a single dose . . . the fevers subside immediately and never return." The bark has such an extraordinary and rapid effect, he added, that "it must certainly be included among the mirabilia" (Book 1, Chapter XIV).

He then proceeded to describe in detail the administration of the drug (Book 1, Chapter XXIV): "The bark must be given against tertian and quartan fever in the following manner: 2 drams (3.54 grams) of this bark ($\frac{1}{4}$ oz) should be ground firmly and strained. Three hours before a paroxysm the powder should be mixed in a glass of strong white wine and administered when the chill or the fever begins. The patient should be prepared by giving a purgative beforehand and by abstaining from any other medicine for four days following therapy during which time he should be kept in bed. No treatment should be instituted without prescription of a physician who can be the only one to judge the optimal schedule of administration." He also added that the dose should vary according to the age, the strength of the patient and other factors. "For children and people with more delicate complexions one should use smaller doses since these people weigh less. In such

cases it is enough to use only the supernate of the infusion containing the wine without ingesting the powder."

Throughout his publications, the attention to detail is remarkable. He insisted that "the powder must be mixed with wine three hours before the paroxysm for at least three hours." However, he stated, one should not take this time interval too literally since sometimes a weaker infusion prepared with an interval of short duration is sufficient to destroy fevers. If the attack is sudden, then one may not have time for anything more than promptly mixing the powder with the wine before drinking it. He also observed that the drug was efficacious even after an infusion of three or four days, and in one case he recorded its effectiveness even after an infusion of eight days. The most important point, according to him, was that the drug must be given as soon as the paroxysm begins.

To the objection that this drug could not be an effective antimalarial since relapses often take place, he replied "First of all, one or two doses are usually sufficient to eliminate all relapses. Secondly, such relapses are observed primarily in the quartan fevers which have an obstinate nature. In such cases, the fever will give in only after three or four subministrations." But, after all, he added, this is much better than to have to subject a person to a disease which has a duration of "one, twelve or even in some cases 20 to 30 years" (Book 2, Chapter VIII). On the basis of over 600 treated patients he concluded that the beneficial effects could be observed in all the intermittent fevers, in patients of all ages, both sexes, in diseases of long duration and even during pregnancy.

Francesco Torti was born in 1658 in Modena and developed an interest in medicine after devoting himself for a number of years to studies of literature, philosophy and law. This remarkable, discerning observer should be credited above all others for bringing about the downfall of the Galenic therapeutic method in spite of the opposition of conservative physicians and pharmacists and for replacing it with scientific reasoning. Torti became Court physician of Duke Francesco who apparently was greatly impressed by his humanistic culture, and his ability to enliven their conversations with frequent references to

poetry and witty proverbs, as well as by his medical competence. Through the efforts of Torti the Academy of Anatomy was founded in Modena in 1698 and he became its first Director.

In the course of his clinical experience, Torti had observed that some of the periodic fevers, even if light at the onset, rapidly became malignant and frequently resulted in death. Immediately after diagnosing periodic pernicious fevers he prescribed a discriminating administration of chinchona. His reputation for the nearly miraculous cures which resulted spread rapidly. A summarization of his vast experience with this drug was published under the title of "*Therapeutic specialis ad Periodicas Perniciosas*" (*Special Therapy for Pernicious Intermittent Fevers*), (Torti, 1712). This book was well received all over Europe and became so famous that he was often referred to as "*The Hippocrates of Modena*." In the last ten years of his life, as his health deteriorated, he devoted most of his time to poetry and was particularly successful in his satirical style, refined acumen and pungent humor. To our misfortune, he destroyed a large number of his poems before he died in 1741.

Like Badi, Torti observed that when chinchona was ineffective, the failure was due to mixing the bark with walnut powder, cypress bark, peach nut and many other plants. He often denounced the merchants who adulterated the drug as "unscrupulous people who speculate on human health." The main emphasis of his publication was on the classification and treatment of the intermittent fevers and on the effect of therapy on relapses. Somewhat at variance with Badi's conclusions, he stated that a single dose of chinchona frequently will be insufficient to prevent relapses, but that after treatment they always tend to be of shorter duration and less severe. Torti stated specifically that it would be foolish to withhold prescribing the drug for fear that the treated patient would relapse since "relapses will appear regardless of whether or not therapy is instituted." According to him chinchona should be administered in small doses for several days after the disappearance of fever and then again some time later, by alternating periods of treatment with periods of rest. He also suggested that relapses could be

prevented by prophylactic chemotherapy (Book I, Chapter I) and disclosed that the drug had no depressing influence on the nervous system, but that in fact it had "an opposite effect of opium" (Book I, Chapter I). Moreover, since it did not produce blood dyscrasias or intestinal irritation, it did not cause vomiting, excessive sweating or copious urination. However, he reported that occasionally after the use of chinchona there was some transitory deafness which disappeared immediately after withdrawal of the drug. Like Badi, he seldom observed serious incidents deriving from its use and stated that in those three or four cases in which the chinchona bark disappointed its users, the tertian fevers were complicated by other infections such as syphilis.

He frequently emphasized the need for basic research. "No one can assume the role of honest judge if he does not comprehend the cause and does not listen to the defense—physicians many times will act on the basis of preconceived notions without a knowledge of the composition and of the effectiveness of the drug" (Book I, Chapter II).

As one would anticipate, it was not so easy for Torti to meet the most common objection against the use of this drug, i.e. since it does not produce abundant evacuations the damaging humors of the disease could not be eliminated. He admitted that the therapy removes the symptoms without stimulating the secretions nor altering the normal physiology other than producing a minor dilatation of the small vessels, but re-emphasized "that chinchona does not stop the febrile fermentations by means of astringent or opposite action: it destroys them because it possesses a specific power as an antidote" (Book I, Chapter IV). After a detailed discussion speculating as to the possible causes of the fever, Torti suggested that the agent must be a very small substance so powerful as to produce fever every time it is in the blood. With amazing insight he concluded that the medicine specifically acts against the pyrogenic substance separating it from other humors which may have already invaded the blood.

Torti did not hesitate to follow up his working hypotheses with persistent and careful laboratory experimentation. To determine whether the drug could be administered topically, he

mixed quinine powder and wine and applied the poultice on the arm of a woman affected with intermittent fever. Although for eight consecutive days he added a fresh mixture in the same location, the fevers continued. The patient then agreed to take by mouth the same powder which for eight days had been uselessly applied on the arm. Since this brought about an immediate cure without any relapses, he concluded that the drug must be given orally.

In order to conciliate the absence of abundant evacuations with the beliefs held by the followers of Galen and to determine the possible effect of chinchona bark on different substances, he mixed the powder *in vitro* with lemon juice, orange juice, vinegar, water, oil, bile, lymph, urine, blood and other substances. All this, however, resulted in no detectable change. At first, he observed that the powder was delaying the coagulation of blood, but more careful studies revealed that this was due only to partial defibrinization produced by shaking the blood and the powder in order to mix them thoroughly. Once this was avoided, the coagulation time in the tubes containing the bark was not significantly different from that of the controls. These observations reinforced his belief that it was not necessary to expel the ferment which produced the fever, but rather to attack the source. However, he was aware of the dangers of extrapolating laboratory data to clinical situations. Therefore, he warned against placing too great reliance on the definition of biological phenomena in what he referred to as "mathematical" terms. Some of his statements might be considered by some of our contemporary molecular biologists and biostatisticians. "When practicing medicine, as one approaches the bed of the patient he must avoid being more of a mathematician than a physician. . . . Even if the physician is well versed in mathematics, whether young or adult, he should not . . . apply the mathematical laws to medicine unless he has had at least 40 years of professional practice. I do not mean that the principles of the mathematicians are not precise. Indeed, they are true and infallible, but I object to their inopportune application to complex problems which are insoluble under those circumstances. How could a mathematician,

no matter how expert, measure with his precise laws the inordinate movements of running water which deviates its course, backtracks and splashes?" (Book 1, Chapter VI).

Torti indicated that chinchona is more effective against the future symptoms than against those which are already occurring. Therefore, the medicine should not be given just at the beginning of the benign intermittent fevers, but it may be preferable to wait until the host has an opportunity to respond by his own power. For these cases he suggested that therapy be continued for eight consecutive days after the fever subsides and that even if the patient seems to be completely recovered, treatment should be resumed for at least six days after an interval of fifteen days. In some cases, he added, one may wish to continue the drug for preventive treatment. However, he cautioned that since the action of the drug requires a certain amount of time before it becomes effective, this waiting period does not apply to the management of the malignant pernicious intermittent fevers. From the beginning these fevers can produce symptoms which are very dangerous, due to the occlusion of some vessels and should be treated immediately, particularly since profound alterations of the brain may arise if the disease is brought by the blood to the meninges or to the central nervous system. He reiterated that "it is of maximal importance to remember that chinchona does not act immediately even when given in very large doses, but requires usually at least 24 hours to show its effects." Therefore, when prescribing it one must keep in mind that the patient must still be in sufficient health to be able to resist with his own force for at least 24 hours.

Unlike Badi (Book 1, Chapter XX) who speculated that this bark might be effective in many other acute and chronic diseases, Torti insisted that the specific power of this drug is limited to the intermittent fevers and has little or no value in any of the other conditions. Although Torti was adamant in the use of chinchona as a suppressive drug, he was somewhat uncertain as to its value as a chemoprophylactic agent. He indicated that in some cases it seemed to work very well whereas in others he observed individuals who developed the fevers even after having used it for a long

time. Therefore, although preventive therapy was based on logical criteria and was sometimes successful, "for reasons of prudence and not knowing the activity of this medicine in healthy individuals," he maintained some reservations until better elements for a definitive judgement would become available.

Torti's classification of the intermittent fevers is most impressive. He was able to describe them precisely by their symptomatology even though he had no other foundations on which to base his observations. The secret could perhaps be found in his statement: "Nothing in the whole world is more useful than the order, the progression and the evolution of the scientific observations . . . frequently, to obtain perfection little is needed. A painting is beautiful not just for the exact choice of colors but for the right quality. Few simple strokes form an image . . ." (Book 3, Chapter IV). Confident in the value of his accomplishments, he admonished one not to feel badly if occasionally he does not receive due credit, since "As physicians we receive most of the time excessive praise for very little or nothing at all."

The impact of these two men on chemotherapy, and on improved methods of management of diseases was very great indeed. Together, they succeeded in casting aside strict adherence to previously held Galenic theories, thus providing the necessary foundations for modern chemotherapy.

By pausing a moment to look into the past and by reading the documents of these scientists who have preceded us, there are some important lessons that can be learned. I hope that an additional case has been made for a close partnership between basic and disease-oriented research. As stated in a previous workshop, "a balanced combination of biological research and of empirical trials for the development of new antimalarial drugs is necessary. A malariologist whose interests lie only in some obscure mechanism of action and who fails to relate his findings to malaria as a medical problem is as incomplete as the brilliant diagnostician who does not care for his patients" (Sadun, 1966).

It is obvious from this brief historical review that facts which permit significant generalizations are frequently more important

than those of apparently greater immediate relevance to a particular problem. Badi and Torti placed themselves in conflict with the currently held Galenic theories, thus contributing to the development of modern chemotherapeutic approaches. Their courageous conclusions demonstrated that "the highest allegiance of science must continue to be to truth as defined by the validation procedures of the scientific process itself. Even when this runs contrary to correct popular beliefs—the distortion of scientific results or the selective use of evidence for political purposes, no matter how worthy, is unforgivable insofar as it is presented clothed by the authority and imputed objectivity of science" (Brooks, 1971).

At this time in our history, we are required to balance expenditures for care of current illness against equally important, although less definable, costs of basic research on health problems. This can be a very frustrating exercise since as stated by an ancient Thai proverb: "Only a fool can attempt to balance an elephant with a donkey." Failure to relate the past to the present may tempt us to be unduly concerned with "relevant research" which by being so discernible is frequently pedestrian. Memory and communication are the essential steps which made us civilized and on which science must base the advances that society needs. By pausing to examine where we have been, and why we are where we are, we may gain a historical perspective enabling us to find invaluable guides as to where we should go. By meditating over past accomplishments, we become aware that, as stated by Bernard de Chartres in the 12th Century "we are dwarfs perched on the shoulder of giants. We see more and farther, not because our vision is better or our height is greater, but only because they lift us." However, as in the previous workshop (Sadun, 1969), let me remind you of some of the drawbacks in making a patient, detailed research of the early literature; it is time consuming, it may have an ego-damaging effect by showing that many of our observations had already been made a long

time ago, and by exalting the ancient achievements it may lead us to disregard those pertaining to the present time "dum vetera extollimus recentium incuriosi." Tacitus-Annals (II, 88).

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Basic Research Priorities from the Perspective of National Malaria Programs in Developing Countries

L. M. HOWARD

Director, Office of Health Technical Assistance Bureau, Agency for International Development, Washington, D. C. 20523

In his Presidential address to the American Society of Tropical Medicine and Hygiene last December, Dr. William Reeves expressed a growing uneasiness about the outcome of efforts to contain the major infectious diseases of tropical and temperate regions. "I fear that society and the scientific community have become complacent with the advances made in research and their relative freedom from epidemics. I contend that while we have won many battles in our research efforts, the longer range and bigger war to contain certain infectious diseases can still be lost. I believe there are a variety of technical, economic, social and political forces that will influence the outcome of the contest. We, as scientists, tend to forget that the majority of these factors can be quite independent of the status of our scientific knowledge or competence."¹

The basis for this concern is well understood by those familiar with the current status of national malaria programs overseas such as my colleague, Dr. Robert Kaiser, and in particular by such outstanding authorities on malaria research needs for developing countries as Professor Bruce-Chwatt, Dr. Tibor Lipes, and Dr. Martin Young.

Many of you are aware that the worldwide program is primarily an effort of developing countries themselves with the assistance of the World Health Organization, UNICEF, and the Agency for International Development which administers all United States bilateral assistance. The WHO has invested 109 million dollars between 1958–1971 in technical coordination and policy guidance. The Agency for International Development, with the assistance of the U.S. Public Health Service, has contributed 375 million dollars in the last 20 years (1950–1971) for commodities and technical advisory services to 36 national programs. These programs have included approximately 80 percent of the total population participating in national programs. Developing

countries administer their own programs and provide approximately two-thirds of all costs.

However incomplete and inadequate the accomplishment so far, it must be candidly noted that the progress attained in reducing malaria throughout the world since World War II would not have occurred without the above magnitude of external support.

The maintenance of present gains as well as progress in the future will depend in large measure on the results of basic research to solve current technical problems. For this reason, we are keenly interested in the proceedings of this Workshop and are indebted to Dr. Sadun for his kind invitation.

The WHO Director-General's report of May 1972 indicates that 1.826 billion population live in areas originally at risk to malaria. Within this population, which includes 37 countries, 728 million are reported to be free of the disease.² Active programs cover an additional population of approximately 600 million. The total population under complete or partial protection is 1.346 billion, approximately one-third of the world's population. In the delivery of public health services on a global scale, this quantitative accomplishment still remains unparalleled in history. (See Table 1.)

Since 1967, populations-at-risk without any programs have been reduced from 421 million to 270 million. Today nevertheless, populations with limited or no programs still amount to 480 million, principally in Africa, but including countries along a wide equatorial belt covering parts of Asia and the Americas.

Out of 146 countries or territories originally at risk to malaria, only 53 countries have actually attempted an eradication program. Twenty-seven countries today have limited antimalaria programs. As of 2 years ago, 34 countries were not participating in any type of program.

For the non-participating or partially partic-

ipating countries, inability to meet exacting technical, administrative or operational criteria has precluded full-scale programs.

Of more importance, perhaps, has been the outcome of national programs which were judged to have met initial criteria for operational feasibility. In analysis of 42 such programs by the WHO Expert Committee on Malaria in 1966, 12 programs representing 70 percent of the population were found to be progressing satisfactorily, the other 30 being affected primarily by poor management, or planning, or insufficient government support.³

One cannot turn the clock back. We will perhaps never know what might have happened if governments had vigorously supported their programs. We will never know if resistance to insecticides and to drugs would have become the problems they are today if national programs had been pressed efficiently and rapidly during the first 10 years of operation.

Waterston, the economic planner at the World Bank, has observed that "Examination of available evidence makes it clear that in countries with development plans, lack of adequate government support for the plans is the prime reason why most are not carried out successfully."⁴

In the application of scientific technology on a mass scale, one learns that the impact of biological science on a given society is limited by the social values and commitments of that society.

The general conclusion from these few comments on national malaria programs is that extraordinary progress has indeed been achieved, but the pace of progress is slowing down due to biological and social resistance. New research must take into account not only specific biological problems but the need to develop methods which compensate for social and administrative constraints.

The remaining risk from malaria is not limited to the effect on local populations only, but also to foreign populations, visitors, and armed forces which may be based in that country. In comparison to the extraordinarily high levels of morbidity prevailing at the end of World War II, malaria has been dramatically reduced in many countries through the efforts of national governments with external

Table 1. World-wide distribution of population at risk to malaria by program phase.

Phase of programme	Population in millions			
	1957	1961	1966	1971
Malaria eradication programmes:				
Maintenance	220	317	619	728
Consolidation	-	75	335	299
Attack	569	575	234	315
Preparatory	-	59	39	4
Total	789	1 026	1 227	1 346
Where no malaria eradication in operation but where limited control measures instituted	-	-	-	210
Without specific antimalaria measures	421	393	365	270
Total	421	393	365	480
Grand total	1 210	1 419	1 592	1 826

assistance. In spite of continuing low endemicity in the Philippines, for example, the residents of Clark Air Force Base are beneficiaries of a Philippine Government program which has greatly reduced the risk of malaria since the day that General MacArthur observed that it was going to be a long war if, for every division in combat readiness, there was a second division down with malaria, and a third division convalescing from malaria.

It is equally true that where governments or assisting agencies are unable to undertake programs, for whatever reason, as in Africa and Southeast Asia, the risk to foreign residents and troops remains high. In the face of the continuing high risk from malaria in many parts of the world today, there has been no greater effort to find solutions than the outstanding basic malaria research program of the Walter Reed Army Institute of Research. Acknowledging the major research achievements of this program during the past decade, and the invaluable contributions to international malaria programs, one may take advantage, however, of hindsight to conjecture the possible advantages which might have accrued through an improved appreciation of the priorities of the developing countries.

For example, during the 1969 Panel Workshop, Col. Gochenour reported 12,000 cases of malaria among U.S. armed forces in Vietnam in 1968.⁵ He estimated direct medical treat-

ment costs at approximately 11 million dollars. Col. Canfield's data show the 1965–1971 case total to be 81,885.⁶ If the 1968 medical cost estimates were to be applied, one might derive a total cost estimate of approximately 60 million dollars. As I understand, no such estimate has actually been determined. The accuracy is not so important as the order of magnitude. Prior to 1964, a highly effective malaria campaign in South Vietnam did not require more than 2 million dollars in commodity assistance per year. Is it possible that a program of basic research to interrupt malaria in the general population-at-risk in Southeast Asia during the 1960's might have yielded, at less cost, an equally effective end result in the 1970's? Although the question is, in a sense, rhetorical, a cost benefit analysis might have justified such an effort.

With this perspective, the basic research programs of both developed and developing countries themselves might still include an orientation towards efforts to interrupt transmission at points in the cycle other than those which offer direct human protection.

However selfevident it may appear that there is logic to the coordination of research priorities among different organizations with international health interest, there are in fact significant differences in determination of those priorities today.

Professor Abel-Smith of the London School of Economics in a recent discussion on health priorities, noted that public decisions are ultimately value decisions taken through the normal political process.⁷ Informed decision-taking is of course to be preferred over uninformed decision-taking—but the sense of direction or preference precedes the choices of alternatives which must be made to achieve that purpose.

Basic research priorities in malaria, as in other fields of science, are subject to the same processes of value-decisions and understandably so. The Department of Defense must be concerned with the health and protection of its manpower. Thus value is placed on measures which provide direct protection of men whether or not such measures influence morbidity in the general population. Research priorities have emphasized the parasite-man interaction, chemotherapy, mosquito control

measures, and more recently, the emphasis on vaccine development. There emerges an assumption that research towards direct antiparasitic agents is the most logical path to achieve protection of limited population groups.

In non-endemic countries such as the United States, domestic malaria research priorities have focused on accurate understanding of epidemiological variables in order to train a cadre of public health officers with the capability for rapid epidemiological surveillance and treatment in the event of reintroduction of malaria.

Neither of these perspectives adequately fit the problem faced by countries with populations-at-risk which may number as high as several hundred million. The ultimate objective here is also human protection, but not through measures which provide individual human protection as a primary measure. The limited resources of developing nations require a simple, low-cost technology which can interrupt transmission within the whole population-at-risk. For this purpose, interruption at any point in the transmission cycle produces the same end result. Research priorities have emphasized development and testing of long-acting residual insecticides as a primary measure and chemotherapy as an important but secondary tactic for the reduction of the human parasite reservoir.

Assuming that nation-wide measures suggest a favorable cost-benefit ratio, what general characteristics would describe antimalaria measures designed to interrupt transmission within the significant population at large?

1. Operational Simplicity

A vaccine, for example, offers the hope that one might avoid the need for the complex current campaign strategy of geographic reconnaissance, sophisticated logistics and high manpower requirements for insecticide application, chemotherapy, health education, epidemiological assessment, and laboratory support. The technical agent should be stable under tropical conditions, ship easily, and preclude the need for refrigeration. The technology should permit application with considerably less than 100 percent efficiency.

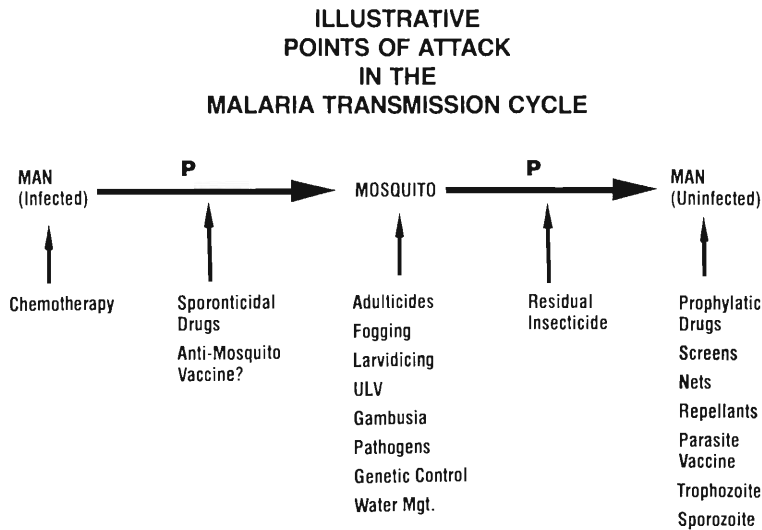


Figure 1. Illustrative points of attack in the malaria transmission cycle.

2. Low Selection Pressure

The agent should apply to the transmission cycle in ways which stimulate minimal selection pressure on the parasite or mosquito.

3. Duration of Effect

Any new agent should have a residual effect of at least 3–6 months. The mobilization of manpower to cover an entire country at more frequent intervals is expensive and difficult to administer. The same principal applies to chemotherapeutic agents because of relatively short duration of effect. No drug, regardless of effectiveness or price, has so far been consistently or effectively applied to larger populations over a long period of years as a primary antimalaria measure. Extensive experience in mass drug application in the Americas should be persuasive on this point. The place of chemotherapy is not challenged as a supportive measure or as a specific in the case of illness.

4. Low Cost

Present technological agents, whether insecticides or drugs, have cost an average of 10–15 cents per capita per year. More expensive agents are not likely to be supported in the long run. This suggests the need for bio-

logical products or agents which can be produced in the national laboratories of more than one country. It suggests products or agents which can be mass-produced, a barrier which still impedes the development of antigen from the malaria parasite itself in either trophozoite or sporozoite form.

5. Social or Cultural Acceptability

Recent African experience with smallpox programs suggests that vaccination still remains one of the most widely accepted methods for administration of specific agents in developing countries. On the whole, insecticide application has been remarkably well tolerated, considering the inconvenience required in moving all household material out of the house prior to spraying. Nevertheless, the housespray refusal rates are increasing in many countries.

A substitute for residual insecticides would allay the concerns of those, both in the United States and abroad, who fear the potential adverse environmental consequences of non-biodegradable insecticides. The demand for a biodegradable insecticide would please the ecologist but still annoy the housewife whose home must be treated.

How do these general characteristics apply to current research efforts?

In Figure 1, which illustrates familiar points of attack on the transmission cycle, a few of the current and anticipated future technologies are listed. From a preventive point of view, present measures for protection of uninfected man are difficult to apply effectively on a national scale. Short duration drugs cannot be effectively administered nationwide over long periods of time as a primary preventive measure. Screening, nets, and repellents are prohibitive in cost except for a small minority. Development of a parasite vaccine still faces the hurdle of adequate antigen supply. Individually, these are effective measures in a selected population, but they offer little optimism in the near future, for the application to large populations.

For the same reasons, chemotherapy for infected man is an important individual measure and supportive in the treatment of residual infections in a mass campaign. Yet, applicability to a large national campaign is limited unless a long acting drug can be found.

Residual insecticides have induced the selective or repellent actions that are now creating an increasingly difficult problem. Over 1500 alternatives insecticides have been tested with only 2 resulting candidates, OMS-33 and OMS-43, to replace DDT. The potential for resistance remains. In much of Africa, where outdoor biting predominates, insecticides are inapplicable as a primary measure.

Antimosquito measures are highly effective when selectively used. As primary measures, they tend to be expensive and logistically difficult for national applicability in developing countries. Furthermore, effective use of such entomological measures requires highly skilled professional knowledge of local vector habits by locality. Such skills are difficult to provide on a national scale.

At the point of transmission between infected man and the mosquito, effectiveness of sporonticidal drugs is known. Mass applicability is difficult for the same reasons which preclude efficient mass administration of any short-duration drug.

What alternatives remain? With the objective of interrupting transmission at any point in the cycle, is it possible, for example, that human populations might be immunized with uninfected mosquito tissue antigens in order to induce an immunological reaction within the

midgut of the mosquito which feeds on man? Is it possible that such reaction within the mosquito might interfere with the development of the sporogonic cycle?

At the University of Illinois, Jakstys obtained photomicrographs of the normal whorl configuration of ribonucleoprotein-containing endoplasmic reticulum within the cytoplasm of *A. stephensi* midgut cells within 50 hours after feeding on a normal rabbit. Such configuration fails to recur in midgut cells following a blood meal on rabbits immunized with antigen prepared from mosquito midgut cells.⁸ The ultimate effect of this change on sporogony is yet to be determined.

I am not suggesting that this particular experiment be pursued. The approach however serves to illustrate the point that basic research in malaria should not overlook innovative ways to interrupt the transmission cycle at any point even though the primary effect may not be immediately protective of man. The indirect approach may provide the more permanent solution. As a further illustration, the use of a mosquito antigen suggests the potential for vaccination as an ultimate procedure, the use of an antigen supply which exists in abundance, the avoidance of selective pressure on either malaria parasites or mosquitoes, the potential for low-cost production in countries of need, and the possible stimulation of long-duration effects. Interruption of transmission for three years or longer should allow natural attrition of the main reservoir of *vivax* and *falciparum* malaria to levels small enough to permit effective chemotherapy of residual cases.

Antimalaria programs of the future will in all probability require a far greater spectrum of technology and procedures than we now possess. If we consider therefore the direction of future basic research priorities in malaria, I should think that we would have to consider more carefully the perspective of the developing countries. Our failure to do so is to risk the continued consequences of infection among our own citizens and manpower both here and abroad.

Malaria is an ancient and respected enemy. It is not about to fade away without greater innovation and effort than we have been able to mobilize thus far.

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Malaria in U. S. Military Personnel 1965-1971

C. J. CANFIELD

Walter Reed Army Institute of Research, Washington, D. C. 20012

Brigadier General Tigertt (1966) addressed the second panel workshop on Malaria Research here at the Walter Reed Army Institute of Research. The title of his paper was the "Present and Potential Malaria Problem." At that time he reviewed the history of drug-refractory falciparum malaria and the impact of this disease on U. S. forces during the escalation of military activities in Vietnam during the Fall of 1965 and Spring of 1966. Six years have passed and it now seems appropriate to review subsequent events in the light of historical perspective.

In Vietnam malaria was the third most frequent medical cause of hospitalization of Army personnel behind respiratory and diarrheal diseases. However, the longer time required for treatment and recuperation made malaria the major cause of medical disability (Canfield, 1969).

Most cases of malaria were in soldiers and marines. The total number of cases in these two groups by year is shown in the Table 1. Although these data represent worldwide figures, the majority of cases were from Vietnam. The death rate of 1.7/1000 cases is an astonishingly low figure. If deaths due only to falciparum malaria are considered, the death rate is 0.3%. This can be compared with deaths

due to falciparum malaria in Britain during 1954-1969 of 16% (Bruce-Chwatt, 1971) and deaths in civilian hospitals in the United States during 1965-1969 of 10% (Neva, *et al.*, 1970). Rapid diagnosis and treatment probably were the major causes of the low fatality rate in Vietnam.

Attack rates for U. S. Army personnel in Vietnam are shown in the Figure 1. From a high of nearly 50 cases/1000/annum, there was a gradual decline to 17 cases/1000/annum. There is probably no single explanation for this decline, but better education of personnel on protective measures, more effective treatment, changes in military tactics and the introduction of daily chemoprophylaxis with dapsonc during 1966 all probably played a role.

The chemoprophylactic regimen used in Vietnam (weekly chloroquin/primaquin [C-P] and daily dapsonc) has been shown in volunteers not to be completely protective for strains of falciparum malaria from Southeast Asia (Willerson, *et al.*, 1972). However, there is no evidence that chloroquin, when ingested, is not completely suppressive for vivax malaria (Hiser, *et al.*, 1971). Despite this, approximately 40% of malaria cases in Vietnam were due to *Plasmodium vivax*. It is assumed that these cases of vivax malaria resulted from fail-

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Table 1. Malaria cases, worldwide (1965–1971).

Year	Army Cases* (Deaths)	Navy Cases** (Deaths)	Total Cases (Deaths)
1965	2080 (16)	297	2377 (16)
1966	7070 (14)	2079 (3)	9149 (17)
1967	11856 (11)	2767 (4)	14623 (15)
1968	10734 (15)	6133 (11)	16867 (26)
1969	10339 (10)	9809 (22)	20148 (32)
1970	9637 (12)	3504 (5)	13141 (17)
1971	5249 (9)	331 (5)	5580 (14)
	56965 (87)	24920 (50)	81885 (137)

* Data provided by Patient Administration & Biostatistics Branch, Office of The Surgeon General, Washington, D. C.

** Data provided by Naval Medical Data Services Center, Bethesda, Md.

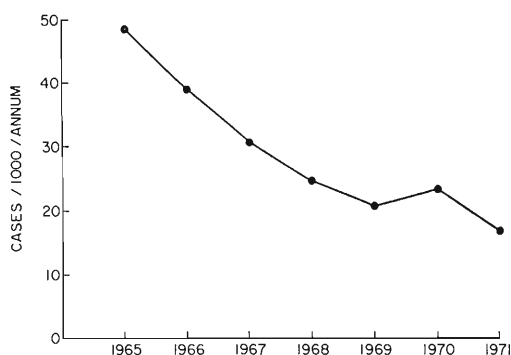
ure to take the prescribed C-P tablet. Many reasons have been advanced for these failures: temporary unavailability of tablets; a desire to have a non-fatal disease to avoid field duty; poor acceptability of the C-P tablet because of gastrointestinal side effects, and others.

Plasmodium vivax, in addition, accounted for over 14,000 cases of malaria in the United States since 1965—more than 80% of the total cases of malaria.* That military personnel were not radically cured of vivax malaria when they left Vietnam was probably due in most instances to failure to take the prescribed course of C-P tablets (Barrett, *et al.*, 1969) and, in others, the use of a regimen not curative in all cases (Martelo, *et al.*, 1969). These experiences point out the need for better chemoprophylactic drugs and improved methods of drug administration.

Treatment of patients with acute falciparum malaria infections in Vietnam underwent a major change during late 1965 and early 1966. Although chloroquin resistance was known in Southeast Asia, the severity of the problem was not appreciated until many military hospitals were full of patients with recrudescence chloroquin-treated malaria. Treatment with quinine alone or quinine and chloroquin provided little improvement. Pyrimethamine, however, in combination with quinine and dapsone or chloroquin and sulfisoxazole finally provided cure rates of nearly 100% (Neva, *et al.*, 1970).

The next significant therapeutic change occurred in 1969–1970 when a repeat study of the above combinations showed cure rates had

* Center for Disease Control Malaria Surveillance, 1970 Annual Report, May 1971.

**Figure 1. Attack rates for U. S. Army personnel in Vietnam, 1965–1971.**

decreased to less than 90% (Hall, in prep.). Treatment of patients who recrudescence required the use of intravenous quinine by constant infusion in combination with oral pyrimethamine and sulfisoxazole for complete cures. Some patients had repeated recrudescences despite multiple courses of combination therapy and were evacuated to Walter Reed General Hospital for treatment with new investigational drugs. These drugs were available because the U. S. Army Malaria Research Program had screened thousands of chemical compounds in search of new agents effective against the multi-drug resistant falciparum malaria from Southeast Asia. Two drugs which had originally been synthesized but not tested during the World War II program surfaced—a phenanthrene methanol and quinoline methanol. Finally tested in volunteers against strains of falciparum malaria from Vietnam in 1968–1969, these agents proved wholly effective in the patients who were evacuated to the United States with multiple recrudescences. A limited field test in Vietnam in 1971 was somewhat disappointing in that cure rates were only 90% in naturally acquired acute infections. However, the virtually complete absence of even minor side effects made these drugs an important milestone. At present the U. S. Army is testing analogs of these compounds which have shown from 8–80 times more activity in animal test systems. There is little doubt that at least one of these compounds will provide us with an effective single drug for treatment of all strains of falciparum malaria presently characterized.

However, we cannot be complacent about these encouraging drug results. The military experience with malaria from Vietnam has re-emphasized several key facts. First, drug development similar to basic research does not lend itself to heroic short-lived measures. The research period from first recognition of biologic activity to a useable drug is often many years. Now, more than ever before, intensive scrutiny of the efficacy and safety of drugs is required prior to their use in man. Secondly, the malaria parasite has repeatedly shown a remarkable ability to adapt to new drugs and the continued use of any drug will predictably be associated with parasite resistance to the drug. Thirdly, chemoprophylaxis alone will probably never provide the solution to the Army's malaria problem. Even with a completely effective agent such as chloroquin for prevention of vivax malaria, there was still a significant loss of military manpower in Vietnam. What is needed is a new approach to this old disease. Any new approach will probably arise from application of studies in the basic sciences, such as these about which we will be hearing during these meetings. Many of the recent studies have been of great interest to those of us involved in clinical malarology. Studies of immunology in malaria have made remarkable advances in the last few years. Few would have predicted the successes that have been observed with irradiated trophozoite and sporozoite immunization of experimental animals (Wellde, *et al.*, 1969, Nussenzweig, *et al.*, 1969).

Biochemical studies of plasmodium metabolism have given us new insight into the nutritional requirements of the parasite. Refined culture systems for asexual parasite development have resulted from these studies although the ideal system is still elusive. However, our understanding of drug action has greatly benefited from these studies. In addition, simple culture techniques have been used to determine relative drug resistance of various strains of *P. falciparum* and have been applied to the evaluation of the relative efficacy of similar chemical compounds within a class (Rieckmann, *et al.*, 1968).

Better test systems for drug evaluation have resulted from basic research. Foremost among these has been the successful transmission of human falciparum malaria to the *Aotus* mon-

key (Geiman, *et al.*, 1967). This accomplishment gave us the ability to test new chemical compounds against the ultimate target, *P. falciparum*, in a biologic system other than man.

Studies of the pathophysiology of human malaria have also advanced significantly in the last 6 years. Refined techniques were applied to U. S. military personnel in Vietnam with acute malaria infections. These included renal function studies, electrolyte studies, endocrine status evaluations, small intestinal biopsies and function studies, and ferrokinetic studies. Results from these studies have led to a more rational approach to the non-chemotherapeutic management of individual patients with the disease.

In summary, the last few years have been extremely fruitful for malaria research. Although I have alluded primarily to studies of particular interest to those of us in clinical malarology, many other important observations have been made in the basic sciences. We all look forward with interest to a productive exchange of ideas during the workshop.

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II

HOST-PARASITE RELATIONSHIPS

A. Primate Models

Trophozoite Induced Infections of *Plasmodium falciparum* in *Saimiri sciureus* (Squirrel Monkeys)

R. N. ROSSAN, M. D. YOUNG, AND D. C. BAERG

Gorgas Memorial Laboratory, Apartado 6991, Panamá 5, Rep. de Panamá*

ABSTRACT: Unaltered *Saimiri sciureus* were susceptible to the trophozoite stages of chloroquine sensitive (Uganda-Palo Alto) and chloroquine resistant (Vietnam-Oak Knoll) strains of *Plasmodium falciparum*. The infections were serially transferred in these hosts.

High parasitemias, reaching more than 400,000 asexual parasites per cmm of the Uganda-Palo Alto strain, were sustained.

Gametocytemias were produced in 2 hosts but failed to infect 3 species of anophelines.

The monkeys adapted well to captivity and survived long periods following the infections.

The monkeys of Panama have failed generally to be good hosts for indigenous strains of *Plasmodium falciparum*. The Taliaferros (1934) reported only short periods of parasitemias in *Alouatta villosa* (black howler monkeys). Porter and Young (1967) were able to produce moderate infections of *P. falciparum* in *Saguinus geoffroyi* (the marmoset), which persisted for as long as 15 days, but serial passages were not achieved. Many other attempts in this laboratory to infect different species of Panamanian monkeys with parasitized human blood have not resulted in establishing infections which could be maintained serially.

Geiman and Meagher (1967), using an African strain of *falciparum* (Uganda-Palo Alto) directly from man, adapted this parasite to *Aotus trivirgatus* (night monkey) and established serial passages. Using this adapted strain, we found that it would grow well and could be passed serially in *Cebus capucinus* (the white-faced capuchin) (Young and Baerg 1969). Only transitory parasitemias were produced in *Ateles fusciceps* (the black spider monkey) and *A. villosa* (Baerg and Young 1970). Subsequently, a preliminary report indicated that this strain would grow in *Saimiri sciureus* (the squirrel monkey) (Young and Rossan 1969). The potential of *Saimiri* as a model for *P. falciparum* infections has been investigated further using the African strain (Uganda-Palo Alto) as well as a strain from

Vietnam (Vietnam-Oak Knoll). The results are presented in this report.

Material and Methods

Panamanian *Saimiri* monkeys used in this study were collected in the Chiriqui Province, Panama, from an area about 35 km east of the Costa Rican border. None of these animals were found to be harboring naturally acquired plasmodial infections.

Initially, the monkeys were housed in groups of 20 to 30 in out-door gang cages. After inoculation, the animals were moved into individual cages located in indoor laboratory rooms. As *Saimiri* monkeys are fastidious eaters, diverse foods were offered. The diet consisted of bananas, canned fruit cocktail, cottage cheese, raw peanuts, monkey chow (Wayne Monkey Diet) and a canned preparation (Science Diet®). This ration was supplemented occasionally with live, neonatal mice and weekly with a vitamin mixture (Octavitamin). Water was available ad libitum. The monkeys adjusted well to laboratory conditions.

All *Saimiri* were healthy adults and subadults, of either sex, and weighed approximately 300 to 500 gms. The monkeys initially were inoculated with the Uganda-Palo Alto strain of *P. falciparum* from *Aotus* bearing the 32nd and 33rd passages at Gorgas Memorial Laboratory (Young and Baerg 1969). Subsequent serial trophozoite transfers then were continued between *Saimiri*. The Vietnam-Oak Knoll strain, which had been adapted to *Aotus* monkeys, was provided kindly by Dr. W. Sidiqui of the University of Hawaii. *Saimiri*

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* Mailing address: Gorgas Memorial Laboratory, P. O. Box 2016, Balboa Heights, Canal Zone.

Table 2. *Plasmodium falciparum* infections in squirrel monkeys—after intraperitoneal rechallenge with 5 X 10⁶ trophozoites of the Uganda-Palo Alto strain.

Monkey no.	Subpatent period prior to rechallenge—days	Prepatent and (Subpatent) periods—days	Patent period—days	Maximum observed parasitemia		Comments
				Per cmm	Patent day	
4792 ^a	61	—	—	—	—	Negative 263 days
4687 ^a	72	3	3	40	2	Negative 383 days
"	—	(18)	26	1,140	4	
"	—	(47)	11	5,440	7	
4800 ^a	62	4	8	1,840	6	
"	—	(34)	3†	30	3	
4805	42	6	17†	148,860	17	

^a Amodiaquine (10 mg base/kg) administered days 17, 16, 15 prior to rechallenge.
† Died.

aqueine (a total of 30 mg base per kg over a 3 day period). One of these subjects (4792) did not develop an infection during a 263 day observation period. Parasitemias appeared in the remaining 3 *Saimiri* after prepatent period of 3 to 6 days. Although a high parasitemia resulted in 1 *Saimiri*, relatively low grade infections persisted for 3 and 18 days, respectively, developed in the other 2 recipients. In the latter 2 monkeys, relapses occurred after subpatent periods of 18, 47 and 34 days. Two monkeys, 4805 and 4697, evidenced gametocytemias during the 26th to 28th day and the 7th to 39th day (intermittently) of the primary attack. Three species of colonized mosquitoes, *Anopheles albimanus*, *A. aztecus* and *A. pseudopunctipennis*, were fed upon these hosts at gametocyte concentrations of 10 to 30 per cmm. Dissections of anophelines from a total of 25 fed lots were negative for oocysts and sporozoites.

The Vietnam-Oak Knoll strain of *P. falciparum* inoculated intraperitoneally (90 × 10⁶ parasites) into 2 normal *Saimiri* produced patent infections 5 and 6 days later, respectively. The parasitemias were patent for 11 and 17 days, and maximum parasitemias were 210 and 5,610 per cmm. There were no gametocytemias, and no relapses were recorded during subsequent observation periods of 50 and 57 days. Subinoculation was not attempted from these *Saimiri*.

Discussion

Both the Uganda-Palo Alto and Vietnam Oak Knoll strains had been adapted to normal *Aotus* monkeys prior to inoculation into *Sai-*

miri hosts. There was no difficulty in initially establishing significant infections in the unaltered *Samiri* recipients, and in continuing a passage line with the Uganda-Palo Alto strain. In these passages, fulminating parasitemias were achieved in unaltered recipients. However, some of the infections in the *Saimiri* were self-limiting, while the *Aotus* monkeys required chemotherapeutic intervention to sustain the life of the host. Infections of the Uganda-Palo Alto strain in intact or splenectomized *Cebus* (Young and Baerg 1969), in contrast, were generally lower than in *Saimiri*. Although patent periods as short as 12 days were observed, parasites persisted in several *Saimiri* at high levels for more than 4 weeks during the primary attack, signifying a tolerance of this host species for *P. falciparum*. Further, persistence of the parasites at subpatent levels was shown by the fact that relapses did occur.

The results obtained after rechallenge of a limited number of subjects were variable, but indicated that 3 monkeys were protected as evidenced by either complete absence of parasitemia or by low maximum parasitemias and patent periods of short duration. This host-parasite combination may be useful in further studies of immunity acquired against the erythrocytic phase of *P. falciparum*.

Limited trials with 3 species of anophelines, fed upon 2 monkeys that showed low gametocyte concentrations of the Uganda-Palo Alto strain, did not yield infected mosquitoes. These results are in accord with the failure to infect experimental vectors from *Aotus* and *Cebus* monkeys bearing this strain (Baerg and Young 1969).

The small size and availability of *Saimiri* and its relative ease of adaptation to the laboratory environment may offer further advantages for this species as an experimental model.

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Characterization of *Plasmodium vivax* Infections in *Saimiri sciureus* (Squirrel Monkeys)

R. N. ROSSAN, D. C. BAERG AND M. D. YOUNG

Gorgas Memorial Laboratory, Apartado 6991, Panamá 5, Rep. de Panamá*

ABSTRACT: Infections with a monkey-adapted strain of human *Plasmodium vivax* (Achiote) were established in *Saimiri sciureus* and were serially transferred 26 times in this host species by trophozoite inoculation. Patent infections were produced in all of the 42 unaltered and 1 splenectomized recipients.

Primary infections persisted for as long as 72 days and maximum parasite concentrations reached more than 100,000 per cmm. Relapses occurred, and were usually of shorter duration with parasite densities lower than in the primary attack.

Eleven unaltered *Saimiri* were infected by the sporozoite stages in *Anopheles albimanus* derived from *Aotus trivirgatus* carrying this vivax strain. Although prepatent periods were longer than for trophozoite induced infections, most of the characteristics of the parasitemias were similar.

Oocysts were demonstrated in *Anopheles albimanus* and *A. aztecus* following feeding upon several *Saimiri* with trophozoite induced infections.

It has been shown that infections with *Plasmodium vivax* can be induced in 5 species of New World monkeys by the trophozoite and sporozoite stages of the parasite. The most widely used experimental host is *Aotus trivirgatus* (the night monkey); vivax strains are

easily adapted and maintained in this model (Young et al. 1966; Porter and Young 1966; Hickman 1969; Baerg et al. 1969; and Ward et al. 1969). Vivax malaria also has been transferred from *Aotus* by trophozoites and sporozoites to *Saguinus geoffroyi* (the Panamanian marmoset) (Porter and Young 1966; Baerg et al. 1969; Baerg et al.—unpublished; and Porter 1970) and to *Ateles fusciceps* and *A. geoffroyi* (spider monkeys) (Baerg et al. 1969; Baerg et al.—unpublished; Young and

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* Mailing address: Gorgas Memorial Laboratory, P. O. Box 2016, Balboa Heights, Canal Zone.

Table 1. Serial trophozoite passages of *Plasmodium vivax* in *Saimiri sciureus*—primary parasitemia.

No. of monkeys	Inoculum × 10 ⁶ range	Prepatent period —days Mean (Range)	Patent period —days* Mean (Range)	Parasitemia				Remarks
				1,000 per cmm		Maximum per cmm		
				No. of monkeys	Patent day Mean (Range)	Mean (Range)	Patent day Mean (Range)	
<i>Passage Nos. 1–5</i>								
12	1.1–12.5	7 (1–18)	32 (23–72)	11	8 (4–13)	30,455 (910–86,290)	15 (11–18)	2 died patent days 21, 38
<i>Passage Nos. 6–10</i>								
11	<1–12.5	6 (1–21)	27 (24–28)	11	6 (3–15)	16,841 (1,720–39,940)	13 (3–20)	3 died patent days 14, 29, 59. 4 sacrificed when parasitemia approximated 40,000 per cmm
<i>Passage Nos. 11–15</i>								
5	1.3–2.1	8 (4–19)	28 (25–34)	5	7 (5–8)	48,138 (16,570–111,070)	14 (10–16)	2 died patent days 17, 19
<i>Passage Nos. 16–20</i>								
6	<1–9.9	6 (2–11)	22 (18–27)	5	9 (3–19)	43,360 (6,030–87,130)	14 (6–29)	2 died patent days 3, 39
<i>Passage Nos. 21–26</i>								
9	<1–10.9	4 (1–7)	35 (20–55)	9	6 (4–8)	48,955 (14,700–93,210)	14 (11–18)	2 died patent day 34

* Monkeys dying during patency are not included in tabulation of length of patent period.

Porter 1969; and Porter and Young 1970). The latter 2 hosts require splenectomy to produce significant infections.

Deane et al. (1966) found that a splenectomized *Saimiri sciureus* (squirrel monkey) would support an infection of vivax malaria derived directly from man. We since have determined that intact *Saimiri* will sustain infections of a monkey adapted vivax malaria introduced by blood stages or mosquitoes (Young et al. 1971). Subsequent to our preliminary report, we continued our investigations on the characteristics of the infections in *Saimiri* monkeys. The results are reported here.

Materials and Methods

Procedures concerning the procurement, handling and husbandry of *Saimiri* monkeys at Gorgas Memorial Laboratory were given by Rossan et al. (1972).

The human malaria strain, Achioté, was isolated in Panama and has been serially transferred by blood or sporozoites in monkeys since 1966 (Porter and Young 1966). Several passage lines in *Aotus* and *Saimiri* were maintained to achieve further parasite adaptation and to study the course of infection in these primates. In most cases unaltered *Aotus* and *Saimiri* served as recipients. Inoculation pro-

cedures, staining of blood films and counting of parasites were as described previously, with some modifications (Porter and Young 1966).

Infected *Aotus* served as donors for sources of mosquito infections as given in previous reports (Baerg et al. 1969). Only *Anopheles albimanus* was used as the vector for sporozoite transmissions. Sporozoites were introduced either by the interrupted bite technique or by intravenous inoculation of a sporozoite suspension. Animals inoculated intravenously were administered a single dose of penicillin (400,000 units). *Anopheles aztecus*, *A. pseudopunctipennis*, and *A. punctimacula* were utilized for their comparative susceptibilities to the induced malaria in the monkeys.

Results

Trophozoite Induced Infections

After 78 consecutive blood passages in *Aotus* monkeys, infections were established in *Saimiri* by subinoculation into 1 splenectomized and 2 unaltered animals. Passage lines were continued in intact *Saimiri* from the splenectomized recipient and from 1 unaltered recipient; these were carried through a total of 8 and 26 passages, respectively. Data on the primary parasitemias of the infections in the monkeys comprising the 2 lines are grouped and summarized in Table 1 according

Table 2. Characteristics of relapses in *Saimiri sciureus* with trophozoite induced infections of *Plasmodium vivax*.

No. of monkeys	Subpatent period —days Mean (Range)	Patent period —days Mean (Range)	Maximum parasitemia	
			Per cmm Mean (Range)	Patent day Mean (Range)
<i>1st Relapse</i>				
19	29 (5-66)	14 (3-24)	915 ($<10-10,910$)	9 (4-14)
<i>2nd Relapse</i>				
9	29 (5-51)	17 (12-21)	992 ($<10-5,430$)	10 (8-14)
<i>3rd Relapse</i>				
5	31 (10-77)	18 (3-28)	1,816 ($<10-6,240$)	13 (4-21)
<i>4th Relapse</i>				
1	24	15	390	10

to the number of serial transfers. Parasitemias were produced in all of 43 monkeys inoculated (40 intraperitoneally and 3 intravenously).

Overall prepatent periods ranged from 1 to 21 days, and were as short as 1 day in both the early and late passages. There was no apparent correlation between the prepatent period and numbers of parasites inoculated. Generally, there was a relatively rapid ascent of parasite numbers. In 41 of the 43 recipients parasitemias reached 1,000 per cmm between the 6th and 9th day of patency with the maximum concentration about 1 week later.

In the initial passage into 3 *Saimiri*, the splenectomized recipient experienced a maximum parasitemia of 86,290 per cmm, whereas maxima of 910 and 2,360 per cmm were achieved in the unaltered, companion subjects. However, within a few subsequent passages, the infections in unaltered recipients reached as high as 84,360 per cmm. The average maximum parasitemias, with a peak in one animal of 111,070 during the 15th passage, were consistently higher after 10 serial transfers.

The initial patent periods in 28 monkeys (all unaltered) were as short as 18 days and as long as 72 days, averaging 30 days. Eleven other monkeys died while showing parasites, but 9 of these subjects succumbed during a descending phase of the infection. An additional 4 monkeys were sacrificed for tissue studies when parasitemias approximated 40,000 per cmm.

Blood films were examined daily from the

Table 3. *Plasmodium vivax* infections in *Saimiri sciureus*. Gametocyte infectivity to mosquitoes.

Mosquito species	Hosts	Lots	
	Yielding pos. Mosq./Total	Pos./ Tot.	Percent infected
Trophozoite Induced Infections			
<i>Anopheles albimanus</i>	1/20	2/141	4-9
<i>Anopheles aztecus</i>	2/11	4/49	9-71
<i>Anopheles pseudopunctipennis</i>	0/5	0/12	0
Sporozoite Induced Infections			
<i>Anopheles albimanus</i>	0/7	0/50	0
<i>Anopheles aztecus</i>	0/3	0/23	0
<i>Anopheles punctimacula</i>	0/1	0/3	0

28 monkeys following termination of the primary attack. No parasites were detected in 6 of these animals which died from the 15th to the 87th day post patency and in 3 surviving for more than 500 days. However, the infections in 19 monkeys (68%) relapsed 1 or more times after subpatent periods ranging from 5 to 77 days (Table 2). One relapse only was recorded in 10 monkeys, while as many as 4 were observed in the remaining animals. The mean duration of the subpatent periods between the relapses were similar. In all cases, the maximum parasitemia achieved during relapse was markedly lower than in the primary attack. Additionally, the patent periods in most instances were shorter for relapses than in the primary attack.

Three species of mosquitoes were fed upon *Saimiri* hosts harboring trophozoite induced infections. As indicated in Table 3, although some *A. albimanus* and *A. aztecus* showed oocyst development, the gametocytes in *Saimiri* were poorly infective for these vectors. No sporozoites were seen in the salivary glands of the infected mosquitoes, however mature oocytes, containing sporozoites, were observed on the stomach wall of *A. aztecus*.

Sporozoite Induced Infections

A total of 49 normal *Saimiri* in 10 experiments, and 2 splenectomized *Saimiri* in 1 experiment, received sporozoites from *A. albimanus* mosquitoes which had been infected on malarious *Aotus* monkeys. Patent infections have developed in 11 unaltered recipients from 7 of these trials. The remaining 40 monkeys were followed 30 or more days and 6 of these are continuing to be examined. Trans-

Table 4. Sporozoite induced infections of *Plasmodium vivax* in *Saimiri sciureus*.

Monkey	Prepatent and (Subpatent) periods—Days	Parasitemia			Remarks
		Maxima per cmm	Patent day	Duration	
5984	20	17,620	20	30	*Parasitemia continuing
	(14)	540 R	12	19	
	(17)	290 R	22	34	
	(33)	330*R	9	>20	
4792	37	260	12	30	Alive 10 days post patency
	(7)	80 R	7	15	
5918	28	37,410	17	20*	*At death
6295	15	<10	—	3	Died 39 days post patency
5915	15	1,030*	10	14	*Treated with chloroquine for chemotherapy studies
5912	26	1,010*	11	16	
6298	29	1,870*	13	14	
6270	48	1,260*	19	>31	
6296	29	7,620*	15	17	
4806	43	<10*	—	>10	*Parasitemia continuing
5988	26	40*	3	>4	

R Relapse.

missions were achieved by both mosquito bite and by intravenous inoculation of sporozoites. Some of the infection characteristics are shown in Table 4. Prepatent periods were variable, ranging from 15 to 48 days. Parasitemias as high as 37,410 per cmm were recorded. Infections in 5 *Saimiri* were treated with chloroquine in the early stages of the parasitemia for use in another study; patency is continuing in 2 additional recipients. The infections relapsed in 2 of 3 untreated subjects, 7 and 14 days after the primary attack. A total of 4 relapses has been experienced by one of these hosts. As in relapses observed for trophozoite induced infections, the primary parasitemias were greater than in subsequent attacks. No oocysts were seen in 3 species of anophelines fed upon *Saimiri* with sporozoite induced infections (Table 3).

Discussion

The Achioté strain of *P. vivax* had been maintained for 6 years, by serial transfer in *Aotus* monkeys, before inoculation into Panamanian *Saimiri*. After the initial passage the parasites were readily adapted to the *Saimiri* model and produced, in some cases, very high parasitemias in unaltered subjects. No infection failures resulted by trophozoite passage to

43 *Saimiri* monkeys in a total of 26 serial passages. The inoculum size used was within the normal range of that for routine transfer in *Aotus*. There appeared to be no appreciable change in most of the characteristics of the infections after adaptation to *Saimiri*, although the highest maximum parasitemias in normal recipients developed after the 10th passage. In an initial report (Young et al. 1971) we showed that *P. vivax* could be transmitted to *Saimiri* by sporozoites from *Aotus* donors. Infections now have been produced in 11 more subjects by this method. It was indicated that *Saimiri* monkeys were poor hosts for infecting experimental vectors. Further, no positive mosquito lots were obtained from *Saimiri* harboring sporozoite induced infections. These results contrast with our findings that the majority of *Aotus* hosts, with sporozoite induced infections, will infect mosquitoes. Relapses occurred in both trophozoite and sporozoite induced infections. It was not determined if the relapses in the latter infections were initiated by persisting exoerythrocytic stages or from subpatent erythrocytic forms. The maximum parasite concentrations achieved during these relapses were lower than during the corresponding primary infection, which indicated that the hosts had acquired some degree of immunity. Also, in

most instances the patent periods during relapses were shorter. These animals were not rechallenged.

During the course of the experiments with vivax infections in *Saimiri*, 12 of 54 (22%) succumbed during patency. This mortality rate is much lower than corresponding data which showed that 67% of 387 *Aotus* hosts died during patency (unpublished data). In *Saimiri*, although in some cases the deaths could have been attributed to high parasite concentrations, other factors such as stress from handling and the presence of naturally occurring parasites (esp. *Acanthocephala* sp.) may have contributed to this mortality.

The above findings demonstrate that *Saimiri* will support development of vivax malaria, either through serial transfer or by sporozoite transmission. This New World primate represents another experimental model for the study of human malaria.

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Plasmodium falciparum in the White-handed Gibbon: Effect of Prolonged Infection on Serum Biochemistry Values

F. C. CADIGAN, JR.,¹ P. K. IBER, AND V. CHAICUMPA

U. S. Army Medical Component, SEATO Medical Research Project, Bangkok, Thailand

It has been previously shown that *P. falciparum* can be transmitted easily by blood

inoculation (Ward *et al.*, 1965; Ward and Cadigan, 1966; Cadigan *et al.*, 1969) and somewhat less easily by mosquito transfer (Gould *et al.*, 1966) to the white-handed gibbon (*Hylobates lar lar*). Parasitemias in the intact animal are of a low order, but in splenectomized animals high levels (up to 8% parasit-

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¹ Present address: U. S. Army Medical Research Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. Reprint request should be directed to address mentioned above.

emia) can be achieved (Cadigan *et al.*, 1969). The infection persists, on the average, for seven months. The shortest period in our experience was six weeks and the longest was 72 weeks. Parasitemia is usually constantly patent for eight to ten weeks and is intermittently patent thereafter. Despite high levels of parasitemia, no overt disease symptoms or signs are produced. If one compares the mean rectal temperatures of infected animals with those of uninfected animals, a statistically significant difference can be shown. However, the two ranges overlap very considerably, particularly in terms of the maximum temperature so that one can not determine from data on a single animal whether or not it was infected. Further, in an infected animal, one can not show a correlation between parasitemia and body temperature. The problem is that gibbons have extraordinarily labile body temperatures particularly in response to exercise.

In other papers we have described the course of parasitemia (Ward and Cadigan, 1966; Cadigan *et al.*, 1969) effect of rapid passage, blood group compatibility and other factors (Cadigan *et al.*, 1969). In this paper we shall present the changes in selected blood chemistry values.

Materials and Methods

The present data are derived chiefly from two groups of infected animals numbering seven and six respectively and four control animals. Of the first group, six had previously been infected with a different isolate of *P. falciparum*. All were successfully infected in this test with an identical isolate and showed the same parasitemic response as the "virgin" animal. All animals used were splenectomized white-handed gibbons (*Hylobates lar lar*) from northern Thailand. The strain of *P. falciparum* used in this study had been isolated by us from a patient in central Thailand and had been maintained for a period of slightly over fifteen months by serial passage in gibbons. All passages had been done by intravenous inoculation of heparinized blood. The animals in the present study received approximately one hundred million parasites each.

After inoculation, the animals were examined daily for evidence of overt disease. Thick and thin blood smears were obtained daily by

finger puncture and were stained by the Giemsa method. Parasite counts were made in terms of the number of asexual forms per 500 WBC.

Blood was drawn at semi-weekly or weekly intervals for determination of hematocrit, BUN, cholesterol, bilirubin, thymol turbidity, alkaline phosphatase, SGOT, SGPT, creatinine, total protein and serum electrophoresis, and lactic dehydrogenase determinations. The group of seven animals was studied for six months, the others for three months.

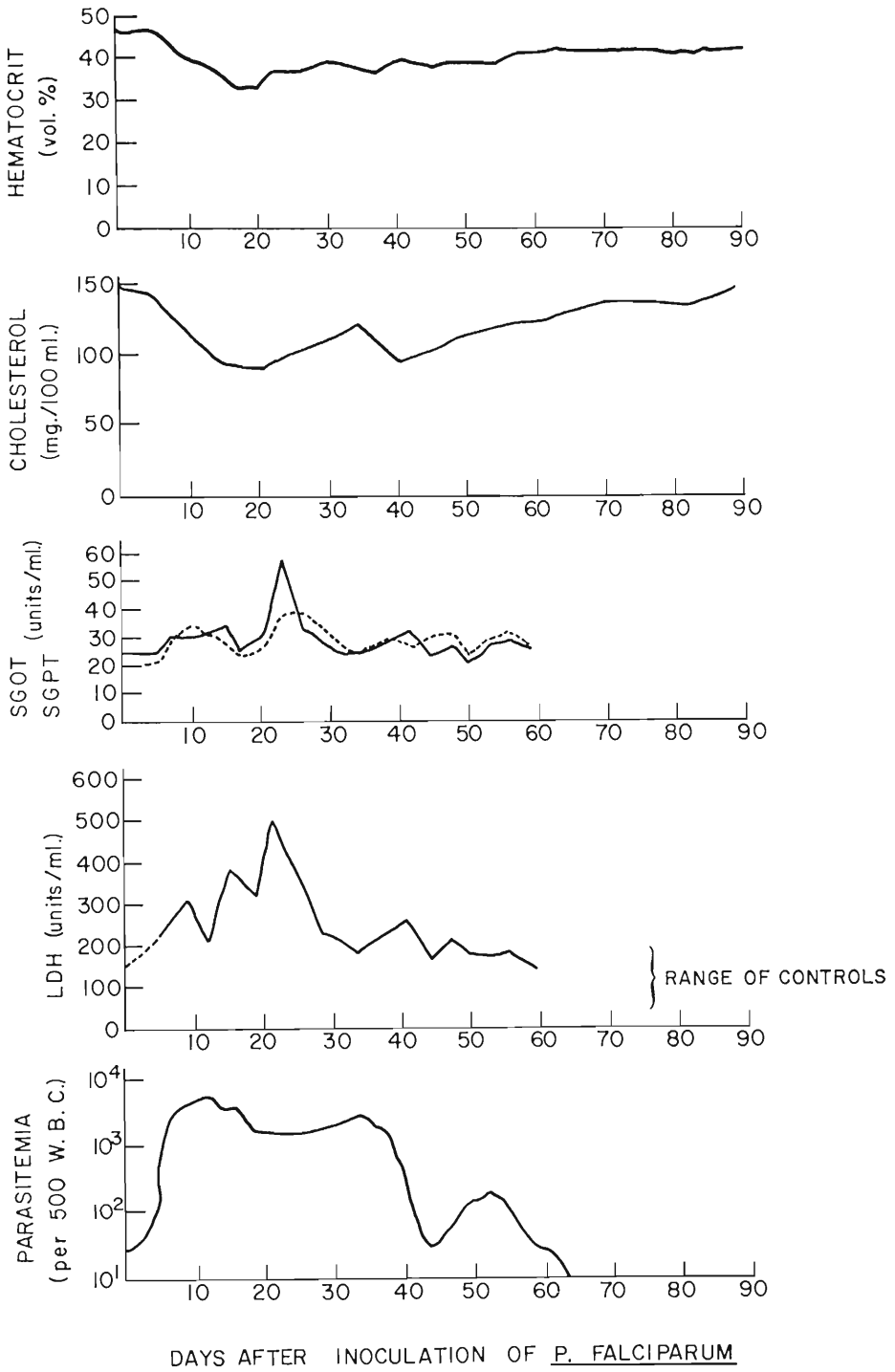
Results

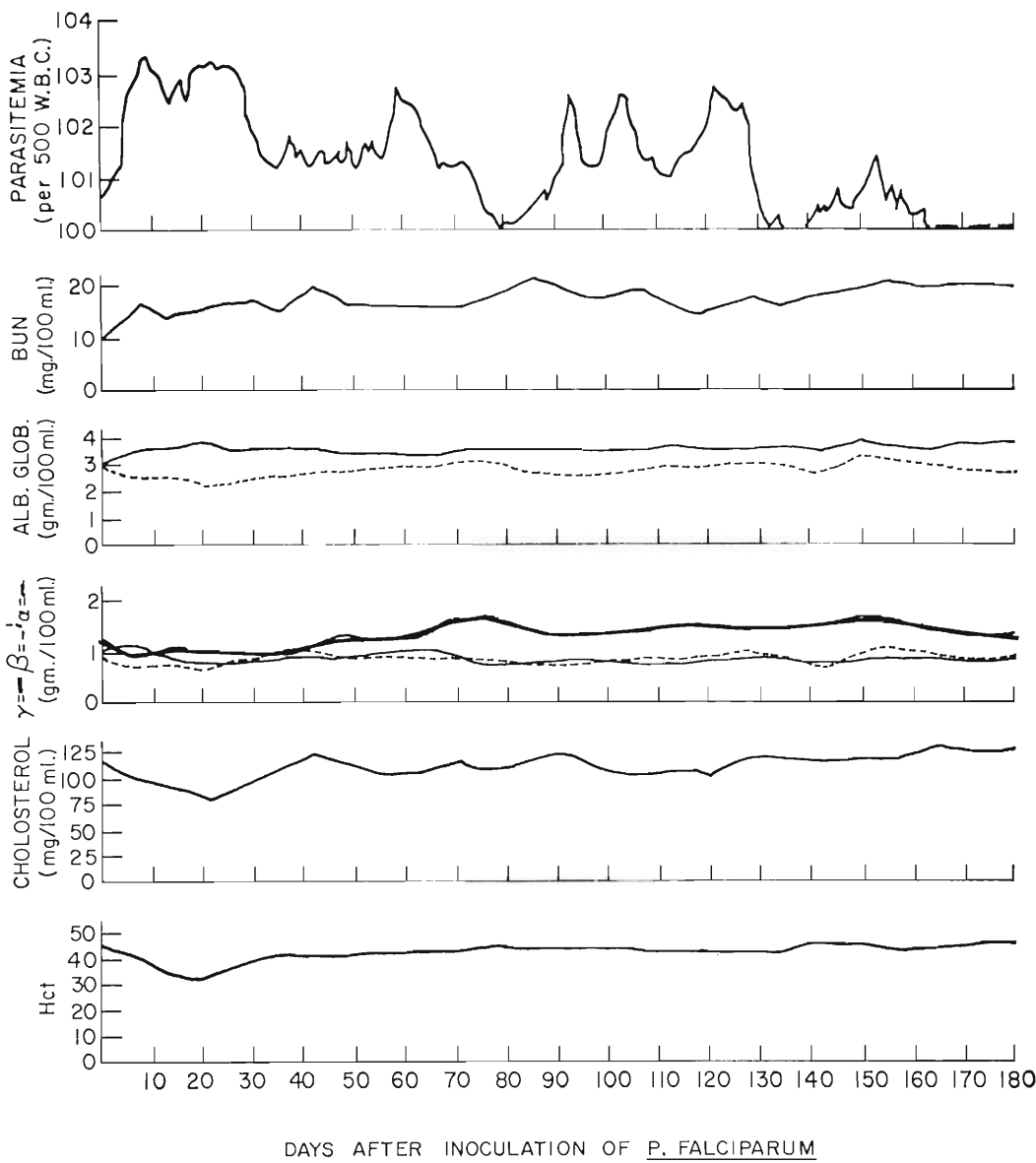
At no time did any gibbon show evidence of symptoms attributable to malarial infection. No change in behavior, appetite or attitude was noted. Minor fluctuations in body temperature occurred but no clinically significant correlation could be made either with the stage or magnitude of parasitemia.

In every instance, a drop in hematocrit followed the peaking of peripheral parasitemia. The lowest hematocrit noted was 28%. No evidence of clinical embarrassment could be noted. Detailed analysis of the hematologic response of gibbons to *P. falciparum* infection has been given elsewhere (Cadigan *et al.*, 1969). The direct and total bilirubin, thymol turbidity, and creatinine levels fluctuated slightly during the observation period but showed no correlation with parasitemic curves and no overall trend.

The blood urea nitrogen showed no dramatic changes except that it showed a tendency to rise throughout the observation period and to remain elevated. Figure 1 shows the mean values of weekly determinations of BUN of the seven gibbons inoculated simultaneously with the same dose of *P. falciparum* and which showed approximately the same course of parasitemia. The parasitemic curve is based on daily mean parasitemia. It can be seen that there is no apparent correlation between the BUN and the level of parasitemia. At the end of the observation period, the mean BUN was approximately double the pre-infection level.

Approximately three-quarters of the animals studied showed a slight but definite rise in total protein during the study period. An unexpected finding was that serum albumin levels showed a consistent rise during the first





←
Figure 1. Serum biochemical values from gibbons infected with *Plasmodium falciparum*.

few weeks of infection and then remained constant throughout the observation period at a level slightly higher than that prior to infection. On the other hand, globulin levels decreased slightly during the first three weeks

and then gradually rose. It can be seen from Fig. 1 that the mean did not return to the pre-infection level until day 60. With minor fluctuations it then stayed at approximately that level for the remainder of the six months. As

a result, the A/G ratio increased slightly during the first few weeks and then returned to near pre-infection levels.

In the globulin fraction, no major change or trend could be discerned in the alpha-2 globulin. Beta-globulin decreased slightly during the first two weeks in most animals and then, with minor fluctuations, remained at the same level. Gamma globulin showed a slight decline during the first ten days, then began to rise after forty days and stabilized at about 100 days. In most instances the gamma globulin increased 50–100% above pre-infection levels.

Alkaline phosphatase levels showed slight increases in some animals and slight decreases in others. In only two animals did a marked rise (to approximately double normal levels) occur between seven to twenty-one days after infection followed by a return to normal.

Transaminase levels did not show significant fluctuation during parasitemia in 10 animals which had been previously infected with either homologous or heterologous isolates. In 15 animals not previously infected with falciparum parasites, 13 showed an increase in SGOT/SGPT. Figures 2 and 3 show representative curves. Since determinations were not done on a daily basis, it is not possible to say what was the duration or maximum level of rise.

Cholesterol levels dropped consistently in all the study animals at approximately the same time that the hematocrit dropped. The bottom levels were as low as one-third of pre-infection levels. Although the levels dropped slightly with subsequent peaks of parasitemia, the fluctuation was minor compared to the original drop. The return to normal levels is slow.

In eight animals in which LDH levels were determined, there was a definite rise during the parasitemia. Since these determinations were done at 7 day intervals, it is not possible to make precise correlations, but it is obvious from the figure that the peak is after the peak of parasitemia by at least seven or eight days and roughly relates to the lowest hematocrit and cholesterol levels and the highest transaminase levels. LDH fractions 1, 2 and 5 remain fairly constant. Fractions 3 and 4 parallel each other closely and are about equally responsible for the increase of total LDH.

Discussion

It appears from these data that the liver of gibbons is affected by blood induced falciparum infection. The alteration of function is not great but is consistent. It is particularly interesting that the transaminase rises occurred only in animals not previously infected, even those which had been infected previously with heterologous isolates had parasitemias indistinguishable from "virgin" animals. The decrease in cholesterol is similar to that described by Desowitz (Desowitz *et al.*, 1967) in animals infected with a gibbon malaria, but the degree of anemia is much less. The lack of change in bilirubin in our animals is probably related to the lower level of hemolysis. The slow but persistent increase in BUN is interesting and suggests that further studies of kidney function should be done.

The rise in albumin was not expected and can not be explained at this time. This increase has been our experience consistently with gibbons infected with falciparum malaria. It would take a very marked level of hemoconcentration to achieve this increase in albumin and the other indices give no suggestion of marked hemoconcentration and certainly not for such a prolonged period. Unpublished data by Cadigan *et al.* show that liver explants *in vitro* have a marked increase in the production of albumin when cultured in the presence of serum from animals which have undergone subtotal hepatectomy. It is conceivable that the alteration of liver function by malaria is analogous to partial hepatectomy. Investigations of this matter are under way.

The gibbon is a good model for the study of the effects of falciparum malaria since the parasitemia is as prolonged as in untreated humans and does not have the very severe and short duration which it has in *Aotus* monkeys. Since the gibbons are not ill, there is no change in their food and water intake or activity. Also, there is no need to give anti-malarial treatment and thus the unaltered infection can be studied over a prolonged period.

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Infectivity of *Plasmodium falciparum* Gametocytes from *Aotus trivirgatus* to Anopheline Mosquitoes

R. A. WARD, D. E. HAYES, S. C. HEMBREE, L. C. RUTLEDGE, S. J. ANDERSON AND A. J. JOHNSON

Department of Entomology and Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D. C. 20012

ABSTRACT: Three new Oriental strains of falciparum malaria Vietnam (Brai.), Vietnam (Smith) and Philippines (Per.) were adapted to splenectomized *Aotus trivirgatus* monkeys. The course of infection of each of these strains through various blood passage levels is described with particular emphasis on gametocyte development. An immunosuppressant drug, Imuran®, was shown to influence gametocyte maturation and subsequent mosquito infectivity in the Per strain. Changes in mosquito infectivity as related to gametocyte developmental patterns are analyzed. Evidence is presented that mosquito susceptibility to *P. falciparum* in *Aotus* is related to early passage history of a particular strain rather than to any inherent qualities of the strain. With increased blood passage, *Aotus*-adapted strains become more virulent and the infection is fatal before gametocyte maturity is achieved.

In most respects, with the exception of immature forms in the peripheral circulation, the pattern of gametocyte maturation, infectivity to anophelines and duration of gametocyte infectiousness in *Aotus* parallels the situation in man. It is concluded that the *Aotus* system will provide the optimal model for studying human falciparum malaria upon the demonstration of a complete exoerythrocytic cycle in this host.

Since Geiman and Meagher (1967) reported that the night monkey *Aotus trivirgatus*, was susceptible to *Plasmodium falciparum* from man, numerous workers have infected this host by serial passage of parasites (Voller, et al. 1969; Hickman, 1969; Baerg and Young, 1969; Young, M.D., 1970). Attempts to establish the sporogonous cycle of falciparum malaria in this host have been largely unsuccessful. Collins and Contacos

(1968) recorded the infection of *Anopheles freeborni* mosquitoes from *Aotus* with the Malayan IV strain of *P. falciparum* and subsequent transmission to human volunteers by mosquito bites. Further details of this experiment were described by Collins et al. (1968). Jeffery (1969) relates that a Cambodian strain of *P. falciparum* produced rare infections in *A. balabacensis* and *A. freeborni*. The Chamblee group was unable to effect monkey to monkey transmission by sporozoite passage (Sodeman et al. 1969) and since that time has not published further information on mosquito infec-

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tions of *P. falciparum* from this host. Baerg and Young (1969) were unable to infect *Anopheles albimanus* in 46 feeding trials on Panamanian *Aotus* infected with Malayan Camp and Uganda Palo Alto falciparum malaras. This confirmed the earlier observations of Contacos and Collins (1968) who were also unable to infect anophelines with these parasite strains. Ward and his colleagues at WRAIR were unable to infect *Anopheles stephensi* and *A. balabacensis* with the Camp/W strain of falciparum malaria (Hickman, 1969).

A comparison of the single successful experiment of Contacos and Collins (1968) with those of other workers revealed that the former group of workers used a parasite strain in the first monkey passage from man while other investigators used parasites in 5th or higher passage levels from chimpanzees or night monkeys (Ward, 1969). At that time Ward stressed that it was too early to ascertain whether gametocyte infectivity is a characteristic of certain parasite strains in *Aotus* or an easily lost attribute following several blood transfers in heterologous host systems.

The present series of experiments were designed to test the hypothesis that there would be a stronger probability of establishing mosquito infections of falciparum malaria from *Aotus* with parasite isolates which had a recent history of mosquito transmission among human volunteers rather than with falciparum strains which had an extensive history of consecutive blood passage in subhuman primates. If this hypothesis were shown to be valid, then subsequent studies would be designed to study the pattern of mosquito infectivity, determine maximal gametocyte levels for donor monkeys and develop procedures for enhancing gametocyte production.

Materials and Methods

The *Aotus trivirgatus* monkeys* were procured primarily from a single supplier¹ who imported *Aotus* directly from Colombia. Virtually all animals for malaria studies at WRAIR

have been taken from the Magdalena River basin and appear to be *Aotus trivirgatus griseimembra* Elliot (Cadigan, F.C., personal communication). This is the same subspecies which occurs in Panama. Procedures for housing, feeding and handling *Aotus* are described by Hickman (1969). Most animals were splenectomized 4–7 days prior to exposure to malarial parasites. Prior to mosquito feeding, animals were anesthetized with 0.2 ml Sernylan® (Phencyclidine hydrochloride, 20 mg/cc) administered intramuscularly. At appropriate dosage levels Sernylan produces the desired effect without affecting blood pressure nor reducing body temperature, both of which appear to affect mosquito feeding response. In many of the experiments, an immunosuppressant Imuran (Azathioprine) was administered by intraperitoneal inoculation at a daily dose rate not in excess of 5 mg/kg body weight.

Three species of anopheline mosquitoes have been utilized in vector studies: *Anopheles stephensi* (India strain), *A. quadrimaculatus* and *A. balabacensis*. The rearing procedures are described by Esah and Scanlon (1965) and Gerberg (1970). Infected mosquitoes are maintained within a temperature range of 24–27° C. Oocyst counts were made on a sample of 12 mosquitoes 7 days subsequent to gametocyte exposure and sporozoite levels were assessed 7 days later. Rutledge et al. (1970) described procedures for handling malaria infected mosquitoes in detail.

D. F. Clyde and V. C. McCarthy, University of Maryland School of Medicine, Baltimore, provided fresh isolates of *Plasmodium falciparum* infected blood of the Vietnam (Brai.), Philippines (Per.) and Vietnam (Smith) strains from male volunteers, inmates of the Maryland House of Correction, Jessup. On days when the appropriate parasitemia levels were observed, 10cc of heparinized blood were collected under sterile precautions and carried on wet ice to WRAIR for inoculation into *Aotus*. In most instances, less than 2 hours lapsed between the transfer of infected blood from man to monkey. Usually, $4-9 \times 10^7$ parasites were inoculated intraperitoneally into splenectomized monkeys. For transfer between monkeys to maintain the parasite strain or provide donors for mosquito infectivity studies, the inoculum contained 2.4×10^5 –

* In conducting the research described in this report, the investigators adhered to the 'Guide for Laboratory Animal Facilities and Care,' as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

¹ Tarpon Zoo, Tarpon Springs, Florida.

Table 1. Infection of splenectomized *Aotus trivirgatus* with *Plasmodium falciparum* (Vietnam, Brai., strain).

Passage level	<i>Aotus</i> no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm ³	Day of first gametocytes	No. of mosquito pools infected	Day of death
						No. of pools fed	
1 ¹	G-369	10	24	1,662,000	17	0/8	24
2	G-396 ²	11	23	212,000	11	0/11	—
2	G-985	13	23	1,673,000	15	0/3	24
3	G-984 ³	2	7	100,170	3	2/11	—
3	G-992 ⁴	2	13	1,320,000	3	—	14
4	228 ⁵	5	14	1,140,000	0 ⁶	—	14

¹ One other *Aotus* inoculated did not exhibit patent parasitemia.
² Treated with quinine on day 23.
³ Treated with Imuran, 2 mg/kg × 3 days.
⁴ Treated with Imuran, 3.3 mg/kg × 3 days.
⁵ Treated with Imuran, 1 day at 5 mg/kg and 1 day at 2.5 mg/kg.
⁶ Gametocytes not observed.

8.3 × 10⁵ parasites. Standard malarial procedures were followed in enumerating parasites.

Results

During April 1970, isolates of the Vietnam (Brai.) and Philippines (Per.) falciparum strains were inoculated into splenectomized *Aotus* monkeys. Both these isolates were from sporozoite-induced infections from volunteers at the Maryland House of Correction.

The details of the 4 blood transfers of the Brai. strain are summarized in Table 1. This strain appeared to be extremely virulent from the first passage level; 4/6 *Aotus* exposed developed parasitemia in excess of 10⁶ parasites/mm³ and died of malarial infections. The gametocytes appeared relatively immature during the first 2 passage levels and dissection of 22 lots of mosquito pools of *Anopheles stephensi* and *A. quadrimaculatus* disclosed no oocyst development.

Monkeys of passage levels 3 and 4 were given small doses of an immunosuppressant drug, Imuran, on the assumption that such treatment might prolong gametocyte survival until a degree of maturity was achieved that would be suitable to infect anophelines. Small doses were administered 1 day prior to infection and 1–3 days subsequent to infection. It was observed that *Aotus* G-984, which received the above treatment, infected small numbers of *Anopheles stephensi* 1216 days after the first appearance of gametocytes. The last 2 *Aotus*, G-992 and 228 died before mosquito feeds could be achieved.

The behavior of the first 3 passages of the

Philippine (Per.) strain are reviewed in Table 2. Three *Aotus*, 2 of which were splenectomized were inoculated with infected human blood on 3 April 1970. Although all monkeys became infected, the non-splenectomized G-967 only exhibited a transitory parasitemia for a single day. This strain was not as virulent as the Brai. strain and peak parasitemias (6.7–7.5 × 10⁵ parasites/mm³) only reached a level half that of the former strain. Although most gametocytes were immature during the first passage level, 1/32 pools fed contained infected anophelines. *Aotus*, H-233, infected with 3rd passage parasites was treated with Imuran to determine whether the observation with the Brai. strain could be repeated. Mosquito infections were found on 3/9 days that *Anopheles stephensi* cages were applied to *Aotus* H-233. The first mosquito infection occurred on day 31 of the infection or 17 days after gametocytes first appeared in the peripheral circulation.

Studies on the Brai. and Per. isolates indicated that treatment of monkeys with an immunosuppressant rendered them more suitable as gametocyte carriers. One alternate possibility would be that as a parasite strain became better adapted to a host following consecutive transfer, gametocytes would persist for a longer period of time, and thus attain sufficient maturity to be infectious to mosquitoes. Another possibility might be that inherent differences in *Aotus* monkeys, such as prior malarial exposure or length of time in a laboratory colony might alter the course of a subsequent malarial infection and the resultant production of gametocytes.

Table 2. Infection of splenectomized *Aotus trivirgatus* with *Plasmodium falciparum* (Philippine, Per. strain).

Passage level	<i>Aotus</i> no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm ³	Day of first gametocytes	No. of mosquito pools infected	Day of death
						No. of pools fed	
1	G-388	14	26	752,400	21	0/8	27
1	G-067	17	27	166,650	24	1/24	—
1	G-967 ¹	12	13	25	—	—	—
2	G-983	13	40	667,500	14	0/6	41
3	H-233 ²	5	22	490,140	16	3/9	—

¹ Non-splenectomized.² Treated with Imuran, 5 mg/kg × 6 days and 2.5 mg/kg × 5 days.

To test these hypotheses, an experiment was conducted with the Per. strain at the 4th passage in 6 splenectomized *Aotus* which had been received in a single shipment and maintained at WRAIR for 30 days prior to infection. The monkeys were divided into 3 treatment groups: the 1st, a control, received a 1 ml saline injection for 17 days; the 2nd was injected with Imuran at a dosage of 5 mg/kg for 17 days and the 3rd received Imuran 5 mg/kg for 8 days and then 2 mg/kg for 9 days. In all instances, the inoculations started 2 days prior to infection. All drugs were administered in saline solution by the intraperitoneal route. The dosage range selected was one that did not exceed the maximum used for immunosuppressant purposes in humans.

All animals were examined for a period of 35 days following intraperitoneal inoculation 4×10^6 parasites from *Aotus* H-233. The infections became patent within a period of 9–14 days irrespective of treatment (Table 3). There was a significant difference in the level

of maximum parasitemia achieved, with untreated controls markedly lower than treated *Aotus*. Gametocytes were far more abundant in animals receiving immunosuppressants and only in these individuals were mature gametocytes observed. Similarly, only in treated monkeys was it possible to infect *Anopheles stephensi* and *A. quadrimaculatus* mosquitoes with this parasite. *Aotus* H-254 was a particularly good gametocyte donor. This monkey 1st infected anophelines on day 33 of the infection (14 days after the 1st appearance of gametocytes). The highest oocyst counts (20.7/midgut) were observed on day 40 and the gametocytes remained infective to mosquitoes through the 61st day of the infection. Examination of the oocysts revealed normal morphology and subsequent dissection of salivary glands disclosed motile sporozoites.

In order to support studies on the chemotherapy of malaria, three isolates of the Vietnam (Smith) strain were obtained from the University of Maryland School of Medicine between December 1970 and April 1971. The

Table 3. Effect of an immunosuppressant upon *Plasmodium falciparum* (Philippine, Per. strain) infection in splenectomized *Aotus trivirgatus*.

Treatment	Animal no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm ³	Day of first gametocytes	No. days gametocytes observed	No. mosquito pools infected
						No. days examined	No. pools fed
Control	H-234	13	13	120	— ¹	0/13	— ¹
	H-250	14	26	33,960	26	3/17	0/3
Imuran (5 mg/kg × 17 days)	H-251	12	16	340,800	22	7/18	0/7
	H-252	11	19	187,680	19	11/22	2/17
Imuran (5 mg/kg × 8 days, 2 mg/kg × 9 days)	H-253	12	26	95,760	19	5/16	0/4
	H-254	9	19	457,560	19	16/24	14/23

¹ Gametocytes not observed; no mosquito pools fed.

Table 4. The course of infection of *Plasmodium falciparum* (Smith strain) in splenectomized *Aotus trivirgatus* and subsequent susceptibility of anopheline mosquitoes to falciparum gametocytes in this host.

Passage	<i>Aotus</i> no.	Day patent	Day highest parasitemia	Maximum no. parasites/mm ³	Day of first gametocytes	Day of first mature gametocytes	No. days mature gametocytes/mm ³	Day of first mosquito infection	Day of last mosquito infection	No. of mosquito pools infected	
										No. of pools fed	No. of pools infected
1	546 ¹	12	14	36,570	14	22	0	—	—	—	0/20
2	383	3	20	22,730	14	19	5	20	39	—	12/18
3	700 ²	7	20	17,650	20	28	0	—	—	—	0/2
4	387 ²	4	14	203,070	4	13	17	18	39	—	9/29
5	388 ²	1	11	177,800	2	8	12	21	38	—	9/21
6	593	3	61	67,080	3	Not determined	Not determined	53	73	—	5/31
6	927 ²	7	20	299,720	7	16	Not determined	—	—	—	0/6
7	922 ²	2	19	102,110	15	17	20	28	51	—	15/25
7	876 ^{2,3}	9	17	355,600	14	23	0	—	—	—	0/1
8	923 ²	18	29	122,430	23	31	1	—	—	—	0/5
9	983 ⁴	2	15	882,650	4	—	0	—	—	—	—
10	971	7	17	171,700	12	18	21	21	34	—	13/31
11	S-2 ⁵	4	10	377,190	8	—	0	—	—	—	—

¹ *Aotus* 545, which was infected at the same time did not develop a patent parasitemia.

² Imuran administered 5 mg/kg daily for 10–40 days.

³ Died day 24.

⁴ Died day 18; mature gametocytes not observed.

⁵ Died day 12; mature gametocytes not observed.

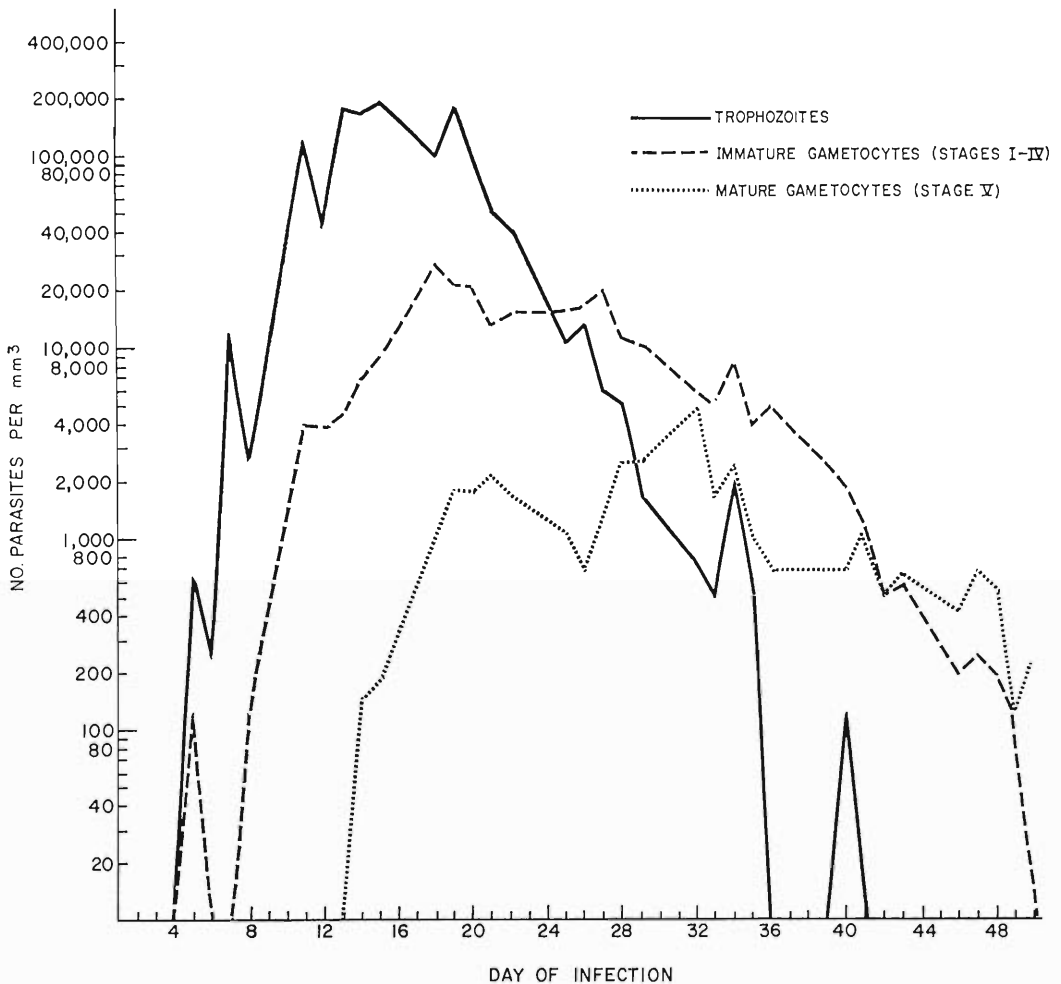


Figure 1. Course of parasitemia of *P. falciparum* (Smith strain) in splenectomized *Aotus trivirgatus* 387 during the fourth passage.

Smith strain, which was isolated at the end of 1968 from a patient who acquired the infection near the Cambodian border, was of special interest due to the resistance to chloroquine, pyrimethamine and chlorguanide (Clyde et al., 1971). Successful adaptation of this strain to *Aotus* would provide material for antimalarial drug evaluation that could be compared with studies in human volunteers.

The first 2 inoculations of Smith strain *falciparum* isolates did not establish patent parasitemias. On the 3rd attempt, 1 of the 2 *Aotus*

(546) became patent on day 12. Since then, 9 blood transfers of the isolate have been made in splenectomized *Aotus* monkeys as indicated in Table 4. After the 1st passage, all animals developed patent infections. 7/12 *Aotus* were treated with the immunosuppressant, Imuran, for varying periods of time (10–40 days) at a dose of 5 mg/kg daily. Unlike the previously described experiment with the Per. strain, this treatment did not significantly change the course of parasitemia nor affect gametocyte development and maturation.

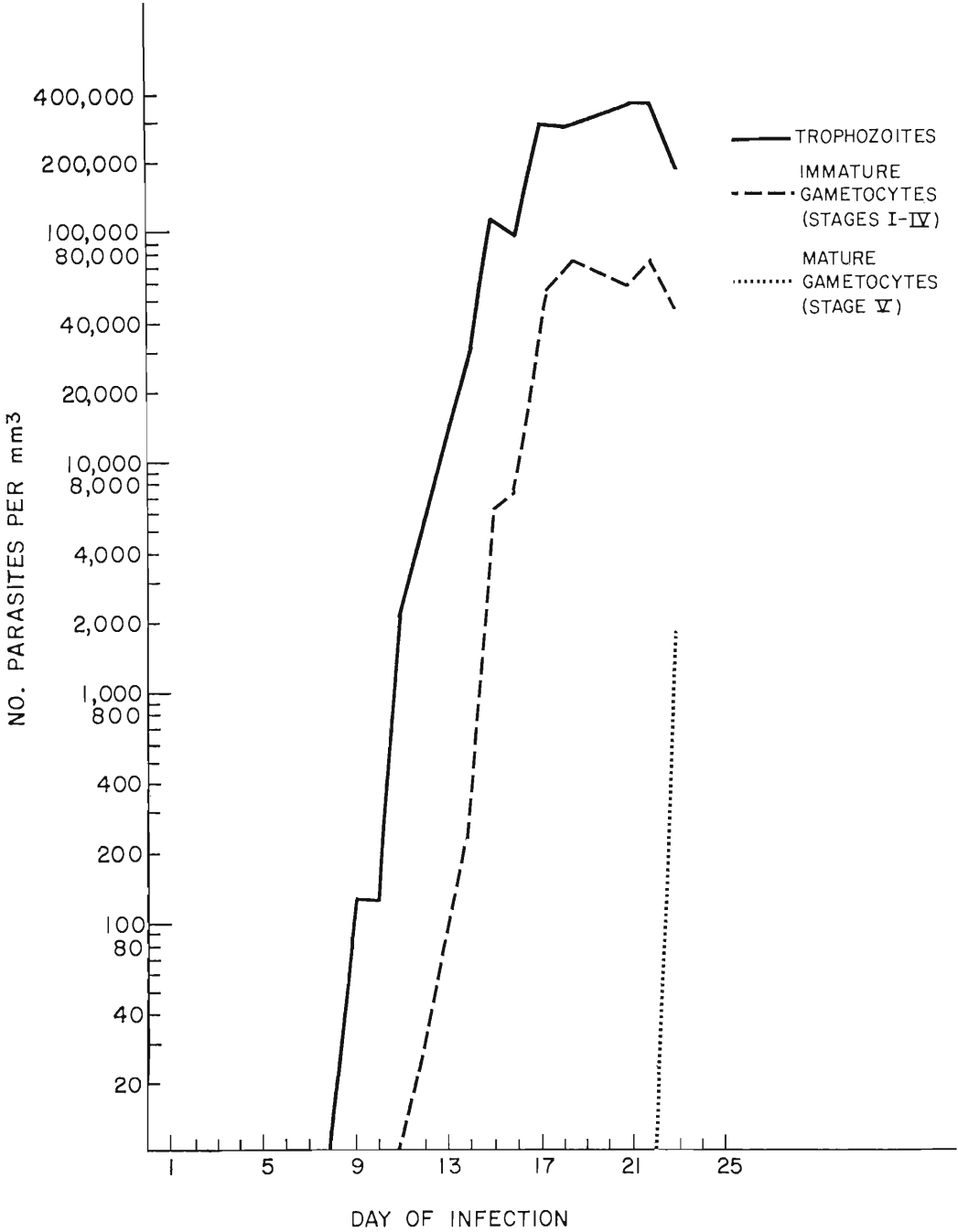


Figure 2. Course of parasitemia of *P. falciparum* (Smith strain) in splenectomized *Aotus trivirgatus* 976 during the seventh passage.

Maximal levels of parasitemia in the Smith strain were similar to the Per. strain and only half those observed in the Brai. strain.

Two general patterns of parasitemia were observed in the Smith strain. The first was exemplified by *Aotus* 387 (Figure 1). This animal attained a peak parasitemia of 200,000 parasites/mm³, then gradually decreased to extremely low levels over the next 3 weeks. The immature gametocytes (stages I–IV) gradually increased and remained at levels between 1,000–10,000/mm³ for a month. Mature gametocytes (stage V) were visible for a similar period. *Aotus* 976 was typical of the 2nd pattern in which the parasitemia attained an extremely high peak, over 350,000/mm³ (Figure 2). Abundant immature gametocytes were present but mature gametocytes only appeared in low numbers prior to the death of the monkey from the malarial infection. This second pattern occurred with greater frequency at higher passage levels.

Gametocyte development was examined in detail in the studies of the Smith strain to determine whether there were any relationships between gametocyte production, maturation of gametocytes and levels of mosquito susceptibility to infection. In this study, gametocytes were examined daily on thin smears prepared at 0730 hrs. The gametocytes were classified into stages of development as outlined by Field and Shute (1956) and emended by Hawking et al. (1971). Five developmental stages were recognized, i.e.

- I Rounded forms, do not fill erythrocyte
- II Irregular shape (sometimes triangular), slightly elongated.
- III Elongated, pointed or slightly rounded ends; erythrocyte slightly distorted.
- IV Elongated, rounded ends, erythrocyte very distorted.
- V Mature, sausage-shaped; rounded ends.

Unlike human falciparum infections, all developmental stages of the gametocyte of this species occur in the peripheral circulation of *Aotus*. Gametocytes appeared as early as the

first day of a patent infection or as late as 13 days after the first appearance of ring-stage trophozoites. 75% of the animals had circulating gametocytes within the first 5 days of a patent infection. The general pattern of gametocyte development is summarized in Figures 3–5 which show this pattern in respect to time and level of mosquito infection in *Aotus* 388, 922 and 971. An analysis of these distributions and those observed in other *Aotus* listed in Table 4 shows that the very immature stages (I and II) peak earlier than stages III–V and disappear from circulation sooner. Sometimes stages III and IV reach peak numbers the same day as in *Aotus* 922 while in other animals the peak frequencies were separated by 2–4 days (*Aotus* 388 and 971).

The ability of gametocytes to infect anopheline mosquitoes was related both to the stage of the gametocyte, its maturity and frequency. Mosquito infections occurred 1–13 days after mature gametocytes first were detected. Peak mosquito infections appeared 3–9 days later. With the exception of *Aotus* 593, all peak oocyst counts in anophelines occurred in feeds made 27–31 days after the animal was infected. In most instances, mosquitoes could be infected daily for a 13–23 day period after the first day gametocytes were infective in a monkey.

On several occasions, 2 or 3 species of *Anopheles* were simultaneously fed on the same monkey to compare the relative susceptibility of different species to infection. *Anopheles stephensi* and *A. balabacensis* showed similar levels of infection while *A. quadrimaculatus* was refractory to infection (Table 5).

Four attempts were made to transmit *P. falciparum* back to splenectomized monkeys through the bites of *Aotus*-infected mosquitoes or the inoculation of infected salivary glands. Each *Aotus* received a subcutaneous or intravenous inoculation of 20 heavily infected salivary glands or a feed of 50–200 infected anophelines. The first 2 experiments were conducted with anophelines infected with the Per. strain while the latter trials were conducted with the Smith strain. During a 50 day pe-

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Figure 3. Development of *P. falciparum* (Smith strain) gametocytes in splenectomized *Aotus trivirgatus* 388 in relation to anopheline susceptibility.

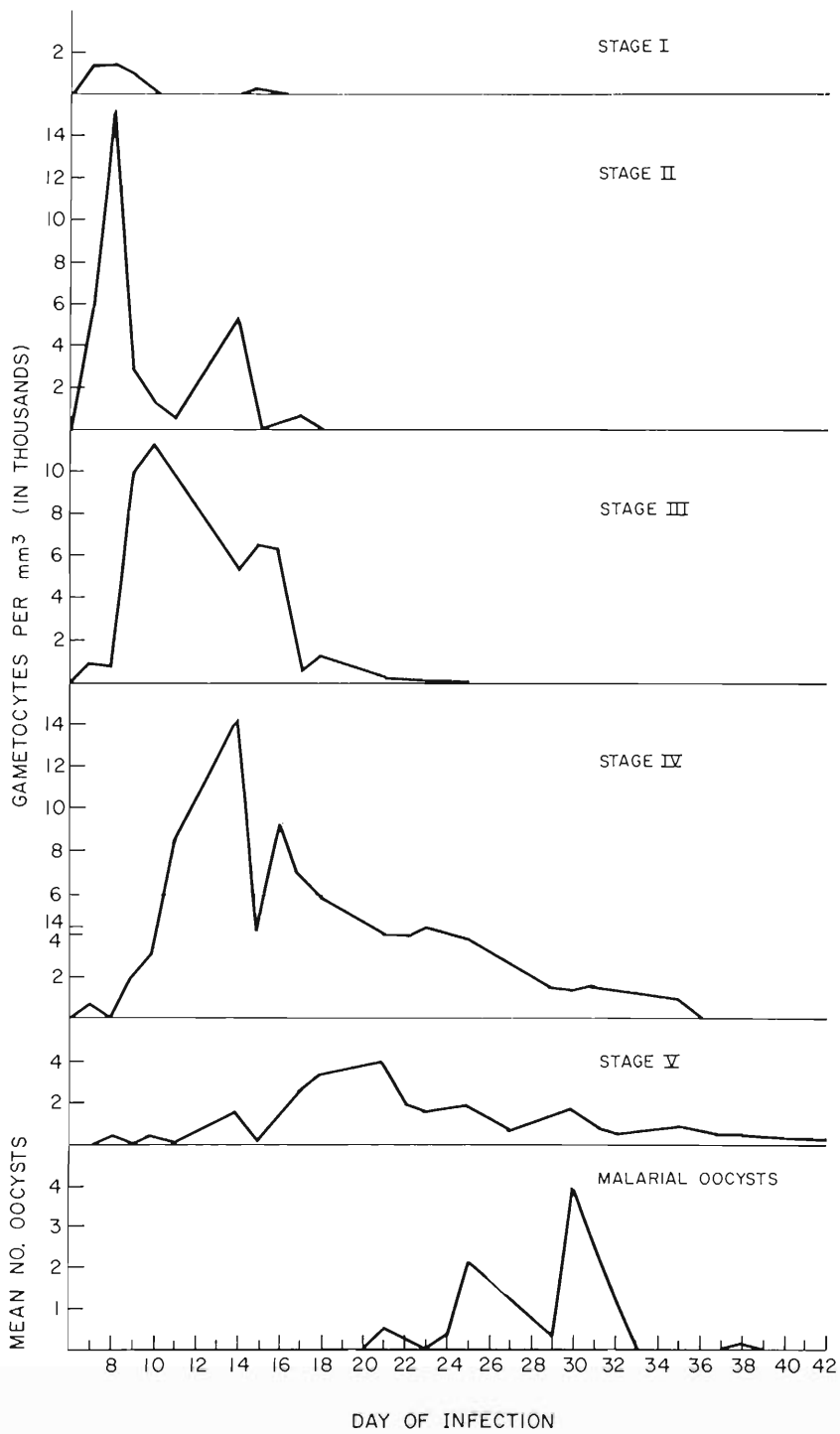


Table 5. Comparative susceptibility of *Anopheles* species to Smith and Per. strains of *falciparum* malaria in *Aotus trivirgatus*.

Aotus no. & strain	Day of feed	Mean no. malarial oocysts/midgut		
		A. <i>stephensi</i>	A. <i>balabacensis</i>	A. <i>quadrinaculatus</i>
971 (Smith)	21	0.7	0.8	0
	24	1.6	10.8	0
	25	1.4	1.5	0
	26	6.3	1.9	0
254 (Per.)	40	20.7	—	0
	41	0.8	0.2	0
	55	1.8	—	0
	56	3.7	—	0

riod, the *Aotus* were examined twice weekly to detect patent infection. In addition, a splenectomized chimpanzee, 578, served as a control to verify the infectivity of sporozoites of the Per. *falciparum* strain in the 2nd experiment. None of the exposed *Aotus* developed a patent infection. However, chimpanzee 578 became positive 13 days after exposure to infected *Anopheles stephensi* and attained a peak parasitemia of 320,000/mm³ on day 21. Immature *falciparum* gametocytes were observed 2 days later, thus confirming the identity of the transmission.

Discussion

The facility with which splenectomized *Aotus* monkeys can be infected with Asian and African isolates of *falciparum* malaria presents a contrast to the inability of this host to sustain high level infections with New World isolates of this parasite. The ready adaption of 3 new Asian isolates to *Aotus* affirms this premise. A possible explanation may be that an insufficient number of isolates from different geographical areas in the New World have been inoculated into *Aotus*. Young (1970) states that in the many attempts to establish *P. falciparum* of Panamanian origin in the local *Aotus*, none have become well established. The McClendon strain (isolated from a South Carolina patient in 1940) was not able to infect *Aotus* (Jeffery, 1969). However, the Central America Malaria Research Station

(1970) reported infection of *Aotus* with *falciparum* strains from Honduras and El Salvador. These are apparently the only geographical areas from which *falciparum* isolates have been collected for inoculation into *Aotus*.

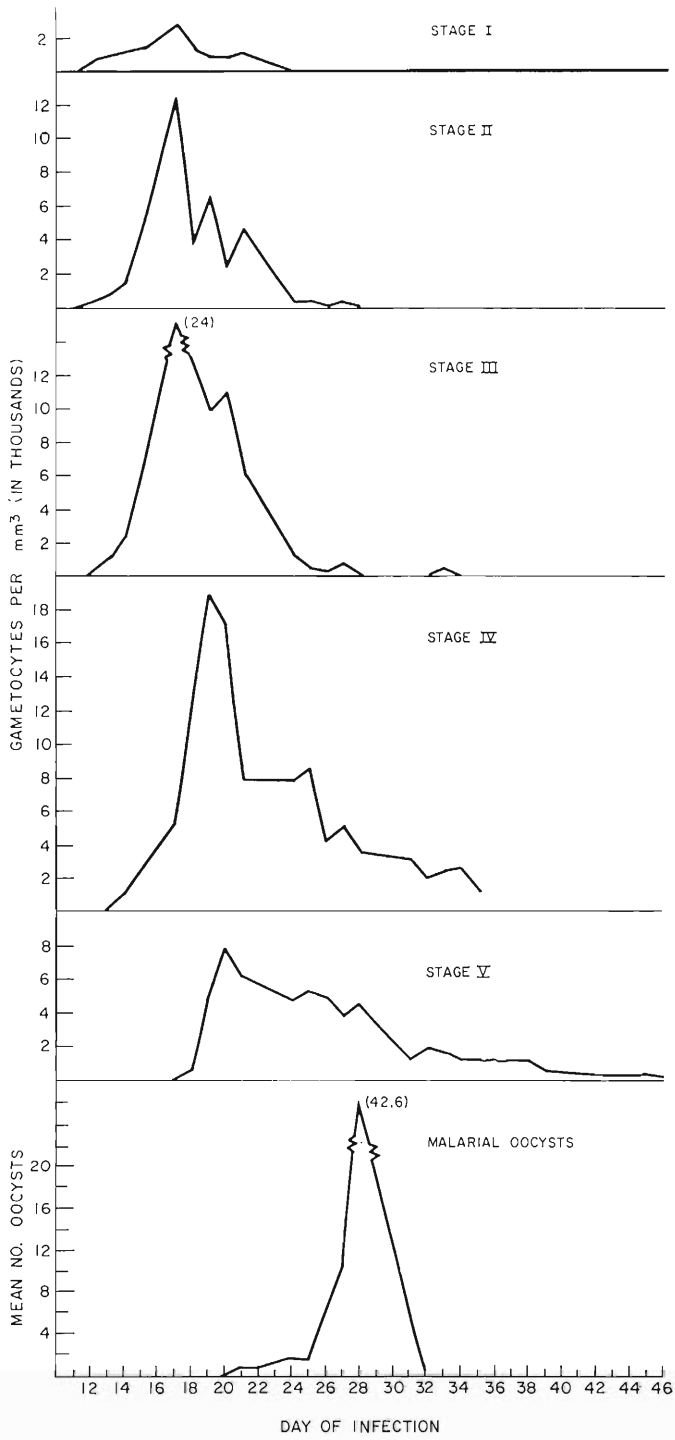
An alternate hypothesis is that *falciparum* malaria strains from the Western Hemisphere differ at an antigenic or enzymatic level from Old World parasite strains. The 400 or so years which have lapsed since the probable introduction of *falciparum* strains from Africa via the Spaniards and their slaves (Bruce-Chwatt, 1965), the geographic isolations of clones of malarial parasites in a new environment and the selection within a new set of anopheline vector species are all very favorable attributes for rapid selection. Hence it would not be unexpected to find such a difference in susceptibility of *Aotus* to these isolates.

At the Teheran International Congresses on Tropical Medicine and Malaria the question of gametocyte infectivity was raised (Ward, 1969). Two alternate hypotheses were proposed. The first was that gametocyte infectivity to anophelines is a characteristic of certain parasite strains of *P. falciparum* in *Aotus* and could probably be observed more often if different parasite isolates were studied. A 2nd hypothesis was that gametocyte infectivity was an easily lost attribute following several consecutive blood transfers in heterologous host systems.

The recorded observations on 2 Vietnam isolates (Brai. and Smith) and 1 from the Philippines (Per.) indicated the gametocytes of an Oriental region *falciparum* strain can probably produce infective gametocytes in *Aotus* during the early passage history from man. In the above strains it occurred during the first 3 passage levels. The point of loss of gametocyte infectivity is substantiated by the history of the Smith strain (Table IV). In the latter passages, as more virulent clones of the parasite were selected, insufficient time was usually available for full maturation of gametocytes to stage V levels. Consequently, parasitemia attained such high levels (over 200,000/

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Figure 4. Development of *P. falciparum* (Smith strain) gametocytes in splenectomized *Aotus trivirgatus* 971 in relation to anopheline susceptibility.



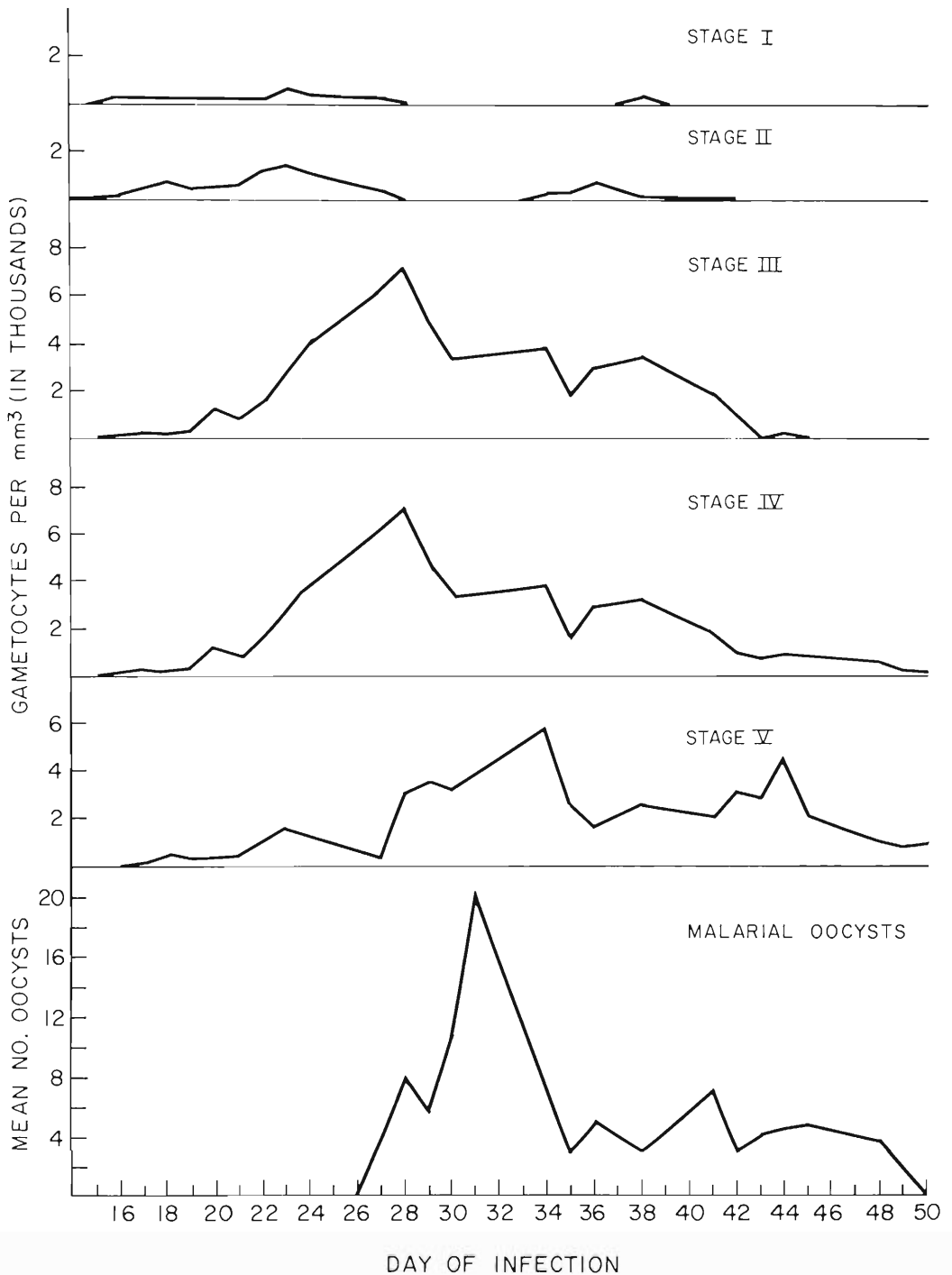


Figure 5. Development of *P. falciparum* (Smith strain) gametocytes in splenectomized *Aotus trivirgatus* 922 in relation to anopheline susceptibility.

mm³) that the *Aotus* died of acute malarial infection before mosquito exposure could be effected. With further blood passage, the Smith strain may lose the ability to develop even early stage gametocytes as has occurred in certain isolates of the Malayan Camp Strain.

The application of the stabilate principle as proposed by Cunningham and Lumsden (1965) to the low temperature preservation of malarial parasite isolates at passage levels which favor the production of moderate parasitemias at a non-lethal level and maturation of infective gametocytes should prevent the above cited problems. It is quite obvious that a stabilate of 5th passage Smith material will differ from material preserved at the 11th passage.

Immunosuppressant drugs were shown to exert an influence on gametocyte infectivity in the experiment with the Per. strain when parasite dosage and other factors were well controlled. In routine passages, such an effect was difficult to detect due to possible interaction between level of passage and drug dosage. As an example, *Aotus* 971 (Smith strain) exhibited the best gametocyte donor pattern observed to date despite the lack of Imuran treatment.

The pattern of mature gametocyte production in *Aotus* as related to mosquito infectivity showed a close parallel to the situation in non-immune humans. Pampana (1963) points out that during the primary attack, gametocytes do not appear before the 7th day of the parasitemia, and usually only are visible 10–12 days after the asexual parasites become patent. Similarly, falciparum gametocytes are not infective to mosquitoes until 2 days after they are observed in the peripheral circulation of man (Jeffery and Eyles, 1955). Gametocytes remain infective for a period of several weeks in man. Boyd (1949) discusses a patient who produced infectious gametocytes 4 days after the first appearance of this stage, and served as an infectious donor to anophelines for a 19 day period. He points out that mosquitoes were only infected when gametocyte levels were between 40–400/mm³. These observations closely parallel our results in *Aotus*. In the Smith strain, where gametocyte maturity was assayed daily, the first mosquito infections occurred at an average of 12.6 days after the infection became patent (range = 7–21 days).

The duration of mosquito infectivity averaged 19 days (range = 13–23 days). Similar mature gametocyte levels are observed in *Aotus*.

The failure of Philippines (Per.) and Vietnam (Smith) strains to undergo sporogonous development in *Anopheles quadrimaculatus* parallels the experience of Clyde, et al. (1971). As *A. quadrimaculatus* is a North American vector, and *A. stephensi* and *A. balabacensis* Asian vectors, this is not unexpected. This observation has been noted numerous times during the study of falciparum malaria from different geographic areas and has epidemiological significance.

The fact that Contacos et al. (1968) and ourselves were able to cyclically transmit falciparum infections from *Aotus* to humans and a chimpanzee respectively indicate that the sporogonous cycle of the parasite is compatible with that in anophelines infected on man. Since Sodeman, et al. (1969) have observed partial development of the exoerythrocytic schizont of *P. falciparum* in *Aotus* it is predicted that complete development of this parasite in the hepatic tissues will be demonstrated within the very near future. When this has occurred, it may be stated unequivocally that *Aotus trivirgatus* affords the closest known simulation for the mosquito transmission of falciparum malaria.

Acknowledgments

We wish to thank David F. Clyde and Vincent C. McCarthy, University of Maryland, School of Medicine for providing fresh isolates of human infections. The technical assistance of Leroy H. Bell and Henry J. West is gratefully acknowledged.

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Comments on Primate Models

W. D. TIGERTT

Commanding General, Madigan General Hospital, Tacoma, Washington 98431

We have been considering human malaria plasmodia infections in three sub-human primates. During the course of blood passaged *Plasmodium falciparum* infection in the splenectomized gibbon Cadigan noted various changes in serum biochemical values. Many of these seem comparable to those reported in other hosts with malaria, but in the gibbon these alterations are not associated with any

evident illness. His observation that animals on re-infection did not show a change in transaminase values deserves additional study as does his report that blood urea nitrogen values increased during the course of the infection.

Rossan and co-workers have expanded the earlier observations and describe the serial transfer by blood inoculation of *Plasmodium vivax* and of two strains of *falciparum* in the

unaltered *Saimiri sciureus* (squirrel monkey). The initial inoculum in each instance was obtained from *Aotus* at a fairly high passage level. Vivax infections showed relapses, while the response to falciparum was variable ranging from self limiting disease to death with fulminating parasitemia. They also describe infection of this animal with sporozoites of *P. vivax* derived from *Aotus*. They were successful in infecting certain anophelines with *P. vivax* by feeding on animals infected by blood passage.

Ward and associates report considerable success in infecting certain anophelines by feeding on the modified *Aotus* with a blood induced *P. falciparum* infection. It is difficult for me to offer an opinion as to the identity of the critical factor or factors in their success. All of their animals were splenectomized and many received Imuran (R) during the period just before and for some days after blood inoculation. Possibly the drug acted as an immunosuppressant and delayed the initial curbing of the rate of increase of the asexual forms or perhaps it suppressed Garnham's hypothetical crescent antibody. Certainly in some of the treated animals there was a rapid development of asexual forms and a very early appearance of gametocytes.

However, it must be remembered that in some species, including man, Imuran (R) fails

to suppress antibody response to some antigens and has numerous actions including a modification of the inflammatory response and of cell-mediated immunity, as well as having hepatic and marrow toxicity. Upon termination of the drug, a paradoxical antibody response may occur. The final complexity concerning the role of Imuran (R) lies in Ward's observation that the "best" gametocyte production, as measured by mosquito infectivity, was in a non-drug treated animal.

I find it equally difficult to clearly relate gametocyte production to passage history. Certainly, repeated blood passage of the drug-resistant Smith strain of *P. falciparum* in splenectomized *Aotus* was not associated with a loss of infectivity for mosquitoes.

The only conclusion that I can draw at this time is that the production of infectious gametocyte, or to use the terminology of earlier workers—gametocytes of "good quality," is associated with an initial fairly high level of asexual parasitemia without a fatal outcome. Perhaps any method of insuring such an event will increase the likelihood of "good quality" gametocytes.

Finally, Ward and associates are to be congratulated on their demonstration that *P. falciparum* sporozoites derived from *Aotus* are infectious for the methionine treated *Aotus*.

II

HOST-PARASITE RELATIONSHIPS

B. Clinical Aspects

Clinical Aspects of Acquisition of Immunity to Falciparum Malaria¹

R. D. POWELL,² J. V. MCNAMARA,³ AND K. H. RIECKMANN⁴

The purpose of this report is to present information about certain clinical aspects of the acquisition of immunity to falciparum malaria. The data presented pertain to initial infections and to reinfections in persons who were initially nonimmune and who received intermittent, subcurative doses of antimalarial medication. Instances in which such persons acquired immunity sufficient to result in asymptomatic persisting asexual parasitemia afforded an opportunity to gain insight into the circumstances relating to parasitemia and fever preceding the onset of such a state during initial infections and during reinfections.

Materials and Methods

Studies were conducted at the University of Chicago-Army Medical Research Project at the Illinois State Penitentiary, Stateville Branch, Joliet, Illinois. Conditions and general methods of study have been described previously (1, 2). Male inmates, 21 to 45 years old, volunteered to participate in investigations pertinent to chloroquine-resistant *P. falciparum* and to chloroquine-sensitive *P. falciparum*.

Volunteers: Volunteers were accepted for study only when careful medical evaluation indicated that they were in good health, after the nature of the planned studies had been explained and discussed at length, and after the volunteers then indicated that they wished

to participate in the investigations. Two volunteers were Negro men who had both sickle cell trait and glucose-6-phosphate dehydrogenase (G6PD)-deficiency; standard techniques, involving assays of erythrocytic G6PD and the 2% sodium metabisulfite method and paper electrophoresis, were used to document the presence of G6PD deficiency and of hemoglobin S (hemoglobin S and hemoglobin A were detected in both men; activity of G6PD in hemolysates was about 10% of normal in both men). All other volunteers had no evident abnormalities involving red cells or hemoglobin. All volunteers were considered non-immune at the outset of the studies. Prospective volunteers were excluded from study if they had previously resided in areas where falciparum malaria has been or is endemic or if they had had illnesses suggesting falciparum malaria.

Strains of *P. falciparum*: The studies involved 5 strains of chloroquine-resistant *P. falciparum* (one from Colombia, South America, termed the Colombia strain, and 4 from Southeast Asia, termed the Thailand (JHK), the Vietnam (Sn.), the Vietnam (CV), and the Malayan (Camp.) strains) (2-12) and 2 strains of chloroquine-sensitive *P. falciparum* (the well-known McLendon strain, isolated originally in the southeastern part of the United States, and a strain from Uganda termed the Uganda strain) (13). No strain-related or inoculum-related differences with respect to the parameters focused on in this report were noted. Volunteers were infected either by bites of infective mosquitoes (*Anopheles quadrimaculatus* or *A. stephensi*) or by intravenous inoculation of small samples of blood containing asexual erythrocytic parasites (14).

Observations: The observations that form the substance of this report were made during investigations that were carried out primarily to obtain information about the therapeutic effects of synthetic antimalarial agents and of quinine. A secondary objective was to de-

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² Present address: Department of Internal Medicine, The University of Iowa, and Veterans Administration Hospital, Iowa City, Iowa 52240.

³ Present address: Sansum Medical Clinic, Inc., 317 W. Pueblo St., Santa Barbara, California 93102.

⁴ Present address: Rush-Presbyterian-St. Luke's Medical Center, Laboratory for Tropical Diseases, Box 112, Joliet, Illinois 60434.

velop "carriers" in whom gametocytes would provide a source for obtaining infective mosquitoes that were required for studies relating to chemoprophylaxis. Volunteers who were reinfected with homologous or heterologous strains of *P. falciparum* often were men who had served as "carriers" during initial infections. Notable findings pertinent to chemoprophylaxis or chemotherapy have been or will be reported elsewhere.

Volunteers were hospitalized, and were cared for under very close medical supervision, when patent parasitemia was present. Early during the clinical course, parasite counts, performed with a modification (2) of the method of Earle and Perez (15), were determined at least twice daily (morning and afternoon), and the volunteers' temperatures were determined 2 to 4 times a day. Parasite counts were performed at more frequent intervals when acute attacks were present. Temperature was determined at more frequent intervals when fever was present or suspected. Later during the clinical course, after substantial partial immunity had been acquired, parasite counts were usually performed only once a day and temperatures were determined at least twice daily. After treatment regarded as generally adequate to effect radical cure had been given, follow-up blood smears were examined either daily or 3 times a week for 2 to 3 months. In volunteers who had had stubbornly recrudescing infections, the follow-up period during which serial blood smears were examined often exceeded 2 to 3 months.

Many of the volunteers who were infected with chloroquine-resistant *P. falciparum* received conventional therapeutic doses of widely-used synthetic agents (including chloroquine, amodiaquine, hydroxychloroquine, mepacrine, pyrimethamine, and proguanil) that failed to effect radical cure. When acute attacks were present, these volunteers, as well as other men infected with chloroquine-resistant *P. falciparum* and men infected with chloroquine-sensitive *P. falciparum*, often received intermittent subcurative doses of quinine administered orally to control parasitemia and to effect temporary relief of fever and symptoms. Levels of asexual parasitemia infrequently exceeded 10,000 per cu mm. The early clinical and parasitological course was often characterized by a series of recrudescences

with intervening periods during which only subpatent parasitemia was present.

Data pertinent to initial infections noted in this report reflect information relating to all volunteers who participated in studies at this project between July 1, 1961, and July 1, 1969, who received no chemoprophylactic medication, and who had patent infections for more than 2 weeks. About half of these men acquired partial immunity sufficient to result in asymptomatic or near-asymptomatic persisting asexual parasitemia during initial infections; about half received curative treatment before such a state had developed. The point at which asymptomatic or near-asymptomatic persisting parasitemia first became evident was selected as an indicator of the acquisition of substantial partial immunity. Criteria used to determine the day of onset of such a state were as follows: in the face of continuously patent asexual parasitemia for at least 7 subsequent days, in the absence of treatment, levels of parasitemia displayed no overall increase and temperature did not exceed 100° F. on any of these 7 subsequent days. Parameters determined in relation to initial infections and to reinfections included: the number of days that elapsed between the onset of patency and the onset of such a state or the onset of curative treatment; the number of days on which patent parasitemia was present during such intervals; the number of days on which maximal detected levels of asexual parasitemia exceeded either 1,000 or 10,000 per cu mm; and the number of days on which the maximal temperature detected exceeded either 100 or 102° F. The statistical significance of differences between corresponding mean values in different groups of volunteers was determined with the t-test for small samples as described by Bancroft (16).

Conventional therapeutic doses of chloroquine (a total of 1500 mg of chloroquine base orally over 3 days) were employed to effect radical cure of infections with chloroquine-sensitive *P. falciparum*. Pyrimethamine (a total of 150 mg orally over 3 days) was used to effect radical (complete) cure of infections with 2 of the strains of chloroquine-resistant *P. falciparum* (the Colombia strain and the Vietnam (Sn.) strain) that proved sensitive to pyrimethamine. Seven-, 10-, or 14-day courses of quinine (10 grains of quinine sulfate orally

Table 1. Group A.*

	Negro men (19)	Caucasian men (62)	Mean difference	t	p
Duration (days)	43.3	42.9	0.4	0.091	p > 0.9
Days Patent	31.5	31.6	0.1	0.029	p > 0.9
C > 1,000 (days)	6.0	8.8	2.8	1.783	0.05 < p < 0.1
C > 10,000 (days)	0.5	0.9	0.4	1.134	0.2 < p < 0.3
T > 100° F. (days)	14.4	17.5	3.1	1.270	0.2 < p < 0.3
T > 102° F. (days)	5.2	8.3	3.1	2.899	0.001 < p < 0.01

* Numerals in parentheses indicate the number of volunteers. Data indicate the mean number of days elapsing between the onset of patency and the onset of curative treatment ("Duration"), the mean number of days on which patent asexual parasitemia was detected ("Days Patent"), the mean number of days on which temperature exceeded 100° or 102° F., and the mean number of days on which counts of asexual parasites exceeded 1,000 or 10,000 per cu mm.

every 8 hours), alone or in conjunction with other agents, were generally used to effect radical cure of infections with the Thailand (JHK), the Vietnam (Sn.), or the Malayan (Camp.) strains. Against these 3 strains, the particular regimens employed were ones that proved effective in achieving radical cure of infections in nonimmune volunteers. As a precautionary measure, in an effort to reduce even a slim possibility that radical cure had not been achieved, a second course of such treatment was usually administered 4 to 6 months later even though follow-up in the interim provided no indication that the previous course of treatment had failed to effect radical cure. Unless otherwise indicated, volunteers exposed to reinfection received at least 1 such course of treatment between infections. In most instances, 2 such courses of treatment were given before re-exposure.

Observations

Initial Infections

One hundred and sixty volunteers (100 Caucasian men and 60 Negro men) had initial infections in which patent asexual parasitemia was present for more than 2 weeks. Seventy-nine volunteers (38 Caucasian men and 41 Negro men) acquired immunity sufficient to result in asymptomatic or near-asymptomatic persisting parasitemia during initial infections. Eighty-one volunteers (62 Caucasian men and 19 Negro men) received curative treatment before such a state had developed.

Group A: Table 1 presents information concerning the 81 volunteers who received curative treatment before a state of asymptomatic or near-asymptomatic parasitemia had

developed (Group A). The average interval between the onset of patency and the onset of curative treatment slightly exceeded 6 weeks both in Negro men and in Caucasian men. During such intervals, blood smears were negative about 27% of the time, maximal parasite counts were between 10 and 1,000 per cu mm about 53% of the time, maximal parasite counts ranged between 1,000 and 10,000 per cu mm about 18% of the time, and parasite counts exceeded 10,000 per cu mm about 2% of the time. The average number of days of patent parasitemia in Negro men closely paralleled that in Caucasian men. Parasite counts exceeding 1,000 or 10,000 per cu mm and fever exceeding 100 or 102° F. occurred more often in Caucasian men than in Negro men. The mean difference with respect to the number of days on which temperature exceeded 102° F. was statistically significant. The other mean differences noted were not statistically significant.

Group B: Table 2 presents information concerning the 79 volunteers who acquired immunity sufficient to result in asymptomatic or near-asymptomatic persisting asexual parasitemia during initial infections (Group B). Intervals from the onset of patency to the onset of persisting asymptomatic parasitemia ranged from 11 to 120 days. The average interval was about 8 weeks. During such intervals, blood smears were negative about 14% of the time, maximal parasite counts were between 10 and 1,000 per cu mm about 60% of the time, maximal parasite counts ranged between 1,000 and 10,000 about 24% of the time, and parasite counts exceeded 10,000 per cu mm about 2% of the time. The average number of days on which parasitemia exceeded 1,000 or 10,000 per cu mm during such intervals in

Table 2. Group B.*

	Negro men (41)	Caucasian men (38)	Mean difference	t	p
Interval (days)	49.2	62.1	12.9	2.607	0.01 < p < 0.02
Days Patent	43.8	52.6	8.8	2.226	0.02 < p < 0.05
C > 1,000 (days)	15.1	16.0	0.9	0.401	0.6 < p < 0.7
C > 10,000 (days)	1.6	1.1	0.5	0.777	0.4 < p < 0.5
T > 100° F. (days)	21.1	29.1	8.0	2.999	0.001 < p < 0.01
T > 102° F. (days)	7.6	11.0	3.3	2.825	0.001 < p < 0.01

* See footnote to Table 1. The interval noted refers to the interval between the onset of patency and the onset of persisting asymptomatic or near-asymptomatic persisting asexual parasitemia.

Negro men was not significantly different from that in Caucasian men. The average number of days that elapsed after the onset of patency, the average number of days on which patent parasitemia was present, and the average number of days on which fever exceeded 100 or 102° F. before the onset of asymptomatic persisting parasitemia were significantly greater in Caucasian men than in Negro men (Table 2).

One of the 2 Negro men with sickle cell trait and G6PD deficiency was in Group A. The other Negro man with sickle cell trait and G6PD deficiency was in Group B. Both were infected with the Uganda strain of chloroquine-sensitive *P. falciparum*. In the former volunteer, fever first occurred on the fourth day of patency after asexual parasitemia had increased to 680 per cu mm. Symptoms abated after administration of a relatively small dose of quinine; parasitemia temporarily decreased, and then increased again, reaching 2,990 per cu mm on the 10th day of patency and 3,450 per cu mm on the 11th day. Acute attacks, with fever between 103 and 104.4° F., occurred on the 10th, 12th, and 13th days of patency. Small doses of quinine were given on the 10th and 12th days of patency. Symptoms abated, asexual parasitemia decreased transiently and then increased again, to 12,400 per cu mm, on the 25th day of patency, at which time another acute attack occurred, with fever of 103.4° F. The acute attack subsided after administration of a small amount of quinine. After another temporary decrease in levels of parasitemia, levels of asexual parasitemia again increased, to 6,580 per cu mm, on the 39th day of patency, and another acute attack of malaria occurred, with fever of 103.8° F., at which point curative treatment with chloroquine was given. The clinical and parasito-

logical course in this volunteer was comparable to that noted in other Negro men in Group A who were infected with this strain.

The clinical and parasitological course in the second Negro man with sickle cell trait and G6PD deficiency closely paralleled that in other Negro men in Group B who were infected with the Uganda strain. In this volunteer, the interval between the onset of patency and the onset of asymptomatic persisting asexual parasitemia was 65 days. During this interval, patent asexual parasitemia was present on 60 days, parasitemia exceeded 1,000 per cu mm on 17 days and exceeded 10,000 per cu mm on 1 day, and fever exceeded 100° F. on 28 days and exceeded 102° F. on 18 days. Administration of small amounts of quinine, to control parasitemia and to effect temporary relief of symptoms, was required on 6 occasions during this interval. Gametocytes were often evident; they proved infective for mosquitoes on 8 separate occasions. No evidence was obtained to suggest that with respect to the course of symptoms, the course of asexual parasitemia, the course of gametocytemia, or infectivity of gametocytes, the situation in this volunteer differed notably from that in other Negro men who were infected with *P. falciparum* and who had neither sickle cell trait nor G6PD deficiency.

In volunteers in Group B, the duration of patent parasitemia after the onset of asymptomatic or near-asymptomatic persisting parasitemia was highly variable. In one volunteer, asexual parasitemia remained patent for only 6 days after such a state had occurred and blood smears then proved persistently negative even though no treatment was given. The interval between the first and last days of patency in this man was only 28 days. In another volunteer, after the onset of asymp-

Table 3. Initial infections in Groups C and D.*

	Group C (35)	Group D (18)	Mean difference	t	p
Duration (days)	23.8	106.9	83.1	10.597	p < 0.001
Days Patent	18.7	95.1	76.4	11.011	p < 0.001
C > 1,000 (days)	4.9	17.4	12.5	3.421	0.001 < p < 0.01
C > 10,000 (days)	0.8	1.9	1.1	1.018	0.3 < p < 0.4
T > 100° F. (days)	11.1	29.3	18.2	4.740	p < 0.001
T > 102° F. (days)	5.3	9.4	4.1	2.898	0.001 < p < 0.01

* See footnote to Table 1. Duration refers to the interval between the first and last days of patency of the initial infections.

matic persisting parasitemia, asexual parasitemia remained continuously patent for 47 days, and blood smears then proved intermittently positive for asexual parasites for the next 187 days. The interval between the first and last days of patency in this man was 344 days.

In general, after the onset of asymptomatic or near-asymptomatic persisting parasitemia, asexual parasitemia, as well as gametocytemia, gradually decreased, to barely patent levels, over several weeks to several months. Subsequently, in the absence of curative treatment, blood smears often proved only intermittently positive for the next several weeks and then became persistently negative. The clinical course after the onset of asymptomatic or near-asymptomatic parasitemia was usually characterized by a continued absence of symptoms. Some volunteers, although afebrile, experienced occasional headaches, myalgia, minor gastrointestinal symptoms, or fatigue that gradually lessened in frequency or degree with time. Physical findings remained normal; detectable splenomegaly was consistently absent. Levels of the hematocrit, if normal at the outset of asymptomatic persisting parasitemia, remained normal. If slight to moderate anemia had occurred, levels of the hematocrit gradually returned to normal over several weeks. Occasionally, after asymptomatic persisting parasitemia had been present for several weeks, the clinical and parasitological course was punctuated by a slight increase in levels of asexual parasitemia with a recurrence of fever and of symptoms. Such recurrences were both mild and transient. They subsided quickly, either spontaneously or after administration of small amounts (5 or 10 grains, for example) of quinine. In 2 instances, transient recurrences of fever and symptoms occurred

after more than 4 months had elapsed following the onset of patency.

Second Exposures

Fifty-three volunteers who had previously had patent infections with *P. falciparum* were subsequently re-exposed to infection with homologous or heterologous strains of *P. falciparum*. This group included 35 men (Group C) who had not developed sufficient partial immunity to result in asymptomatic persisting parasitemia during their initial infections, and 18 men (Group D) in whom such a state had developed during initial infections. Group C included some volunteers in whom patency during initial infections had lasted less than 2 weeks. In general, the volunteers in this group had had initial infections that were considerably shorter in duration, with less parasitemia and less fever, than those in Group D (Table 3).

Group C: Group C included 22 volunteers (15 Caucasian men and 7 Negro men) who were re-exposed to infection with homologous strains, and 13 volunteers (9 Caucasian men and 4 Negro men) who were re-exposed to infection with heterologous strains. Re-exposures involved mosquito-induced infections in 7 men and blood-induced infections in 28 men. At the end of patency of the initial infections, each man in Group C had received a course of chemotherapy regarded as generally adequate to effect radical cure of infections with the particular strain of *P. falciparum* involved. Intervals between completion of such treatment and the day of re-exposure ranged from 5 days to 5.5 years. The average interval was 11.9 months; the median interval was 7.5 months.

The volunteer in whom this interval was only 5 days was re-exposed to an infection

Table 4. First and second infections in volunteers in Group C who developed asymptomatic persisting parasitemia during second infections.*

No.	First infection						Interval between infections (months)	Second infection					
	D	DP	C > 1,000	C > 10,000	T > 100°	T > 102°		I	DP	C > 1,000	C > 10,000	T > 100°	T > 102°
1.	5	5	2	2	4	3	0.5	41	41	20	7	11	4
2.	4	4	0	0	5	3	13.0	44	39	19	0	20	7
3.	4	4	0	0	5	1	5.0	59	49	19	0	20	8
4.	62	34	3	0	9	4	17.0	14	14	7	0	8	1
5.	41	40	13	1	19	12	5.0	22	22	10	1	11	1
6.	40	40	13	0	22	10	6.0	20	20	5	0	4	4
7.	14	14	0	0	9	3	2.5	38	38	12	1	22	6
Mean	24.2	20.1	4.4	0.4	10.4	5.1	6.9	34.0	31.9	13.1	1.3	13.7	4.4
8.	18	15	3	1	5	2	7.0	94	85	24	1	31	0
9.	1	1	0	0	0	0	65.0	33	33	17	1	21	11
10.	7	7	3	0	4	2	9.0	116	55	18	6	15	8
11.	11	4	0	0	1	1	45.0	25	24	6	0	13	4
12.	4	4	0	0	4	1	3.5	47	46	17	0	18	4
13.	35	19	0	0	11	3	3.0	46	46	24	0	21	14
Mean	12.7	8.3	1.0	0.2	4.2	1.5	22.1	60.1	48.2	17.7	1.3	19.8	6.8

* See footnote to Table 1. Data pertinent to initial infections indicate the duration of the initial infection (D), the number of days on which patent parasitemia was present (DP), the number of days on which temperature exceeded 100° F. or 102° F. (T > 100° or T > 102°) and the number of days on which parasite counts exceeded 1,000 or 10,000 per cu mm (C > 1,000 or C > 10,000). The interval between infections is indicated in the middle column. Data pertinent to second infections indicate the interval between the onset of patency and the onset of persisting asymptomatic parasitemia (I) and the numbers of days of patent parasitemia (DP), of fever exceeding 100 or 102° F., or of asexual parasitemia exceeding 1,000 or 10,000 per cu mm during such intervals. Volunteers 1-7 were reinfected with homologous strains. Volunteers 8-13 were reinfected with heterologous strains of *P. falciparum*.

with a homologous strain of chloroquine-sensitive *P. falciparum* soon after completion of a conventional therapeutic course of chloroquine. He did not develop patent parasitemia after this re-exposure. In all other instances, at least 2 weeks elapsed after completion of chemotherapy of initial infections before re-exposure. All other men in Group C developed patent parasitemia after re-exposure. One man, who received treatment soon after the onset of patency, did not develop fever. All other volunteers developed fever within 5 days after onset of patency.

Prepatent periods and incubation periods were within the range of those observed, with comparable parasite inocula, during initial infections with nonimmune volunteers reported previously (14). Conspicuous prolongations of prepatent periods or of incubation periods were not noted. In the 7 men whose reinfections involved mosquito-induced infections, for example, prepatent periods ranged from 7 to 12 days (mean: 9.7 days), and incubation periods ranged from 7 to 13 days (mean: 10.6 days). Most volunteers in Group C first developed fever on the first, second, or third days after the onset of patent parasitemia. Incubation periods exceeded prepatent periods by an average 2.2 days (range: 0 to 5 days) in men reinfected with homologous strains and

by an average of 1.3 days (range: 0 to 3 days) in men reinfected with heterologous strains. The mean difference, however, was not statistically significant ($0.2 < p < 0.3$). Observations relating to the early course of parasitemia, fever, and symptoms after reinfection in volunteers in Group C did not suggest that prior infections in these men had resulted in the acquisition of immunity sufficient to modify the initial clinical course of reinfection with either homologous or heterologous strains of *P. falciparum*.

Twenty-two volunteers in Group C received curative treatment before they developed sufficient partial immunity during reinfections to result in asymptomatic persisting parasitemia. In these instances it usually was not possible to determine whether prior infections influenced the rapidity with which such partial immunity developed during reinfections. One volunteer was reinfected with a homologous strain 2 weeks after an initial infection during which patent parasitemia had been present for 49 days. The initial acute attack during reinfection in this man subsided after the administration of amounts of quinine much smaller than those that had been necessary to control parasitemia and fever during his initial infection. This man, however, received curative treatment soon thereafter, so that it was not

Table 5. Intervals preceding the onset of asymptomatic persisting parasitemia during second infections in volunteers in Group C. Comparison of reinfection with homologous and heterologous strains.*

	Reinfected with homologous strain (7)	Reinfected with heterologous strain (6)	Mean difference	t	p
Interval (days)	34.0	60.1	26.1	1.631	0.1 < p < 0.2
Days Patent	31.9	48.2	16.3	1.643	0.1 < p < 0.2
C > 1,000 (days)	13.1	17.7	4.5	1.267	0.2 < p < 0.3
C > 10,000 (days)	1.3	1.3	0.1	0.035	p > 0.9
T > 100° F. (days)	13.7	19.8	6.1	1.660	0.1 < p < 0.2
T > 102° F. (days)	4.4	6.8	2.4	1.024	0.3 < p < 0.4

* See Table 4 and the footnote to Table 4. Data indicated represent mean values.

possible to make a satisfactory assessment of the acquisition of partial immunity during reinfection in this volunteer.

Such assessment was possible during studies with 13 men in Group C, 7 of whom were reinfected with homologous strains, and 6 of whom were reinfected with heterologous strains (Table 4). Three of the 7 men who were reinfected with homologous strains (Table 4: Volunteers 4, 5, and 6) had had initial infections that were relatively long compared to those in the other volunteers noted in this table. The average number of days on which temperature had exceeded 102° F. during the initial infections in men reinfected with homologous strains was significantly greater than that in men reinfected with heterologous strains (0.02 < p < 0.05). Other mean differences noted with respect to initial infections in these men were not statistically significant. A state of asymptomatic persisting parasitemia during reinfection developed sooner, after

fewer days of parasitemia and of fever, in men reinfected with homologous strains than in men reinfected with heterologous strains. However, none of the mean differences noted in this regard proved to be statistically significant (Table 5).

The findings during reinfection in the volunteers in Group C noted in Table 4 were compared with those observed during initial infections in volunteers in Group B (Table 6). Findings in men in Group C who were reinfected with heterologous strains were not significantly different from those detected in men in Group B. In men in Group C who were reinfected with homologous strains, however, the average interval, the average number of days of patent parasitemia, and the average number of days on which fever exceeded 100 or 102° F. before the onset of asymptomatic persisting parasitemia proved to be significantly less than the corresponding mean values noted during studies with volunteers in Group

Table 6. Acquisition of immunity sufficient to result in asymptomatic persisting parasitemia during reinfection in volunteers in Group C compared to that during initial infections in volunteers in Group B.*

	Group B (79)	Group C		Mean difference	t	p
		Volunteers reinfected with homologous strains (7)	Volunteers reinfected with heterologous strains (6)			
Interval (days)	55.4	34.0		21.4	3.268	0.001 < p < 0.01
Days Patent	48.0	31.9		16.2	3.032	0.001 < p < 0.01
C > 1,000 (days)	15.4	13.1		2.3	0.868	0.3 < p < 0.4
C > 10,000 (days)	1.4	1.3		0.1	0.080	p > 0.9
T > 100° F. (days)	24.9	13.7		11.2	3.783	p < 0.001
T > 102° F. (days)	9.2	4.4		4.8	3.980	p < 0.001
Interval (days)	55.4		60.1	4.8	0.318	0.7 < p < 0.8
Days Patent	48.0		48.1	0.1	0.017	p > 0.9
C > 1,000 (days)	15.4		17.6	2.2	0.739	0.4 < p < 0.5
C > 10,000 (days)	1.4		1.3	0.1	0.033	p > 0.9
T > 100° F. (days)	24.9		19.8	5.1	1.743	0.05 < p < 0.1
T > 102° F. (days)	9.2		6.8	2.4	1.099	0.2 < p < 0.3

* Data for Groups B and C represent mean values presented as noted in the footnote to Table 4.

Table 7. Data pertinent to first and second infections in volunteers in Group D.*

No.			First infection				Interval between infections (months)	Second infection					
	D	DP	C > 1,000	C > 10,000	T > 100°	T > 102°		I	DP	C > 1,000	C > 10,000	T > 100°	T > 102°
1.	28	28	9	0	22	13	0.7	NP					
2.	117	109	27	0	60	15	0.1	NP					
3.	112	97	6	0	18	7	13.5	1	1	0	0	0	0
4.	114	94	8	0	14	5	8.5	1	1	0	0	0	0
5.	62	54	11	0	28	11	9.0	12	9	2	0	5	1
6.	137	107	16	1	18	7	34.0	40	24	2	0	3	1
7.	133	108	23	4	16	6	11.5	8	5	1	0	1	0
8.	119	92	3	0	15	5	11.5	1	1	0	0	0	0
9.	99	86	5	0	43	9	14.0	13	11	2	0	3	1
10.	126	113	12	0	42	6	16.5	9	9	3	0	3	1
11.	77	71	0	0	14	3	5.5	1	1	0	0	0	0
12.	108	103	35	4	40	10	8.0	9	9	3	0	4	2
13.	97	97	4	0	36	15	13.0	22	22	4	0	1	1
Mean	102.2	89.2	12.2	0.7	28.2	8.6	11.4	10.6	8.5	1.6	0.0	1.8	0.6
14.	134	134	54	18	45	18	13.0	8	8	3	0	2	1
15.	75	75	45	7	53	15	11.5	25	22	7	0	11	4
16.	145	144	19	0	15	4	53.0	16	16	5	0	4	2
17.	109	86	16	0	23	7	9.0	47	40	8	0	18	8
18.	132	113	20	0	26	13	2.5	43	23	8	0	9	2
Mean	119.0	110.4	30.8	5.0	32.4	11.4	19.8	27.8	21.8	6.2	0.0	8.8	3.4

* Data are presented as indicated in the footnote to Table 4. Volunteers 1-13 were reinfected with homologous strains. Volunteers 14-18 were reinfected with heterologous strains. NP indicates not patent. With respect to initial infections in these volunteers, the mean number of days on which parasitemia exceeded 1,000 per cu mm in men later reinfected with heterologous strains was significantly longer than that in men later reinfected with homologous strains ($0.02 < p < 0.05$). Other mean differences noted in relation to the initial infections were not statistically significant. The median interval between infections in men reinfected with homologous strains and the median interval between infections in men reinfected with heterologous strains were 11.5 months.

B (Table 6). The latter differences reflected primarily the rapidity with which such a state developed during reinfection in the 3 men (Table 4: Volunteers 4, 5, and 6) in Group C who had had rather lengthy initial infections.

Group D: Group D included 13 volunteers (8 Negro men and 5 Caucasian men) who were re-exposed to infection with homologous strains, and 5 volunteers (all Negro men) who were re-exposed to infection with heterologous strains. Re-exposures involved mosquito-induced infections in 2 men and blood-induced infections in 16 men. Intervals between the last day of patency of initial infections and the day of re-exposure ranged from 4 days to 4.5 years. The average interval was 11.4 months. The median interval was 11.5 months.

Two volunteers (Table 7: Volunteers 1 and 2) were re-exposed to blood-induced infection with homologous strains of *P. falciparum* relatively soon (4 days and 22 days) after immunity sufficient to result in persistently negative blood smears (in the absence of treatment) had occurred during initial infections. Neither man had received treatment in the interim. Neither man developed patent parasitemia after re-exposure. In all other instances, at least 2.5 months elapsed between infections, and treatment considered generally adequate

to effect radical cure of infections had been given at the end of the initial infections. Each of the other 16 men in Group D (11 infected with homologous strains and 5 with heterologous strains) developed patent parasitemia after re-exposure.

Of the volunteers in Group D who developed patent parasitemia after re-exposure to infection with homologous strains of *P. falciparum*, 4 had only asymptomatic asexual parasitemia from the outset of patency of the reinfection. Fever occurred in the other 7 volunteers who were reinfected with homologous strains and in all 5 volunteers who were reinfected with heterologous strains.

Prepatent periods of reinfections in men in Group D were at times prolonged. The prepatent period in 1 volunteer exposed to a mosquito-induced reinfection with a homologous strain, for example, was 22 days. Prolongations of the prepatent period, however, were very inconsistent. More conspicuous, and more consistent, was an alteration in the relationship between prepatent periods and incubation periods in the 7 men in Group D who developed fever after reinfection with homologous strains. Incubation periods exceeded prepatent periods in these men by an average of 5.1 days (range: 3 to 7 days). In-

Table 8. Intervals preceding the onset of asymptomatic persisting parasitemia during second infections in volunteers in Group D. Comparison of reinfection with homologous and heterologous strains.*

	Reinfected with homologous strain (11)	Reinfected with heterologous strain (5)	Mean difference	t	p
Interval (days)	10.6	27.8	17.2	2.059	0.05 < p < 0.1
Days Patent	8.5	21.8	13.3	2.292	0.02 < p < 0.05
C > 1,000 (days)	1.6	6.2	4.7	4.382	0.001 < p < 0.01
C > 10,000 (days)	0.0	0.0	—	—	—
T > 100° F. (days)	1.8	8.8	7.0	2.431	0.02 < p < 0.05
T > 102° F. (days)	0.6	3.4	2.9	2.255	0.02 < p < 0.05

* Data represent mean values of studies pertinent to reinfections noted in Table 7.

cubation periods exceeded prepatent periods by an average of 2.2 days (range: 1 to 4 days) in men in Group D who were reinfected with heterologous strains. The difference was statistically significant (0.01 < p < 0.02).

Even more conspicuous was the rapidity with which volunteers in Group D who had fever after reinfection developed partial immunity sufficient to result in asymptomatic persisting parasitemia (Table 7). This was especially noticeable in volunteers who were reinfected with homologous strains. Mean values in relation to fever and the duration of parasitemia before such a state was first evident in men in Group D who were reinfected with homologous strains were significantly less than the corresponding mean values noted in men in Group D who were reinfected with heterologous strains (Table 8) and significantly less than the corresponding mean values detected in men in Group C who were reinfected with

homologous strains (Table 9). Although such mean values in men in Group D who were reinfected with heterologous strains were less than the corresponding mean values in men in Group C who were reinfected with heterologous strains, only 3 of the mean differences detected in this regard proved to be significant at the 5% level (Table 9).

Third, Fourth, or Fifth Exposures

Five volunteers participated in studies involving third, fourth, or fifth exposures to infection with homologous or heterologous strains of *P. falciparum*.

Volunteer 120: This volunteer, a Negro man, was exposed on 4 occasions to blood-induced infection with the McLendon strain of chloroquine-sensitive *P. falciparum* and on a fifth occasion to a blood-induced infection with the Thailand (JHK) strain of chloroquine-resistant *P. falciparum*.

Table 9. Acquisition of immunity sufficient to result in asymptomatic persisting parasitemia during second infections in volunteers in Group C compared to those in Group D.*

	Group C	Group D	Mean difference	t	p
No. Reinfected with Homologous Strains	7	11			
Interval (days)	34.0	10.6	23.4	3.338	0.001 < p < 0.01
Days Patent	31.9	8.5	23.4	4.244	p < 0.001
C > 1,000 (days)	13.1	1.6	11.6	4.864	p < 0.001
C > 10,000 (days)	1.3	0.0	1.3	1.327	0.2 < p < 0.3
T > 100° F. (days)	13.7	1.8	11.9	4.434	p < 0.001
T > 102° F. (days)	4.4	0.6	3.8	3.568	0.001 < p < 0.01
No. Reinfected with Heterologous Strains	6	5			
Interval (days)	60.1	27.8	32.4	1.944	0.05 < p < 0.1
Days Patent	48.2	21.8	26.4	2.610	0.02 < p < 0.05
C > 1,000 (days)	17.7	6.2	11.5	4.008	0.001 < p < 0.01
C > 10,000 (days)	1.3	0.0	1.3	1.397	0.1 < p < 0.2
T > 100° F. (days)	19.8	8.8	10.0	2.884	0.01 < p < 0.02
T > 102° F. (days)	6.8	3.4	3.4	1.403	0.1 < p < 0.2

* Data represent mean values presented as indicated in the footnote to Table 4.

Patency first developed 5 days after the initial exposure. An acute clinical attack occurred with spiking fever on the third and fifth days of patency. Asexual parasitemia reached 151,000 per cu mm on the fifth day of patency. A conventional 3-day course of chloroquine (total dose: 1500 mg of chloroquine base) was administered orally. Parasitemia decreased, symptoms subsided, and blood smears became negative 5 days after initiation of treatment with chloroquine. The volunteer had had patent parasitemia for 9 days, temperature exceeding 100° F. on 5 days, temperature exceeding 102° F. on 4 days, parasitemia exceeding 1,000 per cu mm on 5 days, and parasitemia exceeding 10,000 per cu mm on 3 days.

This volunteer then received intravenous inoculations of freshly-obtained blood containing asexual erythrocytic forms of the McLendon strain 2 days, 9 days, and 25 days after the last day of patency (4, 11, and 27 days after completion of treatment with chloroquine). These 3 inocula contained 430,000, 430,000, and 3,000,000 asexual parasites, respectively. Daily blood smears remained negative until the 36th day after completion of treatment with chloroquine, at which point patent parasitemia recurred. During the next 2 days, parasitemia increased to 14,000 per cu mm, and an acute clinical attack occurred, with fever of 104.6° F. Symptoms subsided, parasitemia decreased, and blood smears again became negative 3 days after administration of subcurative doses of blood schizontocidal medication. A recrudescence of asexual parasitemia occurred 6 days later. Levels of asexual parasitemia promptly increased to 5,500 per cu mm, and another acute attack of malaria ensued. Curative treatment with chloroquine was then administered.

Four months later, the volunteer was exposed to a blood-induced infection with the Thailand (JHK) strain of chloroquine-resistant *P. falciparum*. Patent parasitemia and fever ensued soon thereafter. Administration of intermittent, subcurative doses of blood schizontocidal medication was frequently required during a subsequent 27-day interval before curative treatment was given. Levels of asexual parasitemia repeatedly displayed a tendency to increase rather rapidly, in association with fever and symptoms, during this interval.

No evidence was obtained to suggest that the prior infections with the McLendon strain resulted in a modification of course of parasitemia, fever, or symptoms during the subsequent infection with the Thailand (JHK) strain.

Volunteer 136: This volunteer, a Caucasian man, was exposed to blood-induced infection with the Vietnam (Sn.) strain of chloroquine-resistant *P. falciparum* on 2 occasions and to a mosquito-induced infection with the Malayan (Camp.) strain of chloroquine-resistant *P. falciparum* on 1 subsequent occasion. All 3 infections were of brief duration. Patent parasitemia and fever ensued promptly after the initial exposure. Curative treatment was given during an acute clinical attack after patent parasitemia had been present for 4 days. The volunteer was re-exposed to blood-induced infection with the Vietnam (Sn.) strain 11.5 months later. Patent parasitemia and an acute clinical attack ensued soon thereafter. Spiking fever developed on the first day of patency, and curative treatment was given. Patent asexual parasitemia was present for 6 days during the initial infection and for only 1 day during the second infection. Fifteen months after the second infection, the volunteer was bitten by mosquitoes heavily infected with the Malayan (Camp.) strain. Patent parasitemia first occurred 9 days later, and an acute clinical attack of malaria, with fever of 103.4° F., occurred on the following day, at which point curative treatment was given. No evidence was noted to suggest that the brief infections with the Vietnam (Sn.) strain resulted in modification of either the prepatent period or incubation period of the subsequent infection with the Malayan (Camp.) strain.

Volunteer 263: This volunteer, a Negro man, had 3 sequential infections with the Malayan (Camp.) strain of chloroquine-resistant *P. falciparum* followed by an infection with the Uganda strain of chloroquine-sensitive *P. falciparum*. All 4 infections were blood-induced.

He acquired immunity sufficient to result in asymptomatic persisting parasitemia during his first infection. Such a state was first evident on the 70th day of patency of the initial infection. Curative treatment was given beginning on the 133rd day of patency of the initial infection, at which point patent parasitemia

had been present on 108 days, parasitemia had exceeded 1,000 per cu mm on 23 days, parasitemia had exceeded 10,000 per cu mm on 4 days, temperature had exceeded 100° F. on 16 days, and temperature had exceeded 102° F. on 6 days.

After re-exposure to infection with the Malayan (Camp.) strain 11.5 months later, patent parasitemia ensued but was asymptomatic from the outset. Overt gametocytemia developed; gametocytes repeatedly proved infective for mosquitoes. Levels of asexual parasitemia, which remained less than 1,000 per cu mm even though no treatment was given, gradually decreased. Subsequently, blood smears proved intermittently positive and then persistently negative. Curative treatment was given on the 63rd day after the onset of patency, at which point patent parasitemia had been present on 47 days with no associated fever or symptoms.

After re-exposure to infection with the Malayan (Camp.) strain 14.5 months later, patent asexual parasitemia again occurred and again remained asymptomatic from the outset of patency. Although no chemotherapeutic agents were administered, levels of asexual parasitemia never exceeded 50 per cu mm. Blood smears proved only intermittently positive after the 16th day of patency. Curative treatment was given beginning on the 27th day of patency, at which point patent asexual parasitemia had been present on 18 days with no associated fever or symptoms.

Thirteen months later, the volunteer developed patent parasitemia after exposure to a blood-induced infection with the Uganda strain. Low-grade fever occurred on the first day of patency. During the second to 6th days of patency, levels of asexual parasitemia increased to 11,190 per cu mm. Fever exceeding 102° F. occurred on the 3rd, 5th, 6th, and 7th days of patency and reached a maximum of 104° F. on the 8th day. Although no chemotherapeutic measures were employed, levels of asexual parasitemia decreased, and fever subsided, the next day. Gametocytemia became evident, and gametocytes proved infective for mosquitoes. After the 8th day of patency, relatively low levels of asexual parasitemia remained evident for 33 days, but fe-

ver or symptoms did not recur. Curative treatment was then administered.

Thus, in this volunteer, who had previously acquired a high degree of partial immunity to infection with the Malayan (Camp.) strain, subsequent infection with the Uganda strain was characterized by an initial course comparable to that in nonimmune persons, and by a subsequent course in which immunity sufficient to result in asymptomatic persisting parasitemia was rapidly acquired.

Volunteer 413: This volunteer, a Caucasian man, had 2 sequential infections with the Malayan (Camp.) strain followed by an infection with the Uganda strain. The first infection was mosquito-induced; the second and third infections were blood-induced.

He received curative treatment on the 41st day of patency of the initial infection, during which time immunity sufficient to result in asymptomatic persisting parasitemia had *not* been acquired. Patent parasitemia was present on 40 days, parasitemia exceeded 1,000 per cu mm on 13 days and exceeded 10,000 per cu mm on 1 day, and temperature exceeded 100° F. on 19 days and exceeded 102° F. on 13 days during the first infection.

Re-exposure 5 months later resulted in patent parasitemia, associated with fever and symptoms. During the second infection with the Malayan (Camp.) strain, immunity sufficient to result in asymptomatic persisting parasitemia was evident by the 22nd day of patency. Curative treatment was given beginning on the 55th day of patency, at which point patent parasitemia had been present on 55 days, parasitemia had exceeded 1,000 per cu mm on 12 days and 10,000 per cu mm on 1 day, and temperature had exceeded 100° F. on 13 days and 102° F. on only 1 day.

Re-exposure 3 months later to a blood-induced infection with the Uganda strain, resulted in patent parasitemia that was initially associated with low-grade fever and symptoms. Small doses of quinine were given on 2 occasions. Immunity sufficient to result in asymptomatic persisting parasitemia was evident by the 19th day of patency, at which point patent parasitemia had been present on 19 days, parasitemia had exceeded 1,000 per cu mm on 7 days and 10,000 per cu mm on 2 days, and temperature had exceeded 100°

F. on 3 days and 102° F. on 1 day. Asymptomatic parasitemia then persisted for 5 weeks, at which juncture curative treatment was given.

It appeared that in this man, as in the previous volunteer, immunity acquired during prior infections with the Malayan (Camp.) strain was associated with a reduction in the time that elapsed before the onset of asymptomatic persisting parasitemia during a subsequent infection with the Uganda strain.

Volunteer 419: This volunteer, a Negro man, had 2 infections with the Malayan (Camp.) strain, the first mosquito-induced and the second blood-induced, followed by a blood-induced infection with the Uganda strain. The sequence of events observed was very similar to that noted in Volunteer 413.

He received curative treatment on the 40th day after the onset of patency of the initial infection. Patent parasitemia had been present on each of these days, parasitemia had exceeded 1,000 per cu mm on 13 days and had not exceeded 10,000 per cu mm, and temperature had exceeded 100° F. on 22 days and 102° F. on 10 days. Immunity sufficient to result in asymptomatic persisting parasitemia had not been acquired. After re-exposure 6 months later, patent parasitemia, initially associated with fever and symptoms, ensued. Immunity sufficient to result in asymptomatic persisting parasitemia was evident by the 20th day of patency of the second infection. Curative treatment was initiated on the 47th day of patency, at which point patent parasitemia had been present on 47 days, parasitemia had exceeded 1,000 per cu mm on 8 days and had not exceeded 10,000 per cu mm, and temperature had exceeded 100° F. and 102° F. on 4 days.

After exposure to infection with the Uganda strain 11 months following the second infection, patent parasitemia developed that was associated initially with fever and symptoms. Parasitemia increased to 30,510 by the 5th day of patency, and a single dose of 540 mg of quinine base was given. Spiking fever occurred on the 5th, 7th, 8th, and 9th days of patency. After fluctuating between 260 and 18,000 per cu mm on the 6th to 8th days of patency, levels of parasitemia then decreased, and fever subsided, without further chemo-

therapy. After the 12th day of patency, asexual parasitemia persisted at relatively low levels, with no associated fever or symptoms except for transient headache on the 20th day of patency. Asexual parasitemia then remained evident, with no associated fever or symptoms, and gradually decreased over the next 4 months, at which point curative treatment was given. As in Volunteers 413 and 419, it appeared that in this man immunity acquired previously during infections with the Malayan (Camp.) strain resulted in a shortening of the time required before the onset of asymptomatic persisting parasitemia during a subsequent infection with the Uganda strain.

Discussion

These studies involved falciparum malaria in well-nourished American men who were otherwise healthy and who were cared for under close medical supervision in a hospital setting in a non-endemic area. Considerable caution is warranted with respect to possible extrapolation of these findings to apply to other circumstances. The situation relating to acquisition of immunity to falciparum malaria in infants or children who reside in endemic areas, who may not be well nourished, and who may have other afflictions, for example, may differ considerably from that noted during these investigations.

Although these studies provide no information about untreated falciparum malaria, they provide considerable insight into the acquisition of immunity in persons who receive intermittent, subcurative doses of antimalarial agents. The volunteers who participated in these studies often had repeated recrudescences that reflected administration of doses of antimalarial drugs that were adequate to effect a temporary or "clinical cure" but that were inadequate to effect a complete or "radical cure." Clinical cure without radical cure is particularly apt to occur in patients who are infected with strains of *P. falciparum* that are resistant to chloroquine and to other widely used synthetic antimalarial agents. The findings noted during these studies are, therefore, especially pertinent to situations that may obtain in relation to treatment of patients who are or may be infected with chloroquine-resistant *P. falciparum*.

In volunteers who developed immunity sufficient to result in asymptomatic persisting asexual parasitemia, there was marked individual-to-individual variation in the time that elapsed before such a state was first evident during initial infections or during reinfections. Although the average interval after the onset of patency before such a state was first evident during initial infections was 8 weeks, this interval was less than 3 to 4 weeks in some instances and as long as 10 to 12 weeks in other instances. Intervals preceding the onset of asymptomatic persisting parasitemia during reinfections tended to be remarkably short in volunteers who had had rather lengthy initial infections or who had developed high degrees of partial immunity during initial infections and who were reinfected with homologous strains of *P. falciparum*. In some of the latter volunteers, parasitemia resulting from reinfection was and remained asymptomatic from the outset of patency.

Instances in which asymptomatic persisting parasitemia developed relatively soon after the onset of patency emphasize the need to be alert to the possibility of encountering asymptomatic recrudescences after treatment of patients who are or may be infected with chloroquine-resistant *P. falciparum*. With time, and with repeated recrudescences or reinfections, the likelihood of asymptomatic or near-asymptomatic recrudescences following subcurative treatment steadily increases. Unless follow-up examinations of blood smears are performed, such recrudescences may be easily overlooked.

Our findings with volunteers in Group B indicate that during initial infections, the average interval between the onset of patency and the onset of asymptomatic persisting parasitemia in Negro men was significantly shorter than that in Caucasian men. Such a state developed during initial infections considerably more frequently among Negro men than among Caucasian men. It is conceivable that had curative treatment not been given prior to the onset of such a state in volunteers in Group A, Caucasian men in Group A would have developed persisting asymptomatic parasitemia sooner than those in Group B, or Negro men in Group A would have developed asymptomatic persisting parasitemia later than those in Group B.

Several factors resulted in the administration of curative treatment to volunteers in Group A before the onset of asymptomatic persisting parasitemia. Most frequently, such treatment was given simply because the studies planned had been completed. Such treatment was necessary on rare occasions for administrative reasons. In other instances, curative treatment was given to men in Group A because some volunteers in this group, primarily Caucasian men, were not tolerating *falciparum* malaria as well as were others. Such volunteers were ones who, over several weeks, appeared to be acquiring little if any clinically discernible partial immunity. It is likely that had more men in Group A been followed until they developed asymptomatic persisting parasitemia, mean intervals between the onset of patency and the onset of asymptomatic persisting parasitemia would have been longer both in Caucasian men and in Negro men, and the difference between the mean interval in Caucasian men and that in Negro men would have been even more marked than it was. These observations indicate that, under comparable circumstances, Negro men with *falciparum* malaria acquire immunity sufficient to result in asymptomatic persisting parasitemia more quickly than do Caucasian men.

Recent studies involving American military personnel infected with *P. falciparum* in Southeast Asia revealed that recrudescing infections with drug-resistant parasites were detected predominantly in Caucasian men (17). The rarity of detection of such infections in Negro soldiers suggested that Negro men might be relatively refractory to infection with drug-resistant *P. falciparum*. Our findings suggest that earlier acquisition of immunity in Negro men may warrant consideration with respect to interpretation of these observations.

A rather striking aspect of our findings related to the number of days on which temperature exceeded 102° F. before the onset of asymptomatic persisting parasitemia during initial infections (Table II). Such a state often developed even though volunteers had had fever exceeding 102° F. on only 5 to 10 days. Prolonged periods of high, spiking fever were clearly not a prerequisite for the acquisition of immunity sufficient to result in asymptomatic persisting parasitemia. In general, prior to the onset of such a state, although the situation

with respect to levels of parasitemia in Negro men was comparable to that in Caucasian men, Negro men experienced considerably less fever than did Caucasian men. Our findings indicate that both in Caucasian men and in Negro men, but in Negro men in particular, alertness to the possibility of asymptomatic recrudescences after treatment of drug-resistant infections is warranted even if patients have not had extended periods of high, spiking fever during previous clinical attacks.

During studies with the 2 Negro volunteers with sickle cell trait and G6PD deficiency, we obtained no evidence that the erythrocytic abnormalities in these men conferred protection against falciparum malaria. Our observations in this regard, however, provide no information about situations that may obtain in the face of high levels of parasitemia. Our studies do not provide evidence consistent with the thesis that the presence of hemoglobin S or of G6PD deficiency confers a biological advantage against falciparum malaria (18, 19). Although these very limited observations by no means represent evidence adequate to render this thesis invalid, it may be of interest that in neither volunteer was there even a slight suggestion that the clinical or parasitological course was modified as a result of the presence of hemoglobin S or G6PD deficiency.

Our observations pertinent to reinfection generally coincide with and extend those of many other investigators, as reviewed and discussed by Redmond (20) and, more recently, by Jeffery (21). Initial infections of relatively short duration appeared to have little if any modifying influence upon the course of subsequent reinfections with homologous or heterologous strains of *P. falciparum*. If initial infections were lengthy, and, in particular, if a high degree of immunity had been acquired during initial infection, immunity sufficient to result in asymptomatic persisting parasitemia was often evident relatively soon after the onset of patency of reinfections, especially when reinfections involved homologous strains. Such modification of the course of reinfection with heterologous strains, although of lesser degree than that noted during reinfection with homologous strains, was unequivocal (Table VII). Studies involving third infections in Volunteers 263, 413, and 419 serve to underscore this point. Although some workers have not

noted protection with respect to reinfection with heterologous strains of *P. falciparum* (20), our findings in this regard are entirely in accord with observations recently presented and discussed by Jeffery (21).

"Solid" immunity (immunity sufficiently marked to prevent patency of reinfections) as it relates to reinfection with homologous strains, although noted frequently by some earlier workers, was consistently not evident during the studies presented by Jeffery (21). We observed such "solid" immunity in only 2 instances. In both instances, volunteers had acquired a sufficiently high degree of immunity during initial infections that blood smears had become persistently negative, and re-exposure took place soon thereafter without intervening treatment. In both instances, persisting subpatent parasitemia from the initial infection may have been present at the time of re-exposure. In all of the studies presented by Jeffery, curative treatment had been given before reinfection. He pointed out that during the investigations of other workers who found that prior infections completely prevented re-establishment of infections with homologous strains, the presence of subpatent parasitemia at the time of re-exposure was a possibility in many cases (21). During our investigations, except in 1 man re-exposed to infection with a chloroquine-sensitive strain very soon after administration of chloroquine, patent parasitemia consistently occurred after re-exposure when treatment regarded as generally adequate to effect radical cure of initial infections had been given in the interim. These findings are consistent with the notion that "solid" immunity, when observed, may at times reflect primarily a persistence of subpatent parasitemia from preceding infections.

Although some volunteers in Group D had only asymptomatic parasitemia from the outset of patency after reinfection, the early clinical course after reinfection of volunteers in this group was usually characterized by the occurrence of fever and of symptoms. In several instances, levels of asexual parasitemia, after initially increasing over several days, decreased, and fever and symptoms subsided, spontaneously. Had antimalarial drugs been administered during the mild to moderate acute attacks that resulted from reinfection of these

men, the impression gained clinically would have been that these agents were highly effective even if the agents exerted no blood schizontocidal effects whatsoever. These observations illustrate certain of the difficulties inherent in evaluation of antimalarial agents in persons who are or may be partially immune, and the need for carefully controlled studies, involving comparable groups of patients, particularly when the issue at stake relates mainly to efficacy rather than lack of efficacy.

In agreement with observations of many other workers, we noted that after volunteers had acquired clinically discernible partial immunity during initial infections or reinfections, very small doses of antimalarial agents (5 or 10 grains of quinine, for example) often sufficed to control parasitemia and to terminate fever and symptoms. In many instances, levels of parasitemia decreased, and fever and symptoms abated, with no treatment. The transient symptoms that occurred were indistinguishable from those of many other infections or diseases. Fever and symptoms in such persons were sufficiently mild, brief, and minimally bothersome that had those infected been in civilian life, they probably very seldom would have taken the trouble to consult a physician. Patent gametocytemia was very frequently present, and gametocytes often proved infective for mosquitoes, under such circumstances. Such persons represent a potential source for transmission of malaria either by mosquitoes or by transfer of asexual parasites, as may occur via transfusions of blood or via sharing of unsterilized needles or syringes among narcotics addicts. The same is true of persons who are initially nonimmune and who develop asymptomatic parasitemia in the absence of intervening fever or symptoms because of intermittent ingestion of antimalarial agents that suppress but do not eliminate asexual parasites (22). Persons who have acquired partial immunity to falciparum malaria and who have persisting parasitemia pose not only practical clinical problems but also formidable epidemiological problems that are of immense importance with respect to public health. The detection and appropriate treatment and follow-up of such persons represents a major challenge.

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Resistant Falciparum Malaria in Vietnam: Its Rarity in Negro Soldiers

A. P. HALL

C. J. CANFIELD

Walter Reed Army Institute of Research, Washington, D. C. 20012

ABSTRACT: Falciparum malaria in white soldiers in Vietnam often ran a protracted course characterized by repeated recrudescences of the infection. In sharp distinction, the black patients did not develop infections that were difficult to eradicate. This genetic difference may offer some explanation for the striking absence of chloroquine-resistant falciparum malaria in Africa.

Black men are less susceptible than white men to infection with *P. vivax*, however, little is known about the comparative incidence and clinical course of falciparum malaria in these two ethnic groups. In Vietnam, U.S. Army troops infected with *P. falciparum* were admitted to acute care hospitals and treated with quinine, pyrimethamine and dapson. The majority were transferred to the (6th) Convalescent Center for completion of therapy and recuperation. Between November 1969 and

February 1970, a prospective ethnic study was performed at this hospital on all such patients. Patients whose recrudescences were diagnosed after discharge were recycled to the Center. Followup was thus unusually complete and few recrudescences would have been missed. Preliminary findings from the survey have been reported (Hall and Canfield, 1971).

Results

A survey of the patients at the Center who did not have malaria showed that 11.7% (117/1000) were black. This figure correlated

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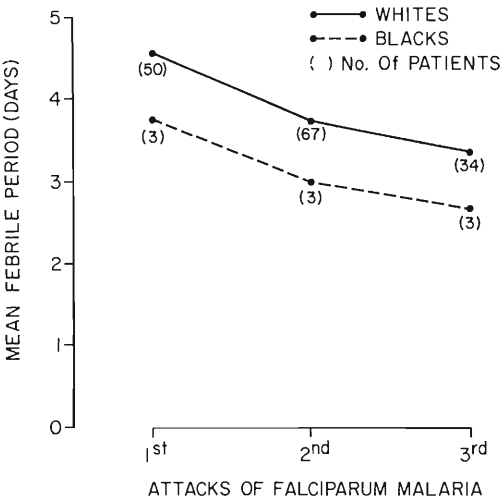


Figure 1. In patients with recrudescent falciparum malaria, the mean febrile period was progressively shorter with each successive attack. This sequential decrease probably denotes acquired immunity. The vertical difference between the mean febrile periods for the black and white patients, presumably represents a difference in innate immunity.

well with official Department of Defense estimates. Thus, if falciparum malaria affected black (negro) men and white (caucasian) men equally, it would have been expected that about 11.7% of the patients with malaria would have been black. However, of 788 patients suffering their first attack of falciparum malaria, only 7.9% (62) were black (Table 1).

Some of these patients recrudesced. At the

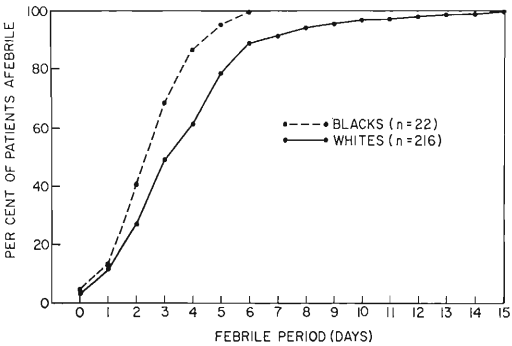


Figure 2. In patients with only one attack of *P. falciparum*, the mean febrile period was shorter in the black men than in the white men.

Convalescent Center and at the Walter Reed Army Institute of Research, 206 patients with recrudescent infections have been treated, of whom only 4.4% (9) were black (Table 1). There were 55 of these 206 patients who were diagnosed as having severely resistant infections; none of these 55 men were black (Table 1).

The clinical picture of the acute attack in the two ethnic groups was compared. The mean highest temperature recorded was the same, but the duration of fever following initiation of therapy was greater in the whites. In patients with recrudescent infections, the febrile period after beginning therapy for each attack was less in the black men (Figure 1). The number of blacks is small, since so few of them had recrudescences. Similarly, in patients with only one attack of the disease, the

Table 1. Ethnic difference in falciparum malaria.

Falciparum malaria in Vietnam 1970: clinical characteristic	Patient group (% black men)	Control group (% black men)
Initial attack rate	7.9% (62/788) (1)*	11.7% (117/1000)
Patients with at least 1 recrudescence	4.4% (9/206) (2)**	"
Patients with severely** resistant infections	0% (0/55) (3)*	"

* Difference between patient and control groups
(1) $\chi^2 = 6.72$ $P < 0.01$
(2) $\chi^2 = 9.04$ $P < 0.005$
(3) Binomial Probability $P < 0.005$

** A patient was defined as having a severely resistant infection if (1) a continuous infusion of quinine for 10 days given for an acute attack was followed by a recrudescence or (2) 4 or more recrudescences occurred following 4 or more courses of oral therapy.

mean febrile period was less (2.91 ± 0.307 versus 4.07 ± 1.73 days) in the black men (Figure 2).

Discussion

A unique feature of this study was that it was possible to compare the clinical picture of naturally acquired (autochthonous) falciparum malaria in an endemic area in black men and white men not previously exposed to the disease, i.e. non-immune people. The black men were less liable to develop the disease, which was also less difficult to eradicate as shown by the shorter mean febrile period after beginning therapy, the lower recrudescence rate, and the absence of severely resistant infections.

Complementary evidence has accrued in controlled infections in volunteers artificially inoculated with mosquitos or blood infected with *P. falciparum*. Boyd and Kitchen (1945) reported that the black volunteers tolerated falciparum malaria better, had lower parasite counts and a shorter duration of fever. In the white men the infections frequently consisted of 2 or even 3 waves of trophozoites each with its following wave of gametocytes. In the black men the infection was commonly limited to one wave of each. Powell, et al. (1972), in the next paper, have also found that blacks experienced a shorter duration of fever and more rapidly developed asymptomatic parasitemia. Kitchen (1949) reported that the pre-patent and incubation periods for mosquito-induced falciparum malaria did not differ in the two ethnic groups; whereas Powell and McNamara (1970) found these 2 periods to be longer in black men following mosquito induced but not blood induced infections.

Most of these studies suggest that the "African gene" imparts a higher degree of immunity to falciparum malaria. This may help explain the striking absence of chloroquine-resistant falciparum malaria in Africa (Bruce-Chwatt, 1970).

The incidence and severity of falciparum malaria is less in those African Negroes with the sickle cell trait (Allison, 1954; Luzzatto, et al., 1970), but may not be in those with sickle cell disease (Adeloye, et al., 1971; Pichanick, 1971). However, only 9% of American blacks have this trait (Myerson, et al., 1959). Thus, a partial protection in 9% of the black

soldiers at risk could not have accounted for all the differences reported in the present study. Glucose-6-phosphate-dehydrogenase (G6PD) deficiency occurs in about 10% of American Negroes (Carson and Frischer, 1966). There is conflicting evidence as to whether this abnormality protects against malaria (Motulsky, 1960; Allison and Clyde, 1961; Kidson and Gorman, 1962; Powell and Brewer, 1965; Siniscalco, et al., 1966; Gilles, et al., 1967; Bowman, 1967; and Bienzle, et al., 1972). One group reported that the red cell adenosine triphosphate (ATP) level is lower in blacks and related this to protection against malaria (Brewer and Powell, 1965; Brewer and Coan, 1969). Malaria cannot be induced in dogs (Boyd, 1949), a species noted for a high red cell sodium/potassium ratio (Richmond and Furchner, 1970). Thus, the report of a high red cell sodium in black people (Munro-Faure, et al., 1971) may therefore be relevant. It is probable that there are other factors, presently unknown, to account for the ethnic difference in falciparum malaria.

Equally obscure is the explanation for the lower susceptibility of black men for *P. vivax* demonstrated, during malariotherapy for neurosyphilis (Mayne, 1932; Boyd and Stratman-Thomas, 1933; Becker, et al., 1946; Young, et al., 1946; and Young, et al., 1955) and from military experience (Trager, 1947; Hankey, et al., 1953; and Fisher, et al., 1970). Both Boyd (1949) and Coatney, et al. (1971) present much interesting information on the ethnic aspects of malaria. For example, clinical attacks caused by *P. malariae* are apparently somewhat less prolonged in blacks than in whites. However, blacks are more immune to vivax than to falciparum or malariae malaria (Boyd, 1934).

Since white men are relative genetic strangers to malaria, it may be more accurate to say that they are more susceptible rather than that black men are less susceptible. Resistant infections of *P. falciparum* are usually attributed to strain differences of the parasite in its susceptibility to drugs. This study emphasizes that strain differences of the human host may also be relevant. Thus, in some instances, "resistant infection" may be a more accurate term than "drug resistant infection." The ethnic difference in malaria remains largely unexplained and merits elucidation.

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Some Characteristics of *Plasmodium vivax* from Vietnam

D. F. CLYDE

University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT: A strain of *Plasmodium vivax* from the Central Highlands of southern Vietnam, studied in non-immune Caucasian volunteers at Maryland, has the short incubation period and early relapse pattern characteristic of the tropical "Vivax" type of which the Chesson strain is an example. This Vietnam strain may be distinguished from the Chesson, however, by its greater sensitivity to chloroquine and primaquine. Treatment with chloroquine of attacks of Vietnam vivax malaria resulted in radical cure in 3 of 15 cases, but such cures always followed the administration of 15 mg (base) of primaquine daily for 14 days. The use of chloroquine alone at weekly intervals for prophylaxis of the Vietnam strain provided suppressive cure in 4 of 6 volunteers, and such cures occurred in all 7 men receiving chloroquine with 45 mg of primaquine weekly for 6 or 7 weeks after exposure to heavily infected mosquitoes.

Vivax and Hibernans patterns of behavior of *Plasmodium vivax* infections are exemplified in the Western Pacific region by, respectively, the tropical Chesson strain from New Guinea, having a short incubation period and an interval between primary attack and relapse usually no longer than 2 months (Coatney et al., 1950; Jeffery, 1956), and temperate Korean strains with a long incubation and an interval to relapse of 9 or more months (Arnold et al. 1956). Since these and other strains may behave in an irregular and atypical manner, it is

of considerable epidemiological and clinical interest to ascertain the characteristics of strains transmitted in areas geographically and climatically intermediate to New Guinea and Korea. The incubation, first and second relapse and drug response patterns of a strain of *P. vivax* from Vietnam, studied in non-immune Caucasian volunteers, are described here, but data relating to later relapses, longevity of the parasite and immunological characteristics are not included. Interest is enhanced by the fact that 80 per cent of the cases of malaria currently imported into the United States are attributable to *P. vivax* acquired mainly in the same general locality.

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Table 1. Prepatent Period and Relapse Activity of Vietnam Vivax.

Volunteer, age and weight (kg)	Day of primary attack (and treatment days)	Day of first relapse (and treatment days)	Day of second relapse
EC, 26, 86	14 (C:35-37)	57 (D:61-66)	87
JC, 30, 83	15 (C:17-19)	64 (C:71-73)	—*
JG, 34, 78	15 (C:26-28)	96 (C:97-99)	201
LM, 25, 70	15 (C:22-24)	67 (C:76-78)	142
PN, 43, 63	14 (C:22-24)	49 (CP)	
RN, 35, 73	12 (C:16-18)	—*	
MO, 25, 67	13 (C:22-24)	62 (C:70-72)	98
JS, 29, 75	18 (C:39-41)	52 (C:54-56)	—*
ST, 26, 92	13 (C:65-67)	84 (C:85-87)	140

* RN was followed without relapse to Day 233, JC to Day 426 and JS to Day 676, when the studies were terminated and primaquine given as a final precaution.
Treatments: C = Chloroquine 1.5 gm (base) over 3 days, D = Doxycycline 0.2 gm daily for 6 days, CP = Chloroquine for 3 days with primaquine for 14 days, terminating the study.

Method

The strain of *P. vivax* described here is a composite of several lines isolated from American soldiers infected in the Central Highlands of the Republic of Vietnam in 1966-68, the behavior of these lines when studies in non-immune volunteers being identical. Between passages, the strain has been maintained in a glycerolized, frozen state, alongside a line of Chesson vivax with which its characteristics have been directly compared, and with which it is indistinguishable morphologically and in its developmental stages in the principal colonized vector used, *Anopheles stephensi*. The methods used in the care and maintenance of the volunteers participating in these studies have been described elsewhere (Clyde et al., 1970); it is only necessary to stress again that clinical precautions are taken to prevent high levels of parasitemia, severe symptoms and possible complications, and that the ethical guidelines and restrictions recommended by an independent University committee are strictly observed.

Results

Prepatent period

Twenty-three non-immune Caucasian volunteers, including those listed in Table 1, were exposed to 10 or more mosquitoes heavily infected with the Vietnam strain of *P. vivax*. Parasitemia was first observed from 12 to 18 days after exposure, the mean being 14.6 days. Chesson vivax, transmitted to 67 Caucasian volunteers in the Maryland system by methods identical to those used for the Vietnam strain, had a prepatent period of 13.0 days (range

10-16 days). However, in two additional cases receiving very small sporozoite inocula from one or two lightly infected mosquitoes, the periods were 36 and 168 days.

Period to first relapse

Primary attacks of mosquito induced Vietnam vivax malaria occurring in 9 non-immune Caucasian volunteers were treated with chloroquine 1.5 gm (base) during 3 days. All infections had been initiated on Day 0 by exposure of the volunteer to 10 or more heavily infected mosquitoes. The period to first relapse is shown in Table 1, and ranged from 49 to 96 days (mean 66) after initiation; from subsidence of the primary attack to beginning of relapse the range was 12 to 68 days. One patient (RN) did not have a relapse although followed for 233 days.

Period to second relapse

Seven of the first relapses shown in Table 1 were treated with the schizontocidal drugs indicated, the remaining patient receiving primaquine in addition in order to terminate his participation in the study. Of the 7 cases not given primaquine, two (JC and JS) did not develop a second relapse during follow-up periods of 426 and 676 days. Relapse occurred in the remainder from 87 to 201 days (mean 134) after initiation of the infections; from subsidence of the first relapse to beginning of the second the range was 23 to 103 days.

Period to third relapse

Only one of the cases shown in Table 1 as developing a second relapse could be followed

Table 2. Vietnam Vivax Relapses following Suppressive Prophylaxis.

Volunteer, age and weight (kg)	Days from final prophylactic dose to:	
	Appearance of parasitemia	End of follow-up when no parasitemia
RB, 24, 76	41	—
WB, 28, 99	—	146
TE, 31, 82	23	—
DH, 25, 66	—	186
LH, 42, 79	21	—
MN, 26, 71	52	—
DO, 32, 87	11	—
DP, 32, 82	—	191
RR, 38, 75	—	1278
CS, 21, 74	—	189
HT, 30, 70	—	146

for third relapse, the remainder terminating their participation in the study and receiving primaquine. This individual, LM, was given the 3 day course of chloroquine as treatment for his second relapse, and was followed until Day 450 without further relapse. At the end of this period he received primaquine in order to release him from the study.

Release following suppressive prophylaxis

The effective suppressive prophylactics chloroquine 300 mg (base) or the quinoline-methanol compound WR-30090 690 mg were administered to 11 Caucasian volunteers at weekly intervals for 8 weeks, commencing on the day of or the day before exposure to 10 or more mosquitoes heavily infected with the Vietnam strain of *P. vivax*. Three individuals exposed at the same times and not given prophylactic treatment developed vivax malaria in 12–15 days. Following completion of suppressive treatment, infections appeared in 2 of the 6 men who had received chloroquine and 3 of the 5 who had received WR-30090. The results are shown in Table 2.

When the same course of chloroquine was given to 6 volunteers exposed to the Chesson strain, infections appeared in all the men from 13 to 61 days after the final dose.

Relapse following use of primaquine

Primaquine 45 mg (base) was given weekly with chloroquine 300 mg (base) to 7 Caucasian volunteers for 6 or 7 weeks, commencing on the day of or 2 days before exposure to 10 or more mosquitoes heavily infected with the

Vietnam strain of *P. vivax*. Two men exposed at the same times and not given prophylactic treatment developed vivax malaria 14 days later. Suppression was effective during the course of treatment, nor did infection develop thereafter when they were followed for 112 days.

Primaquine 15 mg (base) was given daily for 14 days to 11 volunteers experiencing attacks of malaria caused by the Vietnam strain of *P. vivax*. The infections had been induced by exposure to infected mosquitoes, and were treated also with chloroquine. No relapses occurred, the individuals being followed for periods of 173 to 970 days after ingestion of the last dose of primaquine.

The 14-day course of primaquine was also administered in this study to 31 men infected with the Chesson strain. Nine of the infections relapsed (29 per cent), parasitemia appearing from 60 to 149 days after the last dose. The cases that did not relapse were followed for 165–1,561 days.

Discussion

Some of the characteristics by which strains of *P. vivax* may be distinguished are the length of the prepatent period, the interval between primary attack and relapses, and the response of exoerythrocytic stages to primaquine. The two extreme types, the tropical "Vivax" with short prepatent period, frequent relapses and a relative insensitivity to primaquine (this last characteristic possibly being limited to the Chesson strain), and the temperate "Hibernans" with a protracted prepatent period and long intervals between relapses, are exemplified in the Western Pacific region by the Chesson strain, originating in New Guinea in 1944, and Korean strains isolated in 1951–53. Intermediate types exist, while the Chesson and Korean strains have themselves behaved in atypical fashion in the hands of different investigators working in comparable non-immune volunteer systems.

The prepatent period in Maryland volunteers of a strain of *P. vivax* from the Central Highlands of southern Vietnam was 12–18 days (mean 14.6), and of Chesson vivax 10–16 (13.0). Other investigators have found this period to be 8–19 days (12.6) for the Chesson and another New Guinea strain of similar vintage (Coatney et al., 1950; Cooper

et al., 1953; Engstrom et al., 1947; Whorton et al., 1947; and Fairley, 1947); longer periods occasionally occurred, often being attributable (as in two of our cases) to exposure of the volunteer to a sub-standard inoculum of sporozoites (Coatney et al., 1950; Jeffery, 1956). A strain transmitted in the Solomon Islands in 1943 had a prepatent period of 10–19 days, the average being 12.9 days (Engstrom et al., 1947). North Vietnamese strains isolated around 1954 were found to have a range of 14–25 days (Tiburskaja et al., 1967).

While the strains from New Guinea, the Solomons and Vietnam had the short prepatent periods described, this was not the case with Korean strains similarly studied in non-immune volunteers. Although in one study 4 of 6 volunteers receiving a South Korean strain had short prepatent periods, parasitemia did not develop in the other two men for 287 and 315 days (Arnold et al., 1954), while only 6 of 38 receiving a North Korean strain experienced a short incubation and the remainder did not show parasites until 250–390 days had elapsed (Tiburskaja, 1962).

The relapse pattern of the Vietnam strain studied at Maryland also matched that of the tropical group. The first relapse following schizontocidal treatment of the primary attack occurred 49–96 days (mean 66) after exposure of the volunteers to the heavily infected mosquitoes or, expressed as the interval from subsidence of the primary attack to relapse, 12–68 days (mean 34). One patient in nine did not suffer a relapse. Second relapses were seen in 5 of 7 patients whose first relapses had been treated with schizontocides but not primaquine; the infections reappeared 76–201 days (mean 134) after exposure, or 23–103 days (mean 54) after subsidence of the first relapse. A third relapse did not occur in the only patient in whom this could have been expected, although he was followed for 450 days.

Since the period from treatment to relapse varies with the type and dosage of schizontocide used (Coatney et al., 1950), the Maryland data can only be compared with those of studies in which use was made of similar courses of chloroquine. For Chesson vivax in Caucasian non-immune subjects, the interval between subsidence of primary attack and

first relapse was found to be 43–50 days (mean 47) (Coatney et al., 1950), and in another study 43–380 days (mean 106) (Jeffery, 1956), all cases in both studies relapsing; the interval between first and second relapse was, respectively, 49–98 days (mean 68), all cases again relapsing (Coatney et al., 1950), and 48–284 days (mean 99), 19 of 23 cases relapsing (Jeffery, 1956). Third relapses occurred in 1 of 3 patients (Coatney et al., 1950 and in 12 of 14 patients (Jeffery, 1956). For a North Vietnamese strain, 3 of 4 patients experienced a first relapse following malariatherapy (Tiburskaja et al., 1967). For a South Korean strain, 3 volunteers treated in the initial attack developed first relapses 273–313 days (mean 288) after exposure, the interval between primary attack and relapse being about 9 months (Arnold et al., 1954).

Relapses (or delayed primary attacks) developed upon completion of suppressive prophylaxis without primaquine in 5 of 11 Maryland volunteers protected for 8 weeks following exposure to the Vietnam strain. This result may be compared with the consistent appearance of delayed attacks following similar treatment of the Chesson strain; volunteers given chloroquine 300 mg (base) for 8 weeks at Maryland, and at other centers for up to 52 weeks (Lints et al., 1950), all subsequently developed parasitemia.

Relapses following administration of primaquine are of great practical importance. The prophylactic use of 45 mg (base) of primaquine with chloroquine at weekly intervals for 6 or 7 weeks provided suppressive cures in all 7 Maryland volunteers exposed to the Vietnam strain, in contrast to results with the Chesson strain which, in the hands of other investigators (Alving et al., 1960), emerged in 1 of 29 men after the conclusion of 8 weeks of the same regimen. The radically curative use of 15 mg of primaquine daily for 14 days, usually during an attack in association with the 3 day course of chloroquine, was successful in all Vietnam vivax cases treated at Maryland, but relapses occurred with the Chesson strain in 29 per cent of Maryland volunteers and in 27–35 per cent of the volunteers studied elsewhere (Alving et al., 1960; Coatney and Getz., 1962).

It may be concluded that the strain of *P.*

vivax from Vietnam studied at Maryland resembled the Chesson rather than the Korean strain in its incubation and relapse characteristics, but is more sensitive than the Chesson strain to chloroquine and primaquine.

Acknowledgments

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Comments on Clinical Aspects

R. L. KAISER

Director, Malaria Program Center for Disease Control, Atlanta, Georgia 30333

Each of the three papers under discussion speaks to a subject of timely importance in present-day malaria research. Striking increases of malaria are being reported cur-

rently in several tropical areas of the world. In addition the vast reservoir of malaria on the African continent has yet to be confronted.

In the early years of the global malaria eradication effort, essentially all reliance was placed on vector control through use of residual insecticides.

With the recent realization of the limitations and risk inherent in dependence on a single methodology, it is most encouraging that greater attention is being directed towards studies of the immune mechanism and the host-parasite relationship.

The paper by Powell, McNamara and Rieckmann presents a wealth of data and experience accumulated over many years on the clinical facets of acquisition of immunity to falciparum malaria.

It was of particular interest and relative to the report of Hall and Canfield, that the mean interval of parasitemia in excess of 1000 and 10,000 parasites per cubic millimeter in group B was equal in Caucasians and Negroes. The clinical manifestations were strikingly dissimilar in the two groups, with the Caucasian group experiencing longer periods of patency and fever before onset of asymptomatic parasitemia. Rather than resistance to infection as suggested by Hall, the Negro may merely have greater tolerance and more rapidly acquire clinical immunity.

The study by Powell and his colleagues provides valuable information on the acquisition of immunity in persons receiving intermittent subcurative doses of antimalarial agents. Somewhat similar circumstances prevail in many areas of the world where malaria transmission is of moderate to low intensity and where antimalarial drugs are commonly employed in less than fully curative doses. It is therefore likely that infections similar in character to those described, particularly repeated challenges by homologous parasite strains intermittently modified by subcurative doses of drugs, occur frequently. The implications of asymptomatic parasitemia are most significant. Clearly a case detection method based on a history of fever and clinical illness will miss cases if the process of acquiring clinical immunity described by the authors is operative.

The studies further point out the need to carefully review the potential role of any proposed malaria vaccine based on antigen derived from the blood stages of the parasite. Rather than development of "solid immunity"

as is associated with various viral immunizing agents, such a vaccine would appear likely to modify the clinical manifestations of the disease without eliminating parasitemia or infectivity.

The authors appropriately caution against conduct of chemotherapeutic evaluations in volunteers who have acquired levels of tolerance and point out the danger of relying on the absence of symptoms as criteria for clinical cure in attacks of long duration. This is of particular importance in cases of drug resistant falciparum malaria.

The discussion succinctly summarizes the present day knowledge of the development of clinical immunity or tolerance to human malaria.

The report by Hall and Canfield suggests a decreased incidence of resistant falciparum malaria in Negroes in Vietnam and is based on a very critical methodologic assumption. The assumption is that in the absence of a protective factor in Negroes, the proportion of Negroes to Caucasians in the population admitted for falciparum malaria to the 6th Convalescent Center would be the same as the proportion of Negroes to Caucasians in the population admitted to the Center for all other causes. Because the percentage of Negroes experiencing their first attack of malaria (7.9%) is less than the percentage of Negroes (11.7%) in the total patient group admitted for all other causes, it is inferred that Negroes are less susceptible to falciparum infections than Caucasians. This reasoning may be somewhat precarious in that it assumes equal influence of a series of factors of critical importance to the hypothesis. These include assumptions: that Negroes and Caucasians comply equally conscientiously with directives on chemoprophylaxis; that Negroes and Caucasians are at the same risk of exposure to falciparum malaria; that Negroes and Caucasians are equally attractive to the vectors; and that Negroes and Caucasians equally employ ancillary protective measures such as repellents and nets. Further as the literature amply supports that falciparum malaria is better tolerated in Negroes than Caucasians it is possible that fewer infections came to clinical notice in Negroes than in Caucasians. Other observations under controlled conditions indicate that race does not play a

prominent role in susceptibility of non-immunes to falciparum infection; but Negroes do appear to possess greater clinical tolerance than Caucasians to infections with falciparum malaria.

Although the paper notes that the mean febrile period subsequent to initiation of therapy was shorter in Negroes than Caucasians, the small number of Negroes renders this comparison questionable particularly in the group of recrudescence infections. In addition to measurement of susceptibility to falciparum infection in Negroes and Caucasians by the method employed in this report it would be desirable to examine well defined populations with documented equal risk factors and calculate attack rates for Negroes and Caucasians based on parasitologic, serologic, and clinical parameters. The difficulties of carrying out such a study are clearly recognized.

The report by Clyde characterizing a composite strain of *P. vivax* from Vietnam is of great practical importance. Consistent with reports of other Southeast Asia strains of vivax, the Vietnam strain studied has definite Chesson characteristics, particularly with respect to its incubation and relapse characteristics.

The paper concludes that the Vietnam strain is more sensitive than the Chesson strain to chloroquine and primaquine. The author also reported successful suppressive prophylaxis with chloroquine in 4 of 6 volunteers infected with the Vietnam strain. In order to further clarify the implication of this observation it would have been interesting to treat and follow the three controls who developed infection and observe them for relapses.

The results of the study of weekly chloroquine-primaquine and of the 14-day course of primaquine revealed a high degree of effectiveness and supports the view that the number

of cases of vivax, particularly in military returnees from Vietnam, reflects failure to adhere to the drug regimen rather than ineffectiveness of primaquine against Vietnam vivax. Although the numbers of cases of vivax in military returnees to the United States are decreasing, the data on primaquine are most reassuring from a therapeutic and public health standpoint.

Remarks by Dr. Clyde

Nikolaiev's differentiation into *P. vivax hibernans* and *P. vivax vivax* is generally criticized on the grounds that (1) bimodal behavior patterns occur in some strains—that is, some strains sometimes behave like hibernans, sometimes like vivax, and sometimes in an intermediate fashion; (2) in climatic areas suitable to hibernans, the vivax behavior pattern is often found (although it is noteworthy that in tropical areas the hibernans pattern is exceedingly rare).

During the Korean War both hibernans and vivax patterns were seen in strains isolated for volunteer study. It should be remembered that following World War II, Korea ceased to be ecologically isolated. Only 4 or 5 years before the Korean War, large numbers of World War II Korean soldiers returned home having served throughout South East Asia even into Burma with the Japanese Army. Undoubtedly, a great volume of the relapsing vivax pattern came back with them to be mingled with the indigenous strains. Whether the vivax and hibernans would have hybridized, and whether the imported vivax pattern would gradually have disappeared, is impossible to tell since the Korean War and the malaria eradication program intervened. The point I am making is that it might be unwise to use the large volume of studies on Korean *P. vivax* to challenge the hibernans hypothesis.

II

HOST-PARASITE RELATIONSHIPS

C. Blood Enzyme Deficiencies

Glucose-6-Phosphate Dehydrogenase Deficiency and Falciparum Malaria in Two Northeast Thai Villages

H. E. SEGAL, W. W. NOLL AND W. THIEMANUN

U. S. Army Medical Component, Southeast Asia Treaty Organization, APO San Francisco 96346

ABSTRACT: *Plasmodium falciparum* infection rates, average number of parasitemic episodes, parasitemia-associated fever, and parasite densities among G-6-PD deficient, heterozygous, and normal villagers were studied during the conduct of a prospective longitudinal study of malaria transmission in Northeast Thailand.

The falciparum malaria experience of deficient and heterozygous persons did not differ significantly, in any of the measured variables, from that of their G-6-PD normal relatives or the remainder of the population under study.

This study thus is in agreement with studies of Thai hospital and clinic populations in its failure to demonstrate a protective effect of G-6-PD deficiency against *P. falciparum* infection.

Possible explanations for the findings and for apparent differences between Thai and African populations are advanced.

The positive geographical correlation between high *Plasmodium falciparum* malaria endemicity and high frequency of the glucose-6-phosphate dehydrogenase (G-6-PD) deficient gene suggested the hypothesis that deficient persons may be more than normally resistant to falciparum malaria infection and morbidity (Allison, 1960; Flatz and Sringam, 1963). Enzyme-deficient African children reportedly have lower rates of infection and lower levels of parasitemia than do enzyme-normal controls (Allison and Clyde, 1961; Gilles et al., 1967). Strains of *P. falciparum* endemic in Africa appear to preferentially infect enzyme-normal erythrocytes (Luzzatto et al., 1969). In Thai populations, however, studies of infection rates, parasite densities, and parasite erythrocyte preference have not, in general, supported the hypothesis that G-6-PD deficiency is protective against falciparum malaria (Kruatrachue et al., 1962; Kruatrachue et al., 1966; and Kruatrachue et al., 1970).

Prospective longitudinal studies of malaria transmission and morbidity in two Northeast Thai villages, in which *P. falciparum* and *P. vivax* are endemic, provided the opportunity to measure the frequency of G-6-PD deficiency and assess its possible protective effect. We studied *P. falciparum* infection rates, number

of parasitemic episodes, parasitemia-associated morbidity, and parasite densities in enzyme-deficient and enzyme-normal subjects.

Materials and Methods

Data were accumulated on two groups of subjects. The "Family Group," a cluster random sample of forty-six families resident in Ban (Village) Bu Phram and Ban Tablan, Prachinburi Province, Northeast Thailand, included 252 persons. This sample was comparable and representative of the total population (Segal, unpublished). Investigators visited each study family sixteen times, usually at two week intervals, between late April 1971 and early January 1972. During each visit they inquired of a history of fever and/or headache and collected capillary blood for quantitative parasite counts, and, on one occasion, for G-6-PD determination and hemoglobin electrophoresis.

The "Sickcall Group" consisted of 303 villagers, not included in the Family Group, who presented at weekly sickcall with signs and symptoms of malaria. Capillary blood specimens were drawn from members of the Group who were available at the completion of the study; these were processed identically to those of the Family Group.

The methemoglobin elution test (Gall et al., 1965) which histochemically distinguishes G-6-PD deficient from normal erythrocytes,

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Table 1. Observed and expected frequencies of G-6-PD deficiency and hemoglobins A and E in a subsample of Family Group females.

Frequency	Homozygous deficient	Heterozygous	Normal
Observed	1	4	39
Expected*	1.1	11.9	31.0

Frequency	Hemoglobin EE	Hemoglobin AE	Hemoglobin AA
Observed	4	15	25
Expected°	2.6	17.1	24.2

* $\chi^2 = 7.36$ df = 2 0.05 > p > 0.02.
° $\chi^2 = 0.78$ df = 2 0.70 > p > 0.50.

was used to identify deficient, heterozygous, and normal persons. Subjects with fewer than 15% deficient erythrocytes were classified as enzyme-normal and those with 85% or greater deficient erythrocytes as enzyme-deficient. Females with intermediate percentages of deficient cells were classified as heterozygotes. Parasite densities were determined by relating the parasite-leukocyte ratio on the blood films to the total leukocyte count. Hemoglobin electrophoresis was carried out on cellulose acetate strips. The high prevalence of hemoglobin E (45%) proved useful in checking family trees and determining the validity of sampling procedures.

The accumulated data permitted the comparison of infection rates, average number of parasitemic episodes per infected person, parasitemia-associated morbidity, and parasite densities in G-6-PD deficient and normal persons. The Family Group and Sickcall Group both contributed data on infection rates and

parasite densities. The Family Group only contributed data on the number of parasitemic episodes and parasitemia-associated morbidity. A history of fever preceding or fever coincident with documented parasitemia was recorded as evidence of symptomatic infection since other symptoms, complications, and mortality from falciparum malaria were not observed.

Results

The methemoglobin elution test was done on blood samples from 203 of the 252 Family Group members (81%). Twelve of 102 males were classified as deficient, 88 as normal, and 2 could not be classified. The frequency of the deficiency gene among males, calculated from a sub-sample of the father and eldest son from each study family, was 16.1% (10/62).

The test identified two of 101 females as enzyme-deficient, eight as heterozygous, and 91 as normal. Using family pedigrees and the results of G-6-PD testing of male relatives as references, nine of 15 females deficient or heterozygous by pedigree were correctly classified (sensitivity = 60%), as were 73 of 74 normal females (specificity = 99%). Using the frequency of the deficiency gene in males (p = 0.161), the expected numbers of deficient and heterozygous females could be calculated from $p^2 + 2pq + q^2 = 1$. Expected numbers were compared with observed numbers of enzyme-deficient, heterozygote and normal persons in a subsample of 44 unrelated Family Group females (Table 1). The test identified the one deficient and four of 11.9

Table 2. *P. falciparum* infection rates, average number of parasitemic episodes, and parasitemia-associated fever in G-6-PD deficient, heterozygous, and normal Family Group members.

Population	Sex	No. persons	No. (proportion) infected	No. of parasitemic episodes		No. (proportion) of episodes of parasitemia- associated fever
				Total	Average†	
Deficient	Male	13	9 (0.69)	45	5.0	4 (0.09)
Deficient	Female	2	0	0	0	0
Heterozygous (test)	Female	9	2 (0.22)	4	2.0	1 (0.03)
Heterozygous (all)*	Female	15	4 (0.27)	10	2.5	1 (0.01)
Enzyme-normal	Male	24	9 (0.38)	31	3.4	4 (0.13)
relatives	Female	20	4 (0.20)	8	2.0	2 (0.25)
Remainder of	Male	73	39 (0.53)	105	2.7	10 (0.09)
Family Group	Female	72	30 (0.42)	129	4.3	10 (0.08)

* This group includes individuals classified by pedigree as well as by the methemoglobin elution test.
† Number of parasitemic episodes per infected person.

(33.6%) heterozygotes expected. The distribution of Hemoglobins A and E in this subsample suggested that the sample was unbiased. Since the methemoglobin elution test appeared to underestimate the number of heterozygotes, data from women identified as heterozygous by pedigree, as well as by test, were included in the following analyses.

Infection rates, average number of parasitemic episodes per infected person, and rates of parasitemia-associated fever observed in deficient and heterozygous Family Group members were compared with those of their normal relatives and the remaining Family Group members (Table 2). Normal relatives were maintained as a distinct group to eliminate the variable of inter-familial risk of infection. Since the risk of parasite acquisition was higher among males (Segal, unpublished), the data were further divided by sex.

Although deficient males seemed to acquire falciparum infection in greater proportion and be parasitemic more often, on the average, than either their enzyme-normal male relatives or the remaining males in the Family Group, the differences, using the chi-square test with Yates correction, were not statistically significant. The proportion of heterozygotes infected and the average number of parasitemic episodes they experienced were very similar to those of their enzyme-normal female relatives and less than found for the remaining normal females. The differences were also not statistically significant. The fever data were based on small numbers and complicated by the fact that five of the seven instances recorded for enzyme-normal relatives were contributed by members of a single family.

Eighty-two members (27.1%) of the Sickcall Group who were available at the comple-

Table 3. *P. falciparum* parasitemias among a sample of G-6-PD deficient and heterozygous, and normal Sickcall Group villagers.

G-6-PD status	Deficient and heterozygous No.	Normal No.	Total
Smear result	(proportion)	(proportion)	
Positive for <i>P. falciparum</i>	4 (0.09)*	42 (0.91)	46
Negative for <i>P. falciparum</i>	3 (0.08)†	33 (0.92)	36

* 1 deficient, 3 heterozygotes.
† 1 deficient, 2 heterozygotes.

tion of the study were tested for G-6-PD deficiency. These eighty-two persons are considered to be representative of the entire group, as the median ages (9.3 years versus 8.3) and the proportion of males (0.50 versus 0.53) were similar. The proportion of deficient and heterozygous persons with parasitemia (0.09) was not different from the proportion of deficient and heterozygous persons without parasitemia (0.08) (Table 3).

The data describing parasite densities in parasitemic members of both the Family Group and Sickcall Group were pooled (Table 4). Since every episode of parasitemia experienced by a member of the Family Group during the study was included, Table 4 would have the effect of accentuating differences in parasite densities between deficient, heterozygous, and normal persons. Nevertheless, considering parasite densities both below and above 10,000 per cubic millimeter, there were no differences, using the chi-square test with Yates correction, in the number of episodes of parasitemia between either deficient and normal males or between heterozygous and normal females.

Table 4. Parasite densities in G-6-PD deficient, heterozygous, and enzyme-normal persons.

Population	Sex	Number (proportion) of parasitemias by range							Total
		1-499*	500-999	1000-4999	5000-9999	10,000-49,999	50,000-99,999	≥ 100,000	
Deficient	M	11 (0.28)	4 (0.10)	14 (0.35)	7 (0.17)	3 (0.08)		1 (0.02)	40 (1.0)
Normal	M	97 (0.62)	19 (0.12)	20 (0.13)	6 (0.04)	10 (0.06)	4 (0.02)	1 (0.01)	157 (1.0)
Heterozygous (test)	F	5 (0.72)		1 (0.14)		1 (0.14)			7 (1.0)
Heterozygous (all)†	F	10 (0.84)		1 (0.08)		1 (0.08)			12 (1.0)
Normal	F	78 (0.50)	21 (0.14)	31 (0.20)	15 (0.10)	7 (0.04)	2 (0.01)	2 (0.01)	156 (1.0)

* Parasites per cubic millimeter.
† This group includes individuals classified by pedigree as well as by the methemoglobin elution test.

Discussion

The variables of falciparum malaria measured infection rates, average number of parasitemic episodes, parasitemia-associated morbidity, and parasite densities did not vary significantly between G-6-PD deficient and heterozygous persons and enzyme-normal controls. This study of village populations is thus in agreement with reported studies of hospital and clinic populations in Thailand in its inability to demonstrate a protective role for G-6-PD deficiency against falciparum malaria.

Treatment of all villagers with symptomatic infections aborted possible development of very high levels of parasitemia. Aside from parasitemia-associated fever and occasional headache, no symptoms, complications, or mortality from falciparum infections were observed. It is thus conceivable that potential differences between G-6-PD deficient, heterozygous, and normal persons were not allowed to develop. Organization of the parasite density data, designed to compensate for the above and accentuate differences, nevertheless failed to show any.

It is not clear whether immunity might have masked otherwise observable differences between enzyme-deficient and normal persons. This possibility has been considered in reference to studies elsewhere (Allison and Clyde, 1961; Kruatrachue et al., 1962). Isolation of the variable of immunity was not feasible because of the small number of falciparum infections in the infant population (Segal, unpublished).

It has been suggested that the protective effect of G-6-PD deficiency is expressed only in heterozygotes, not in enzyme-deficient males (Bienzle et al., 1972; Hendrickse et al., 1971; Luzzatto et al., 1969). Evaluation of this possibility was particularly difficult because of the small numbers of heterozygotes and the decreased risk of parasite acquisition by village females in general (Segal, unpublished). Nevertheless, experience with falciparum infection by all heterozygotes identified was no different from that of their normal female relatives.

Because parasite densities were generally very low, it was not possible to determine if falciparum parasites preferentially infected the enzyme-normal erythrocytes of heterozygous

females. A hospital-based study is in progress to answer this question. Preliminary data indicate that parasite preference for normal erythrocytes, if present at all, is small (Noll, unpublished). If the conclusions of studies in Thai populations are valid, another explanation for the survival of the abnormal gene in this population must be found.

The studies of African populations, in general, do not agree with those in Thais. This does not necessarily mean, however, that the different conclusions are incompatible. There are many genotypic variants of G-6-PD and the abnormal genes present in African and Thai populations are probably not the same. Furthermore, *P. falciparum* in Thailand is almost entirely chloroquine-resistant, while in Africa it is uniformly chloroquine-sensitive. It has been shown that chloroquine resistance in *P. berghei* is accompanied by major metabolic differences (Howells et al., 1970). The Southeast Asian host as well as the infecting parasite may thus be different in important respects from their African counterparts. It should not be surprising to find that the host-parasite interaction might differ as well.

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Hemolytic Side Effects on Some Antimalarial Drugs*

E. SALVIDIO, I. PANNACCIULLI, F. AJMAR, G. GAETANI,
R. GHIO, M. MOLININO AND C. GARRE

Department of Hematology, The University of Genoa, Italy

ABSTRACT: G6PD deficiency of the B- variant is more severe in its clinical manifestations than the A- variant. In fact, not only are the hemolytic crises more severe in affected Caucasians but smaller dosages of antimalarial drugs are also capable of inducing rather severe hemolytic episodes.

This is the case of primaquine, which at 15 and 7.5 mg daily dosages still induces hemolysis in affected Caucasians whereas its effects on affected Negroes are relatively mild. Also the association of daily dosages of DDS and of weekly dosages of primaquine, 45 mg seems to enhance the destruction of G6PD deficient red cells of the B- variant. DDS has a mild hemolytic action at low dosages.

Of the relatively new antimalarial drugs used, DFD, a sulfone derivative, is hemolytic toward G6PD deficient red cells (B- variant) at single dosage of 200-400 mg, whereas G6PD deficient red cells belonging to the A- variant seem resistant up to the 600-800 mg dosages of DFD.

The only new antimalarial drugs devoid of hemolytic side effects on the G6PD B- variant are WR 33063 and WR 30090. If their antimalarial action were satisfying, as it seems, this is a major breakthrough in the prophylaxis of malaria.

The most common defect of red cell metabolism is glucose 6 phosphate dehydrogenase deficiency (G6PD). More than 100 million individuals are affected by this abnormality with a world-wide distribution (WHO Sci Grp, 1967). From a clinical point of view

G6PD deficiency is a major handicap for affected individuals; its main effect being an increased susceptibility to numerous drugs.

The discovery of G6PD deficiency by Carson et al., (1956) was directly related to clinical investigations of the hemolytic effect of primaquine, a drug widely used in malarial areas. Transfusion of 51 Cr tagged, G6PD deficient red cells into normal recipients receiving primaquine made it possible to ascer-

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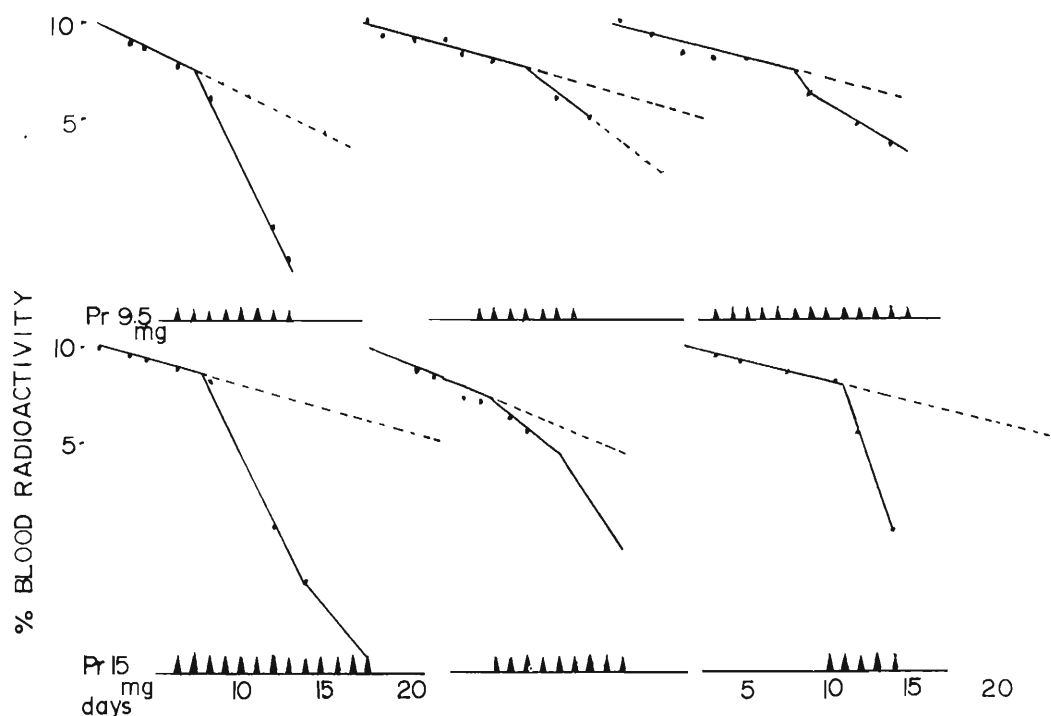


Figure 1. G6PD deficient ^{51}Cr tagged red cells transfused into six normal recipients receiving 7.5 or 15 mg of Primaquine daily. Destruction of G6PD deficient red cells follows in each instance after the administration of the drug.

tain that primaquine sensitivity was due to an intrinsic abnormality of the red cells (Dern et al., 1954).

Soon other substances were shown to be able to destroy G6PD deficient cells; such compounds involving also commonly employed drugs as sulfanilamide, acetanilid, phenacetin, nitrofurantoin (Dern and Bentler, 1955). It should also be remembered that G6PD deficient individuals are susceptible to episodes of hemolysis precipitated by infections and metabolic diseases, which may be complicated further by the administration of potentially hemolytic drugs (Larizza et al., 1960; Chatterji and Das, 1963).

It is now apparent that G6PD deficiency is not a single defect. It is a group of alterations due to numerous mutations affecting the G6PD molecule. In fact, more than 50 genetic variants of the G6PD molecule have been identified (Kirkman, 1971).

Each variant can be explained by a single amino acid substitution in the structure of the G6PD molecule. The principal variants are the G6PD A- affecting the American and African Negroes, the G6PD B- which is prevalent among affected Caucasians of Mediterranean origin, G6PD Canton, G6PD Panay, G6PD Seattle or Seattle-like.

Some differences have been found, from a biochemical and clinical point, among the two most common variants of G6PD deficiency, the A- and the B-.

The degree of red cell deficiency of the enzyme is moderate in the A-, severe in the B- variant. Drug sensitivity involves individuals affected by the two variants, although in different measures.

In Caucasians affected by the B- variant G6PD deficiency is markedly decreased also in leukocytes, platelets, liver cells (Brunetti et al., 1960; Pannacchiulli et al., 1966) and also in

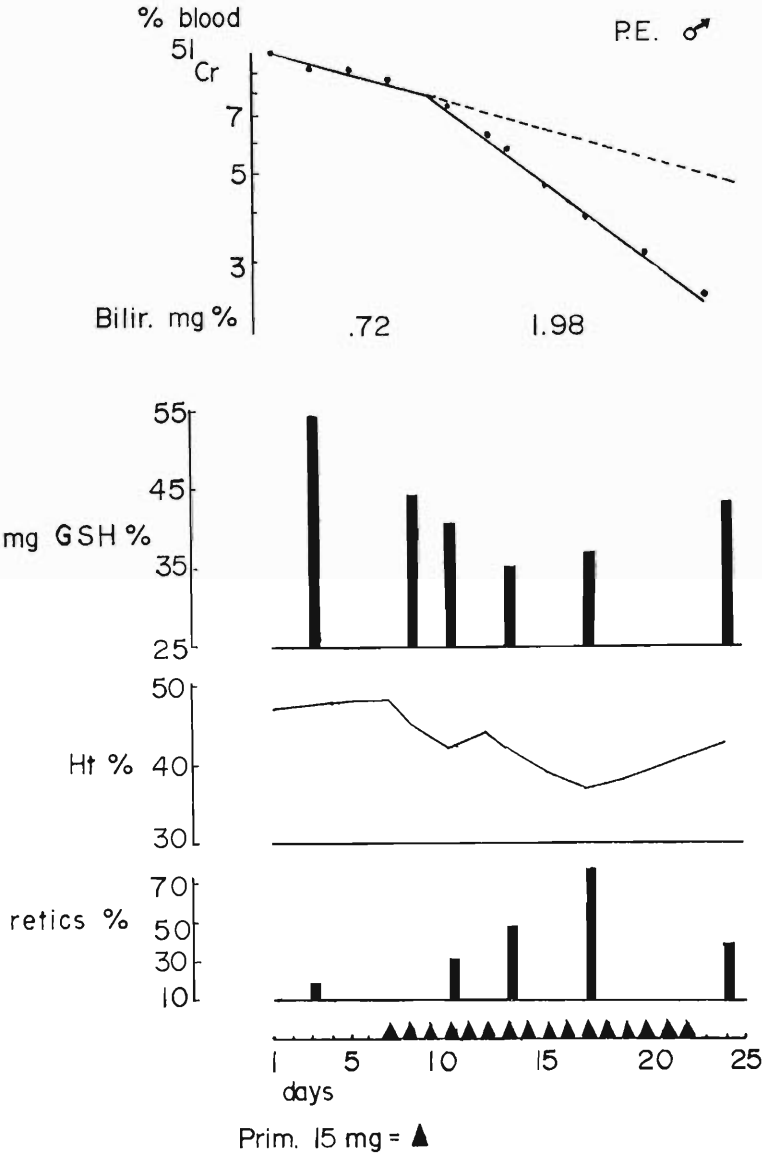


Figure 2. G6PD deficient Sardinian male receiving daily doses of 15 mg of Primaquine for 16 days. ^{51}Cr tagged red cell survival, bilirubinemia, red cell GSH levels, hematocrit and reticulocyte counts show the occurrence of a self-limited hemolytic crisis.

the skin in gastric and duodenal mucosa (Pannacciulli et al., 1966), in the kidneys and adrenal glands (Chan et al., 1965), in the spleen and in bone marrow granuloblasts and erythroblasts (Salvidio et al., 1967). Color

blindness occurs in individuals affected by the two variants but chronic non-spherocytic hemolytic anemia is peculiar only for individuals affected by the B-variant. In contrast to the self-limited course of acute hemolysis and the

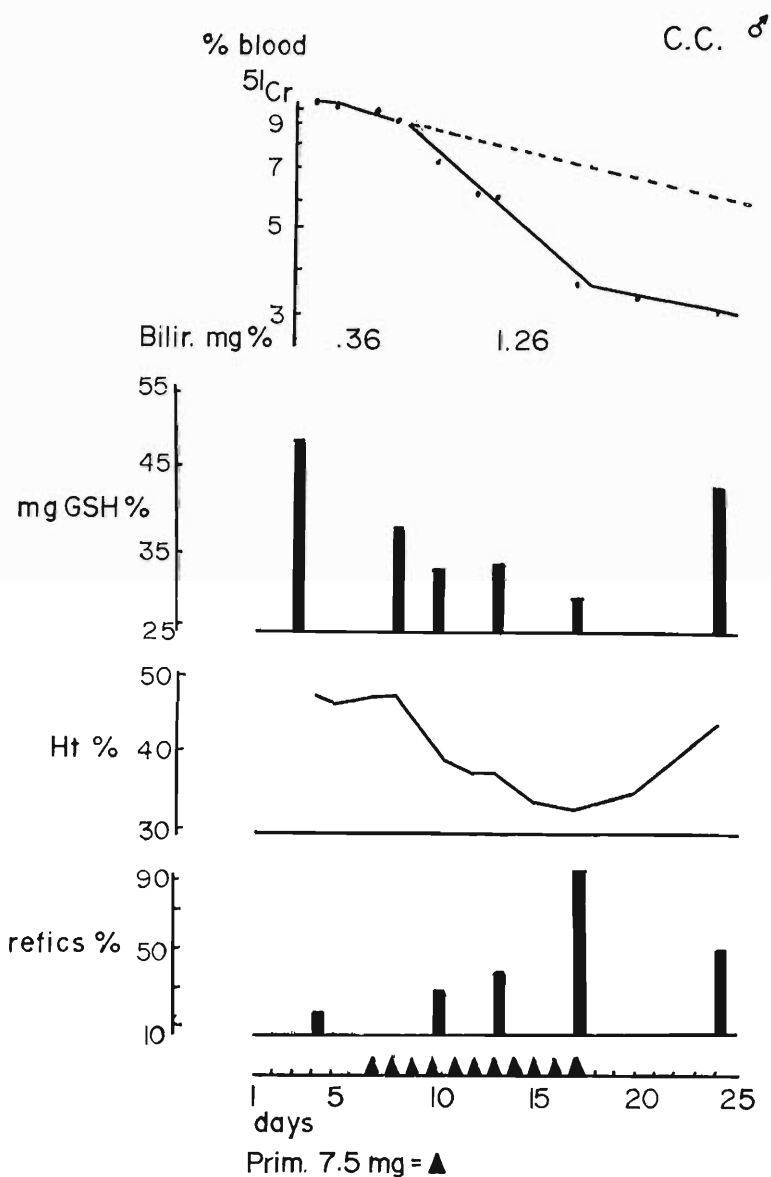


Figure 3. G6PD deficient Sardinian male receiving daily doses of 7.5 mg of Primaquine for 11 days. All data show the occurrence of a rather severe, self-limited hemolytic crisis.

relative resistance to continued drug exposure which characterizes the Negro (A-) type of deficiency, Caucasian individuals with the B-variant undergo a more severe, practically non limited hemolytic episode after exposure to fava beans or to daily dosages of 30 mg

of primaquine base (Pannacciulli et al., 1965; Salvidio et al., 1967).

In fact, in such an individual with G6PD deficiency of the red cell of the B- type, the acute hemolytic phase continued until the drug was withdrawn at a point when more than

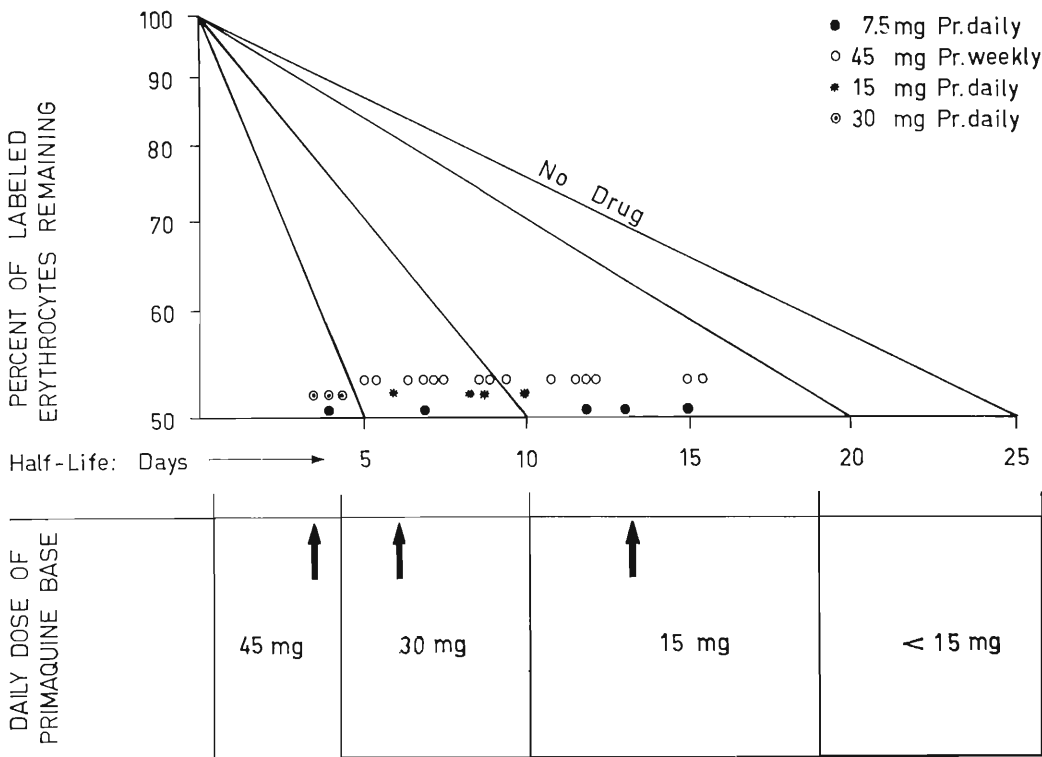
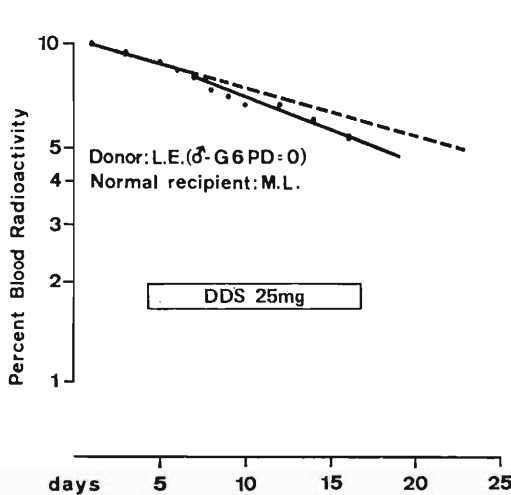


Figure 4. Hemolytic effects of various doses of Primaquine in G6PD deficient Sardinians, compared to the effects caused in hemizygous Negro males. The general outline of the graph, referring to the hemolytic effects of Primaquine in Negroes is taken from Kellermeyer et al.: there is a shift to the left in the cases of treated G6PD deficient Sardinians. (Symbols in the upper right corner.)

half the red cell mass had been destroyed. At this point 59 Fe was administered intravenously in order to label the newly formed red cells. When these young red cells were 7 to 14 days old, a second course of 30 mg primaquine base was administered and this was followed by a renewed drop in the hematocrit and the simultaneous destruction of the young red cells labelled with 56 Fe.

Thus in this subject with the Mediterranean B- variant not only the old but also the young red cells (aged from 10 to 15 days) were susceptible to drug induced hemolysis.

Figure 5. G6PD deficient 51Cr tagged red cells transfused into a normal recipient, to whom DDS was administered for 12 days at 25 mg dose. Moderate destruction of the transfused red cells occurred.



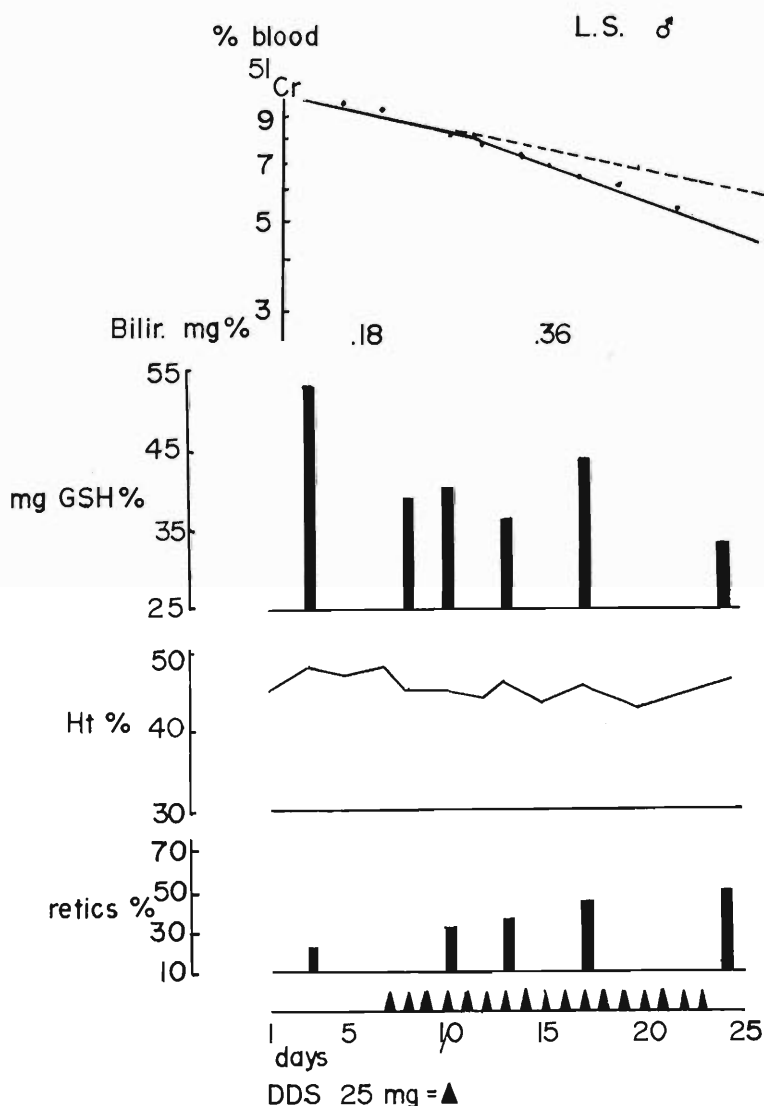


Figure 6. G6PD deficient Sardinian male receiving daily doses of 25 mg of DDS for 17 days. ^{51}Cr tagged red cell survival, bilirubinemia, red cell GSH levels, hematocrit and reticulocyte counts show the occurrence of hemolysis of moderate degree.

The greater susceptibility of individuals affected by the B- type variant to drug induced hemolysis is further confirmed by the administration of weekly dosages of 45 mg of primaquine base and 300 mg of chloroquine. This combination, employed as malaria prophylaxis, generally produces only mild hemolysis in Negro individuals affected by G6PD defi-

ciency of the A- variant (Brewer and Zaronetis, 1967). In Caucasian males with the B- variant, weekly administration of primaquine-chloroquine results often in hemolytic episodes of unpredictable severity, so that this regimen seems unadvisable for Caucasians with the enzyme deficiency of the B- type (Pannacciulli et al., 1959).

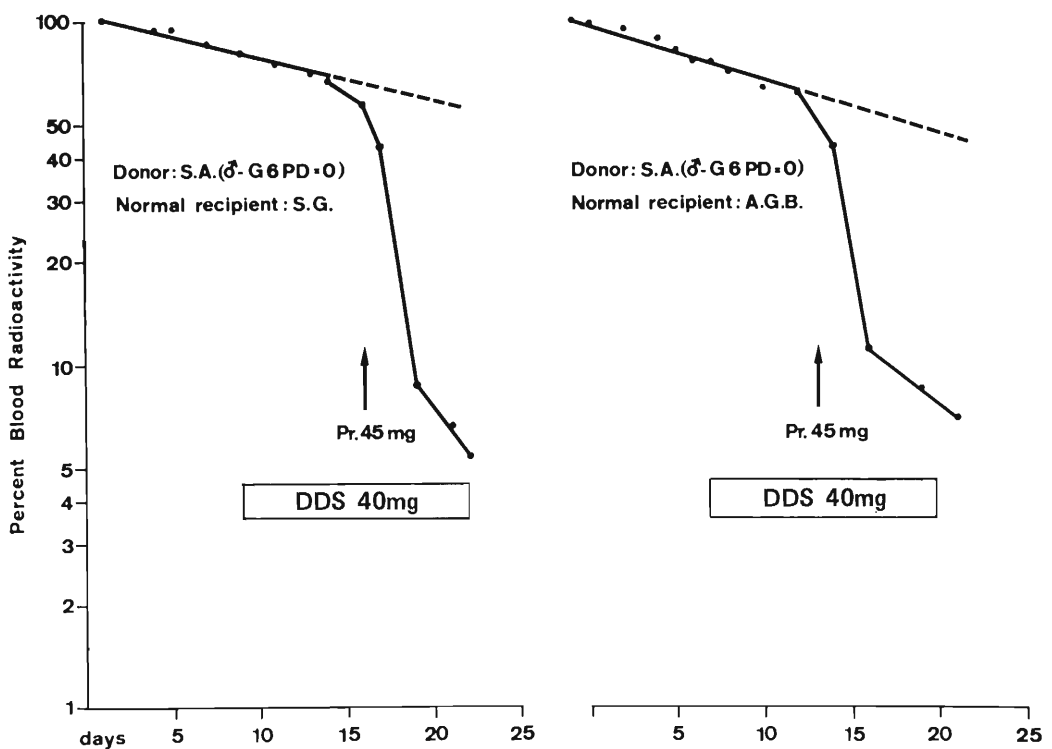


Figure 7. G6PD deficient 51 Cr tagged red cells transfused into two normal recipients receiving 40 mg of DDS daily and then (arrow) a single dose of 45 mg of Primaquine and 300 mg of Chloroquine. Sudden and complete destruction of the deficient cells follows the administration of the single dose of Primaquine.

Table 1. 51 Cr red cell survival studies in G6PD deficient Sardinian receiving 15 mg Primaquine daily.

N.	Cases	51 Cr T/2 before Primaquine (days)	51 Cr T/2 after Primaquine (days)	Levels of the Hematocrit		Peak	
				Mean baseline level (%)	Lowest value observed (%)	retics count (%)	bilir. mg (%)
1	P.E.	22	10	47	37	7.6	1.98
2	P.G.A.	21	9	44.5	34	7.2	0.72
3	P.P.	21	8.5	46	33	7.2	1.10
4	Z.A.	21	6	45	31	11.2	0.55
		21.2	8.3	45.8	33.6		

Table 2. 51 Cr red cell survival studies in G6PD deficient Sardinians receiving 7.5 mg Primaquine daily.

N.	Cases	51 Cr T/2 before Primaquine (days)	51 Cr T/2 after Primaquine (days)	Levels of the Hematocrit		Peak	
				Mean baseline level (%)	Lowest value observed (%)	retics count (%)	bilir. mg (%)
1	S.C.	23	12	35	21	—	—
2	M.G.	28	9	40	33	—	—
3	L.P.	26	13	45	39	4.2	0.72
4	C.C.	30	7	47	33	9	1.26
5	R.G.	21	4	57	44	—	—
		25.6	9	44.8	34		

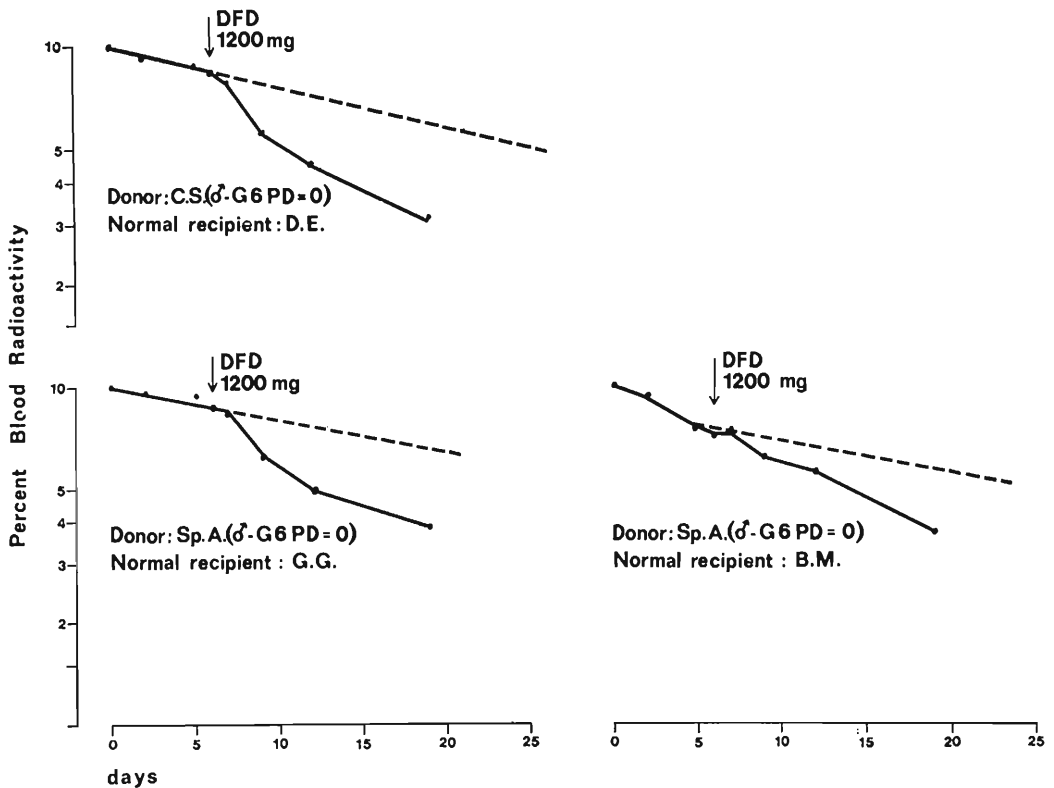


Figure 8. G6PD deficient ^{51}Cr tagged red cells transfused into three normal recipients receiving a single dose of 1200 mg of DFD. The G6PD deficient donors were of the B-variant. In all instances destruction of the G6PD deficient, transfused red cells followed the administration of the single dose of DFD.

In view of the greater severity of G6PD deficiency of the B-variant (Salvidio et al., 1969), Caucasian red cells affected by this inborn error of metabolism seem particularly suited for the study of the possible hemolytic side effects of antimalarial drugs.

Methods

Hematocrit, hemoglobin, reticulocytes, blood group determinations, plasma bilirubinemia were performed according to standard procedures. Methemoglobinemia was measured according to a modified Evelyn Malloy method. The biochemical methods used were the Glock and McLean for G6PD and 6PGD and the Long and Carson for GSSGR. An automatic Gilford 2000 recording spectrophotometer was used. GSH was measured with DTNB method of Beutler and associates.

Red blood cells were tagged with radioactive sodium chromate according to the technique of Mollison and Veall (1955).

Results

Studies with small dosages of Primaquine

It is known that primaquine at low dosages may have important indirect antimalarial effects as it was recently reported that individuals treated with small amounts of this compound failed to transmit malaria to mosquitoes (Carson, personal communication).

Starting from this consideration, G6PD deficient red cells of the B-variant, tagged with ^{51}Cr were transfused into normal recipients to which primaquine in daily dosages of 15 to 7.5 mg was administered. In all recipients the drug caused a rapid destruction of the transfused G6PD deficient red cells (Fig. 1).

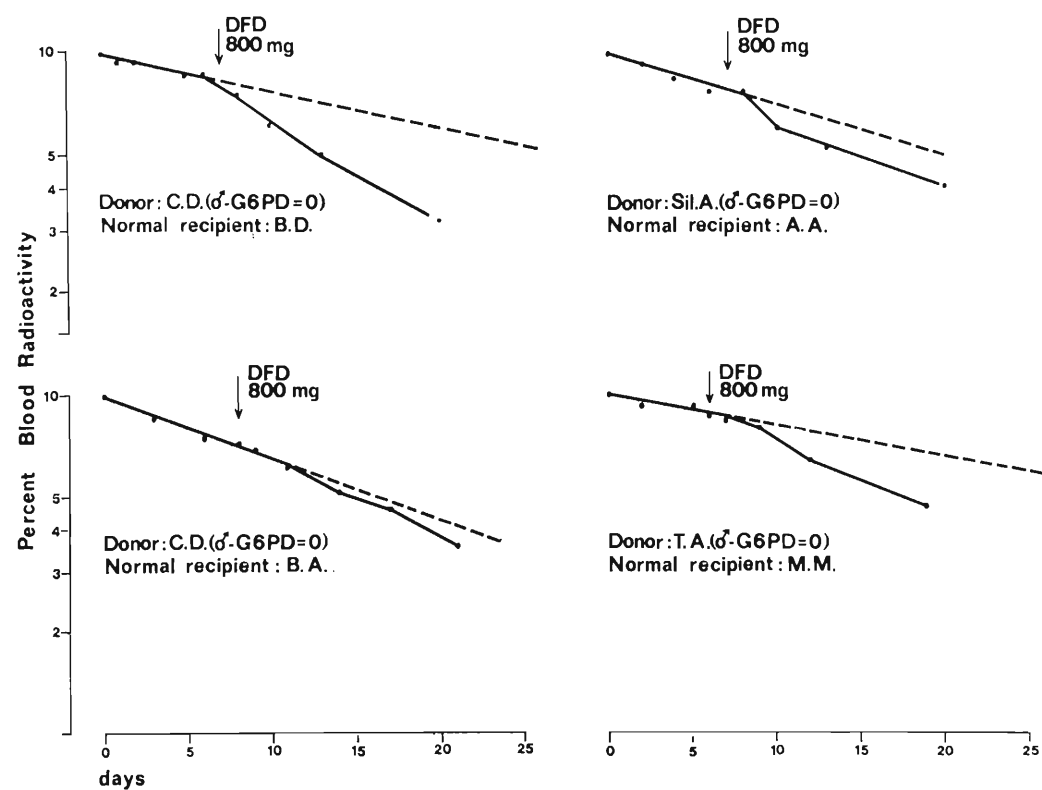


Figure 9. G6PD deficient (B- variant) ⁵¹Cr tagged red cells transfused into four normal recipients receiving 800 mg of DFD in a single dose. Destruction of the transfused red cells followed the administration of the drug.

Furthermore, 9 G6PD deficient volunteers (B-) had their red cells tagged with ⁵¹Cr. After baseline assessment of the ⁵¹Cr red cell survival, primaquine base was administered in daily dosages of 15 or 7.5 mg (Tables 1 and 2).

In all cases, 2-3 days after the administration of the drug was started, there was a marked drop in the hematocrit and a clear-cut shortening of the survival of the tagged red cells. Bilirubinemia and reticulocytosis increased, GSH content of the red cells decreased and returned to normal values only after the drug was withdrawn. There was no marked difference using the 7.5 mg daily dosage instead of the 15 mg daily dosage (Figs. 2 and 3). It can be concluded therefore that G6PD deficient Caucasians (B-

variant) are very sensitive not only to the 30 mg daily dosages of primaquine but also to the 15 and 7.5 mg daily dosages. It should be noted that, according to Alving and associates, hemizygous Negro males react to the 15 mg daily dosages of primaquine with only moderate hemolysis and mild anemia and to

Table 3. ⁵¹Cr red cell survival studies in G6PD deficient Sardinians receiving Dapsone.

N.	Cases	mg DDS daily	Baseline ⁵¹ Cr T/2 (days)	⁵¹ Cr T/2 after Dapsone (days)
1	C.P. a) b)	25 50	25	15 8
2	L.S.	25	30	17
3	M.G. a) b)	40 80	23	15

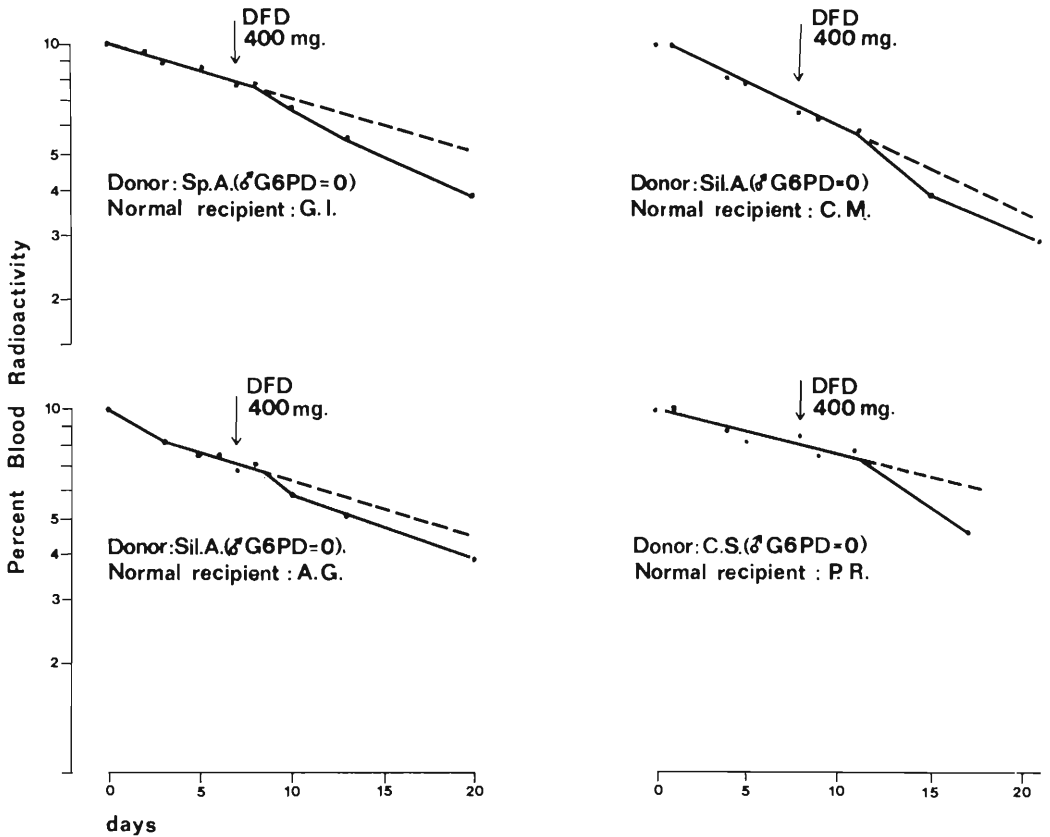


Figure 10. G6PD (B- variant) 51Cr tagged red cells transfused into four normal recipients receiving 400 mg of DFD in a single dose. Some destruction still follows the administration of the drug.

the 7.5 daily dosages with mild hemolysis (Fig. 4) and no anemia at all (Kellermeyer et al., 1962).

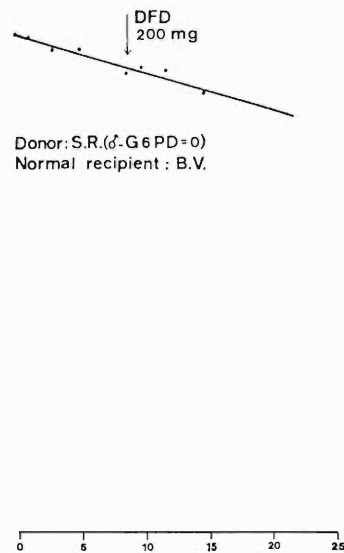
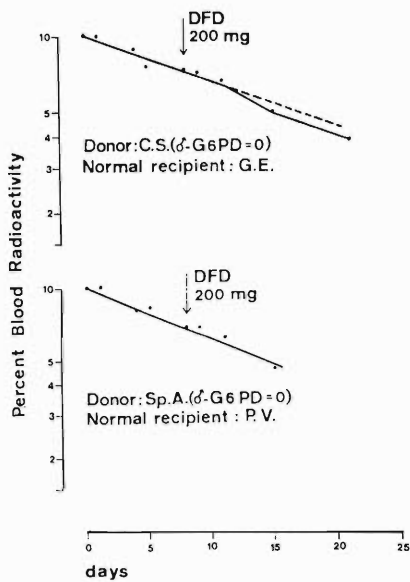
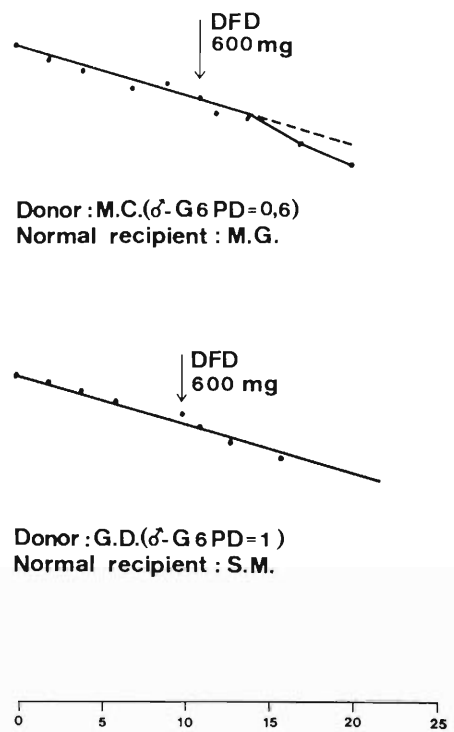
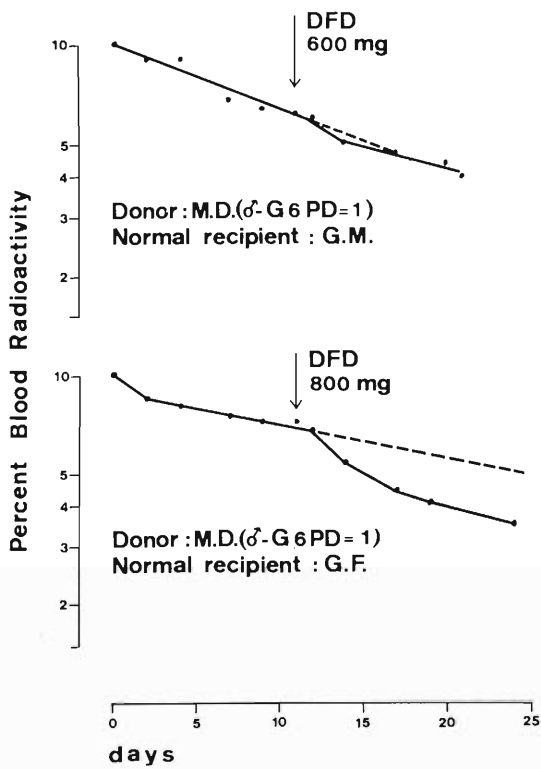
Studies with DDS on transfused, G6PD deficient red cells (B- type)

a) Diaminodiphenylsulfone (DDS) has been used in recent years as an antimalarian agent

(Degowin et al., 1966). G6PD deficient Negroes (A-variant) receiving small daily dosages of DDS showed some hemolysis. Being interested in the side effects of this sulfone on G6PD deficient cells of the B- variant we started at first to transfuse G6PD deficient cells belonging to Caucasians into normal recipients to which DDS was administered in

Figure 11. G6PD deficient (B- variant) 51Cr tagged red cells transfused into three normal recipients receiving 200 mg of DFD in a single dose. Minimal or no destruction of the transfused red cells follows the administration of the drug.

Figure 12. G6PD deficient (A- variant) 51Cr tagged red cells transfused into four normal recipients receiving 800 or 600 mg of DFD in a single dose. Destruction of the transfused red cells follows the administration of the 800 mg dose of DFD, whereas minimal or no destruction can be observed after the administration of DFD at single doses of 600 mg.



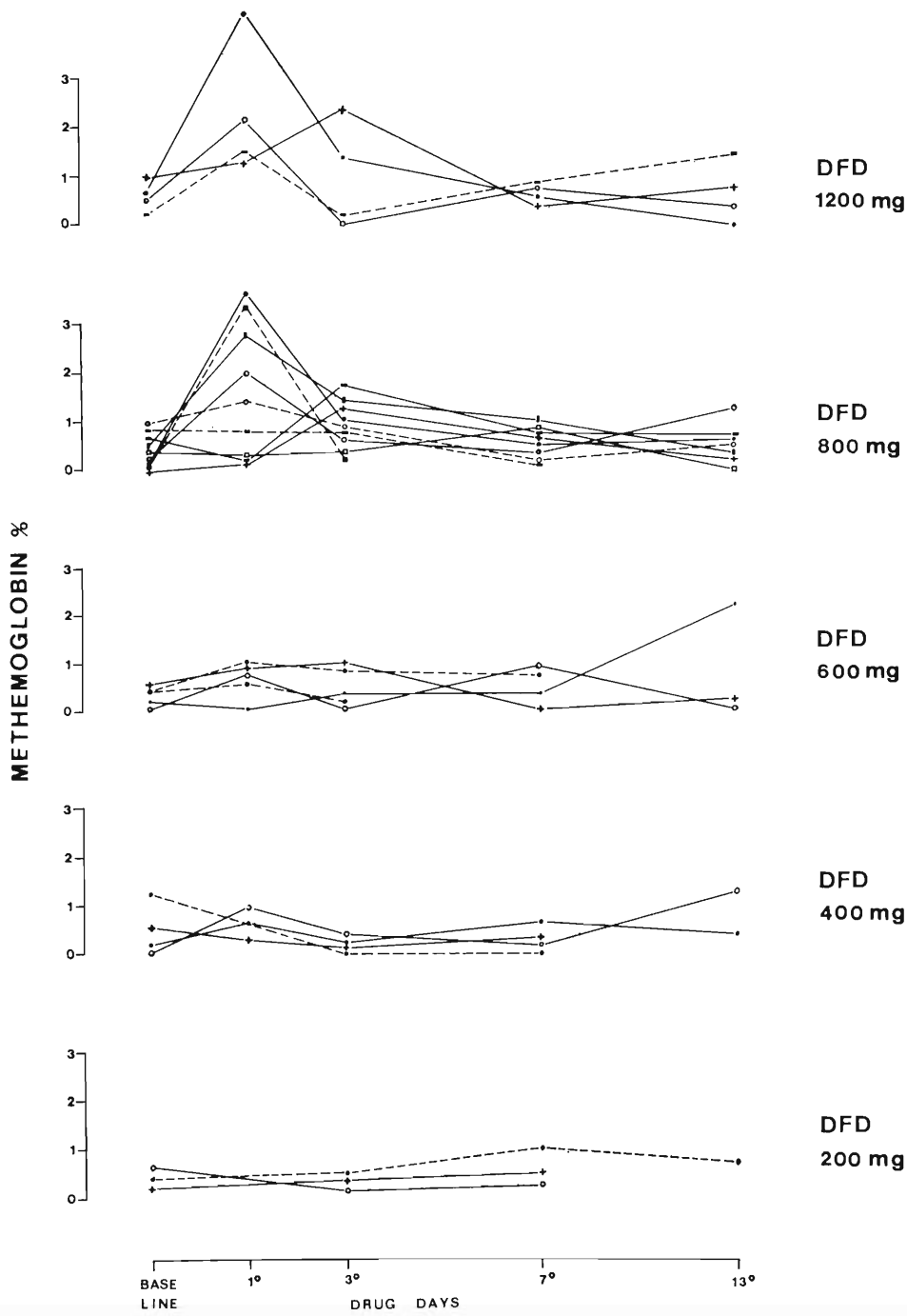


Figure 13. Methemoglobinemia increased (in normal subjects) only when 1200, 800 and 600 mg of DFD in single doses was administered.

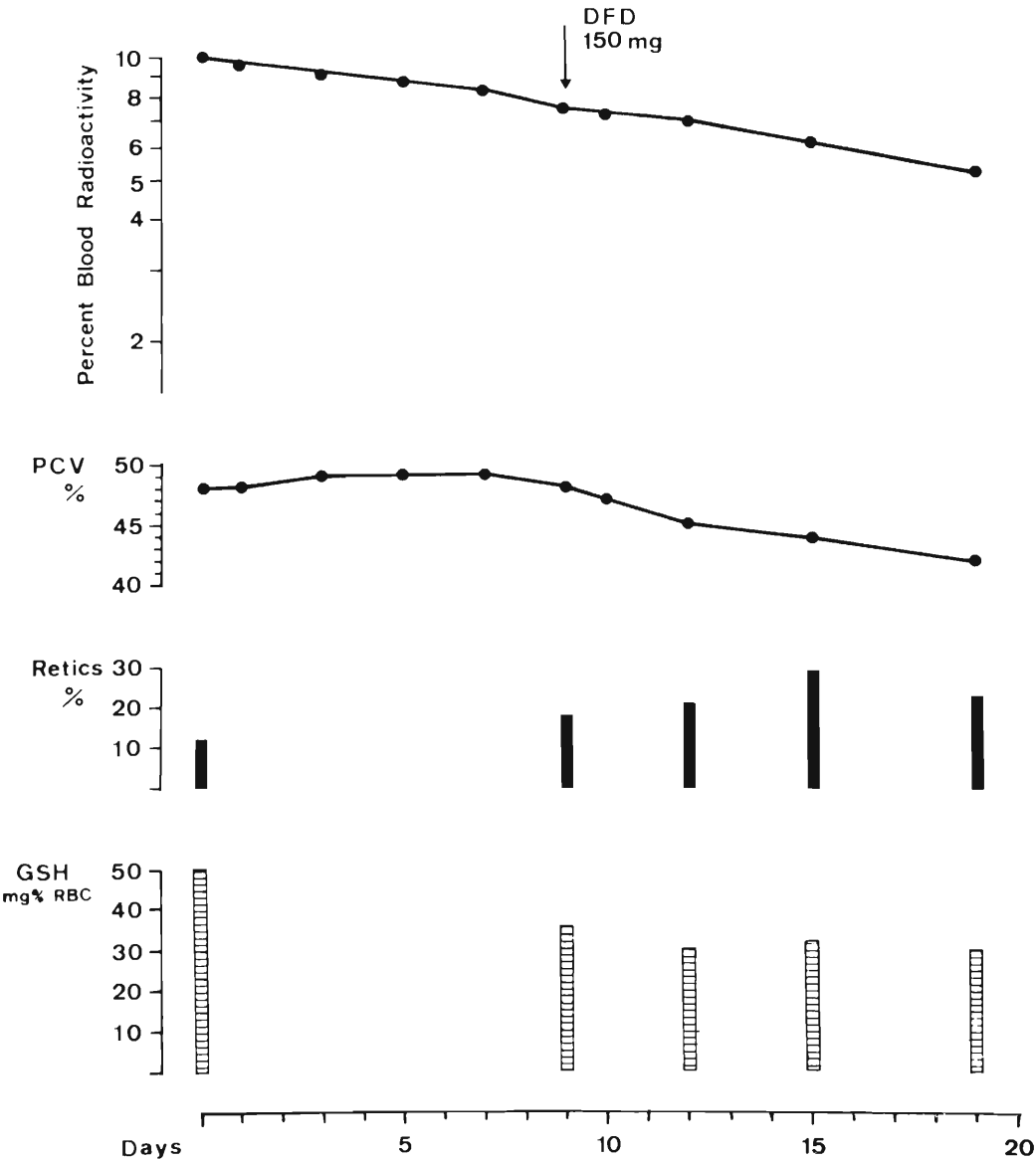


Figure 14. G6PD deficient Sardinian male receiving a single dose of DFD. There is no modification of the 51 Cr tagged red cell survival, and in the hematocrit and hemoglobin values, whereas bilirubinemia, GSH levels of the red cells and reticulocytes increased.

different dosages. The G6PD deficient tagged red cells of 8 Caucasian males and of 2 females were transfused into 15 normal recipients, who received thereafter daily dosages of Dapsone (25, 40, 50, 80 mg) for periods

ranging from 10 to 19 days. The normal recipients did not show any modifications of their own hematological baseline values. In 11 cases the daily administration of DDS was associated with a shortened survival of

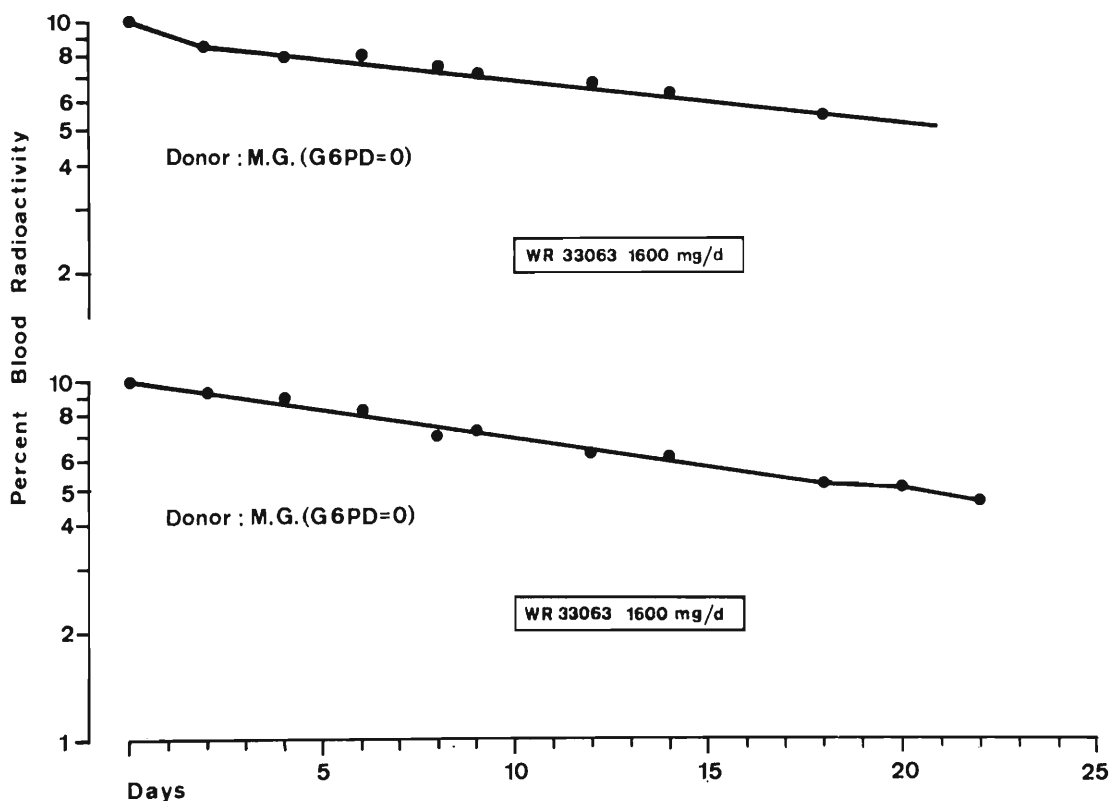


Figure 15. G6PD deficient (B- variant) ^{51}Cr tagged red cells transfused into two normal recipients to which WR 33063 was administered for six days at 1600 mg/day. No destruction of the transfused red cells followed after the administration of the drug.

the ^{51}Cr tagged, G6PD deficient, transfused red cells (Fig. 5). In only 4 out of 15 cases no shortened survival of G6PD deficient red cells could be detected during the administration of the drug. It must be observed, however, that 3 of these cases received 40 mg of DDS daily (French brand, Disulone) and one case received 25 mg of DDS daily (American brand).

b) Five G6PD deficient Sardinian male volunteers received various, sometimes increasing, amounts (25, 40, 50, 80 mg) of DDS daily. In each case a moderate but evident hemolysis followed the administration of the drug (Fig. 6). There was also some modification in the hematocrit. The shortening of the ^{51}Cr tagged, G6PD deficient red cells seemed to be dose related. There was a constant rise in reticulocyte counts and in plasma

bilirubinemia. Red cell GSH decreased during the administration of the drug.

Studies on the synergistic effects of the joint administration of DDS and primaquine

Investigations by Eppes and associates (1967) have indicated that DDS administered in small daily doses together with weekly concurrent administration of 45 mg of primaquine, has protective effects against *Plasmodium falciparum*.

In view of these facts ^{51}Cr tagged, G6PD deficient red cells belonging to 5 Sardinian males were transfused into eight normal recipients who received daily dosages of 25 or 40 mg of DDS. After several days (from 7 to 12 days) of DDS administration, a single

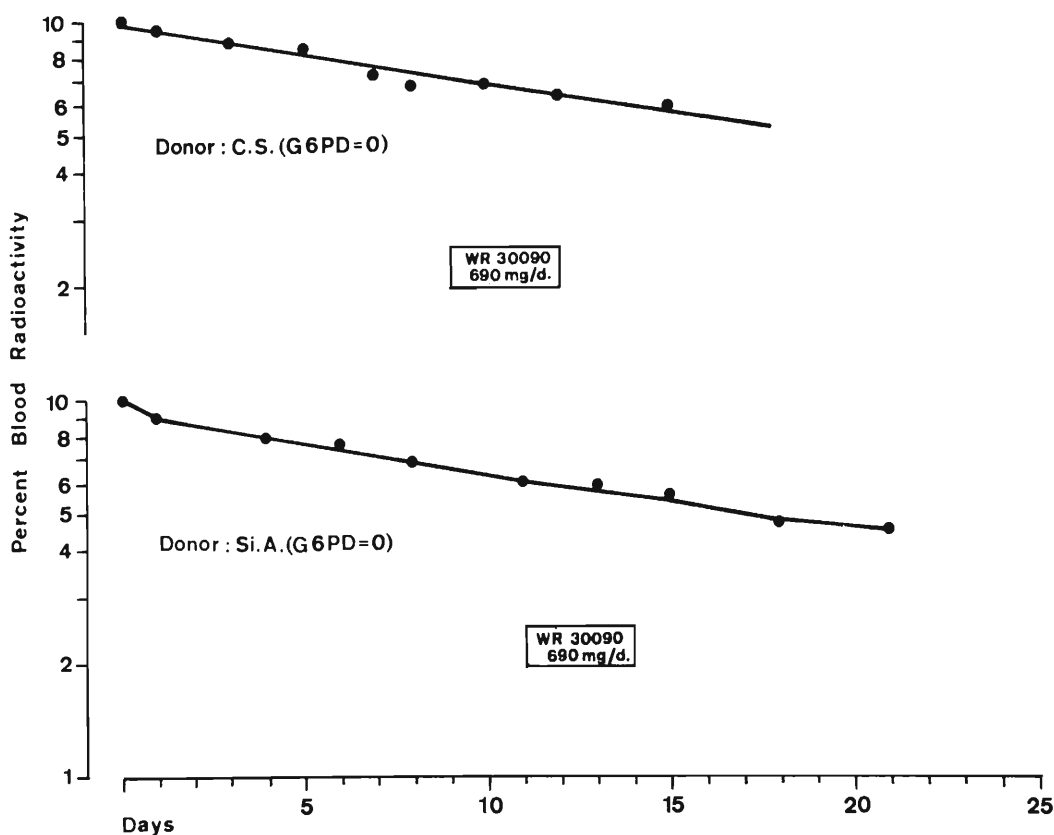


Figure 16. G6PD deficient (B- variant) ^{51}Cr tagged red cells transfused into two normal recipients to which WR30090 was administered for three days at 690 mg/day. No destruction of the transfused red cells followed after the administration of the drug.

dose of 45 mg of primaquine base was also administered to the normal recipients. Whereas DDS alone resulted mainly in a moderate shortening in the survival of the G6PD deficient, transfused red cells, as already described before, the administration of the single dose of primaquine, added to the sulfone, resulted (Fig. 7) in sudden destruction of the G6PD deficient red cells (Salvidio et al., 1970).

Studies on DFD (Di-N-formyl-diamino phenyl-sulfone)

a) ^{51}Cr tagged red cells belonging to G6PD deficient Caucasians (B- variant) and transfused into normal recipients, are destroyed by the administration to the recipients

of single dosages of 1200, 800, 600 and 400 mg of DFD (Figs. 8, 9 and 10).

No definite destruction of G6PD deficient red cells could be detected after the administration of DFD in single dosages of 200 mg (Fig. 11).

b) Comparative studies on the fate of Negro G6PD deficient red cells (A- variant) transfused into normal subjects receiving the drug, seem to indicate that single dosages of 800 mg of DFD destroy the G6PD deficient red cells, whereas single dosages of 600 mg of DFD do not seem to have similar hemolytic effect (Fig. 12).

Methemoglobinemia in normal subjects receiving DFD increased only when 1200, 800 and 600 mg (single dosages) were administered.

Table 4. 51Cr red cell survival studies in G6PD deficient Sardinians receiving varying amounts of Primaquine.

	Prima- quine mg.	G6PD deficient subjects	Mean T/2 Cr 51 r.b.c. in G6PD deficient subjects after administration of Primaquine (days)	Levels of the Ht in G6PD deficient subjects receiving Primaquine		Mean T/2 of Cr 51 G6PD deficient r.b. cells transfused into normal recipients after the administration of Primaquine (days)
				Mean baseline level before Primaquine (%)	Mean lowest value observed after Primaquine (%)	
Daily	7.5	males	9	44.8	34	7.3
	15	males	8.3	45.8	33.6	4.1
		males	3.8	42	26.6	4.3
	30	females	7.2	40	27.8	5.2
Weekly	45(*)	males	9.2	42.8	34.4	6.9

(*) With the exclusion of those G6PD deficient Sardinian males who failed to show any hemolysis after the administration of single or multiple doses of the drug.

c) Five young G6PD deficient male volunteers, otherwise healthy, received a single dose of 50, 100, 100, 150, 150 mg of DFD (Fig. 14). In all cases there were no great modifications of the hematocrit and hemoglobin values, whereas reticulocytes increased almost constantly. There was also a consistent increase of bilirubinemia, while methemoglobinemia rose in some cases. GSH content of the red cells decreased in some cases, while other red cell enzymes tested showed no significant modifications.

The Cr 51 half-time of the autologous G6PD deficient red cells showed only a moderate decrease after the administration of DFD.

In conclusion, with every single dosage of the drug, all subjects showed a mild hemolytic reaction, well compensated at the given dosages.

This study confirms our findings on the hemolytic action of DFD on transfused G6PD deficient red cells.

As far as we know these are the first cases of G6PD deficient volunteers treated with DFD.

Studies on antimalarial drug WR 33063*

51 Cr tagged, G6PD deficient red cells belonging to male donors (B- type) were transfused into 6 normal recipients who, after baseline assessments, received the new drugs.

The 6 cases who received WR 33063 (1600 mg daily for 6 days) did not show modifications of any biochemical test performed.

In 5 cases the 51 Cr half-time of the transfused G6PD deficient red cells was not modified by the drug (Fig. 15). In one recipient, soon after the administration of the drug, there was an increased rate of disappearance of the radioactivity from the blood.

No increase in blood methemoglobinemia was observed.

Studies on antimalarial drug WR 30090**

G6PD deficient (B- type), 51 Cr tagged red cells belonging to three male donors were transfused into five normal recipients.

Three recipients received WR 30090 at 690 mg per day for three days. The last two recipients received the drug at the same dosage for six days, according to the instructions sent from the Walter Reed Army General Hospital (Col. Conrad).

In four out of five cases the 51 Cr tagged, G6PD deficient transfused red cells were apparently not damaged by the drug, even at the prolonged schedule of administration (Fig. 16). In one case the transfused red cells disappeared from the circulation before the administration of the drug, all other parameters remaining normal.

* 6-Bromo- -di-n-heptylaminomethyl- γ-phenantrene-
methanol-hydrochloride.

** 6,8-Dichloro-2-(3', 4'-dichlorophenyl) -(Di-n-butyl-
aminoethyl)-4-quinoline methanol hydrochloride.

In one case there was a slight increase in methemoglobinemia.

Studies on the effects of single dosages of Sulfalene on transfused, G6PD deficient red cells of type B- (Mediterranean)

Sulfalene (Kelfizin) was administered at a single dosage of 1.5 g to five normal recipients to whom G6PD deficient, 51 Cr tagged red cells had been previously transfused.

In no instance was the 51 Cr half-life of the G6PD deficient, transfused red cells, shortened by this dosage of Sulfalene. All other biochemical tests performed on the recipients (including methemoglobin determinations) were normal throughout this study.

Studies on the effects of single dosages of Sulfalene on G6PD deficient volunteers of the Mediterranean variant

Two Sardinian males with G6PD deficiency of their red cells (type B-) volunteered to take a single dose of 1 g and 1.5 g of Sulfalene (Kelfizin) respectively.

In both cases hematocrit, hemoglobin, reticulocytes, bilirubinemia, methemoglobinemia, and all other biochemical tests performed, remained unchanged after the administration of the drug. The volunteer who received 1.5 g of Sulfalene had his red cells tagged with 51 Cr. The survival of the labelled cells, however, was normal before and after the single dosage of the drug.

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Comments on Blood Enzyme Deficiencies

L. LUZZATTO

Department of Haematology, University College Hospital, Ibadan, Nigeria

Judging from the programme, the emphasis in this symposium, is clearly—and rightly—on how malaria can be controlled either by manipulating to our best advantage natural immunological processes, or by combining a variety of old and new chemotherapeutic agents. The impact of both these methods of approach has already been impressive in the past, and the present strides are exciting. Yet, the extent to which either can be perfected will depend on our ability to explore and exploit the genetic properties of the organisms involved: and this includes, in the characteristic dualism of parasitology, both the parasite and the host. For instance, it is now clear that genetic factors in the host can influence markedly resistance to malaria, whether directly by offering a less hospitable environment to the parasite, or indirectly by affecting immune processes. On the other hand, when we use chemotherapy, we must constantly come to grips with the limitations imposed by the arising of resistant plasmodia and by the possible side effects in the human host.

The work done over the years, amongst others by Salvidio and his co-workers indicates clearly how major side effects of antimalarial agents are a function of the genetic constitution of the host. Not only does the haemolytic potential of numerous drugs characteristically depend on deficiency of erythrocyte glucose

6-phosphate dehydrogenase (G6PD): it is further a function of particular types of this deficiency. Thus, it has been known for some time that chloroquine and quinine are essentially safe in subjects with the type of deficiency designated A⁻ (see Table 1), whereas they can be quite dangerous in persons with the Mediterranean type of deficiency. This is not surprising, since it is now quite clear that all common types of G6PD deficiency are not due to a complete absence of the enzyme, but rather to the presence of a structurally abnormal enzyme having either decreased activity, or decreased stability, or both (see review by Kirkman, 1971). In all populations where suitable genetic studies have been conducted, the abnormal enzyme could be shown to be the product of a mutant gene at the G6PD structural locus. Moreover, in each of the major population groups where G6PD-deficiency is prevalent, the common abnormal allele turns out to be a different one (see Table 1)—without prejudice to the possibility that even within a certain population group, several common deficient alleles might exist: this indeed seems to be the case, for instance, in Greece (Kirkman *et al.*, 1965; Stamatoyannopoulos *et al.*, 1972) and in Thailand (Flatz and Tantachamroon, 1970; Panich, 1972). Whereas most of the variants listed in Table 1 have been well characterised at the biochemical and haematological level, detailed studies on drug-induced haemolysis, of the kind presented today, seem to have been confined almost exclusively to subjects with either the

(Portions of the experimental work carried out in the University of Ibadan and referred to in this paper have received financial support from the United States Public Health Service (Grant GM 17261), and from the World Health Organization).

Table 1. Heterogeneity of “common” variants of Glucose 6-Phosphate Dehydrogenase associated with enzyme deficiency.*

Name of variant	Activity in RBC (approx. % of normal)	Population	Highly endemic malaria ⁺	Severity of drug-induced Haemolysis
A-	8-20	Africa	Yes	Self-limited†
Mali [‡]	5	W. Africa	Yes	Probably self-limited
Mediterranean [§]	0-7	Mediterranean	"Till recently	Unlimited**
Athens	20-25	Greece	"Till recently	Not known
§	5-10	Thailand	Yes	Not known
Taiwan-Hakka	2-9	Hakka-Chinese	No	Probably unlimited¶
Canton	4-24	S. China	Yes	Probably unlimited¶
Union	0-3	Philippino	Yes	Not known
Indonesia	0-5	Indonesia-Thailand#	Yes	Not known
Markham	1.5-10	New Guinea	Yes	Not known

* All data are from Yoshida, Beutler & Motulsky (1971), except where stated otherwise.
+ Data after WHO survey, 1967, in Pampana (1969). [‡] Kahn *et al.* (1971).
† Tarlov *et al.* (1962).
** See Pannaciuili *et al.* (1965).
§ Probably heterogeneous; see Kirkman *et al.* (1965), Stamatoyannopoulos *et al.* (1971).
§ "Fast-moving" variant described by Flatz and Tantachamroon (1970); may be identical to Canton variant.
¶ See McCurdy *et al.* (1970).
Panich, V. (1972).

A- or the Mediterranean type of G6PD deficiency (previous data tabulated in WHO, 1967). Similar work in other populations is much needed, especially because the “severity” of G6PD deficiency is not merely a function of the residual level of enzyme found in the erythrocyte. Rather, the impact of deficiency on red cell physiology will depend critically on the qualitative properties of the abnormal enzyme. For instance, both Mediterranean and A- have some essentially advantageous kinetic properties which offset, to a significant extent, the adverse effects of deficiency (Kirkman *et al.*, 1965; Luzzatto and Afolayan, 1971). Other variants, not listed in Table 1 because they are not common, for instance Chicago (Kirkman *et al.*, 1964), Tripler (Engstrom and Beutler, 1970), Alhambra (Beutler and Rosen, 1970), Freiburg (Busch and Boie, 1970) are associated with more severe manifestations (chronic not drug-dependent haemolysis), in spite of levels of activity in the erythrocyte comparable to that found in A- and Mediterranean, or even higher, presumably because they have unfavourable physico-chemical properties. In this respect, one would like to know which type of deficient erythrocytes are to be used as the most sensitive indicator while screening for the haemolytic potential of new drugs. Whereas not much can be said about the deficient variants common in Asia and in the Pacific, there is no doubt that, between the two thor-

oughly investigated variants, the Mediterranean is more sensitive than A-, and it is encouraging to learn that some new agents have shown no haemolytic effect by the elegant and safe technique used by Salvadio and collaborators. Because of the heterogeneity of the “Mediterranean” variant revealed in certain areas by electrophoretic and kinetic studies, it would be interesting to know whether similar studies have been conducted in Sardinia. This might be of more than academic importance when deficient cells from a particular subject are being used as indicators of haemolysis.

The mechanism of drug-induced red cell destruction in G6PD deficiency has been the subject of numerous investigations and speculations (see Jaffe, 1972), but it has not yet been elucidated in detail and it will not be discussed here. One intriguing question is whether there is any relationship between the protective effect of G6PD deficiency against malaria (see below) and the fact that anti-malarial drugs are prominent amongst those causing haemolysis of G6PD-deficient cells. Since the protective effect against malaria is not fully understood either, one can hardly expect a clearcut answer. However, no matter how ultimately similar the action on the red cell of drugs widely different in structure, like primaquine and DDS, may be, the mechanism of their antimalarial action is certainly quite different. Thus, one is led to

Table 2. Schematic summary of data on increased resistance against malaria conferred by different genes.

	Thalassaemia	Glucose 6-phosphate dehydrogenase deficiency	Haemoglobin S
Geographic distribution ^a	+	++	+++
Gene frequency in Nigeria	0.004 ^b	0.21 ^c	0.13 ^d
"Micro-mapping" ^e	+	+	+
Age Stratification	?	?	+
Parasite rate/density	?	+	+
Mortality from malaria	?	+	+
Mechanism	?	+	+

(^a) See Livingstone (1967).
(^b) Esan (1970).
(^c) Luzzatto, Allan and De Flora (1965).
(^d) See Table III.
(^e) Correlation between malaria endemicity and frequency of abnormal gene *within* a restricted geographical area: See for instance data by Allison (1954) for haemoglobin S in East Africa, Siniscalco *et al.* (1966) for thalassaemia and G6PD deficiency in Sardinia, Stamatoyannopoulos, Panayotopoulos and Motulsky (1966) for G6PD in Greece, Bienzel, Okoye and Goegler (1972) for haemoglobin S in Togo.
(^f) Gilles (1964).
(^g) Bienzle *et al.* (1972).
(^h) Gilles (1964).
(ⁱ) Raper (1956).
(^j) Luzzatto, Usanga and Reddy (1969).
(^k) Mackey and Vivarelli (1954); Miller, Neele and Livingstone (1956); Luzzatto, Nwachuku-Jarrett and Reddy (1970).

conclude, perhaps with slight reluctance, that for the same enzyme abnormality in the red cell to be underlying haemolysis by anti-malarial drugs and relative resistance against malaria is a sheer coincidence.

Coming to the evidence for this resistance against malaria, and to its possible basis, the paper by Segal, Noll and Thiemanun contains important new information. Indeed, while the remarkable geographical correlation between world distribution of malaria—especially of the subtertian variety—and that of G6PD deficiency was amply documented and discussed by the mid-sixties (*e.g.* by Livingstone, 1967), direct evidence of protection by G6PD deficiency against malaria has been obtained only rather recently. To a large extent, the methods used have been similar to those that have proven valuable in the analogous study of the sickle-cell trait (see Table 2). However, the case of G6PD deficiency has been more difficult to unravel for a variety of reasons, some of which have been discussed elsewhere (Luzzatto, 1972; Bienzle *et al.*, 1972). In essence, it is clear that protection afforded against *P. falciparum* by any genetic trait is always only a relative one. Therefore, cross-sectional studies have consistently failed to reveal significant differences in prevalence of parasitaemia between G6PD-deficient subjects and controls, and Segal's series is no exception. By contrast, analysis of parasite densities in

a large group of female Nigerian children heterozygous for the A⁻ type of G6PD deficiency has revealed significant protection against high rates of parasitaemia (Bienzle *et al.*, 1972). These data are being complemented convincingly, in my own view, by the longitudinal study of Segal *et al.*, from which we learn that adult subjects with the appropriate genetic constitution suffer less episodes of parasitaemia: thus, not only life-threatening attacks, but also milder morbidity seems to be modified by the trait. Again, it is the heterozygous "intermediate" females, and not the hemizygous males that are protected. Since G6PD deficiency in Thailand is heterogeneous (see Table 1), it would be interesting to analyze further the data by subdividing the subjects according to the different electrophoretic variants found in their red cells. At any rate,—while similar studies in yet other populations would be desirable—since all Thai variants are probably different from the A⁻ type found in Nigeria, it appears that the protection of heterozygous females may be a general feature of this human polymorphism. Its biological and perhaps sociological implications may be more far-reaching *that they* appear at first sight. For instance, one could ask whether a higher mortality from malaria of female children in areas of holoendemic malaria will significantly contribute to the sex-ratio being imbalanced in adults in favour

Table 3. Decreased prevalence of the sickle cell trait in various clinical syndromes (Ibadan, Nigeria).

	Number studied	% AS	Significance of difference from controls	Reference
Cerebral malaria	29	0	P < 0.02	Edington & Watson-Williams (1965)
Tropical splenomegaly syndrome	44	7	P < 0.01	Sagoe (1971)
Burkitt tumor	100	17	P < 0.1	Williams (1969)
Nephrotic syndrome	72	12.5	P < 0.02	Adeniyi (1972)
CONTROLS	100,000	25.4		Unpublished*

* From the Records of the Blood Bank, University College Hospital, Ibadan, 1961–1970—See also Watson-Williams, 1965; Esan, 1970.

of females, and whether this imbalance might in turn help to stabilize a polygamous mating pattern in a number of populations.

Whereas from the point of view of natural selection a genetic trait will be favoured substantially only if the selective force is important, as in lethal malaria, its relevance to human pathology may be wider. Several years before extensive studies on G6PD were carried out, the protective effect of the sickle-cell trait, not discussed here, had already been demonstrated with respect to heavy *P. falciparum* parasitaemia (see Table 2). It has later become apparent that, based on its prevalence, the sickle-cell trait may have protective value against certain special conditions which are known or are thought to be somehow related to malaria (Table 3). Amongst these, the nephrotic syndrome in childhood deserves special mention, not only because of its high incidence in many parts of Africa, but also because there is overwhelming evidence that *P. malariae* is important in its pathogenesis (see Edington & Gilles, 1969). Thus, one might infer that the protective effect of the haemoglobin S trait is not limited to *P. falciparum*. With respect to thalassaemia (see Table 2), it is noteworthy that, although the hypothesis of a malaria-balanced polymorphism was first formulated for this trait (Haldane, 1949), direct studies of parasite rates and densities in thalassaemia carriers *versus* controls have been lacking, nor has any detailed mechanism for possible protection been visualized. While the Mediterranean areas, whence “thalassaemia” derived its name, are now essentially free of malaria, portions of Asia, where *P. falciparum*, α - and β -thalassaemia are all prevalent, would seem apt for such studies.

Since a number of genes at several genetic loci seem to be involved in malaria protection (apart from those in Table II, the ABO locus may also be included) one wonders naturally about their relative weights in those populations in which more than one is encountered. Precise measurements are difficult to carry out, for a variety of largely obvious reasons. Absolute gene frequencies in the population are not by themselves a useful guide, since they will depend on the balance between selection *against* certain genotypes (*e.g.*, AA and SS) and *for* other genotypes (*e.g.*, AS). Clearly, selection against sickle cell anaemia must be greater than against any common type of G6PD deficiency. Thus, on a semi-quantitative level, if in a certain population the frequency of AS heterozygotes and of G6PD deficiency heterozygotes were the same, and if malaria is the main selective force favouring those genes, one could conclude that in that population the protective effect of S is greater than that of G6PD deficiency. From data on the Bamba people of Uganda, who have one of the highest known frequency of the haemoglobin S gene, Livingstone (1967) has calculated that the biological fitness of an AS person is on the average 25% higher than the fitness of an AA person in that environment. At the opposite extreme, we can safely predict that the protective effect of the ABO blood group, if any, must be very small indeed: otherwise, since no substantial selection is likely against any of the genes involved, the favoured blood group would have replaced all others in many heavily malarious areas. Earlier in this meeting Hall and Canfield, and Powell, McNamara and Rieckmann have provided impressive evidence of greater resistance to *P. falciparum* in one ethnic group compared

to another. Since no single gene seemed to be responsible for the observed differences, it is possible that these resulted in fact from the composite advantage afforded by multiple genes, each one of which might have indeed only a very slight protective effect by itself.

While the differences amongst various protective genetic factors may be only quantitative in terms of their survival value, they are likely to be qualitative in terms of host-parasite relationship. In this respect, G6PD deficiency is perhaps unique, as protection seems to depend specifically on the dual erythrocyte population of heterozygous females. Normal cells in normal people, and G6PD-deficient cells in deficient people can be very heavily infected, but when both types coexist in the same person, normal cells are preferred by the parasite to deficient cells and, as a result, very high levels of parasitaemia seldom occur in heterozygotes. A purely hypothetical model to explain this somewhat paradoxical finding could be based on the concept of the parasite becoming adapted to a particular type of erythrocyte. Specifically, we are suggesting that when schizogony has taken place in a normal cell, most of the resulting merozoites are now more competent to infect normal cells than deficient cells. The opposite will be true, but perhaps to a lesser extent, when schizogony has taken place in a deficient cell. While this model is somewhat reminiscent of restriction in bacteriophage (see in Hayes, 1968), a taxonomically closer analogy can be found in the gradual adaptation of *P. lophurae* to mouse erythrocyte (McGhee, 1956). It is probably not useful to refine this model, until more is known of the genetic make-up of the parasite and of the changes it undergoes during the asexual cycle. Presently, as far as I am aware, there is no evidence against the possibility that considerable portions of the parasite's genome may be lost during schizogony, or unevenly distributed amongst merozoites. A particular erythrocytic environment is very likely to impose constraints on which portions of the parasite's genome can be allowed to drop out without loss of viability, and such a selective process with respect to parasite structure will be amplified through successive asexual cycles (obviously, only the rare Plasmodium with a complete genome will successfully mature into a gametocyte).

If these successive schizogonic cycles take place, for instance, in cells with a certain level of G6PD, loss of the plasmodium's own G6PD might be permissible. However, emerging merozoites, while fully competent to undergo a new cycle in similar red cells, will not be capable to thrive in G6PD-deficient red cells, although they could have done so, had they not previously foregone their own enzyme. While other, more sophisticated models could certainly be considered in order to explain existing findings, the present one exhibits, perhaps together with several flaws, the advantage that it can be experimentally tested, and this we are currently endeavoring to do.

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III

PLASMODIAL ULTRAMICROSCOPY

Morphological Divergence in a Mammalian Malarial Parasite: The Fine Structure of *Plasmodium brasilianum*

C. R. STERLING, M. AIKAWA AND R. S. NUSSENZWEIG

From the Institute of Pathology, Case Western Reserve University, Cleveland, Ohio, 44106, and the Department of Preventive Medicine, New York University School of Medicine, New York, New York, 10016

ABSTRACT: The fine structure of the erythrocytic stages of *Plasmodium brasilianum* and their host cells was studied. Unlike most other mammalian malarial parasites, *P. brasilianum* possesses mitochondria with prominent cristae and a nucleolus. Ingestion of host cell cytoplasm occurs only at the cytostome. These observations are similar to those of the quartan malaria parasite of man, *P. malariae*, and support the contention that *P. brasilianum* and *P. malariae* are closely related. Also, as in *P. malariae*, excrescences are observed on the surface of all parasitized erythrocytes. They are suggested to be the means by which parasitized cells are sequestered during the development of the immune response in infected monkeys.

Plasmodium brasilianum Gonder and von Berenberg-Gossler, 1908, is a quartan malarial parasite having a wide distribution among New World monkeys of the family Cebidae. Interest has been generated in studying this blood parasite because morphologically it resembles the quartan malarial parasite of man, *P. malariae* (von Berenberg-Gossler, 1909; Garnham, 1966; Garnham et al. 1963). *P. brasilianum* is pathogenic to monkeys (Taliaferro and Taliaferro, 1934) and man has been shown to be a susceptible host for *P. brasilianum* following the bite of sporozoite infected *Anopheles freeborni* mosquitos (Contacos et al. 1963). The potential of *P. brasilianum* as a true zoonosis has been discussed by Contacos and Coatney (1963). Interestingly, Old World monkeys are apparently insusceptible to infection by *P. brasilianum* (Garnham, 1963).

Numerous ultrastructural investigations on the erythrocytic stages of malarial parasites have been made in recent years (see Aikawa, 1971 for a review). Few of these reports, however, have dealt with the fine structure of the erythrocytic stages of simian malarial parasites (Aikawa et al. 1966, 1969a, b; Rudzinska and Trager, 1968). The present study

describes the ultrastructure of the asexual and sexual stages of *P. brasilianum* from the squirrel monkey *Saimiri sciureus* and correlates the fine structure of this parasite with other primate *Plasmodia* particularly *P. malariae* of man. The parasite-host cell to host relationship is discussed and the similarities of the human and simian quartan malarial parasites are emphasized, with the hope of directing new research toward comparing these groups.

Materials and Methods

Blood collected from infected splenectomized squirrel monkeys, *Saimiri sciureus*, was fixed for electron microscopy in 1.25% glutaraldehyde, 0.1 M phosphate buffer and 4% sucrose (pH 7.3) for one hour. After briefly rinsing in 0.1 M phosphate buffer, the blood was post-fixed for one hour in 1% OsO₄ in phosphate buffer. The blood was briefly rinsed in 30% and 50% ethanol to remove traces of osmium and was stained *en bloc* in a 1% uranyl acetate-50% ethanol mixture for 30 minutes. The blood was further processed and embedded in Epon as previously described (Aikawa et al. 1966). Small pieces of liver from infected squirrel monkeys, were fixed, stained *en bloc* and processed as above. Thin sections were cut on a Porter Blum-MT-2 ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 101 electron microscope.

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Results

Trophozoites:

Erythrocytic trophozoites of *P. brasilianum* are surrounded by two unit membranes. The outer membrane surrounding the trophozoite, as in other mammalian malarial parasites, is presumably derived from the plasmalemma of the host cell (Ladda et al. 1969). In very young trophozoites the host cell and parasite membranes are separated by a narrow intracellular space (Fig. 1). This intracellular space diminishes with the growth and expansion of the trophozoite so that eventually the two membranes closely appose one another (Fig. 2). The two unit membranes appear indistinguishable, each measuring 7 nm in thickness. They are separated by a dense zone measuring 4 nm in thickness.

In young trophozoites, the spherical body of asexual blood merozoites may still be found associated with the single mitochondrion (Fig. 1). The ground substance of the cytoplasm contains numerous ribosomes and a well de-

veloped network of rough and smooth endoplasmic reticulum (Fig. 1). The nucleus of young trophozoites has so far not been observed to contain a distinct nucleolus or clumping of chromatin material.

A prerequisite for growth of the trophozoite is the obtainment of nutrient material from the host cell. The main site of trophic activity in *P. brasilianum* trophozoites appears to be centered around the cytostome, which in longitudinal section is bounded by two dense parallel segments (Figs. 3, 4). The cytostome of *P. brasilianum* measures 170 nm in its inner diameter and 250 nm in its outer diameter. Invaginations derived from the cytostome are bounded by a double membrane (Figs. 4, 5). The digestion of hemoglobin by the malarial parasite presumably leads to the formation of insoluble hemozoin granules which are observed within smaller single membrane bound food vacuoles (Fig. 5). These smaller vacuoles appear to be derived from the cytostomal invaginations. The food vacuoles are not to be

→

Figure 1. Young trophozoites of *Plasmodium brasilianum*. The parasite plasma membrane (Pm) is separated from the host cell plasma membrane (Hm) by a narrow intracellular space (IS). The plasma membrane of the erythrocyte shows areas of excrescence formation (arrows). Mitochondrion (M), Nucleus (N), Spherical body (S). 33,000X

Figure 2. The parasite limiting membranes of a mature trophozoite. The plasma membranes of the host cell (Hm) and parasite (Pm) closely appose one another. 150,000X

Figure 3. A longitudinal section through a cytostome (C) of a trophozoite. 60,000X

Figure 4. A longitudinal section through two cytostomes (C) of a schizont. The invaginations formed at the cytostomes are bounded by two membranes. 50,000X

Figure 5. Single membrane bound food vacuoles (F) containing malarial pigment (P). One food vacuole (arrows) appears to be in the process of pinching off from a double membrane bound invagination. 67,500X

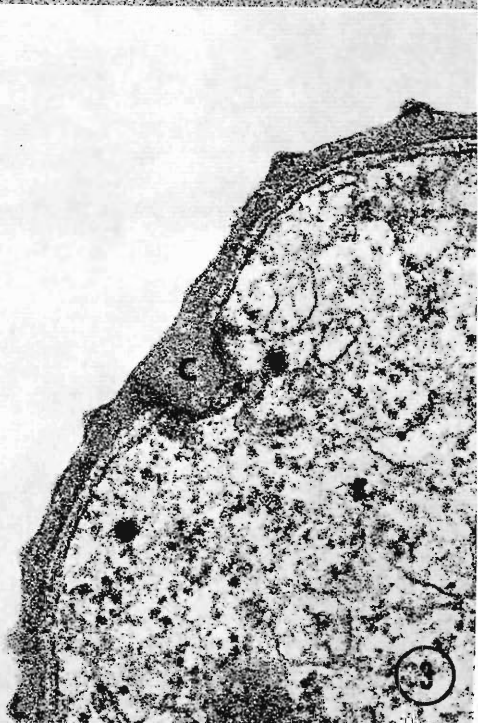
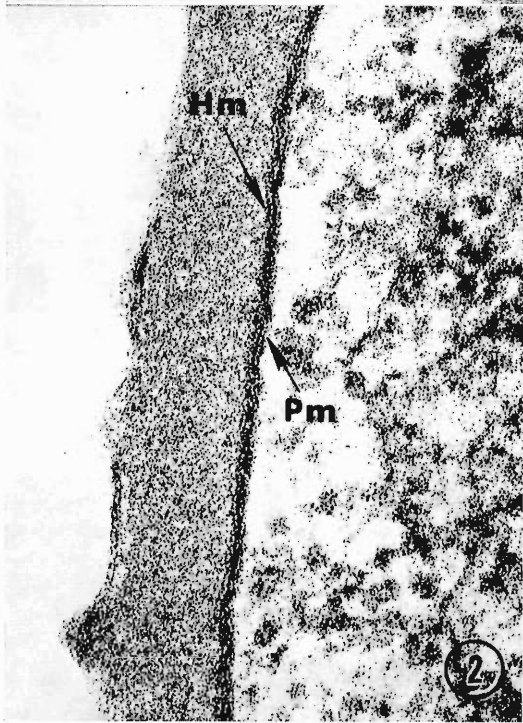
Figure 6. An elongate mitochondrion (M) of a mature trophozoite. Dense body (arrow). 40,000X

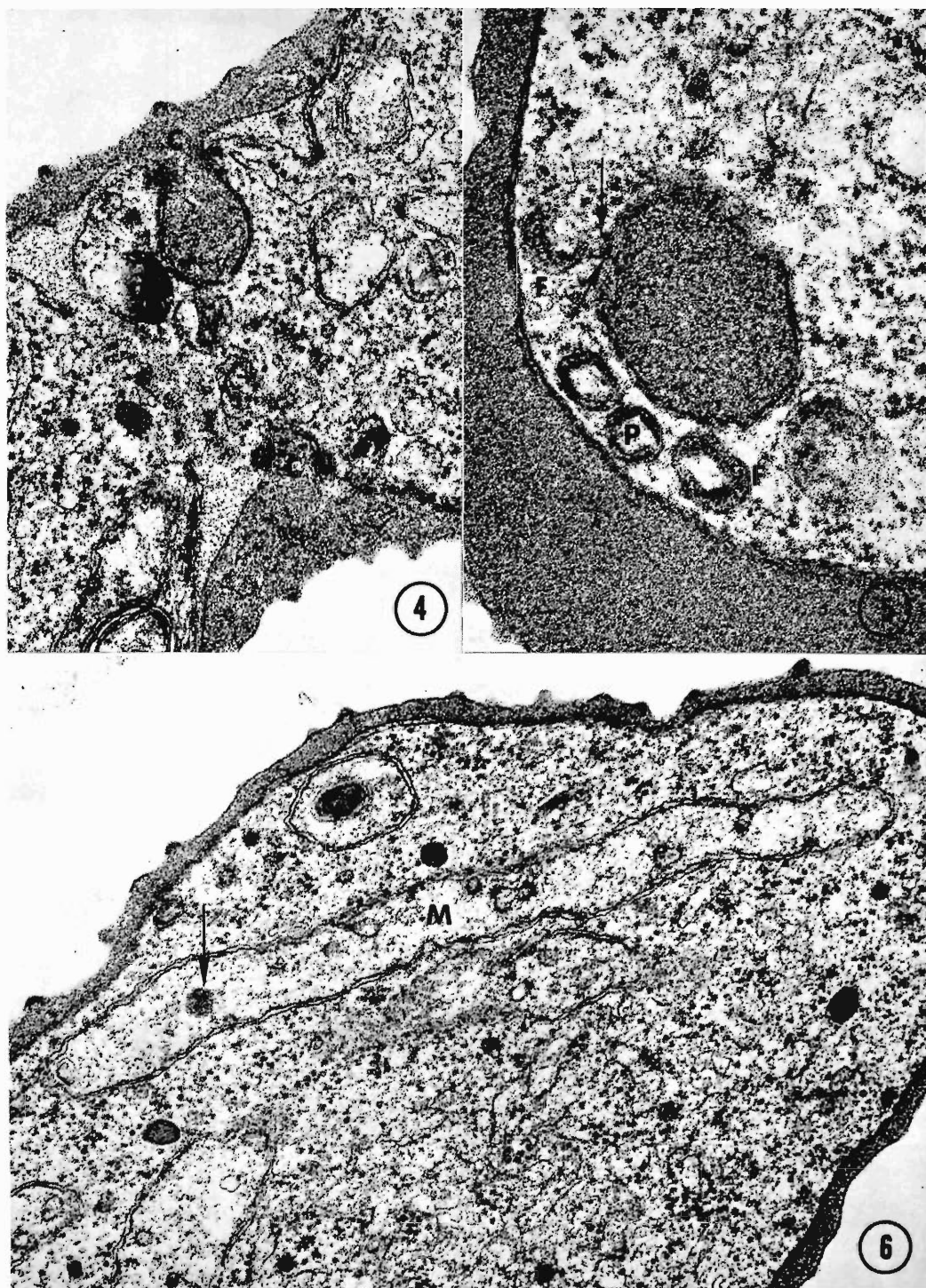
Figures 7 and 8. A distinct nucleolar region (Nu) is found within the nucleus (N) of trophozoite. Fig. 7—32,500X. Fig. 8—26,000X

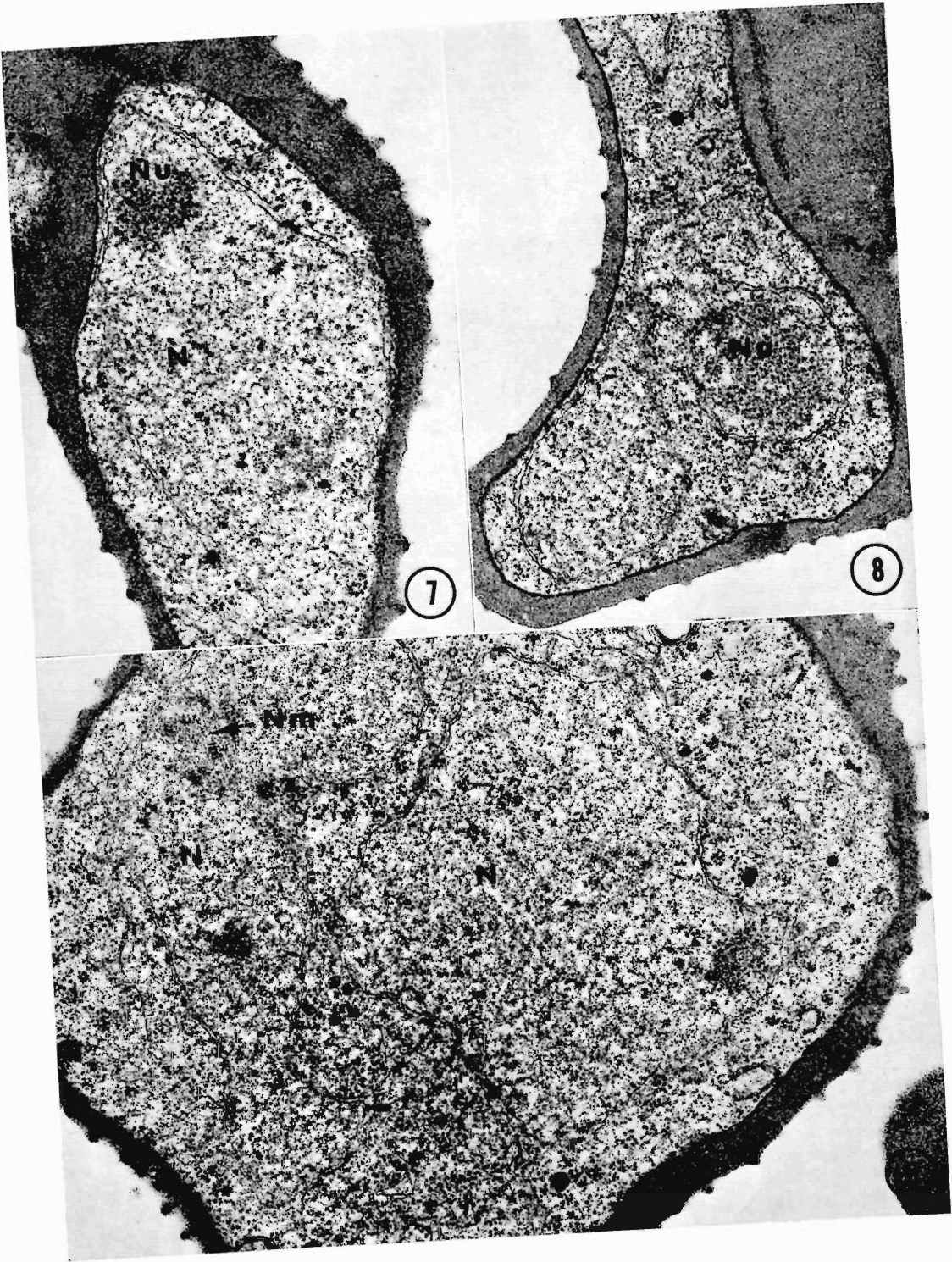
Figure 9. A binucleate schizont of *Plasmodium brasilianum*. The parasite's cytoplasm nearly fills the host cell. Nuclear division is indicated in one of the schizont's nuclei by the presence of nuclear microtubules (Nm). 26,000X

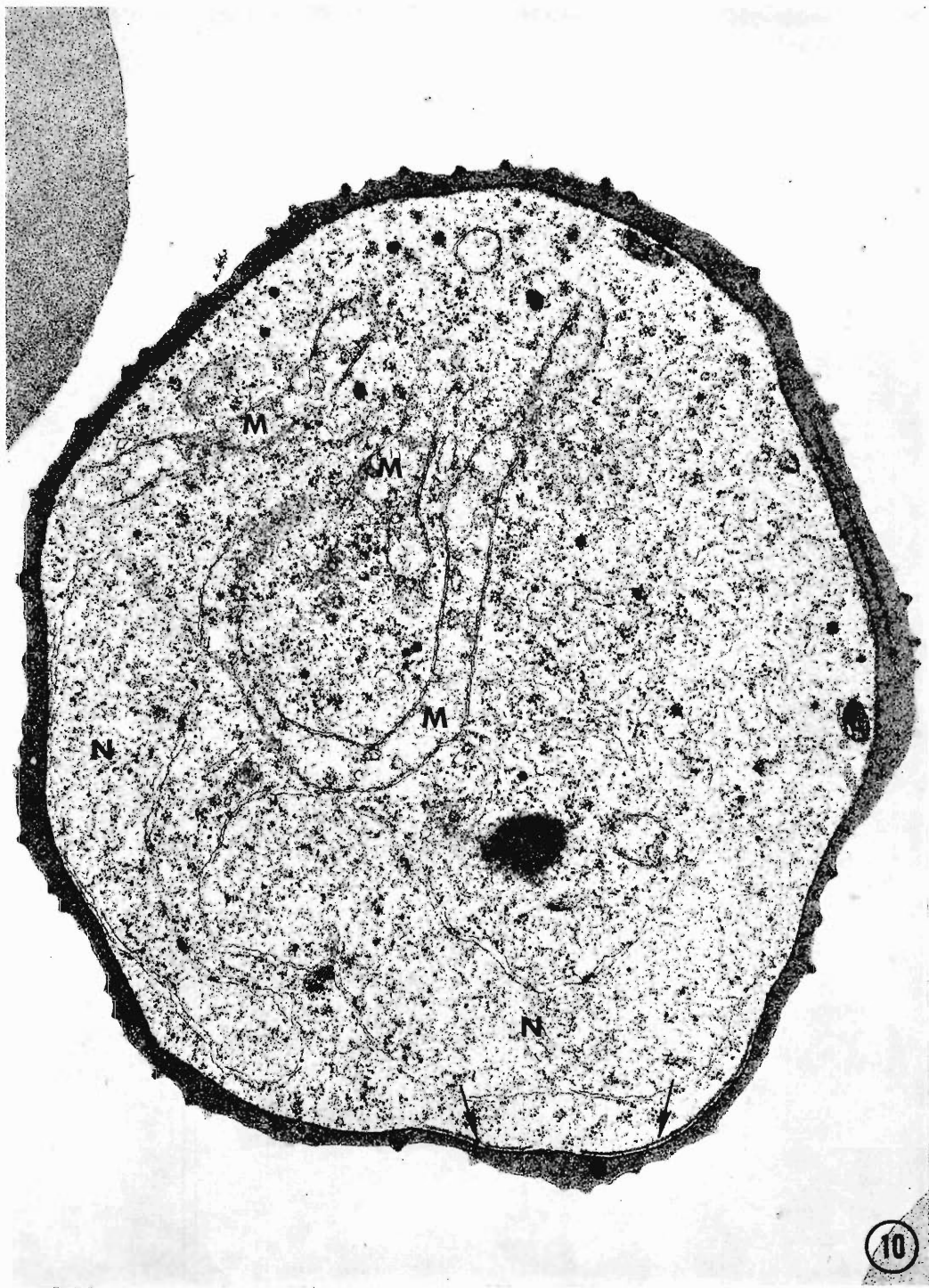
Figure 10. A young schizont with long cristate mitochondria (M). Regions of membrane thickening (arrows) appear beneath the parasite's limiting membranes. The surface of the erythrocyte possesses numerous excrescences. Nucleus (N). 24,500X

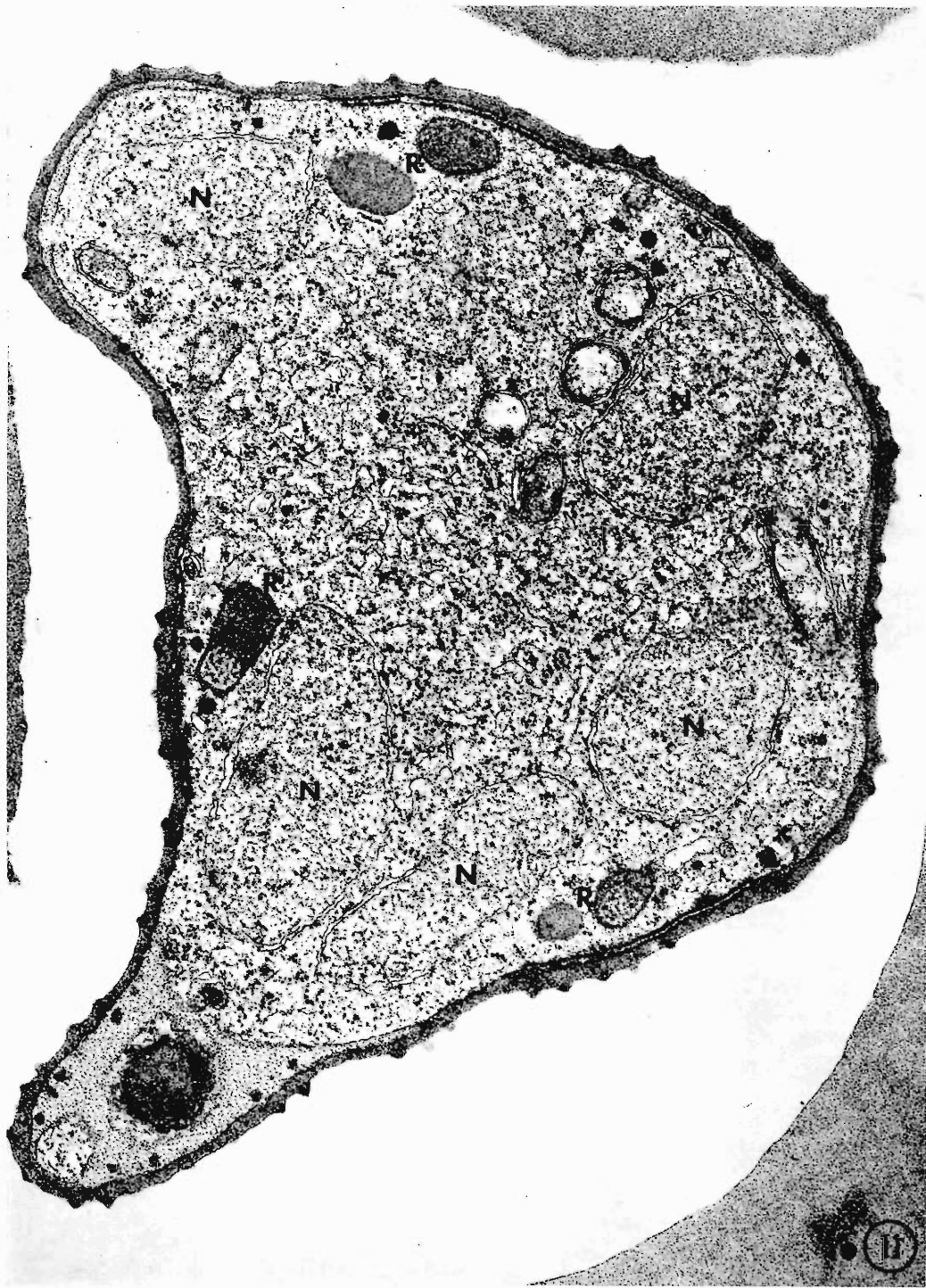
Figure 11. An advanced schizont with 5 nuclei (N). The rhoptries (R) are associated with areas of membrane thickening. 31,000X











confused with other double membrane bound vacuoles surrounding hemoglobin material of the same consistency as that of the host cell's cytoplasm. Presumably, these vacuoles are the result of the amoeboid activity of the trophozoite's cytoplasm (Aikawa, 1971).

The mitochondria of maturing *P. brasilianum* trophozoites are unusual among the mammalian species of malarial parasites in that they contain tubular cristae (Fig. 6). These cristae, however, are never as numerous as in the asexual blood stages of the avian and reptilian *Plasmodium* species (Aikawa, 1966; Aikawa and Jordan, 1968). The mitochondria of *P. brasilianum*, like other protozoan mitochondria, are surrounded by a double membrane, the inner membrane giving rise to the tubular cristae. Occasionally, a large aggregate of dense material is observed within these mitochondria (Fig. 6).

Unlike most other mammalian malarial parasites of the genus *Plasmodium*, the nucleus of *P. brasilianum* mature trophozoites contains a distinct nucleolar-like area (Figs. 7, 8). The granular components of this nucleolar region measure 20 nm in diameter and appear similar to ribosomes of the trophozoite's cytoplasm. Smooth and rough endoplasmic reticulum abound within the cytoplasm of maturing trophozoites (Fig. 10).

The formation of stumpy pseudopodia seems to characterize the growth of *P. brasilianum* trophozoites (Fig. 8). When mature, however, nearly all trophozoites tend to be rounded in appearance, their cytoplasm nearly filling the host cell (Figs. 9, 10). At this stage of development, the complex series of events leading to the formation of asexual merozoites is initiated.

Schizogony:

Nuclear and mitochondrial divisions are the first evident features of schizogony (Figs. 9–13). As observed in other species of *Plasmodium*, nuclear division is indicated by microtubules radiating from a poorly defined electron dense centriolar plaque on the nuclear membrane of the parasite (Figs. 12, 13). Banded structures, suggestive of kinetochores, (Aikawa et al. 1972), are observed along the nuclear microtubules and occasionally beneath the centriolar plaque (Figs. 12, 13). The nuclear membrane does not disappear during nuclear division. Mitochondrial division is indicated by their increase in length and eventually numbers (Fig. 10). As noted in trophozoites, the mitochondria of schizonts possess prominent tubular cristae.

The differentiation phase of schizont growth, in which cytoplasmic elements of the schizont

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Figures 12 and 13. Advanced stages of nuclear division. Kinetochores (K) are seen beneath the poorly defined centriolar plaques (Cp). Nuclear microtubules (Nm) are also attached to the centriolar plaques. Fig. 12—80,000X. Fig. 13—102,000X

Figure 14. Advanced stage of merozoite formation. The budding merozoite remains attached to the residual cytoplasm of the mother schizont (Rb) by a narrow isthmus (arrows). Mitochondrion (M), Nucleus (N), Spherical body (S). 30,000X

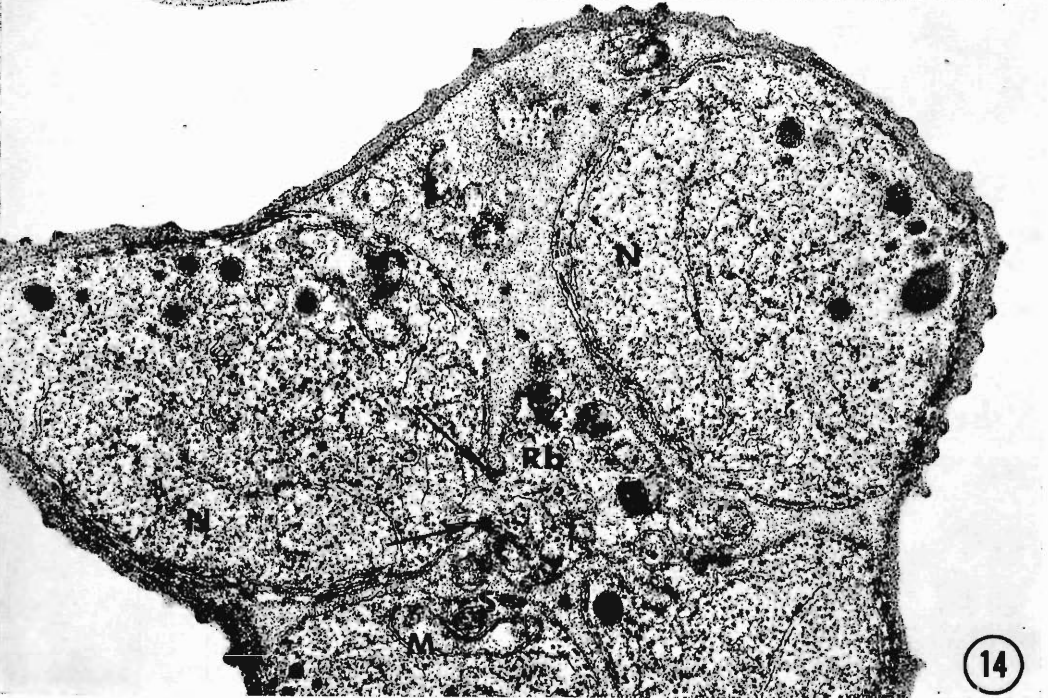
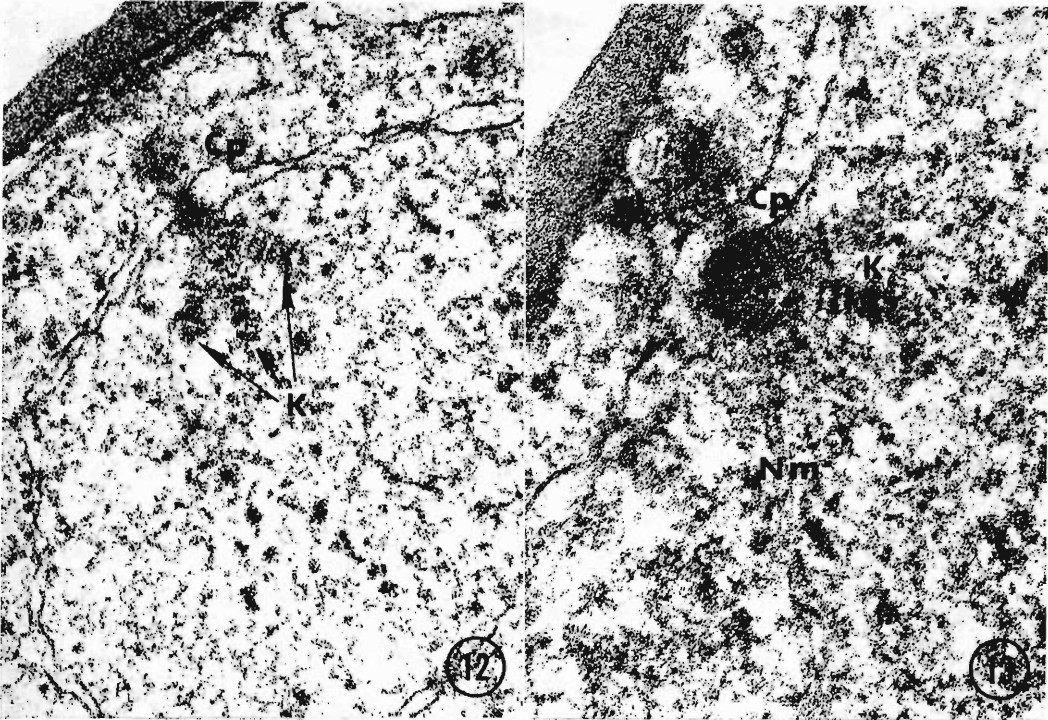
Figure 15. A tangential section of one of the polar rings (Pr) at the anterior end of the merozoite. The subpellicular microtubules (Mt) appear to be connected to this polar ring. The ducts of the rhoptries (R) extend through the polar ring. 85,000X

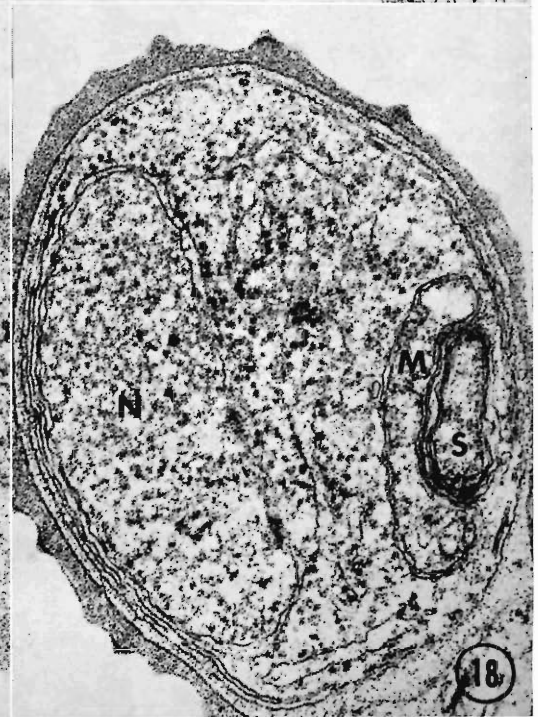
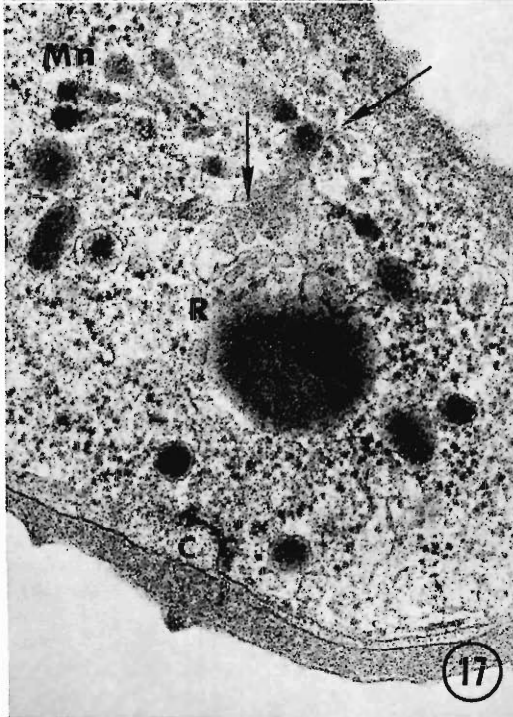
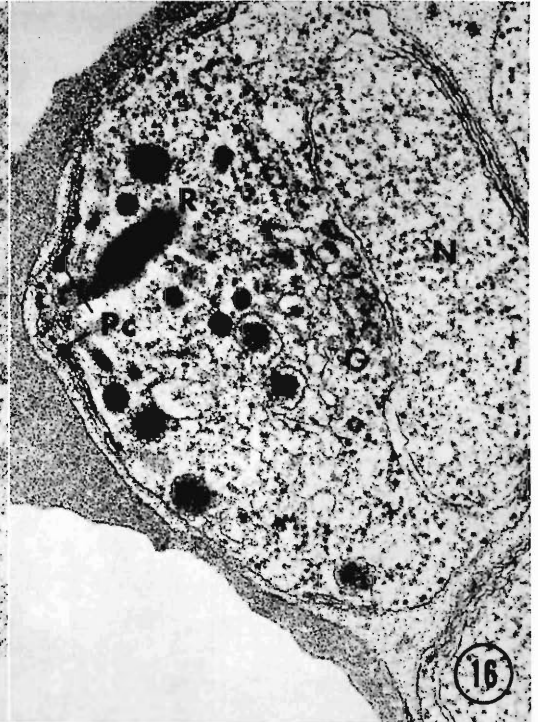
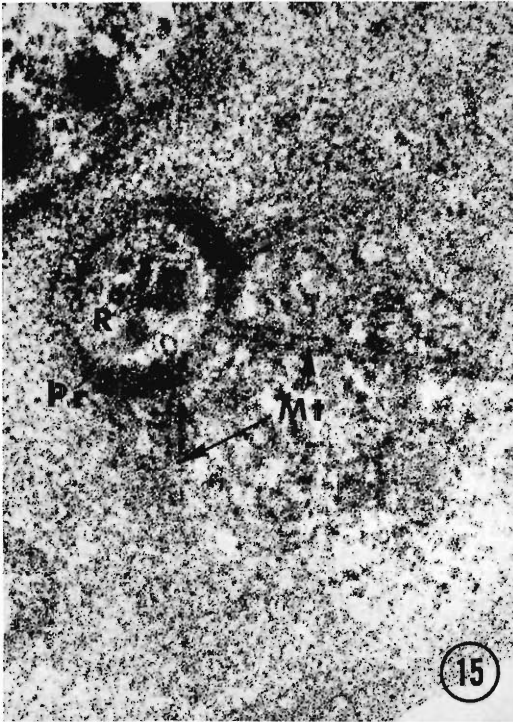
Figure 16. Longitudinal section of a merozoite. The rhoptries (R) extend toward the anterior end of the merozoite. A subpellicular cavity (Pc) is also associated with the anterior end. A prominent Golgi region (G) is present just anterior to the nucleus (N). 41,000X

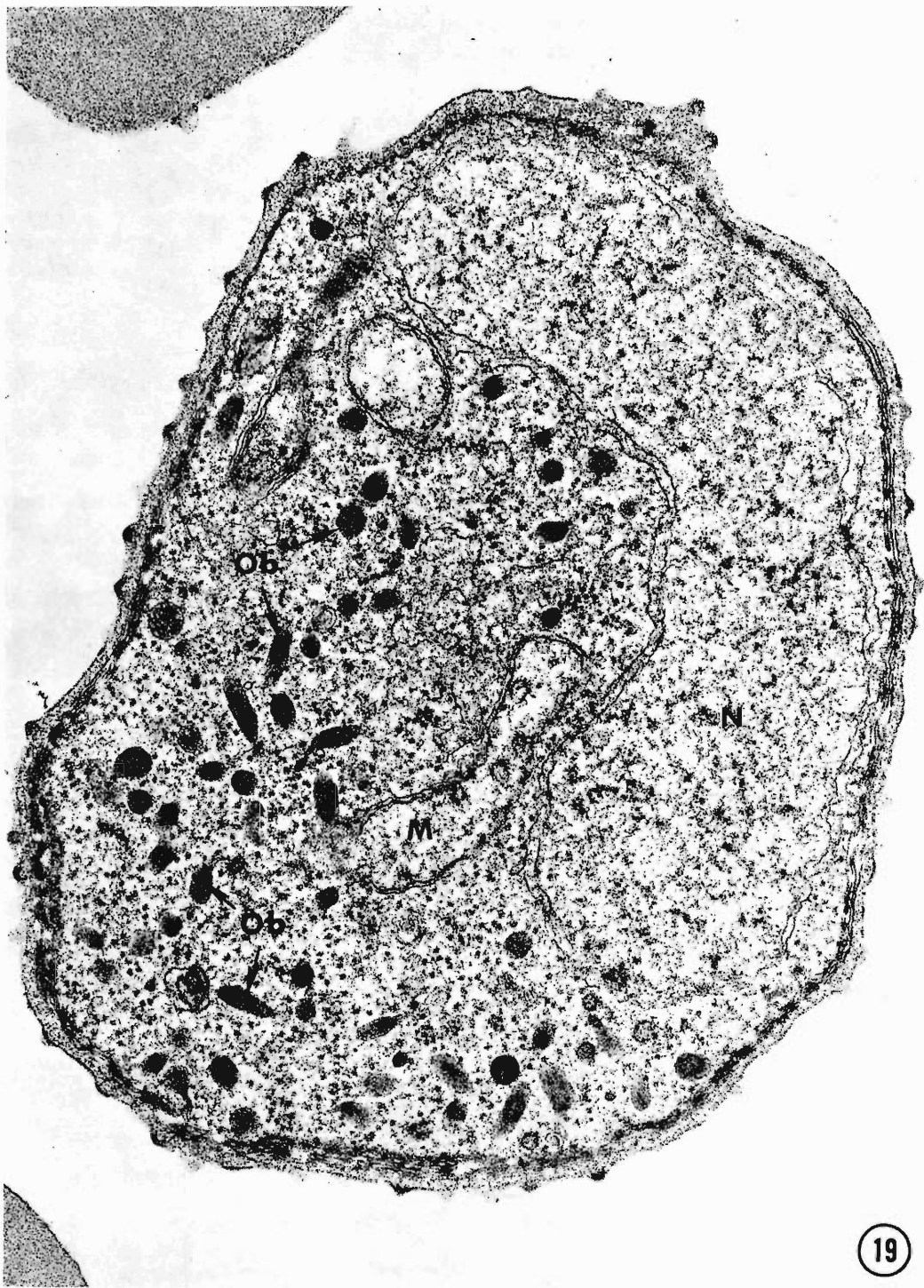
Figure 17. A tangential section of one of the rhoptries (R). Ducts leading from the micronemes (Mn) appear to connect with the rhoptries (arrows). Cytostome (C). 46,000X

Figure 18. A cross section through a merozoite. A mitochondrion (M) partially encircles the spherical body (S). Nucleus (N). 52,000X

Figure 19. A macrogametocyte of *Plasmodium brasilium*. The cytoplasm contains numerous osmophilic bodies (Ob). Mitochondrion (M), Nucleus (N). 46,500X







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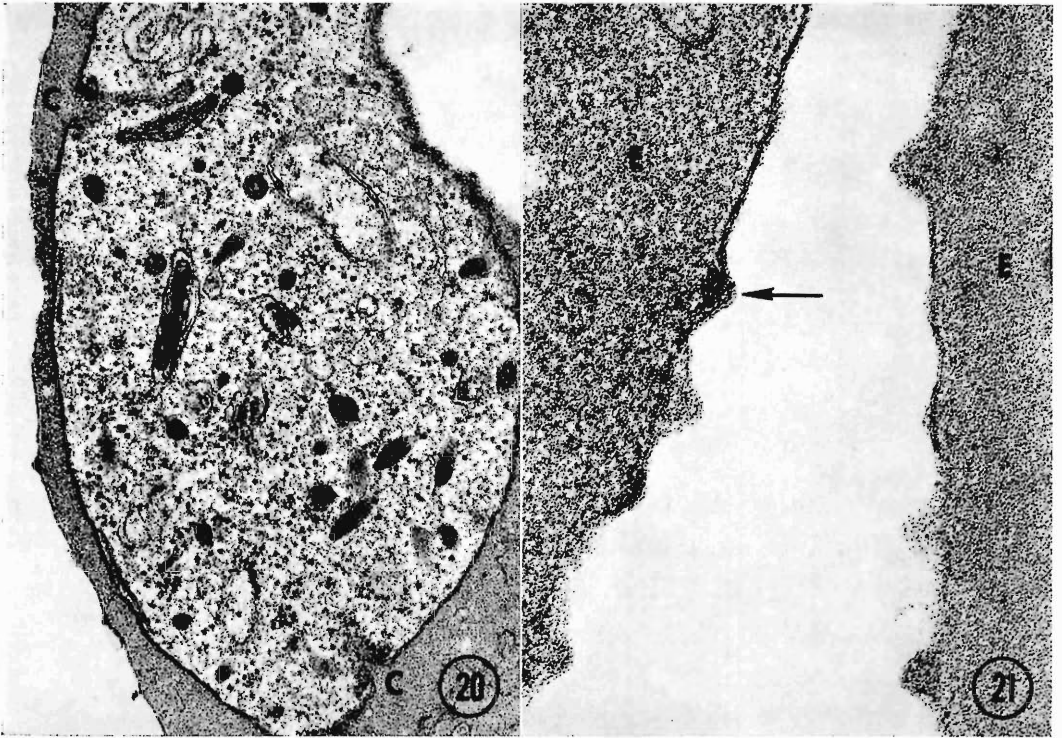


Figure 20. Two cytosomes (C) of a macrogametocyte viewed in longitudinal section. 34,000X

Figure 21. A surface view of 2 parasitized erythrocytes (E). The excrescence (arrow) is bounded by the plasma membrane of the erythrocyte. 108,000X

are organized into asexual merozoites, commences with the development of a double inner membrane in the region of eventual merozoite budding (Figs. 10, 11). Immediately beneath this doubled inner membrane the precursors of the rhoptries and micronemes are observed (Fig. 11). These structures develop in close proximity to Golgi-like vesicles observed near the nuclei of presumptive merozoites.

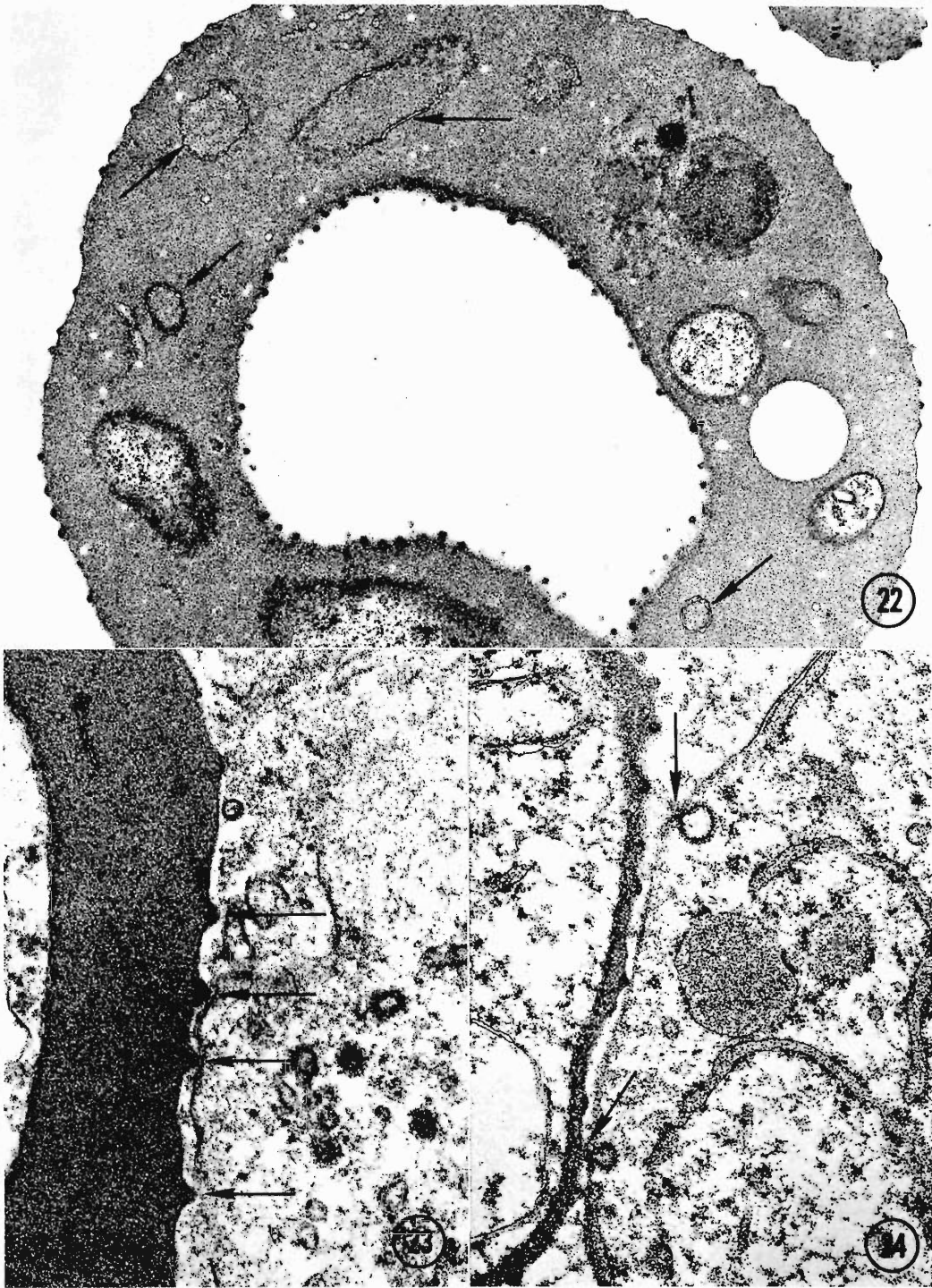
With the progression of merozoite formation, the outer and doubled inner membrane

differentiate into the polar rings. Subpellicular microtubules associate with the polar rings thus forming a part of the budding merozoite's cytoskeleton (Fig. 15). The rhoptries and micronemes associate with the anterior region of the merozoite marked by the polar rings and various organelles, including the nucleus, mitochondrion and associated spherical body of unknown origin, endoplasmic reticulum and ribosomes migrate into budding merozoites. When the budding process is completed, all that remains of the mother schizont is a resid-

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Figure 22. A cross section of a parasitized erythrocyte. Membrane bound clefts (arrows) are present in the cell. 20,000X

Figures 23 and 24. Cone shaped excrescences (arrows) of infected erythrocytes in contact with the plasma membrane of Kupffer cells. Fig. 24. Pinocytotic vesicles (arrows) at the junctional site. Fig. 23—52,000X. Fig. 24—32,000X



ual body containing numerous pigment particles (Fig. 14).

Merozoites:

Fully formed merozoites of *P. brasilianum* contain the characteristic organelles found within the merozoites of other mammalian malarial parasites. Thus, they contain a nucleus, mitochondrion, spherical body, cytostome, rhoptries, micronemes, subpellicular microtubules, Golgi vesicles, ribosomes and endoplasmic reticulum.

The doubled inner pellicular membrane surrounding merozoites often show interruptions. This double membrane terminates in the region of the polar rings near a pellicular cavity (Fig. 16). The micronemes and paired rhoptries associated with the anterior region of the merozoite appear to form a large interconnecting network of tubular canals with the rhoptries acting as large connecting reservoirs (Fig. 17). Sections cut tangential to the surface of the rhoptries reveal the presence of pore-like structures which may represent the entry site of collecting tubules from the micronemes into the rhoptries (Fig. 17).

The mitochondrion, which is located in the posterior half of the merozoite, partially encircles the spherical body and contains tubular cristae (Figs. 14, 18). The cristae, however, are fewer in number than observed in the mitochondria of other blood stages of this parasite's life cycle. The nucleus of *P. brasilianum* merozoites, which is also located posteriorly, does not display the prominent nucleolar region evident in trophozoites. The Golgi-like network of vesicles is located just anterior to the nucleus (Fig. 16).

The cytostome, which is located approximately midway between the merozoite's anterior-posterior axis, is similar in structure and size to the cytostome of erythrocytic trophozoites (Fig. 17).

Gametocytes:

The electron microscopic examination of gametocytes was made difficult due to their scarcity. However, they were readily identifiable since they differed greatly from trophozoites and schizonts. Using the morphological criteria set forth by Rudzinska and Trager (1968) and Aikawa et al. (1969b),

we were only able to distinguish macrogametocytes in our preparations. The mature macrogametocytes nearly fill the cytoplasm of the host cell (Fig. 19). They are surrounded by a three layered membrane, the inner one of which often shows disruptions. The main features which distinguish the macrogametocytes from other erythrocytic stages are the presence of numerous osmophilic bodies and a well developed system of endoplasmic reticulum within the cytoplasm (Fig. 19). The mitochondria of macrogametocytes possess tubular cristae as observed in the other erythrocytic stages. The nucleus is always located to one side of the gametocyte. In a young macrogametocyte, two cytostomes were observed in a single section plane (Fig. 20).

The Host Cell:

Single and double membrane bound clefts are observable within the cytoplasm of infected host cells (Figs. 10, 22). These clefts may be small and circular, or elongate in shape. They are usually more evident in cells infected with the more mature parasites.

Uninfected erythrocytes of *Saimiri sciureus* are surrounded by a single plasma membrane displaying a smooth contour. Parasitized erythrocytes, however, show an irregular contour due to the presence of small electron dense protrusions. These protrusions, or excrescences, are more numerous about the surface of erythrocytes containing mature trophozoites, schizonts and gametocytes than in erythrocytes which contain trophozoites and young gametocytes. In a single section as many as 60 excrescences have been observed about a parasitized host cell containing a mature parasite (Fig. 11), while cells infected by young trophozoites may show only 5 to 10 excrescences (Fig. 1).

The excrescences are generally cone-shaped and measure 45 nm in height and 100 nm in width. They are bounded by the plasma membrane of the host cell and possess a matrix which is generally denser than that of the host cell's cytoplasm (Fig. 21). However, the base is not sharply demarcated, but gradually merges into the erythrocyte cytoplasm.

Primarily observations on the liver of splenectomized squirrel monkeys infected with *P. brasilianum* show numerous parasitized

erythrocytes within the sinusoidal spaces. The excrescences of these parasitized erythrocytes frequently form a focal tight junction with the plasma membrane of Kupffer cells lining the sinusoids (Fig. 23). Pit-like depressions similar to pinocytotic vesicles are occasionally observed at the point of contact between the excrescences of the infected erythrocytes and the Kupffer cells (Fig. 24). Similar contact is also noted between parasitized erythrocytes and macrophages within the liver sinusoids.

Discussion

Investigations of malarial parasite ultrastructure have revealed several differences between the avian and reptilian *Plasmodium* spp. on the one hand and mammalian *Plasmodium* spp. on the other (Aikawa et al. 1969b; Aikawa, 1971; Rudzinska and Vickerman, 1968; Rudzinska, 1969). These differences, which include mitochondrial systems, the mode of uptake and digestion of hemoglobin and the presence or absence of a nucleolus, are most apparent in the erythrocytic phase of the malarial parasite's life cycle. Rudzinska and Vickerman (1968) and Rudzinska (1969) relate the differences between the *Plasmodium* spp. primarily to the dissimilarity of hosts and host cells, while other workers have stressed differences between life cycle stages and metabolic requirements (Aikawa, 1966; Peters, 1969; Blackburn and Vinijchaikul, 1970; Howells, 1970). With respect to the differences mentioned above, *P. brasilianum* appears to be a most interesting mammalian malarial parasite since it displays characteristics similar to the avian and reptilian malarial parasites.

The presence of mitochondria within the erythrocytic stages of avian (Aikawa, 1966; Aikawa et al. 1966a, b, 1967, 1969a) and reptilian (Aikawa and Jordan, 1968; Scorza, 1971) malarial parasites has been well established. Evidence for the existence of mitochondria within the parasites of these groups is based on the presence of distinct cristae in a double membrane bounded structure. The identification of mitochondria within the erythrocytic stages of mammalian malarial parasites has been more difficult. But to this difficulty, organelles such as double membrane bounded structures and multilaminated

membrane organelles are suggested to be mitochondrial equivalents in mammalian malarial parasites (Aikawa et al. 1966b, 1969b; Blackburn and Vinijchaikul, 1970; Howells et al. 1969; Ladda, 1966, 1969; Ladda et al. 1966; Rudzinska and Trager, 1968; Rudzinska and Vickerman, 1968). The evidence suggesting that these structures represent the mitochondria of mammalian malarial parasites comes from cytochemical studies demonstrating the presence of cytochrome oxidase (Howells et al. 1969; Theakston et al. 1969), NADH- and NADPH-dehydrogenase (Theakston et al. 1970) activity within the multilaminated and double membrane systems. Cristate mitochondria have been observed however, in certain primate *Plasmodium* spp. These cristate mitochondria were observed in gametocytes of *P. falciparum* (Smith et al. 1969; Kass et al. 1970) and trophozoites of *P. malariae* (Smith and Theakston, 1970).

In the present study, there can be little doubt as to the existence of distinct mitochondria in the erythrocytic stages and gametocytes of *P. brasilianum*. However, the significance of cristate mitochondria in the erythrocytic stages of this mammalian parasite species and their apparent absence in most others, is difficult to explain.

Biochemical studies on the avian parasite *P. lophurae* indicate that glucose is catabolized aerobically principally via the Embden-Meyerhoff glycolytic and tricarboxylic acid (Krebs) cycle routes (Sherman et al. 1970). On the other hand, the importance of the Krebs cycle in various mammalian malarial parasites has been questioned (Bowman et al. 1961; Bryant et al. 1964; Peters, 1969; Howells, 1970; Scheibel and Pflaum, 1970). With respect to these differences, it must be remembered that the enzymes of the Krebs cycle are principally located within the mitochondrial matrix (Ernster and Luylensteirna, 1970). Functionally linked with these enzymes are the enzymes of the inner mitochondrial membranes including the cytochromes of the electron transport system and the necessary entities for coupled phosphorylation.

Studies with certain yeast cells grown anaerobically show that mitochondrial cristae may be entirely absent, while the same yeast cells grown under aerobic conditions may show prominent mitochondrial cristae develop-

ment (Swift and Wolstenholme, 1969). To some degree then, the functional and structural state of mitochondrial development is linked with its respiratory activity. In support of this, Howells (1970) was unable to demonstrate succinic dehydrogenase activity or cristate mitochondria within the erythrocytic stages of the mammalian malarial parasite *P. berghei*. He used this enzyme system "since it is considered to be exclusively mitochondrial and integrated in the Krebs citric acid cycle." However, in the sporogonic stages of *P. berghei*, where cristate mitochondria are evident, Howells successfully demonstrated succinic dehydrogenase activity.

In *P. lophurae*, which utilizes the Krebs' cycle, and in other avian malarial parasites, well developed mitochondrial cristae are evident in the erythrocytic portion of the life cycle (Aikawa, 1966), whereas in the erythrocytic stages of most mammalian malarial parasites, where the importance of the Krebs cycle is highly questionable, cristate mitochondria are absent. The presence of cristate mitochondria in *P. brasilianum* and certain other primate malarial species may therefore imply that these parasites utilize the Krebs' cycle to a greater extent during the erythrocytic phase than other mammalian malaria parasites. Evidence for this will have to await further biochemical studies. It is interesting to note that the sporogonic stages of development of the mammalian malarial parasites in the insect intermediate hosts possess prominent cristate mitochondria (Vanderberg et al. 1967; Howells, 1970; Terzakis, 1967). Howells (1970) suggests that this change from acristate mitochondria in the erythrocytic phase to cristate in the sporogonic phase reflects differences in the parasite's metabolism in the mammalian and insect host.

The second point of difference between the avian and mammalian malarial parasites, one which is the subject of much controversy, concerns the mode of uptake and ingestion of hemoglobin. To account for these differences, three mechanisms of feeding have been described for malarial parasites. These include: 1) phagotrophy (Rudzinska and Trager, 1957, 1959), 2) pinocytosis (Rudzinska and Trager, 1965; Cox and Vickerman, 1966) and 3) cytotosomal feeding (Aikawa et al. 1966a, b).

Cytosomal feeding has been firmly estab-

lished as the means by which avian and reptilian malarial parasites feed on host cell cytoplasm (Aikawa, 1966, 1971; Aikawa, et al. 1966a, b, 1967, 1969b; Aikawa and Jordan, 1968; Scorza, 1971). The cytostome has also been observed to be the means by which host cell ingestion is accomplished by some mammalian malarial parasites (Aikawa et al., 1966b, 1969a, b). However, many workers consider phagotrophy (Rudzinska and Trager, 1957, 1959; Fletcher and Maegraith, 1962; Blackburn and Vinijchaikul, 1970), or other combinations of the three methods mentioned above to be important in the feeding process of mammalian malarial parasites (Cox and Vickerman, 1966; Rudzinska and Vickerman, 1968; Scalzi and Bahr, 1968; Theakston et al. 1968; Killby and Silverman, 1969; Rudzinska, 1969). In *P. brasilianum*, cytotosomal feeding was the only method of ingestion of host cell cytoplasm observed. Evidence indicating that phagotrophy does not occur in this parasite can be attributed to the following: 1) young trophozoites of *P. brasilianum* rarely show extensive pseudopod development, 2) the more mature trophozoites almost always tend to be rounded and 3) multiple cytostomes ingesting host cell cytoplasm have been observed. Interestingly, the cytotosomal orifice of *P. brasilianum* trophozoites is similar in size to that of avian *Plasmodium* spp; (Aikawa et al. 1966a, b), measuring 180 nm.

The digestion of hemoglobin in the erythrocytic stages of *P. brasilianum* occurs in small single membrane bounded food vacuoles which presumably originate from cytotosomal invaginations. Occasionally, double membrane bounded vacuoles are observed within the parasite's cytoplasm. However, these vacuoles are interpreted as being "false food vacuoles" (Aikawa, 1971) which result from surface indentations viewed in cross section. Evidence suggesting that these double membrane bound vacuoles are not true food vacuoles comes from serial section (Vivier and Petitprez, 1970), isolation (Aikawa et al. 1969a) and cytochemical studies (Aikawa and Thompson, 1971) of other mammalian malarial parasites.

The presence of nucleolus in the avian malarial parasites and its absence in the mammalian malarial parasites has been suggested to constitute a difference between these groups



(Rudzinska, 1969). However, a clearly defined nucleolar region has been observed in *P. falciparum* (Smith et al. 1969; Kass et al. 1971), *P. malariae* (Smith and Theakston, 1970) and *P. brasilianum*. The significance of its presence in some mammalian malaria parasites and its absence in others remains obscure. The granular component of the nucleolus of *P. brasilianum* and other malarial parasites is composed of aggregates of ribonucleoprotein granules and resembles the nucleolonema region of nucleoli of other cell types. Evidence suggesting that this represents the nucleolus of malarial parasites is the inability of DNAase to digest this structure (Aikawa et al., 1972). Unlike many other cell types, a pars amorphi has not been described in the nucleoli of malarial parasites.

Of interest in this study was the presence of pore-like structures observed in sections cut tangential to the surface of the rhoptries. Since we have observed the ducts of micronemes to be connected to the large paired rhoptries, we have interpreted these pore-like structures as possibly representing the entry site of the microneme ductules into the rhoptries. Scholtyssek and Mehlhorn (1970) diagrammatically represented the rhoptries and micronemes as a single function complex. The observation of this study tends to support their hypothesis. The large number of Golgi vesicles located anterior to the nucleus and near the micronemes also appears to be a part of this large functional complex. A clear pellicular cavity which lies just inside the inner membrane of merozoites of *Haemoproteus metchnikovi* (Sterling, 1972) and sporozoites of *H. columbae* (Klei, 1972) and is suggested to provide additional support for the anterior end of the organism, has also been identified in merozoites of *P. brasilianum*. Since the fine structure of other cellular organelles within the erythrocytic stages of *P. brasilianum* is essentially similar with other *Plasmodium* sp., their structure and function will not be reviewed here (see review by Aikawa, 1971).

Certain primate malarial parasites, including *P. falciparum*, *P. coatneyi* and *P. knowlesi*, display a propensity to withdraw to internal organs of the body to undergo schizogony. Ultrastructural studies by Merrill and Welde (1970), Luse and Miller (1971) and Aikawa

et al. (1972) on *P. falciparum* within internal organs of monkeys and by Rudzinska and Trager (1968) on *P. coatneyi*, have revealed the presence of extrusions, or excrescences, on parasitized erythrocytes. These authors have suggested that the excrescences represent the mechanism by which parasitized cells are sequestered or possibly agglutinated. *P. knowlesi*, which is also sequestered during schizogony, does not display prominent excrescences (Miller et al. 1971a). However, rheologic studies by Miller et al. (1971b) revealed that *P. knowlesi* infected cells were less deformable, a condition which they suggested may lead to capillary obstruction.

Smith and Theakston (1970) demonstrated the presence of "symmetrical tubules" (excrescences) in all parasitized cells from the peripheral blood of two patients infected with *P. malariae*. However, they did not attempt to interpret this erythrocyte morphology. *P. brasilianum*, like *P. malariae*, displays excrescences on the surface of all parasitized erythrocytes of the peripheral circulation. Cells infected by all stages of parasite development, including gametocytes, display these structures. In *P. brasilianum* and *P. malariae* infections, schizogony is not confined to the microcirculation of internal organs, therefore, the presence of excrescences in these infections cannot be used to explain the sequestration of parasitized erythrocytes as it relates to schizogony.

Taliaferro and Cannon (1936) studied the cellular reactions to *P. brasilianum* in Panamanian monkeys with reference to the development of immunity. They noted that during the crisis stage of the infection, marked by either a sharp or gradual diminution in the number of parasites in the peripheral blood, large numbers of parasitized cells become packed in the Billroth cords of the spleen and were scarce in the sinuses. "This finding indicates that normal and infected cells enter the cords from the artery, but that only normal cells can pass into the venous sinuses. It seems probable that the parasitized red cells are held in the cords by some affinity with the macrophages. . . ." These workers also noted that following splenectomy there was a compensatory increase in phagocytosis within the liver and to a lesser extent the bone marrow.

In the present study, parasitized cells were sequestered in great numbers within the liver. These cells formed focal junctions with Kupffer cells and macrophages of the liver sinusoids. At the junctional site, small vesicles in the Kupffer cells are often observed opposite the excrescences of the infected erythrocytes. They appear to be formed in response to the presence of excrescences. This observation may indicate that these excrescences stimulate reticuloendothelial cells to become active for phagocytosis of the infected erythrocytes. In *P. brasilianum* infection, the absence of sequestration during schizogony, the pronounced suppression of parasitized erythrocytes during the crisis stage of the infection, the apparent affinity of infected cells with phagocytic cells and the development of a strong immunity in primary infections suggests that the excrescences of parasitized erythrocytes may play an important role in the immune reaction.

The presence of specialized components on the surface of certain cells, for example, the polysaccharide capsular material of *Diplococcus penunoniae*, endows these cells with the capacity to resist phagocytosis unless acted upon by specific antibodies, the opsonins. Taliaferro and Cannon (1936) suggested that the active capture of *P. brasilianum* parasites may possibly be aided by "the cooperation of an opsonizing antibody". They suggested that "such an antibody would give a satisfactory basis for the undoubted humoral effect noted in the present species at the time of crisis and would supply a plausible explanation for the specificity of malarial immunity...". Recent studies on *P. knowlesi* have demonstrated the presence of antigen on the surface membranes of infected cells and have shown that opsonic activity increases as the level of parasitemia decreases (Brown and Brown, 1965; Brown et al. 1970). In *P. brasilianum* infections, red cell excrescences increase in number with the maturity of the parasite within the cell. They appear, therefore, to be a direct response of the host cell to the presence of the parasite, and like the specialized surface components of other cells, they may possibly represent the specific surface sites against which opsonins are directed during the crisis stage of infection. Needless to say, further work is needed to clarify this hypothesis.

The striking similarity between *P. brasilianum* and *P. malariae* cannot be overlooked. Both parasites display a quartan periodicity and similar morphology in their erythrocytic and exoerythrocytic phases (Garnham, 1966). The similarities in morphology of the erythrocytic stages become most apparent when they are compared ultrastructurally. Smith and Theakston (1970) reported the following observations in their brief description of *P. malariae*: 1) the presence of surface excrescences in all parasitized cells, 2) mitochondria with tubular cristae, 3) the presence of a nucleolus, 4) clefts, morphologically similar to Mauer's clefts and 5) a large number of dense bodies (micronemes) in merozoites. Similar observations were made on *P. brasilianum* in this study. *P. brasilianum* and *P. malariae* are also immunologically related. Antigen preparations of *P. brasilianum* are routinely used for the fluorescent antibody test of *P. malariae* in man. The similarity between *P. brasilianum* and *P. malariae* is even more interesting when one also considers the fact that man is susceptible to *P. brasilianum*, while the Old World monkeys apparently are not (Garnham, 1966). As Garnham (1966) points out, "On an evolutionary basis, it seems quite impossible to reconcile the similarity of the quartan parasite of man and New World monkeys, and the striking differences between them and species such as *P. inui* in Old World monkeys." Future investigations in our laboratory are being aimed at resolving some of the similarities and differences between *P. brasilianum* and *P. malariae*.

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Virus-like Particles and Sporozoite Budding

J. A. TERZAKIS

Department of Pathology, Lenox Hill Hospital,
100 East 77th Street, New York, New York

ABSTRACT: Ultrastructural events in sporozoite differentiation of the *Plasmodium gallinaceum* oocyst are reviewed. The presence of virus-like particles in drug-treated and untreated oocysts is shown in the nucleus, cytoplasm and capsule. The particles in the capsule are seen in oocysts with mature sporozoites thus indicating that the particles are not associated with oocyst degeneration. An hypothesis is forwarded that some of the virus-like particles give rise to linear densities which are known to be important in sporozoite budding.

Studies on the sporogonous development of untreated and drug-treated *Plasmodium gallinaceum* oocysts have revealed interesting observations and raised unanswered questions with regard to oocyst differentiation and sporozoite budding. Earlier studies (Terzakis et al., 1966, 1967) have shown a rather well-ordered sequence of changes in the *P. gallinaceum* oocyst. For the first five days following the infective blood meal, there is little observable morphological change save for cytoplasmic enlargement as evidenced by increasing oocyst diameter and a great increase in the number of randomly dispersed oocyst nuclei. About the sixth day of the usual eight-day cycle, there is peripheral, subcapsular vacuolization and coalescence with

cleft formations and subdivision of oocyst cytoplasm into sporoblast islands. The nuclei migrate to linear densities located beneath the sporoblast plasma membrane and the sporozoite buds at precisely such areas. The present work seeks to explore the relationship to this differentiation of three distinct observations. These are: virus-like particles within oocysts; membrane-bounded virus-like particles upon and within the oocyst capsule, and the above-mentioned linear densities appearing beneath the oocyst plasma membrane.

Materials and Methods

The administration of specific antimalarial agents and the fixation of both treated and untreated infected *P. gallinaceum* midgut material was done as noted in previous works by Terzakis (1968, 1971). The tissue was embedded in Epon. Sections 1-2 microns in

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thickness were stained with a 0.5% alcoholic solution of toluidine blue and examined in the phase-contrast microscope. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome. Sections were stained with either alcoholic 0.5% uranyl acetate or saturated uranyl acetate followed by staining with lead citrate. The grids were carbon filmed and examined either in an RCA-EMU -3G operating at 100 kV or an Elmiskop 101 at 80 kV accelerating voltage respectively.

Results

Aggregates of virus-like particles are found within drug-treated and untreated *P. gallinaceum* oocysts (Figs. 1–5). In untreated oocysts studied to date, the particle aggregates have not been seen later than 48–54 hours following the infective blood meal; whereas in drug-treated oocysts, they are seen as late as seven days following the infective blood meal (Terzakis, 1969). The aggregates are enclosed by a pair of trilaminar membranes. Particle diameter is about 415 Angstroms. Some aggregates have no apparent relation to oocyst nuclei, while some are located close to a nucleus. In Fig. 3, an aggregate of virus-like particles is found within the outer nuclear membrane. The innermost of the membranes surrounding the aggregate and the inner nuclear membrane are distinctly seen and separate. Each particle has a dense central region and an outer, limiting trilaminar membrane best seen in Fig. 5. In an oocyst treated with 0.001% pyrimethamine, a partly disrupted aggregate of virus-like particles is noted adjacent to a nucleus. Some membrane-bounded particles are seen in nearby oocyst cytoplasm and a trail of them is noted deeply in the

cytoplasm close to the oocyst plasma membrane and within the oocyst capsule (Fig. 4). These particles exhibit some variability in size and some appear to be enclosed in a budding cytoplasmic vesicle (Fig. 4).

Linear densities are seen beneath the oocyst plasma membrane in untreated oocysts (Figs. 6–8). Such densities are noted in oocysts 48 hours following the infective blood meal as well as in older oocysts and in drug-treated oocysts. The densities are composed of variable lengths of parallel dense membranes separated by a small space of lower density. In some instances, each dense membrane appears single while in others, each is trilaminar. The linear densities vary in length from about 1380 to 9200 Angstroms although these longer lengths are unusual.

In both treated and untreated oocysts which have differentiated to form sporoblasts and sporozoites, round virus-like particles are seen on the inner surface of and within capsule substance (Fig. 9). The particles are enclosed by a trilaminar membrane. The core of the particles is of moderate density and there is some variability of particle size ranging from about 280 to 670 Angstroms. The close proximity of the virus-like particles to the oocyst plasma membrane and the linear densities is shown in Fig. 8. The particles have a thin trilaminar covering similar to a viral envelope in this instance.

Discussion

Important among the details of sporogonous development of *P. gallinaceum* oocysts outlined above was the apparent tropism between oocyst nuclei and linear densities occurring just below the oocyst plasma membrane. Such

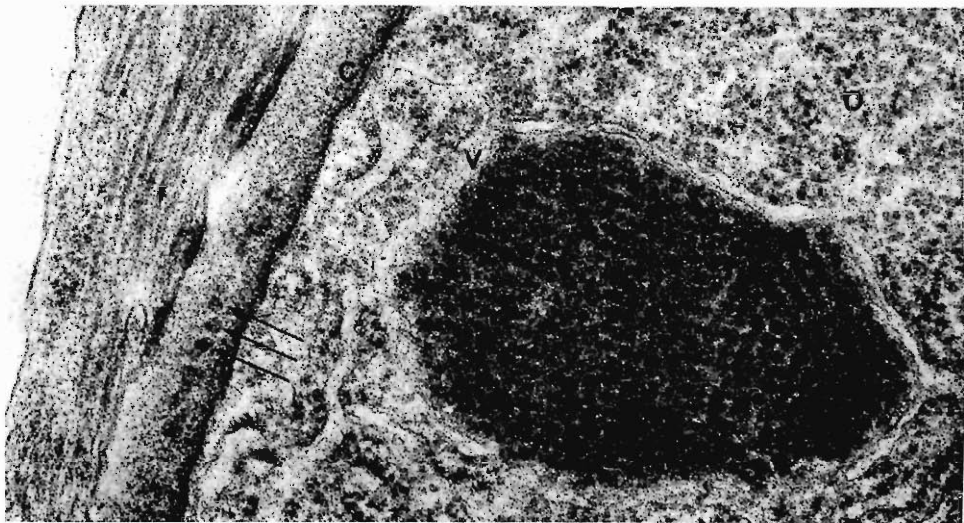
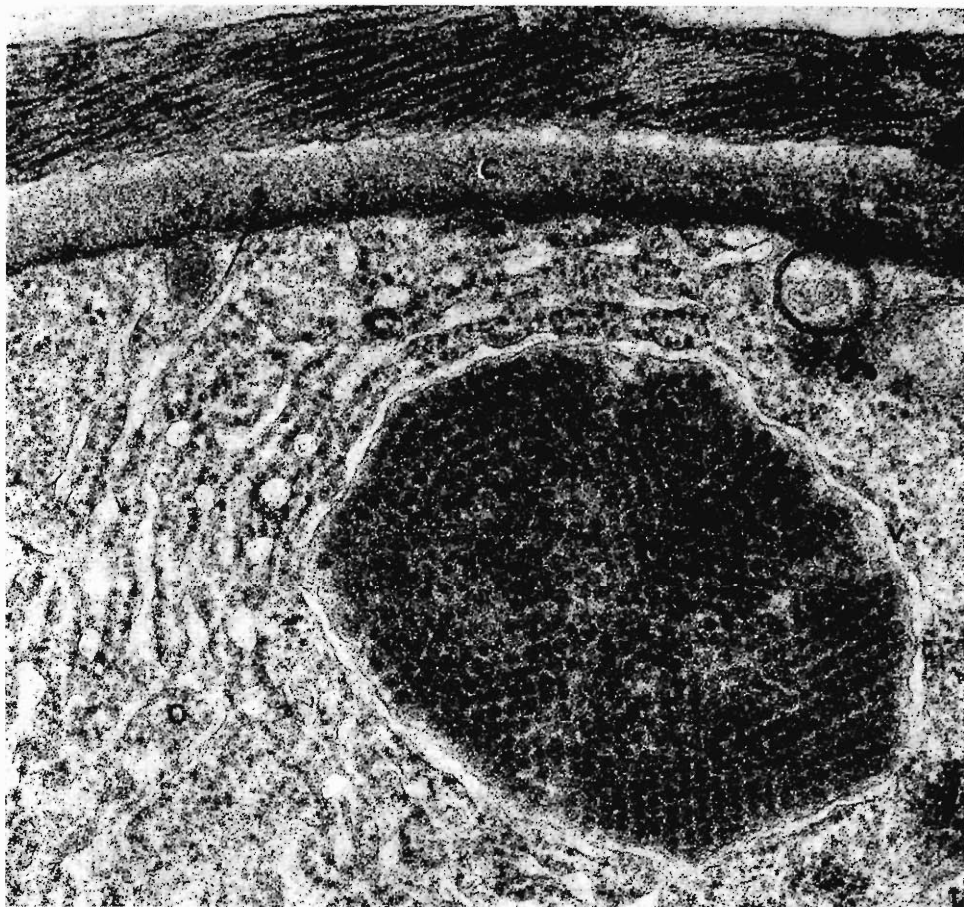
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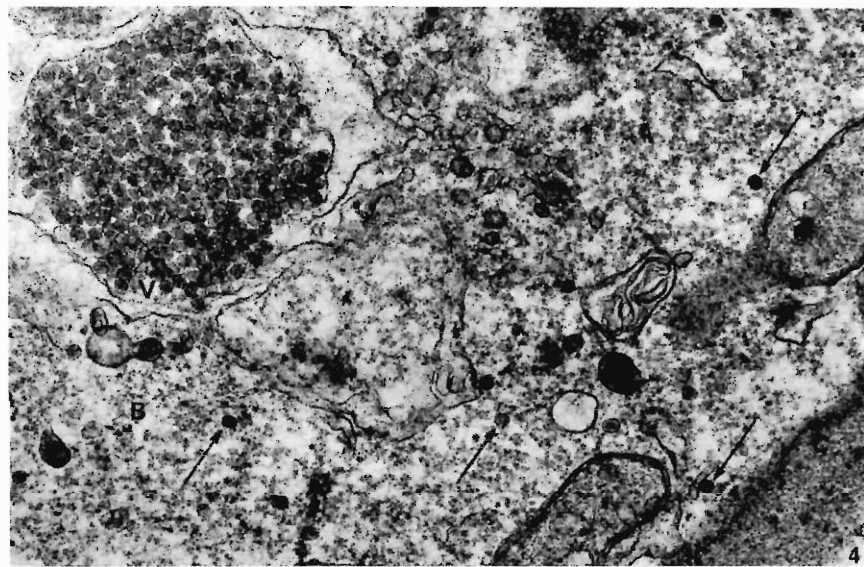
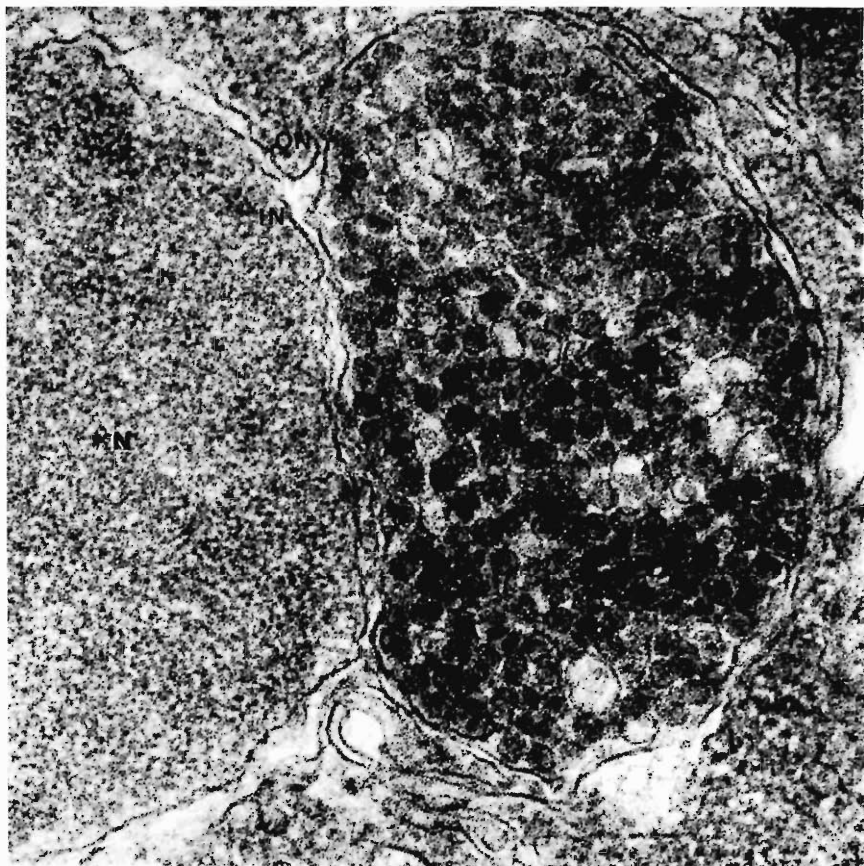
Figure 1. A crystalline array of virus-like particles (V) in an untreated oocyst (O) 48 hours following the infective blood meal. One particle (arrow) is seen within the oocyst capsule (C). 56,250X.

Figure 2. The virus-like particles (V) with oocyst cytoplasm (O) measure about 415 Angstroms in diameter. Several particles (arrows) are seen in oocyst capsule (C). 55,000X.

Figure 3. An aggregate of virus-like particles (V) is seen within a pair of membranes, one of which is the outer nuclear membrane (ON). The innermost membrane surrounding the particles is separate from the inner nuclear membrane (IN). Nucleus (N). Oocyst treated with 0.1% Primaquine. 105,000X.

Figure 4. A partly disrupted aggregate of virus-like particles (V) from which a trail of individual particles is seen in the cytoplasm and extending into the oocyst capsule. One particle is also seen in a budding cytoplasmic vesicle (B). Oocyst treated with 0.1% pyrimethamine. 40,000X.





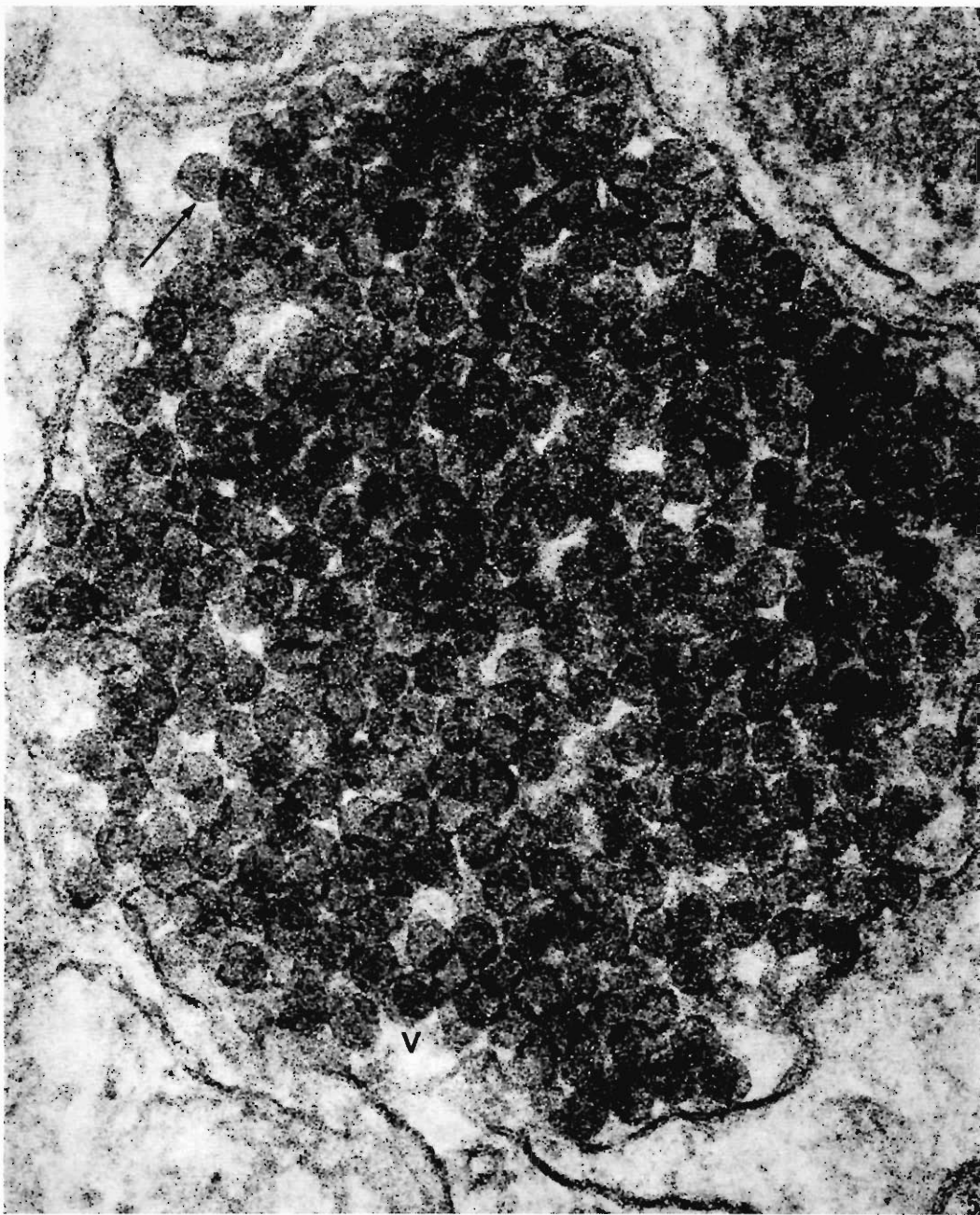
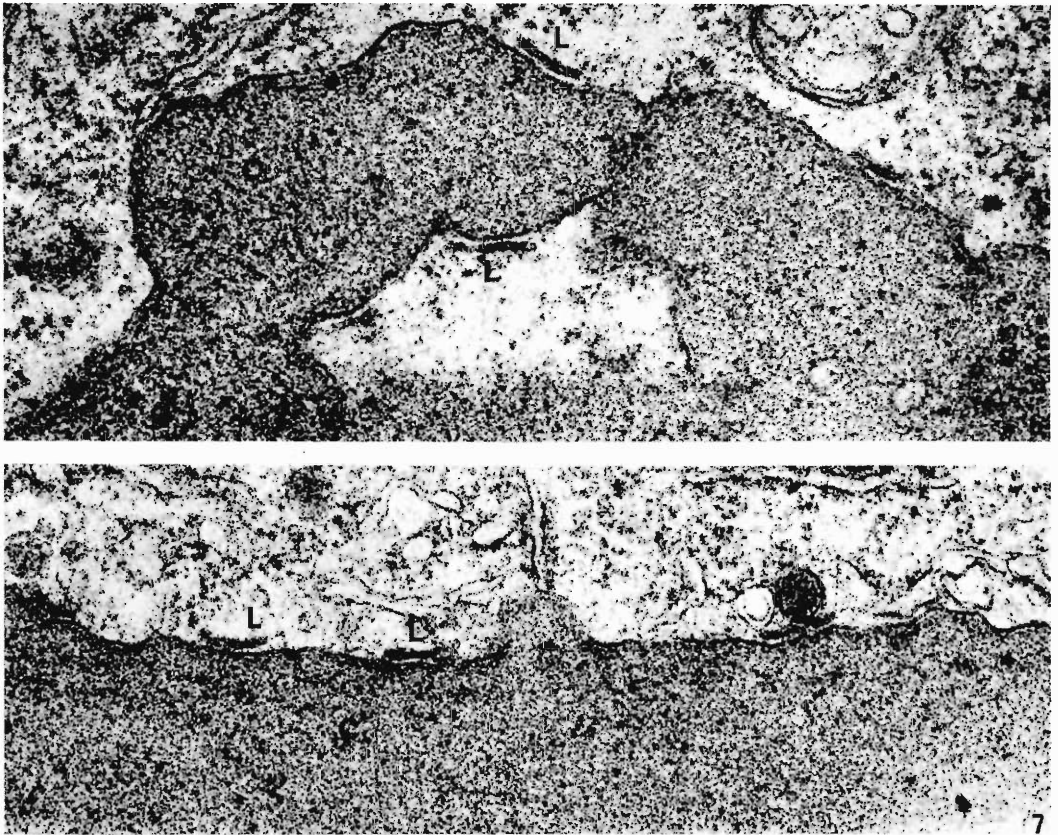


Figure 5. A higher magnification of particle aggregate (V) seen in Fig. 4. Particles have well-defined trilaminar membrane (arrows). 153,000X.



Figures 6 and 7. Linear densities (L) are seen in oocyst cytoplasm just interior to the oocyst plasma membrane and capsule (C). Untreated oocysts 48 hours. Fig. 6—65,000X; Fig. 7—57,000X.

densities serve as precursors of the inner pellicular membrane of each budding sporozoite in addition to being a focal point for a migrating oocyst nucleus. Furthermore the longitudinal axis of the mitotic spindle of such a nucleus is directed perpendicular to these linear densities. The origin of the densities has remained quite mysterious. The present work documents their presence at least as early as forty-eight hours following the in-

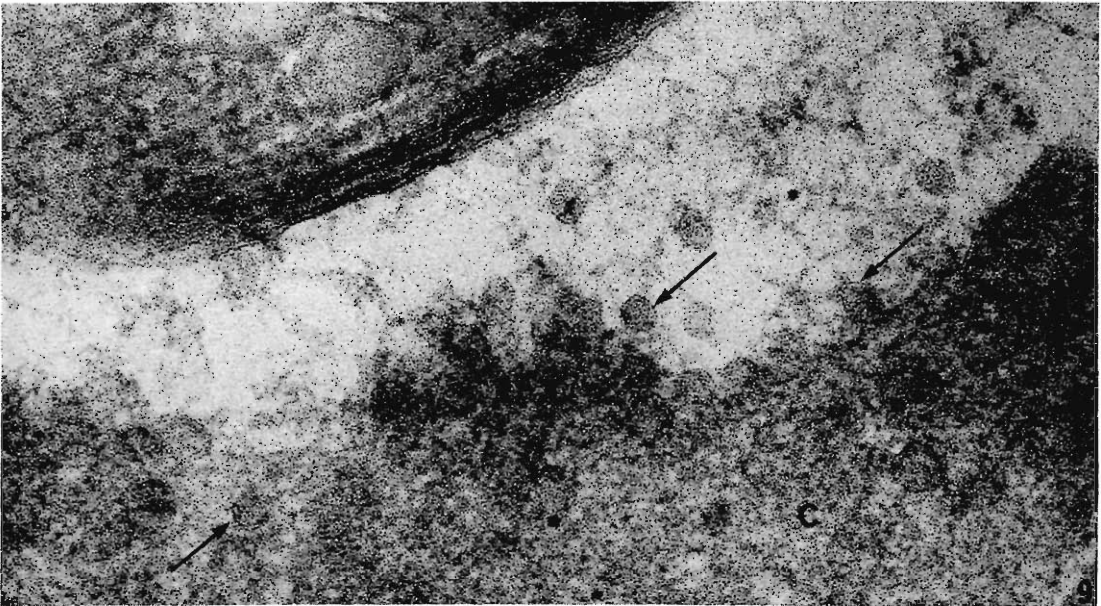
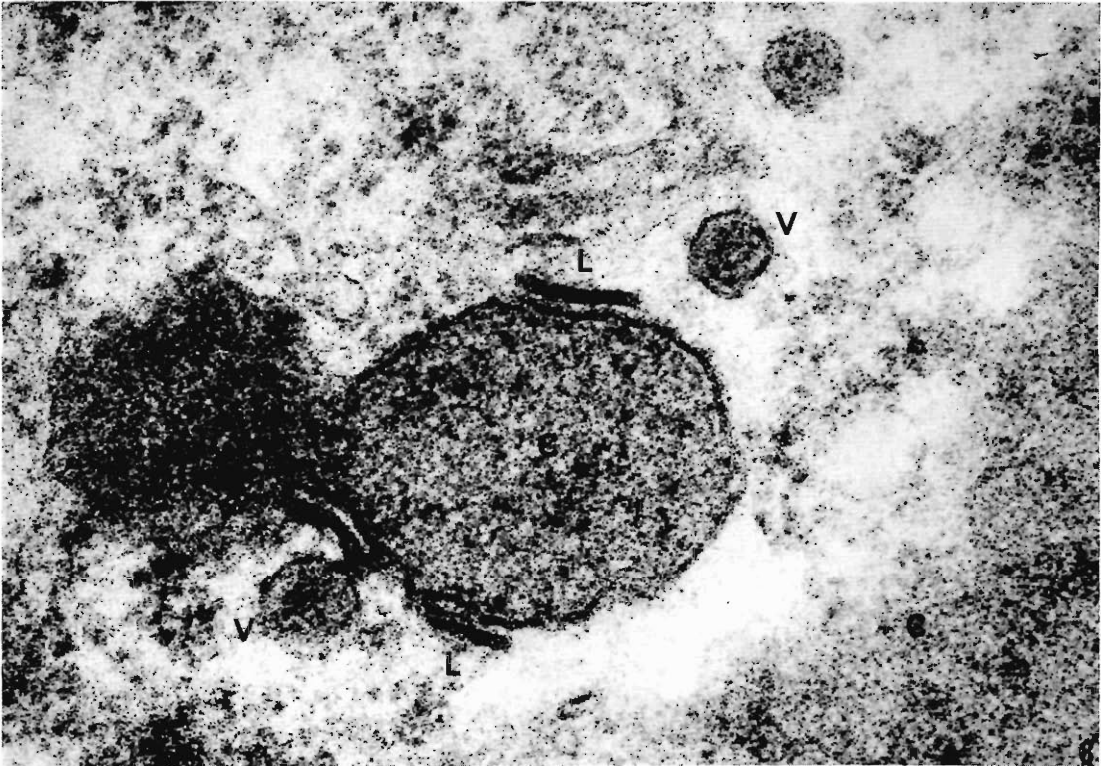
fective blood meal and indicates that some may be considerably longer than previously noted.

Virus-like particles have been reported in malarial parasites, particularly in the sporogonous cycle. Garnham (1961) and Garnham et al. (1962) described a crystalloid inclusion in ookinetes of *P. gallinaceum* and *P. c. bastienelli*. Brief mention of a virus affliction of plasmodia was made by Dasgupta in 1968.

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Figure 8. Virus-like particles (V) in close proximity to linear densities (L). The particles appear to have an additional envelope membrane about them. Oocyst capsule (C). Untreated oocyst 48 hours following infective blood meal. 110,000X.

Figure 9. Virus-like particles (arrows) on and within oocyst capsule (C). Maturing sporozoite (S). Untreated oocyst. 130,000X.



Terzakis (1969) described virus-like particles in retarded ookinetes and early untreated oocysts of *P. gallinaceum*. More recently, virus-like particles were demonstrated in exoerythrocytic infection of *P. b. berghei* by Bafort (1971). Davies et al. (1971) described virus-like particles in *P. b. berghei* oocysts and in *Anopheles stephensi* mid-gut epithelium. These workers also postulated that the virus-like particles may have a role in oocyst development.

One strong indication that the virus-like particles are not simply a reflection of imminent oocyst degeneration is their appearance in and upon the capsule in untreated and treated differentiated oocysts, i.e. those containing mature sporozoites.

The present work demonstrates the location of virus-like particles within oocyst nuclei in some instances. Furthermore, a limiting or capsid-like membrane is noted about each particle and strengthens the view that the particles are indeed viruses. The membrane-bounded particles seen individually in the cytoplasm and on the inner surface of and within the oocyst capsule closely resemble the virus-like particles within the nucleus. The data strongly suggests that the virus-like particles are released from the nuclear aggregate and make their way through the oocyst plasma membrane. The least length of the linear densities seen measures about 1380 Angstroms. This length corresponds closely to the circumference of the virus-like particles. It is suggested that some of the virus-like particles in passing through the cytoplasm to the oocyst capsule remain interior to the plasma membrane, open up, and that their capsid-like membranes become the linear densities.

Observations of budding sporozoites indicate that accretion of linear densities, which will become the sporozoite inner pellicular membranes, occurs in situ, i.e. growth of such membranes is not due to simple addition of other observable cytoplasmic components. Therefore, linear density growth is apparently autonomous and the synthetic apparatus must be located in close proximity. Suggestions by Vanderberg and Rhodin (1967) and Vanderberg et al. (1967) that blebbing of the nuclear membranes might serve as a precursor to the linear densities in the sporogonous cycle of *P. berghei* are not tenable in *P. gal-*

linaceum infections. The present work demonstrates linear densities as early as forty-eight hours following the blood meal when oocyst nuclei are randomly disposed in the cytoplasm and not particularly close to oocyst plasma membrane. Furthermore, no blebbing of oocyst nuclear membranes has been observed to date in this material.

The budding of malarial sporozoites brings to mind work done on a virus-mammalian epithelial cell system. From a study of parainfluenza virus SV5 in monkey kidney cells, Choppin et al. noted that viral envelope proteins are incorporated into discrete regions of the host cell membrane. Those areas serve as gathering points for virus particles. The mature virion is released after a budding process in which the virus particle is enveloped by host cell membrane. Figure 6 of their work shows a regular profile of budding cytoplasm and shares similarities to budding sporozoites.

The foregoing observations indicate that a symbiotic relationship may exist between the virus-like particles and the protozoan parasite.

Acknowledgments

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Observations on the Fine Structure of *Plasmodium traguli*

F. C. CADIGAN, JR., F. C. COLLEY, AND V. ZAMAN
Institute for Medical Research, Kuala Lumpur, Malaysia and Faculty of Medicine, University of Singapore, Republic of Singapore

ABSTRACT: Examination of the fine structure of *Plasmodium traguli* revealed the presence of mitochondria in schizonts and gametocytes, Golgi bodies and tubule complexes. In addition to the usual malaria pigment bodies two other inclusions were seen. One is in an aggregate of small dark granules, the other type has a lucent "crystalline" appearance.

Little is known of the biology of *Plasmodium traguli* because of the great difficulty in keeping the host, the lesser mouse deer (*Tragulus javanicus*), alive in the laboratory and because of the tiny size of the parasite. We have been able to overcome the first problem by establishing a successful colony of *T. javanicus* (details will be reported elsewhere). The tiny size of the parasite remains a source of frustration for light microscopy (Fig. 1). The erythrocytes of most mammals tend to be of uniform size, regardless of the body size of the host; however, the erythrocytes of the lesser mouse deer are unusually small, averaging

1.75 μ in diameter. In the present study the fine structure of *P. traguli* is compared with that of other *Plasmodium* species.

Materials and Methods

Blood obtained by ear puncture of several *T. javanicus* (of which one had been splenectomized), was allowed to clot for two minutes. The clots were fixed in 6% glutaraldehyde in phosphate buffer at pH 7.2, and then post-fixed in OsO₄. After dehydration in a graded series of ethyl alcohol, the blood was infiltrated with Spurr's (1969) low viscosity embedding medium. Thin sections were cut with glass knives on a Porter-Blum microtome, stained with uranyl acetate and lead citrate, and studied with an Hitachi H5-8 electron microscope.

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ABBREVIATIONS FOR ALL FIGURES

ER	ENDOPLASMIC RETICULUM	M	MEMBRANE	P	PIGMENT
FV	FOOD VACUOLE	MC	MEMBRANE COMPLEX	R	RIBOSOMES
D	DENSE INCLUSION	MH	HOST MEMBRANE	RBC	RED BLOOD CELL
G	GOLGI BODY	MI	MITOCHONDRION	RH	RHOPTRY
GR	GRANULAR INCLUSION	MP	PARASITE MEMBRANE	T	TUBULES
I	INCLUSION	N	NUCLEUS	V	VACUOLE

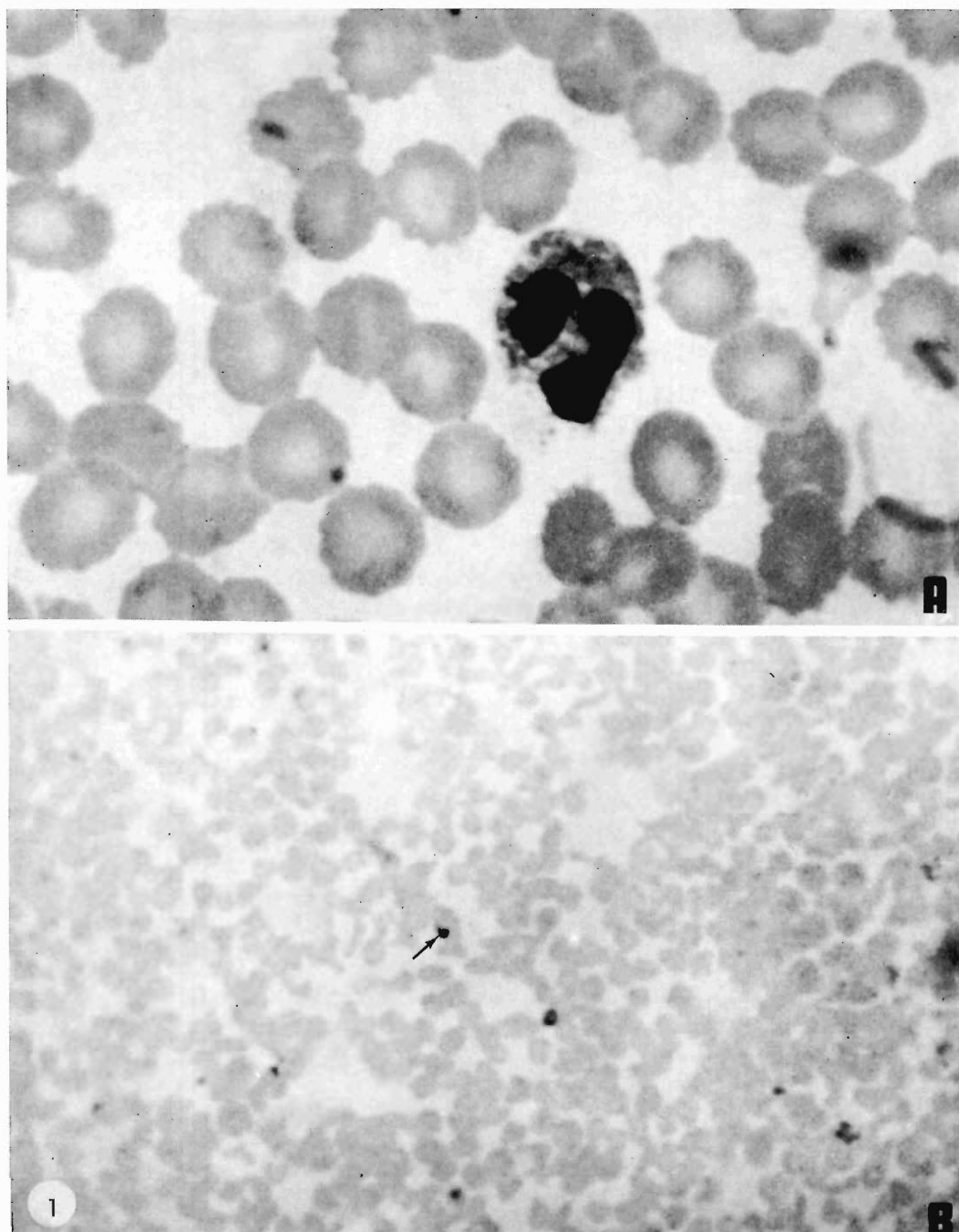


Figure 1. A. Human blood. B. Mouse deer blood (note malarial parasite at arrow). Giemsa stained. Approx. magnification X 2,300.

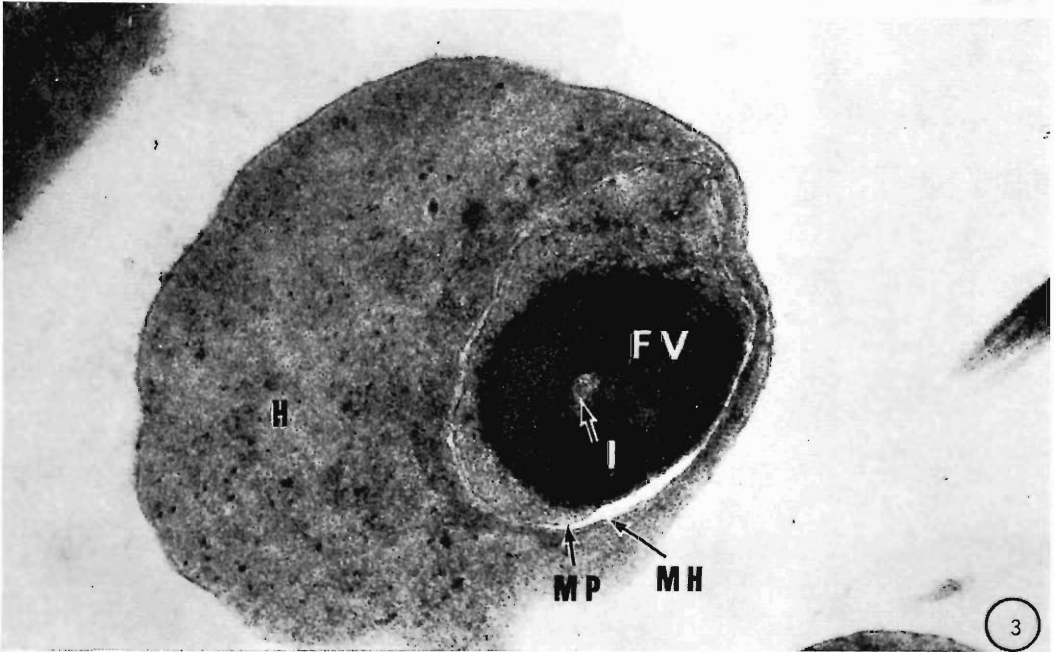
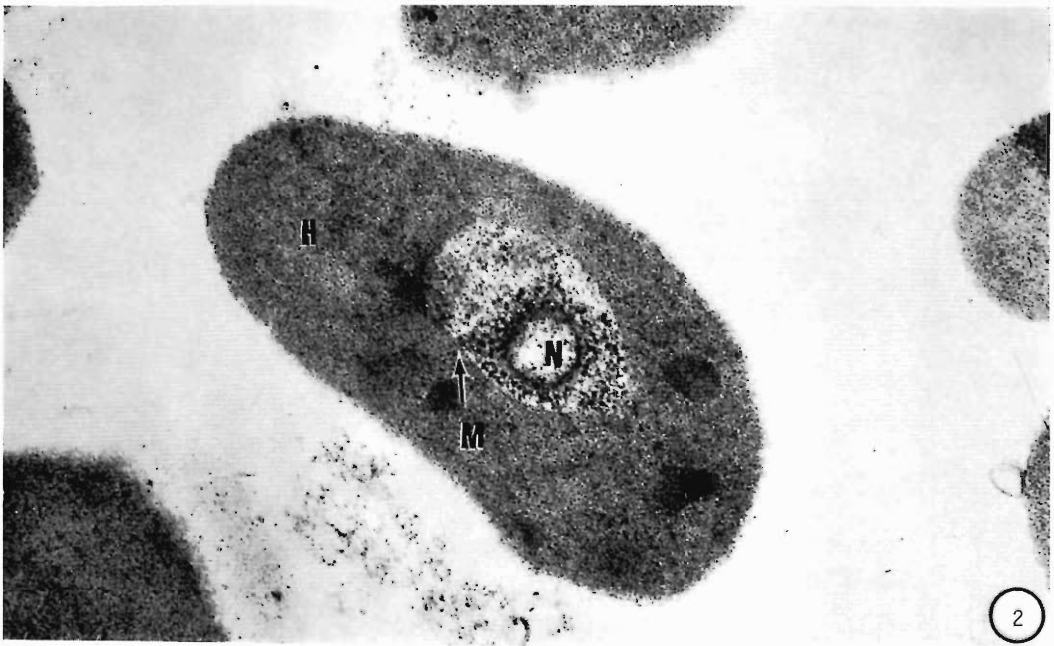


Figure 2. Young trophozoite. X 62,000.

Figure 3. Young trophozoite containing a food vacuole (FV) with a lucent inclusion (I). Note membranes of the parasite (MP) and host (MH). X 68,000.

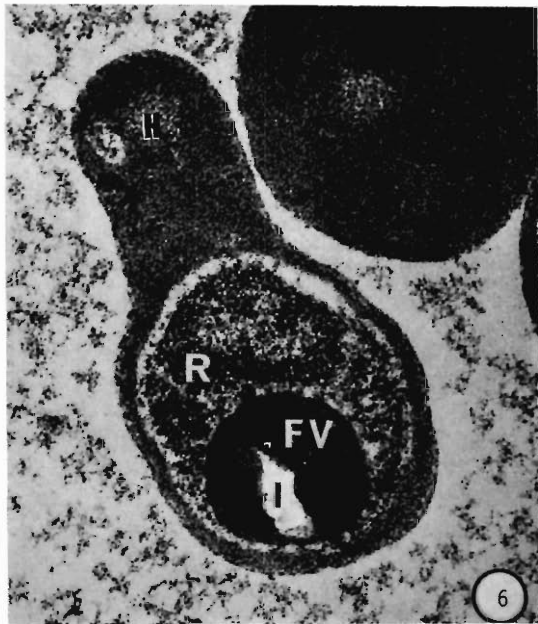
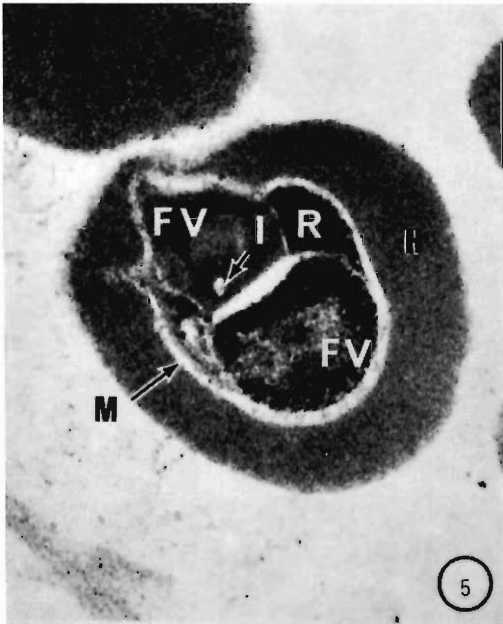
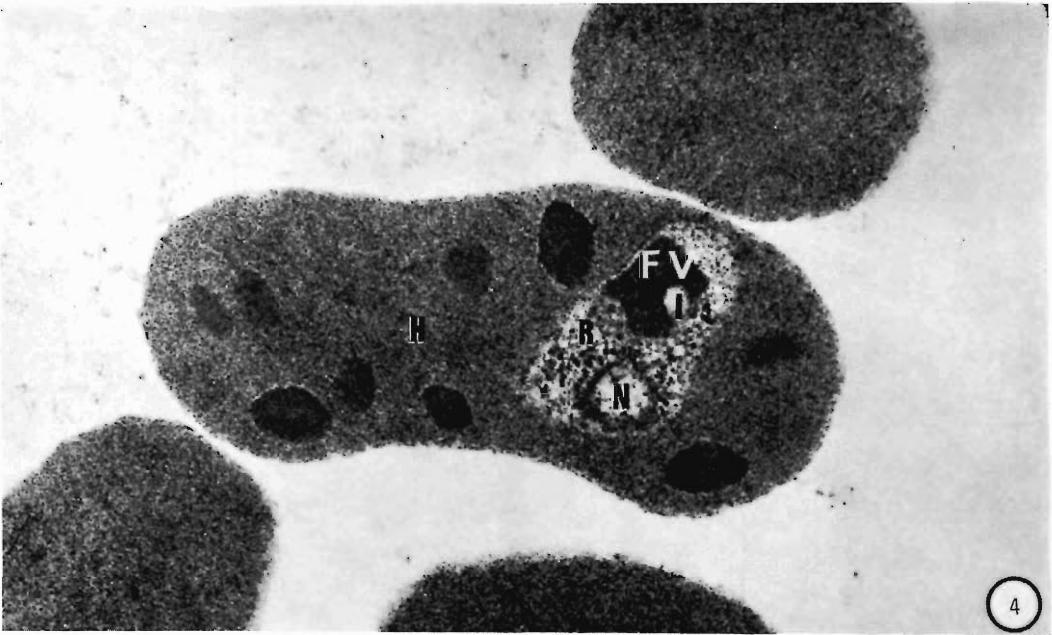


Figure 4. Young trophozoite. X 65,000.

Figure 5. Young trophozoite. X 65,000.

Figure 6. Young trophozoite. Note inclusion (I) in food vacuole (FV). X 72,000.

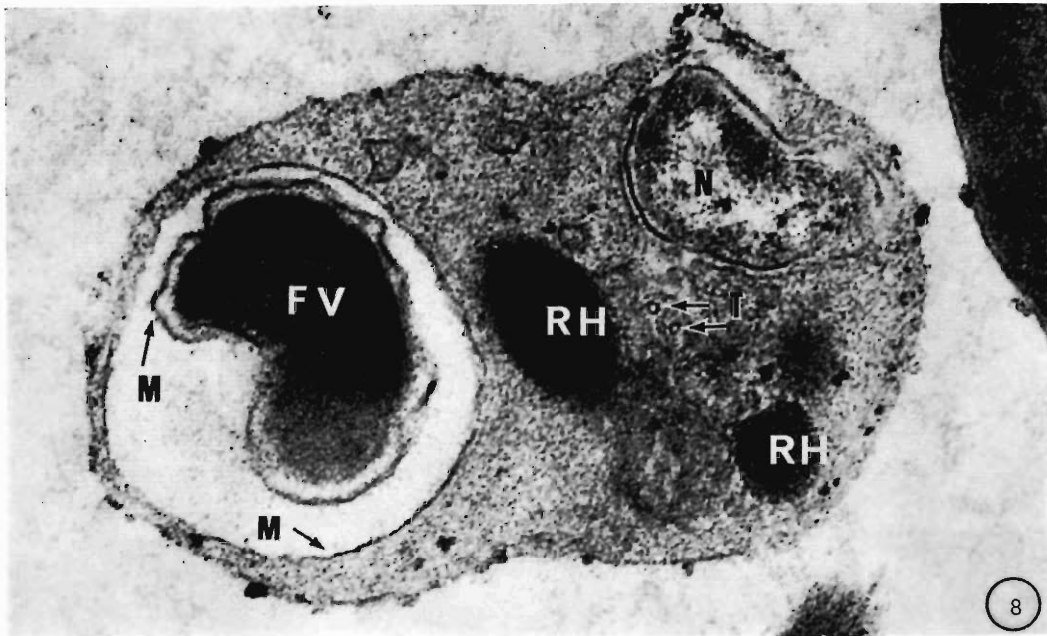
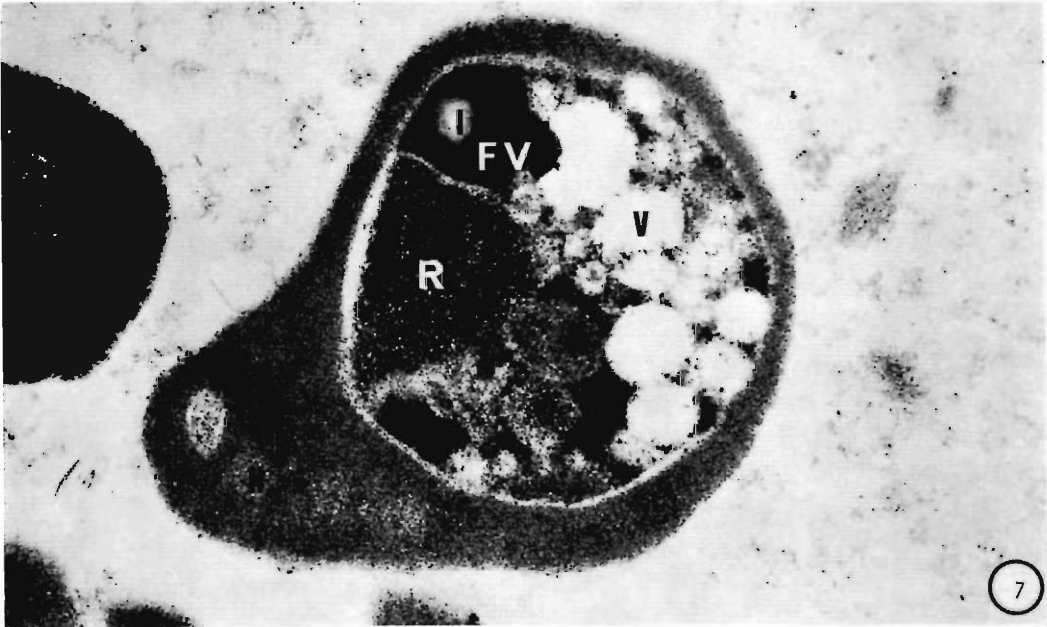


Figure 7. Young trophozoite. Note vacuoles (V). X 70,000.

Figure 8. Older trophozoite. Rhoptries (RH), and tubules (T) may be seen in the parasite cytoplasm. X 58,000.

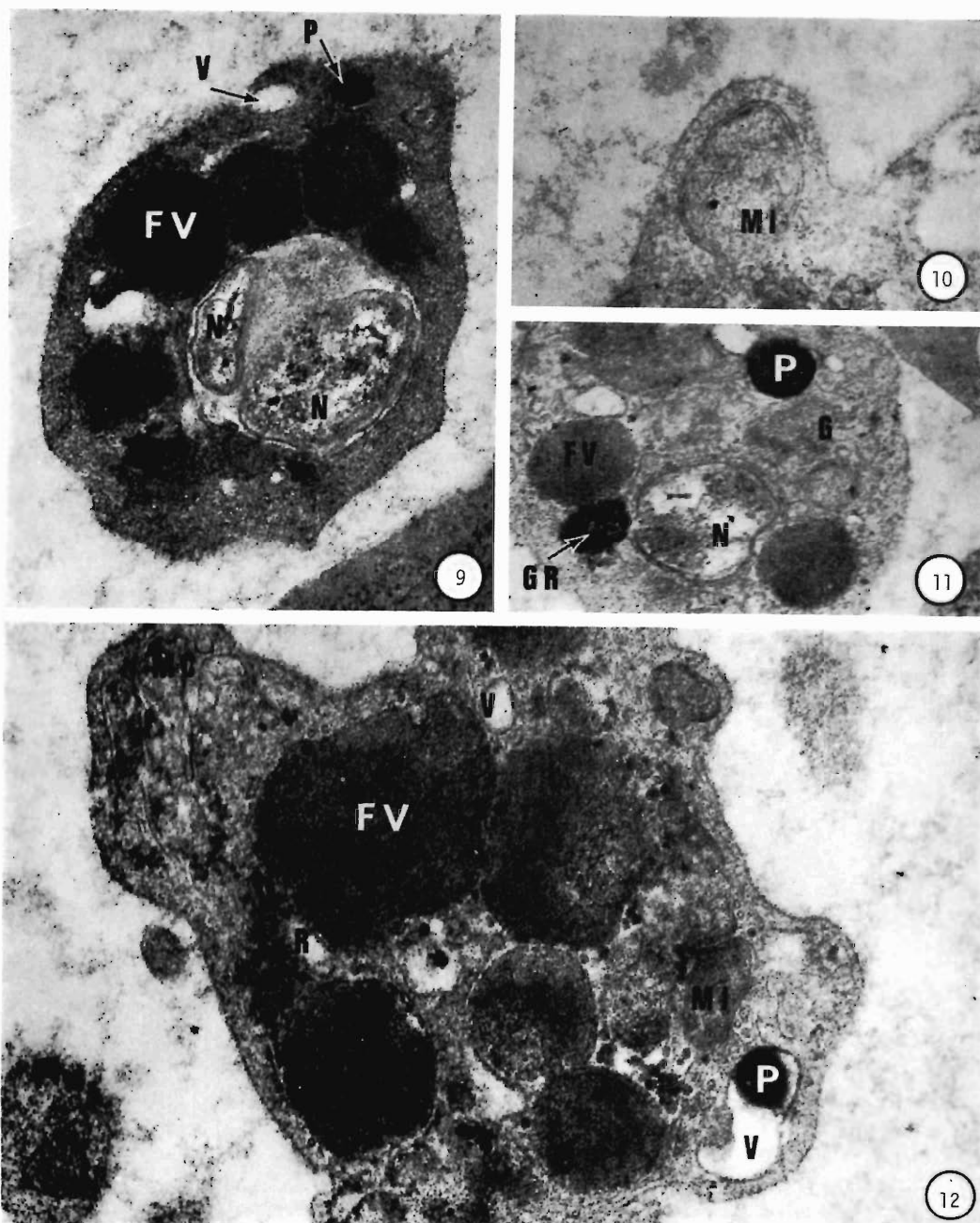


Figure 9. Young schizont. X 55,000.

Figure 10. Mitochondrion of schizont. X 55,000.

Figure 11. Schizont containing Golgi body (G) and granular inclusion (GR). X 41,000.

Figure 12. Schizont containing a membrane complex (MC) and mitochondria (MI). X 55,000.

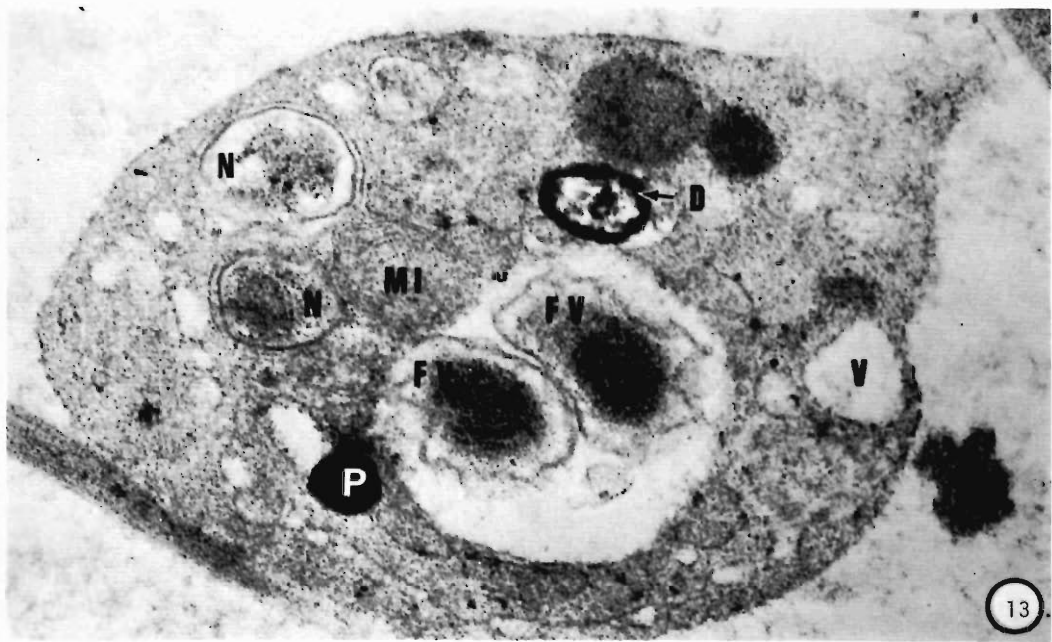


Figure 13. Young schizont with two nuclei (N). X 55,000.

Figure 14. Older schizont with three nuclei (N). X 63,000.

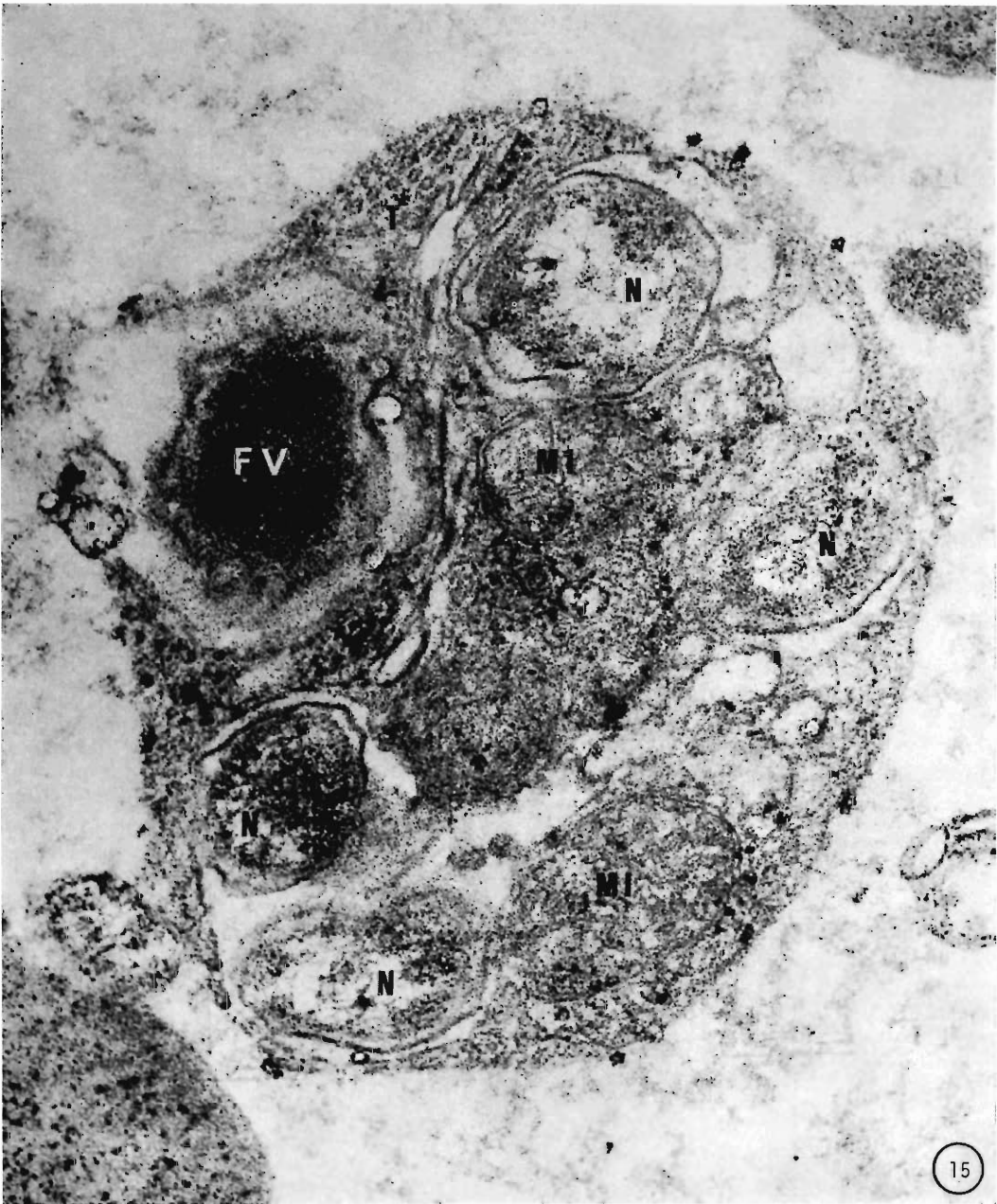


Figure 15. Older schizont with four nuclei (N) and large mitochondria (MI). X 55,000.

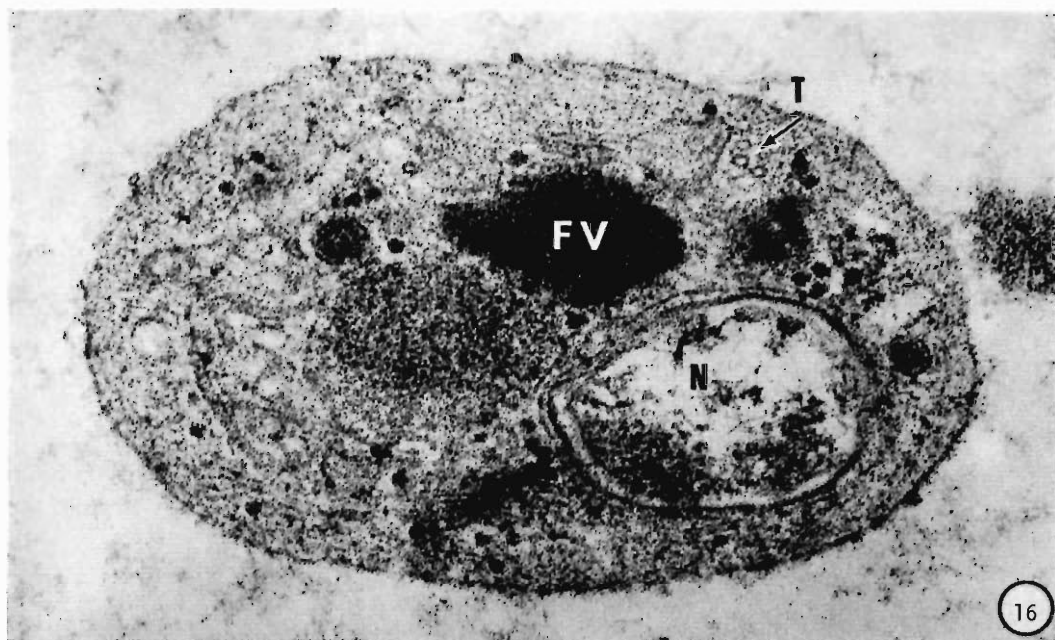


Figure 16. Gametocyte. X 55,000.

Results

Trophozoites (Fig. 2-8)

The smallest ring form of *P. traguli* observed by electron microscopy was approximately $0.61\ \mu$ by $0.4\ \mu$. At this stage the nucleus is surrounded by dense granular material, presumably ribosomes. A unit membrane surrounds the parasite and a similar membrane separates it from the host cell. Large, dense food vacuoles are seen in most of the early trophozoites. Some of these vacuoles contain a small lucent inclusion. In larger trophozoites this clear inclusion has a crystalline appearance. The parasite membranes are not always easily seen in early stages. This may be due to the pH of the fixative (7.2). Smith *et al.* (1969) have indicated that a fixative with a pH of 8.0 results in better membrane preservation in *P. falciparum*.

Older trophozoites occupy most of the host erythrocyte and electron lucent vacuoles appear in the cytoplasm. The parasite nucleus at this stage is large and distinct. Rhoptries and small tubules are also present at this stage.

Schizonts (Fig. 9-15)

Young schizonts contain multiple food vacuoles, membrane bounded vesicles, pigment granules, and mitochondria. A group of membranes resembling a Golgi body may sometimes be seen near the nucleus. Inclusions consisting of clusters of dense granules which are distinct from the homogeneous pigment granules are present. In addition, inclusions which are similar to the crystalline inclusions seen in earlier stages are present and are surrounded by a very dense ring. Distortion of the shape of the erythrocyte is common and the host cell is definitely enlarged. Pigment is usually seen in or at the edge of lucent membrane bounded vacuoles. Many ribosomes are present, usually in clumps in several locations. Mitochondria with membranous cristae are common in late schizonts and clusters of tubules are sometimes seen.

Gametocytes (Fig. 16-18)

Gametocytes contain tubules and mitochondria as well as ribosomes. The host cells are definitely enlarged. What appears to be a

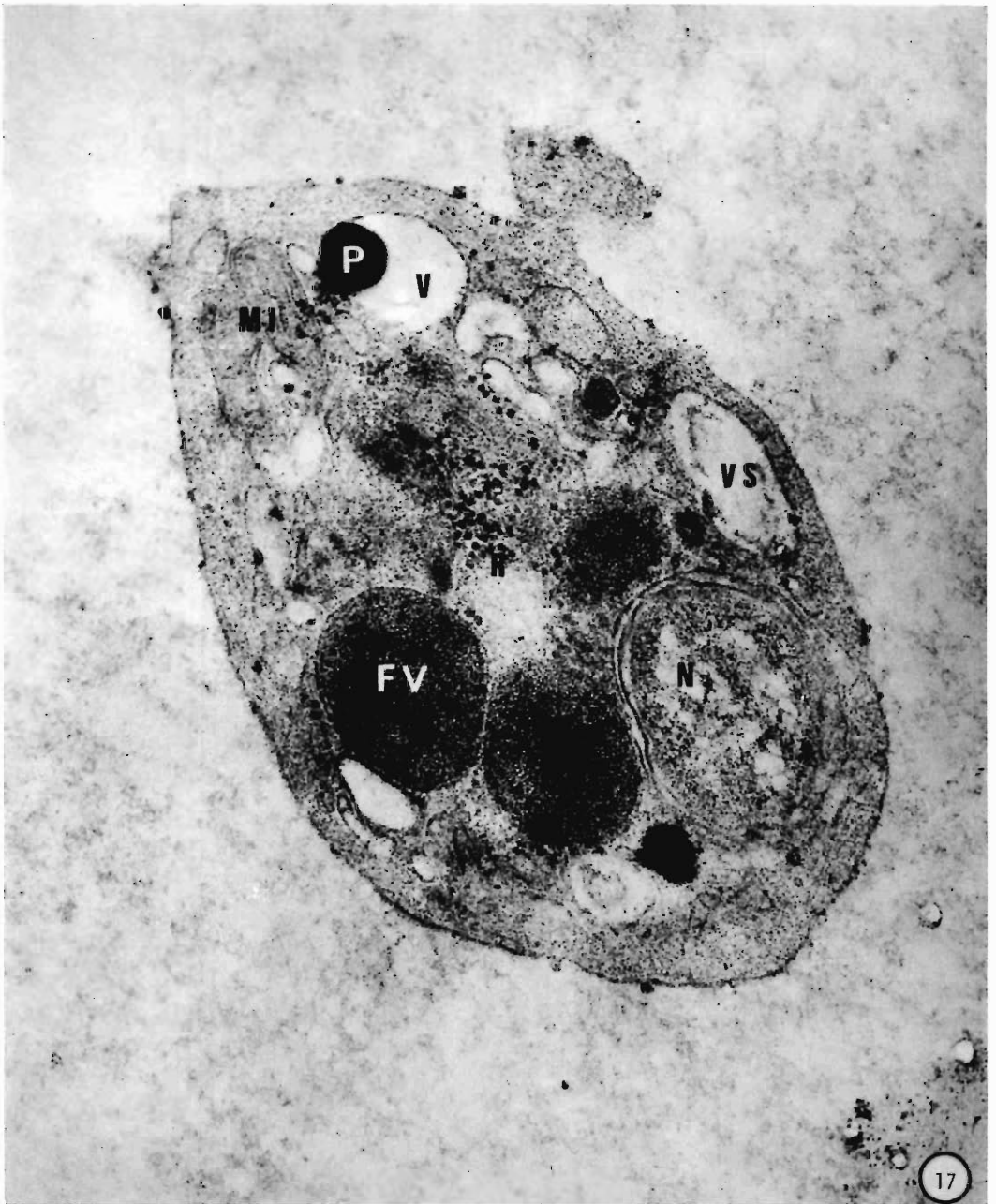


Figure 17. Gametocyte containing ribosomes (R) and mitochondria (MI). X 70,000.

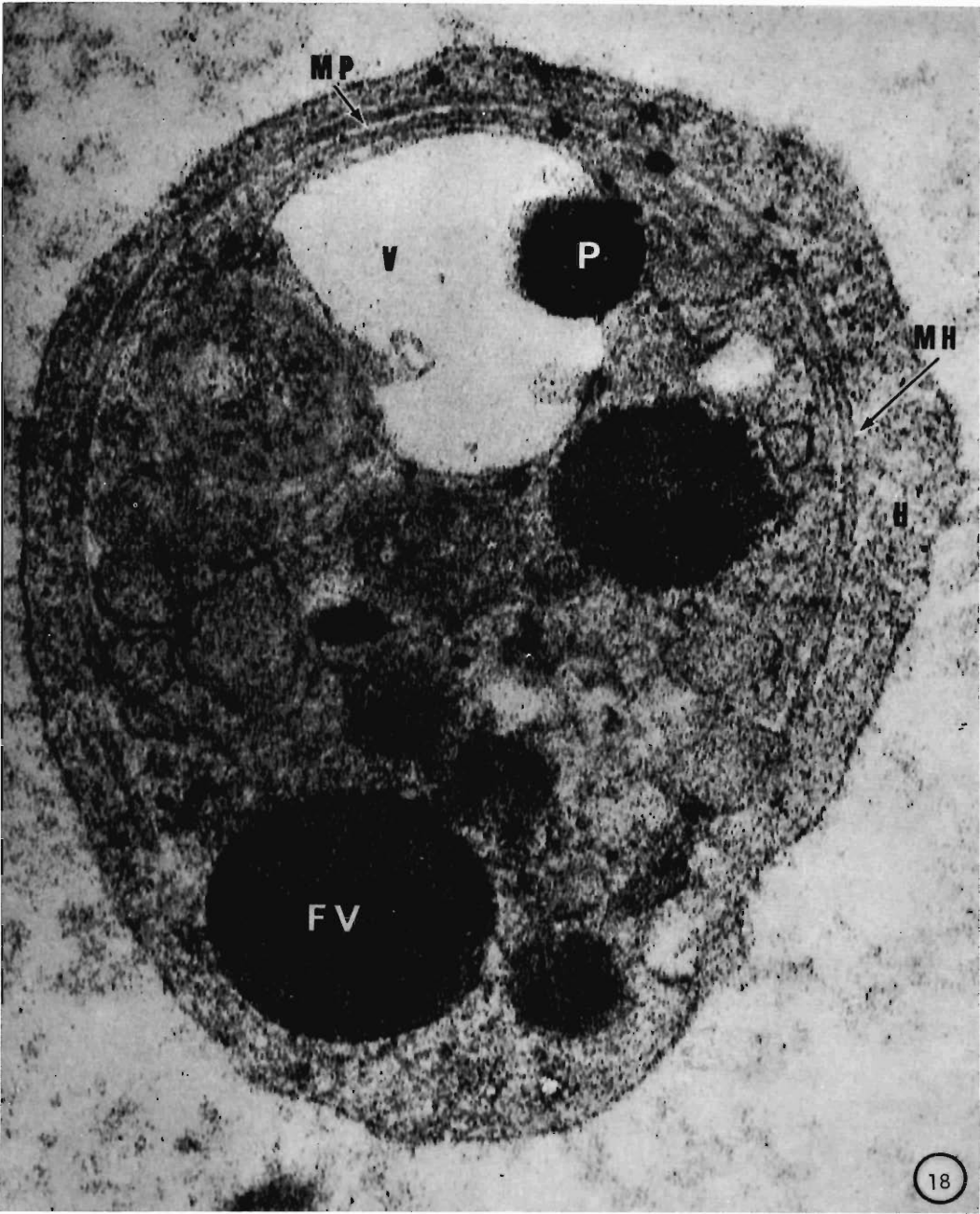


Figure 18. Gametocyte. Note membranes of parasite (MP) and host (MH). X 60,000.

mature gametocyte is limited by a unit membrane and is separated from the host cell by a similar membrane. The largest gametocyte seen measures $2.4 \mu \times 3.1 \mu$.

Discussion

Although mitochondria are common in avian malarias they seem to be very uncommon in mammalian malarias; however, they have been reported in *P. falciparum* (Smith *et al.*, 1969). The mitochondria of *P. traguli* have membranous cristae rather than the tubular cristae which are characteristic of protozoan mitochondria. The number of mitochondria appears to increase during schizogony. We have not seen the concentric membrane complex and "sausage-shaped" double membrane structure seen in other mammalian malarias (and which is thought to fulfill the function of mitochondria in them).

Structures resembling Golgi bodies have been seen. Golgi bodies have also been seen in *P. falciparum* and *P. berghoi* (Rudzinska, 1969). Multiple tubules are seen in rows in trophozoites and young schizonts and in clusters in other schizonts. Further study of the nature of these large clusters of tubules seen in the late schizont will be necessary in order to determine their function. No nucleoli or

aggregates of chromatin material have been seen.

As has been described for other malarias, there is considerable variation in the shape of erythrocytes parasitized by trophozoites but the outlines of cells containing gametocytes are regular. The four-fold increase in volume which the gametocyte causes in the host cell may be the cause of this regular surface. The density of the host cell cytoplasm decreases as the parasite develops until its cytoplasm is virtually indistinguishable from that of the parasite. The two double walled membranes surrounding the older gametocyte are characteristic of mammalian malarias (Rudzinska, 1969).

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Morphologic Studies on the Freeze-Etched Avian Malarial Parasite *Plasmodium gallinaceum*^{1,2,3}

C. A. M. MESZOELY,⁴ R. L. STEERE AND G. F. BAHR

Armed Forces Institute of Pathology, Washington, D.C. 20305, and Plant Virology Laboratory, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT: The malarial parasite *Plasmodium gallinaceum* is a unicellular, intracellular blood parasite inhabiting the nucleated erythrocytes of chickens. This ultrastructural study on material prepared with improved freeze etching techniques emphasizes the third dimensional aspects of the parasite complementing and confirming earlier morphological studies on thin sections. Our study demonstrates a very intimate relationship between the conoid region and the pellicular complex suggesting that this region is highly specialized, differing markedly from the surrounding surface areas. An estimated nuclear pore count of between 30–40 is obtained for the parasite and a count of over 100 for the host erythrocyte nucleus. A surface view of the cytostome shows it to be a simple depression on the plasma membranes with an orifice of 800 Å. The surface appearance of the outermost trophozoite membrane is smooth, while the inner surface of the innermost membrane, where it contacts the cytoplasm, is rough. The interphase membrane surfaces appear also smooth.

Since the first electron microscopic study of the erythrocytic forms of *Plasmodium lophurae* by Rudzinska and Trager (1957), much has been learned about the ultrastructure of the avian malarial parasite. With the aid of improved glutaraldehyde fixatives and better embedding media a number of new structures and organelles have been discovered and their organization more accurately described by Ristic and Kreier (1964) and especially by Aikawa (1966, 1967), Aikawa et al. (1966a, 1966b, 1967, 1969) and Aikawa and Jordan (1968). All of the above studies were limited to the examination of stained ultrathin sections, except one by Aikawa (1967) in which negative staining was also employed.

Now the freeze-etching technique first developed by Steere in 1957 and significantly improved (Steere, 1969a and Steere and Moseley, 1969) allows for the direct study of avian parasites in the third dimension. Not

only a much clearer understanding of the spatial arrangement and configuration of cell organelles is obtained, but the technique also exposes membrane and intermembrane surfaces for detailed study of their mutual relationships. It was particularly for this latter reason that this study was undertaken, since these membranes can be assumed to exert decisive control over the passage of antimalarials into the parasite cytoplasm and nucleus. The morphology of asexual erythrocytic forms of the avian parasite *Plasmodium gallinaceum* is described in this paper for the first time as revealed through freeze etching and compared with what is already known from studies on ultrathin sections.

Methods

P. gallinaceum was obtained for the purpose of this study through the kind cooperation of Dr. Richard L. Beaudoin of the Naval Medical Research Institute, Bethesda, Maryland, where this parasite is maintained through weekly blood passages in 2- to 3-week-old White Rock chickens. The parasitized blood was fixed for one hour in 1.25% glutaraldehyde in 0.05M phosphate buffer as described by Aikawa (1969). Some of this fixed material was placed in ascending glycerol series until a 40% concentration was reached, then frozen and fractured at -190°C under vacuum. The

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³ The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

⁴ Present address: Department of Biology, Northeastern University, Boston, Massachusetts 02115.

exposed fractured surface was subsequently shadowed with platinum and carbon. The replica thus obtained was freed from the adhering specimen in a chromic acid solution and mounted on collodion-coated 75-mesh copper grids. This is essentially the method described by Steere (1957). In a recently introduced variation of the technique by Steere (1969b) both surfaces originating along the plane of fracture are shadowed simultaneously, and replicas of corresponding opposing surfaces are obtained. This double replica system (see Figs. 4, 5, 7, and 14) allows for a more precise position of subcellular components. A control sample was post-fixed in 2% OsO_4 , dehydrated in an ascending alcohol series and propylene oxide, and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). The replicas from cell fractures and thin sections were studied and recorded with an RCA EMU IV or the Siemens 101 electron microscope.

Observations and Discussion

From studies on stained ultrathin sections of the asexual erythrocytic forms of the avian malarial parasite the following cell compo-

nents have been identified: 1) two plasma membranes in the trophozoite and an additional inner microtubular pellicle in the merozoite, 2) a cytostome (or micropyle), 3) mitochondria, 4) a spherical body, 5) an endoplasmic reticulum, 6) a ribosome, 7) a nucleus with a double nuclear envelope, 8) a nucleolus, 9) food vacuoles, 10) a residual body, 11) paired organelles, 12) dense bodies (toxonomes), and 13) a conoid. The last four structures occur only in the dividing schizont and merozoites and in the very early trophozoites. Most of the listed structures and organelles are identifiable in the freeze-etched parasite, but endoplasmic reticulum, ribosomes, and microtubules are rare. A nucleolus or a mitotic apparatus has not been identified in such preparations. The identifiable structures are usually prominent and will be described below.

Pellicular System

The pellicular system of the mature trophozoite appears in thin sections to consist of two plasma membranes (Fig. 3). Recent studies (Ladda, 1969; Ladda et al., 1969; Ladda and Steere, 1969) on the penetration of erythrocytes by malarial parasites indicate that the

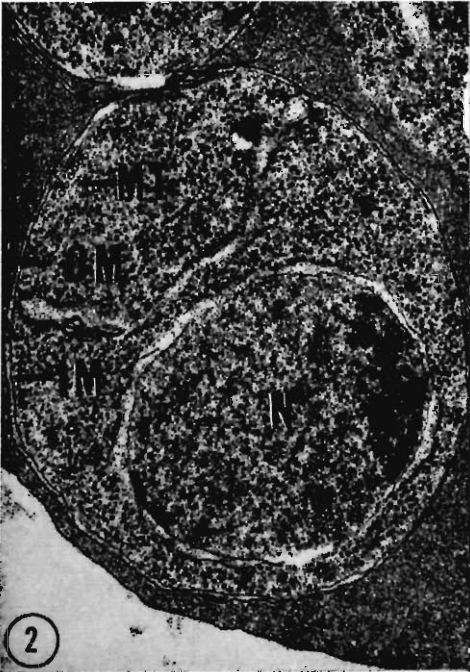
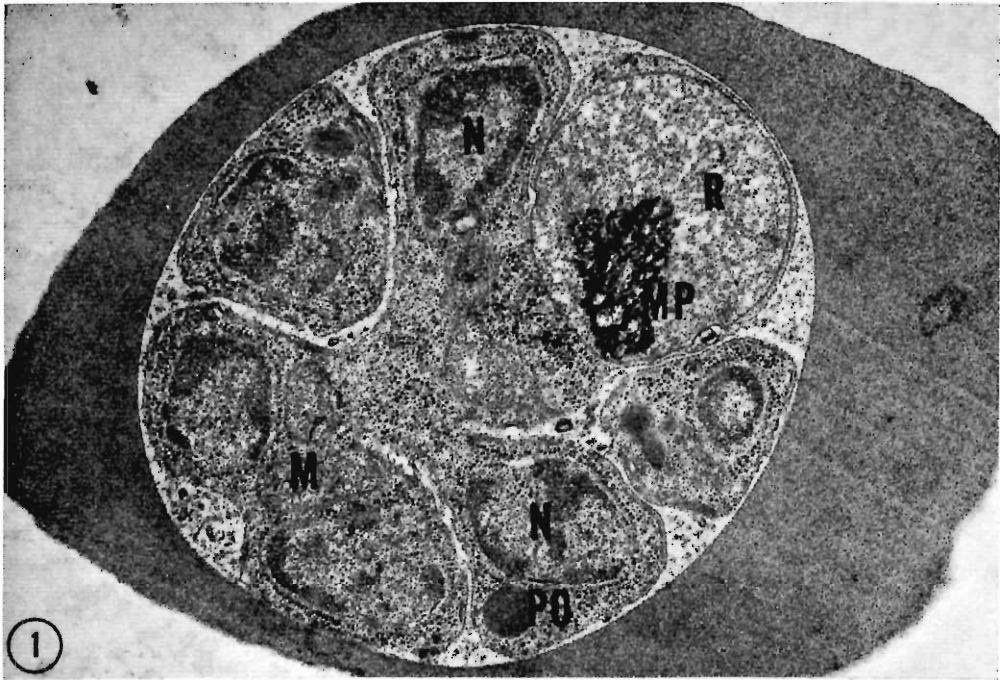
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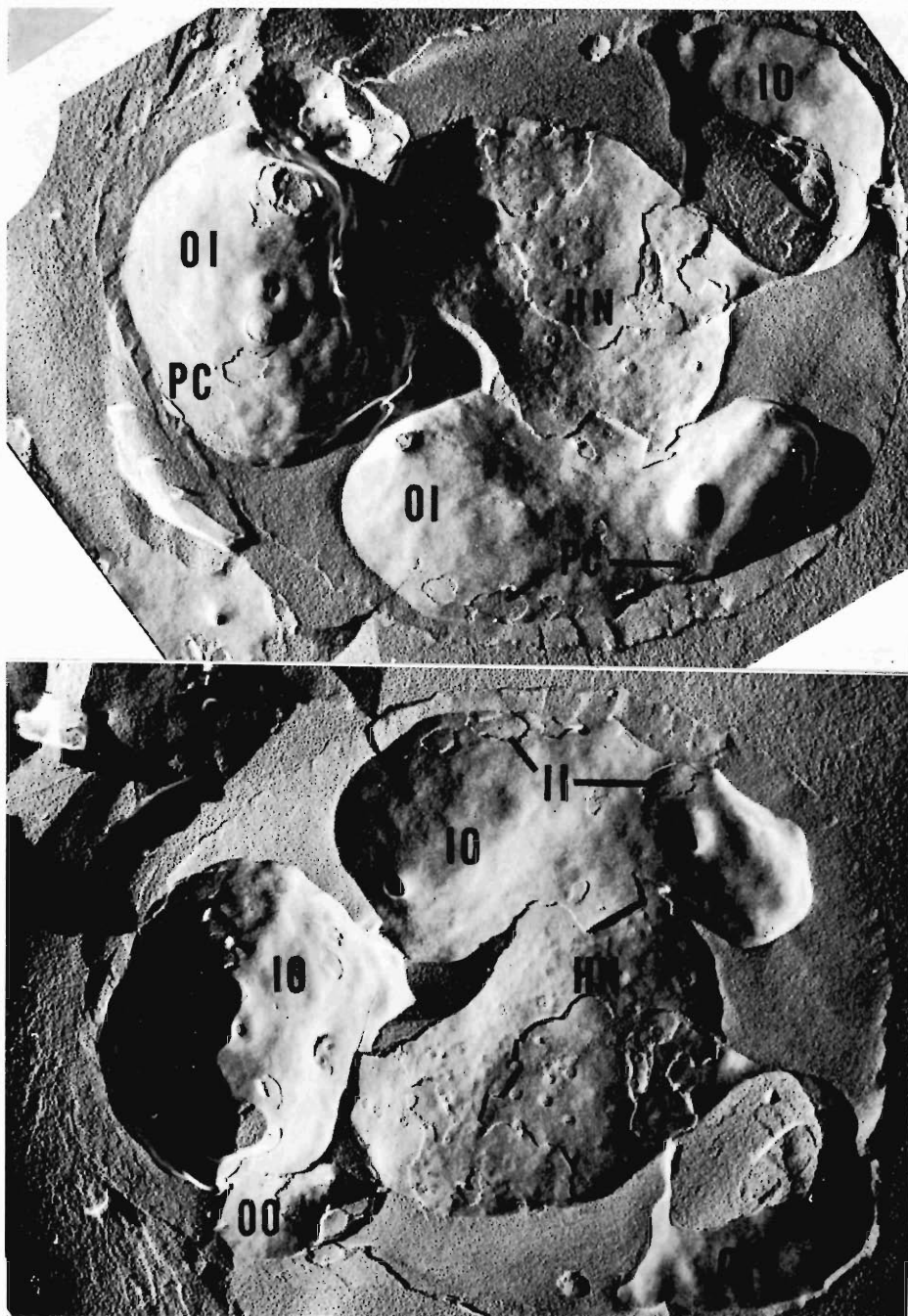
Figures 1-3. Photomicrographs of stained thin sections of erythrocytic stages of *Plasmodium gallinaceum* presented for comparison with the freeze-etched preparations. Fig. 1. Mature schizont of *P. gallinaceum* with budding merozoites. *M*, mitochondrion; *R*, residual body containing darkly staining malarial pigment (*MP*); *N*, nucleus; *PO*, paired organelles. Ribosomes and the pellicular complex are also shown. $\times 22,400$. (AFIP Neg. 70-5833-1.) Fig. 2. An early trophozoite shortly after penetration of the host erythrocyte, as indicated by its pellicular complex. *IM*, inner membrane; *MT*, microtubules; *OM*, outer membrane. $\times 30,000$. (AFIP Neg. 70-5833-2.) Fig. 3. A feeding trophozoite with functional cytostome (*CT*) and food vacuoles (*F*) with malarial pigment; *SB*, spherical body. $\times 25,000$. (AFIP Neg. 70-5833-3.)

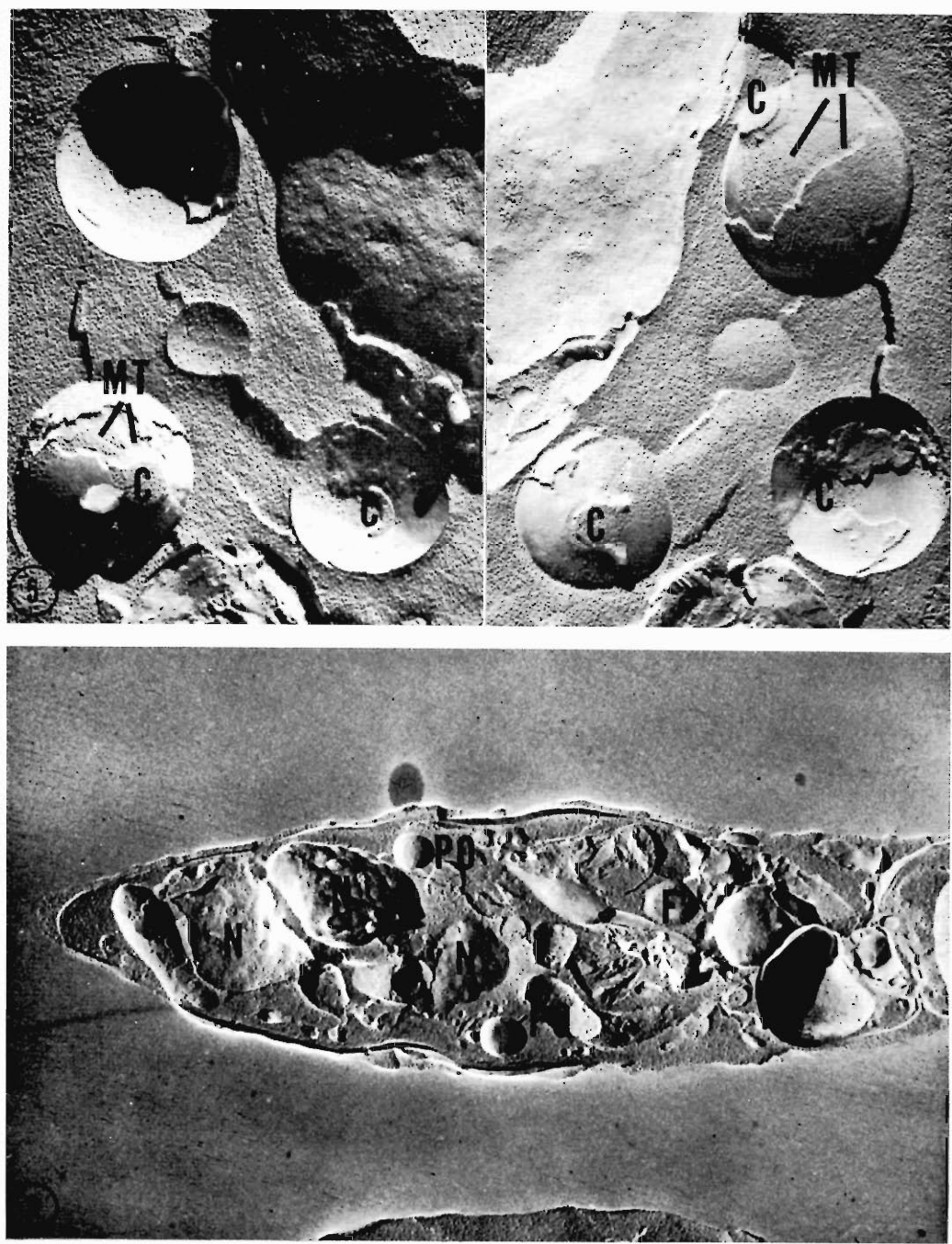
Figures 4A and B. Double replica of a freeze-etched nucleated erythrocyte containing three parasites. The micrograph shows the surfaces of the various membranes of the malarial parasites. *II*, inner surface of the inner membrane; *IO*, inner surface of the outer membrane; *OI*, outer surface of the inner membrane, *OO*, outer surface of the outer membrane. *HC*, host cytoplasm. Note that opposite the latter no membrane is visible in the top photograph, but only the host's own cytoplasm. *PC*, parasite cytoplasm; *HN*, erythrocyte nucleus. $\times 17,600$. (4A.—AFIP Neg. 70-5833-4; 4B.—AFIP Neg. 70-5833-5.)

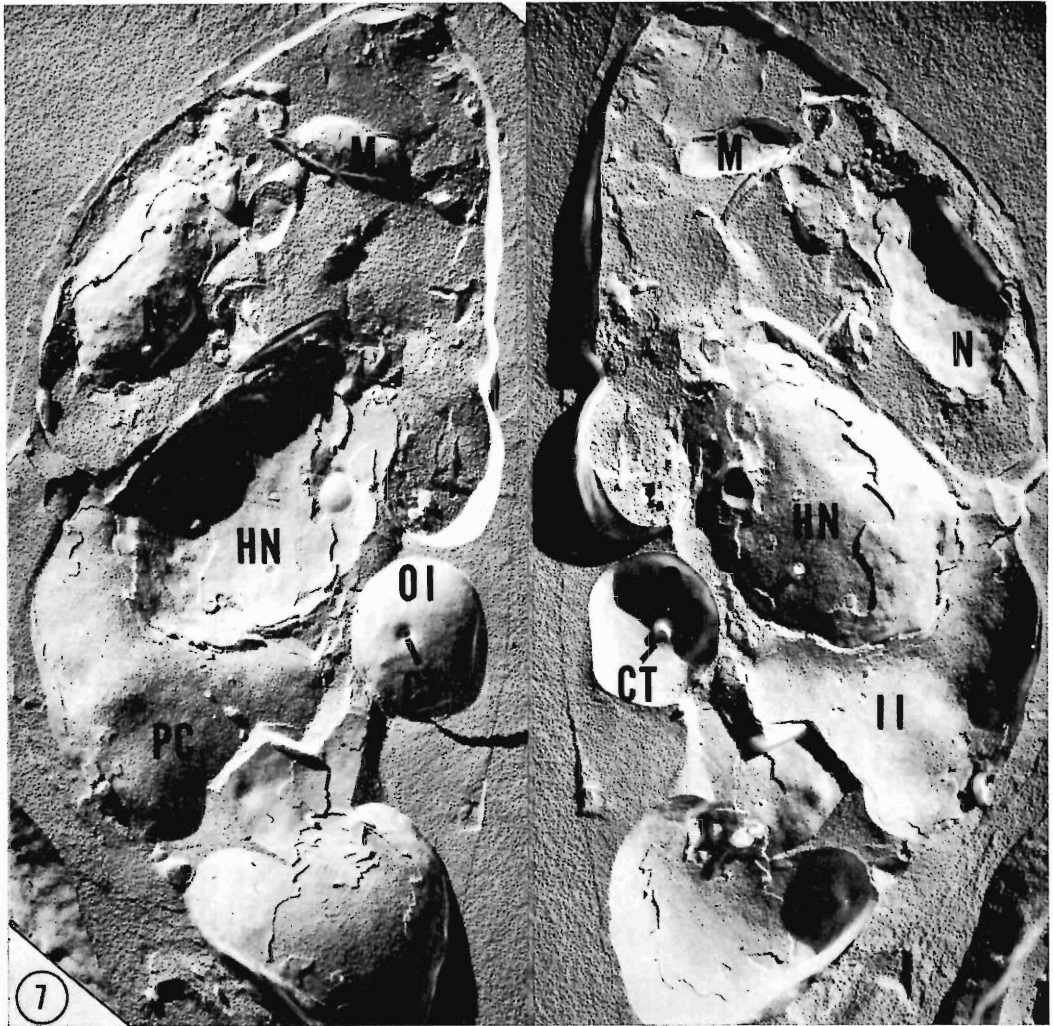
Figures 5A and B. Double replica of three young parasites that just completed invasion of the host as indicated by their small size, spherical shape, presence of microtubules (*MT*), and conoid (*C*), with polar ring. Note that even though the membranes of the parasite are fractured away from surrounding areas, they remain firmly attached to the conoid region. $\times 26,400$. (5A.—AFIP Neg. 70-5833-6; 5B.—AFIP Neg. 70-5833-7.)

Figure 6. An early schizont with several nuclei (*N*). Paired organelles (*PO*) can also be distinguished, and the lower one is surrounded by smaller spherical dense bodies or toxonomes. *F*, food vacuoles; *M*, mitochondrion. $\times 19,000$. (AFIP Neg. 70-5833-8.)









Figures 7A and B. Double replica of an erythrocyte with several parasites and a host nucleus (HN). *II*, inner surface of the inner parasite membrane; *IO*, inner surface of the outer membrane; *OI*, outer surface of the inner membrane. Note that the micrographs show that two membranes are involved in the formation of the cytostome (*CT*). A small cytoplasmic plug in the left picture fits into a pore in the replica on the right. *M*, mitochondrion; *N*, nucleus; *PC*, parasite cytoplasm. X 17,600. (7A.—AFIP Neg. 70-5833-9; 7B.—AFIP Neg. 70-5833-10.)

outer membrane is of host origin; that is, plasmalemma invaginates and finally surrounds the parasite as it invades the erythrocyte.

In freeze-etched preparations, the outer surface of the outer membrane, as well as the opposing surfaces between outer and inner membranes, is smooth (Figs. 4, 6 and 14). The surface of the membrane in con-

tact with the cytoplasm of the parasite, however, is deeply pitted. The pits are impressions of granular particles of the order of 80 to 100 Å that cover the cytoplasmic surface (Fig. 7). Occasionally some of these granules remain attached to the peeled surface of the inner membrane.

The fracture planes produced during speci-

men preparations most frequently occur between outer and inner membranes in the trophozoite. Next in frequency are fracture planes that follow the surface of the cytoplasm, while fracture planes along the outermost surface of the parasite are rare. This phenomenon in our freeze-fractured material appears to support the current concept that the outer membrane is of host origin, since the outer membrane of the trophozoite shows a stronger affinity toward the host than toward the parasite. Ladda and Steere (1969) briefly described the morphologic characteristics of the rodent malaria, *P. berghei*. They suggested that this parasite is surrounded by two membranes—an outer membrane with a smooth outer surface and an inner membrane with a rough granular surface. The rough granular surface shown in their micrograph is nearly identical to what we describe as the cytoplasmic surface, and we suggest that it is the surface of the cytoplasm that they depicted in their paper. In merozoites and very early trophozoites the pellicular system is more complex than that of the mature trophozoite (Aikawa, 1967). The free merozoite is surrounded by two outer membranes and an inner microtubular layer (Fig. 2). After penetration the parasite acquires one additional membrane (for complete discussion of the fate of membranes in the malarial parasite see Aikawa et al. (1969).

Because of the complexities of the pellicular system in the merozoite (or early trophozoite) arising from erythrocyte penetration and the great number of fracture planes possible, the surface properties of various membrane components have not been fully resolved in these areas. The innermost microtubular skeleton, however, has been observed (Fig. 5). Tubules radiate outward from the conoid region much like longitudinal lines subdividing the globular parasite. If the microtubules have been peeled off from the cytoplasmic surface, impressions are left which are devoid of cytoplasmic granules.

Conoid Region

The conical anterior end of the merozoites and sporozoites of the malarial parasite is termed "conoid" (Aikawa et al., 1969a, 1969b; Garnham et al., 1960). In section, the trun-

cated tip of this structure appears to be encircled by densely staining polar rings from which the above-mentioned microtubules radiate. Also closely associated with the conoid are the strongly osmophilic paired organelles and smaller dense bodies (toxosome) (Fig. 1). Recent studies (Ladda et al., 1969) convincingly demonstrated that the conoid region of the free merozoites is the structure primarily responsible for the process of invasion of the erythrocyte host. It appears that when the tip of the conoid end contacts the plasmalemma of the erythrocyte, this membrane invaginates, creating a cavity that eventually surrounds the parasite. Both paired organelles and small dense bodies are presumed to contain enzymes that aid in the process of host invasion.

Conoid, paired organelles and dense bodies are also clearly visible in freeze-etched preparations of merozoites (Figs. 6 and 11). The conoid region can still be identified after host invasion, by the presence of a polar ring and associated microtubules (Fig. 5), even though the shape of the parasite has changed from pear-shaped to globular. It is highly significant that in a distinct number of the young trophozoites the pellicular system remained firmly attached to the conoid region while all other membranes appeared to have been peeled from the surrounding cytoplasmic surface of the parasite by the fracturing process (Fig. 5). This intimate relationship between the conoid region and the pellicular system suggests that this is a highly specialized area with surface properties markedly different from the remaining cytoplasmic surfaces of the parasite. When one considers the importance of the conoid during the process of penetration this observation is not surprising.

Cytostome (Micropyle)

The cytostome of the malarial parasite in sectioned material appears as a depression in the pellicle, having two opposing sets of darkly staining bands in its wall near its opening (Fig. 3). Aikawa and co-workers (1966a, 1966b) showed the cytostome to be a feeding structure through which the parasite ingests the cytoplasm of the host. This structure appears to be functional only in the more advanced stages of the trophozoite, when

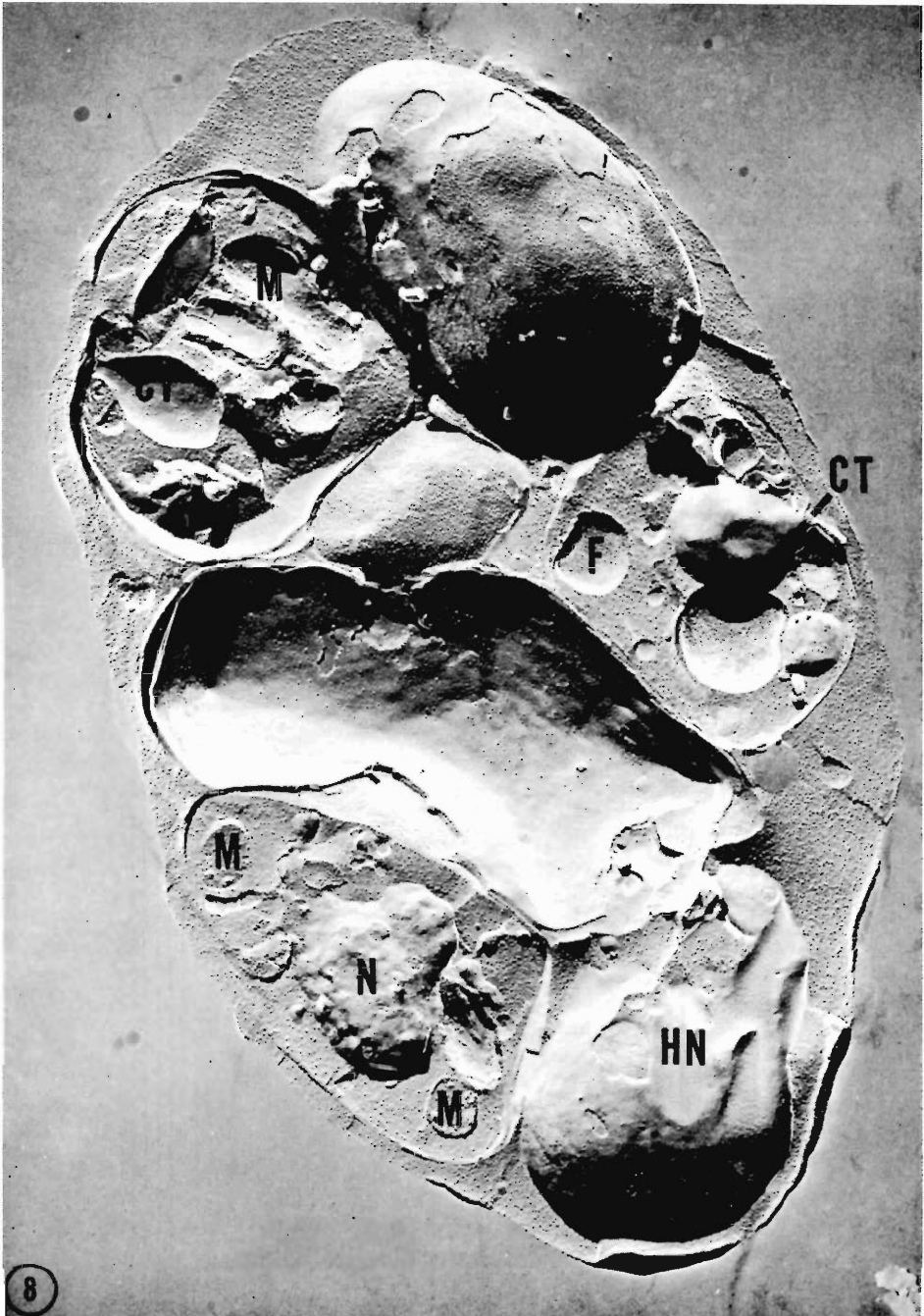


Figure 8. An erythrocyte of a chicken with several parasites and host nucleus (*HN*). Two cystosomes are shown which resemble flasks with narrow necks. Isolated food vacuoles (*F*), mitochondria (*M*), and nucleus (*N*) with nuclear pores are also visible. X 23,000. (AFIP Neg. 70-5833-11.)

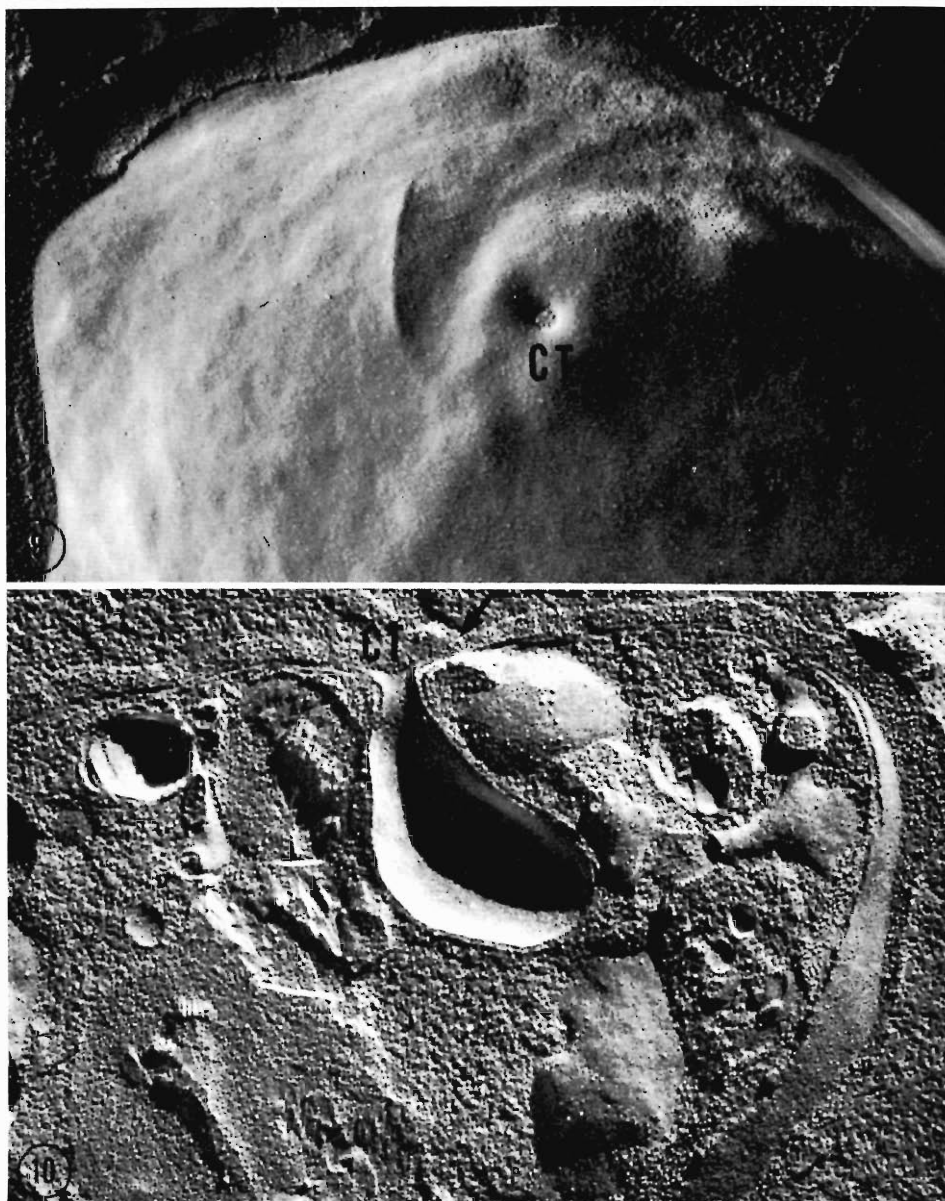


Figure 9. Surface view of the cytostome (CT), showing it to be a simple depression on the plasmalemma of the parasite with a pore at its center leading to the interior of the parasite. Note that the cytostome is located on an elevated area of the parasite. X 60,000. (AFIP Ng. 70-5833-12.)

Figure 10. A sagittal fracture through the cytostome (CT). The cytostome has a narrow neck, but it expands into a sizable sac. X 54,000. (AFIP Neg. 70-5833-13.)

the posterior portion of the cytostome is expanded into a saclike structure filled with hemoglobin. Presumably food vacuoles bud off from this cytostomal sac (1966a, 1966b).

In freeze-fractured malarial cells, the cytostome can be observed in a surface view (Fig. 9) as well as in sagittal section (Fig. 10). In a surface view the cytostome appears as a simple depression on the plasmalemma, the center of which is occupied by a pore with an average diameter of 600–900 Å. Frequently the cytostome has been observed on an elevated portion of the parasite. These circular surface elevations surrounding the cytostomal pore, may correspond to the dark bands seen in thin sections. In sagittal fractures the cytostome looks like a flask-shaped sac with a smooth outer membrane.

When the fracture plane passes through the cytostomal sac its contents, presumably host hemoglobin, are dislodged, leaving the smooth inner lining of the sac free of any debris. The lining of the cytostome is textured much like the outermost plasma membrane from which it is apparently derived. Occasionally, however, the lining may show some pitting, which may be a preparation artifact, but may also reflect functional differences between outer plasma membrane and cytostome. Counterparts for the dark bands found in sectioned material could not be found in the freeze-fractured preparation.

Nucleus

The nucleus of the avian malarial parasite as seen in thin sections is surrounded by a double nuclear membrane, and in some sections nuclear pores can be seen as small interruptions along these membranes. The heterochromatin, especially in the merozoite, is prominent and is arranged peripherally (Fig. 1). Occasionally a nucleolus is identifiable. Aikawa (1966) and Scalzi and Bahr (1968) described, in the dividing trophozoite, a mitotic apparatus consisting of spindle fibers radiating outward from two darkly staining

plaques, or nuclear poles, located along the nuclear membrane. Small dark bodies on the spindle fibers were suggested by Aikawa to be chromosomes.

The most prominent feature of the nucleus of the freeze-cleaved parasite are the nuclear pores. These pores are irregularly arranged and have an average of 1000–1200 Å (Figs. 6–8 and 12). A maximum count of 15 nuclear pores was obtained on a nucleus with nearly one half of its surface exposed, suggesting a maximum count of 30–40 nuclear pores in the avian parasite. The number of nuclear pores follows the size of the parasite nucleus. The number of pores is less in the early stages, when the parasite is also smaller. The nucleus of the host erythrocyte is estimated to have over 100 nuclear pores, from an actual count of 51 pores. The nuclear pores of the host are of the same size as those found in the nucleus of the parasites and are irregularly arranged. Ladda and Steere (1969) noted that the nuclear pores of the malarial parasite of the rodent are also irregularly arranged. The appearance of the nuclear membranes bears some resemblance to the pellicular system of the mature trophozoite; e.g., all membrane surfaces are relatively smooth except for the inner surface of the inner membrane which is in contact with the granular surface of the nucleoplasm. This surface is deeply pitted. Sites of nuclear pores are occasionally marked on the surface of the nucleoplasm as small projecting plugs of nucleoplasm (Fig. 4).

Mitochondria and Spherical Body

The mitochondrion is a distinct feature of the thin-sectioned avian parasite (Figs. 1 and 3). It is surrounded by a double membrane and has microtubular cristae, as is generally the case in most protozoans. Each merozoite and early trophozoite has a single mitochondrion, but during nuclear division mitochondria also increase in number to provide each budding merozoite with organelles.

The spherical body is closely associated

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Figure 11. A mature schizont with several merzotes (*Me*). One merozoite has paired organelles (*PO*) with ducts leading toward the conoid end (*C*). A young trophozoite (*T*) showing nucleus (*N*) and mitochondrion is also seen in the same red blood cell. X 16,000. (AFIP Neg. 70-5833-14.)

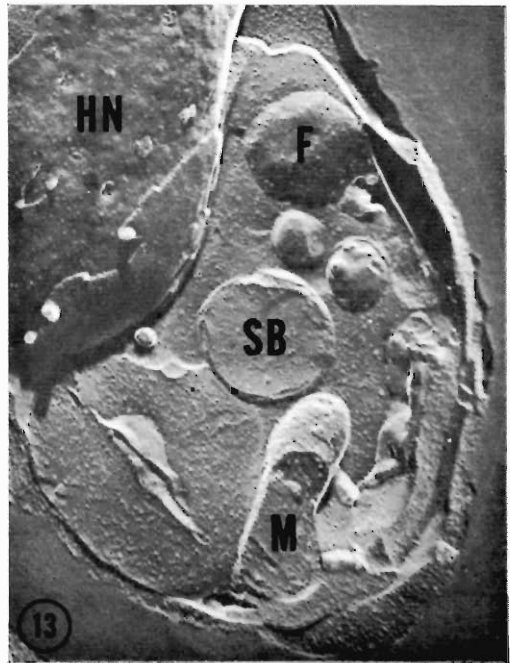


Figure 12. A trophozoite with a food vacuole (*F*), a mitochondrion (*M*) with its membrane completely stripped away, showing its granular matrix. Nuclear pores are irregularly arranged on the surface of the nucleus (*N*). X 19,000. (AFIP Neg. 70-5833-15.)

Figure 13. Micrograph of a freeze-fractured malarial parasite depicting the close association of the spherical body (*SB*) with the mitochondrion (*M*). Note the double membrane surrounding the spherical body. *HN*, erythrocyte nucleus; *F*, food vacuole. X 25,850. (AFIP Neg. 70-5833-16.)

with the mitochondrion. This organelle is surrounded by a double membrane and occasionally it appears to contain a myelin figure structure. Both the mitochondrion and the spherical body appear to be unique to the avian and the reptilian erythrocytic stages of *Plasmodium* (Aikawa and Jordan, 1968). The mammalian parasite has no typical mitochondrion but only a double-membraned vesicle without cristae of any kind. This structure may perform mitochondrial functions, as suggested by the experiments of Howells et al. (1969). Ladda (1969), Ladda et al. (1969), and Ladda and Steere (1969) pictured some structures resembling cristae in *P. berghei* and in *P. yoeli*, but since cristae are absent in the majority of preparations, more evidence is needed to confirm this observation. No counterpart for the spherical body has been identified in the mammalian form.

In the freeze-etched preparations, mitochondria appear as elongate sausage-shaped structures (Figs 12 and 13) with a granular matrix surrounded by two smooth membranes. In specimens fractured in cross section, cristae are arranged in a circular manner near the membrane and appear as small circular bumps or depressions (Fig. 8). The microtubular cristae seen in our parasite preparations differ markedly in this respect from the lamellar cristae of most mammalian tissues, such as those shown in the freeze-etched renal cells by Friederici (1969). We did not observe the morphologic differences between outer and inner mitochondrial membranes reported by this author. This may reflect further differences between mammalian and protozoan mitochondria, or it may be the result of differences in techniques. The spherical body associated with the mitochondrion has also a

granular matrix but lacks cristae (Fig. 13). It, too, is surrounded by two smooth membranes.

Residual Body and Food Vacuoles

In sections of the maturing trophozoite, a number of vesicles containing dark pigment granules surrounded by a single membrane can be observed (Fig. 3). Aikawa et al. (1966a, 1966b) observed vacuoles being pinched off from the cytostome and regarded them as food vacuoles and the pigment particles as residue of hemoglobin digestion. In the dividing schizont, the smaller food vacuoles fuse to form one giant pigment-containing residual body (Fig. 1). The residual body is left behind when the newly formed merozoites leave the erythrocytic host.

In the freeze-fractured parasite the residual body appears as a spherical body with an average diameter between 20,000 and 25,000 Å. It is surrounded by a single membrane and filled with many spherical pigment granules of various sizes. The size of these pigment granules ranges from 500 to 2500 Å (Fig. 15). The matrix of the residual body is granular, similar to the cytoplasm of the merozoites. The food vacuoles appear as spherical bodies with somewhat pitted membranes, in freeze-fractured material (Fig. 8).

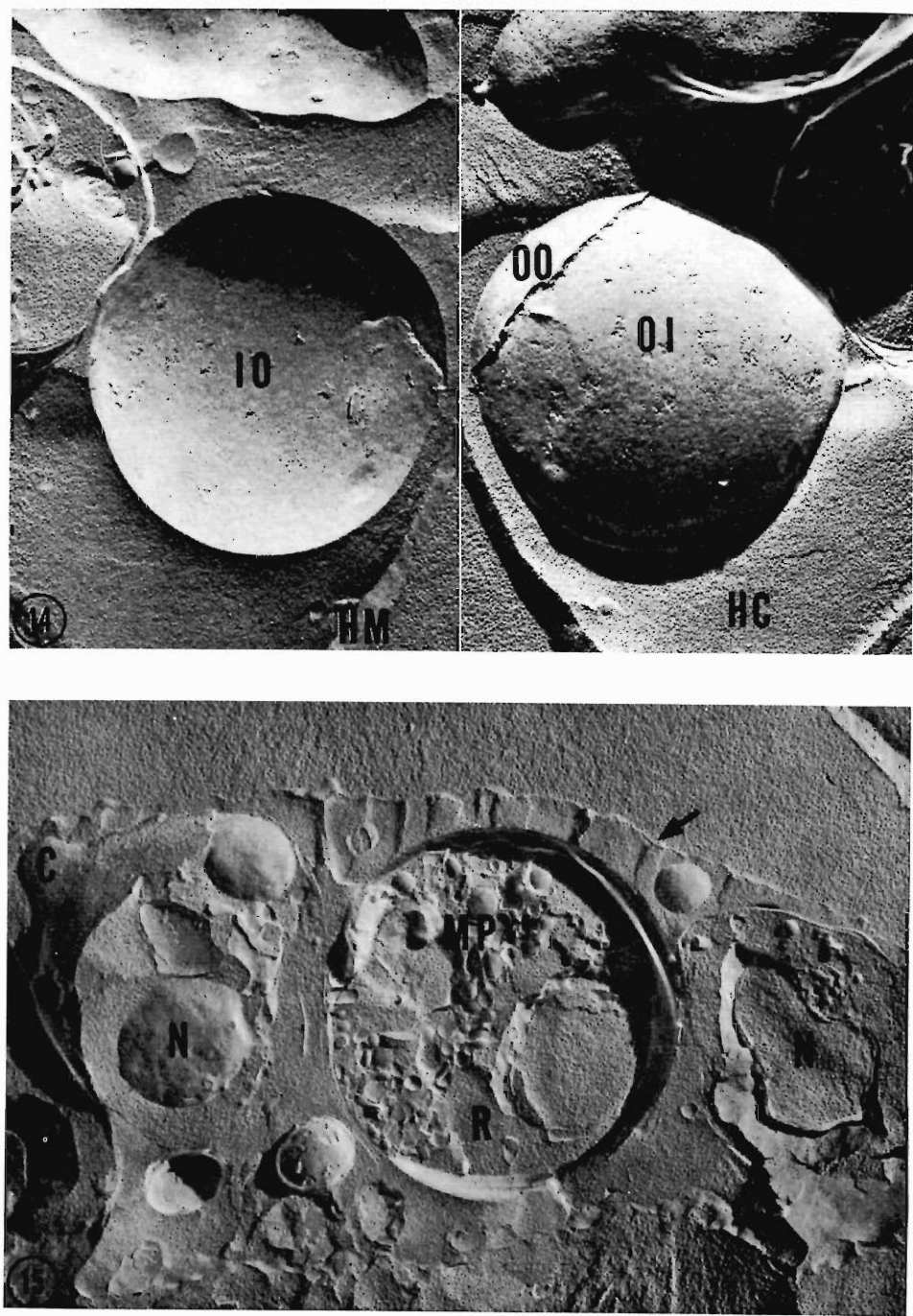
Conclusions

Our studies on freeze-etched malarial parasites demonstrated this technique to reveal useful and unique morphologic information on this important blood parasite. Freeze-etching complements thin sectioning by permitting the visualization of the parasite and its organelles in partial third dimension, thereby clarifying spatial relationships and configurations. Application of this technique to the

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Figures 14A and B. Double replica of a freeze-fractured parasite in which the fracture plane followed the outermost surface of the parasite. *HC*, host (or erythrocyte) cytoplasm; *HM*, host membrane; *IO*, inner surface of the outer membrane; *OI*, outer surface of the inner membrane; *OO*, outer surface of the outer membrane. The arrow points to the impression in the host cytoplasm left by the parasite. X 20,000. (14A.—AFIP Neg. 70-5833-17; 14B.—AFIP Neg. 70-5833-18.)

Figure 15. Micrograph of a freeze-fractured preparation of a red blood cell with two parasites and a residual body (*R*). The residual body contains numerous spherical and subspherical particles of malarial pigment (*MP*) and some other unidentified membranous structures. *N*, parasite nucleus; *C*, conoid. The arrow marks the outer limit of the host erythrocyte. X 19,200. (AFIP Neg. 70-5833-19.)



study of the malarial parasite also yielded the following information not obtainable from thin sections: 1) We observed the presence of irregularly arranged nuclear pores with a diameter of 1000 to 1200 Å and arrived at an estimate of 30 pores per mature trophozoite nucleus and over 100 pores per host erythrocyte nucleus. 2) There is an intimate relationship between the conoid and the pellicular complex of this region, emphasizing the specialized role of the conoid during penetration of the host. 3) The cytostomal feeding structure was found to be a simple depression on the surface of the pellicle with a centrally located pore leading into the interior of the parasite (Fig. 9). 4) When the double replica technique was used, we observed surfaces of four membranes in the trophozoite, the outer membrane of which appeared to have a relatively smooth outer as well as inner surface, while the inner membrane had a smooth outer but a rough inner surface, resulting from its contact with the granular cytoplasm of the parasite. We are aware of the controversy among researchers employing freeze-etched techniques, as in the case of fracture of the plasma membranes during this procedure. Some maintain that fracture planes follow the outer surface of membranes, while others maintain that the fracture plane separates each membrane into two leaflets, thus fracturing them down the middle.

Evidence from double replicas of freeze-etched erythrocytes indicates that we fractured along the outside of the outermost membrane, in some instances (Figs. 4 and 14). The presence of clearly definable microtubules in some fracture planes suggests, however, that fractures between the cell membrane proper and the surface of the cytoplasm do occur (Fig. 5).

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The Feeding Process in the Exoerythrocytic Stages of *Plasmodium lophurae* Based Upon Observations with the Electron Microscope

R. L. BEAUDOIN AND C. P. A. STROME

Division of Experimental Parasitology, Naval Medical Research Institute,
National Naval Medical Center, Bethesda, Maryland 20014

ABSTRACT: The feeding process of exoerythrocytic stages of *Plasmodium lophurae* cultivated in brain cells is reconstructed from the evidence of fine structure microscopy. Preparation of materials for sectioning was accomplished *in situ*. A modification of existing techniques is described which eliminates artifacts created during removal of specimens from the culture flask and their subsequent processing. The technique further allows selection and orientation of individual cells for sectioning and study.

Using these methods, the exoerythrocytic parasite was observed actively ingesting fluid filling the intermembrane space through the cytostome and incorporating it into small digestive vacuoles. The limiting membrane at the interface of the parasite and host cell is never seen entering the cytosomal cavity. This suggests that the host supplies nutrients which diffuse across the limiting membrane into the intermembrane fluid bathing the exoerythrocytic parasite.

Feeding by exoerythrocytic forms differs from feeding by the erythrocytic stages which apparently ingest the limiting membrane through the cytostome together with unaltered hemoglobin of the host red blood cell. The size of the cytostome in exoerythrocytic stages is similar to that reported in sporozoites and about one-half the size of the cytostome in blood stages of *P. lophurae*.

The disintegration of ribosomes and other host cell organelles at the periphery of the parasite suggests that lytic enzymes are released by the parasite. Similarities between the appearance of the intermembrane fluid and the surrounding host cytoplasm support this view.

The mechanisms by which intracellular malaria parasites feed has been the subject of intense interest for more than a decade. Rudzinska and Trager (1957, 1959) first demonstrated that the intra-erythrocytic stages of *P. lophurae* and *P. berghei* phagocytize the surrounding cytoplasm of the host cell by ingesting hemoglobin which is then digested within food vacuoles. These observations were extended by Aikawa, *et al.* (1966a, 1966b) who demonstrated that hemoglobin enters the parasite through a specialized structure which they called the cytostome. This structure had been reported in sporozoites of *P. falciparum* several years earlier by Garnham *et al.* (1961).

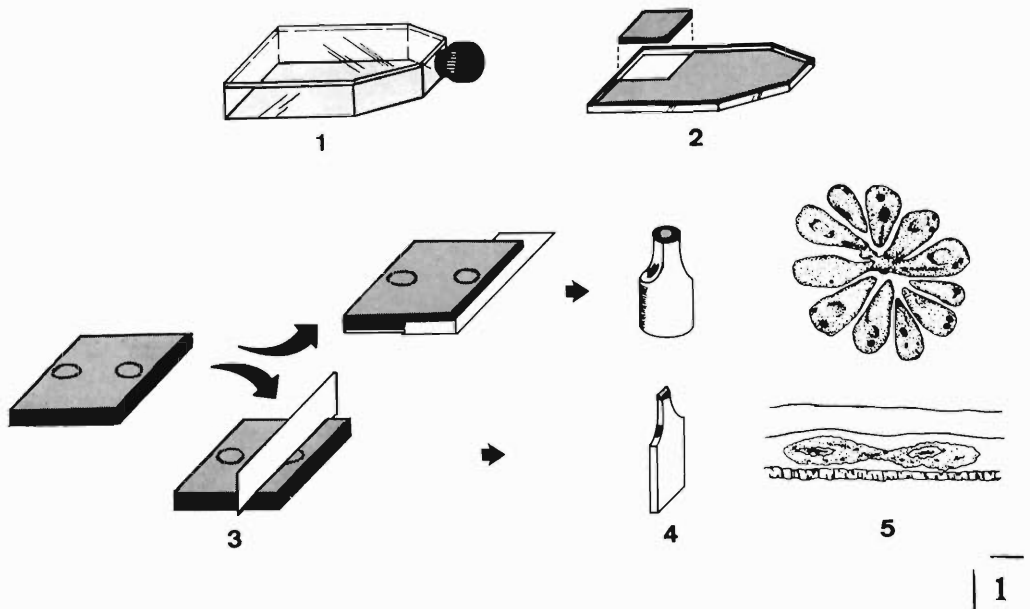
The studies by Aikawa *et al.* (1966a) included detailed observations of the cytostome in exoerythrocytic stages of *P. fallax*; how-

ever, as feeding was not observed, it was concluded by these authors that the cytostome was non-functional in these stages. This view was strengthened by the report of Hepler *et al.* (1966) who concluded that food must enter the parasite by diffusion of small molecules from the surrounding host cytoplasm. Direct evidence for this was lacking and the mechanism by which exoerythrocytic parasites feed remained unknown up to the present.

Materials and Methods

Cells infected with exoerythrocytic forms of *P. lophurae* (Beaudoin and Strome, 1970) were cultivated using techniques described previously for *P. fallax* (Davis *et al.*, 1966). Infected cell sheets selected as study specimens were fixed and embedded *in situ* in the plastic t-flasks in which they were grown (Brinkley *et al.*, 1967). When care is taken in the embedding procedure to cover the specimen with a very thin layer of Epon, the parasitized sheet may be studied with phase contrast optical lenses. This permits selection of

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LEGEND

Ct = Cytostome
 ER = Endoplasmic reticulum
 Fv = Digestive vacuole
 IM = Inner membrane of parasite pellicle
 IR = Inner ring of the cytostome
 LM = Limiting membrane

M = Mitochondrion
 Mf = Membrane fragments in the host cell
 N = Nucleus
 OM = Outer membrane of the parasite pellicle
 OR = Outer ring of the cytostome
 RBC = Erythrocyte

Figure 1. Illustration of the procedure followed in selecting and orienting specimens for sectioning. Monolayer of parasitized cells is fixed *in situ* in plastic t-flask (1). Dehydration and embedding are carried out in the flask. Top of flask is cut away and a portion of the embedded monolayer is removed (2) and examined with phase contrast optics. A parasite is selected and marked by ringing with a dye. Ringed area is cut out (3) and cemented to an appropriate block (4). Specimens are then sectioned in the desired pre-determined orientation (5). A diagram compares the view of a schizont obtained from a surface section (top) with that obtained from a cross section (bottom).

Figure 2. Young exoerythrocytic trophozoite infecting a turkey cell in tissue culture. 50,000X. This trophozoite has initiated the feeding process. The outer membrane of the pellicle (OM) is invaginating from the base of the cytostome (Ct) into the parasite cytoplasm to form what will become the digestive vacuole. Note that the limiting membrane (LM) does not enter the cytostomal cavity. The cavity is filled with the contents of the space between the outer membrane and the limiting membrane. The intermembrane space has begun to distend. Note the condition of the host cell. Vacuoles are already visible and cisternae appear swollen.

Figure 3. Trophozoite of the erythrocytic cycle in a turkey erythrocyte. 70,000X. This parasite has also begun to feed by invagination of the outer membrane (OM). Note that the limiting membrane (LM) has likewise entered the cytostomal cavity (Ct) followed by hemoglobin of the host erythrocyte (RBC) which at this point appears undamaged. Thus, initially the digestive vacuole of the erythrocyte is bounded by two membranes, an inner membrane corresponding to the limiting membrane (LM) and the outer membrane (OM) derived from the parasite pellicle. It appears to enclose intact hemoglobin. Next to the cytostome is a large digestive vacuole (Fv) in which the contents are already partially digested. Note that only a single membrane persists, presumably the outer membrane, and the remaining fragments of the limiting membrane can now be observed within the partially digested hemoglobin in the vacuole.



individual cells for sectioning. The area selected may then be cut out of the flask and cemented to a standard block in the desired orientation. Thus, not only can an individual cell be chosen for study, but its orientation for sectioning can be easily and accurately manipulated (Fig. 1).

Briefly, the preparation of specimens was as follows. The medium overlying the monolayer cultures was decanted from the t-flask and replaced with a 1.25% glutaraldehyde fixative in 4% sucrose adjusted to pH 7.2 with phosphate buffer. The cell layer was post-fixed in the flask with 1% OsO_4 and washed three times in phosphate buffer followed by a 1–2 minute wash in 0.1 N sodium acetate. The tissue was then overlayed with 0.5% aqueous uranyl acetate, pH 3.9, for 20 minutes and washed again for 1–2 minutes in 0.1 N sodium acetate and three times in phosphate buffer, allowing 20 minutes per wash. Preparations were dehydrated in four progressive changes of ethanol (35%-70%-80%-90%) followed by three changes each of 90%, 95% and 97% hydroxypropyl methacrylate (HPMA), three mixtures of HPMA/Luft's Epon 812, and finally three changes of the Epon alone. Excess Epon was drained from the monolayer until a layer about the thickness of a coverslip remained. Holes were then burned in the top of the plastic flask and the preparations were allowed to harden in air for three 24-hour periods at 37°, 45° and 60°C respectively.

Sections were cut with a diamond knife using a Porter Blum MT-2 ultra-microtome, mounted on single hole formvar grids and

stained in 2% aqueous uranyl acetate and Reynold's lead citrate. Specimens were examined with a Siemens 1A electron microscope operated at 80 kv.

Observations

A study of the fine structure of the exoerythrocytic stages of *Plasmodium lophurae* in tissue culture has provided the first insights into the manner of feeding by these parasites. Evidence has established that feeding occurs by means of the cytostome, a specialized structure occurring in the pellicle of the parasite. Cytostomes, first reported only in the exoerythrocytic merozoite (Hepler *et al.*, 1966), have now been observed in all stages of this cycle including merozoite (Fig. 4), trophozoite (Fig. 2) and schizont (Fig. 5).

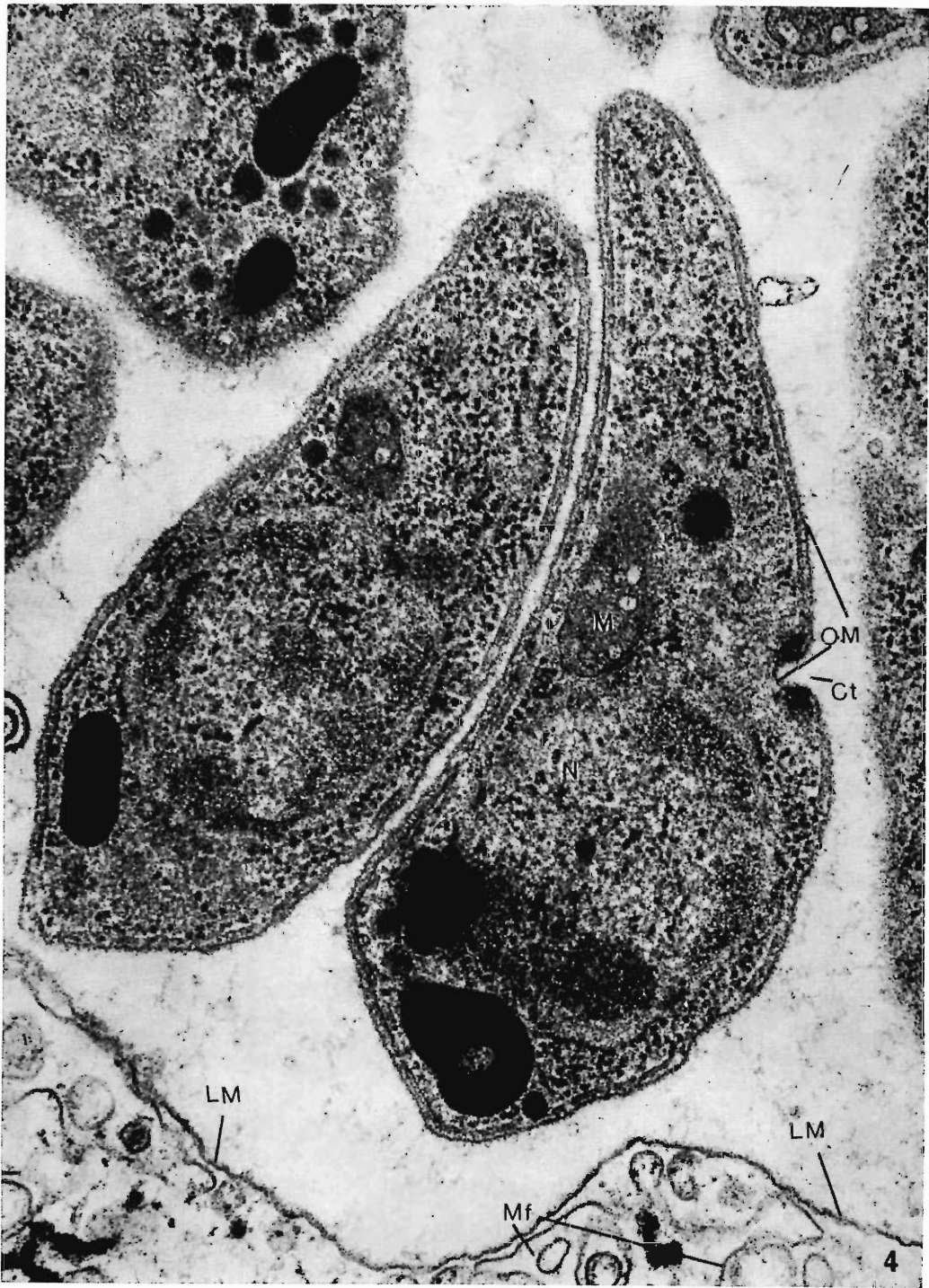
In surface view, the cytostome appears as two concentric rings surrounding an opening into the cytoplasm of the parasite (Fig. 11, 12). In sagittal section, these rings appear as double dense bars formed by thickenings on the inner surface of each of the infolded pellicular membranes (Fig. 5). These thickenings are structurally similar in every detail to the desmosomes which occur on the inner surface of the plasma membrane of mammalian cells (Porter and Bonneville, 1968). Thus, the inner ring is the thickened inner surface of the outer membrane of the pellicle, and the outer ring is a thickening of the inner surface of the inner membrane. In intracellular forms, the outer ring of the cytostome is the only portion of the inner membrane which remains (Fig. 5). In exoerythrocytic stages of

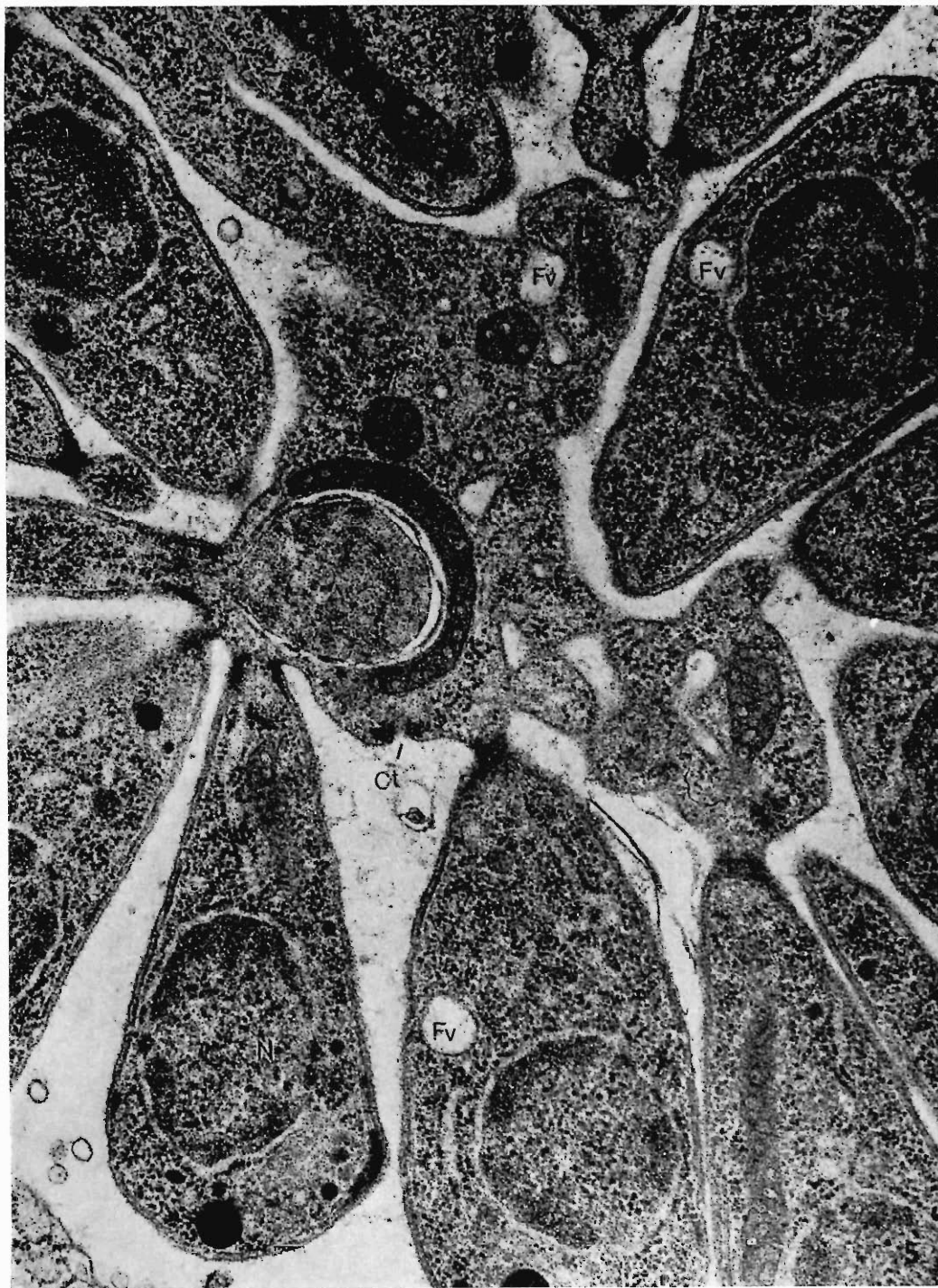
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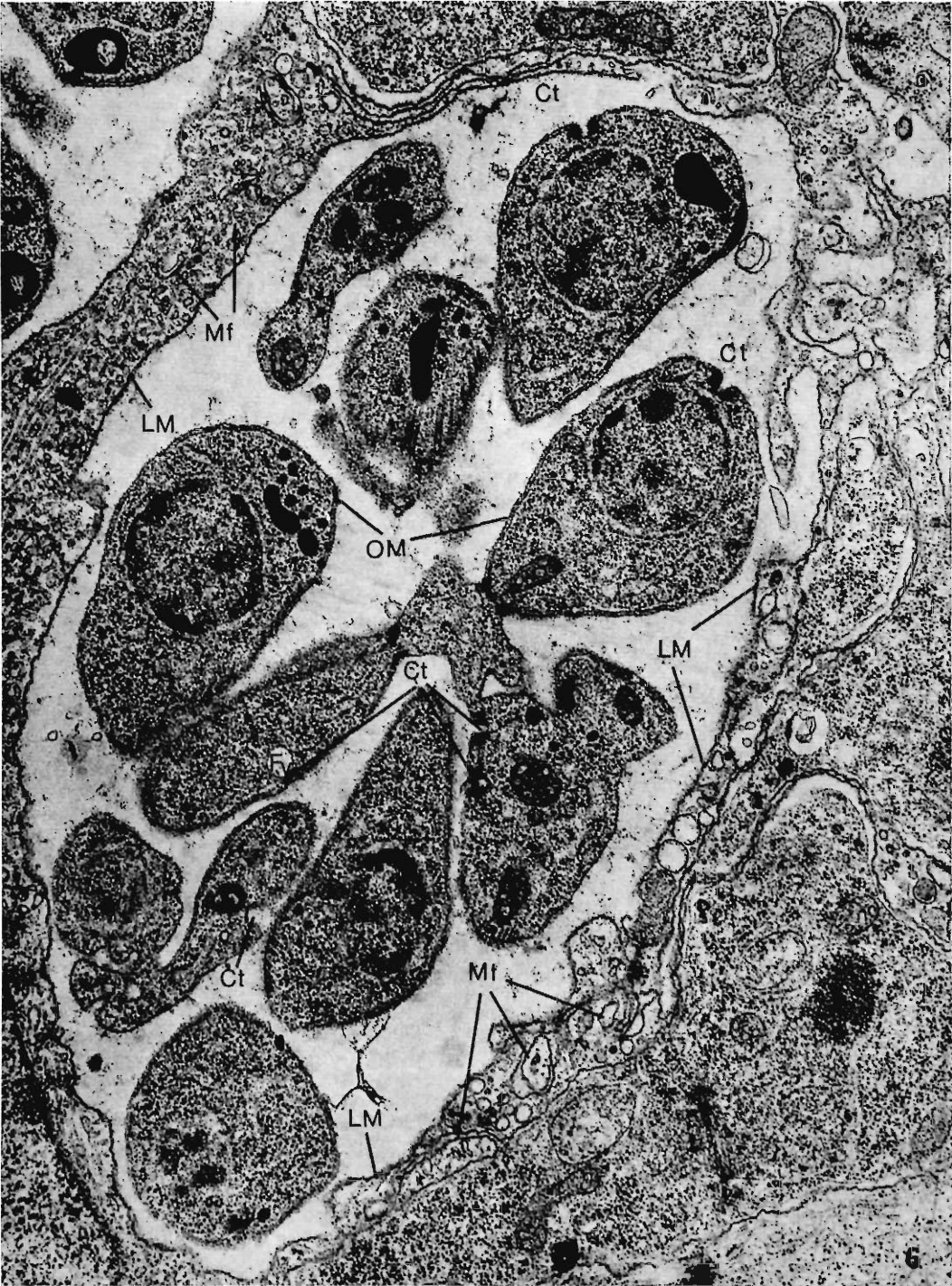
Figure 4. Exoerythrocytic merozoites within a mature schizont. 56,000X. The intermembrane space is greatly distended, and the contents can be observed entering the cytostomal cavity (Ct). The membrane at the base of the cytostome can be observed to be continuous with the outer membrane of the parasite pellicle. Note the condition of the host cell. Density of the intermembrane space approaches that of the adjacent cell cytoplasm. Ultrastructures of the host cell are no longer recognizable with only fragments of membranes (Mf) still visible.

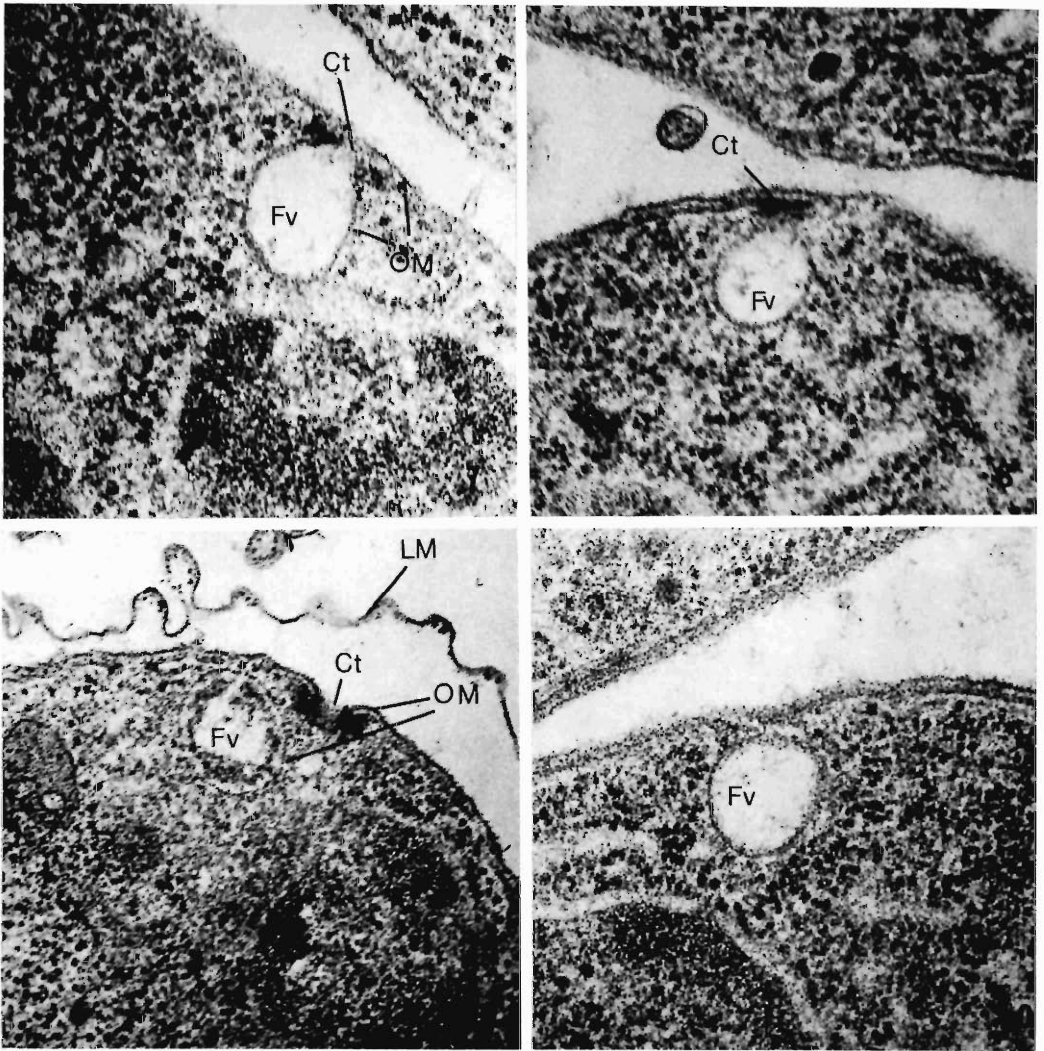
Figure 5. Exoerythrocytic schizont in the final stages of segmentation. 35,000X. Both the schizont and the merozoites are actively feeding, as evidenced by the number of digestive vacuoles (Fv). Note the cytostome (Ct) in the lower edge of the schizont. The digestive vacuole (Fv) is just being pinched off from the cytostome in the merozoite at the bottom of the plate.

Figure 6. Exoerythrocytic schizont. 21,000X. Several cytostomes (Ct) can be seen in various stages of ingestion. Note the condition of the host cell. Ribosomes are no longer visible and host organelles have deteriorated beyond recognition, with only membrane fragments (Mf) remaining.









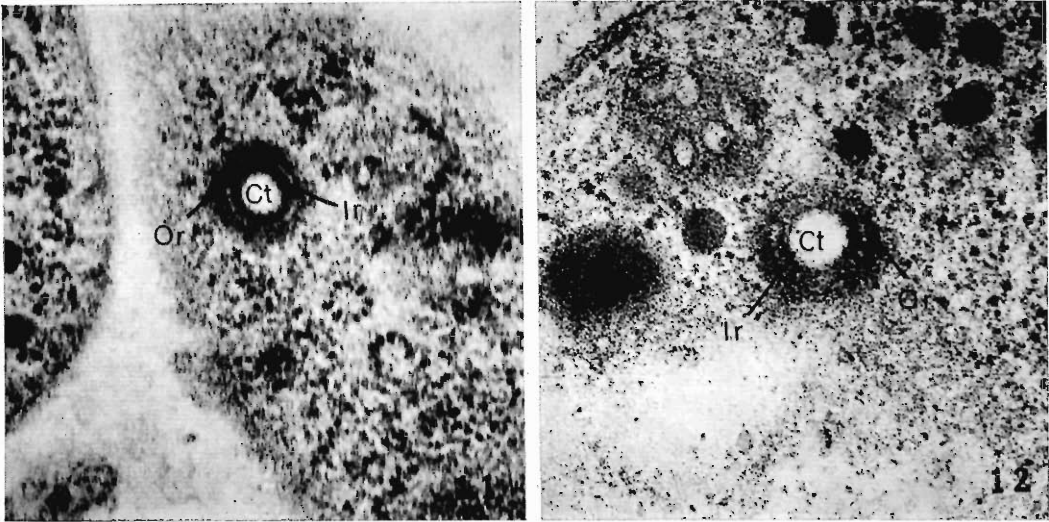
Figures 7-10. Examples of cytosomal feeding by exoerythrocytic parasites. Fig. 7. Section through the cytosome of a microzoite. 86,500X. A digestive vacuole (Fv) is being formed by the cytosome (Ct). Fig. 8. Oblique section through the cytosome of a microzoite. 70,000X. The digestive vacuole (Fv) is being pinched off from the cytosome (Ct). Fig. 9. Section through the cytosome of a schizont. 70,000X. This cytosome (Ct) has just pinched off a digestive vacuole (Fv) just below it. The contents of the vacuole have begun to condense. Fig. 10. Section of a digestive vacuole within a microzoite. 70,000X.

P. lophurae, measurements of the orifice circumscribed by the rings ranged from 70-115 μ for the inner diameter, approximately 200-240 μ for the outer diameter and from 60-70 μ for the depth of the cytosomal cavity.

Because the cytosome of the exoerythrocytic parasite is considerably smaller than that of

the erythrocytic parasite, the digestive vacuoles produced by the former are correspondingly much smaller. (Compare digestive vacuole in Fig. 3 with vacuoles in Figs. 9 and 10.)

The process of feeding by the exoerythrocytic parasites, while superficially resembling that reported in the erythrocytic cycle, differs sig-



Figures 11 and 12. Surface sections of cytostomes of merozoites. 70,000X. The cytostome (Ct) is composed of an opening into the parasite cytoplasm. This opening is bounded by two concentric rings which are actually thickenings, similar to desmosomes, on the inner surface of the two membranes of the parasite pellicle which have turned inward. The inner ring (Ir) is derived from the outer membrane of the pellicle, and the outer ring (Or) from the inner interrupted membrane of the pellicle. In the intracellular stages, the outer ring of the cytostome is the only portion of the inner membrane which remains.

nificantly from the latter in several respects. Like parasites of the red cell cycle, material to be ingested enters the lumen of the cytostomal cavity (Fig. 4). Then the outer membrane of the parasite invaginates further permitting entry of the food material into the body of the parasite until (Fig. 2, 7) a vacuole is eventually formed and pinched off (Fig. 7, 8). Thus, the outer membrane of the parasite pellicle becomes the membrane surrounding the digestive vacuole. Immediately after pinching off has been completed, the base of the cytostomal cavity begins to thicken and a new membrane forms (Fig. 5, 9).

The most striking difference between the feeding process in the two cycles is that the so-called host cell membrane is never observed entering the cytostomal cavity in the exoerythrocytic parasite, whereas in the red blood cell stages, the cytoplasm of the host cell enters the parasite bounded by the limiting membrane (Fig. 3). Initially, therefore, the digestive vacuole of the erythrocytic parasite is bounded by two membranes: an inner one derived from the limiting membrane and an outer one derived from the outer membrane

of the parasite pellicle. (Compare Figs. 2 and 3.) In the exoerythrocytic forms, the ingested material appears to consist of a fluid which fills the space between the outer membrane of the parasite pellicle and the limiting membrane of the host cell.

The nature of this fluid is not known. It appears to be electron transparent (Fig. 4), and observations of living schizonts made with phase contrast time-lapse cinemicrography also suggest that the fluid is relatively clear. As the volume of the fluid-filled space increases with maturation of the parasite, regions of the host cell which border on the limiting membrane become progressively less dense. (Compare condition of the host cells in Fig. 2 with that in Figs. 4 and 6.) This decrease in density appears to be due to the gradual disappearance, possibly the lysis, of host ribosomes and other organelles. Often, the only visible structures are membrane fragments, presumably the remains of these organelles (Figs. 4 and 6). Cells infected by younger parasites show changes that are more subtle, such as enlarged cisternae and cytoplasmic vacuolation (Fig. 2). On the other hand,

the erythrocytic parasite which directly ingests host cell hemoglobin does not produce similar "lytic" changes (Fig. 3). Cytostomal feeding was observed in all stages of the exoerythrocytic cycle, including the newly formed merozoites (Figs. 5, 6, 7, 8 and 10). The number of digestive vacuoles seen in merozoites suggests that active feeding is a normal occurrence even in this earliest stage.

Discussion

Although it has been recognized for years that the intraerythrocytic stages of the malaria parasite feed upon hemoglobin of the host cell, the details of the feeding process remained unknown until recently when studies were made with the electron microscope. Rudzinska and Trager (1957) were the first to describe the engulfment of host cell cytoplasm in the erythrocytic stages of *Plasmodium lophurae*. These workers demonstrated that cytoplasm of the host cell becomes enclosed within the substance of the parasite by an infolding of the limiting membrane to form a pouch which fills with hemoglobin. Eventually, this pouch pinches off to become a food vacuole, and subsequent digestion of the hemoglobin contained within it proceeds to the formation of hemozoin, or malaria pigment. The same authors later extended these findings to the mammalian parasite, *P. berghei* (1959).

The reports by Rudzinska and Trager were later confirmed by Aikawa *et al.* (1966a, b). These authors, using new and improved fixatives, extended the observations made in the earlier work to show that invagination of the parasite pellicle during formation of food vacuoles occurs at specific points in the plasma membrane of the parasite. They further demonstrated that a special organelle of the parasite pellicle functions as a submicroscopic mouth. The structure is formed by concentric thickenings of the two pellicular membranes which have turned inward to surround an opening into the body of the parasite. The organelle was first described in sporozoites of *P. falciparum* by Garnham *et al.* (1961) who called it a micropyle on the assumption that it functioned as a pore through which infective sporoplasm was extruded into the host cell. Aikawa *et al.* renamed this structure the cytostome in keeping with its demonstrated func-

tion as an organelle for feeding. Subsequently, they reported the presence of the structure and its relation to feeding in the erythrocytic forms of several avian and mammalian malaria parasites (Aikawa *et al.*, 1966a, b).

The cytostome has been reported from many sporozoans, and its occurrence in this group has been discussed by Scholtyseck (1970) who refers to it as the micropore, following the suggestion of Levine (1969). We agree with Aikawa (1971), however, that a functional name is preferable to a descriptive one, and we have retained the term cytostome in referring to this structure in plasmodia.

The cytostome was first reported from the exoerythrocytic merozoite of *P. fallax* by Hepler *et al.* (1966). Aikawa *et al.* (1966a) included observations on exoerythrocytic parasites in their study on the feeding function of the organelle, but, failing to observe actively feeding parasites, they concluded that the cytostome was non-functional during this cycle.

Later, the same workers (1967) observed feeding by the cytostome in exoerythrocytic as well as erythrocytic stages of *P. elongatum*. This species presents a unique case since the parasites of both cycles infect cells of the erythrocyte series, although at different stages of maturation. Thus, exoerythrocytic parasites infect erythroblasts and other cells of the bone marrow, while the erythrocytic cycle occurs in mature cells containing hemoglobin. Possibly as a result of this singular circumstance, the fine structure and feeding activities of parasites in the bone marrow and red blood cell cycles appear to be identical. The only difference is in the composition of the ingested cytoplasm which is rich in ribosomes and other structures in parasites of stem cells, but consists solely of hemoglobin in parasites of mature red blood cells. Feeding in the exoerythrocytic stages of *P. lophurae* is significantly different from that observed in *P. elongatum*. While cytostomal ingestion occurs in the exoerythrocytic stages of *P. lophurae*, the material ingested consists of a fluid which occupies the intermembrane space between the limiting membrane and the outer membrane of the parasite pellicle.

It has been suggested by several workers that the exoerythrocytic stages obtain nourishment from the diffusion of small molecules across the intermembrane space and directly into the parasite (Hepler *et al.*, 1966; Aikawa

et al., 1966a). Our observations support this view in part, but it is clear that the parasite takes more than a passive role in the feeding process. Diffusion appears to occur in both directions across the limiting membrane and the intermembrane space. The appearance of portions of the host cell adjacent to the parasite suggests that cytolysis of host cytoplasm is brought about by substances elaborated by the parasite. Enlargement of the intermembrane space and the accumulation of fluid within it, is accompanied by the apparent dissolution and disappearance of host ribosomes and other organelles. In advanced stages of parasite development, the adjacent host cell structures are usually reduced to fragments of membranes and recognizable organelles are rarely observed.

The fluid within the intermembrane space probably contains the dissolved substances resulting from the extra-parasite hydrolysis of host cytoplasm which have diffused across the limiting membrane. The electron density of the fluid is similar to that of the hydrolyzed portions of the host cell. Nutrients dissolved in the intermembrane fluid are taken up by the parasite through the cytostome into digestive vacuoles and incorporated into the cytoplasm of the parasite. This type of feeding might be described as pinocytosis rather than as phagotrophy which occurs in the red blood cell stages of malaria parasites (Rudzinska and Trager, 1957).

The fluid nature of the ingested material may account for the small size of the exoerythrocytic cytostome which is approximately one-half the size of the cytostome of erythrocytic forms of this species. Likewise, the digestive vacuoles of the former are much smaller than those observed in the blood forms. Digestive vacuoles are usually seen in the vicinity of a cytostome and are not numerous in any one parasite. This would be expected considering the small volume of the vacuoles and the partially digested nature of the material ingested.

Acknowledgments

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Cytochemistry of the Nucleus of Malarial Parasites

M. AIKAWA, C. R. STERLING AND J. RABBEGE

From the Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT: The characterization of the nucleus of the erythrocytic stages of *P. berghei* and *P. gallinaceum* was aided by using pyrimethamine administration and DNAase or EDTA extraction. Studies combining these methods were particularly useful for the study of nuclear division of these parasites. Clumped granules often seen along the nuclear membrane of the merozoites and uninucleate trophozoites were extracted by DNAase and EDTA. This observation demonstrates that they contain deoxyribonucleoproteins. On the other hand, the nucleolus of *P. gallinaceum* was not extracted by DNAase or EDTA.

During nuclear division, centriolar plaques, nuclear microtubules and electron dense bars located on the nuclear microtubules become apparent. They were not extracted by DNAase or EDTA. Because of their location and inability to react with DNAase or EDTA treatment, the electron dense bars on the nuclear microtubules are suggested to be kinetochores. Ill-defined electron dense materials were noted at the equatorial region of the metaphase nucleus and were extracted by DNAase and EDTA treatments. It is suggested that they may be the poorly organized chromosomes of malarial parasites. The nuclear microtubules gradually disappear in the telophase nucleus. Resulting daughter nuclei eventually repeat the process of nuclear division.

Although the presence of a nucleus in malarial parasites was recognized at the time of the identification of malarial parasites, the detailed nature of the parasite's nucleus has never been well elucidated (Garnham, 1966). Jirovec and Cerny, in 1932, first reported Feulgen-positive material in the dividing nuclei of malarial parasites, thus demonstrating the presence of deoxyribonucleoproteins in the parasite. Since then, many investigators have attempted to study the morphology of the nucleus of malarial parasites by similar methods (Thomson, 1932; Chen, 1944; Dasgupta, 1964). Recently, with the aid of electron microscopy, the morphology of the nucleus of

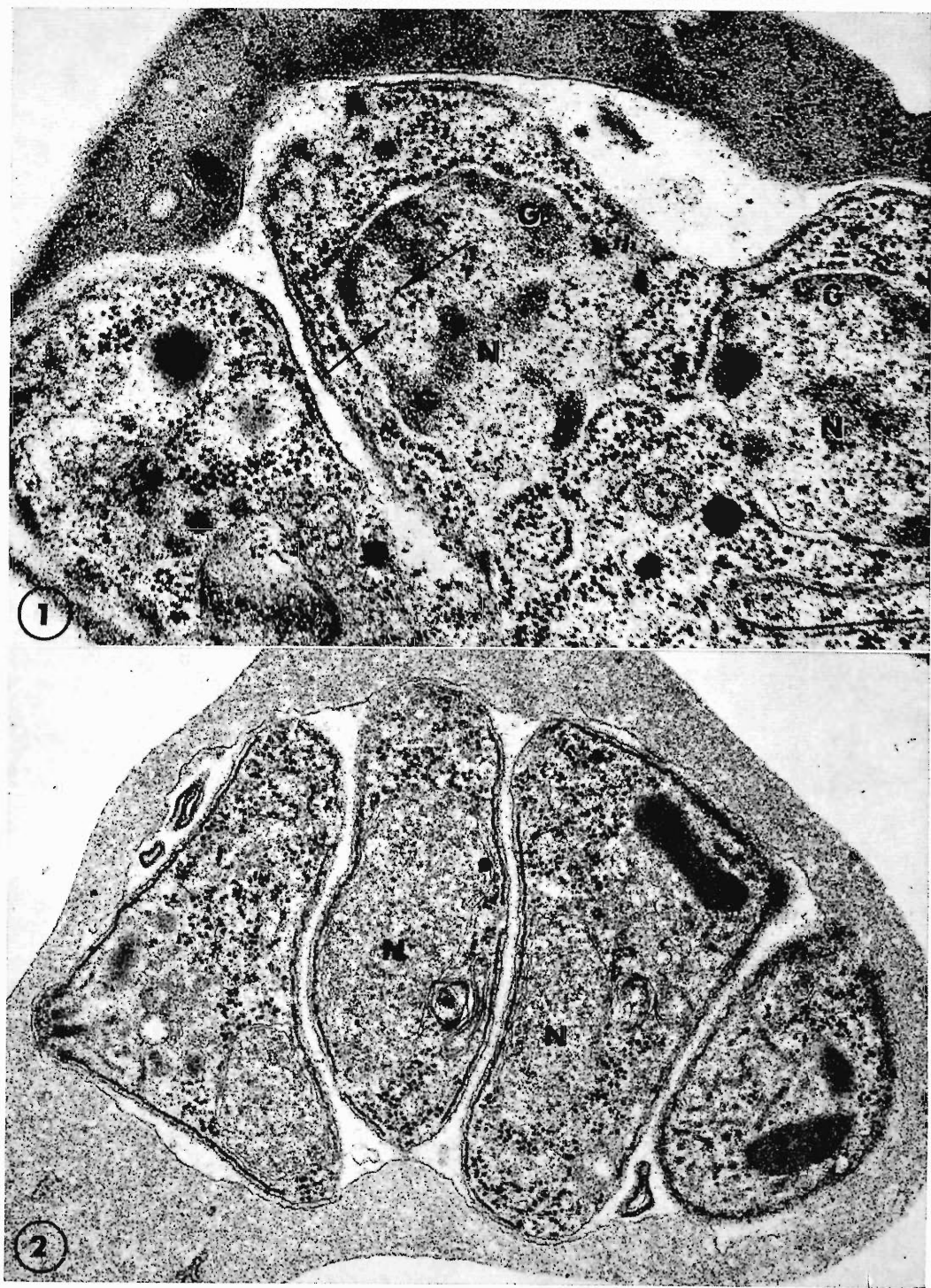
malarial parasites has been described in more detail (Aikawa, 1966; Ladda, 1969; Scalzi and Bahr, 1968). The nucleus of various species of malarial parasites contains filamentous materials and electron dense granules which tend to accumulate along the nuclear membrane. The condensed electron dense granules are considered to be heterochromatin on the basis of morphology. Also, an aggregate of electron dense granules of 15 nm, found in the nucleus of some malarial parasites, has been called a nucleolus. However, there have been no cytochemical studies to support these assumptions.

Similarly, the nuclear division of malarial parasites has not been well understood. For example, Wolcott (1957) described "chromosomes" in the schizonts of malarial parasites by phase contrast microscopy, but the structures have now been established by electron

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Figure 1. Budding merozoites of *P. gallinaceum*. Nuclei (N) are round to oval and fine electron dense granules (G) are heavily clumped forming irregular bands along the nuclear membrane. Between these bands are less electron dense zones in which large dense granules of 18–22 nm (arrows) and fine fibrils are scattered. X 45,000.

Figure 2. Erythrocytic merozoites of *P. berghei*. Nuclei (N) show less clumping of the fine granules than those of *P. gallinaceum*. X 43,000.



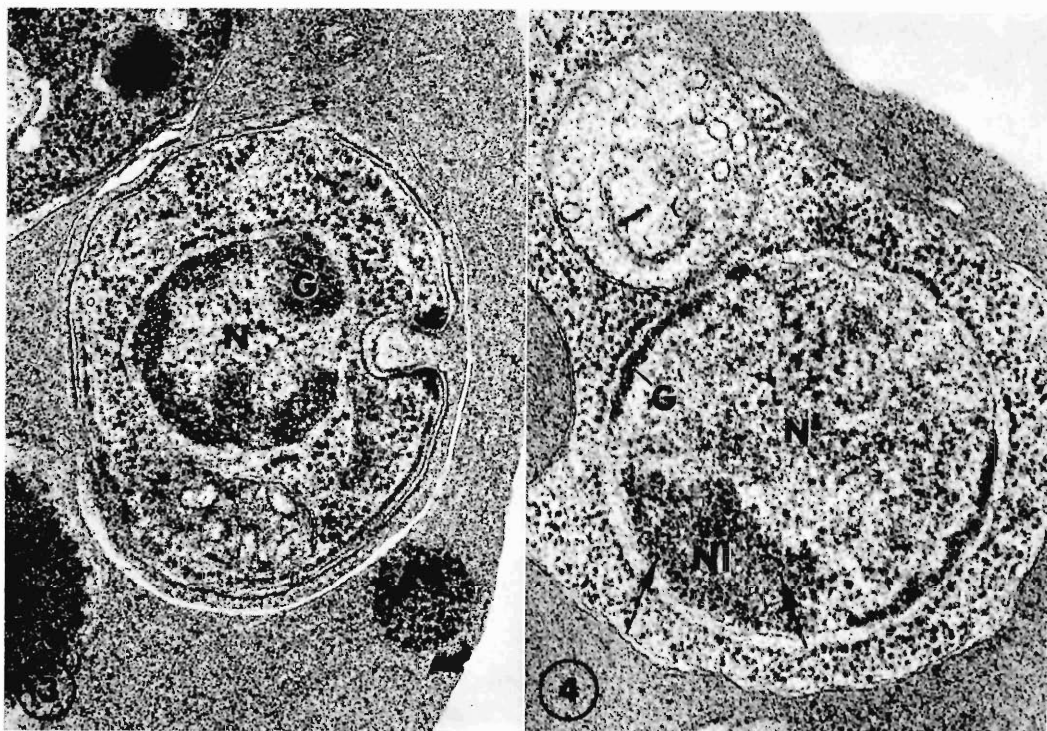


Figure 3. A uninucleate trophozoite of *P. gallinaceum*. The morphology of the nucleus (N) is similar to that of the merozoite. A heavy clumping of fine granules (G) can be seen along the nuclear membrane. X 47,000.

Figure 4. A uninucleate trophozoite of *P. gallinaceum*. As the parasite grows, the clumping of fine granules (G) is seen as a thin layer along the nuclear membrane. A distinct nucleolus (NI) composed of granules of 15 nm (arrows) is also seen. X 45,000.

microscopy as paired organelles (rhoptries) (Aikawa, 1971). Electron microscopic studies on the dividing nuclei of several species of malarial parasites revealed that these nuclei possess nuclear microtubules of 22 nm diameter. The nuclear microtubules (spindle fibers) become more prominent when the parasites are treated with pyrimethamine, which appears to arrest nuclear division at metaphase (Aikawa and Beaudoin, 1968). This study demonstrated minute electron dense structures along the spindle fibers which have been suggested to be chromosomes of the malarial parasite. Again, however, there is no cytochemical data to support this suggestion.

In recent years a technique has been developed for determining the chemical composition and physiochemical state of organelles in

tissue prepared for electron microscopy by means of the enzymatic or chemical extraction of specific cellular components from thin sections of tissue embedded in epoxy resin (Anderson and André, 1968; Monneron and Bernhard, 1969; Franke and Falk, 1970; Dupuy-Coin et al., 1972). With this technique, using DNAase and EDTA, we attempted to characterize the nuclear structure of the erythrocytic stages of the malarial parasites, *P. berghei* and *P. gallinaceum*. Since dividing nuclei are rarely observed in these parasites, pyrimethamine was used in order to obtain substantial numbers of dividing nuclei for this study.

Materials and Methods

Mice of the ICR strain, each weighing an average of 25 g, were inoculated intraperi-

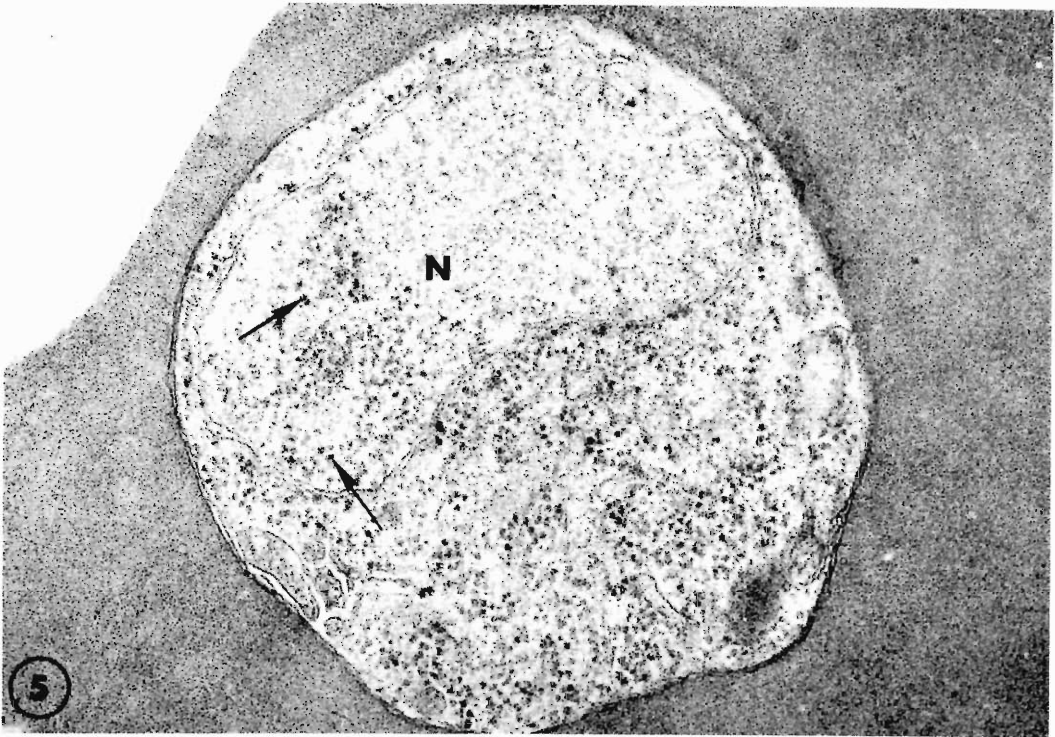


Figure 5. A uninucleate trophozoite of *P. berghei*. The clumping of fine granules in the nucleus (N) is not prominent. Instead, the nucleus is scattered with large granules (arrows) and fibrils. X 42,000.

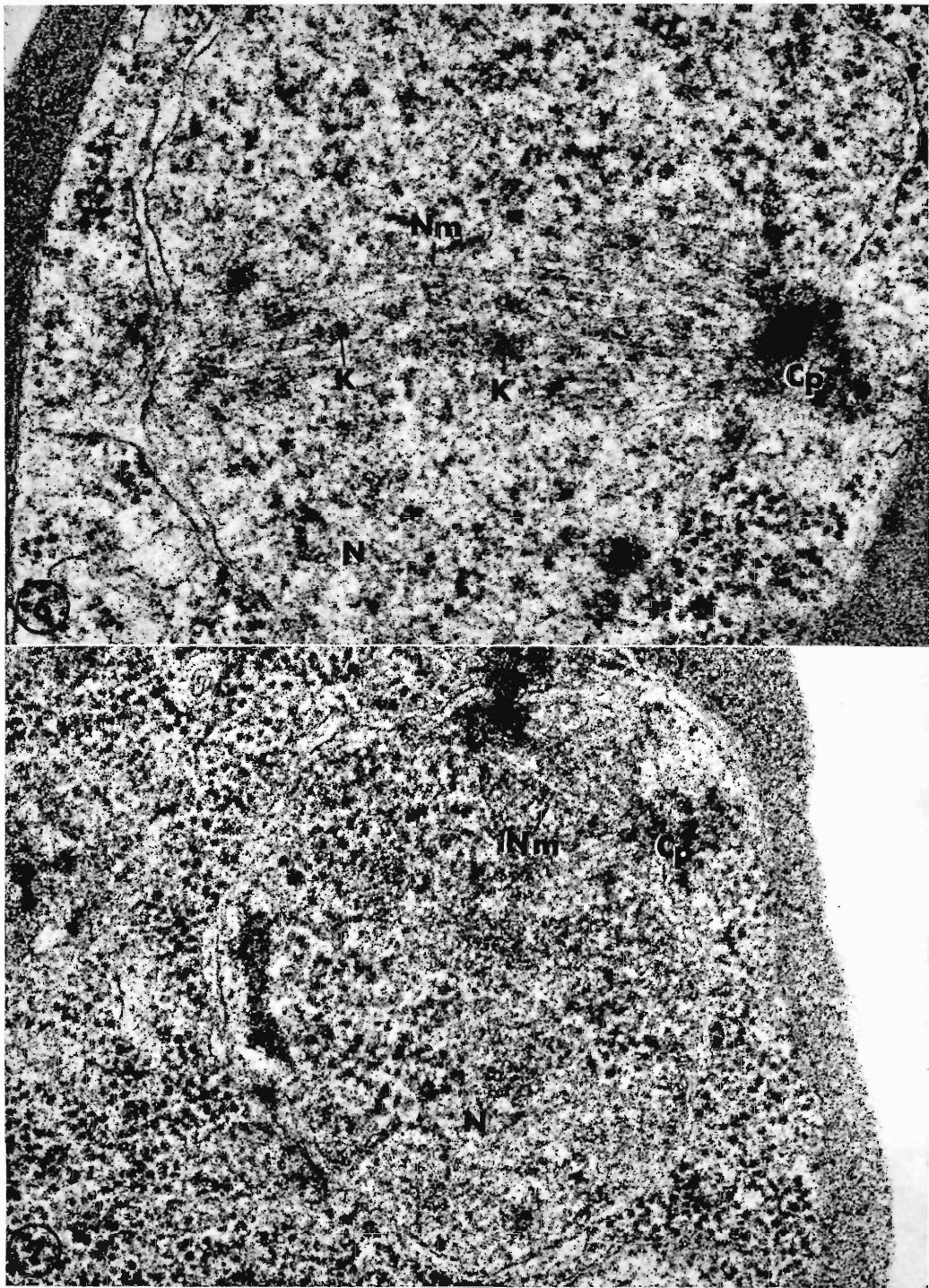
toneally with the erythrocytic stages of *P. berghei* (NYU-2 strain). Two week old chicks, weighing an average of 400 grams, were inoculated intravenously with the erythrocytic stages of *P. gallinaceum* (obtained from Dr. J. Kreier). When the parasitemia in mice and chicks reached approximately 60%, a single dose (10 mg/kg) of pyrimethamine was given orally to some of the mice and chicks. The controls did not receive pyrimethamine. Blood samples were obtained at two and five hours from these animals treated with pyrimethamine. The blood samples were prepared for electron microscopy in the manner previously described (Aikawa, 1966).

For the experiment of DNA (deoxyribonucleic acids) digestion by DNAase, blood samples from the untreated and pyrimethamine treated animals were incubated for one hour at 37°C in DNAase solution after fixing in 1.25% buffered glutaraldehyde solution. The DNAase solution contained DNAase (pancreas

DNAase, Calbiochem, L. A., Calif.) 1mg/ml and 0.0025 M MgSO_4 in 0.1M Tris-HCl buffer. The control samples were incubated in a solution without DNAase. After incubation, they were washed in 0.1M Tris-HCl buffer and processed for electron microscopy.

The resulting blocks were cut on a Porter-Blum MT-2 ultramicrotome and the sections were mounted on 300 mesh copper grids. Thin sections of the blocks which were previously made for an experiment on pyrimethamine effects on the erythrocytic stages of *P. gallinaceum* (Aikawa and Beaudoin, 1968) were also used for the succeeding extraction experiments.

For the experiment of DNA digestion from the thin sections of tissue by DNAase, the thin sections of the untreated and pyrimethamine treated parasites were incubated in 5% H_2O_2 at 37°C for 15 minutes (Monneron and Bernhard, 1969). These sections were then washed in distilled water and were



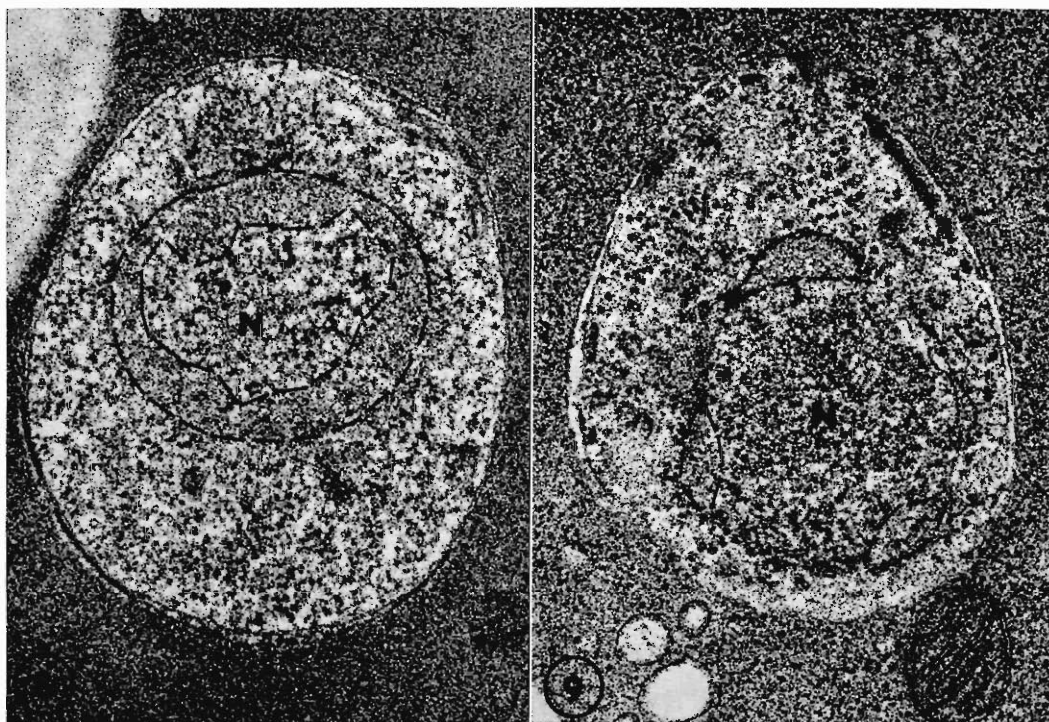


Figure 8. A uninucleate trophozoite of *P. gallinaceum* after DNAase treatment. The heavily clumped granules seen in Fig. 3 are extracted and only fine fibrillar material occupies the bleached out areas (encircled with dotted lines) in the nucleus (N). X 52,000.

Figure 9. A uninucleate trophozoite of *P. berghei* after EDTA treatment. The result is similar to that shown in Fig. 8. Areas encircled with dotted lines represent the extracted areas in the nucleus (N). X 46,000.

further incubated in the DNAase solution as described above for 30 minutes to 1 hour at 37°C. They were stained with 1% uranyl acetate and lead citrate. For the extraction experiment by EDTA (sodium ethylenediamine tetraacetate), the sections were stained with 1% uranyl acetate after incubation in 5% H₂O₂ and were incubated in 0.2M EDTA solution for 30 minutes at 37°C (Dupuy-Coin

et al., 1972). After this treatment, the sections were washed in distilled water and were stained with lead citrate. All of these sections were examined with a Siemens Elmiskop 101 electron microscope.

Observations

A. Nuclear morphology of untreated *P. berghei* and *P. gallinaceum*

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Figure 6. A uninucleate trophozoite of *P. gallinaceum*. The nucleus (N) is scattered with granules and fine fibrils, and shows bundles of nuclear microtubules (Nm), which radiate from poorly delineated centriolar plaques (Cp). Along the nuclear microtubules are electron dense bars (K) located in the equatorial region. X 65,000.

Figure 7. A nucleus (N) of *P. berghei* shows bundles of nuclear microtubules (Nm). They radiate from centriolar plaques (Cp) located on the nuclear membrane. X 64,000.

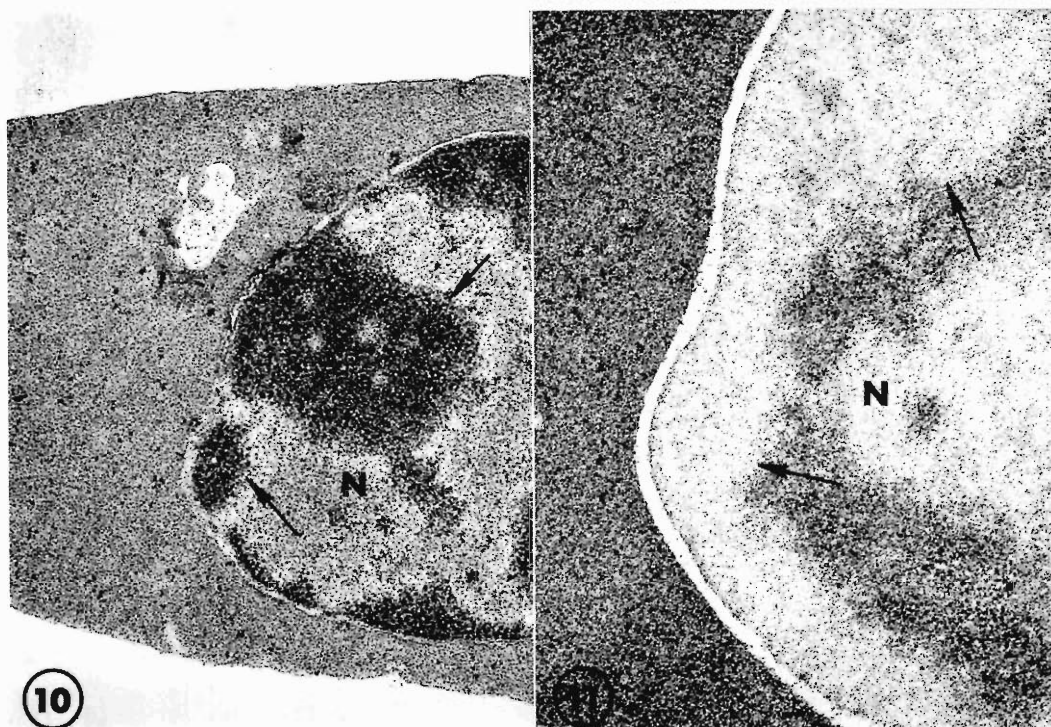


Figure 10. A chick erythrocyte without DNAase or EDTA treatment. The nucleus (N) shows a prominent clumping of chromatin (arrows). X 40,000.

Figure 11. A chick erythrocyte after EDTA treatment. The clumped chromatin in the nucleus (N) is now extracted, leaving behind a less electron dense zone (arrows). X 42,000.

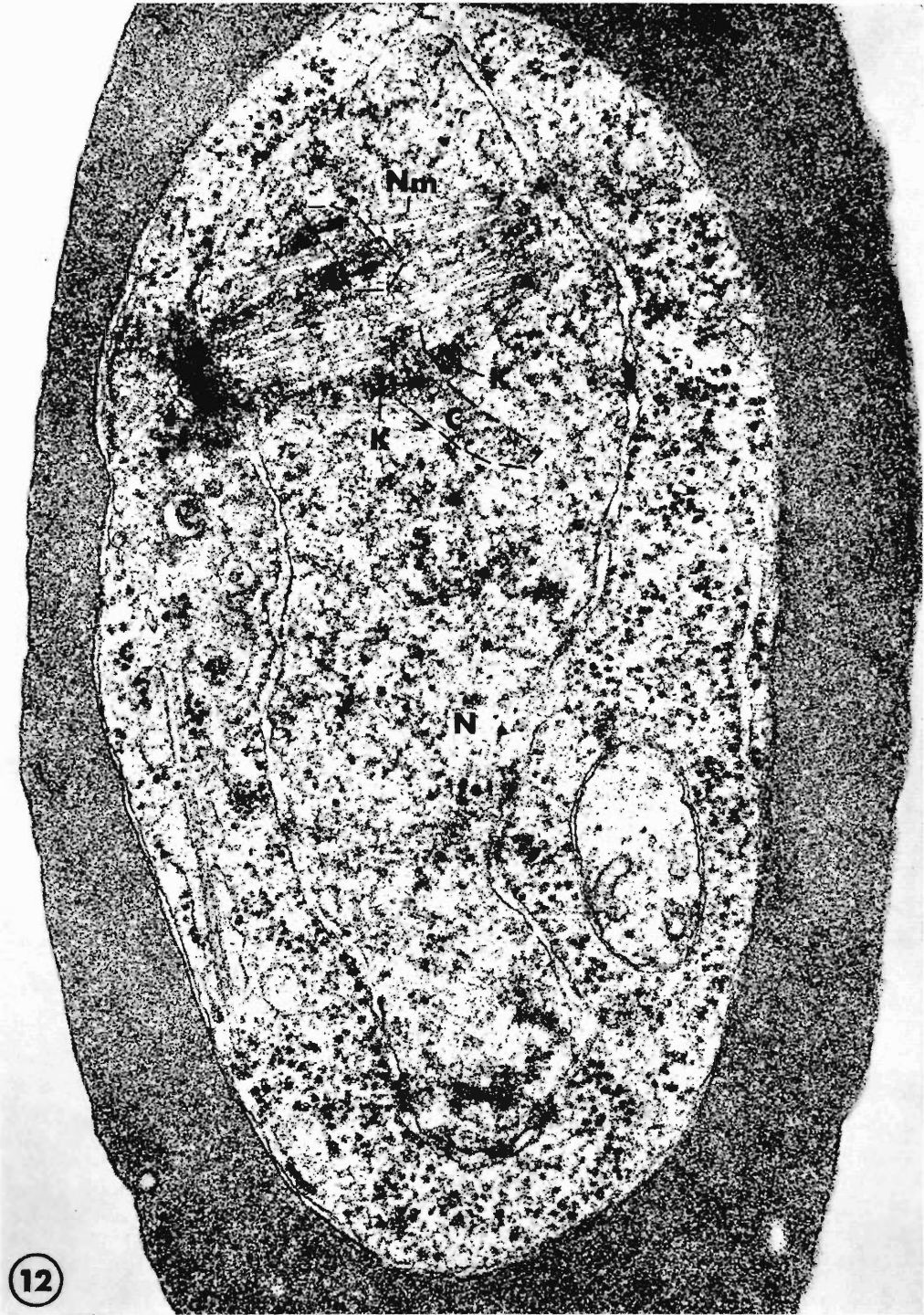
a) Before extraction

The nucleus of the erythrocytic merozoites of *P. gallinaceum* and *P. berghei* is round to oval in shape, measures 0.7×1 nm on the average, and is surrounded by two distinct nuclear membranes (Figs. 1, 2). In the erythrocytic stages of *P. gallinaceum*, fine electron dense granules measuring about 4 nm in diameter are heavily clumped forming an irregular band along the nuclear membrane (Fig. 1). The clumped granules in the merozoite of *P. gallinaceum* extend toward the center of the nu-

cleus in some parasites. Between these bands of clumped granules are irregularly shaped less electron dense zones in which large dense granules and fine fibrils are scattered (Fig. 1). These large granules vary in size and measure 18–22 nm in diameter. On the other hand, the merozoites of *P. berghei* show less clumping of the fine granules than in those of *P. gallinaceum* (Fig. 2). No nucleoli are observed in the erythrocytic merozoites of either parasite.

The nucleus of a uninucleate trophozoite of

Figure 12. A uninucleate trophozoite of *P. gallinaceum* after pyrimethamine administration. Nuclear microtubules (Nm) radiate from centriolar plaques (Cp) located on the nuclear membrane. Paired electron dense bars (K) located on the microtubules are observed midway between two centriolar plaques. Ill-defined darkly stained material (C) occupies the equatorial region (encircled with dotted lines) and is in contact with the electron dense bars (arrows). X 62,000.



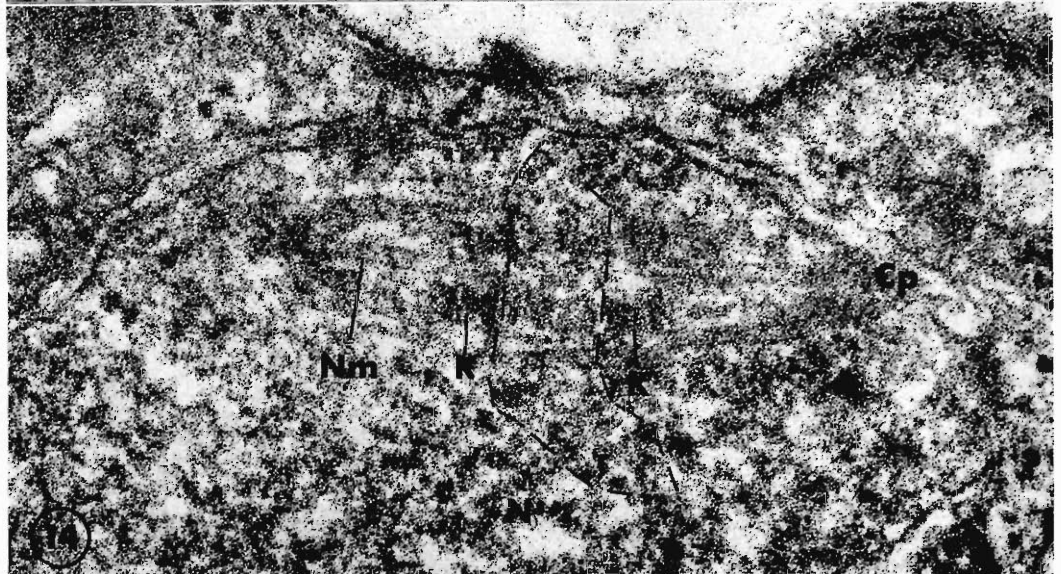
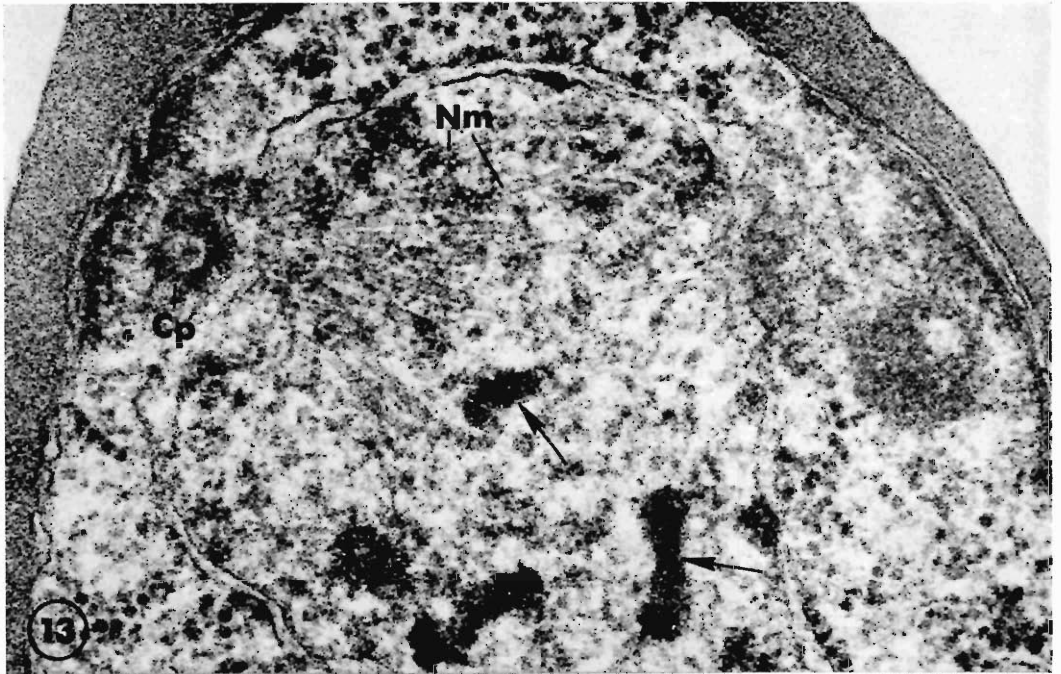


Figure 13. Another example of *P. gallinaceum* after pyrimethamine treatment. Bundles of nuclear microtubules (Nm) radiate from centriolar plaques. One centriolar plaque shows a circular face view (Cp). Irregularly shaped electron dense materials (arrows) are often noted in the nucleus after pyrimethamine treatment. X 67,000.

Figure 14. The mitotic apparatus of *P. berghei* after pyrimethamine treatment. Nuclear microtubules (Nm), centriolar plaques (Cp), electron dense bars (K) and ill-defined electron dense areas (encircled with dotted lines) can be observed in the nucleus (N). X 85,000.

P. berghei and *P. gallinaceum* is similar to that of the merozoite (Fig. 3). As the parasite grows the clumping of the fine granules is observed as a thin layer along the nuclear membrane, and rarely extends toward the center of the nucleus (Fig. 4). The degree of granular clumping appears to decrease gradually and some nuclei do not show any clumping of granules. Instead only large granules of 18–22 nm in diameter and fine fibrils are found in such nuclei (Fig. 5). Occasionally a distinct nucleolus composed of aggregates of 10–15 nm granules is seen in the uninucleate trophozoites of *P. gallinaceum* (Fig. 4), but not in *P. berghei*. The pars amorpha of the nucleolus described in many other cell types is not apparent in the nucleolus of *P. gallinaceum*.

When the nucleus of a uninucleate trophozoite starts to divide to become a schizont (defined as a parasite having more than one nucleus), clumping of fine electron dense granules can no longer be observed (Fig. 6). Instead, a bundle of nuclear microtubules (spindle fibers) is noted radiating from a poorly delineated centriolar plaque which is located on the nuclear membrane (Figs. 6, 7) or within the nuclear pore. However, the occurrence is not frequent. This may be due to the rapidity of nuclear division of malarial parasites and the chances of obtaining this particular structure in a thin section of a large nucleus are rare.

The nuclei of schizonts of *P. berghei* and *P. gallinaceum* vary in size, shape and character. Smaller sized nuclei are round in shape and have the same size and morphological character as that of the merozoites. A prominent clumping of fine electron dense granules can be noted as observed in the merozoites. On the other hand, larger nuclei are irregular in shape and show less clumping of fine electron dense granules. In these nuclei, bundles of nuclear microtubules originating from a centriolar plaque are frequently observed. The nuclear microtubules in these nuclei are often shorter than those seen in the dividing nuclei of the uninucleate trophozoite. However, minute electron dense bars are always present at the terminal end of each microtubule. In mature schizonts, the nuclei become uniform in size and morphology and are similar to those in the merozoites.

b) Nuclear morphology after extraction with DNAase or EDTA

The hydrolytic extraction of nuclear components from thin sections of tissue embedded in epoxy resin depends on penetration of DNAase or EDTA to the tissue through the epoxy resin (Monneron and Bernhard, 1969). Treatment of epoxy resin embedded tissue with 5% H_2O_2 for 10 minutes appears to be sufficient to make the epoxy resin porous enough to permit adequate penetration of these agents to the reaction sites. However, treatment of the sections with 5% H_2O_2 alone did not alter the morphology of the malarial parasites. Yet, without H_2O_2 treatment, DNAase and EDTA are not effective for extraction of nuclear components.

The results obtained by DNAase treatment on thin sections are similar to those obtained by the DNAase treatment before embedding the tissue in the epoxy resin, although more extraction of nuclear components occurred when thin sections are used. This may be due to the thinness of the nucleus in the thin sections, whereas the treatment of whole parasites with DNAase may slow the access of the enzyme to the nucleus. The extraction of the nuclear components by EDTA also gives similar results to those obtained by DNAase, though the degree of extraction is not as prominent as that of DNAase. Since the results from both of these methods are essentially similar, they will be described together.

The prominent extraction of the nuclear material by DNAase and EDTA occurred in the clumped electron dense granules of *P. berghei* and *P. gallinaceum* (Figs. 8, 9). This is particularly noticeable in the nuclei of merozoites, uninucleate trophozoites, and the later stage of schizonts in which granular clumping is a prominent feature. The areas in which the clumped fine granules are bleached out show fine fibrillar materials (Figs. 8, 9). The space between the clumped fine granules shows aggregates of electron dense granules of 18–22 nm, some of which are linked together by fibrils. The background of the intragranular space is less electron dense than that seen in the untreated nuclei. The nucleoli remain unchanged after the treatment and the granules which composed the nucleolus are unchanged in their site. The bleach-

ing of clumped granules is also observed in the nuclei of the host erythrocytes and leukocytes (Fig. 11—Fig. 10 is a control). The granules of these cells appear to be more sensitive to the action of DNAase because it is readily extracted by the treatment when there is very slight extraction in the nuclei of malarial parasites.

B. Nuclear morphology after exposure to pyrimethamine

The most prominent changes in the erythrocytic stages of *P. berghei* and *P. gallinaceum* after exposure to pyrimethamine are seen in the nucleus. The nucleus of uninucleate trophozoites and schizonts of both *P. berghei* and *P. gallinaceum* is generally less electron opaque than in the control group. This change appears to be due to fewer numbers of granules of 18–22 nm and fibrils. Also, there appears to be less granular clumping in the treated parasites than in the control group.

Instead, irregularly shaped electron dense amorphous materials are observed in the nucleus of the uninucleate trophozoite and schizonts after pyrimethamine treatment (Fig. 13). The clumped fine granules and electron dense amorphous materials are bleached out by DNAase and EDTA treatments (Figs. 21–23).

Many nuclei of uninucleate trophozoites and schizonts of *P. berghei* and *P. gallinaceum* demonstrate bundles of nuclear microtubules, which radiate in fan-shaped fashion from a centriolar plaque located on the nuclear membrane or in a nuclear pore (Figs. 12–17). Though these nuclear microtubules are also observed in the untreated parasites, they appear much more frequently in the pyrimethamine treated parasites.

Each nuclear microtubule (spindle fiber) is composed of a densely stained cortex and a lightly stained central core and measures 11–13 nm across the inner diameter and 19–22

→

Figure 15. A nucleus (N) of pyrimethamine-treated *P. gallinaceum* after EDTA treatment. The centriolar plaques (Cp), nuclear microtubules (Nm) and electron dense bars (K) remain unchanged. However, the ill-defined electron dense zones located at the equatorial region are extracted (encircled with dotted lines). X 56,000.

Figure 16. A nucleus (N) of *P. berghei* after DNAase treatment. The centriolar plaques (Cp), nuclear microtubules (Nm) and electron dense bars (K) can still be seen after the treatment. Zones surrounded by dotted lines indicate the areas extracted by DNAase. X 68,000.

Figure 17. A nucleus (N) of pyrimethamine-treated *P. gallinaceum* in an advanced stage of nuclear division. Two centriolar plaques (Cp) are now separated from each other and the nucleus begins to become dumb-bell shaped. A bundle of nuclear microtubules (Nm) is no longer closely associated with the bundle that originated from the opposite centriolar plaque (arrow). X 64,000.

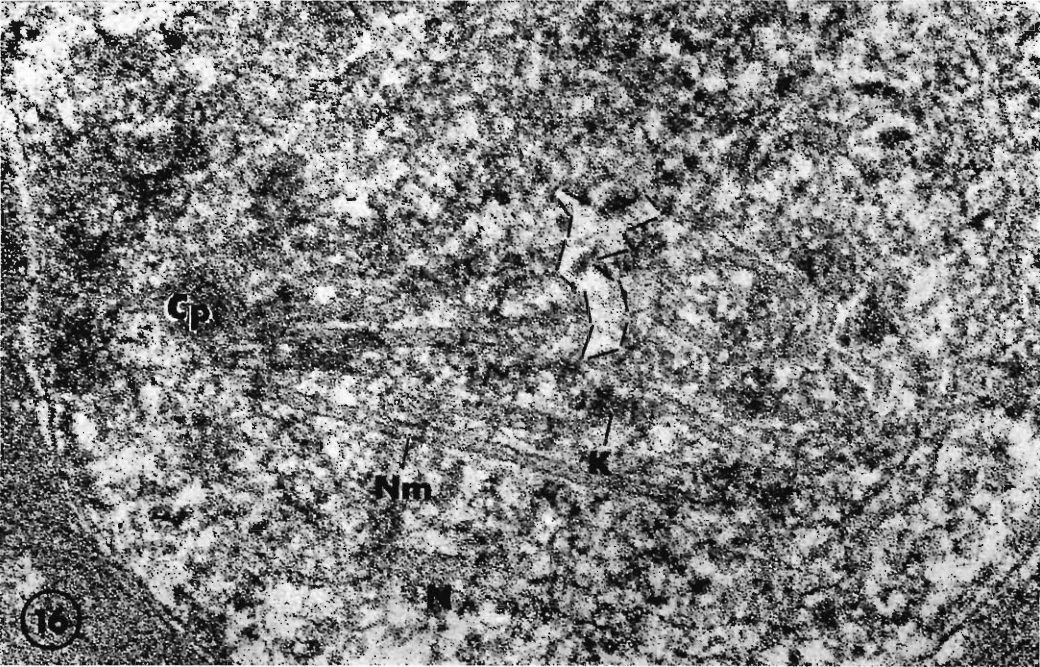
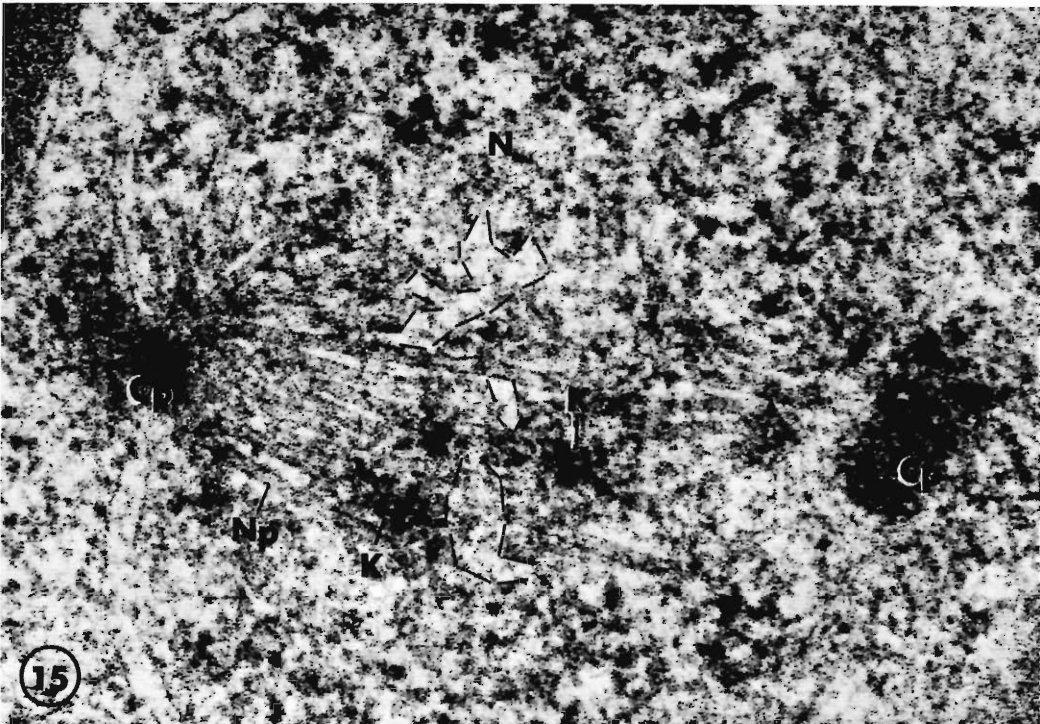
Figure 18. A telophase nucleus (N) of *P. gallinaceum*. The nuclear microtubules (Nm) are fragmented. A distinct nucleolus (NI) is located at the narrow isthmus of the nucleus. X 41,000.

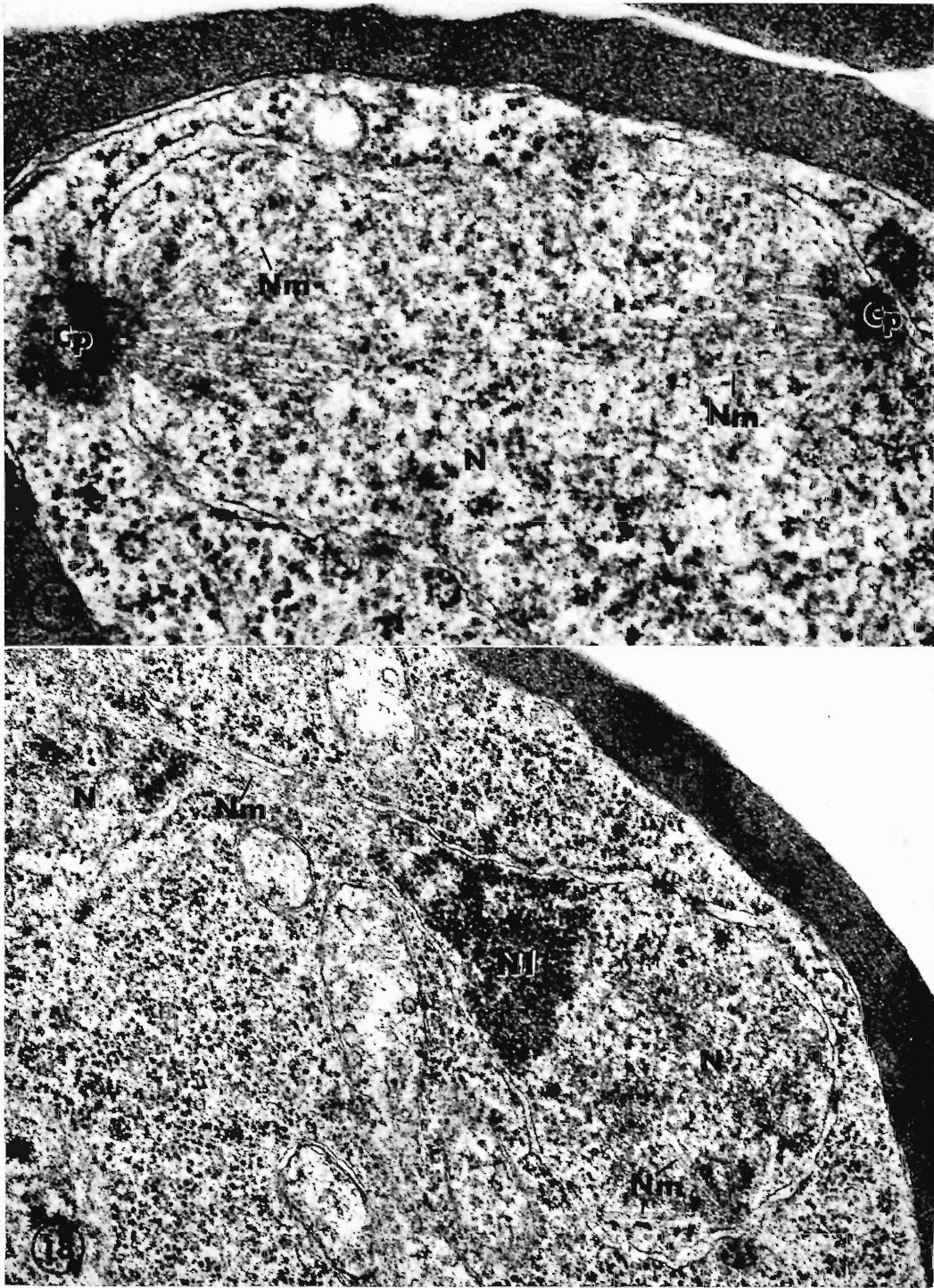
Figure 19. A schizont of *P. berghei* after pyrimethamine treatment. The nuclei (N) vary in shape and size. Short segments of nuclear microtubules (Nm) radiate from a centriolar plaque (Cp), but rarely meet with the other set of microtubules originating from the opposite centriolar plaque. X 45,000.

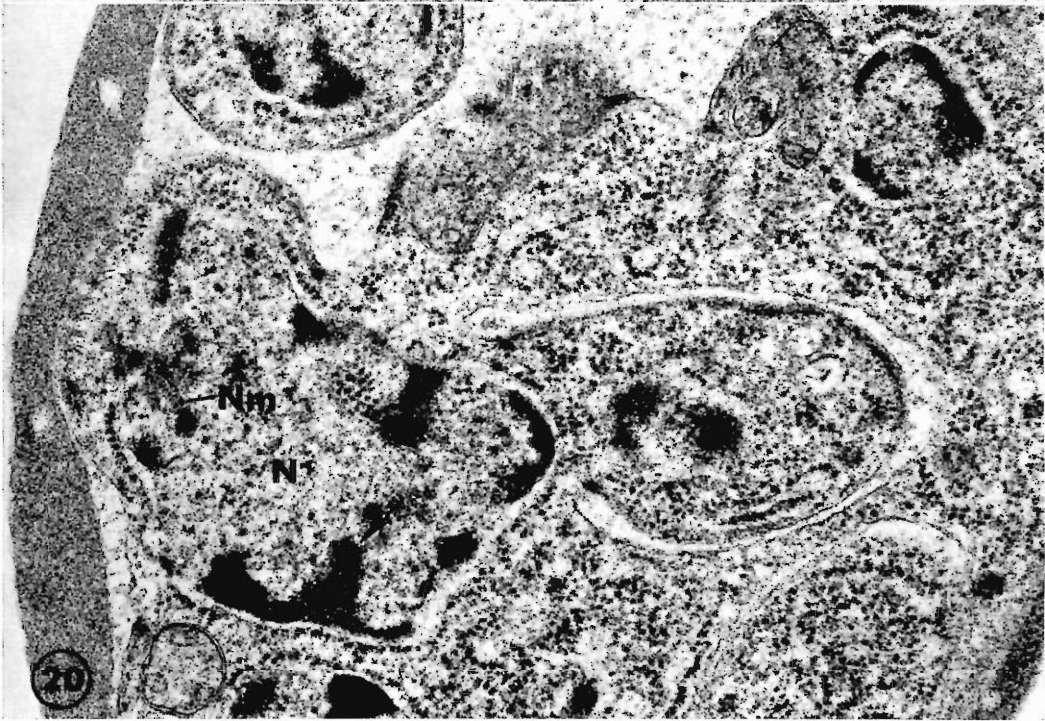
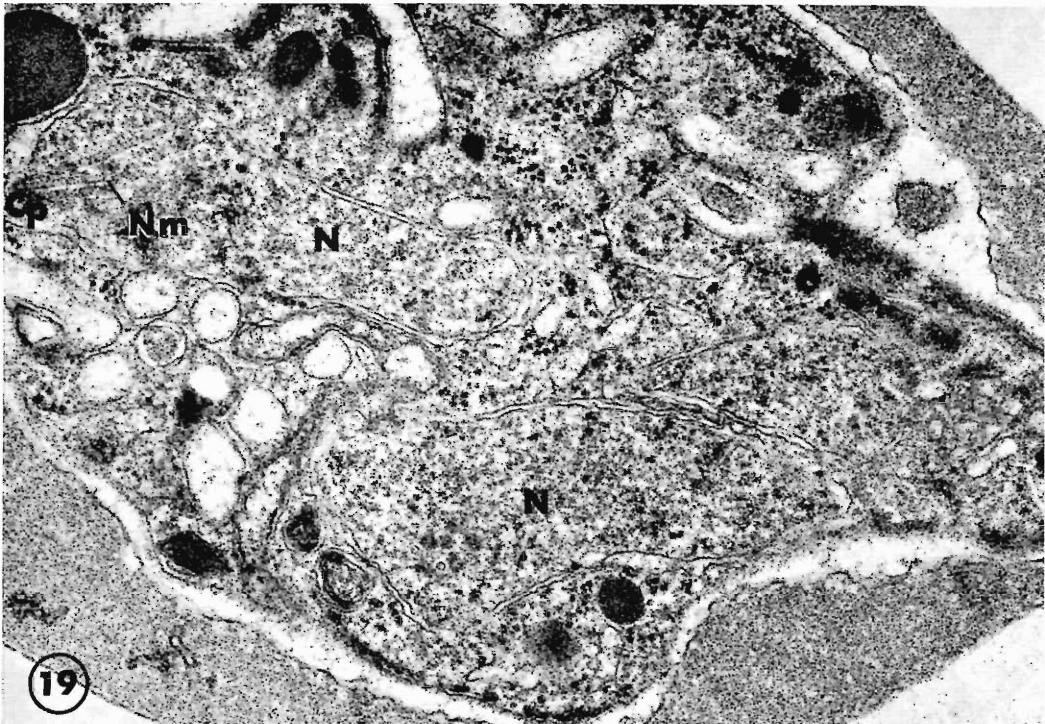
Figure 20. A schizont of *P. gallinaceum* after pyrimethamine treatment. The morphology of nuclei (N) is similar to those in Fig. 19, except that the former shows a prominent clumping of granules (arrow). X 39,000.

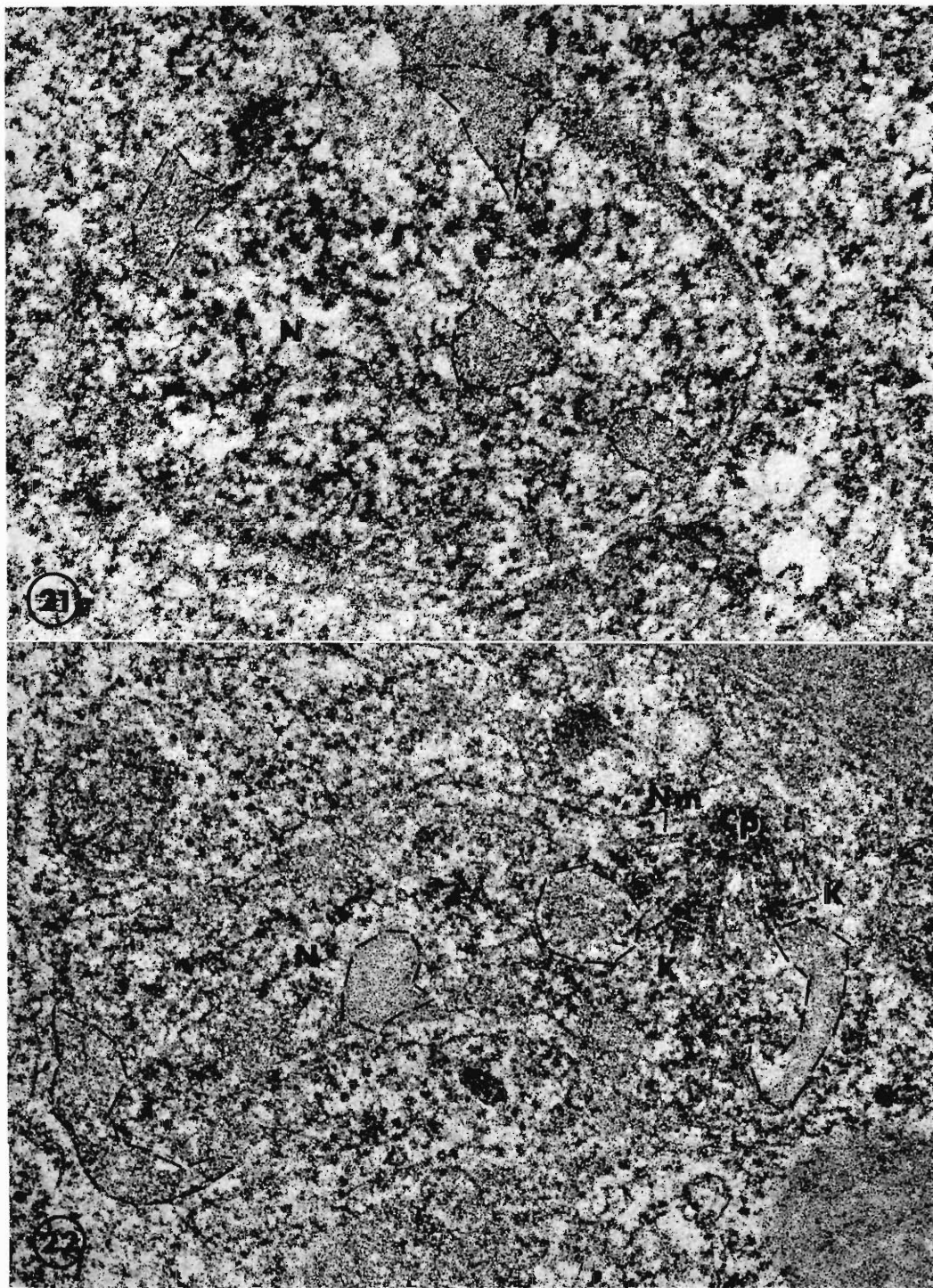
Figure 21. A nucleus (N) of pyrimethamine-treated *P. gallinaceum* after EDTA treatment. The nuclear matrix is less electron dense and the clumped granules after EDTA treatment are replaced with fine fibrillar material (encircled with dotted lines). X 56,000.

Figure 22. A nucleus (N) of pyrimethamine-treated *P. gallinaceum* after DNAase treatment. A bundle of nuclear microtubules (Nm) radiates from a centriolar plaque (Cp). The ends of the microtubules are in contact with one side of the electron dense bars (K), while the other side is in contact with a zone extracted by DNAase (encircled with dotted lines). X 60,000.









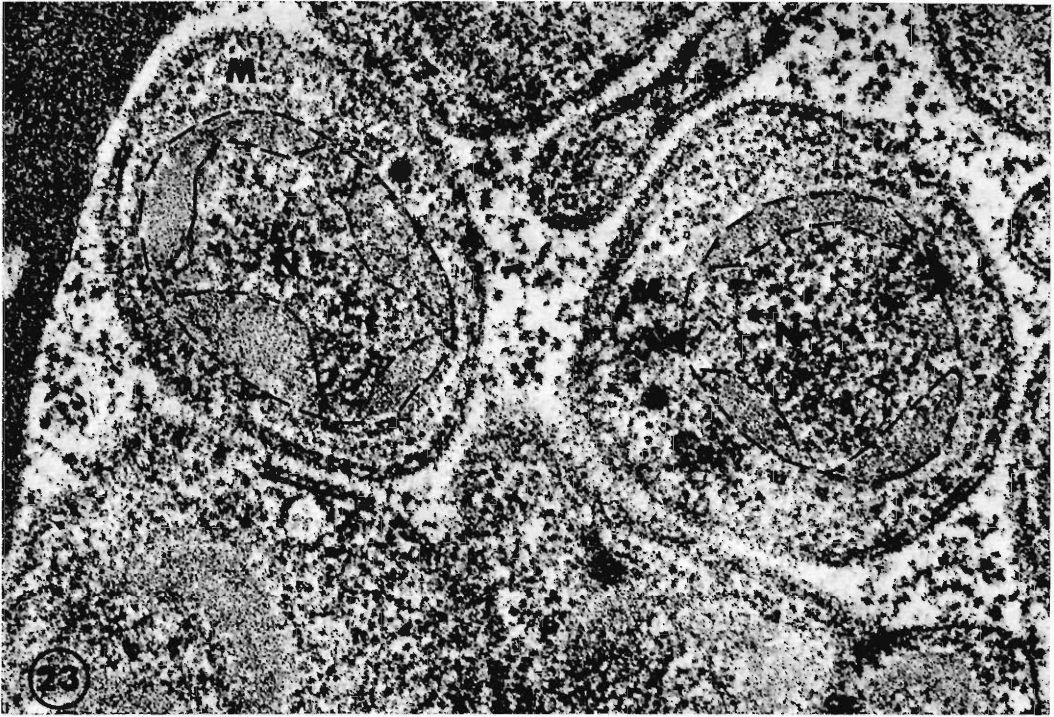


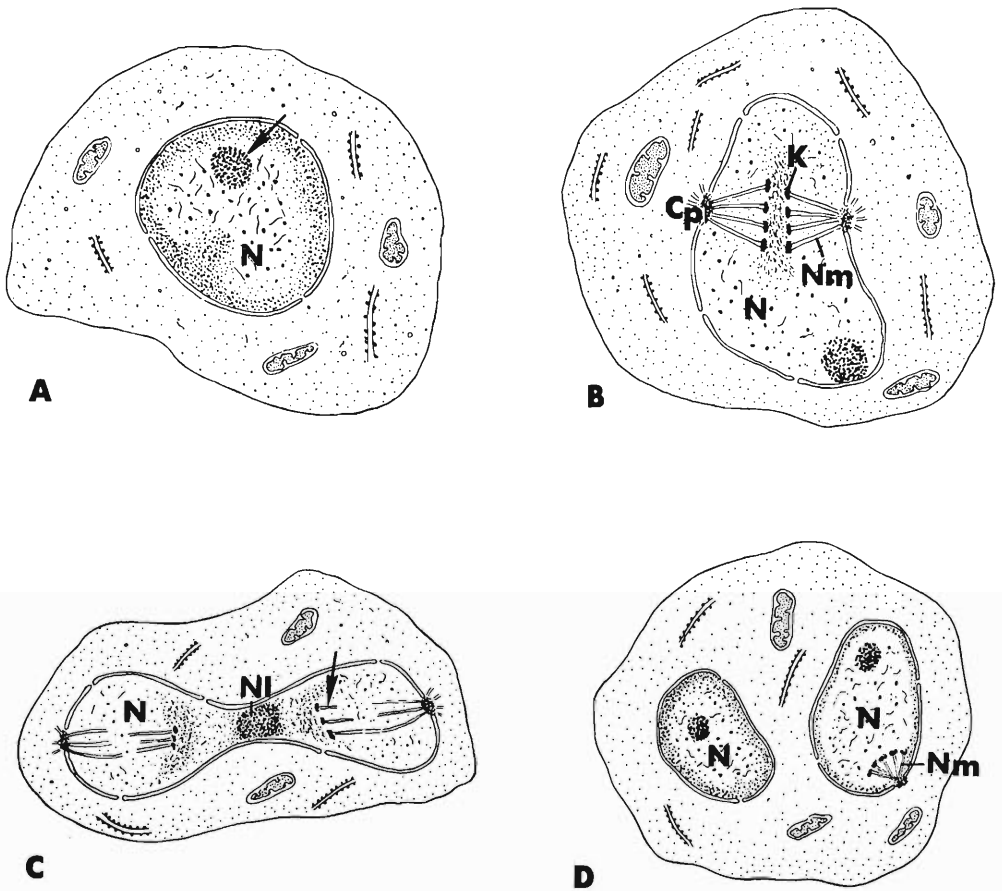
Figure 23. Merozoites (M) of pyrimethamine-treated *P. gallinaceum* after EDTA treatment. Extracted areas (encircled with dotted lines) in the nucleus (N) correspond to the clumped granular zones before EDTA treatment. X 44,000.

nm across the outer diameter (Figs. 12–14). When a full view of the mitotic apparatus in metaphase is obtained in a nucleus, two centriolar plaques are seen in the same plane of section and their bundles of microtubules radiate toward each other and meet at a point roughly midway between the two centriolar plaques (Figs. 12, 14). In general, the microtubules are not connected with those originating from an opposite centriolar plaque, although on rare occasions one microtubule connects two centriolar plaques.

At the midpoint in which bundles of microtubules from opposite plaques meet, paired electron opaque structures on and perpendicular to the microtubules are observed (Figs. 12, 14). Each of these paired, dense structures is a short bar that measures approximately 75 nm in length and 74 nm in width. Each bar is composed of three darkly stained subunits separated by lightly stained spaces. Though these structures are usually seen mid-

way between two centriolar plaques, occasionally they occur close to each centriolar plaque. In this instance, the distance from one centriolar plaque to its bar is approximately the same as the distance from the opposite plaque to its bar. Ill-defined darkly stained materials are often observed in the equatorial plate of the metaphase, where paired electron dense bars are present (Figs. 12–14). These mitotic figures are often off-center within the nucleus and occasionally more than one mitotic figure in a given nucleus is observed (Fig. 13).

DNAase and EDTA treatments do not bleach out the mitotic apparatus, such as centriolar plaques, nuclear microtubules (spindle fibers), and minute electron opaque structures (Figs. 15, 16, 22). Actually, they become more prominent after DNAase and EDTA treatments. This is because some of the electron dense background of the nucleus is extracted by DNAase and EDTA. In these



24

Figure 24. Schematic diagrams of the nucleus of malarial parasites. A) The interphase nucleus (N) shows clumped chromatin along the nuclear membrane and the interchromatin space is scattered with large granules. A nucleolus (arrow) is particularly prominent in *P. gallinaceum*. B) The metaphase nucleus (N) shows a mitotic apparatus composed of centriolar plaques (Cp), nuclear microtubules (Nm) and kinetochores (K). Ill-defined electron dense material is noted at the equatorial region and is closely associated with kinetochores. C) The telophase nucleus (N) is dumb-bell shaped and possesses fragmented nuclear microtubules (arrow). A nucleolus (Nl) is located at the narrow isthmus of the nucleus. D) Resulting daughter nuclei (N) after nuclear division vary in size. The larger nucleus often shows a bundle of nuclear microtubules (Nm).

sections, the detailed structure of the centriolar plaque can be obtained. The face view of an electron dense centriolar plaque shows a circular profile, from the periphery of which nuclear microtubules radiate (Figs. 15–17). There is no clear demarcation between the

centriolar plaque and the microtubules. At the midpoint at which bundles of microtubules from opposite plaques meet, paired electron opaque structures on the microtubules are clearly observed. Though the equatorial plate of metaphase is often occupied with a darkly

stained material before extraction with DNAase or EDTA, this area becomes electron transparent after digestion (Figs. 15, 16, 22). The extracted area is irregularly outlined and extends beyond the area where microtubules are present.

During the nuclear division of *P. berghei* and *P. gallinaceum*, the nuclear membrane does not disappear. From the centriolar plaques a few microtubules also radiate toward the cytoplasm where cytoplasmic differentiation is taking place.

As nuclear division progresses, the nucleus becomes dumb-bell shaped and the nuclear microtubules become fragmented (Fig. 18). A nucleolus in *P. gallinaceum* can be observed at the narrow isthmus of the dumb-bell shaped nucleus (Fig. 18). This represents telophase of nuclear division. However, it is difficult to determine if the nuclear division of these malarial parasites proceeds synchronously in a manner such as 2, 4, 8 and so on.

There are various sized nuclei in the late stage of schizonts as observed in the control group. However, pleomorphism of the nuclei is greater in the parasites treated with pyrimethamine. The presence of nuclear microtubules is particularly pronounced in the irregularly shaped nuclei of the budding merozoites (Figs. 19, 20). In these nuclei, short segments of microtubules radiate from a centriolar plaque toward the center of the nucleus (Figs. 19, 20). However, they rarely meet with the other set of microtubules which originate from the opposite centriolar plaque. The end of each microtubule possesses a minute electron dense structure which again is situated perpendicular to the axis of the microtubule. Because the distance between the centriolar plaque and the minute electron dense structure is short and the microtubules radiate from the centriolar plaque in fan-shaped fashion, these electron dense structures appear to surround the centriolar plaque in a semi-circular fashion.

The irregular shaped nucleus often extends into an adjacent budding merozoite and sometimes a large nucleus is seen halfway between a newly forming merozoite and part of the mother schizont (Fig. 20). All of these nuclei possess nuclear microtubules. Also, a large nucleus disproportionate to the size of the

parasites is observed in those parasites exposed to pyrimethamine.

Discussion

The enzymatic and chemical extractions of specific cellular components from thin sections of tissues embedded in water-insoluble epoxy resins have been established as a useful method for electron microscope cytochemistry (Monneron and Bernhard, 1969; Franke and Falk, 1970; Dupuy-Coin et al., 1972). Monneron and Bernhard (1969) demonstrated that tissue reactive sites and enzyme activities are not all irreversibly altered even after treatment with routine electron microscope techniques. After oxidation, the polymerized epoxy resin appears to be sufficiently porous to permit adequate diffusion of hydrolases to the embedded tissue. Though DNA can be expected to be digested by DNAase, EDTA can also be used for the location of DNA in thin sections of tissues. EDTA is generally considered to bleach out uranyl stain fixed on deoxyribonucleoproteins while structures containing ribonucleoproteins retain the uranyl stain (Dupuy-Coin et al., 1972).

By using DNAase or EDTA the clumped fine granules located along the nuclear membrane of the erythrocytic stages of *P. berghei* and *P. gallinaceum* were bleached out. This result confirms that the clumped fine granules are deoxyribonucleoproteins and correspond to the clumped chromatin described in many other cell types. The degree of extraction of nuclear chromatin by DNAase and EDTA appears to correspond to the degree of chromatin clumping in the nucleus. Prominent extraction of nuclear chromatin is particularly noticeable in the merozoites and uninucleate trophozoites which show the most prominent clumping of chromatin. The clumped chromatin is generally considered as metabolically inactive. However, its reactivity with DNAase and EDTA apparently still remains, as observed by the drastic extraction of the chromatin granules.

After extraction of the clumped chromatin in the parasites' nucleus intrachromatin granules become more prominent. Monneron and Bernhard (1969) similarly described prominent intrachromatin granules in the nucleus after EDTA treatment. Because of the non-

bleaching character of the interchromatin granules under treatment, it was suggested that they do not contain DNA or may be coated by some proteins which prevent the reaction between DNAase and EDTA with deoxyribonucleoproteins. The biological function of intrachromatin granules is still not clear. Perichromatin fibrils which were not prominent before extraction of DNA from the nucleus, become apparent after the bleaching of chromatin. Perichromatin fibrils have been suggested as representing the morphological expression of transcription along the derepressed regions of nucleohistones (Monneron and Bernhard, 1969). The characterization of interchromatin granules and perichromatin fibrils in the parasite's nucleus after DNAase or EDTA treatment suggests that the parasite's nucleus is not markedly different from that of eukaryotic cells. Furthermore, it indicates that this method is useful for the detailed study of the morphology of nuclear components. The negative reaction of an aggregate of granules of 15 nm by these treatments similarly identifies this structure as one that does not contain DNA and is most likely a nucleolus which contains RNA.

In 1968, Aikawa and Beaudoin demonstrated that pyrimethamine arrests or retards the nuclear division of the erythrocytic stages of *P. gallinaceum* at metaphase. This conclusion was supported by the observation of a higher percentage of schizonts with their nuclei showing metaphase and fewer resultant merozoites as well as the interference with nuclear migration into newly forming merozoites. Similar findings were obtained for the erythrocytic stages of *P. berghei* as well as *P. gallinaceum* in the present study. It should be noted, however, that the mode of action of pyrimethamine has been biochemically demonstrated to inhibit folic acid metabolism of malarial parasites by binding with folic acid reductase, leading to a block of the biosynthetic pathway to nucleic acid (Hitchings, 1960; Ferone et al., 1969). The cytological effect of pyrimethamine appears to differ from those biochemical data in that it appeared to interfere at metaphase rather than at prophase in which DNA synthesis is believed to occur. However, Gutteridge and Trigg (1971) recently reported that pyrimethamine did not significantly affect the incorporation of ^3H -

adenosine into DNA and RNA of the erythrocytic stages of *P. knowlesi*. Thus, they suggested that the thymidylate for DNA synthesis in *P. knowlesi* is synthesized by a pathway not involving dihydrofolate reductase. Though this observation does not fully explain our findings, it may explain the occurrence of mitotic apparatus in the parasites treated with pyrimethamine. Whatever the modes of action of pyrimethamine might be on malarial parasites, it appears to be a useful drug for studying the nuclear division of malarial parasites because it arrests the nuclear division at metaphase.

The mitotic apparatus seen in the dividing nuclei of malarial parasites is composed of centriolar plaques, spindle fibers (microtubules), and electron dense structures located on the spindle fibers. Neither DNAase nor EDTA treatment bleached out these structures. This indicates that the electron dense structures seen along the spindle fibers are not chromosomes, contrary to the previous suggestion made by Aikawa and Beaudoin (1968). Scalzi and Bahr (1968) speculated that these organelles may be kinetochores. The electron microscopic image of the kinetochore has been well described in various cell types (Luykx, 1965; Jokelainen, 1967; Bajér and Mole-Bajér, 1969; Journey and Whaley, 1970; Comings and Okada, 1971). Luykx (1965) reported that the kinetochore of *Urechis* eggs is composed of two layers of dense material separated by an intermediate less dense layer. The outer layers are about twice as thick as the middle layer. One surface of the kinetochore is attached to the chromosomes and the other is attached to the spindle fibers. It has been reported that the kinetochore region contains little or no DNA and that what DNA is present is more resistant to the enzyme treatment than is the rest of the chromosome (Luykx, 1965). Thus, the morphology, interrelationship with nuclear microtubules (spindle fibers) and cytochemical reaction indicate that the electron dense structures described here are the kinetochore of malarial parasites, though the detailed morphology is somewhat different from those in eukaryotic cell types.

If these structures are kinetochores, a question arises as to the morphological characteristics of the malarial parasite's chromosomes.

Though it is ill-defined, there are electron dense granular materials often seen near the kinetochore and along the equatorial plate. These electron opaque materials bleach out after DNAase or EDTA treatment. Thus, these electron dense, yet ill-defined structures may be poorly organized chromosomes of malarial parasites. They may correspond to a weak Feulgen reaction in light microscopy (Chen, 1944). Uninuclear organisms having a nucleus of less than 4 nm in diameter were reported to have diffuse chromosomes with poorly defined boundaries (Moens and Perkins, 1969). Similar poorly defined electron dense material in dividing nuclei of *Saprolignea* has been reported by Heath and Greenwood (1970). They suggested that they are the chromosomes of *S. ferax*. However, it may be worthwhile to mention that irregularly shaped more electron dense material is seen in the nuclei of the parasites treated with pyrimethamine. These dense structures are again associated with the nuclear microtubules and are bleached out by DNAase and EDTA. Therefore, it may represent a condensed form of the chromosomes produced by pyrimethamine.

Centriolar plaques, which are located on the nuclear membrane, show a close relationship with the nuclear microtubules. In some sections, there is a direct connection between these two structures. Symmetrical and cylindrical structures of the centriolar plaques indicate that they are centriolar equivalents (Robinow and Marak, 1966; Aikawa and Beaudoin, 1968). Recently McCully and Robinow (1971) described a similar structure in *Schizosaccharomyces pombe* and considered it a kinetochore equivalent because microtubules connect two of these structures located at the opposite poles of the nucleus. However, in the malarial parasites, these structures appear to be centriolar equivalents as discussed above. The centriolar plaque may also be the place where synthesis and organization of the nuclear microtubules occurs because during the beginning of nuclear division, short segments of the nuclear microtubules attached to the centriolar plaque grow outward to meet the other set of microtubules originating from the opposite plaque. In snail (Gall, 1961) and chick cells (Kalnins and Porter, 1969), an electron dense area similar to the centriolar plaque

is located close to the centriole and has been reported to synthesize the subunits of nuclear microtubule. These subunits are suggested to be organized into microtubules by the centriole (Heath and Greenwood, 1970). The centriolar plaque of malarial parasites may possess the centriolar function as well as the ability to synthesize and organize the nuclear microtubules.

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Comments on Plasmodial Ultramicroscopy

P. C. C. GARNHAM

Senior Research Fellow, Imperial College Field Station, Ascot, Berkshire, England

My first comment is to express surprise that none of the papers in this section deals directly—as they have in earlier workshops—with the general biological characters of malaria parasites. The present papers are concerned with fine structure and its implications in the physiology, chiefly of the erythrocytic stages, of the organisms. Probably this is the result of the many questions raised in 1966 and 1969 regarding the subject, and you will have noticed that some of the puzzles are now being elucidated. These workshops are of particular value to the research worker, because they

pinpoint the actual advance he has made in the preceding three years.

The subject matter however is not entirely devoted to electron microscopy and the paper on *P. brasilianum* contributes further evidence of the relationship between this quartan parasite of New World monkeys and *P. malariae* of man. Also, Terzakis continues his fascinating study of viruses and *Plasmodium*. I intend to discuss these two papers in a little detail in view of their wide interest, but will have further questions to put to the authors of the four others.

P. brasilianum: *Fine Structure* (Sterling, Aikawa and Nussenzweig). *P. brasilianum* differs from most other mammalian species by possessing (like avian and reptilian parasites) cristate mitochondria, by feeding almost exclusively through the cytostome, and by exhibiting a nucleolus. The authors also suggest that the paired organelles of *P. brasilianum* differ by having certain pore-like structures. I would call their attention to the triple, instead of the paired organelle, to which we ascribed in the sporozoite a possible specific significance. *P. brasilianum* produces on the host cell, knob-like processes, which are well known in *P. coatneyi* and *P. falciparum*. I might mention that the new malaria parasite of the orang-utan, *P. silvaticum*, also induces this characteristic distortion of the erythrocytic envelope. Similarly, like *P. coatneyi* and *P. falciparum*, the orang-utan parasites retreat to the internal organs after the first half of their growth in the peripheral blood. Sterling and his colleagues state that excrescences on the red blood cells are also prominent in infections with *P. malariae* and *P. brasilianum*. Although such abnormalities are not accompanied by the disappearance of the quartan parasites from the peripheral blood, it is suggested nevertheless that at a later stage when immunity commences, sequestration of the parasite occurs in the liver, where they form a tight complex with the Kupffer cells. May I suggest also that such sequestration may explain the extraordinarily long duration of quartan infections which thereby evade the effects of humoral immunity?

This paper provides further evidence of the close affinity between *P. brasilianum* and *P. malariae* and of their possibly common origin. May I suggest that the ultrastructure of *P. inui* (the quartan parasite of Asian monkeys) should be similarly investigated? It should be quite unlike the other two.

Virus-like particles and sporozoite budding (Terzakis). I have been unable to determine from this paper how frequent is the presence in his material of the so-called virus. From earlier work e.g. on ookinetes, it seemed that these "crystalline" structures were always present. I feel that the only way in which to reach a firm conclusion as to their nature, would be to apply classical virological tech-

niques, both in the mosquito host and in the sporogonic stages of the parasite—perhaps in the vertebrate stages also. We might recall Col. Sprintz's remarks at the last workshop.

Fine structure of P. traguli (Cadigan, Colley and Zaman). In this paper there is again a mention of "lucent crystalline" bodies in food vacuoles in young trophozoites in the blood. Are these similar to the crystalloids described by Terzakis and others? In my opinion probably not. This paper is interesting as there is a real *raison d'être* for the use of the electron microscope in the examination of the blood of the chevrotain whose red blood corpuscles are almost beyond the resolution of the light microscope. Observations should continue in order to clarify the relationship between *P. traguli*, *Hepatocystis fieldi* and possibly a new species of *Plasmodium*. I was fortunate in being able to see this material recently in Malaya, and feel that the opportunity should not be missed to carry out transmission experiments with suitable mosquitoes and laboratory bred animals.

Freeze-etched technique in P. gallinaceum (Meszoely, Steere and Bahr). Although three dimensional techniques have been in use for some years in the study of parasitic protozoa, they have done little more than confirm the interpretation of ultrastructure as determined by the examination of ultra thin sections. The paper by Meszoely and his colleagues demonstrates however a number of new features particularly in relation to the surface membranes, and the pores in the nuclear membrane. It has long been known that the subpellicular microtubules are attached to the polar ring, but these new observations demonstrate how this complex is formed and its function in penetration. The posterior end of the microtubules has never been properly demonstrated, although serial sections indicate that they terminate only slightly posterior to the nucleus—at least in sporozoites. The application of freeze-etching might help in the solution of this problem. It is interesting to hear that the cytostome is elevated above the surface of the plasmalemma and presumably this is due to the thickened double collar which projects outwards. This technique could well be applied to the extracellular stages of the

parasite e.g. the sporozoite, where the outermost membrane cannot be part of a host cell.

The feeding process of the exoerythrocytic stages of Plasmodium lophurae (Beaudoin and Strome). Cytostomes of the exoerythrocytic stages of the avian malaria parasite are compared with the larger ones of the erythrocytic stages, and the authors suggest that, in the E E forms, the host cell membrane persists over the opening of the micropore, while in the erythrocytic form the membrane is drawn into the cavity which in consequence is larger. In the former, presumably digestion takes place in the narrow cytostome, while in the latter the haemoglobin is more directly exposed to the enzymes of the parasite. A diagram would be useful. I wonder how much the consistency of the cytoplasm of a tissue cell as compared with that of an erythrocyte affects this question. It would be interesting to compare these phenomena in mammalian species which, in so many ways, differ from avian or reptilian malaria parasites. There have been few observations on the ultrastructure of their exoerythrocytic stages, though my colleagues and I described rather inadequately some features of these stages in *P. berghei*. We found that the internal diameter of the cytostome was 75 nm in the exoerythrocytic merozoite, whereas in the trophozoite in the blood, the diameter was greater (90 nm). The Liverpool workers obtained similar figures.

Beaudoin and Strome draw attention to the presence of structures resembling cytostomes or micropores on the inner surface of the plasmalemma of mammalian cells and named "desmosomes". Actually, these are thickenings of the surface of two contiguous cells, which form an attachment plate and have a totally different function (Ham, 1969). Moreover they do not possess the essential characters of the cytostome *viz.* (1) it dips at right angles to the surface into the interior of the parasite, (2) it lies at a relatively fixed position near the nucleus, and (3) the organelle is single or less often two or three may be present.

Cytochemistry of nucleus of P. gallinaceum and P. berghei as determined by electron microscopy (Aikawa, Sterling and Rabbege). Cytochemical techniques for the ultrastructure of

parasitic protozoa have been limited in the past largely to the detection of phosphatases. These observations of Aikawa and his colleagues bring us a step nearer to the definition of chromosomes in malaria parasites, and the details of nuclear division. Prior treatment with pyrimethamine "fixes" the process in metaphase, but one might ask what other damage is caused, and can one recognise drug-induced abnormalities? The very irregularity and amorphous nature of the electron-dense masses suggest "decomposition". The nuclear microtubules or spindle fibres are however shown up clearly in contrast, partly as the result of the extraction of DNA. The centriolar plaques and spindles remain unaffected—but what is surprising in the latest results, is that the electron dense structures are also untouched i.e. they do not contain DNA: They are not chromosomes (as was previously thought) but are kinetochores—the connecting link between the microtubules and the chromosome. The chromosome itself, is still poorly defined even though pyrimethamine may increase its density.

I should like to make one general comment on any work involving *P. berghei*. In contrast to *P. vivax*, *P. cynomolgi*, or *P. gallinaceum*, *P. berghei* is quite unpredictable in its sporogonic behavior; it sometimes produces viable sporozoites, but often they are non-infective, and even under the best conditions only about one sporozoite in five is capable of developing in the liver of mice. Very often, the percentage of viable forms is less than 0.1 per cent (see Wéry). This is often not realised, because the introduction of, say 100,000, of such sporozoites into a mouse will produce a perfectly good infection although the inoculum will contain only 100 living sporozoites. Apart from other considerations, it is obviously important to know whether the parasite you are observing—e.g. for fine structure—are viable or non-viable. This applies only to observations on the sporozoites, because the subsequent forms are the product of the viable sporozoites and are presumably normal.

Such a condition of non-viability should be reflected in the fine structure and for some years, I have been struck by the virtual absence of the cytostome in the sporozoites of *P. berghei*. On the other hand, this organelle

has been repeatedly seen in the other stages. During the last 1½ years, Dr. Sinden and I have attempted to assess the presence or absence of the organelle in the sporozoites of a relatively “good” strain of *P. berghei* (Nigeria) and have compared the results with *P. vivax*, *P. cynomolgi*, and *P. gallinaceum*. I thought at first that the great rarity of the cytostome as reported by Vanderberg in the sporozoites of ripe oocysts was due to the possibly immature state of the sporozoites. Accordingly our observations were made both on bursting oocysts and on infected salivary glands. The work is incomplete, but the results so far are quite impressive.

It is tempting to ascribe the poor viability of the sporozoites of *P. berghei* to a genetic defect, reflected in a failure of development of an essential organelle, and I should like to ask anyone working on the fine structure of the rodent parasites to confirm this or any other features in their material. It would be

Table 1. Incidence of Micropore in Sporozoites of Plasmodium spp.

Species	Oocyst	Salivary glands
<i>P. vivax</i>	21/1617	under examination
<i>P. cynomolgi</i>	109/2200	29/2121
<i>P. berghei</i>	0/1815	0/7000
<i>P. gallinaceum</i>	8/810	under examination

useful also to extend the work to other notoriously “bad” species, such as *P. gonderi*, *P. knowlesi*, etc.

Vanderberg, Nussenzweig and Most carried out quantitative experiments and found that only a small and variable fraction of the sporozoites of *P. berghei* in a given inoculation is actually infective. They showed clearly that the natural host (*G. surdaster*) was a better recipient than “laboratory rodents,” so the host factor as well as the intrinsic viability of the sporozoite must be taken into account.

IV

IN VITRO STUDIES

The Effects of Antibiotics on ^{14}C -isoleucine Incorporation by Monkey Erythrocytes Infected with Malarial Parasites

J. V. SCHNELL AND W. A. SIDDIQUI

Department of Tropical Medicine and Medical Microbiology, University of Hawaii,
3675 Kilauea Avenue, Honolulu, Hawaii 96816

ABSTRACT: Puromycin and cycloheximide inhibit the incorporation of ^{14}C -isoleucine into protein by *Plasmodium falciparum*-infected owl monkey erythrocytes, while chloramphenicol does not. Similarly, protein synthesis by *P. knowlesi*-infected rhesus monkey erythrocytes is inhibited by puromycin, but not by chloramphenicol. These results indicate that the malarial parasites respond to these antibiotics as do other eukaryotic organisms.

A number of studies have been made on amino acid incorporation by intraerythrocytic malarial parasites; Butcher and Cohen (1971) Gutteridge and Trigg (1971); Polet and Conrad (1969); Sherman *et al.* (1969, 1971a & b); and Trager (1971). We have been investigating the incorporation of ^{14}C -isoleucine into protein by *P. knowlesi*-infected rhesus erythrocytes and *P. falciparum*-infected owl monkey (*Aotus trivirgatus*) erythrocytes. In order to establish the nature of the product of the incorporation reaction we have tested several antibiotics known to inhibit protein synthesis in other biological cells. These experiments have yielded results that are of interest in comparing the parasitized erythrocyte to other organisms.

Materials and Methods

The procedures for maintaining the blood stages of the malarial parasite were the same as those outlined by Siddiqui and Schnell (1972). All inorganic reagents used were reagent grade. Biochemicals were products of Nutritional Biochemicals Corp. Cleveland, Ohio. The ^{14}C -isoleucine and the organic scintillators were purchased from New England Nuclear, El Cerrito, California. The filter paper discs used for the isotope experiments were Whatmann 3MM, circles 2.3 centimeters in diameter. Cycloheximide and chloramphenicol were products of Sigma Chemical

Co. St. Louis, Missouri; puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp.

The parasitized blood was drawn into a syringe containing heparin, and immediately centrifuged at $700 \times g$ for 8 minutes in a refrigerated centrifuge. The plasma was drawn off, and the cells were washed twice by centrifugation and resuspension in four volumes (relative to volume of packed cells) of 0.85% aqueous NaCl (w/v). The washed cells were suspended in an equal volume of saline and then refrigerated until used.

The incorporation experiments were conducted by first incubating for 10 minutes at 37°C 0.1 ml of the antibiotic being tested, 0.2 ml of the washed blood cell suspension and 0.8 ml of the basal simplified medium of Siddiqui *et al.* (1969) which is a modification of the Harvard medium of Geiman *et al.* (1946). One component of the basal simplified medium, stearic acid, was omitted for these experiments. After this incubation $0.2 \mu\text{C}$ of ^{14}C -isoleucine was added, the suspension rapidly mixed and a 0.1 ml sample pipetted onto a disc and dropped into cold 5% (w/v) aqueous trichloroacetic acid as the zero time sample. Additional samples were taken at desired time intervals in the same manner. The final specific activity of the ^{14}C -isoleucine is 7.4 mC/m mole taking into account the cold isoleucine in the medium. The final concentrations of the two amino acids included in the reaction mixture were: isoleucine, 2.7×10^{-5} moles/liter; and methionine 4.6×10^{-5} moles/liter. Under these assay conditions leucocytes do not show significant incorporation of ^{14}C -isoleucine.

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Table 1. Effects of Antibiotics on Isoleucine Incorporation by *P. falciparum* (FUP) Infected Owl Monkey Blood.

Antibiotic	Concentration (moles/liter)	cpm/10 ⁷ parasitized erythrocytes ^a
None	—	594
Puromycin	1.0×10^{-5}	64
"	9.0×10^{-5}	26
"	1.8×10^{-4}	< 10
Chloramphenicol	3.1×10^{-5}	557
"	15.5×10^{-4}	629
"	3.1×10^{-4}	541
Cycloheximide	3.6×10^{-5}	25
"	1.8×10^{-4}	14
"	3.6×10^{-4}	< 10

^a Parasitemia = 29.1% (R-63%, T-29, S-1, S₂-7). Values given are after a three hour incubation.

Incorporation of ¹⁴C-isoleucine into proteins by the parasitized erythrocytes was measured using the filter paper disc method of Mans and Novelli (1961). The samples were counted in a Packard scintillation spectrometer Model 3320, and quenching measured by the channel ratio method. The scintillation mixture contained 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) p-bis 2-(5-phenyloxazolyl)-benzene in toluene. The values obtained were corrected for the zero time control.

Results

Table 1 represents a summary of an experiment designed to test the effect of three antibiotics on ¹⁴C-isoleucine incorporation by *P. falciparum*-infected owl monkey erythrocytes. In interpreting the results of these experiments differences of less than ten percent are not considered significant. It can be seen from the data of Table 1 that puromycin and cycloheximide are potent inhibitors of protein synthesis by malaria-infected erythrocytes. However chloramphenicol, even at high concentrations, had no effect on the incorporation reaction. The experiment summarized in Table 2 was designed to determine whether *P. knowlesi*-infected rhesus erythrocytes responded to puromycin and chloramphenicol in the same manner. Additionally the amount of ¹⁴C-isoleucine incorporation in relation to parasitemia and growth stages is also indicated in Table 2. The three samples of blood for this experiment were taken from the same animal at various times in the growth cycle: 21a was drawn at 7:00 a.m., 21b at 8:15 p.m. and 21c at 7:00 a.m. the following day.

Table 2. Effects of Antibiotics on Isoleucine Incorporation by *P. knowlesi* Infected Rhesus Monkey Blood.

Experiment No.	Antibiotic	Concentration (moles/liter)	cpm/10 ⁷ parasitized erythrocytes ⁽¹⁾
21-a (2)	None	—	1289 ⁽²⁾
	Puromycin	1.8×10^{-5}	< 10
	Chloramphenicol	3.1×10^{-4}	1168
21-b (3)	None	—	1932
	Puromycin	1.8×10^{-5}	< 10
	Chloramphenicol	3.1×10^{-4}	1904
21-c (4)	None	—	3394
	Puromycin	1.8×10^{-5}	10
	Chloramphenicol	3.1×10^{-4}	3632

¹ Values given are after a three hour incubation.

² Parasitemia 14.3% R 54, % 13, S₂ 14, >S₂ 11, G 8.

³ Parasitemia 24.7% R 1, T 94, S₂ 1, >S₂ 1, G 3.

⁴ Parasitemia 19.2% R 22, T 21, S₂ 31, >S₂ 20, G 6.

The data of Table 2 indicates that puromycin inhibits protein synthesis by *P. knowlesi*-infected rhesus red blood cells, but that chloramphenicol is without effect. The possible interpretation for these results is discussed below. The amount of ¹⁴C-isoleucine incorporation in relation to growth stages indicates that the late growth stages (schizonts and segmenters) synthesize protein more rapidly than young ring stages. A more detailed presentation of incorporation rate in relation to the parasites growth cycle will be published separately.

Discussion

The effects of the three antibiotics on protein synthesis by parasitized monkey erythrocytes have some interesting implications concerning the malarial parasite as a microorganism. Puromycin causes release of the nascent polypeptide chain from ribosomes and apparently inhibits protein synthesis in all biological systems in which it has been tested (Nathans, 1967). Cycloheximide inhibits protein synthesis by the usual cytoribosome-soluble cell material mechanism (Sisler and Siegel, 1967), but does not affect protein synthesis by the ribosomes of isolated mitochondria (Loeb and Hubby, 1968). The incorporation of ¹⁴C-isoleucine by the *P. falciparum*-infected owl monkey erythrocytes we have observed would indicate that mitochondrial protein synthesis is not significant during the three hour experiment.

Chloramphenicol has effects on protein synthesis opposite to those of cycloheximide; i.e. chloramphenicol does not inhibit protein synthesis by cytoribosomes of eukaryotic organisms (Ashwell and Work, 1970), but does inhibit protein synthesis by mitochondrial ribosomes (Firkin and Linnane, 1970). Prokaryotic cells, such as bacteria and blue-green algae, have protein synthesizing mechanisms which are sensitive to chloramphenicol (Vasquez, 1966). Thus, the inability of chloramphenicol to inhibit protein synthesis in our experiments suggests that the parasitized erythrocyte may be considered an eukaryotic organism.

Sherman *et al.* (1971a) have reported that chloramphenicol inhibits ^{14}C -isoleucine incorporation by *P. lophurae*-infected duck erythrocytes. However, the concentration (4×10^{-3} M) they employed is extremely high and may represent nonspecific effects such as those described by Firkin and Linnane (1969). The upper concentration (3.1×10^{-4} M) of chloramphenicol we used in these experiments represents the maximum reasonable level recommended by Ashwell and Work (1970). Therefore, we are confident that chloramphenicol does not inhibit protein synthesis by malarial-infected erythrocytes, and that this indicates that parasitized erythrocytes are analogous to eukaryotic organisms.

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In-vitro and *in-vivo* Studies with *Plasmodium falciparum* and *Plasmodium knowlesi*

SIDDIQUI, W. A. AND J. V. SCHNELL

Department of Tropical Medicine and Medical Microbiology, University of Hawaii,
3675 Kilauea Avenue, Honolulu, Hawaii 96816

ABSTRACT: The results of the comparative studies on *in-vitro* and *in-vivo* growth stages of the erythrocytic cycle of *Plasmodium knowlesi* and *P. falciparum* are reported here. In *P. knowlesi*, the development of the erythrocytic stages of the parasites is highly synchronous, identical and follows a 24-hour cycle in both *in-vivo* and *in-vitro*. Whereas the development of the erythrocytic stages of *P. falciparum in-vivo* (stages seen in the peripheral blood of owl monkeys) and *in-vitro* is not identical and less synchronous although a 48-hour cycle is apparent.

In the present study when plasma was replaced by stearic acid in the modified Harvard medium, development of complete erythrocytic cycle of *P. falciparum* was not achieved. However, the parasites grew from ring to mature trophozoite stage. Furthermore our present study shows that two amino acids, methionine and isoleucine, are required for the growth of the parasites from ring to trophozoite in our plasma-free culture medium. The significance of these results and the present status of *in-vitro* cultivation of *P. falciparum* are discussed.

The characteristics of the intraerythrocytic growth of the asexual forms of malarial parasites are a gradual increase in size and the development of a variable number of nuclei. During growth the asexual form of the parasite progresses through various morphological stages: young and mature ring forms, young and mature trophozoites, schizonts and segmenters with eventual formation of merozoites. Polet and Barr (1968) reported the relationship of the synthesis of DNA, RNA and protein to *in-vitro* developmental stages of *Plasmodium knowlesi*. No such study is available on a species of a human malarial parasite. Since the successful establishment of *P. falciparum* in *Aotus trivirgatus* (Geiman and Meagher, 1967), a regular supply of *P. falciparum* for *in-vitro* cultivation and biochemical studies became available. We report here the comparative studies on *in-vivo* and *in-vitro* growth stages of the erythrocytic cycles of *P. knowlesi* and *P. falciparum*.

In 1970 we published a paper (Siddiqui *et al.*) describing in detail the results obtained on *in-vitro* cultivation of *P. falciparum* from infections in man and experimental animals. In

all of the experiments described in that paper, the parasites could be seen to complete their entire intraerythrocytic cycle using the modified Harvard medium (Geiman *et al.*, 1966). There was invasion of merozoites in some red blood cells but the overall multiplication was not significant at the end of 48-hours incubation period. The same type of results with *P. falciparum* was obtained by Trager (1958, 1966) and Trigg (1967). More recently Trager (1971), using a technique in which a slow flow of culture medium was maintained over the settled layer of red cells, described the development of *P. falciparum* through two cycles of development with no increase in total number of parasites. The presence of plasma in the cultures was found to be required for the growth of the parasites in all the studies described above. In order to study the nutritional requirements for the parasites at each phase of the development, it was thought desirable to replace plasma with known chemical substances. Five years ago we succeeded in replacing plasma by stearic acid for the growth of *P. knowlesi* (Siddiqui, *et al.*, 1967). Trigg (1968) confirmed this result but found that better results were obtained when the lipid was bound to a protein carrier (Trigg, 1969). No such study is reported for *P. falciparum* and therefore it was thought desirable to test the ability of stearic acid to replace plasma for the

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in-vitro cultivation of *P. falciparum*. The results of this study and the requirement for amino acids in our plasma-free culture system are reported here.

Materials and Methods

Plasmodium knowlesi. The strain used for these studies was originally isolated from a monkey (*Macaca irus*) from Malaya and was given to us in 1965 by Dr. E. H. Sadun of Walter Reed Army Institute of Research, Washington, D.C. The parasites were maintained by injection of freshly drawn parasitized blood into a normal rhesus monkey (*Macaca mulatta*) or by injection of parasitized blood which had been stored at -70°C .

Plasmodium falciparum (Uganda-Palo Alto or FUP Strain). This strain was isolated from a patient who contracted the infection in Uganda and was admitted to the Palo Alto-Stanford hospital in 1966. Blood-induced infection of this strain was established in *Aotus trivirgatus* (Geiman and Meagher, 1967). Since then this strain has been maintained in the laboratory of Dr. L. H. Schmidt of the Southern Research Institute, Birmingham, Alabama by serial passages in owl monkeys. Dr. Schmidt sent us a monkey infected with the FUP strain (serial passage no. 164) in 1970. Starting from that infection we have been getting high parasitemias and terminal infections in owl monkeys. Since then we have been maintaining this strain by injection of freshly drawn, parasitized blood into a normal owl monkey or by injection of parasitized blood which had been stored at -70°C . In order to get normal infections in owl monkey, the infected blood should not be kept frozen for more than 3 or 4 weeks.

The basal simplified medium (Siddiqui *et al.*, 1969) and the modified Harvard medium (Siddiqui, *et al.*, 1966) were used for *in-vitro* cultivation of *P. knowlesi* and *P. falciparum* respectively. The techniques used to test the replacement of plasma by stearic acid has been described earlier (Siddiqui *et al.*, 1967 & 1969). In all the experiments, tubes were run in duplicate and the results presented are the averages of these duplicates. For the evaluation of growth and multiplication of the parasites, thin films on four coverslips (representing two independent samples) were pre-

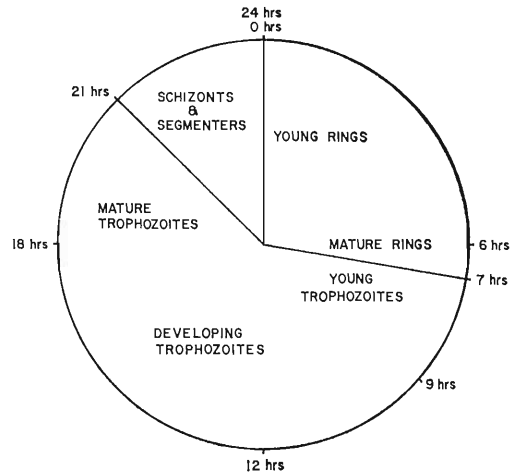


Figure 1. Diagrammatic representation of growth stages of the erythrocytic cycle of *Plasmodium knowlesi* *in-vivo* and *in-vitro*.

pared from each tube. Microscopic evaluation of thin films involved: (i) the number of parasites per 1,000 erythrocytes, (ii) the number of degenerate and extracellular parasites per 100 normal parasites, (iii) the number of developmental stages of parasites, i.e. rings, trophozoites, 2-nucleated schizonts and >2 -nucleated schizonts and segmenters, and (iv) the qualitative morphological features of these parasites.

The comparative studies on *in-vivo* and *in-vitro* growth stages of the erythrocytic cycle of *P. knowlesi* and *P. falciparum* were conducted in the following way: twelve blood samples, two hours apart, were taken simultaneously from a rhesus monkey infected with *P. knowlesi* and from *in-vitro* cultures of parasites from the same animal. Sixteen blood samples, three hours apart, were taken simultaneously from an owl monkey infected with *P. falciparum* (FUP strain) and from *in-vitro* culture of parasites from the same animal. Stained (Leishman Stain) thin blood films of these samples were examined to compare the developmental stages of these parasites growing *in-vivo* and *in-vitro*.

Results

The summary of the results of the comparative study on *in-vivo* and *in-vitro* growth stages

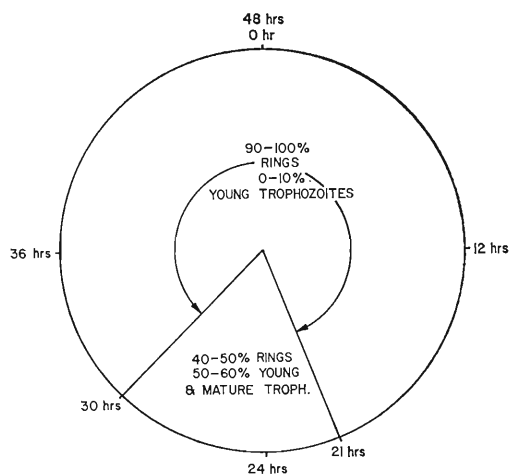


Figure 2. *In-vivo* *P. falciparum* (Uganda-Palo Alto strain) in *Aotus trivirgatus*. Diagrammatic representation of erythrocytic stages found in the peripheral blood during a forty-eight hour cycle.

of the erythrocytic cycle of *P. knowlesi* is illustrated in Fig. 1. The result shows that in both *in-vivo* and *in-vitro* the development of the erythrocytic stages of the parasites is highly synchronous and identical. The process of the development of the trophozoite from the late ring stage to its maturation comprises the major portion (fourteen hours) of the erythrocytic cycle. The process of nuclear multiplication leading to the formation of schizont and segmenter stages takes only three hours.

The summary of the results of the comparative study on *in-vivo* (sample obtained in the peripheral blood) and *in-vitro* growth stages of the erythrocytic cycle of *P. falciparum* is illustrated in Figures 2 and 3 respectively. As is evident from the results illustrated in Fig. 2, the population of parasites in the peripheral blood of owl monkeys consisted predominantly of small, typical ring forms during the 48-hour life cycle. During a short period of 9 hours, specially in monkeys with parasitemias of 10% or more, 50-60% of the parasites in the forms of young and mature trophozoites could be seen in the peripheral blood. The picture with *in-vitro* studies is very different (Fig. 3). The development of the erythrocytic stages was not very synchronous although a 48-hour cycle was apparent. In the first 12-hours of

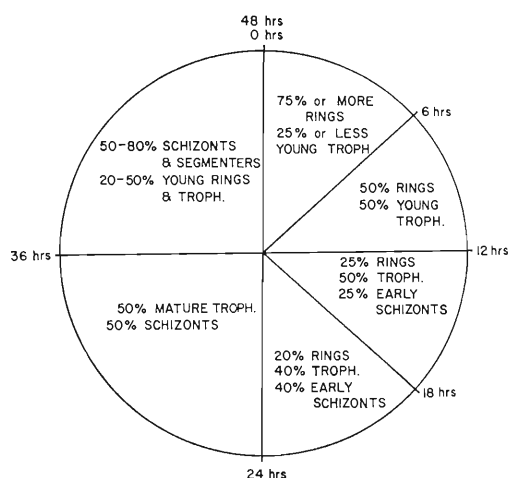


Figure 3. *In-vitro* *P. falciparum* (Uganda-Palo Alto strain). Diagrammatic representation of growth stages of erythrocytic cycle during a forty-eight hour incubation period.

the erythrocytic life-cycle, the process of maturation of ring forms and the development of young trophozoites take place. During the second 12-hours of the cycle, the process of maturation of trophozoites and the production of 2-nucleated schizonts occur. By the end of 36-hours of incubation the population of the parasites predominantly consisted of mature trophozoites and 2 to 4-nucleated schizonts. Beyond the 36-hour incubation period, the blood films are characterized by the presence of predominantly 4-nucleated schizonts and 8- to 16-nucleated segmenters, young rings, trophozoites and degenerate segmenters. It is evident from these results that in order to obtain the later growth stages of *P. falciparum*, the parasites have to be grown *in-vitro*.

In order to study the nutritional requirements of *P. falciparum* at each stage of its development, it was thought desirable to replace plasma with a known chemical substitute. A series of experiments were performed to test the ability of stearic acid to replace plasma for the *in-vitro* cultivation of *P. falciparum* and the results of a typical experiment are summarized in Table 1. The results indicated that in tubes 3 and 4, where plasma was replaced by stearic acid, the para-

Table 1. Effects of Stearic Acid and of Plasma for *in-vitro* culture of *Plasmodium falciparum* (FUP strain).

Incubation period	Tube No.	Addition to the Medium*	Differential counts (per 100 parasites)			
			R	T	S ₂	>S ₂
"0" hr.			68	26	4	2
42 hrs.	1	Plasma	2	34	51	13
"	2	"	4	37	48	11
"	3	Stearic Acid**	2	82	14†	2†
"	4	"	13	74	12†	1†
"	5	No plasma, No stearic acid	28	70††	2†	
"	6	"	27	72††	1†	

* Modified Harvard Medium, (For details see Siddiqui, *et al.* 1970, *Am. J. Trop. Med. & Hyg.* 19(4), 586-591).
** Concentration 0.05 mM.
† Morphology of cytoplasm & nuclei is not as good as in tubes with plasma.
†† Abnormal trophozoites with clumped pigment and less cytoplasm than in the normal trophozoites.
R = Rings; T = Trophozoites; S₂ = 2-nucleated schizonts; >S₂ = >2-nucleated schizonts and segmenters.

sites grew well from rings to trophozoites. Further growth to schizonts and segmenters was not significant but better than that obtained in tubes 5 and 6 with no plasma and stearic acid.

Recently we have conducted a few preliminary experiments on the amino acid requirements for the development of *P. falciparum* in our plasma-free culture system. The results of a typical experiment are summarized in Table 2. Two sets of controls were used: one set of tubes in which the medium was not supplemented with any amino acid, the second set where the medium was supplemented with five amino acids (comprising the Group I amino acids originally formulated for experiments with *P. knowlesi*). It is evident from the results that the development of the parasites was best in those tubes which contained both amino acids, isoleucine and methionine.

Discussion

The results of the comparative study on *in-vivo* and *in-vitro* growth stages of the erythrocytic cycle of *P. knowlesi* and *P. falciparum* show some interesting differences and similarities between these two species with regard to this study. In the case of *P. knowlesi*, the development of the erythrocytic stages of the parasites is highly synchronous, identical and follows a 24-hour cycle in both *in-vivo* and *in-vitro*. Whereas the development of the erythrocytic stages of *P. falciparum* *in-vivo* (stages seen in the peripheral blood) and *in-vitro* is not identical and less synchronous although a 48-hour cycle is apparent.

The identical results obtained in *in-vivo* and *in-vitro* studies with *P. knowlesi* demonstrate that the growth of the parasites in our basal simplified medium (Siddiqui *et al.*, 1969) is not only adequate but duplicates the time sequence of the development of growth stages

Table 2. Requirement for Methionine and Isoleucine for *in-vitro* development of *Plasmodium falciparum* (Uganda-Palo Alto Strain).

Amino Acids added to culture medium ¹					Differential counts/100 parasites ^{2,3}			
Isoleucine	Leucine	Lysine	Valine	Methionine	Ring	Trophozoite	Schizont	>2-nucleated Schizont & Segmenter
+	+	+	+	+	9	78	12	1
+	+	-	+	+	7	66	22	5
+	-	-	-	+	8	60	29	3
+	-	-	-	-	16	70	14	-
-	-	-	-	+	8	77	13	2
-	-	-	-	-	26	65	9	-

¹ Plasma-free culture medium [Modified Harvard Medium + Stearic acid (0.05 mM). For details see Siddiqui *et al.* 1970, *Am. J. Trop. Med. & Hyg.* 19(4): 586-591].
² At "0" hr. 90% parasites were rings.
³ Differential counts were made at the end of 41 hours incubation period.

obtained *in-vivo* through at least one complete erythrocytic cycle. Trigg (1971) reported a delay of approximately two hours in the duration of the asexual cycle *in-vitro* when compared with that observed *in-vivo*. There is a minimum of one to two hours of delay between bleeding the host and inoculating the cultures and hence this observable delay. Our results on the time sequence for the development of the growth stages of erythrocytic cycle of *P. knowlesi*, in general, are in agreement with that of Polet and Barr (1968) and Gutteridge and Trigg (1970).

Hawking *et al.* (1968) reported an extensive study on the purpose, production and control of 24-hour and 48-hour cycle of malarial parasites in the blood. One of the conclusions drawn by Hawking was "that schizogony occurs at a hour constant for each species (mid-day for plasmodia of man and monkeys, early morning or late evening for some plasmodia of birds)." Since 1965 we have been working with a simian malarial parasite, *P. knowlesi* and have observed that timing for the schizogony has fluctuated from monkey to monkey, but generally it has occurred in late evenings or early mornings.

The difference in the growth stages of the erythrocytic cycle of *P. falciparum* observed *in-vivo* (samples taken from peripheral blood) and *in-vitro* at various intervals of time is not surprising. Miller (1969) studied the distribution of mature trophozoites and schizonts of *P. falciparum* in the organs of *Aotus trivirgatus* and found that the heart was a major site for the maturation of trophozoites and deep vascular schizogony. It is also well recognized that *P. falciparum* undergoes schizogony in capillaries of deep tissues in man (Garnham, 1966). Therefore it is not surprising that the trophozoites, schizonts and segmenters stages are usually not seen in the peripheral blood of man or an experimental animal except in overwhelming infections. In our experimental *in-vivo* studies, when the infection reaches 10% or more, we regularly find 50–60% of the population of the parasites in the form of young and mature trophozoites in the peripheral blood during a short period of approximately 9 hours on every other day. This observation is in general agreement with the results reported by Hickman (1969), who studied the course of infection of the Camp

strain of *P. falciparum* in *Aotus trivirgatus*. The persistent presence of ring stage in the peripheral blood, while maturation of trophozoites and schizogony occurring in capillaries of deep tissues, can be explained by the presence of more than one brood of parasites circulating in the blood. This could also be the probable explanation for nonsynchronous development of parasites *in-vitro*. There is no other report available to compare *in-vitro* development of the growth stages of the erythrocytic cycle of *P. falciparum* at every three hours interval period. However the growth stages observed at the end of Day 1 and 2 incubation are comparable to the results reported in earlier studies (Trager, 1966, 1971; Trigg, 1967 and Siddiqui *et al.*, 1970). It is also evident from these results that in order to obtain the later growth stages i.e. trophozoites, schizonts and segmenters of *P. falciparum*, the parasites have to be grown *in-vitro*.

On the basis of these results we are able to predict when we can obtain the desired growth stages from an infected monkey or from an *in-vitro* culture. Based on this information we conducted a few preliminary experiments to compare the ability of various growth stages of the erythrocytic cycle of *P. knowlesi* and *P. falciparum* to incorporate labeled precursors of proteins (^{14}C -isoleucine) and nucleic acids (^{14}C -orotic acid). [This work was done in cooperation with Drs. S. C. Chou and K. A. Conklin of the Department of Pharmacology, University of Hawaii, Honolulu, and the details of the techniques and results will be published later.] The preliminary results of these *in-vitro* incorporation studies with *P. knowlesi* are in general agreement with those of Polet and Barr (1968). The most interesting finding is the observation that DNA synthesis takes place over a relatively short period of time which corresponds to the development of trophozoites to schizonts and segmenters. This finding is in contrast with the conclusion drawn by Gutteridge and Trigg (1970) that "Taken as a whole, our data suggests that in fact, DNA synthesis is linear throughout the cycle". It is also interesting to mention that our preliminary results with *P. falciparum* show the same general incorporation pattern as those reported for *P. knowlesi*. It may be of interest to men-

tion that our general conclusion, that at least in two species, *P. knowlesi* and *P. falciparum*, the DNA synthesis takes place over a short period of time corresponding to the beginning of schizogony, was predicted by Garnham in 1966 when no studies on isotope experiments were available. While commenting on the general pattern of the erythrocytic life-cycle of malarial parasites, he pointed out that shortly before the malarial parasite reaches the schizont stage "it requires deoxyribonucleic acid in large quantities and it can be postulated that the precursor of DNA is produced periodically by the host, e.g. via hormones or through night:day stimulus. It could also be assumed that this would explain the synchronicity of development, for the growing parasites would have to wait until the DNA became available before they could begin their final schizogony and then they would all partake of this substance together".

In-vitro development of the complete intraerythrocytic cycle of *P. falciparum* has been reported earlier (Bass and John 1912; Sinton 1922; Trager, 1966, 1971; Trigg 1967; Geiman et al., 1967 and Siddiqui et al., 1970). In all these studies the presence of plasma was found to be indispensable for the growth of the parasites. Therefore a precise determination of nutritional requirements of these parasites depends to some extent on the replacement of plasma in culture by a known chemical substance. Five years ago we succeeded in replacing plasma by stearic acid (Siddiqui, et al., 1967) for *in-vitro* cultivation of a simian malaria parasite, *P. knowlesi*. Trigg (1968, 1969) confirmed this result but found that better results were obtained when the lipid was bound to a protein carrier. In the present study when plasma was replaced by stearic acid in the modified Harvard medium, development of complete erythrocytic cycle was not achieved. However, the parasites grew from ring stage to mature trophozoite and some nuclear multiplication was also obtained. Furthermore our present study shows that two amino acids, methionine and isoleucine, are required for the growth of the parasites from rings to trophozoites during a 24-hour incubation period in our plasma-free culture medium. The requirements for methionine and isoleucine have been shown earlier

for *in-vitro* cultivation of *P. knowlesi* (Polet and Conrad, 1968; Siddiqui et al., 1969). The only other report of a specific growth requirement for *P. falciparum* was that of Trager (1966), who found that pantothenic acid is required for the intracellular development of this parasite.

These studies show that the status of *in-vitro* cultivation of *P. falciparum* is still at a preliminary stage. In a modified Harvard Medium (Geiman et al., 1966), used by most of the workers in this field, complete development of one intraerythrocytic cycle has been achieved but no overall multiplication of the parasites occurred. In order to obtain a significant increase of parasites at the end of the first generation, a medium has to be formulated which fulfills the nutritional requirements of the parasites and enables them to complete the following essential phases of their developmental cycle: i) growth from rings to trophozoites, ii) growth from trophozoites to schizonts (beginning of the nuclear multiplication), iii) maturation of schizonts to segmenters and eventual formation of merozoites, and iv) intraerythrocytic invasion by merozoites. Experiments with plasma-free culture medium has shown some progress in defining the nutritional requirements to a limited extent. In this connection Trigg's (1969) findings that stearic acid bound to a protein carrier gave better results for *in-vitro* cultivation of *P. knowlesi* merits further study. Besides attempts to formulate an optimum medium, new techniques for *in vitro* cultivation may also play an important role in achieving our immediate goal to obtain a significant increase in the number of parasites (*P. falciparum*) at the end of the first generation. In this regard, Trager (1971) approached the intraerythrocytic cultivation of *P. coatneyi* and *P. falciparum* by combining a stationary layer of cells with a slow and continuous flow of medium. In favorable experiments using this new technique both species of parasites went through two cycles of development, so that late schizonts and young rings were present after two as well as four days of incubation. There was, however, no increase in total parasites number. Hence this new technique, though cumbersome, merits further trials and modifications.

Acknowledgments

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Centrifugal Perfusion Chambers for Incubation of Erythrocytes and Parasites in Fresh Plasma

J. D. TINER

Department of Laboratories, Beth Israel Medical Center, New York, N. Y. 10003

ABSTRACT: Plasma deteriorates during the first few hours *in vitro*: Published biochemical studies indicate that lipid ratios depart irreversibly from normal values and that the trend is measurable after the first half hour of incubation. In general, serum and plasma, pure or in high concentrations, have not been suitable culture media for cells, tissues or parasites. Stale plasma adversely affects red cells but plasma that is fresh or stored less than 24 hours at 0° C is an untested medium for malaria parasites and red cells. For it to be "fresh," at least in terms of sedimentation rate standards, the plasma must remain no more than an hour or two in the 37° C culture chamber. Preliminary observations and calculations indicate that malaria-infected erythrocyte suspensions can be incubated in several changes of fresh plasma per hour when centrifugal perfusion chambers become available.

The previous paper (Tiner, 1969) reported that Trowell's T8, a relatively simple organ culture medium, unexpectedly permitted more intraerythrocytic schizogony than complex commercial tissue culture media such as 135 and 199. That superiority of T8 may have been due to its higher glucose content and/or a more stable pH because of the higher phosphate buffer concentration. A red cell's micro-environment can be stabilized either by periodic additions of alkali, glucose, etc., to counteract metabolism-mediated changes in the medium (Imarisio *et al.*, 1969) or by use of perfusion. Trager (1971) found the latter to be of promise in experiments with two species of primate plasmodia. The present paper describes a way to develop perfusion so that the natural host can serve as the source of labile factors even if these have body temperature half-lives as short as a few minutes.

Trigg (1969) found no major difference between the Harvard medium for malaria culture and commercial tissue culture media. Schizogony and reinvasion of malaria parasites in red cells *in vitro* for all studies to date have been below *in vivo* rates, and with one exception, of limited duration. Anderson (1953) maintained a stable *Plasmodium galinaceum* culture in avian erythrocytes of which 50% were replaced each day with fresh red cells. His medium was heat-inactivated plasma plus freeze-lysed erythrocytes.

Lipids are important cell surface constituents, and some of them are in equilibrium with like molecules in plasma. When blood is incubated for as little as ½ hour *in vitro*, plasma lipid ratios deviate from initial values (Chevallier and d'Hollander, 1971). Esterases mediate a continuing decline in free cholesterol and permit changes in lipid ratios such as have been reported by Tato and Rubaltelli (1970).

Hematology textbooks (Miale, 1967; Wintrobe, 1967) state that a blood sample more than two hours old should not be used to determine a sedimentation rate. Normally shaped red cells (discocytes) have a limited physiological tendency to fit together like stacked pancakes to form the well known rouleaux. The resulting aggregates settle more rapidly than single cells.

A possible morphological basis for alterations in the degree of rouleaux formation is provided by scanning electron microscope studies of red cells in plasma of varied post-collection ages (Bessis and Brecher, 1971; Bessis and Mercier, 1971). Plasma incubated more than 4 hours at 37° is "stale"—it causes erythrocytes to form projections (Leavitt, 1971) that cause them to resemble sea urchins (echinoderms). Bessis and Lessin (1970) call these abnormal red cells "echinocytes", a term to be adopted, if only as a reminder that the cells have become abnormally shaped while immersed in isosmotic plasma. They have not been shrunken or "crenated" by any hypertonic solution.

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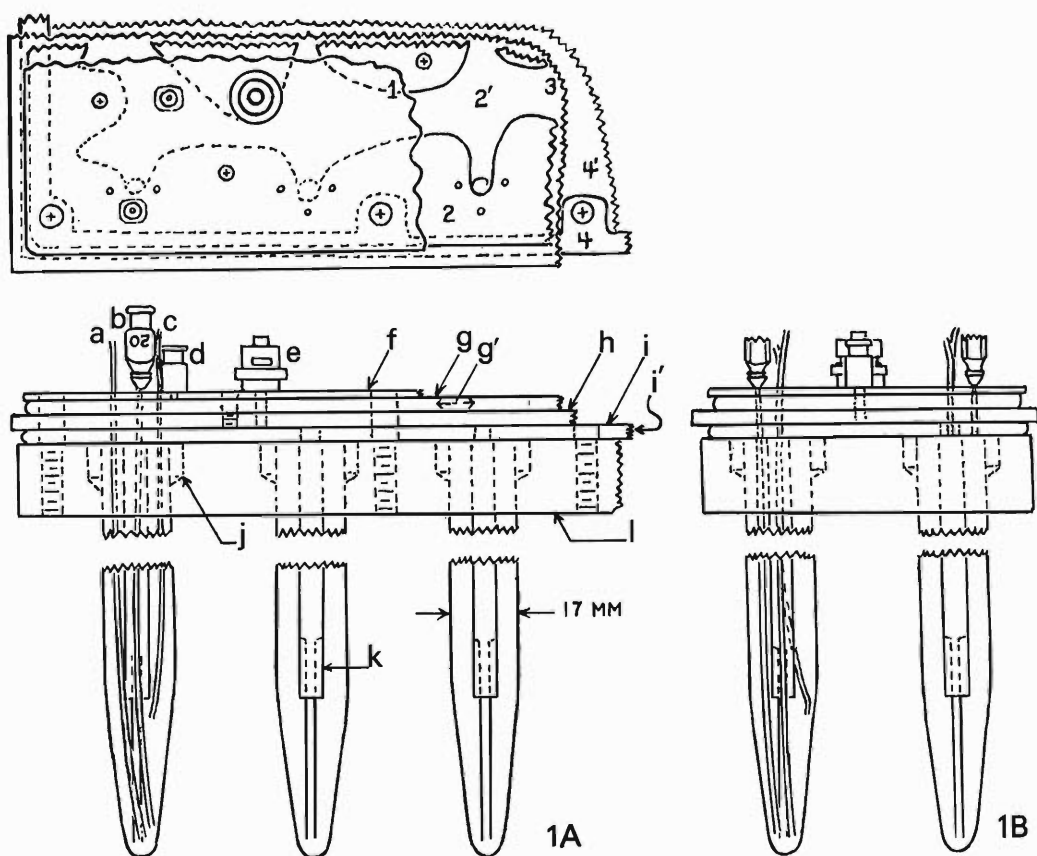


Figure 1. Orthographic projection of manifold for ten culture chambers. Numerals in top view: 1. Cover plate. 2. Upper gasket. 2'. Upper gallery. 3. Intermediate plate for attachment of central cannulae. 4. Lower gasket. 4'. Lower gallery. Letters in side view: a. Infusion line. b. 20 gauge hypodermic needle in inoculation and sample port. c. Effluent line. d. One of pair of gas connections to upper gallery. e. One of gas connections to lower gallery. f. Cover plate. g. Upper gasket. g'. Upper gallery. h. Intermediate plate. i. Lower gasket. i'. Lower gallery. j. Cylindrical rubber washer. j. Polypropylene block. k. Central cannula.

Apparatus

The Tidal System (Tiner, 1969) is based on an aeration principle developed to prolong survival of plant parasitic nematodes in cold traps (Tiner, 1966). The manner of its adaptation to blood cell studies is shown in Figs. 1 and 2. Details of the Tidal culture chambers are shown in Fig. 1. A set of ten conical 15 ml centrifuge tubes is held by cylindrical rubber washers in a plastic block covered with a gasketed manifold. Fluctuations of gas pressure in the central cannulae raises and

lowers a suspension of red cells in the bottoms of the culture tubes. The arrangement of pumps and gas passages to effect this is illustrated in Fig. 2.

The two pumps at *b* operate 180° out of phase and gas is pushed back and forth between them. On each downstroke there is a limited pressure increase and then gas bubbles pass through the mineral oil in the center valve. Irrespective of the pump rate, fresh gas need be fed through only in quantity sufficient to balance the metabolic activity of about 2 ml of infected blood cells. Most of

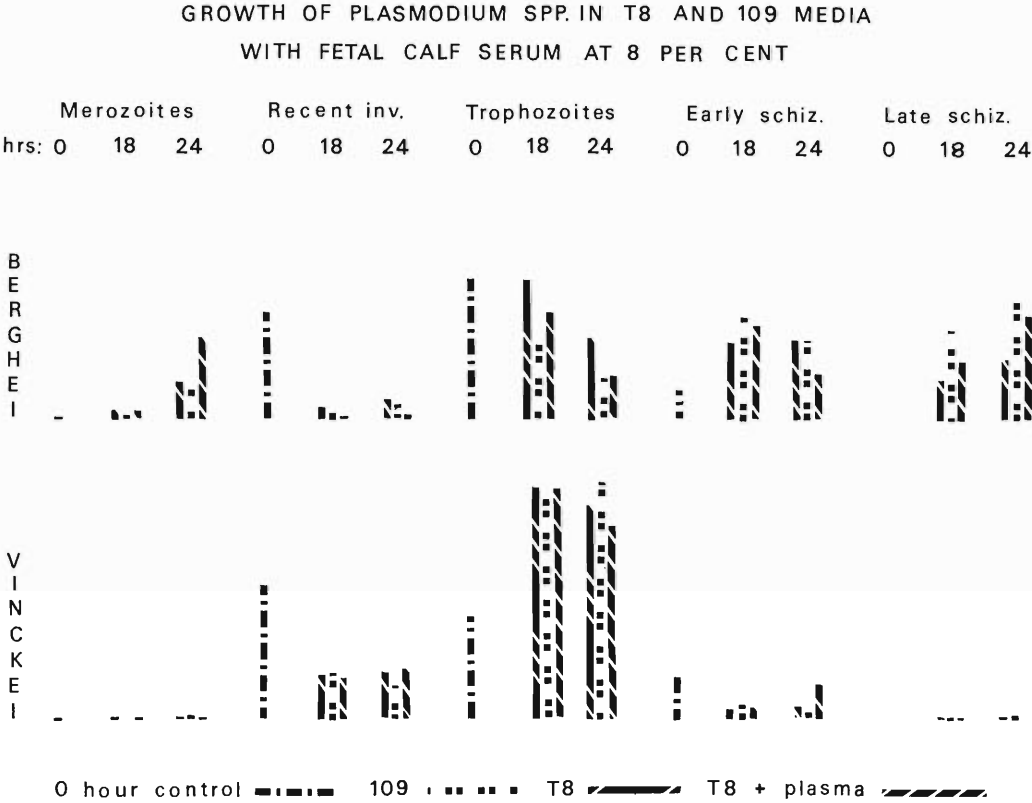


Figure 3. *Plasmodium berghei* and *P. vinckei* schizogony in cultures perfused with T8 and 109 media containing 8 per cent fetal calf serum. The T8 and 109 cultures were started with infected red cells resuspended in T8 medium plus 8% FCS. The T8 + plasma cultures had been resuspended in the supernatant plasma recovered during buffy coat removal plus such plasma removed from the other T8 culture for each species.

and Butcher (1969) reported that a low-oxygen (5% O₂, 5% CO₂, 90% N₂) gas mixture favors development of malaria in red cells.

Low-Oxygen Gas Phase

Culture media in the Tidal System appear to be unusually well equilibrated with the gas phase. No other study has reported a failure of reticulocytes to mature in culture, but previously (Tiner, 1969) they consistently were developing basophilic granules. After 5% oxygen gas was used, those granules were no longer seen except when there was a leak of air into the gas phase, or when failure of an infusion line permitted acidity to develop in

a culture chamber. These observations were made on mouse blood, whereas reticulocyte studies by others have been on cells from different species (Bertles and Beck, 1962).

In the low-oxygen gas mixture, *P. berghei* underwent schizogony irrespective of the presence of serum or plasma in the culture medium. Figs. 4 and 5 show the kind of results that can be obtained when the unit gravity Tidal System is operated on a routine basis. In Fig. 4, the results of Experiment 102 are presented as 100-parasite differential counts. That experiment was started with inoculum from ca. 1% parasitemia mouse blood prepared by buffy coat and supernatant plasma removal followed by resuspension to hematocrit .4 in

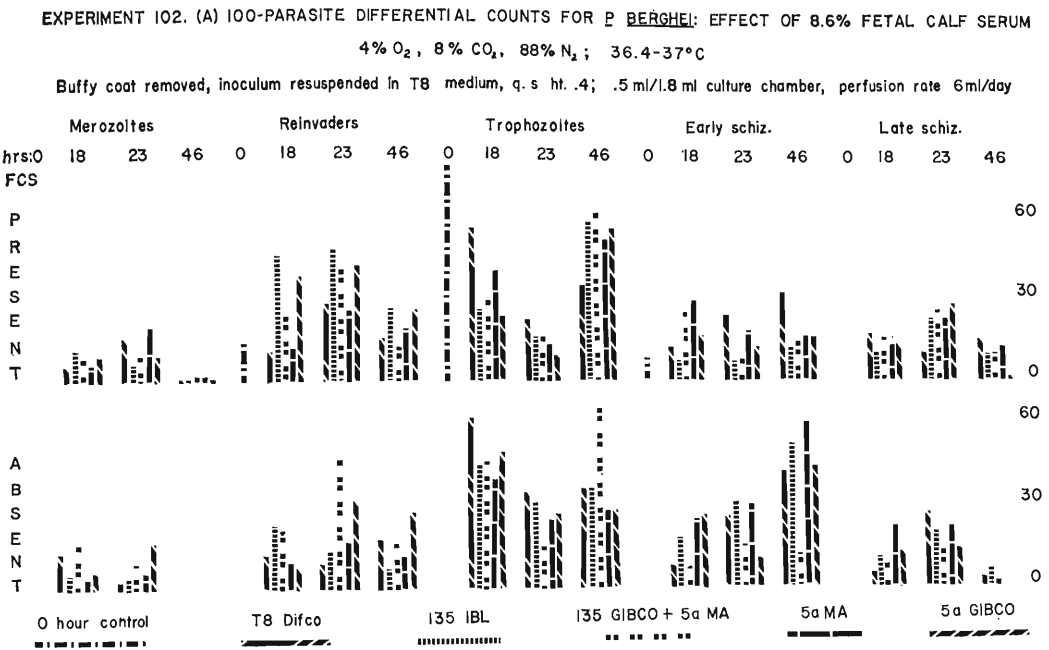


Figure 4. Experiment 102 (A). 100-parasite differential counts for *P. berghei* showing effect of 8.6 per cent fetal calf serum. Conditions: gas phase, 4% O₂, 8% CO₂, 88% N₂; temperature range 36.4 to 37° C.

T8 medium. Each culture chamber was inoculated with 0.5 ml of that infected red cell suspension. The effluent take-off was set at the 1.8 ml mark, and perfusion media were: T8 from Difco Inc., Detroit, Michigan 48201; 135 from Industrial Biological Laboratory (IBL) Rockville, Maryland 20850; 135 from Grand Island Biological Company (GIBCO) Grand Island, New York 14072 mixed with equal parts 5a from Microbiological Associates (MA) Bethesda, Maryland 20014; and pure 5a from MA and GIBCO.

The parasites underwent schizogony and reinvaded on the first day of the experiment. Percentages of trophozoites declined at 18 and further at 23 hours, though not as rapidly in the absence of serum. Reinvasers (merozoite-sized stages apparently inside of red cells) showed approximately corresponding increases in this interval. In the presence of serum, the parasites reached a second generation by 46 hours; whereas in its absence, most of the trophozoites originally present seemed to go only to the early schizont stage.

Mixed 135 + 5a medium supported pro-

liferation in the absence of serum comparable with that in the serum-supplemented complex media. However, if the data are re-figured (Fig. 5) as mm of typical stained smear that must be traversed to find 100 parasites, the mixed medium was not exceptional—certainly not at 46 hours. In other experiments that were performed both previously and subsequently, mixed 135 and 5a media were not superior to either medium alone, provided that 135 contained 300 mg% glucose.

Serum and Plasma

High concentrations of serum or plasma usually were detrimental to the parasites, but in one experiment, concentrations of up to 80% fetal calf serum from a sample stored for most of a year at 4°C permitted intraerythrocytic development of the parasites (Tiner, 1971). A lecture by Dr. Marcel Bessis at the Mt. Sinai School of Medicine in August, 1970, brought attention to the possibility of a new approach: perfuse suspensions with the freshest possible plasma. Accordingly, a re-

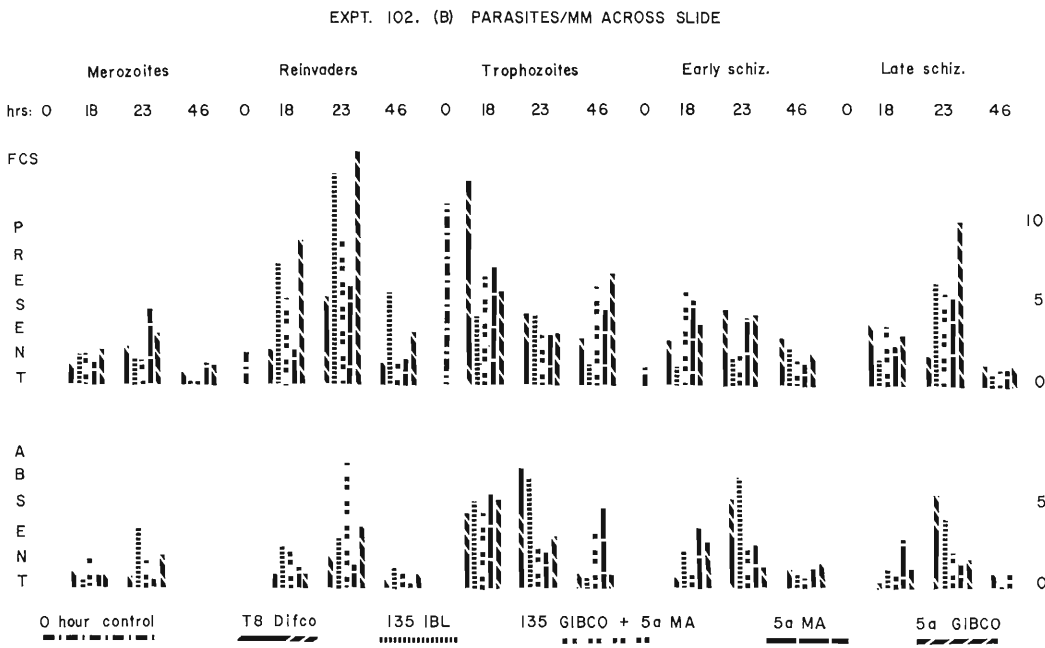


Figure 5. Experiment 102 (B). Results shown in Fig. 4 are re-calculated as parasites per mm of oil immersion traverse across each slide.

frigerator large enough to keep the reservoirs and peristaltic pump at 0°C was acquired. It was placed immediately adjacent to the water bath where the cultures were incubated. Mixtures of 50% and more fresh heparinized plasma in McCoy 5a culture medium were perfused at 4 culture chamber volumes per day (VPD) without improvement in the amount of schizogony or extent of red cell survival over results such as those in Experiment 102.

Flow Rate

The upper limit for rapid perfusion of the culture chambers was re-determined and found to be the rate already in use—4 VPD. Faster flow rates swept the red cells into the effluent stream. At Tidal pump speeds of about 1 cycle per minute which were in routine use, the suspension stayed almost a cm below the effluent take-off and the cells did not pack excessively in the bottoms of the culture chambers. Faster and slower pump speeds did not influence a sometimes observed loss of red cells into the effluent stream.

Bubbles form in a slowly flowing liquid line when air diffuses through an arched polytetrafluoroethylene siphon. Such bubbles move along with the nutrient solution and emerge into the culture chamber. Upon rising to the surface, they disturb the interface between the red cell suspension and the supernatant. To ensure that bubbles did not disturb the cell suspension, a set of culture chambers was modified so that the influent lines, instead of emptying into the bottoms of the culture chambers, were directed down the central cannulae to the meniscus excursion or intertidal zone. Bubbles released there broke directly into the gas phase while the stratified suspensions outside the cannulae remained undisturbed.

Fatigue of the plastic coating on the cam followers of the peristaltic pump led to an observation that precluded further efforts to increase the flow rate of plasma through erythrocyte suspensions in stationary chambers. After about 100 perfusion experiments of about 2 days each, there were deformed plastic coatings on the cam followers of the ef-

fluent side of the peristaltic pump. Some culture chambers in each group of ten did not drain as fast as they were being filled by the perfusion stream with a resulting build-up of extra supernatant. A 6 VPD perfusion rate raised the top of the red cell suspension to the level of the effluent take-off causing red cells to be steadily lost into the waste line. That observation showed that a flow rate above 4 VPD—not bubbles—caused the red cells to enter the effluent. It can be calculated (Tiner, 1971) that at 4 VPD, the median post collection age of plasma in a well mixed 1.5 ml culture chamber is 4 hours—twice the maximum post collection age of blood that is suitable to start sedimentation rate determinations. Those 4 hours represented exposure to a 37°C incubation—rather than a 20° or 25° room-temperature.

Centrifugal Perfusion

The foregoing considerations weigh against further efforts to study at unit gravity the effect of plasma freshness on suspended particles which have a sedimentation rate less than or equal to the few mm/hr of blood cells. Neither filtration nor percolation is a promising alternative. A bacterial membrane filter is a foreign solid surface which may adversely interact with erythrocytes, especially their surface lipids. A sufficient flow of plasma through such filters probably cannot be established, especially when red cells are present to occlude the pores. Accepted procedures for washing erythrocytes are based on use of centrifugal force. Centrifugal perfusion according to the scheme of Fig. 6 will permit fresh plasma studies to proceed.

The cell suspension continues to be agitated by pressure oscillations in the gas phase, but if the centrifuge speed is 300 rmp, then for a 4 place clinical centrifuge head, the force holding the cells in the culture chamber is 10g. The design shown includes a pair of standpipe valves in the swing-out head along with the culture chambers. Whatever the speed of rotation, the pumped gas will bubble through the oil, not the cultures.

Only two centrifugal culture chambers are shown because with the refrigerator and liquid perfusion equipment remaining stationary, each chamber requires separate lines to deliver

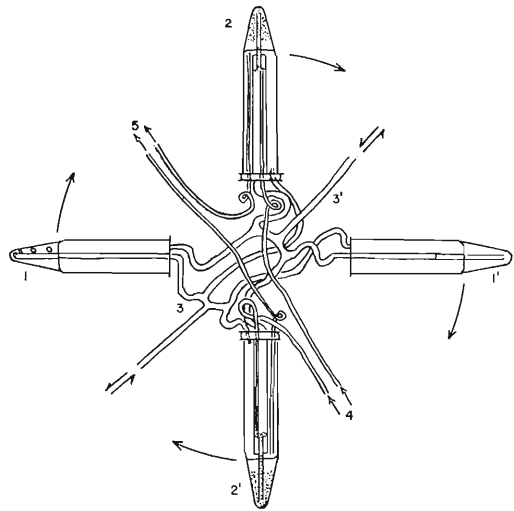


Figure 6. Centrifugal culture chambers and valves. 1. 1' Multi-standpipe valves. 2. 2' Culture chambers. 3. 3' Manifolded gas passages that connect with the Tidal pump. 4. Nutrient infusion lines. 5. Effluent lines.

fresh nutrient and remove supernatant waste-and-nutrient mixture. Also, there should be two lines for a body of gas to continuously provide agitation. As can be seen from Figure 7 a multiple rotary connector (MRC) is needed for six fluid lines.

The first model MRC was operated at up to 284 rpm with seven ½ inch fluorocarbon elastomer (Viton or Fluorel) O-rings serving as separatory seals for the fluid lines. Because of a heat problem, the O-ring grooves in the shaft were deepened to reduce friction. Then, in the absence of metal bearings to stabilize the shaft axis, leaks developed. The connector is being rebuilt with ball bearing races to center each end of the shaft. At the upper end, one removable, prelubricated, sealed race will suffice. It can be manually pressed into place whenever the autoclaved culture assembly is installed. The lower end of the connector shaft is encumbered by six fluid lines. Of these, the four for liquids are too easily contaminated to be interrupted after sterilization. Ball bearing races for medium loads and speeds cannot be steam sterilized, and by definition, split bearings are not sealed. Lubricants from unsealed bearings might con-

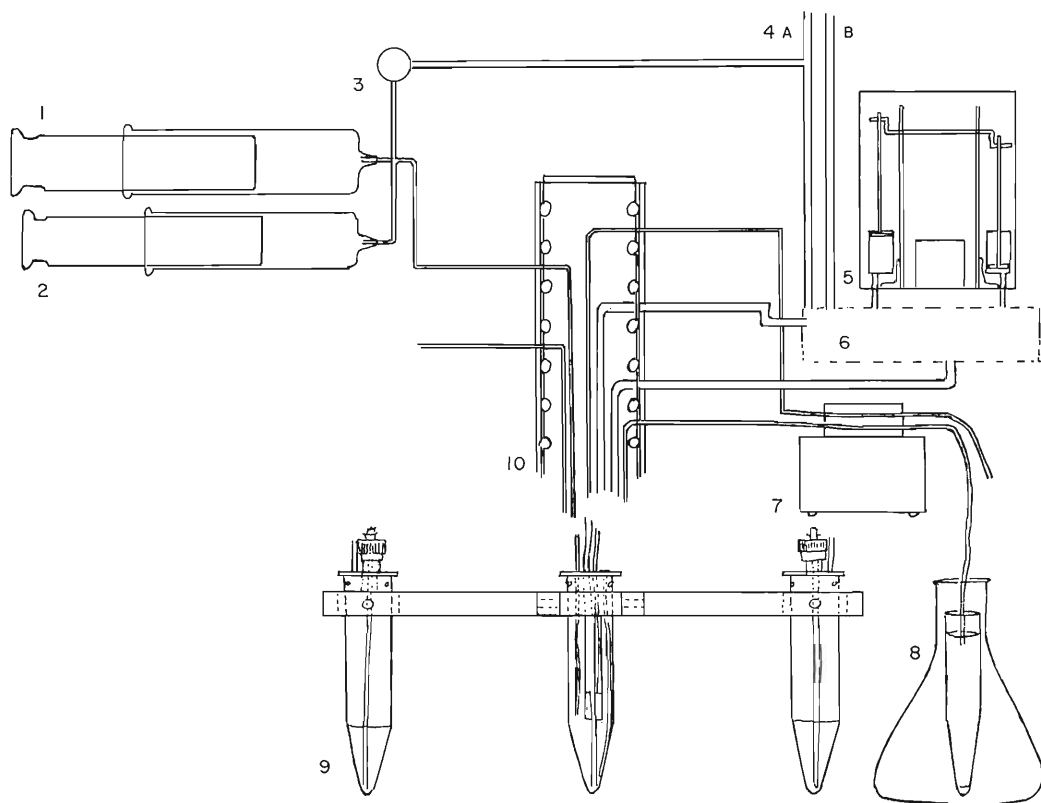


Figure 7. Mechanical components of centrifugal culture chamber system. 1. Plasma infusion syringe (refrigerated). 2. Rinse nutrient syringe. 3. Solenoid valve for low pressure gas to clear nutrient line following rinse. 4. A and B. Low-oxygen gas lines. 5. Paired Tidal pumps. 6. Gas valve and humidification system. 7. Peristaltic pump. 8. Effluent receptacle. 9. Swing-out centrifuge head showing oil pot valves (two) and culture chambers (one in view). 10. Multiple rotary connector for six fluid lines. In the latest design, the two gas lines will enter at the top and pass coaxially down the rotor shaft.

taminate the culture medium. A design that is correct from the standpoints of engineering and axenic culture technique can be achieved if three sealed bearing races are positioned as rollers that contact the lower end of the shaft. Two of these rollers will be permanently mounted in the cross-bar above the spinning head of the centrifuge. The third will be bolted onto that bar after the sterilized culture assembly is positioned.

To keep the fluid lines separated and sealed along the shaft, present specifications call for rotary O-ring seals of less than $\frac{1}{2}$ " inside diameter. They will be mounted in cylinder wall grooves formed by spacing apart stainless

steel plates with chrome plated copper. The design will be repeatedly tested in the course of studies on malaria infected erythrocytes in fresh plasma.

Acknowledgment

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Viability and Fine Structure of Extracellular *Plasmodium lophurae* Prepared by Different Methods

W. TRAGER, S. G. LANGRETH AND E. G. PLATZER*

The Rockefeller University, New York, New York 10021

ABSTRACT: *Plasmodium lophurae* parasites were freed from their host duck erythrocyte by the following method: lysis with antiserum; antiserum followed by trypsin and DNAase, French pressure cell treatment; saponin; saponin followed by trypsin and DNAase. Samples of the parasites were prepared for electron microscopy and their viability was tested by determining the extent of their development and their incorporation of labeled amino acids after one day of incubation *in vitro* in an erythrocyte extract medium.

Parasites prepared by immune lysis alone were fully freed from the erythrocyte, and showed the best viability and a normal fine structure. Free parasites prepared by the French pressure cell were essentially dead; they showed ruptured nuclei and leached cytoplasm and failed to develop *in vitro*. Parasites prepared by immune lysis plus enzymes, or by saponin alone or followed by enzymes, all showed essentially normal fine structure and a somewhat lower viability than those prepared by immune lysis alone. Unlike immune lysis, lysis with saponin gave only a small proportion of parasites fully freed from the external host erythrocyte membrane.

In studies aimed at understanding the physiological relationships between malaria parasites and their host erythrocytes it is essential to have viable parasites freed from their host cells. Several methods for preparing such extracellular parasites have been used: first hemolysis with saponin as used by Christophers and Fulton (1939) and by Sherman and Hull (1960) and others; then, immune hemolysis as used by Trager (1941) and by Speck et al. (1946) and others (Bowman et al., 1960); more recently hemolysis with the French pressure cell first suggested by D'Antonio (1966). These methods of course are also applicable to the preparation of malarial antigens (Stauber et al., 1951; D'Antonio et al., 1970), where viability of the parasites is less important.

Although the fine structure of extracellular malaria parasites prepared in different ways has been compared (Aikawa et al., 1969; Killby and Silverman, 1969) no comparative studies of their physiological activity or viability have been done. The latter would be difficult to do with most species of malaria parasites since one must rely on inoculation of extracellular parasites into a suitable animal host. Walter (1968) has shown for *P. berghei*

that only merozoites are infective, so that such an infectivity test would measure only the number of surviving merozoites. Furthermore, it is known that with some species (as *P. berghei* and *P. knowlesi*) only one or a very few live parasites suffice to produce an infection leading to death of the host (Gamham, 1966).

The bird malaria parasite *P. lophurae*, however, can be kept alive extracellularly *in vitro* up to 4 or 5 days (Trager, 1958). A comparative study of the extent of development of extracellular parasites of this species cultured for one day under optimal conditions would therefore be expected to indicate the effect of the method of preparation on the subsequent viability of the organisms. By the fixation of samples of the freshly prepared free parasites and their study by electron microscopy some correlations between fine structure and viability have been established.

Materials and Methods

I. *P. lophurae* was maintained in ducklings in such a way as to give synchronous heavy infections (Trager, 1950). On the 4th day after inoculation when 90% or more of the parasites were young, uninucleate trophozoites, infected blood was drawn from one or more ducklings and subjected to different treatments for liberation of the parasites.

* Present Address: Dept. of Nematology, University of California, Riverside, California.

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II. A. Immune lysis.

1. Standard method. Infected blood was centrifuged 5 min. at 20° at 1500 rpm. The plasma and much of the buffy coat were discarded and the cells were resuspended in 4 times their volume of duck erythrocyte extract medium full strength (see Trager, 1971). To 6.3 ml of this suspension in a flask were added 0.13 ml guinea pig serum (prepared aseptically and stored in a dry-ice chest) and 0.7 ml antiduck erythrocyte serum (prepared by immunization of rabbits with washed duck blood cells and having a hemolytic titer of at least 1:5000). The mixture was incubated $\frac{1}{2}$ hour at 40°C on a rocking platform with vigorous swirling after the first 15 min. and again at the end of the half hour period. It was then sucked in and out in a pipet and transferred to a centrifuge tube. The centrifuge was started and run so as to reach 500 rpm after 50 seconds, when it was shut off. A clear supernatant contained fully freed parasites and free red cell nuclei whereas the sediment consisted of agglutinated hemolyzed red cell ghosts both with and without parasites. The supernatant was used directly to inoculate culture flasks.

Although the method entails a loss of about half the parasites, it gives free parasites with a minimum of manipulation and therefore constitutes the control method of preparation.

2. Immune lysis followed by trypsin, DNAase and washing. Infected blood was centrifuged 10 min. at 10° at 1500 rpm. The plasma and most of the buffy coat were removed and the cells were resuspended in 4 times their volume of high potassium buffer (K-1) (Trager, 1959), pH 7.4 containing 5% bovine serum albumin. Lysis with antiduck erythrocyte serum was performed as in the standard method above. After the 30 min. incubation with antiserum, trypsin (Worthington, activity 217 U/mg, 0.5 mg/ml final concentration) was added and incubation on the rocker was continued for 60 min. at 37°C. Then DNAase (Worthington, activity 1200 U/mg) was added to a concentration of 0.15 mg/ml. The nuclease treatment on the rocker was for 30 min. The mixture was then transferred to a centrifuge tube and centrifuged for 50 seconds at 400–500 rpm. The clear supernatant containing the free parasites was re-

moved and centrifuged at 2500 rpm for 10 min. The supernatant was discarded. The parasites were resuspended in 40–50 times their volume of K-1 buffer plus 5% bovine serum albumin and washed once by centrifugation.

Finally the parasites were resuspended in duck erythrocyte extract medium and this suspension inoculated to culture flasks.

B. French pressure cell lysis.

A 1:4 suspension of infected erythrocytes in erythrocyte extract medium was prepared as for method A-1. This was placed in the previously sterilized French pressure cell and subjected to a constant pressure of 500 lbs. (625 psi), while the fluid was allowed to emerge dropwise into a sterile tube. The lysate was centrifuged briefly to remove most intact red cells and the supernatant was used for inoculation of culture flasks.

C. Saponin lysis.

1. Saponin followed by washing. Infected blood was centrifuged in order to remove the plasma and buffy coat as in method A-2. To the packed cells was added an equal volume of 0.15% saponin (Eastman, practical grade) in K-1 buffer. The suspension was mixed, transferred to a flask, and incubated on a rocker for 15 min. at 37°. Two volumes of K-1 buffer containing 5% bovine serum albumin were then added and the mixed suspension was transferred to a centrifuge tube. Centrifugation was for 10 min. at 2500 rpm. The supernatant was removed. The cells were washed by centrifugation at 2500 rpm for 10 min. in 4 volumes of K-1 buffer—5% bovine serum albumin 3 times. The resulting pellet was resuspended in 4 volumes of buffer and centrifuged for 50 sec. at 400–500 rpm. The supernatant containing the parasites was then removed and centrifuged at 2500 rpm for 10 min. The parasites were again resuspended in buffer plus albumin and washed once by centrifugation. Finally they were suspended in erythrocyte extract medium.

2. Saponin followed by trypsin, DNAase and washing. The method of saponin lysis was identical to that in C-1. After the first centrifugation following lysis, the pelleted cells were resuspended in K-1 buffer containing 0.003 M MgSO_4 as well as 5% bovine

Table 1. Development of extracellular *Plasmodium lophurae* *in vitro* after different methods of preparation.

Flasks	Method ^a	Parasites per flask × 10 ⁶	Day 0-% parasites with > 1 Nucleus	Day 1-Av. % parasites	
				> 1 Nucleus	Deg.
1-3	Hemolytic serum in (RCE) (A-1)	100	12	47	4
4-6	Same, but centrifuged and resuspended	80	12	39	22
7-9	Hemolytic serum in buffer; trypsin, DNAase (A-2)	160	1	15	20
10, 11	Same, but centrifuged and washed 3 × in buffer	160	1	14	29

^a In each case the parasites were finally resuspended in full strength duck erythrocyte extract (RCE) and inoculated to flasks of complete medium in the same RCE.

serum albumin and washed once. This pellet was resuspended in 4 volumes buffer—Mg—albumin and transferred to a flask. Trypsin (Worthington, activity 217 U/mg, 0.5 mg/ml final concentration) was added and the suspension was incubated at 37° on a rocker for 45 min. DNAase (Worthington, activity 1200 U/mg, 0.6 mg/ml final concentration) was then added. Incubation continued for another 45 min. The mixture was transferred to a centrifuge tube and centrifuged for 50 sec. at 400–500 rpm. The supernatant was removed and centrifuged for 10 min. at 2500 rpm. The free parasites were resuspended in 40 volumes of K-1 buffer—albumin and washed once more by centrifugation. The parasites were finally resuspended in duck erythrocyte extract medium for inoculation to culture flasks.

III. The suspensions of free parasites in erythrocyte extract medium were used not only for inoculation of the culture flasks but also for preparation of stained slides, for counts of the parasites in a Petroff-Hauser bacterial counting chamber (after 1:1 dilution with 10% formaldehyde), and for fixation for electron microscopy.

Electron microscopy. Parasites to be prepared for electron microscopy were centrifuged at 2500 rpm for 10 min. at 20° in order to remove the erythrocyte extract medium. The pellets from each sample were resuspended directly into fixative, which consisted of 2% glutaraldehyde and 2% sucrose in 0.1 M cacodylate buffer, pH 7.2. Fixation was at 0° for 30 min., including a centrifugation at 2000 rpm during the final 10 min. After fixa-

tion the parasites were rinsed 4 times in 0.1 M cacodylate containing 4% sucrose, pH 7.2 and stored in the buffer overnight in the cold. They were postfixed for 1 hr. at 0° in 1% OsO₄ in 0.1 M cacodylate, 4% sucrose, pH 7.2. After rinsing in buffer and water, they were further treated with 0.5% aqueous uranyl acetate for 2 hr. at room temperature. The parasites were dehydrated in ethyl alcohol and embedded in Epon. Thin sections were cut with a DuPont diamond knife on an MT2 ultramicrotome, stained in uranyl acetate and lead citrate, and examined in an RCA EMU 3F or a Phillips 300 electron microscope.

IV. Culture flasks were prepared as described in earlier papers (Trager, 1971). Full strength red cell extract was used, supplemented with ATP, pyruvate, coenzyme A and folic acid.

Development was assessed morphologically after 20 hr. by the examination of stained smears from the scum. The percentages of forms with 2 or more nuclei and of degenerate forms were determined by counting 200 parasites in successive fields (see Trager, 1971).

In addition, in two experiments proline ¹⁴C or methionine ¹⁴C were added after 16 hr. and the extent of incorporation into trichloroacetic acid precipitable material determined 4 hr. later (see Trager, 1971).

Results

I. Viability.

A preliminary experiment (Table 1) indicated that centrifugation and washing in themselves had little effect on viability. In

Table 2. Development of extracellular *Plasmodium lophurae* in vitro after different methods of preparation.

Flasks	Method*	Parasites per flask × 10 ⁶	% free parasites**	Av. % parasites†		cpm†† per 100 × 10 ⁶ parasites
				> 1 nucleus	Deg.	
1-3	Hemolytic serum in RCE (A-1)	57	100	24	6	242
4-6	Hemolytic serum trypsin, DNAase (A-2)	137	100	24	14	133
8, 9	Saponin (C-1)	135	18	14	9	187
10-12	French pressure cell at 500 lbs. (B)	195	18	4	43	113

* In each case the parasites after the hemolytic treatment were suspended in full strength duck erythrocyte extract (RCE) and inoculated to flasks of complete medium in the same RCE.
** Based on counts of the suspension at the time of inoculation.
† Based on counts after 20 hr. At 0 time there were 5% with > 1 nucleus.
†† Proline-U-¹⁴C (2.5 μc) added to each flask after 16 hr and incorporation into trichloroacetic acid-precipitable material determined 4 hr later.

this experiment Flasks 1-6 cannot be strictly compared to Flasks 7-11 since blood from different infected ducks was used for the two methods of preparation.

In the experiments shown in Tables 2 and 3 all of the blood needed for all four methods of preparation was drawn from a single duck at the same time (into 1/10 its volume of heparinized saline—30 mg heparin and 0.85 g NaCl per 100 ml H₂O). This was kept in a refrigerator and needed samples removed at such times that all methods of preparation were completed and the suspensions were ready for inoculation at the same time.

In both of these experiments the standard method of lysis directly into erythrocyte extract medium gave the best viability of the free parasites as judged by both morphology and extent of incorporation. Parasites lysed by immune serum and then subjected to enzyme

treatments and washing seemed to develop nearly as well as judged morphologically but incorporated much less of the labeled amino acids. Somewhat similar results were obtained with the saponin prepared parasites, but it should be noted that less than 20% of these parasites were outside a ghost red cell, whereas 100% of the parasites prepared by either method of immune lysis were free.

Surprisingly, the parasites prepared by saponin followed by enzyme treatment showed better development and incorporation than those prepared by saponin and washing alone (Table 3). However, 90% of these parasites could be seen to be surrounded by a collapsed ghost red cell membrane, even though the nuclei had been removed by the enzyme treatment.

Parasites prepared by the French pressure cell (Table 2) were clearly dead; they showed

Table 3. Development of extracellular *Plasmodium lophurae* in vitro after different methods of preparations.

Flasks	Method*	Parasites per flask × 10 ⁶	% free parasites**	Av. % parasites†		cpm†† per 100 × 10 ⁶ parasites exposure per hr.
				> 1 Nucleus	Deg.	
1-3	Hemolytic serum in RCE (A-1)	45	100	27	6	790
4-6	Hemolytic serum trypsin, DNAase (A-2)	68	100	21	9	432
7-9	Saponin (C-1)	117	17	21	13	390
10-12	Saponin, trypsin DNAase (C-2)	72	10	21	5	830

*, ** See corresponding notes for Table 2.
† Based on counts after 20 hr. At 0 time there were 2% with 1 nucleus.
†† Methionine-methyl-¹⁴C (2.5 μc) added to each flask after 16 hr. and incorporation into trichloroacetic acid-precipitable material determined 4 hr. later. In a series of earlier experiments with labeled methionine (see Trager, 1971) the cpm incorporated per 10⁶ parasites living in full strength RCE, per hr. exposure, ranged from 700 to 1000.

no increase in multinucleate forms and half appeared degenerate. This confirms results of several earlier experiments.

II. Fine structure.

A normal bird malaria parasite living in a duck erythrocyte is separated from the host cytoplasm by two membranes (Rudzinska and Trager, 1957). A typical section of such a parasite demonstrates this membrane relationship and the usual organelles: nucleus, food vacuole, mitochondrion, scanty endoplasmic reticulum, and numerous free ribosomes (Fig. 1).

Parasites prepared by antiserum lysis (method A-1) were morphologically intact (Figs. 3 and 4). They are surrounded by the two membranes which normally divide parasite cytoplasm from that of the host, but they are completely free from any residual red cell material, such as the outer erythrocyte membrane, mitochondria, or nuclei. Red cell nuclei are present in the preparation, but they are not associated with the free parasites. Parasite organelles, such as nucleus, nucleolus, food vacuoles containing hemozoin pigment, lipid inclusions, vacuoles, mitochondria, and even

cystostomes (Fig. 4), present the same appearance in parasites freed by immune hemolysis as they do in parasites in red blood cells which have not been subject to treatment.

Parasites freed by the antiserum-trypsin-DNAase method (A-2) were also well preserved (Figs. 2, 5, and 6). These free parasites are surrounded by the same two membranes as in the standard antiserum preparation. However, the outer membrane was occasionally found not to adhere closely to the parasite and to appear wavy (Fig. 6). Red cell nuclei were not present in these samples because of the DNAase treatment. Parasite organelles, including the cystostome (Fig. 5) appear normal.

The majority of the parasites in samples subjected to the French pressure cell were not freed from their host erythrocytes (see Table 2). Although these parasites are reasonably normal in appearance, the red cells which contain them are not (Fig. 7). The outer erythrocyte membrane is wavy and irregular, and the red cell cytoplasm appears to be leached, as if there had been loss of hemoglobin. Any French press-treated parasite which was found free from its red cell was

→

Abbreviations for all figures: nucleus (N); nucleolus (n); food vacuole (FV); mitochondrion (M); erythrocyte nucleus (EN); erythrocyte cell membrane (EM); lipid inclusion (L); vacuole (V).

Figure 1. Electron micrograph of a malaria parasite *Plasmodium lophurae* in a duck erythrocyte. Two membranes separate the parasite cytoplasm from the red cell cytoplasm. X 26,000.

Figure 2. Electron micrograph of a parasite freed by antiserum-trypsin-DNAase treatment (method A-2). The parasite is surrounded by two membranes. Its organelles (nucleus, lipid inclusions, mitochondria) are well preserved. X 26,000.

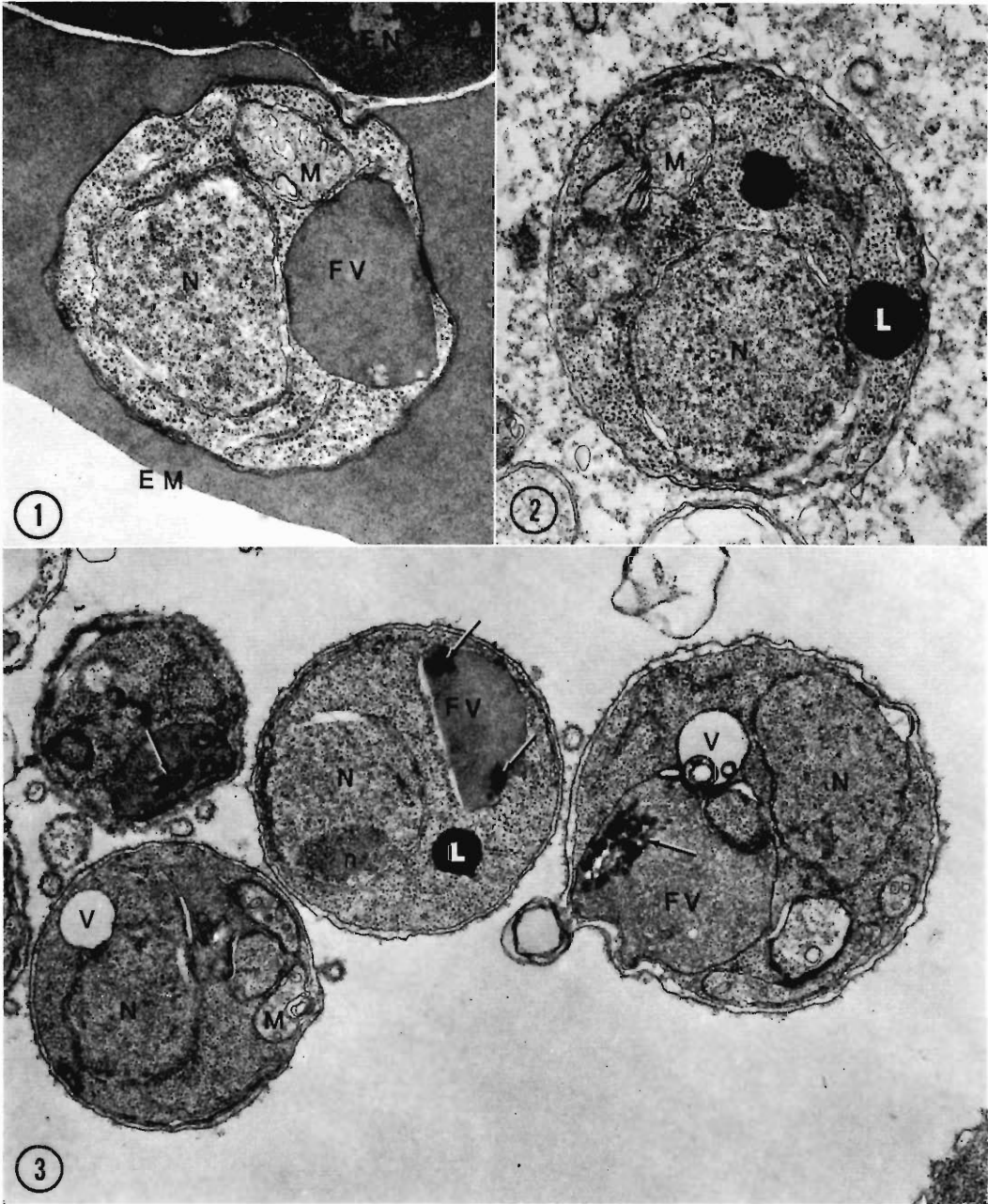
Figure 3. Electron micrograph of parasites freed by antiserum treatment (standard method A-1). The cells are morphologically intact and surrounded by two membranes. Note hemozoin pigment (arrows) in food vacuoles. X 22,000.

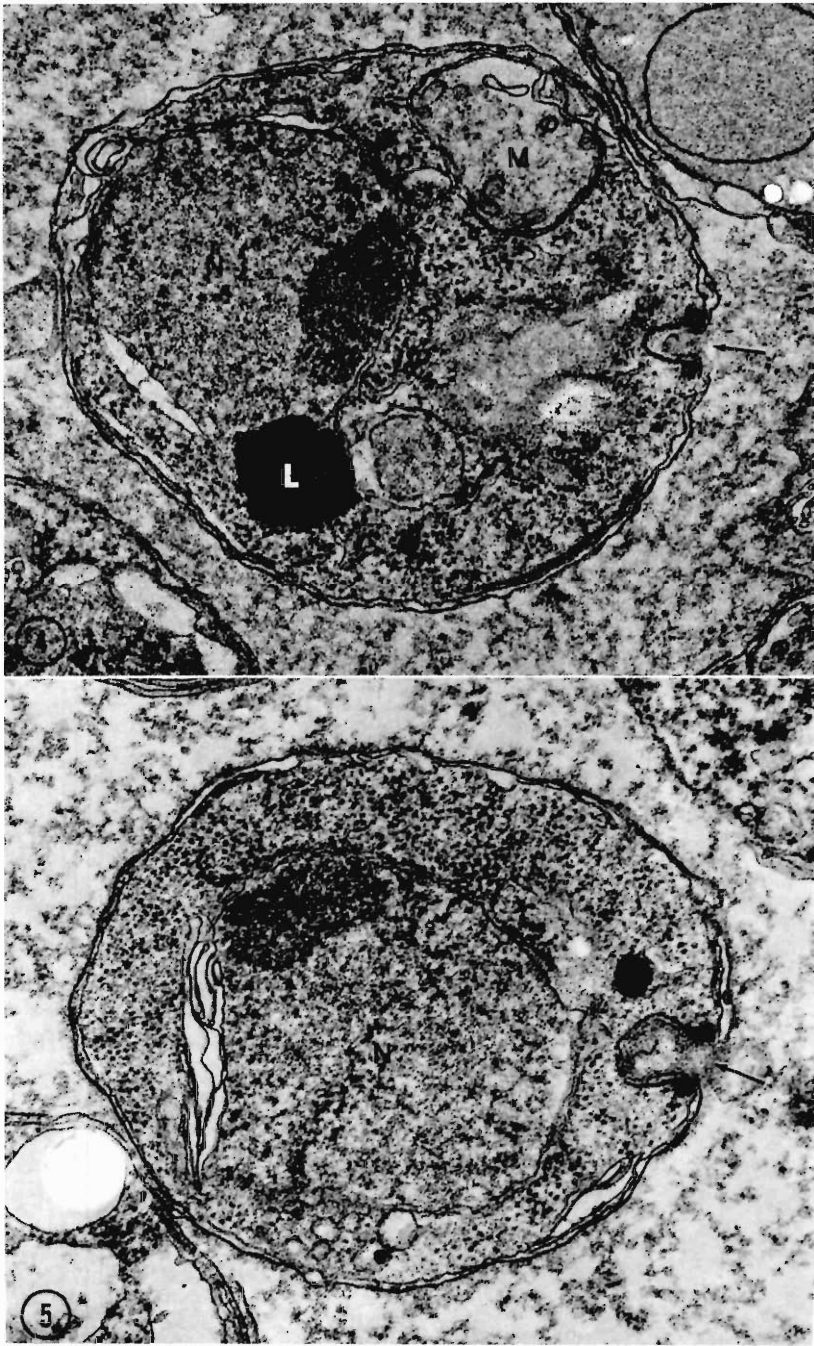
Figure 4. Parasite from an antiserum-treated preparation (standard method A-1) which demonstrates a cystostome (arrow). Two membranes surround the cell. X 35,000.

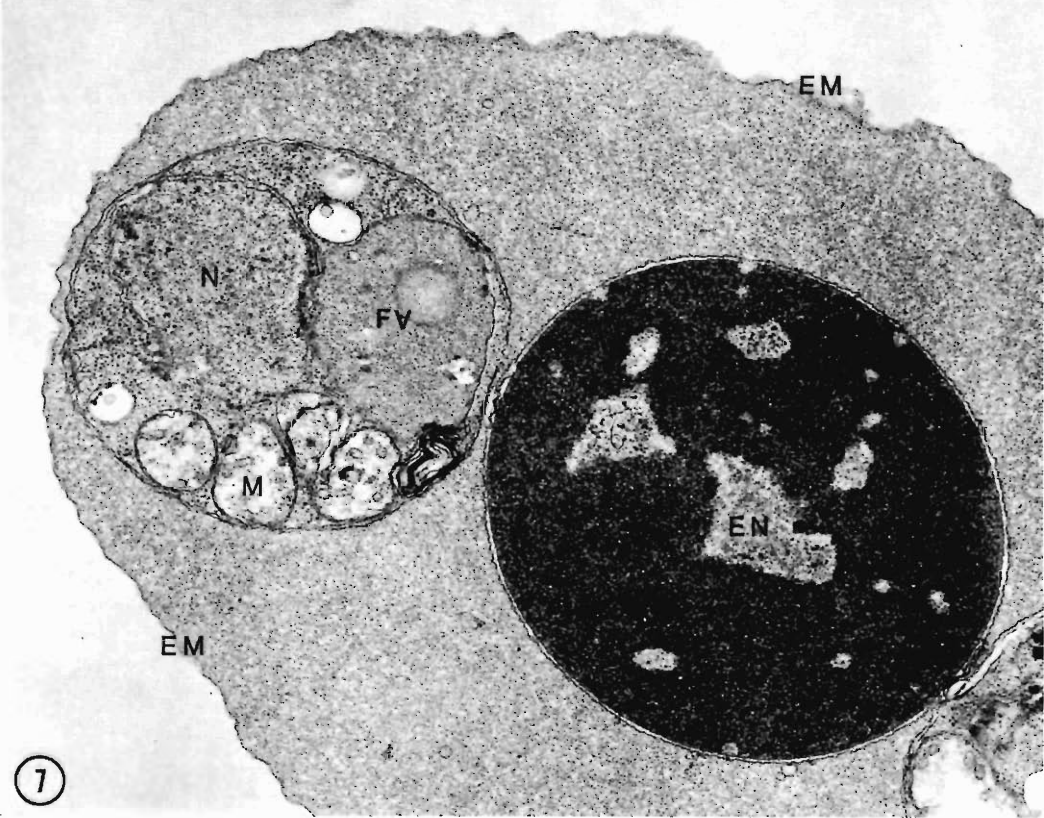
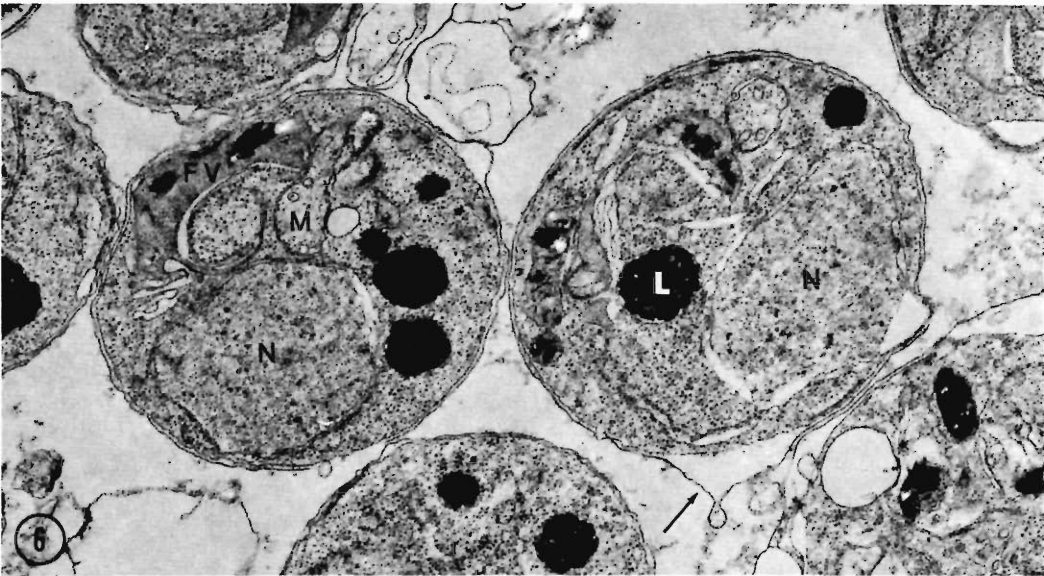
Figure 5. Parasite freed from the red cell by antiserum-trypsin-DNAase. Both membranes of the cystostome (arrow) appear to invaginate. X 37,000.

Figure 6. Electron micrograph of antiserum-trypsin-DNAase treated preparation. Although two membranes surround the parasites, the outer membrane is occasionally wavy (arrow). X 22,000.

Figure 7. Electron micrograph of the French pressure cell treated preparation. The majority of parasites were not freed from their erythrocytes by this method, as is the case here. Note, however, that the red cell membrane appears wavy and that the red cell cytoplasm (hemoglobin) seems to be leached (Compare with Fig. 1). X 23,000.







severely damaged. The nucleus of the parasite in Figure 8 is recognized only because of its nucleolar material. Many ribosomes and other ground substance material appear to be lost. This particular parasite is surrounded by two membranes; in many examples these membranes were also damaged. It is of interest that in Gicsma stained preparations parasites freed by the French pressure cell seemed to have a normal morphology as seen by light microscopy, except that the intensity of coloration of both cytoplasm and nucleus was paler than normal.

As mentioned earlier, neither treatment with saponin alone nor treatment with saponin-trypsin-DNAase produced many truly free parasites. Parasites from saponin preparations were most often found associated with an erythrocyte nucleus and within a red cell ghost (Fig. 10). Whether the parasite was free or within a red cell ghost, however, it was surrounded by two membranes and was morphologically well preserved. Saponin-trypsin-DNAase treated parasites were free from red cell nuclei but retained, to varying degrees, an association with collapsed outer erythrocyte membrane. Fig. 9 is an example of an almost free parasite, which has an amount of extra membranous material so small that it would not be seen by light microscopy. The usual two membranes surround the cell, and all organelles are reasonably intact, which is the general situation in saponin-trypsin-DNAase preparations. Although not depicted, parasites demonstrating cytostomes of normal morphology were found in both types of saponin-lysed samples.

Discussion

It is clear that the best extracellular parasites as judged by both criteria, fine structure at time of preparation and subsequent viability,

were obtained by immune lysis with a minimum of further handling. It is equally clear that parasites freed by the French pressure cell are not viable, in keeping with their disrupted fine structure.

The intermediate results obtained with parasites freed by saponin, saponin followed by enzyme treatments, or immune lysis followed by enzyme treatments are of interest in indicating the sensitivity of the extracellular parasites to small changes in handling. The better viability of parasites prepared by saponin and enzymes than of those prepared by saponin alone might result from deleterious hydrolytic enzyme activities (such as ATPase) of the numerous red cell ghosts within which most of the latter parasites were contained. Early in the work on extracellular development *in vitro* it was observed that erythrocyte extracts contaminated with red cell ghosts did not support good development of the parasites. On the other hand, parasites freed by immune lysis are from the start fully exposed to the suspending medium and perhaps for this reason are more adversely affected by trypsin and DNAase than are those in saponin-lysed cells.

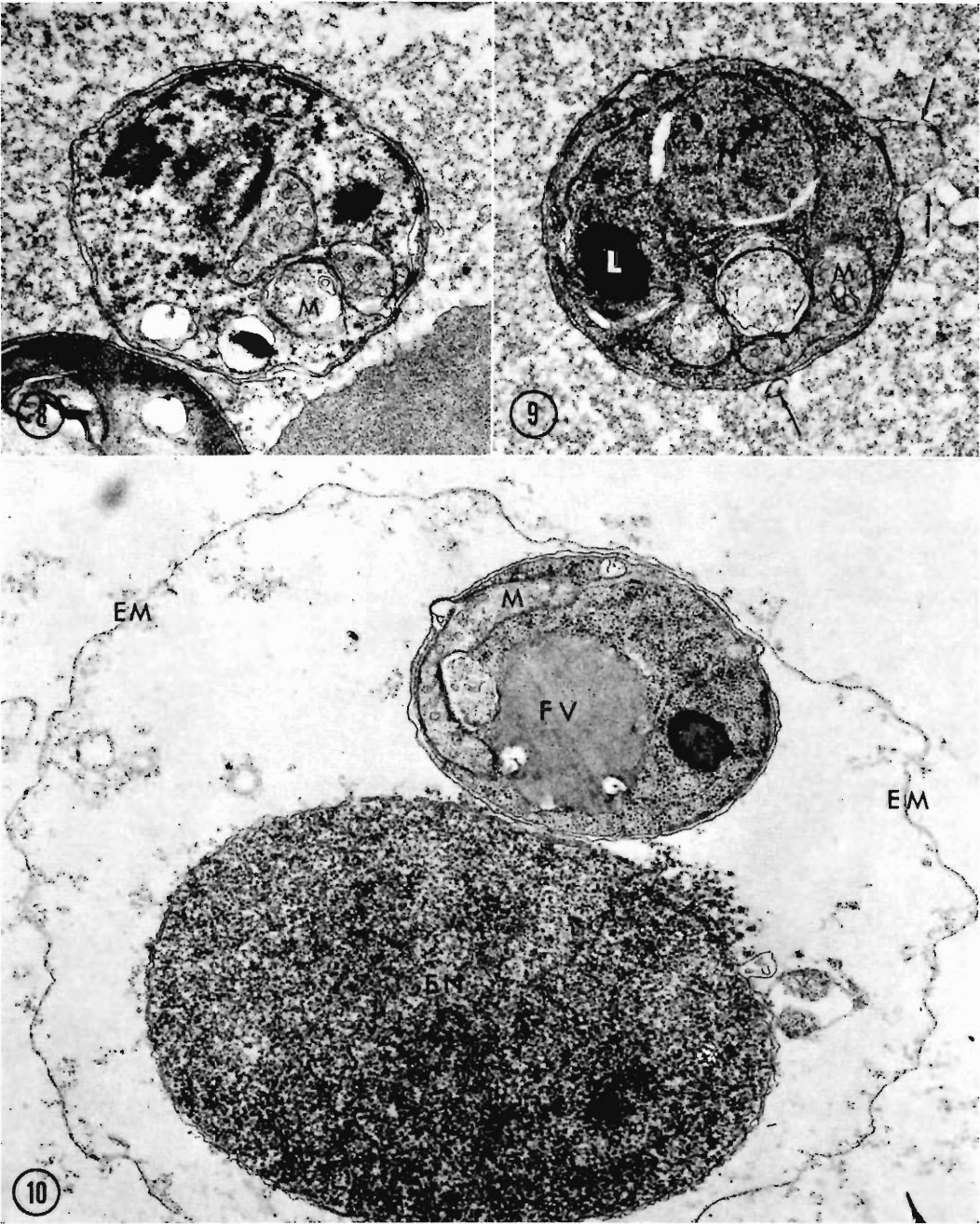
It may be significant that among the parasites liberated by immune lysis and then treated with enzymes more were noted with the outer membrane not adhering closely, than among the parasites liberated by immune lysis alone, or by saponin followed by trypsin and DNAase. Although the outer membrane may be derived originally from invaginated erythrocyte membrane (Ladda et al., 1969) there are strong indications that it becomes closely integrated into both the structure and the economy of the parasite. Thus, erythrocytic malaria parasites, unlike some other intracellular parasites (as *Toxoplasma*, Sheffield and Melton, 1968) never lie in a parasitophorous

→

Figure 8. Parasite freed by French pressure cell method. Although this cell is still surrounded by two membranes, it is clearly badly damaged. The nucleus is smashed and the cytoplasm leached. X 23,000.

Figure 9. Electron micrograph of a free parasite from a saponin-trypsin-DNAase preparation. The cell's organelles are intact, but note the extra membranous material adhering to the surrounding two membranes (arrows). X 23,000.

Figure 10. Electron micrograph of a saponin-treated erythrocyte. The parasite is within a red cell ghost. Although it is well preserved, it is not free. X 22,000.



vacuole. When liberated from the host cell they retain both membranes. Indeed, both membranes are retained after extracellular development *in vitro* (Langreth and Trager, unpublished). The outer membrane may play a major role in controlling entrance of materials to the parasite and certainly deserves special study in this respect.

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In vitro Studies of Malarial Antibody

S. COHEN, G. A. BUTCHER AND G. H. MITCHELL

Department of Chemical Pathology, Guy's Hospital Medical School, London, SE1 9RT.

Little progress has been made towards the goal of continuous *in vitro* cultivation of malaria parasites. This failure can be attributed to the difficulties of providing for the changing metabolic requirements of the plasmodium during its successive stages of growth and development, and of maintaining host red blood cells of 37° *in vitro*, especially in the case of those parasites having relatively prolonged erythrocytic cycles. Some progress has, however, been made in the short-term cultivation of plasmodia having a 24-hour cycle of development. Thus, the growth and multiplication of *Plasmodium knowlesi* in cultures involving a full cycle of development can approximate that observed *in vivo*. This system provides a valuable means for studying the morphology and biochemistry of the parasite, as illustrated by the papers in this section, and can also be used to analyse reactions to anti-malarial drugs (McCormick et al., 1971; Richards & Williams, 1971; Gutteridge & Trigg, 1972) and to specific antibody; the latter subject is considered in this communication.

Mode of Action of Protective Malarial Antibody

Serum from monkeys immune to *Plasmodium knowlesi* has been shown to specifically inhibit the cyclical proliferation of the parasite maintained *in vitro* (Cohen, Butcher & Crandall, 1969). A technique was used which gave mean multiplication rates of 6-fold in 24-hour cultures (Butcher & Cohen, 1971) and parasite growth was assessed by incorporation of ³H-leucine into parasite protein. Pooled immune sera have no effect upon the growth of intra-cellular parasites, but inhibit the cycle of growth which follows parasite division. This effect is not complement dependent and in the sera analysed was associated with IgG and IgM, but not with IgA or IgE. The bivalent peptic fragments of IgG are inhibitory, but univalent Fab fragments are not (Cohen & Butcher, 1970). The similarity between malarial inhibitory anti-

body which appears to block red cell invasion by merozoites, and some viral neutralising antibodies, is apparent from these experiments.

These studies on the nature and mode of action of malarial antibody provide no explanation for the very slow development of malarial immunity in many species including the rhesus monkey, or for the occurrence of periodic low-grade parasitaemia in clinically immune subjects. Several mechanisms may contribute to these phenomena and the most significant appears to be the ability of malaria parasites to evade the host's immune response by undergoing repeated antigenic variation (Brown & Brown, 1965). Such variants are recognised on the basis of the schizont infected cell agglutination test (SICA). In studies summarised below the *in vitro* assay has been used to measure the specificity and levels of malarial inhibitory antibody in rhesus monkeys challenged with defined strains and variants of *P. knowlesi* and results have been correlated with clinical immune status (Butcher & Cohen, 1972).

Variant and Strain Specificity of Protective Malarial Antibody

This has been studied as follows:—

(i) Four rhesus monkeys were given a single infection of 3–5 days duration with a defined variant of *P. knowlesi* followed by cure using chloroquine (5 mg/kg i. m. i.) and sulphadiazine (30 mg/kg i. p. i. daily for 3 days). In three of the monkeys sera collected 4 weeks later contained inhibitory antibody and schizont agglutinins active only against the infecting variant. In the remaining animal infection with variant W4 induced specific schizont agglutinins, but no detectable inhibitory antibody (Table 1).

(ii) One rhesus monkey was challenged successively over a period of 9 weeks with 4 different variants of *P. knowlesi* (W1, 2, 3, 4). Infections were terminated by drug cure when parasitaemia reached 10% and sera were

Table 1. Data for rhesus monkeys (D1 to 4) each infected once with a single variant of *P. knowlesi* and later challenged with the same or a different variant.

Donor	Challenge		Pre-patent (days) (1)	Serum day	W1		W2		W3		W4	
	Day	Variant (2)			SICA	Inhib. % (3)	SICA	Inhib. % (3)	SICA	Inhib. % (3)	SICA	Inhib. % (3)
D1	0	W1	0									
	29	W3	0	<u>29</u>	<u>1250</u>	<u>17</u>	0	0	0	0	0	0
D2	0	W2	0	<u>29</u>	0	0	<u>250</u>	<u>37</u>	0	0	0	0
	60	W2	2	65			50	46				
D3	0	W3	0	<u>30</u>	0	0	0	0	<u>1250</u>	<u>48</u>	0	0
	61	W1	0									
D4	0	W4	0	<u>30</u>	0	0	0	0	0	0	<u>250</u>	<u>0</u>
	73	W1	0									

The sera collected on day 29 or 30 (underlined) were used as variant specific reagents for the SICA test.

(1) Prolongation of pre-patent period beyond normal control (days).

(2) 10^3 parasites.

(3) % inhibition of given variants based on 24 hr radioactivity measurements in duplicate flasks using 0.3 ml serum per culture flask.

taken at intervals for in vitro assay of inhibitory antibody. As shown in Fig. 1 the antibody response at the dose tested was predominantly variant specific since antibody against a given variant became consistently positive only after that variant had been patent. Specific schizont agglutinins showed a similar pattern of development.

(iii) Three rhesus monkeys were repeatedly infected with a single variant and then challenged with a different variant, e.g. Table 2. In each instance the animals were clinically immune to the latter challenge variant as shown by prolongation of the prepatent period and the appearance in the blood of a new variant of *P. knowlesi* distinct from that used for initial challenges. *In vitro* assay of sera during the course of these experiments showed that the inhibitory antibody response was predominantly variant specific, but dose response data revealed that inhibitory antibody against other variants became detectable after the sixth week and increased to a level corresponding to about 2% of that against the original infecting variant (Fig. 2). Schizont agglutination tests, on the other hand, remained negative for such variants (e.g. against W2 and W4, Table 2) despite the presence of specific inhibitory antibody. Challenge with a variant for which low titre inhibitory antibody could be demonstrated induced a rapid increase of inhibitory antibody of corresponding specificity (Fig. 2) and a positive SICA titre (Table 2).

(iv) Six rhesus monkeys were exposed to chronic infections with one strain of *P. knowlesi* (G—provided by Prof. P. C. C. Garnham) and were then challenged with variants of other strains of *P. knowlesi* (W—provided by Dr. E. H. Sadun or Nuri supplied by Dr. K. N. Brown). After chronic infection with the G strain animals were clinically immune to W1 or 'Nuri' strains and their sera before challenge contained inhibitory antibody to both strains and schizont agglutinins against 'Nuri' but not against W1; animals immune to G were fully susceptible to W4 and their sera contained neither inhibitory antibody nor schizont agglutinins (Table 3).

Discussion

Protective malarial antibody has been demonstrated by passive transfer tests in monkey, human, avian and rodent malarias. Such tests are cumbersome and there has consequently been a great need for a dependable in vitro technique for the detection of protective malarial antibody. The schizont infected red cell agglutination (SICA) test (Brown & Brown, 1965) and the in vitro assay of inhibitory antibody (Cohen et al., 1969) have been proposed for this purpose. The study summarised above in which both were employed allows these tests to be assessed as indicators of the immune status of the serum donor.

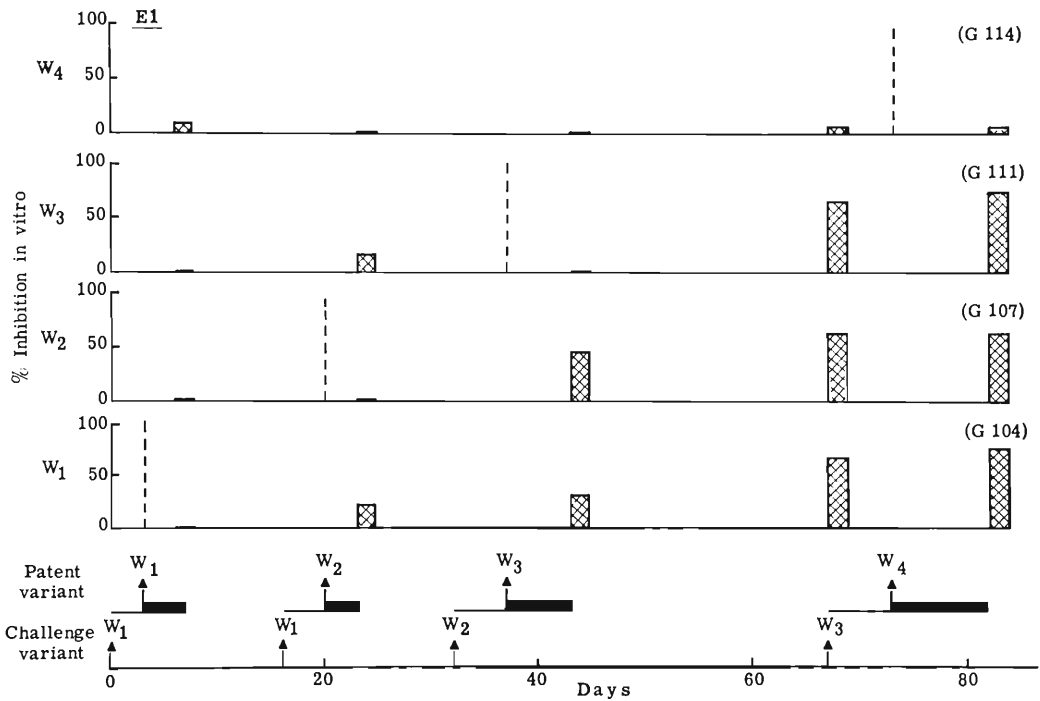


Figure 1. Diagram showing the identity of challenge and patent variants of *P. knowlesi*, duration of parasitaemia (solid blocks) and levels of inhibitory antibody specific for W1-4 (hatched blocks) in a single rhesus monkey. Vertical dotted lines indicate the day on which each variant was first patent. Data for inhibitory antibody represent means of duplicate assays using 0.3 ml serum per culture flask containing 2.5 ml erythrocytes with an initial parasitaemia of 1%.

The specific inhibitory antibody and SICA titres both correlate with immune status: (i) in animals immune to one strain of *P. knowlesi* but fully susceptible to a variant of another strain, when both tests are negative for that variant (Table 3, G56, G59); (ii) in repeatedly challenged animals immune to the challenge variant, when both tests are positive for that variant. However, SICA tests are quantitatively unsatisfactory as compared with inhibitory antibody assays, since the former titres differ considerably with individual variants. In addition, there are circumstances where clinical immunity occurs with positive tests for inhibitory antibody but negative SICA tests. This was observed as follows: (i) animals immune to the G-strain of *P. knowlesi* had negative SICA tests, but high levels of inhibitory antibody against W1 and were clinically immune on challenge with W1

(Table 3); (ii) animals repeatedly immunised with one variant (W1) had negative SICA tests for W2 or W4 but their sera contained inhibitory antibody against both and they were clinically immune when challenged with either (e.g. Fig. 2 and Table 2). These results indicate that the *in vitro* measurement of inhibitory antibody provides a consistently reliable index of the host's immune status and probably measures the protective antibody which initiates specific immune processes *in vivo*.

The relationship between the antibodies responsible for inhibition and those mediating schizont agglutination has not been established. Both have the same overall specificity, since inhibitory antibody is directed predominantly against specific variants identified by schizont agglutination. Although there are instances, mentioned above, in which inhibitory antibody is detectable in the absence of schizont ag-

Table 2. Specific inhibitory antibody and SICA titres against *P. knowlesi* variants W1, 2 and 4 in experimental monkey E2 which was challenged 5 times with *P. knowlesi* variant W1 and then on day 126 with W2.

Challenge (1)			W1					W2					W4						
Day	Variant	Pre-patent (days) (2)	Serum (day)	% Inhibition (3)					% Inhibition (3)					% Inhibition (3)					
				0.01 ml	0.10 ml	0.20 ml	0.40 ml	SICA	0.01 ml	0.10 ml	0.20 ml	0.40 ml	SICA	0.01 ml	0.10 ml	0.20 ml	0.40 ml	SICA	
0	W1	0		0				0											
13	W1	0	13																
41	W1	0 (4)	41	63	71			1250	18	0	22	42	0	1	0	16	20	0	0
63	W1	5	63	67	70			1250	7		38	55	0	4	20	33	0	0	0
97	W1	4	97	53	72			1250	10		48	75	0	0	0	18	36	0	0
			123	56	66			6250	1	23	53	73	0	0	1	11	35	0	0
126	W2	8																	
			133		66				0	20	58	78	50		9	15	22		
			139		67				27	63	83	84	1250			12	22		
			146		67				43	63	80	82	1250			18			
			151		71				45	58	82	85	6250	0	4	12	35		0
			160		68				30	58			1250						
			165						25	60	82	84	1250						

(1) 10⁴ parasites. (2) Prolongation (days) of pre-patent period beyond normal control. (3) Figures show % in vitro inhibition with various doses of serum. (4) Parasitaemia controlled spontaneously (Fig. 5).

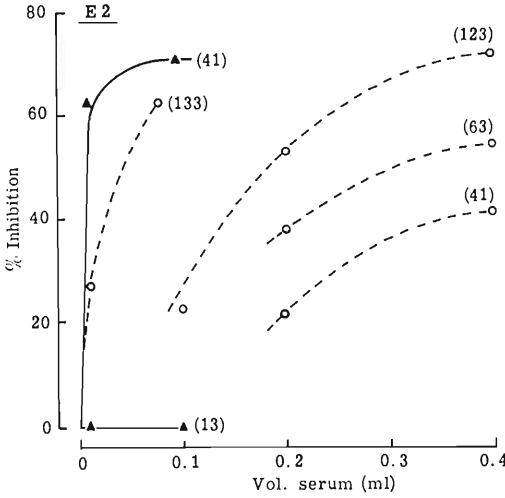


Figure 2. Dose response data for sera of a monkey (E2, Table 2) assayed against *P. knowlesi* variants W1 (continuous line) and W2 (interrupted line). The animal was challenged with 10⁴ W1 parasites on days 0, 13, 41, 63, and 97 and with 10⁴ W2 parasites on day 126. Figures in brackets represent days on which sera assayed were collected.

glutinins, the antibodies active in the two tests might be identical: (i) if the sensitivity of the inhibition test were greater than that of the agglutination test; this is unlikely as equally high levels of inhibition were observed with both negative and positive agglutination (e.g. Table 2); (ii) if surface determinants were distributed on some strains or variants in such a way that these were inhibited but not agglutinated by specific antibody. Alternatively, different antibodies might mediate the two tests; (i) if the Ig classes differ in their activity for SICA and inhibitory tests; this is unlikely since IgG and IgM are active in both tests (Cohen & Butcher, 1970); (ii) if agglutinating and inhibitory antibodies were directed against different specific antigens; it is likely, for example, that inhibitory antibodies are directed against merozoite antigens (Butcher & Cohen, 1971) and agglutinating antibodies against different determinants on the surface of red cells containing schizonts. The isolation and characterisation of agglutinating and inhibitory antigens should establish their relationship.

The appearance of antigenic variants dur-

Table 3. Monkeys immune to *P. knowlesi* (G strain) challenged with *P. knowlesi* W or Nuri strains.

Animal	Immunisation with G-strain			Challenge			Pre-challenge serum	
	Duration (days)	Times immunised	Days since last infect.	Strain (variant)	No. parasites	Delay in patency (3)	Inhibition % (5)	SICA titre
M65 ⁽¹⁾	1010	16	49	W(1)	10 ⁴	0 ⁽⁴⁾	43	0
G50 ⁽¹⁾	98	3	14	W(1)	10 ⁵	5	47	0
G56 ⁽¹⁾	460	5	140	W(4)	10 ⁶	0	0	0
G59 ⁽²⁾	300	2	140	W(4)	10 ⁶	0	0	0
G53 ⁽¹⁾	280	4	41	Nuri	10 ⁴	N	52	250
G54 ⁽²⁾	252	4	49	Nuri	10 ⁴	N	40	50

(1) Infections in these animals were controlled when necessary with mepacrine 6 mg/kg i. m. i. daily for 4 days.
(2) Infections in these animals were controlled when necessary with chloroquine 7.5 mg/kg followed by 4 injections of 2.5 mg/kg daily.
(3) Delay (days) beyond normal control animal. N = parasitaemia not detectable within 2 weeks of challenge.
(4) On 5th day patency M65 showed spontaneous decrease in parasitaemia.
(5) Based on 24 hr radioactivity values in duplicate flasks; sera tested at dose of 0.3 ml per flask containing 0.25 ml red cells (initial parasitaemia 1%).

ing the course of the asexual erythrocytic cycle has been observed in several species of malaria. This phenomenon was first described in *P. berghei* infections of mice (Cox, 1959; Welldé & Sadun, 1967) and subsequently in the monkey malarias, *P. knowlesi* (Brown & Brown, 1965) and *P. cynomolgi bastianelli* (Voller & Rossan, 1969a) and more recently in the human malaria, *P. falciparum* (Voller, 1971) and the avian malaria, *P. lophurae* (Corwin et al., 1970). The schizont agglutination test (Eaton, 1938) has revealed a very wide spectrum of variants of *P. knowlesi* (Brown, 1969) and their successive appearance seems responsible for persisting infections in immunised animals (Brown & Brown, 1965; Voller & Rossan, 1969a). However, certain observations require explanation: (i) spontaneous relapses in *P. knowlesi* infections are usually well controlled by the immunised host and parasitaemia rarely rises above 1%; nevertheless, the relapse variant produces a rapidly fatal infection if inoculated into an unimmunised recipient; (ii) after repeated challenge with a single variant of *P. knowlesi*, rhesus monkeys are resistant to several other variants (Voller & Rossan, 1969b). These findings have prompted the view that malarial infection induces a variant-transcending immunity which inhibits, but does not entirely prevent, the multiplication of new variants (Brown, Brown & Hills, 1968). The specificity and nature of this broad spectrum response were undetermined. More recently, Brown (1971) has postulated that the protective antibody response is entirely variant specific,

but certain surface antigens common to all variants sensitise thymus-derived lymphocytes; these act as "helper" cells in antibody production and enable the host to respond more quickly to new variants and control their proliferation.

The *in vitro* assays summarized above (Butcher & Cohen, 1972) show that inhibitory antibody is predominantly variant specific. Four weeks after a single infection of 3–5 days duration, followed by cure, inhibitory antibody was detectable only against the infecting variant (Table 1). Similarly, in a monkey exposed successively over a period of 9 weeks to variants W1, W2 and W3, specific inhibitory antibody was consistently positive only after the corresponding variant had become patent (Fig. 1). However, when animals were repeatedly challenged with a single variant, W1, inhibitory antibody against other variants, e.g. W2 and W4, became detectable after the sixth week at levels corresponding to about 2% of that for W1 (e.g. Fig. 2).

The presence of inhibitory antibody directed against a variant other than that used for challenge might indicate that this variant had appeared in the host as a result of antigenic variation. This seems unlikely in the present instances because such inhibitory antibody was associated with negative SICA tests for the corresponding variants (Butcher & Cohen, 1972). Brown & Brown (1965) have shown that patent infections consistently evoke specific schizont agglutinins in easily measurable titre and our data illustrate the same

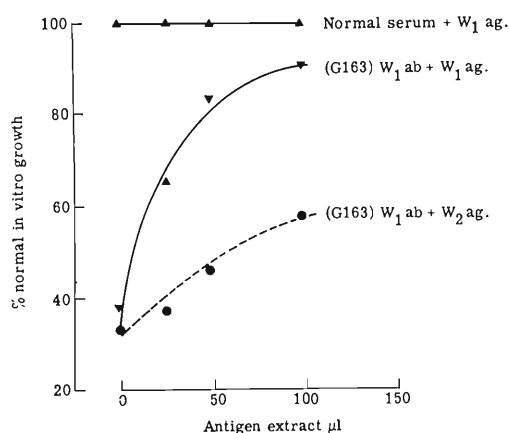


Figure 3. Data showing absorption of antibody against variant W1 by a soluble antigen extract of W1 and partial absorption by a soluble antigen extract of W2. In these assays a dose of immune serum was used which, in the absence of antigen, inhibited parasite growth to 30% of that observed in normal serum.

fact. It appears therefore that chronic malarial infection induces the synthesis of inhibitory antibody active against variants which the host has never harboured. Such activity could be attributed to low-affinity cross-reacting antibody but, since levels increase with repeated challenge, it is likely that parasites within a strain carry common antigenic determinants. The fact that distinct strains of *P. knowlesi* may cross-immunise for one another (Table 2) also increases the probability that variants within a strain carry common protective antigens. Direct support for this is provided by the finding that W1 specific inhibitory antibody can be completely absorbed by soluble W1 antigen extracts and partly absorbed by W2 extracts (Fig. 3). Whether such common antigens occur on all variants of a strain is not known, but the present results are consistent with such a distribution and so is the rarity of severe relapse in chronically infected rhesus monkeys.

It is apparent that the resistance of monkeys to relapse variants arising during chronic infection and the cross-immunity observed after single variant challenge, can be correlated with the presence of inhibitory antibody active against variants not previously patent. Such antibody is detectable about 6

weeks after the first infection and may, by limiting the multiplication rate of most new variants as they arise, account for the progressive lengthening of the pre-patent period characteristically observed with repeated challenge. However, the significance of this latter phenomenon remains uncertain because the mechanism of new variant induction by antibody has not been elucidated. Despite the wide antigenic variability manifest within individual strains of the *P. knowlesi* parasite, the occurrence of cross-immunisation between variants is encouraging from the point of view of vaccine production. A vaccine which contains cross-sensitising antigens may be expected to induce a degree of clinical immunity similar to that observed after repeated challenge or during the course of chronic infection.

These studies show that available in vitro methods of short-term malaria cultivation are useful in analysing mechanisms of malarial immunity. The same methods undoubtedly have considerable potential value for malarial antigen analysis, vaccine development and drug screening.

Acknowledgments

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Comments on *in vitro* Studies

WILLIAM TRAGER

The Rockefeller University, New York, New York 10021

Experiments with malaria parasites developing intracellularly *in vitro* can clearly be of several types and directed toward a variety of ends. The use of cultures of a day's duration for immunological studies has just been beautifully demonstrated by Dr. Cohen's paper. Experiments of even shorter duration also give useful results, particularly if coupled with measurement of incorporation of labeled substrate, as in Dr. Schnell's paper. In such work also the more favorable the *in vitro* conditions for development of the parasites the more likely are the results to be significant. Although those of us who have looked at the cytology of malaria parasites throughout their life cycle have had little doubt that they are eukaryotic cells the biochemical evidence to this effect is reassuring. Dr. Schnell's results are more important in showing the potential usefulness of these methods for work on parasite physiology. Evidently any contribution to protein synthesis that may be made by the membranous structures (thought to possibly have a mitochondria-like function) of *P. falciparum* and *P. knowlesi* must be too small to be detectable. Although the data are not included in the paper, I understand from Dr. Schnell that controls on incorporation by white blood cells gave a negligible value for the experiments with rhesus monkey (*P. knowlesi*) and a value not over 10% of that for the parasitized cells in experiments with *Aotus* monkey blood (*P. falciparum*).

The possibilities with *in vitro* cultures of 1 to 2 days' duration are well illustrated in Dr. Siddiqui's paper. Of special interest are the results with *P. falciparum* in *Aotus* erythrocytes. These parasites evidently require from

outside the erythrocyte (for their initial intracellular growth) only the same two amino acids required by *P. knowlesi*. Also noteworthy is the finding that DNA synthesis in these 2 species occurs only during a short period early in schizogony.

While work such as that of Schnell and Siddiqui aims at simplifying the conditions for intraerythrocytic development *in vitro* during a single reproductive cycle, in another type of approach one can try to duplicate a part of the natural situation in the host animal, where red cells are kept agitated in a medium of whole fresh plasma. This is what Dr. Tiner hopes to be able to do with his centrifugal perfusion chambers.

In his results with his "tidal apparatus" Dr. Tiner has obtained segmentation of *P. berghei* in mouse erythrocytes, actually one of the least favorable systems for *in vitro* work. It would be helpful if he would give counts of the number of parasites per 10,000 red cells at the start and during the course of the experiment.

Dr. Tiner has provided a logical rationale for the replacement of plasma at a rate such that the cells would always be suspended in "fresh" plasma, defined as plasma with minimal changes in its lipid composition and giving a normal sedimentation rate. The centrifugal apparatus is designed to do this. It seems a complex machine, but so is the body of a host animal. If it should show indications of better parasite development than hitherto obtained it would be well worth the effort involved and would constitute a step in the direction of long term culture systems.

V

CHEMOTHERAPY

A. Host-parasite-drug inter-relationships

Energetics of the Malarial Parasite¹

G. CARTER², K. VAN DYKE AND H. F. MENGOLI

Departments of Pharmacology and Microbiology, West Virginia University,
Morgantown, West Virginia 26506

ABSTRACT: We have demonstrated that there exists $1.7 \pm 0.3 \times 10^{-18}$ moles of ATP per malarial parasite (*P. berghei*). This figure represents the amount of ATP in the malarial parasite after incubation with glucose (100 mg%). The endogenous amount of ATP that exists without glucose is about $\frac{1}{2}$ to $\frac{1}{3}$ of the generated amount. If adenosine-2-8-³H in the presence of glucose is added to the preparation of free parasites and the preparation incubated at 20° C for 10 minutes the adenosine is almost completely phosphorylated with the majority of it converted into adenosine-2-8-³H-triphosphate. The production of ATP is almost completely dependent on glucose added to the medium, indicating that there is little or no storage of glucose by the parasite. Once adenosine is phosphorylated it is partially incorporated into nucleic acids. If glucose is not added to the medium no detectable incorporation into nucleic acids occurs. This establishes the link between glycolysis and nucleic acids. We have estimated the amount of cyclic 3',5' adenosine monophosphate (c-AMP) in the malarial parasite to be about 2.2×10^{-21} Moles per parasite. The assay was performed by competitive protein binding.

The demonstration of the amount of ATP, adenosine triphosphate (the important energy storage compound) in the malarial parasite has never been accomplished. The reasons for this relate to the difficulties in separation of parasite from contaminating materials and the lack of sufficiently selectively sensitive methods to measure the small amount of ATP that is present. Also the incorporation of adenosine into ATP of the parasite has never been measured although a host of indirect evidence suggests that this must occur (Bünger and Nielsen, 1968; Van Dyke et al., 1969; Van Dyke and Szustkiewicz, 1969; Lantz and Van Dyke, 1971).

Previous work on the importance of ATP to the malarial parasite by Trager (1964) and the importance of host cell ATP by Brewer (1969) failed to establish the energetics of the parasite itself. A more direct approach to the problem was taken by Nagarajan (1968) with his attempt to incorporate ³²P_i into ATP of the parasite. The methodologies utilized in his study were not sufficiently sensitive or selective to actually demonstrate that incorporation with ³²P_i had occurred into ATP. Furthermore, he found no ATP in the parasite itself nor could he demonstrate a generation of ATP during conditions of incubation. The in-

corporation of ³²P_i into an acid stable preparation and its dependency on glucose do indicate however that some incorporation undoubtedly occurred into ATP.

In this paper we will demonstrate the approximate amount of ATP in the parasite, show that it can be generated under experimental conditions, and indicate that of the amount of adenosine-2-8-³H added almost all is phosphorylated (the majority of which is ATP) and that this phosphorylation is dependent almost completely on glucose.

Incorporation of ATP (from adenosine) into nucleic acids of the parasite is dependent on glucose added to the media. In the absence of added glucose little incorporation into nucleic acids occurs therefore demonstrating the link between glycolysis and nucleic acids.

We will give some preliminary estimations of the amount of cyclic 3', 5' adenosine monophosphate in the preparations. This compound is generated from the ATP manufactured by the malarial parasite.

Methods

Preparation and Counting of Parasites

The preparation of free parasites (*P. berghei*) was as performed in the companion paper. Free parasites were counted (1:10,000 dilution) by assessment in a Coulter counter Model B equipped with an industrial stand. A 30μ aperture was used and both white cells

¹ This investigation received financial support from the World Health Organization and NIH institutional grant.

² This study represents a portion of the work carried out by G. C. in partial fulfillment of the requirement for the degree of Doctor of Philosophy at West Virginia University.

Table 1. Determination of parasite-ATP by the luciferase enzyme.*

Experimental condition	Moles ATP/parasite ^a \pm S.E.
Before Incubation	$0.6 \pm 0.1 \times 10^{-18}$
10 Minute Incubation Without Glucose	$0.6 \pm 0.1 \times 10^{-18}$
10 Minute Incubation With Glucose	$1.7 \pm 0.3 \times 10^{-18}$
10 Minute Incubation with Glucose and ^3H -adenosine	$1.5 \pm 0.4 \times 10^{-18}$

* N = 3 number of observations.

^a Contamination from ATP in white cells less than 1×10^{-12} moles in 6.2×10^6 white blood cells.

and parasites were assayed in appropriate preparations. Electronic noise was compensated by blanking with the diluent (Krebs buffer, pH 7.4, Ca^{++} omitted).

Incubation Conditions

All incubations were carried out as described in the companion paper, except in this study the temperature of incubation was 20°C. (A greater percentage of radioactivity remains as ATP at this temperature.) All assays were performed on the perchloric acid extracts, neutralized with K_2CO_3 .

ATP Measurement

The measurement of non-radioactive adenosine triphosphate (ATP) was accomplished by the method of Stanley and Williams (1969) using non-coincident counting of light emitted (i.e. luciferin-luciferase). All measurements were done at least at two dilutions (1:10) for detection of possible enzyme inhibitors present in biological preparations.

c-AMP Measurement

Cyclic AMP was extracted and measured using the competitive protein-binding method of Gilman (1970).

c-AMP Chromatography

3', 5'-tritiated c-AMP was separated from other phosphorylated derivatives using the method of Makman (1970) on polyethyleneimine thin layer chromatography using ethanol:ammonium acetate.

The chromatography of the other nucleotides AMP, ADP, ATP and adenosine was

performed by the method of Randerath as detailed in the companion paper.

Results and Discussion

Clearly for the estimations of ATP to be reliable the preparation must not contain major sources of ATP other than the parasite. Therefore we studied the quantity of ATP in white cells in normal blood and as shown in the table the contamination is to be disregarded. During the malarial infection the number of white cells does increase as shown by the indicated counts (Table 1) as processed by Coulter counter. However, as shown by the autoradiographs of Büngener and Nielsen (1968), even though there is a major amount of radioactivity from ^3H -adenosine associated with parasites almost no counts are associated with white cells. This lack of phosphorylation relatively speaking is shown in table 2 where the white cells from normal blood phosphorylate adenosine to a minimal extent.

With the contamination both of endogenous ATP in white cells and lack of phosphorylation of adenosine by leucocytes we dismiss their contamination in our preparations. Also, microscopic examination of the saponinlysed preparation has never revealed any contamination by platelets or unlysed red cells. Therefore, we are assuming that they have been eliminated in the parasite-preparation procedure. The contamination by stroma of the freed parasite likewise has been dismissed by Nagarajan (1968). It obviously would contain neither ATP nor be able to phosphorylate adenosine. Any attempt to remove white cells results in damage to the parasite and concomitant loss of adenosine phosphorylation.

From the tabulated results of table 1, it is clear in the presence of glucose the amount of ATP is actually doubled (from 1.2×10^{-9} moles to 2.5×10^{-9} moles per 1.6×10^{-9} parasites). Therefore, the amount of ATP per parasite in the presence of glucose (the more normal situation) can be calculated to be $1.7 \pm 0.3 \times 10^{-18}$ moles). The approximate amount of ATP/reticulocyte calculated from the data of Nagarajan (1968) indicates 1.88×10^{-17} moles per reticulocyte. Roughly speaking, the amount of ATP per parasite might be about 1/10 of the amount per red cell. Naturally this is dependent on the as-

Table 2. The amount of phosphorylation and incorporation into nucleic acids of ³H-Adenosine by *P. berghei* and mouse white cells.

Experimental conditions	Incorp. into Nucleic Acids	dpm/ml \pm S.E. $\times 10^3$ ^a			
		ATP	ADP	AMP	Adenosine
<i>White Blood Cells</i> ^b					
10 minute incubation with glucose (100 mg%)	0.4 \pm 0	56 \pm 24 (1%)*	0 \pm 0 (0%)	340 \pm 50 (4%)	11,607 \pm 42 (96%)
10 minute incubation without glucose	0 \pm 0	30 \pm 3 (0%)	0 \pm 0 (0%)	468 \pm 32 (4%)	11,485 \pm 141 (96%)
<i>Parasites</i> ^c					
10 minute incubation with glucose (100 mg%)	221 \pm 9	9937 \pm 200 (83%)	1093 \pm 15 (9%)	454 \pm 141 (5%)	404 \pm 14 (3%)
10 minute incubation without glucose	10 \pm 1	629 \pm 6 (4%)	18 \pm 2 (0%)	586 \pm 82 (4%)	13,873 \pm 77 (92%)

* Percentage of the radioactivity associated totally with adenosine and its nucleotides.
^a N = 3 number of observations.
^b 6.2 × 10⁶ white cells prepared from normal blood.
^c 1.0 × 10⁹ parasites and 1.4 × 10⁷ white cells in the preparation.

sumption that ATP per reticulocyte done in the rat would be similar to the red cell in mouse blood.

The data in table 2 reveals little incorporation of tritiated adenosine into phosphorylated compounds and into nucleic acids of white cells. Also, without the addition of glucose the observations with free parasites are similar. Once glucose is added and incubation at 20°C for 10 minutes is completed, over 80% of the radioactivity associated with adenosine and its nucleotides is found as ³H-ATP. The incorporation into nucleic acids of the parasite by way of added adenosine appears to be dependent on glucose. This establishes the link between glycolysis and synthesis of nucleic acids.

Utilizing the protein binding assay and extraction of c-AMP of Gilman (1970), we have estimated the amount of cyclic 3', 5' AMP to be 2.2 × 10⁻²¹ moles per parasite. We cannot find the amount of cyclic AMP from added tritiated adenosine because it is too close to the zero time control.

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Drug Effects on the Phosphorylation of Adenosine and its Incorporation into Nucleic Acids of Chloroquine Sensitive and Resistant Erythrocyte-free Malarial Parasites¹

G. CARTER² AND K. VAN DYKE

Department of Pharmacology, West Virginia University, Morgantown, West Virginia 26506

ABSTRACT: Several antimalarial compounds have been shown to inhibit the incorporation of purine nucleosides into the nucleic acids of the malarial parasite. Currently, it is unclear whether this effect is due to inhibition of nucleoside (a) uptake (b) phosphorylation or (c) polymerization. The present experiments were designed to shed some light on the antinucleic acid mechanisms of these antimalarials. Erythrocyte-free malarial parasites were prepared by a saponin lysis technique. Free parasites so prepared were incubated at 37° C in Krebs phosphate buffer with or without drug. ³H-2,8-adenosine, used as the purine precursor was added to each incubation tube. At the designated time (usually 10 minutes) the incubations were stopped by the addition of ice-cold perchloric acid. The incubation tubes were centrifuged to remove the precipitated proteins and nucleic acids, and the supernatant liquid neutralized. The solid pellet, containing nucleic acids was utilized to determine the amount of radioactivity incorporated into parasite nucleic acids. An aliquot of the supernatant liquid was analyzed for its content of radioactive ATP, ADP, AMP and adenosine, by first separating these compounds on polyethyleneimine cellulose thin layer plates. ATP, ADP, AMP and adenosine were eluted and counted using liquid scintillation techniques.

Several of the drugs appear to interfere with phosphorylation of adenosine at high doses. These include quinacrine, ethidium, quinine and adenine arabinoside. Chloroquine and primaquine appear to be inactive in this regard. Inhibition of phosphorylation appears to lag behind the inhibition of polymerization possibly meaning that polymerization is a more sensitive event. In general the inhibition of incorporation into nucleic acids by chloroquine and primaquine is probably not a significant event as evidenced by the high dose (10^{-3} M) required to produce an effect.

Those drugs that produce an effect on nucleic acid polymerization at lower doses (10^{-5} , 10^{-6} Molar) possess structures with three planar rings namely ethidium and quinacrine. Clearly their mechanism must be inhibition of the polymerization of RNA and DNA.

A discussion of the comparison between drug sensitive and resistant strains is included.

Research into the mechanism of action of various antimalarial drugs has yielded little solid information concerning the detailed action of how these drugs may be acting. With the exceptions of those drugs which act on the inhibition of the reduction of folic acid, i.e. tetrahydrofolate reductase inhibitors (Ferone et al., 1969) (e.g. pyrimethamine) the exact biochemical step at which these inhibitors act in the parasite is in question. Although some evidence in bacteria (Hahn et al., 1966) is suggestive that certain drugs may interfere with synthesis of nucleic acids at the polymerase level (either RNA and/or DNA) there is only indirect evidence (Van Dyke et al., 1969, 1970; Van Dyke and Szustkiewicz,

1969; Lantz and Van Dyke, 1971) that this inhibition is important in the malarial parasite (Van Dyke et al., 1969).

With the development of new methodology (Carter et al., 1971) in our laboratory it has been possible to measure the radioactive phosphorylated derivatives of adenosine (i.e. AMP, ADP and ATP). When radioactive adenosine-2,8-³H is added to the parasite the compound is rapidly converted into phosphorylated derivatives (in the presence of added glucose). Simultaneously a portion of the ATP-³H is being incorporated in nucleic acids of the parasite (particularly RNA). If a drug is added to the parasite preparation it is possible to measure inhibition of each phosphorylated derivative of adenosine and inhibition of the polymerization of ATP-³H into nucleic acids.

In this paper we examine these phosphorylation and incorporation processes in both

¹ This investigation received financial support from the World Health Organization.

² This study represents a portion of the work carried out by G. C. in partial fulfillment of the requirements for the degree of Doctor of Philosophy at West Virginia University.

Table 1. Distribution of radioactivity after incubation *P. berghei* with ^3H -adenosine^a dpm/ml \pm S.E. $\times 10^3$.*

ATP	ADP	AMP	Adenosine
5185 \pm 216	1962 \pm 226	2498 \pm 176	1148 \pm 63

^a Free parasite preparations contain approximately 1×10^7 white cells and 1×10^6 parasites per incubation tube.

* N = 10 number of observations.

chloroquine sensitive and resistant malarial parasites (*P. berghei*). Utilizing drugs which have shown important effects against the malarial parasite in previous work, it was our hope that the action of some of these drugs would be elucidated and the proposed mechanism of action of others would be eliminated. No drug has been found to interfere with the parasite's phosphorylation mechanisms and we thought possibly phosphorylation of adenosine might be related to drug resistance. The interference with the energetics of the parasite would of course be an important link in the vital process which gives the parasite its living force. The drugs chosen included quinacrine, quinine, chloroquine, primaquine, ethidium and adenine arabinoside.

Materials and Methods

Maintenance of Parasite Strains

The two strains of *Plasmodium berghei* used in this study, strain-NYU-2 (normal drug sensitivity) and strain-CR (resistant to quinacrine, 4 and 8 amino quinolines), were obtained from Dr. Richard Jacobs, National Institutes of Health.

Parasite infections were maintained by continuous blood passage from infected to healthy white male mice (18–20 g.).

Strain-NYU-2. Infected blood was collected by cardiac puncture into heparinized syringes, from mice which had been inoculated ip. 5–7 days previously with approximately 1 million parasitized red cells.

Strain-CR. Infected blood was collected as described above except that the mice used had been inoculated ip. 12–14 days previously with approximately 5 million parasitized red cells.

All animals were housed in the animal quarters at West Virginia University Medical

Table 2. Distribution of radioactivity after incubating white cells with ^3H -adenosine^a dpm/ml \pm S.E. $\times 10^3$.*

ATP	ADP	AMP	Adenosine
18 \pm 1	12 \pm 9	198 \pm 36	11,030 \pm 1000

^a The white cell preparation (from normal blood) contains approximately 5×10^6 white cells per incubation tube.

* N = 4 number of observations.

Center and had free access to food and water at all times.

Preparation of Free Parasites

Blood was obtained by cardiac puncture from 4–8 parasitized mice. The blood was pooled and centrifuged for 10 minutes at $600 \times g$ in an International Centrifuge at 4°C. The plasma was carefully drawn off, and the packed cells lysed by incubation in a 0.1% solution of saponin in Krebs phosphate buffer (no Ca^{++} , pH 7.4). The concentration was 1 ml of packed cells to 40 ml of this saponin solution and incubation was accomplished for 30 minutes at 37°C. Following this incubation, the parasites were sedimented by centrifugation at $600 \times g$ for 10 minutes and the supernatant solution aspirated and discarded. The parasites were resuspended in Krebs phosphate buffer enriched with 200 mg% glucose, to $2\frac{1}{2}$ times the initial volume of whole blood used. One-half milliliter of this free parasite suspension was used in each reactions tube.

Incubation

All reaction tubes contained the following:

1. 0.5 ml of modified Krebs buffer either with or without drugs.
2. 10 μc of ^3H -2,8 adenosine.
3. 0.5 ml of free parasite suspension (containing 5×10^5 – 1×10^6 free parasites).

Incubations were started by adding 0.5 ml of the free parasite to reaction tubes containing the rest of the mentioned reaction mixture. Incubation was performed at 37°C for a specified time (10 minutes). Reactions were stopped by adding 2.0 ml of ice-cold perchloric acid (0.4N), and the tubes were centrifuged at $1000 \times g$ for 5 minutes. Reaction tubes were kept cold in an ice-water bath for the remainder of the experiment.

Table 3. Effects of quinacrine on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean \pm S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean \pm S.E.) $\times 10^3$	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	12,927 \pm 714	11,552 \pm 714
1 $\times 10^{-3}$	97 \pm 1	99 \pm 1	8,118 \pm 662	6,829 \pm 874
1 $\times 10^{-4}$	87 \pm 1	85 \pm 1	12,209 \pm 588	9,752 \pm 1,188
1 $\times 10^{-5}$	73 \pm 2	72 \pm 1	11,845 \pm 565	10,702 \pm 1,186
1 $\times 10^{-6}$	9 \pm 3	21 \pm 3	13,246 \pm 550	11,300 \pm 518
1 $\times 10^{-7}$	2 \pm 1	10 \pm 1	12,457 \pm 709	11,187 \pm 1,022

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
* N = 6 number of observations.

The supernatant liquid obtained was used to assay the extent of phosphorylation of adenosine by the parasites. The solid pellet was used to determine the amount of radioactivity incorporated into parasite nucleic acids.

Acid-soluble phosphorylated intermediates

A 1 ml aliquot of the supernatant liquid was neutralized with K₂CO₃ (approximately 0.5 ml of a 0.80N solution) and the resultant salt sedimented by centrifugation at 1000 \times g for 5 minutes. A 0.5 ml aliquot of the supernatant liquid was carefully taken and mixed well with 1.5 ml of distilled water.

10 μ l of this solution was spotted on PEI-cellulose thin layer plates, previously washed with distilled water and dried. Volume-equivalent samples of a standard solution (1 mg per ml of ATP, ADP, AMP and Ad) was spotted at the same place for visualization purposes.

Chromatograms were developed by using

two ascending solvent systems. First, water was allowed to ascend the plates for one hour, removing adenosine from the phosphorylated products. Next, the plates were dried and rechromatographed for one hour with 1 Molar LiCl solution. The plates were dried, visualized under U.V.-light (260 m μ). The U.V.-absorbing spots (ATP, ADP, AMP and adenosine) were circled, cut-out and placed in plastic scintillation vials. The radioactivity was eluted in the vial by addition of 1 ml of 0.01 N sodium hydroxide solution to the vial and shaken for one hour. Ten milliliters of scintillation solution was added to vials and radioactivity determined.

Radioactivity incorporated into parasite nucleic acids

Approximately 0.5 ml of heparinized serum was added to the remaining content of each reaction tube. Extra protein was added to produce more insoluble material to handle. Reaction tubes were mixed thoroughly on a

Table 4. Effects of ethidium bromide on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean \pm S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean \pm S.E.) $\times 10^3$	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	12,987 \pm 58	11,876 \pm 60
1 $\times 10^{-3}$	97 \pm 1	97 \pm 1	8,989 \pm 146	4,516 \pm 900
1 $\times 10^{-4}$	93 \pm 1	89 \pm 1	11,640 \pm 92	6,906 \pm 147
1 $\times 10^{-5}$	81 \pm 2	78 \pm 1	12,972 \pm 230	11,403 \pm 115
1 $\times 10^{-6}$	66 \pm 3	68 \pm 1	13,649 \pm 54	12,688 \pm 732
1 $\times 10^{-7}$	25 \pm 2	18 \pm 1	12,844 \pm 120	12,551 \pm 457

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
* N = 6 number of observations.

Table 5. Effects of chloroquine on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean ± S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean ± S.E.) × 10 ³	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	4,703 ± 194	5,770 ± 267
1 × 10 ⁻³	68 ± 2	74 ± 4	4,630 ± 202	5,436 ± 263
1 × 10 ⁻⁴	31 ± 2	45 ± 2	4,955 ± 251	6,089 ± 270
1 × 10 ⁻⁵	10 ± 3	16 ± 2	4,610 ± 138	5,936 ± 278
1 × 10 ⁻⁶	10 ± 3	11 ± 2	5,156 ± 294	5,639 ± 428
1 × 10 ⁻⁷	8 ± 3	7 ± 2	5,115 ± 240	5,574 ± 331

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
* N = 6 number of observations.

Lab Line Vortex Supermixer No. 1290, and centrifuged at 1000 × g for 5 minutes. The supernatant liquid was carefully decanted and solid pellet resuspended in 4 ml of ice-cold perchloric acid. This washing procedure was repeated (a total of three times) so that the last wash was essentially free of acid soluble radioactivity. Then the pellet was resuspended in 4 ml of ice-cold perchloric acid, and the reaction tubes placed in a 90°C water bath for 30 minutes. This heating and acid hydrolysis procedure degrades DNA and RNA of the parasite, and releases the incorporated radioactivity. The tubes are centrifuged at 1000 × g for 5 minutes and 0.5 ml aliquot of the hydrolysis products in the supernatant liquid are counted as previously mentioned.

Liquid Scintillation Technique

All radioactivity was counted on a Packard Liquid Scintillation Spectrophotometer (Model 2002).
The liquid scintillation solution (Carter and

Van Dyke, 1971) used in all experiments was prepared as follows:

- 1. 1,4-bis 2 phenyloxazoyl benzene (POPOP, 0.9 g)
- 2. 2,5 diphenyloxazoyle (PPO, 15 g)
- 3. Biosolv solubilizer (BBS-3, 500 ml)
- 4. Toluene (3 liters)

Samples were counted at least twice to insure reproducibility. The efficiency of counting was determined by adding to each sample 10 µl of ³H-water (2.6 × 10⁶ dpm/ml). This counting media is very efficient and routinely yielded efficiencies between 35 and 42%.

Results and Discussion

The results in table 1 indicate that under the conditions chosen the majority of the adenosine is phosphorylated. That this represents parasite phosphorylation and not contamination from white cells is shown in table 2 by the phosphorylation data of white cells from normal blood. This would be expected

Table 6. Effects of primaquine on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean ± S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean ± S.E.) × 10 ³	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	8,915 ± 295	8,749 ± 504
1 × 10 ⁻³	59 ± 1	81 ± 3	8,424 ± 381	8,886 ± 239
1 × 10 ⁻⁴	13 ± 1	26 ± 3	8,704 ± 324	7,169 ± 920
1 × 10 ⁻⁵	6 ± 2	11 ± 2	8,937 ± 275	8,735 ± 500
1 × 10 ⁻⁶	11 ± 2	3 ± 2	9,019 ± 255	9,147 ± 192
1 × 10 ⁻⁷	9 ± 2	6 ± 2	8,920 ± 190	9,129 ± 367

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
* N = 6 number of observations.

Table 7. Effects of quinine on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean \pm S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean \pm S.E.) $\times 10^3$	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	10,150 \pm 529	8,370 \pm 666
1×10^{-3}	60 \pm 3	83 \pm 2	7,844 \pm 325	3,096 \pm 640
1×10^{-4}	4 \pm 2	20 \pm 5	9,493 \pm 835	7,516 \pm 855
1×10^{-5}	2 \pm 1	10 \pm 4	9,486 \pm 601	8,645 \pm 779
1×10^{-6}	5 \pm 2	8 \pm 4	10,065 \pm 846	8,715 \pm 790
1×10^{-7}	1 \pm 1	6 \pm 3	9,585 \pm 723	8,908 \pm 783

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
^{*} N = 6 number of observations.

because white cells that have not been activated using an outside agent, e.g. phytohemagglutinin, do not phosphorylate and/or incorporate precursors into nucleic acids (Farrow and Van Dyke, 1971). The majority of the phosphorylated derivatives is adenosine triphosphate (ATP). This phosphorylation appears to be almost completely dependent on the metabolism of glucose. A discussion of phosphorylation is shown in the companion paper on parasite energetics (Carter, Van Dyke and Mengoli, 1972).

Quinacrine and Ethidium

Both quinacrine (table 3) and ethidium (table 4) inhibit adenosine phosphorylation at 10^{-3} M concentrations. The effect on the polymerization into nucleic acids occurs at much lower doses (10^{-5} , 10^{-6} Molar). Clearly these intercalating drugs are acting at the level of polymerization well before phosphorylation is affected. In both cases the drug resistant

strain appears to be more sensitive to effects of the drugs on phosphorylation (10^{-4} , 10^{-3} M). There appears to be no difference in the effect of the drugs on polymerization.

Chloroquine and Primaquine

In contrast to quinacrine and ethidium, neither chloroquine (table 5) nor primaquine (table 6) had an effect on phosphorylation of adenosine at the doses tested. The inhibitory effect on the polymerization of adenosine into nucleic acids occurs at higher doses (10^{-4} , 10^{-3} Molar). The interpretation of this lack of inhibition is that these drugs probably do not act at the nucleic acid level (at least in *P. berghei*). It is interesting to note that chloroquine was more effective on nucleic acid polymerization in the resistant strain than in the sensitive. Again, we do not believe that chloroquine acts in an important way on nucleic acids (at least as far as phosphorylation and polymerization are concerned).

Table 8. Effects of adenine arabinoside on phosphorylation and incorporation of adenosine by both drug sensitive and resistant strains of *P. berghei*.

Molar concentration	% inhibition of Adenosine incorporation into Nucleic Acids Mean \pm S.E.*		Total Phosphorylation (dpm/ml) of Adenosine (ATP + ADP + AMP) (Mean \pm S.E.) $\times 10^3$	
	DSS ^a	DRS ^b	DSS	DRS
Control (no drug)	0	0	12,310 \pm 451	13,165 \pm 219
1×10^{-3}	85 \pm 1	77 \pm 1	3,798 \pm 120	4,005 \pm 106
1×10^{-4}	40 \pm 2	26 \pm 2	11,248 \pm 201	10,809 \pm 448
1×10^{-5}	10 \pm 1	8 \pm 3	11,845 \pm 486	12,083 \pm 185
1×10^{-6}	7 \pm 2	6 \pm 2	12,378 \pm 300	12,205 \pm 189
1×10^{-7}	9 \pm 2	8 \pm 1	12,589 \pm 129	11,957 \pm 109

^a DSS = drug sensitive strain (NYU-2).
^b DRS = drug resistant strain (chloroquine resistant).
^{*} N = 6 number of observations.

Adenine Arabinoside and Quinine

Quinine (table 7) and adenine arabinoside (table 8) (ara-A) show inhibition of adenosine phosphorylation at 10^{-3} M. In the case of quinine the drug resistant strain appears to be the more sensitive to inhibition of phosphorylation whereas the two strains are about equally sensitive to the action of ara-A.

Ara-A appears to be the more effective drug against polymerization into nucleic acids of the parasite with significant inhibition at 10^{-4} Molar, with the sensitive strain displaying the greater inhibition. At the dose of 10^{-4} Molar of quinine only the resistant strain showed measurable inhibition toward polymerization into nucleic acids.

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Incorporation of ^{14}C -Amino Acids by Malaria (*Plasmodium lophurae*). V. Influence of Antimalarials on the Transport and Incorporation of Amino Acids*

I. W. SHERMAN AND L. TANIGOSHI

Department of Biology, University of California, Riverside, California 92502, U.S.A.

ABSTRACT: The transport of selected amino acids into normal duck erythrocytes, malaria-infected erythrocytes and erythrocyte-free *Plasmodium lophurae* was studied. Amino acid entry into the normal erythrocyte was by a carrier-mediated process, whereas the mode of entry into the infected erythrocyte was by diffusion (except for glycine). In the free parasites only arginine, lysine, glutamic and aspartic acids showed kinetic patterns characteristic of carrier-mediated entry. Arginine-lysine and glutamic-aspartic acid were competitive inhibitors for entry suggesting that these enter the parasite by distinct and separate loci. An equimolar array of amino acids was inhibitory for amino acid transport into the free parasite, however plasma and red cell amino acid mixtures were without effect on arginine, lysine or glutamic acid entry. The antimalarials quinine, chloroquine and primaquine inhibited the uptake and incorporation of certain amino acids; these effects resulted from an inhibition of energetics rather than a direct block in the uptake process.

The malaria parasite is an obligate intracellular parasite. During its residence within the host erythrocyte the parasite grows rapidly and undergoes multiple fission; to accomplish this the plasmodium must synthesize large amounts of protein. Studies on the protein metabolism of the malarial parasite indicate that there exist three potential sources of amino acids which can be made available for the synthesis of parasite proteins: (a) hemoglobin of the erythrocyte (Fulton and Grant 1956, Sherman and Tanigoshi, 1970), (b) de novo formation of amino acids from glucose (Ting and Sherman, 1966; Polet et al., 1969) and (c) the free amino acid pools of the plasma and the erythrocyte (Fulton and Grant, 1956; Polet and Barr, 1968b, Polet and Conrad, 1969; McKee, 1951; Sherman, et al., 1967, 1969). The present report considers the relationship of the amino acid pools of the host to the ecology of the growing plasmodium. Two questions have been posed: (a) Are the ratios of amino acids available to the parasite critical in determining whether or not a given amino acid mixture will serve as an adequate nutrient source for plasmodial protein synthesis? And, (b), do antimalarials affect the entry of amino acids into the parasite? The experimental model for examining

these questions was the avian malarial parasite, *Plasmodium lophurae*, grown in duckling erythrocytes.

Materials and Methods

Parasitological material. *Plasmodium lophurae* was maintained in white Pekin duck by intravenous inoculation of infected blood (Trager, 1950). Parasitized erythrocytes were used when the parasites were multinucleate, but were not segmenters about to lyse the host erythrocyte. Parasitemia (parasites per 100 red cells) for experiments was not less than 80%.

Transport of single amino acids. Blood removed from the jugular vein of normal and malaria-infected ducklings was collected in syringes containing heparin (Sherman, et al., 1969). The cells were washed three times by centrifugation (800g, for 10 min at 4°C) in glucose-saline (G-S) buffer (Sherman and Hull, 1960), and then the cells were made up to a concentration of 30% (v/v) in this same buffer. "Erythrocyte-free" parasites were prepared by saponin lysis, with the omission of deoxyribonuclease treatment (Sherman and Hull, 1960). Parasites were resuspended in G-S to a concentration of 30% (v/v). Cell suspensions were pre-warmed for 3 min to 37–38°C and then placed in 5ml plastic syringes. A special device (Tracy and Sher-

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man, 1972) permitted the simultaneous addition of cell suspension (5 ml) to a maximum of twenty incubation flasks. Each incubation flask contained 0.1 ml (0.5 μ Ci) of the isotopically labeled amino acid, and 0.1 ml of a non-radioactive amino acid. The final concentration of the amino acid in the incubation flask ranged from 0.01mM–10mM. Incubation of cells and amino acid was carried out by setting the device holding the sample flask in an agitating water bath set at 37–38°C. Incubation was for 5 min after addition of cells to the flasks. The reaction was terminated by the addition of 5ml of ice-cold G-S to the flasks. The rate at which cooling occurs under these conditions is equivalent to about 10 seconds of incubation at 37°C (Winter and Christensen, 1964). The flasks were removed from the water bath and emptied into pre-tared centrifuge tubes set in ice. Following centrifugation (20,000g at 4°C for 5 min), 0.2 ml samples of the supernatant, representing the radioactivity of the extracellular fluid, were pipetted into scintillation vials containing Cab-O-Sil and a PPO-POPOP cocktail (Sherman, et al., 1970). The supernatant was discarded and the pellet washed twice by centrifugation in 10ml G-S. Intracellular radioactivity was determined by lysing the cells in 2ml distilled water, followed by precipitation of proteins with 3ml of 10% (w/v) trichloroacetic acid, and after centrifugation samples of the acid soluble supernatant were removed and placed in scintillation vials. Radioactivity of samples was determined using a Packard Tri-Carb liquid scintillation spectrometer (Model 3375) with an efficiency of 80–85% for carbon 14. Uptake values (V) for amino/acids were obtained by converting the radioactive counts into μ moles taken up/g dry wt/5 min. The transport constants (K_t) and maximal velocity (V_{max}) with their respective standard errors were determined on a Wang 370 Programmed Calculator using the method of Wilkinson (1961).

Transport of amino acids in complex media. "Free parasites" were washed clean of contaminating red cells and hemoglobin in G-S and then made into a 31.5% (v/v) suspension with a high potassium medium (Sherman, et al., 1969). The suspension of parasites was pre-warmed for 3 min, and 5ml aliquots of the suspension were placed in syringes held

in the device cited above. At zero time the warmed suspension was added to pre-tared centrifuge tubes containing 0.25ml of: 50 μ l 0.5 μ Ci of the amino acid under study, 150 μ l of amino acid mixtures in proportions which were equimolar or similar to plasma or that of the red cell, and 50 μ l of the amino acid (non-radioactive) under study in amount to yield a final range of concentrations from 0.1mM–0.8mM. Parasites were incubated at 37–38°C with agitation for 5 min, and the reaction terminated by the addition of 5ml of ice-cold G-S. The remaining procedures as well as calculation of K_t and V_{max} were as described in the previous section. The 5 min incubation period represents the best possible approach to initial rates since shorter incubation periods are technically not feasible with these fragile cells. Although under the present conditions there was probably some back migration which could influence the K_t values these probably do not depart (according to Winter and Christensen, 1964) by more than one-third from those corresponding to initial rates of entry. During the 5 min incubation period there was little metabolism of the amino acids being transported. For example, of the added 14 C-glutamic acid, 89% of the radioactivity was recovered intact, 4% was in CO_2 , 4% in organic acids and 1% in protein; similarly, lysine and arginine both showed recovery of 95% of the added radioactivity in the form of the free amino acid with 3% incorporation into protein. Isotopes were obtained from New England Nuclear Corp., all were labeled with carbon-14 and were L-isomers.

Amino acid transport, antimalarials and ATP levels. "Free parasites" and erythrocytes were prepared as described above. Erythrocyte suspensions (30%, v/v) were made up in a high-sodium medium containing plasma amino acids, and parasites were suspended in a high-potassium medium containing red cell amino acids (Sherman, et al., 1969) at a 30% concentration. Cell suspensions were pre-warmed for 3 min and then 5ml aliquots were added to flasks containing 0.5 μ Ci of the amino acid under study plus an antimalarial drug; a control flask contained only the radioactive amino acid. Incubation was carried out for 2 hrs at 37–38°C in a rocking water bath. Addition of 10ml of ice-cold G-S terminated

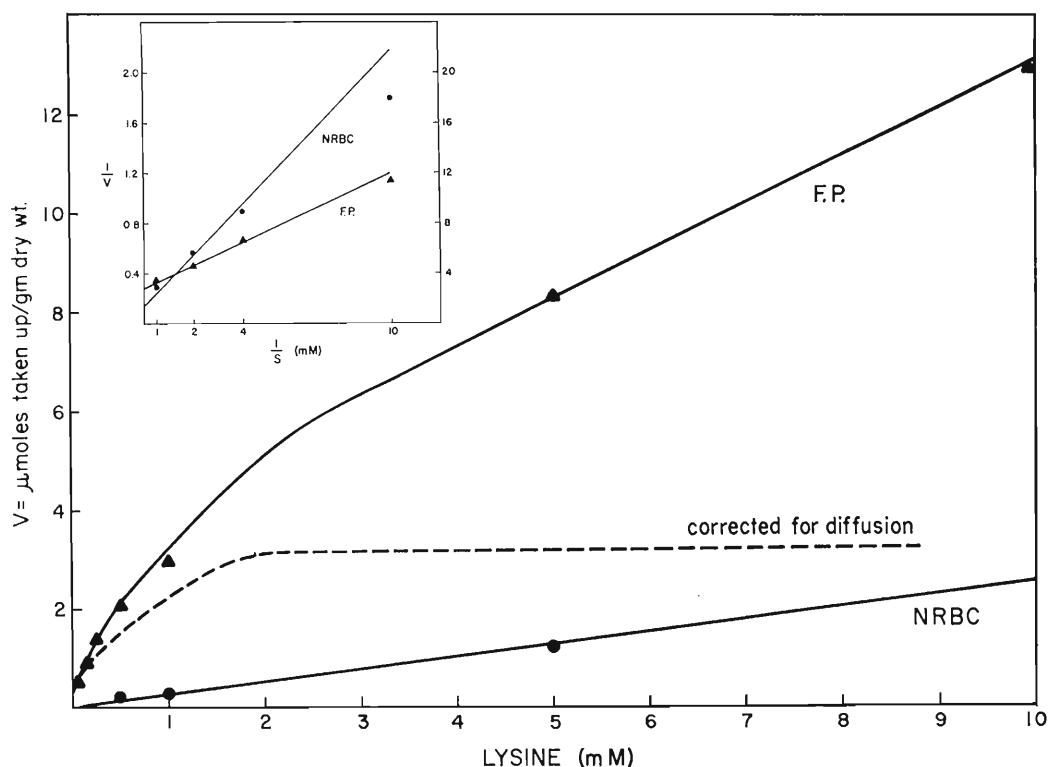


Figure 1. The uptake of lysine by erythrocyte-free *Plasmodium lophurae* (F.P.) and normal duck erythrocytes (NRBC). The incubation time was 5 min at 37° C. The linear portion of the curve for the free parasites was interpreted to be diffusion. To arrive at the diffusion-corrected curve a line was constructed with the same slope as the linear portion of the curve and passing through the origin. Values of this diffusion component were then subtracted from those derived experimentally. The insert shows a double-reciprocal plot (Lineweaver-Burk) of the same data.

the reaction. Methods for calculation of intracellular and extracellular radioactivity and incorporation of isotope into protein were according to previous work (Sherman et al., 1967, 1969). ATP was determined by an enzymatic method (ATP Stat-Pack, Calbiochem). To a neutralized protein-free filtrate of blood the following reagents were added: glucose, hexokinase, glucose-6-phosphate dehydrogenase and NADP. In the presence of hexokinase ATP phosphorylates glucose, the glucose-6-phosphate formed is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase and concomitantly NADP is reduced to NADPH. The increase in absorbance at 340 nm was used for the calculation of ATP levels in the extract of blood.

Results

Transport of amino acids in simple media. When duck erythrocytes were incubated for 5 min in various concentrations of amino acids, the rate of uptake was not a linear function of concentration. Rather, the entry process was saturable and obeyed Michaelis-Menten kinetics, suggesting carrier mediated transport of these amino acids. The K_t (transport constant equivalent to the apparent Michaelis constant) and V_{max} (maximal velocity) were estimated from double reciprocal plots (Lineweaver and Burk, 1934). The K_t and V_{max} values for 6 amino acids and normal duck erythrocytes are presented in Table 1. The kinetic patterns of amino acid entry for mature duck erythrocytes were similar to those re-

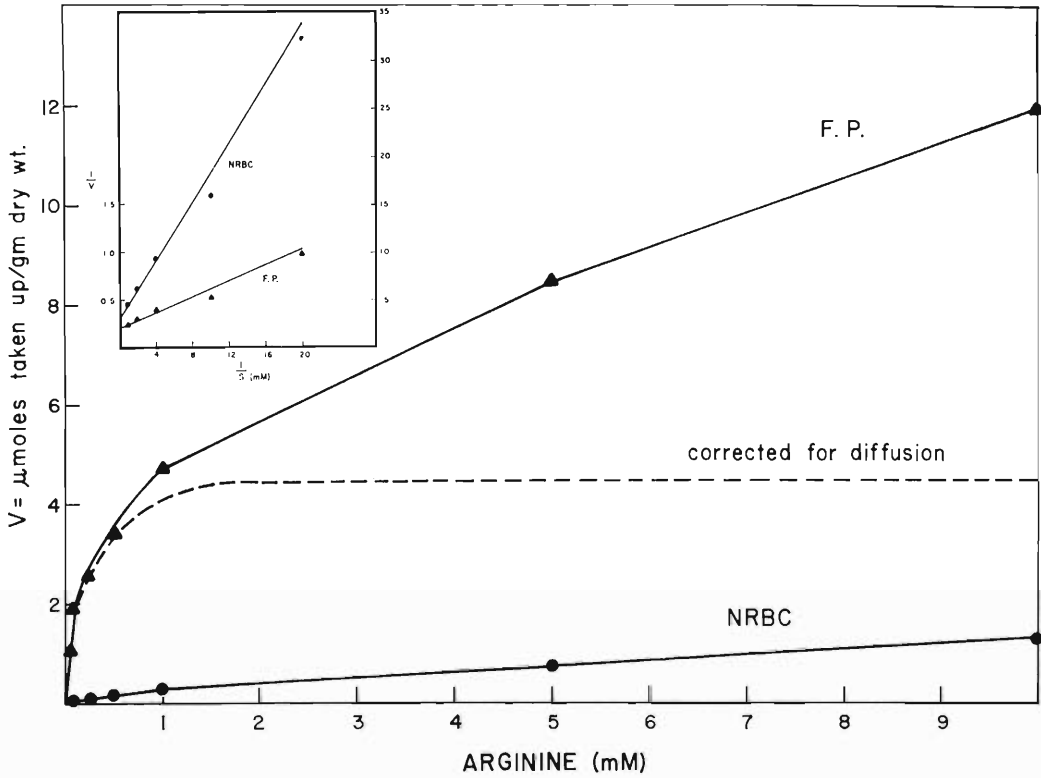


Figure 2. The uptake of arginine by erythrocyte-free *Plasmodium lophurae* (F.P.) and normal duck erythrocytes (NRBC). Incubation time was 5 min at 37° C. Diffusion-corrected curve was obtained as described in Figure 1. The insert shows a double-reciprocal plot (Lineweaver-Burk) of the same data.

ported for mammalian (Winter and Christensen, 1964, 1965) and pigeon erythrocytes (Vidaver, 1964). By contrast, when similar experiments were conducted with *P. lophurae*-infected erythrocytes, the entry process was

not saturable for most of the amino acids examined (glycine was an exceptional case). A non-saturable entry process cannot be used to estimate a transport constant (K_t) and such a kinetic pattern probably represents entry by

Table 1. Kinetic constants for the entry of several amino acids into normal and malaria-infected duck erythrocytes and erythrocyte-free *P. lophurae*. (Values are mean \pm standard error).

	Normal erythrocytes		Infected erythrocytes		<i>Plasmodium lophurae</i>	
	K_t for uptake (mM)	V_{max} μmoles/g dry wt	K_t for uptake (mM)	V_{max} μmoles/g dry wt	K_t for uptake (mM)	V_{max} μmoles/g dry wt
Glycine	0.54 \pm 0.06	0.77 \pm 0.04	1.26 \pm 0.19	0.98 \pm 0.09	Diffusion	—
Alanine	0.99 \pm 0.08	1.79 \pm 0.09	Diffusion	—	Diffusion	—
Isoleucine	8.9 \pm 1.92	8.15 \pm 1.69	Diffusion	—	Diffusion	—
Methionine	2.25 \pm 0.32	2.39 \pm 0.26	Diffusion	—	Diffusion	—
Lysine	1.91 \pm 0.37	0.92 \pm 0.15	—	—	0.37 \pm 0.04	3.82 \pm 0.19
Arginine	0.55 \pm 0.07	0.35 \pm 0.05	—	—	0.25 \pm 0.04	5.31 \pm 0.37
Glutamic Acid	—	—	—	—	1.24 \pm 0.13	20.26 \pm 1.38
Aspartic Acid	—	—	—	—	1.25 \pm 0.075	1.25 \pm 0.048

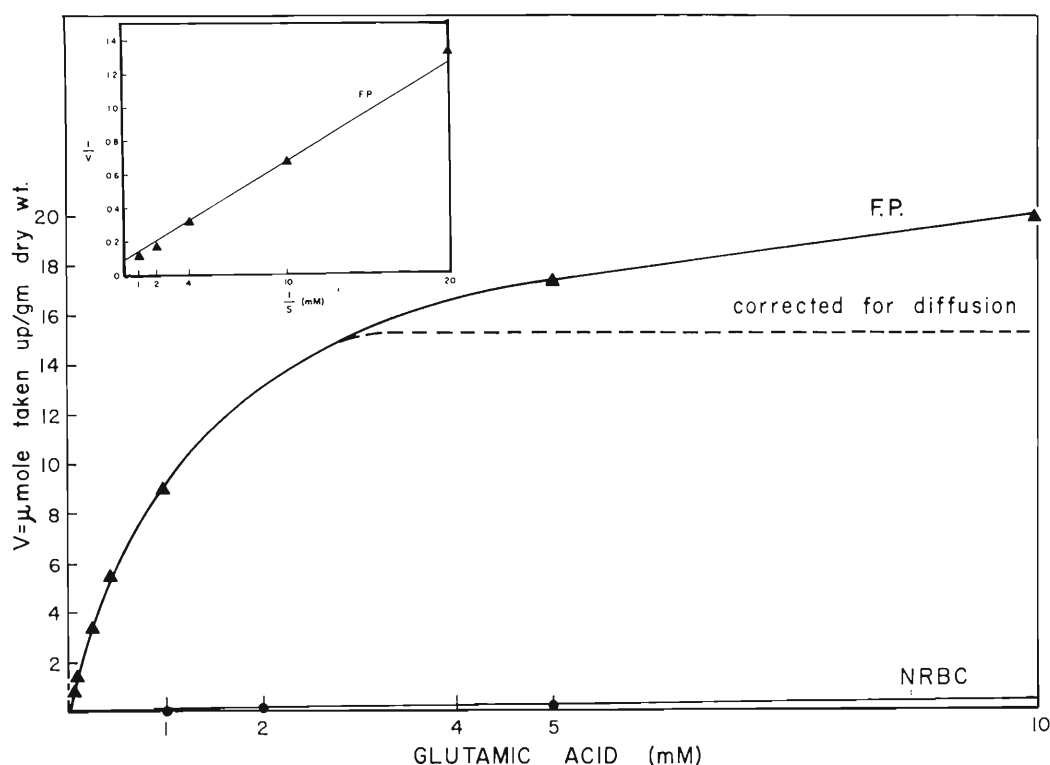


Figure 3. The uptake of glutamic acid by erythrocyte-free *Plasmodium lophurae* (F.P.) and normal duck erythrocytes (NRBC). The incubation time was 5 min at 37° C. Diffusion-corrected curve was obtained as described in Figure 1. Insert shows a double-reciprocal plot (Lineweaver-Burk) of the same data for the free parasites.

diffusion. When erythrocyte-free *P. lophurae* were studied at different amino acid concentrations two patterns of entry were observed: (a) a non-saturable system, presumably representing diffusion and (b) a saturable system. In the latter case (for arginine, lysine, glutamic and aspartic acids) the kinetic plots could be resolved into components, a saturable one at low substrate levels and a non-saturable one at higher amino acid concentrations (Figures 1–3). The non-saturable component is attributed to diffusion, although it is possible that higher concentrations of substrate might reveal saturation conditions. When the kinetic plots were corrected for diffusion, typical saturation curves were obtained. The K_t and V_{max} values for *P. lophurae* (Table 1) were estimated from Lineweaver-Burk plots of the saturable system.

Since arginine, lysine, and glutamic acid appeared to be the only amino acids entering the malarial parasite by a carrier-mediated process, as well as showing uphill transport (Sherman et al., 1971), it seemed of some interest to study the nature of the interactions between these amino acids. When the effects of arginine on lysine uptake and the effects of lysine on arginine uptake were investigated, it was found that each reciprocally and competitively inhibited the uptake of the other (Figure 4). This suggests that arginine and lysine both enter the parasite through the same transport locus. Similar results (not shown here) were obtained with glutamic and aspartic acids. Therefore, it appears that the malarial parasite has two distinct transport loci, one with an affinity for dibasic amino acids (lysine and arginine) and another inter-

acting with dicarboxylic amino acids (aspartic and glutamic acids); all other amino acids appear to enter the parasite by simple diffusion.

Amino acid transport in complex media. Read and his colleagues have published extensively on amino acid transport in tapeworms (Read, et al., 1963; Woodward and Read, 1969; Harris and Read, 1968). As a result of such work it was suggested that if an environment offers a mixture of amino acids, and these compete with or affect the entry of one another into the parasite tissues, then the ratios of amino acids in the mixture could be of importance in determining whether the nutritional requirements of the parasite will be met. To determine whether this contention holds true for malaria parasites, transport studies of glutamic acid, lysine and arginine in the presence of complex amino acid mixtures were conducted. The results of these studies (Table 2) demonstrated that an equimolar array of amino acids was inhibitory for amino acid transport (that is, the K_t was increased); however, there was little or no effect on the K_t and V_{max} values when plasma and amino acid mixtures were used. (An exceptional case was the K_t value for arginine in the presence of plasma amino acids.) The apparent lack of inhibition by differing physiological arrays of amino acids in the malaria parasite and the striking inhibitory effects seen in the tapeworms may be a consequence of the tapeworms showing mediated transport for all amino acids as well as overlapping affinities for the various loci, whereas the plasmodium shows only two transport loci, these do not overlap and the majority of amino acids do not enter by a carrier-mediated process.

Antimalarials and amino acid transport and incorporation. Studies of the antimalarials

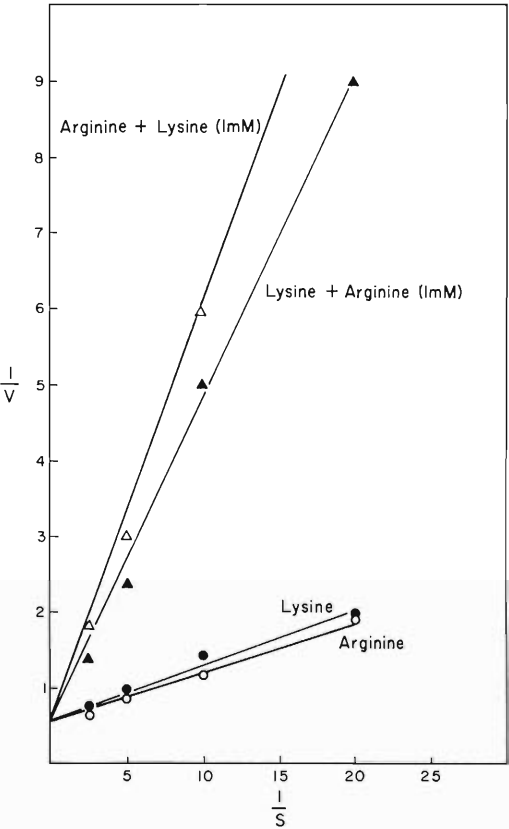


Figure 4. The effect of 1.0mM lysine on the uptake of ^{14}C -arginine, and the effect of 1.0mM arginine on the uptake of ^{14}C -lysine.

quinine, chloroquine and primaquine on the uptake and incorporation of amino acids by synchronized cultures of the free-living ciliate *Tetrahymena pyriformis* showed little effect on cell-free protein synthesis, but did markedly reduce both uptake and incorporation of radioactive amino acids *in vivo* (Conklin and

Table 2. Kinetic constants for erythrocyte-free *P. lophurae* in media containing varied arrays of amino acids. (Values are mean \pm standard error).

Amino acid	Single amino acid	Plasma amino acids	Red cell amino acids	Equimolar amino acids
Glutamic Acid (n = 3)	K_t 1.89 \pm 0.18 V_{max} 17.66 \pm 1.24	1.81 \pm 0.11 17.92 \pm 0.86	2.02 \pm 0.26 20.81 \pm 2.11	2.66 \pm 0.33 25.06 \pm 2.54
Lysine (n = 2)	K_t 0.30 \pm 0.09 V_{max} 3.87 \pm 0.52	0.31 \pm 0.06 3.24 \pm 0.37	0.31 \pm 0.05 3.70 \pm 0.24	0.50 \pm 0.06 4.50 \pm 0.30
Arginine (n = 2)	K_t 0.198 \pm 0.028 V_{max} 2.535 \pm 0.148	0.772 \pm 0.120 2.98 \pm 0.284	0.37 \pm 0.068 2.75 \pm 0.22	0.609 \pm 0.102 3.46 \pm 0.31

Table 3. Uptake and incorporation of ^{14}C -labeled amino acids in the presence of antimalarials and cycloheximide. (Values are mean percent \pm standard error). Incubation time was 2 hrs; drug concentrations are shown in figure 5.

	Antimalarial	Alanine		Arginine	
		% [*] internal	% ^{**} incorporated	% [*] internal	% ^{**} incorporated
Normal red cells	None	27.5 \pm 1.6	26.8 \pm 1.4	22.7 \pm 1.2	47.9 \pm 3.7
	Cycloheximide	25.1 \pm 1.5	6.2 \pm 0.4 ^b	17.3 \pm 0.7	7.2 \pm 0.2 ^b
	Quinacrine	27.1 \pm 1.5	24.8 \pm 1.4	22.0 \pm 1.3	44.1 \pm 4.8
	Primaquine	11.2 \pm 0.4 ^b	3.2 \pm 1.3 ^b	10.8 \pm 0.6 ^b	2.8 \pm 0.5 ^b
	Chloroquine	14.1 \pm 0.5 ^b	3.2 \pm 0.5 ^b	10.8 \pm 0.4 ^b	1.4 \pm 0.4 ^b
	Quinine	13.6 \pm 0.4 ^b	3.8 \pm 0.7 ^b	13.7 \pm 0.8 ^b	4.5 \pm 0.2 ^b
Infected red cells	None	12.0 \pm 0.9	7.3 \pm 1.1	13.9 \pm 2.2	26.7 \pm 3.8
	Cycloheximide	12.1 \pm 0.9	1.9 \pm 0.1 ^b	12.8 \pm 1.8	3.8 \pm 0.4 ^b
	Quinacrine	11.8 \pm 0.9	6.8 \pm 0.4	12.6 \pm 2.2	21.9 \pm 4.2
	Primaquine	7.6 \pm 1.0 [?]	1.9 \pm 0.2 ^b	6.6 \pm 1.2 ^a	2.8 \pm 0.7 ^b
	Chloroquine	8.9 \pm 1.1 [?]	2.5 \pm 0.2 ^b	6.9 \pm 1.2 ^a	2.2 \pm 0.6 ^b
	Quinine	10.0 \pm 1.5	1.6 \pm 0.3 ^b	8.7 \pm 2.4 ^a	5.9 \pm 1.4 ^b
Free parasites	None	8.2 \pm 0.1	31.0 \pm 4.8	22.0 \pm 1.0	38.7 \pm 2.8
	Cycloheximide	6.0 \pm 0.4	1.3 \pm 0.1	17.4 \pm 1.8	0.8 \pm 0.1 ^b
	Quinacrine	8.1 \pm 0.4	31.0 \pm 4.4	13.1 \pm 0.6 ^a	15.8 \pm 1.8
	Primaquine	5.7 \pm 0.2	4.0 \pm 0.5 ^b	7.6 \pm 0.5 ^c	3.2 \pm 0.5 ^b
	Chloroquine	5.6 \pm 0.5 ^a	3.9 \pm 0.5 ^b	6.1 \pm 0.5 ^c	1.0 \pm 0.1 ^b
	Quinine	6.1 \pm 0.4	2.4 \pm 1.1 ^b	7.5 \pm 0.5 ^c	2.1 \pm 0.3 ^b

$$^* \% \text{ internal} = \frac{\text{intracellular free amino acid} + \text{incorporated amino acid}}{\text{total amino acid available}} \times 100.$$

$$^{**} \% \text{ incorporated} = \frac{\text{incorporated amino acid}}{\text{intracellular free amino acid} + \text{incorporated amino acid}} \times 100.$$

^a Significantly different from control at 5% level.

^b Significantly different from control at 1% level.

^c Significantly different from control at 0.1% level.

[?] Although these values are significantly different there is some question as to the validity of such differences in view of the fact that the infected cells contained uninucleate parasites.

Chou, 1970). This suggested that these antimalarial drugs act by directly blocking the uptake of amino acids. Using the *P. lophurae* test system, chloroquine, primaquine and quinine were found to inhibit amino acid incorporation by all cells (Table 3); quinacrine was without effect on protein synthesis. Cycloheximide, a known inhibitor of protein synthesis but not an inhibitor of amino acid transport, also blocked incorporation of radioactive amino acids. Amino acid entry was blocked by antimalarials (excluding quinacrine which had no effect) but this depended on the particular amino acid and the type of cell. For example, alanine uptake was blocked only in the normal erythrocyte and arginine entry was inhibited in all cells (Table 3). These results were anticipated, based on the mode of entry of these amino acids into the various cells. Arginine enters all cells by a carrier-mediated process and alanine demonstrates carrier-mediated entry only in normal cells.

The disparate results for entry and incorporation suggested that the antimalarial effects were indirect, and that these drugs did

not directly block amino acid uptake. To test this possibility, adenosine triphosphate (ATP) levels were determined for normal and malaria-infected erythrocytes in the presence and the absence of antimalarials (Figure 5). The ATP content of the malaria-infected cell was considerably reduced over that found prior to infection, and the degree of reduction was related to parasite size. When normal erythrocytes were incubated for 2 hrs in the presence of the antimalarials primaquine, chloroquine and quinine the ATP levels were depressed approximately 50%. No such decline in ATP levels was evident in erythrocytes containing multinucleate parasites. It should be emphasized that the antimalarials which were inhibitory for amino acid entry were those which depressed the levels of ATP in the normal erythrocyte. These same drugs also blocked amino acid incorporation.

Discussion and Conclusions

Amino acids entered the mature duck erythrocyte by a saturable, nonuphill transport system. Upon infection with *P. lophurae*

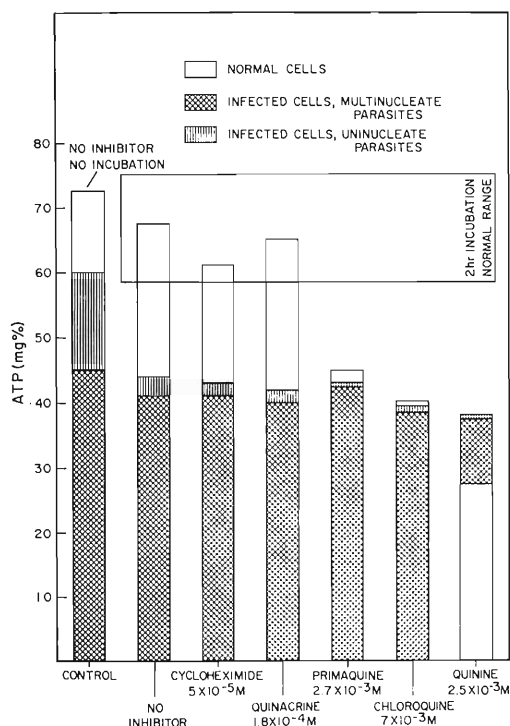


Figure 5. ATP levels in normal and malaria-infected erythrocytes in the presence of antimalarials and cycloheximide.

there was a striking alteration in the mode of entry of amino acids: the entry rate was linear with substrate concentration, following a pattern typical of diffusion kinetics. Only in the case of glycine was the entry process saturable. It would seem that during the parasite's residence within the red cell the amino acid transport carriers of the host cell are either inactivated by the plasmodium, or else it produces some substance which changes the permeability properties of the red cell rendering it highly permeable to most amino acids. In addition, there also exists the possibility that the parasite exerts its effects on amino acid entry into the host red cell by an indirect mechanism, that is by removing some necessary component required by the transport process itself. One component that could play such a role is the cofactor ATP, and indeed there is some evidence (presented below) to support this view. It should be noted that in *P. knowlesi*-infected monkey erythrocytes iso-

leucine and methionine show uphill transport (McCormick, 1970), demonstrating that the entry process of amino acids may differ with the various species of *Plasmodium*.

Previously it was shown (Sherman et al., 1971) that erythrocyte-free *P. lophurae* concentrated only 3 amino acids (arginine, lysine and glutamic acid), and apparently all other amino acids entered the parasite by diffusion. Using an amino acid concentration range from 0.05mM–10mM the uptake of glycine, alanine, isoleucine and methionine by free parasites was a linear function; this supports the view that these amino acids enter the malaria parasite by diffusion instead of by a carrier-mediated process. By contrast, arginine, lysine, glutamic and aspartic acid uptake by the parasite was a saturable process, following Michaelis-Menten kinetics. The competitive inhibition of arginine-lysine and glutamic acid-aspartic acid indicates that the parasite probably has two transport loci, one preferentially transporting dibasic amino acids and the other transporting dicarboxylic amino acids. Further studies will be necessary to demonstrate whether these loci interact with one another or with other amino acids. Additionally, at present there is no evidence for or against the involvement of sodium ions in the transport of these amino acids across the surface of the plasmodium.

Based on the foregoing considerations, namely that most amino acids enter the *P. lophurae*-infected erythrocyte and the parasite itself by diffusion, a direct inhibition of amino acid transport by antimalarials (as proposed by Conklin and Chou, 1970) seemed unlikely. The *in vitro* *P. lophurae*-duck erythrocyte test system indicated that primaquine, chloroquine and quinine inhibited uptake of amino acids only in those cases where amino acid entry was shown to involve a carrier-mediated system (Table 3). Whenever an antimalarial drug inhibited amino acid entry there was also a depression in protein synthesis, a phenomenon that is most likely a result of precursors not being made available to the intracellular protein-synthesizing machinery. The fact that some antimalarials were capable of inhibiting protein synthesis without affecting amino acid entry (e.g. alanine in infected cells and free parasites) suggested that these drugs could exert their effects in an indirect manner.

When normal duck erythrocytes were incubated for 2 hrs in the presence of primaquine, chloroquine and quinine, the ATP levels were severely depressed when compared to control samples incubated without antimalarials. Since the antimalarials which depressed the ATP levels of the uninfected erythrocyte were those most effective in inhibiting amino acid transport it is suggested that their action is due primarily to an inhibition of host cell energetics and not to a direct block of amino acid entry. Although considerable controversy still exists concerning the molecular action of these antimalarials, the present work does reaffirm earlier studies with quinine which indicated a role in blocking cellular energy formation (Moulder, 1948, Silverman, et al., 1944). Trager (1967b) found that chloroquine inhibited the development of *P. lophurae* intracellularly, but had no effect on parasites grown extracellularly, and Clarke (1952) found quinine to have similar effects on the growth of *P. gallinaceum*. Polet and Barr (1968a) showed that chloroquine inhibited protein synthesis in *P. knowlesi*-infected monkey cells growing *in vitro*. In the present system uptake and incorporation were inhibited both intracellularly and extracellularly after *in vitro* incubation with antimalarials for a duration of 2 hours. Perhaps the reason for inhibition of protein synthesis in extracellular *P. lophurae* in the present work and the absence of effect in Trager's system is related to the considerably higher concentrations of drugs used by us. Although the drug concentrations used here were considerably higher than those encountered in the blood plasma of animals treated with such antimalarials there still exists the possibility that some of these drugs are accumulated by infected red cells and thus the parasites are actually exposed to much higher concentrations than those measured in the plasma. Notwithstanding these limitations the *in vitro* *P. lophurae*-duckling erythrocyte incorporation test system could provide a rapid, and relatively inexpensive screening method for potential antimalarials provided the appropriate amino acids are chosen. (Trager (1971) has also used radioisotope incorporation as an indicator of growth in the extracellular cultivation of *P. lophurae*.)

In 1962 James Moulder suggested that

malaria parasites might have a permeability "defect." His reasoning was roughly as follows: malaria parasites, free of their host erythrocyte and grown extracellularly, have the remarkable ability to take up highly polar substances (e.g. ATP, pyridine nucleotides, CoA, acids of the tricarboxylic acid cycle and folic acid) which almost always fail to penetrate intact cells. "This ability," he said, "to take up large, complex and metabolically active molecules is clearly of great advantage in an intracellular existence. In fact, without this ability, little is gained by living inside a cell in preference to outside. Thus, it seems reasonable that, in the course of evolutionary adaptation to life inside the red cell, the malarial parasite may have lost many of the active transport systems regulating passage of molecules in both directions across its cell membrane and have become freely permeable to all sorts of molecules which it derives directly from its host" The pattern of amino acid entry into the erythrocyte-free plasmodium support Moulder's permeability "defect" hypothesis. Of all the amino acids tested only lysine, arginine, aspartic and glutamic acids entered via a mediated (active transport) process, all others were taken up by simple diffusion.

The present work suggests that Moulder's permeability defect hypothesis for the malarial parasite can be extended to the *P. lophurae*-infected erythrocyte. The malaria-infected cell becomes "leaky" to most amino acids, and shows kinetic patterns consistent with those described for diffusion. The malaria-infected red cell shows two other prominent changes (a) an increased sodium ion content (Dunn, 1969; Sherman and Tanigoshi, 1971) and (b) a decreased level of ATP* (Trager, 1967a). To relate these findings to one another, the following hypothesis is proposed: There is an apparent need for ATP in the malaria-infected cell, and this is reflected by decreased levels of ATP in the parasitized erythrocyte. More than 20 years ago Trager (1950) suggested ATP as an important determinant of the parasitic nature of *P. lophurae*, and most recently this ATP-dependence has been ex-

* It is of interest to note that the ATP levels of normal and *P. lophurae*-infected erythrocytes determined by Trager and in the present work were similar despite the use of somewhat different enzymatic methods.

tended to include *P. berghei*, *P. vinckei*, and *P. falciparum* (Brewer and Powell 1965; Brewer and Coan, 1969). Although the exact nature of the role of ATP in the economy of the parasite remains uncertain, there is the distinct possibility that the parasite's ATP requirement may in part be related to its need for preformed purines for nucleic acid synthesis (Tracy and Sherman, 1972). In any event, as the plasmodium grows the ATP pool of the host cell declines, and it is conceivable that this produces a failure in the host cell's sodium pump. This would decrease the efflux of sodium in the malaria-infected cell, and contribute to the increased sodium content of the parasitized erythrocyte. Since the flow of certain amino acids from the plasma into the red cell is a function of the differences in sodium ion concentrations inside and outside the red cell, this failure of the sodium pump could prevent the entry of amino acids coupled to such a system. As a consequence, the parasite would be unable to utilize the amino acid stores of the plasma unless it was able to change the permeability properties of the host cell. This it appears to do, as evidenced by the entry of most amino acids into the infected cell by diffusion. The nature of the material, presumably elaborated by the growing parasite, which effects such changes in the host cell membrane is unknown, but it could be similar to the substance extracted from *P. lophurae*-infected cells which enhanced the osmotic fragility of normal duck erythrocytes (Herman, 1969).

The hypothesis presented above suggests that the primary lesion induced in the host cell by the malarial parasite could be a depletion of host cell ATP. Secondary effects of this would be malfunctions in the ion and amino acid transport systems. The hypothesis further postulates that amino acid transport into the malaria-infected cell can continue only by the utilization of sodium-independent transport systems and/or by the host cell membrane becoming "leaky." Future work will be necessary to validate this hypothesis.

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Studies on the Mode of Action of Primaquine Using *Tetrahymena pyriformis**

K. A. CONKLIN AND S. C. CHOU

Department of Pharmacology, School of Medicine,
University of Hawaii, Honolulu, Hawaii 96822

ABSTRACT: DNA and RNA syntheses were characterized in isolated nuclei from *Tetrahymena pyriformis*. Primaquine was observed to have relatively little inhibitory effect on nucleic acid synthesis in these organelles as compared to previously reported inhibition of nucleic acid precursor incorporation by intact cells. These results indicate that a prominent *in vivo* effect of primaquine in *T. pyriformis* is inhibition of precursor uptake, and not a direct inhibition of DNA and RNA syntheses.

Studies on the mode of action of primaquine have been carried out in several systems, with the results suggesting several possible sites of action. Schellenberg and Coatney (1961) investigated the effects of this drug on ^{32}P -orthophosphate incorporation into nucleic acids by *Plasmodium gallinaceum* and *Plasmodium berghei*. Their results indicated that the mode of action of primaquine was unrelated to any effect on phosphate assimilation into DNA or RNA, and they suggested that this may denote an interference with energy utilization by the parasites. Support for this theory is provided by the investigations of exoerythrocytic stages of *P. fallax* by Aikawa and Beaudoin (1969, 1970). Utilizing high-resolution autoradiography and studying the morphological effects of primaquine on these parasites, they demonstrated that the drug first becomes localized within the parasite mitochondria, where it is often associated with mitochondrial swelling. Skelton *et al.* (1968) have also demonstrated that primaquine inhibits, *in vitro*, the mitochondrial oxidation of DPNH and succinate by coenzyme Q. Whichard *et al.* (1968) and Morris *et al.* (1970), however, have demonstrated by equilibrium dialysis and direct spectrophotometry that primaquine binds to DNA, and they suggested that a portion of the anti-malarial activity of this compound is related to inhibition of DNA function. Another potential mode of action was suggested by Olenick and Hahn (1971), who proposed that the major *in vivo* action of primaquine in

Bacillus megaterium is to block protein synthesis.

Previously, we have shown several effects of primaquine on *Tetrahymena pyriformis*, a ciliate protozoan. Our initial results demonstrated that the drug blocked the incorporation of precursors into DNA, RNA, and protein by intact cells (Conklin and Chou, 1970, 1972a). These results could be interpreted in at least three ways: a) the drug has a direct inhibitory effect on these biosynthetic pathways; b) the drug inhibits the transport of the macromolecular precursors into the cell; or c) the drug inhibits the energy-generating systems of the organism and thus blocks all cellular biosynthetic pathways and energy-dependent membrane transport mechanisms for the macromolecular precursors. To investigate these possibilities, we subsequently utilized several subcellular preparations from *T. pyriformis*, as well as intact cells.

Utilizing isolated mitochondria (Conklin and Chou, 1972b) to investigate energy-generating systems in *T. pyriformis*, primaquine was shown to inhibit the oxidation of several tricarboxylic acid cycle intermediates, and also act as an uncoupling agent (Conklin *et al.*, 1971). However, the drug concentration used in these studies was that which completely blocks cell division, and since the degree of inhibition of mitochondrial functions was considerably less than complete (10–40% inhibition depending on the reaction tested), we felt that these effects did not totally account for the action of primaquine.

We subsequently utilized two experimental procedures to evaluate the effect of primaquine on the transport (or uptake) of the macro-

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Table 1. Requirements for DNA synthesis in isolated nuclei.^a

Incubation mixture	Counts/min/10 ⁶ nuclei	Relative Incorporation
Standard	702	100
Minus ATP	250	36
Minus dATP	428	61
Minus dGTP	260	37
Minus dCTP	253	36
Minus dATP, dGTP, and dCTP	102	15
Minus ATP, dATP, dGTP, and dCTP	35	5
Minus EDTA	477	68
Minus KCl	568	81
Plus DNase I, 0.1 mg/ml	180	27
Plus DNase I, 0.1 mg/ml ^b	56	8
Plus RNase A, 0.1 mg/ml	912	130
Plus RNase A, 0.1 mg/ml ^b	540	78

^a Assay conditions are as described in "Materials and Methods." Each value is the mean of 2 determinations.
^b The nuclei were preincubated with the enzyme for 10 min prior to addition to the reaction mixture.

molecular precursors by intact cells. The first was comparing uptake and incorporation of precursors in the presence of primaquine, and comparing these values to those observed with known inhibitors of synthesis (Conklin and Chou, 1970, 1972a). Nalidixic acid, actinomycin D, and cycloheximide were used as the known inhibitors of DNA, RNA, and protein syntheses respectively, and the results with these agents were compared to those with primaquine. The known inhibitors were found to inhibit incorporation of thymidine (for DNA), uridine (for RNA), and amino acids (for protein) to a considerably greater degree than uptake of these precursors. Primaquine, however, produced the same, or nearly the same, inhibition of uptake as incorporation. Therefore precursor incorporation appeared to be limited by uptake in the presence of this drug, and this indicated that inhibition of precursor uptake is one of the actions of primaquine. The second procedure was carried out utilizing cells in which DNA, RNA, or protein synthesis was blocked (Conklin and Chou, 1972a). With this experimental design it was possible to examine the effects of primaquine on the uptake of precursors independently of incorporation. Each precursor (thymidine, uridine, or amino acids) was examined separately by blocking the appropriate biosynthetic pathway (DNA, RNA, or protein synthesis) with nalidixic acid, actinomycin D, or cycloheximide. In each of these experiments primaquine was observed to block the uptake of the precursor

being studied. Therefore, these results substantiated the above conclusion that primaquine inhibits precursor uptake.

There is an additional line of evidence which would support the hypothesis that inhibition of precursor incorporation by primaquine *in vivo* is due to inhibition of uptake. This would be to demonstrate that the drug does not block the biosynthetic pathways *in vitro*. Using an *in vitro* test system for protein synthesis (Conklin and Chou, 1971), our results indicate that primaquine does not inhibit this pathway in *T. pyriformis* (Conklin and Chou, 1970). Therefore, the inhibitory effect of primaquine on amino acid incorporation by intact cells appears to be due to inhibition of amino acid uptake, and not to inhibition of protein synthesis. Further *in vitro* results, in which we demonstrate the effects of primaquine on nucleic acid synthesis in isolated nuclei, are presented below.

Materials and Methods

Tetrahymena pyriformis, strain GL, was grown and harvested as described previously (Conklin *et al.*, 1969). Nuclei were prepared according to the procedure of Lee and Scherbaum (1965), and the number of nuclei was determined using a hemocytometer.

The standard reaction mixture for assay of RNA synthesis of the nuclear preparation contained in a total volume of 0.25 ml the following: 0.5 mM ATP; 0.05 mM GTP and CTP; 0.5 μ Ci (5-³H)-UTP (17.8 Ci/mM, Schwarz BioResearch); 60 mM KCl and NaCl; 25 mM Tris-HCl, pH 7.5; 1.5 mM MgCl₂; 5 mM mercaptoethanol; 0.25 M sucrose; and 1-3 \times 10⁶ nuclei. The reaction was started by addition of the nuclei, and duplicate 0.1 ml samples were withdrawn after 30 min of incubation at 28°C and processed by the filter paper disc procedure of Byfield and Scherbaum (1966) to determine incorporation of precursors. A blank contained all constituents of the standard incubation mixture except the nuclei. The samples were counted by liquid scintillation as described previously (Conklin *et al.*, 1969).

The standard reaction mixture for assay of DNA synthesis of the nuclear preparation contained in a total volume of 0.25 ml the

Table 2. Requirements for RNA synthesis in isolated nuclei.^a

Incubation mixture	Counts/min/10 ⁶ nuclei	Relative Incorporation
Standard	12,690	100
Minus ATP	252	1
Minus GTP	2,360	19
Minus CTP	2,090	16
Minus ATP, GTP, and CTP	50	0
Minus NaCl and KCl	4,115	32
Plus Actinomycin D, 1.6 × 10 ⁻⁵ M	2,380	19
Plus Actinomycin D, 1.6 × 10 ⁻⁶ M	3,335	26
Plus DNase I, 0.1 mg/ml	2,740	22
Plus DNase I, 0.1 mg/ml ^b	0	0
Plus RNase A, 0.1 mg/ml	0	0
Plus RNase A, 0.1 mg/ml ^b	0	0

^a Assay conditions are as described in "Materials and Methods." Each value is the mean of 2 determinations.
^b The nuclei were preincubated with the enzyme for 10 min prior to addition to the reaction mixture.

following: 0.5 mM ATP; 0.05 mM dATP, dCTP, and dGTP; 2.5 μCi (methyl-³H)-TTP (11.1 Ci/mM, Schwarz BioResearch); 0.3 mM EDTA; 50 mM KCl; 25 mM Tris-HCl, pH 7.5; 1.5 mM MgCl₂; 5 mM mercaptoethanol; 0.25 M sucrose; and 1–3 × 10⁶ nuclei. Other conditions are as described above for assay of RNA synthesis.

Results

Requirements for DNA and RNA syntheses in isolated nuclei are shown in Tables 1 and 2 respectively. Maximum DNA synthesis was dependent upon ATP, dATP, dGTP, dCTP, EDTA, and KCl, since omission of any component decreased incorporation. Treatment with DNase I markedly decreased incorporation, whereas treatment with RNase A had a lesser effect. These results are consistent with a DNA-dependent DNA polymerase reaction (DNA replication), and similar to those described by Pearlman and Westergaard (1969) for DNA synthesis by isolated DNA polymerases. Requirements for RNA synthesis were essentially as described by Lee and Byfield (1970) in that the system required ATP, GTP, CTP, NaCl, KCl, and was markedly inhibited by treatment with actinomycin D, DNase I, or RNase A. These results are consistent with a DNA-dependent RNA polymerase reaction. Both DNA and RNA reaction mixtures showed maximum incorporation after 30 min incubation.

Table 3 shows the effects of primaquine on DNA and RNA syntheses in isolated nuclei.

Table 3. The effects of Primaquine on DNA and RNA syntheses in isolated nuclei.^a

Primaquine concentration (moles/liter)	% Inhibition	
	DNA Synthesis	RNA Synthesis
2.7 × 10 ⁻⁴	18	7
5.4 × 10 ⁻⁴	26	14
2.7 × 10 ⁻³	90	51

^a Assay conditions are as described in "Materials and Methods." Each value is the mean of 2 determinations.

The drug concentrations used were those which inhibit cell division in *T. pyriformis* (Conklin and Chou, 1972a) by 89% (2.7 × 10⁻⁴ M) and 100% (5.4 × 10⁻⁴ M), and a concentration which is ten times the lower dose (2.7 × 10⁻³ M). At 2.7 × 10⁻⁴ M, primaquine inhibited DNA and RNA syntheses 18% and 7% respectively in isolated nuclei (*in vitro*). This same drug concentration in intact cells (*in vivo*), however, has been previously shown to inhibit incorporation of thymidine (into DNA) and uridine (into RNA) by 68% and 88% respectively (Conklin and Chou, 1972a). Twice this level (5.4 × 10⁻⁴ M) produced 26% inhibition, and ten times this concentration (2.7 × 10⁻³ M) produced 90% inhibition of *in vitro* DNA synthesis, whereas, these concentrations inhibited RNA synthesis 14% and 51%. Only at the highest drug level used *in vitro* (2.7 × 10⁻³ M) is the degree of inhibition comparable to the inhibitory effects of one tenth that dose *in vivo*.

Discussion

The inhibitory effect of primaquine on DNA and RNA syntheses in isolated nuclei was considerably less than the inhibition of thymidine and uridine incorporation by intact cells which was previously reported (Conklin and Chou, 1972a). Several factors, however, must be considered if we are to interpret these results as indicating that primaquine does not block nucleic acid synthesis of intact cells. First of all, the possibility exists that the drug may not reach a potential intranuclear site of action in the experiments using isolated nuclei. However, with isolated nuclei the drug need pass only the nuclear membrane, whereas with intact cells the drug must pass, in addition, the cytoplasmic membrane and any cytoplasmic barriers. Therefore, it is more likely that

the drug would reach this site more readily using isolated nuclei.

Another mechanism which may account for greater inhibition of nucleic acid synthesis *in vivo* than *in vitro* is active transport of the drug by intact cells. This could produce a much higher drug concentration in the nuclear environment of intact cells than was used *in vitro*. At this time, however, no such mechanism has been demonstrated in *T. pyriformis*.

A third mechanism which could account for a lesser effect of primaquine on nucleic acid synthesis *in vitro* would be if the drug was metabolized to a more active compound by intact cells. Such a possibility has been considered by Goodwin and Rollo (1955).

Lastly, the discrepancy between the *in vivo* and *in vitro* results may be explained by the difference in precursors utilized in these experiments. Since thymidine triphosphate (TTP), the immediate precursor for DNA, was used *in vitro* whereas thymidine was used *in vivo*, primaquine could block the *in vivo* precursor incorporation by inhibiting conversion of thymidine to TTP.

In conclusion, if we assume that the factors considered above do not account for the effects of the drug *in vivo* and *in vitro*, then the results presented above indicate that the primary effect of primaquine on *T. pyriformis* is not a direct inhibition of nucleic acid synthesis, but instead an inhibition of precursor uptake by the cells as previously suggested (Conklin and Chou, 1972a).

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Chloroquine Resistance in Malaria: Drug Binding and Cross Resistance Patterns*

C. D. FITCH

Departments of Internal Medicine and Biochemistry,
St. Louis University School of Medicine, St. Louis, Missouri 63104

ABSTRACT: A survey of drugs that compete with chloroquine for the high-affinity drug receptor of chloroquine-susceptible (CS) *P. berghei* reveals that the receptor will interact with a diverse group of drugs which have in common a planar ring system of 30 to 40 Å² and a protonated group either in the ring or nearby. Included in this group of drugs are derivatives of 4-aminoquinoline, of quinoline-4-methanol, of pyridine, and of pyrimidine. Although the structural requirements for strong binding to the receptor are incompletely defined, estimates of K_i values indicate that the strength of binding to the receptor depends on the structure of the drug.

The apparent K_i values for amodiaquine, amopyroquine, chloroquine, quinacrine, and quinine vary as would be expected from their therapeutic doses, i.e., when the dose is low the K_i is low. By contrast the apparent K_i for pyrimethamine is high but the therapeutic dose is low, indicating that pyrimethamine acts independently of this receptor. Combining these observations with existing knowledge of the pattern of cross resistance of chloroquine-resistant (CR) *P. berghei* supports the following conclusion: If the therapeutic dose of a drug required to cure infections with CS *P. berghei* and the strength with which the drug binds to the high-affinity drug receptor of CS *P. berghei* are both known, it is possible to accurately predict whether or not CR *P. berghei* will be cross resistant to the drug.

The binding of antimalarial drugs to biological macromolecules has been studied extensively since Ceithaml and Evans (1946) reported that chicken erythrocytes infected with *P. gallinaceum* concentrate quinine to a greater extent than do uninfected erythrocytes. Ceithaml and Evans recognized that the concentrative uptake of quinine might represent adsorption to cellular binding sites, but their data did not permit estimation of the affinity of the binding sites for quinine. Shortly thereafter, Berliner, *et al.* (1948) emphasized the role of plasma proteins in binding antimalarial drugs by measuring the extent of binding of 4-aminoquinoline derivatives in plasma. They found, for example, that approximately 50 percent of chloroquine at therapeutic concentrations in plasma is bound. Parker and Irvin (1952a) subsequently reported that the intrinsic association constant for the binding of chloroquine to albumin is approximately 10^4M^{-1} . Although this association constant indicates relatively weak binding of chloroquine, because of the high concentra-

tion of albumin in blood, relatively weak binding may limit the availability of chloroquine to parasitized erythrocytes.

Parker and Irvin (1952b) also reported that chloroquine binds to nucleic acids, and they suggested that this interaction should be considered in any attempt to explain the chemotherapeutic action of chloroquine. They estimated the intrinsic association constant for the interaction of chloroquine with nucleic acids to be approximately 10^4M^{-1} . Other workers including Kurnick and Radcliffe (1962), Stollar and Levine (1963), Cohen and Yielding (1965), and Allison *et al.*, (1965) have confirmed and extended these observations of chloroquine binding to nucleic acids, but no one has demonstrated binding of chloroquine with an intrinsic association constant of greater than 10^5M^{-1} to purified preparations of DNA or RNA. In addition to chloroquine and other derivatives of 4-aminoquinoline, derivatives of 8-aminoquinoline (Morris, *et al.* 1970) and of quinoline-4-methanol (Estenson, *et al.* 1969, Hahn and Fean, 1969) also are known to bind to nucleic acids. The quinoline-methanols apparently bind to DNA with intrinsic association constants greater than 10^6M^{-1} .

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Recent studies of drug uptake and binding by intact parasitized erythrocytes have used radioactive chloroquine as a tracer and *P. berghei* in mice (Macomber, *et al.* 1966, Fitch, 1969), *P. knowlesi* in rhesus monkeys (Polet and Barr, 1969), or *P. falciparum* in owl monkeys (Fitch, 1970) as experimental models of malaria. In each model system there is a remarkable accumulation of the drug by parasitized cells with concentration gradients (cells:medium) sometimes reaching 500 or greater. Of even more importance is the observation that erythrocytes infected with chloroquine-resistant (CR) parasites have a reduced capacity to concentrate chloroquine in comparison to erythrocytes infected with chloroquine-susceptible (CS) parasites.

My earlier studies of chloroquine resistance in malaria were designed to explain why erythrocytes infected with CR parasites have a reduced capacity to concentrate the drug (Fitch, 1969 and 1970). In those studies, washed uninfected erythrocytes or erythrocytes infected either with CS or with CR parasites were incubated *in vitro* with varying concentrations of chloroquine until steady-state conditions were reached. After incubation the amounts of chloroquine bound to the erythrocytes and remaining in the medium were measured. There was no degradation of chloroquine by these preparations. With a concentration of chloroquine in the incubation medium within the therapeutic range, 10^{-8} M, steady-state concentration gradients were 75 for erythrocytes infected with CS *P. falciparum*, 18 for erythrocytes infected with CR *P. falciparum* and 7 for normal owl monkey erythrocytes. Comparable studies of *P. berghei* in mouse erythrocytes revealed an analogous difference between CS and CR parasites: the gradients were 600 for CS parasites, 100 for CR parasites and 14 for uninfected erythrocytes. The processes responsible for these large gradients were saturable in both model systems, as would be expected for the binding of chloroquine to cellular constituents.

In detailed studies of the *P. berghei* model (Fitch, 1969), erythrocytes infected with CS parasites were found to have at least three classes of binding sites for chloroquine, the apparent intrinsic association constants of which were 10^8 , 10^5 , and 10^3 M $^{-1}$. Normal mouse erythrocytes had a class of binding sites

with low affinity, and erythrocytes infected with CR parasites had binding sites with low and intermediate affinities. The important difference between infections with CS and CR parasites was a deficiency of high-affinity chloroquine binding in the CR infections. This deficiency accounts for the reduced capability of erythrocytes infected with CR parasites to concentrate chloroquine, and it supports the hypothesis that chloroquine resistance is due to a decrease in the amount, affinity or accessibility of a specific class of drug receptor sites. Whether these receptor sites are located on protein, nucleic acid or some other macromolecule has not been determined. It is evident, however, that the receptor has an appreciably higher affinity for chloroquine than the purified albumin and nucleic acids that have been studied previously.

Because this drug receptor apparently has a key role in determining the susceptibility of malaria parasites to chloroquine and presumably to other related drugs, it is desirable to know something about the specificity of the receptor and to know if interaction of a drug with the receptor can be used to predict cross resistance in CR malaria parasites. Cross resistance would be expected if interaction of the drug with the receptor is essential for the chemotherapeutic action of the drug. Therefore, the following studies were undertaken to evaluate a series of drugs for their abilities to compete with chloroquine for the high-affinity drug receptor of CS *P. berghei*.

Methods

Washed mouse erythrocytes infected with CS *P. berghei* were divided into several parts and incubated with a series of concentrations of chloroquine- 14 C, either with or without the addition of a constant amount of a test drug. After steady-state conditions were reached, the erythrocytes were collected by centrifugation and the amounts of chloroquine in the pellet and in the medium were measured radiochemically. Double reciprocal graphs of these data were used to evaluate the type of inhibition caused by the drug and to estimate the K_i for the drug if the graphs were compatible with competitive inhibition.

For these studies blood was obtained from 5–6 week-old white laboratory mice 6 days

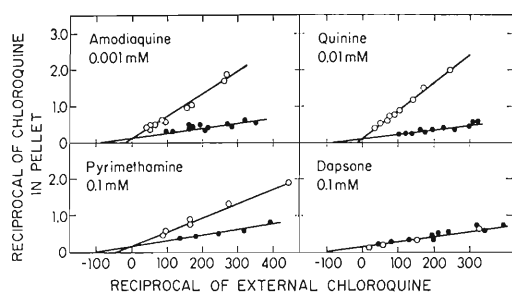


Figure 1. Inhibition of Chloroquine binding to erythrocytes infected with CS *P. berghei*. Five percent suspensions of washed, infected erythrocytes were incubated under room air at 22° C for 1 hour; the initial pH of the incubation mixture was 7.4 and the final pH was 7.2. Open circles indicate the presence of the test drug whose name and initial concentration are shown. The abscissa is the reciprocal of the chloroquine concentration in the medium ($1/\mu\text{M}$) and the ordinate is the reciprocal of the chloroquine bound to the erythrocyte pellet ($1/\mu\text{moles per Kg}$).

after they were inoculated with blood infected with the NYU-2 strain of *P. berghei* (Fitch, 1969). On the 6th day after infection there were approximately as many parasites as there were erythrocytes in the blood. The infections were lethal, killing the mice on the seventh or eighth day after inoculation. The composition of the incubation medium and the methods for measuring chloroquine concentrations have been described previously (Fitch, 1969); the only change was addition of polyoxyethylene sorbitan monooleate (Tween 80) to achieve a concentration in the incubation medium of 0.0125 percent to help keep insoluble drugs suspended. Chloroquine-3- ^{14}C (1.71mCi/mmole) was purchased from New England Nuclear Corporation for these studies.

Results

The graphs in Figure 1 depicting the results from studies of amodiaquine, quinine, and pyrimethamine illustrate the kind of data expected from studies of competitive inhibitors of chloroquine binding. The apparent affinity of the receptor for chloroquine decreases in the presence of inhibitor but the maximal binding capacity of the receptor for chloroquine is unaffected. For comparison, data

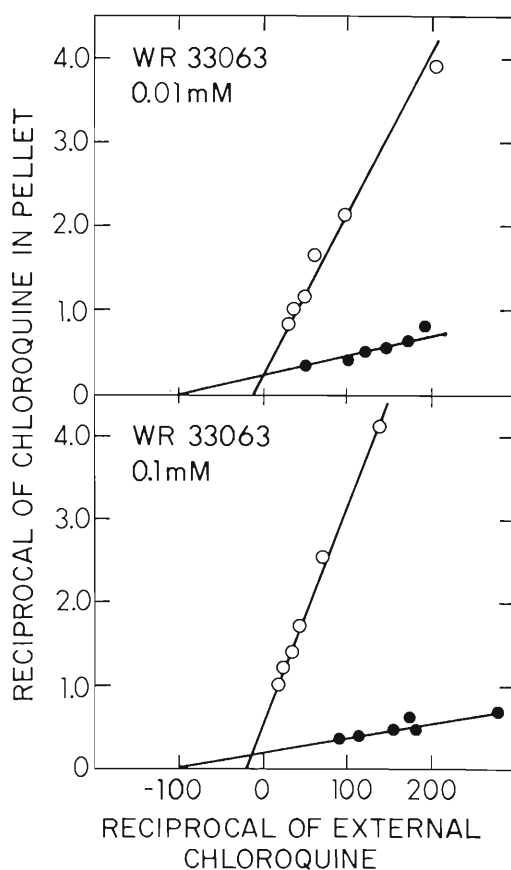


Figure 2. Inhibition of chloroquine binding to erythrocytes infected with CS *P. berghei*. Except for an incubation temperature of 25° C, the details of these studies and the meaning of the symbols are the same as those stated for Fig. 1. The difference in incubation temperature had no demonstrable effect on the results.

from a study using dapsone, which caused no detectable inhibition of chloroquine binding, also are shown in Figure 1.

Figure 2 illustrates studies of the same compound at two different concentrations. The structural formula of this compound, WR 33063, is shown in Table 1. At a concentration of 0.01 mM, WR 33063 appears to be a competitive inhibitor of chloroquine binding; but, at a higher concentration, the same drug causes a mixed type of inhibition. Although this compound may have more than one

TABLE I

COMPETITION OF DRUGS FOR THE HIGH-AFFINITY RECEPTOR OF CHLOROQUINE-SUSCEPTIBLE *P. berghei*:
CORRELATION WITH CROSS RESISTANCE OF CHLOROQUINE-RESISTANT *P. berghei*

DRUG	STRUCTURAL FORMULA	APPARENT K_i	CROSS RESISTANCE
Chloroquine		$5 \times 10^{-7} M$	++++
Amodiaquine		3×10^{-7}	++++
Amopyroquine		1×10^{-7}	++++
WR 30,090		2×10^{-6}	++++
WR 113,618		2×10^{-7}	+++
WR 33,063		1×10^{-6}	+++

TABLE I (Contd.)

DRUG	STRUCTURAL FORMULA	APPARENT K_i	CROSS RESISTANCE
WR 142,410		2×10^{-6}	++
WR 3,863		7×10^{-7}	++
Quinine		2×10^{-6}	++
Quinacrine		5×10^{-7}	+
WR 122,455		7×10^{-7}	+
Pyrimethamine		5×10^{-5}	None
Primaquine		None	None
Dapsone		None	None

Except for incubation temperatures of 25°C, the details of these studies were identical to those stated for Fig. 1. For each compound an average value from two or more studies is shown. The information about cross resistance is from the work of Thompson (1967, 1972): ++++ means a 100-fold or greater requirement of the drug to suppress parasitemia by 75 percent in mice infected with the CR derivative of the KGB-173 strain of *P. berghei* in comparison to the requirement to suppress parasitemia from the parent strain; +++ means a 25-fold or greater requirement; ++ means a 10-fold or greater requirement; and + means a 5-fold or greater requirement.

chemotherapeutic action at high concentrations, for the purposes of this paper it is considered to be a competitive inhibitor of chloroquine binding and its apparent K_i is included in Table 1. The apparent K_i is calculated from the intercept on the baseline of the graph showing chloroquine binding in the presence of 0.01 mM WR 33063 (Fig. 2).

In Table 1 are shown the apparent K_i values of a selected list of antimalarial drugs which were present in the incubation medium at an initial concentration either of 10^{-5} or of 10^{-6} M. Initial concentrations of the drugs were used to calculate K_i . The true values for K_i are probably seriously underestimated since significant amounts of the drugs are undoubtedly bound to these intact erythrocyte preparations—both to the high-affinity drug receptor and to other classes of binding sites. The magnitude of this error may be appreciated by comparing the apparent K_i for chloroquine of 5×10^{-7} M with the apparent dissociation constant of approximately 10^{-8} M

for the chloroquine-drug receptor complex (Fitch, 1969). Theoretically both of these values should be the same. To illustrate the extent of the variability encountered in these estimates of K_i , five independent studies gave the following values for the apparent K_i for amodiaquine: 1×10^{-7} , 2×10^{-7} , 3×10^{-7} , 5×10^{-7} , 5×10^{-7} M. This range of values for amodiaquine was somewhat larger than the range observed for the other drugs. Although the errors involved in estimating the value of K_i may be large, it is still possible to identify those compounds that bind to the drug receptor with high affinity.

An estimate of the degree of cross resistance of the CR derivative of the KGB-173 strain of *P. berghei* to each of the drugs listed in Table 1 was provided by P. E. Thompson (1967, 1972) and is included in the Table. These parasites have a high degree of resistance to derivatives of 4-aminoquinoline and significant resistance to all of the drugs that compete with chloroquine, with a single ex-

ception. Pyrimethamine, the exception, evidently competes with chloroquine, albeit weakly, and yet it is effective in treating infections with the CR strain of *P. berghei*.

Discussion

Competition of a drug with chloroquine for binding to the high-affinity drug receptor of *P. berghei* may be accepted as evidence that both drugs are interacting reversibly with the same binding sites. Accordingly inspection of the list of drugs in Table 1 reveals that the receptor of CS *P. berghei* will interact with a variety of antimalarial drugs, including derivatives of 4-aminoquinoline, of quinoline-4-methanol, of pyridine, of phenanthrene methanol, and of pyrimidine. The structural features of these drugs which enhance binding to the receptor cannot be evaluated completely from the present data, but it is apparent that the binding site will admit molecules processing a 30 to 40 Å² planar ring system (Albert, *et al.*, 1949) with a protonated group either in the ring or nearby. Further work is in progress to determine more completely the structural requirements for strong binding to the receptor.

In the meantime, we may consider the question of whether interaction of a drug with the high-affinity drug receptor of *P. berghei* can be used to predict cross resistance in chloroquine-resistant malaria. At least two criteria must be fulfilled to predict cross resistance: The drug must compete with chloroquine for the high-affinity drug receptor of *P. berghei*, and the therapeutic dose of the drug must be appropriately related to the dissociation constant for the drug-receptor complex. For example, a low therapeutic dose relative to the dissociation constant would indicate that interaction of the drug with the receptor is not essential for the drug's chemotherapeutic action. On the other hand, a high therapeutic dose relative to the dissociation constant might indicate limited delivery of the drug to the receptor *in vivo* rather than an action through a site other than the receptor. For the purpose of this discussion, the apparent K_i may be used as an estimate of the dissociation constant.

Thompson and associates (1967) have reported the therapeutic doses of eight of the

drugs listed in Table 1. In mice infected with the KBG-173 strain of CS *P. berghei*, the doses required to produce 75 per cent suppression of parasitemia were: chloroquine, 2.5; amodiaquine, 2.5; amopyroquine, 2.5; quinacrine, 5.0; quinine, 50; pyrimethamine, 0.31; primaquine, 3.1; and dapsone, 0.5 mg per Kg body weight per day for four days. The therapeutic doses of chloroquine, amodiaquine, amopyroquine, and quinacrine are relatively low, in agreement with their K_i values (Table 1); and the therapeutic dose of quinine is high, in agreement with its K_i value. By contrast the therapeutic doses of pyrimethamine is low whereas its K_i is high. Thus cross resistance of CR *P. berghei* to amodiaquine, amopyroquine, quinacrine, and quinine but not to pyrimethamine would be predicted. Furthermore, cross resistance to primaquine and dapsone would not be predicted as these drugs did not compete with chloroquine for the high-affinity drug receptor.

The pattern of cross resistance predicted in this way from knowledge of therapeutic doses and strengths of binding to the high-affinity drug receptor corresponds to the pattern of cross resistance of various strains of CR *P. berghei* (Peters, 1970). Such good agreement between the predicted and observed patterns of cross resistance demonstrates the power of this method for predicting cross resistance of malaria parasites to drugs, and it provides strong support for the hypothesis that interaction with the high-affinity drug receptor of CS *P. berghei* is essential for the chemotherapeutic action of chloroquine and certain related drugs.

Turning now to the *P. falciparum* model of malaria in the owl monkey (*Aotus trivirgatus*) it is clear that the cross resistance pattern of CR *P. falciparum* is different from that of CR *P. berghei*. Studies of the cross resistance of CR *P. falciparum* to selected drugs are being done by L. H. Schmidt (1972) who provided the following information about chloroquine-susceptible infections with the Uganda-Palo Alto or Malayan Camp-CH/Q strains of *P. falciparum* and with chloroquine-resistant infections with the Vietnam-Monterey or Vietnam-Oak Knoll strains of *P. falciparum* in owl monkeys. Quinacrine, primaquine, and dapsone have not been used in these studies, but data are available for the remaining com-

pounds in Table 1. Both strains of resistant parasites required more than eight times the dose of chloroquine that sufficed to cure infections with the chloroquine-susceptible strains. The degree of resistance to amodiaquine, ampyroquine, and WR 3863 was less; approximately twice as much as these drugs was required to cure infections with CR *P. falciparum* in comparison to infections with chloroquine-susceptible strains. And the degree of resistance to quinine was still less. CR *P. falciparum* did not exhibit cross resistance to WR 30,090, WR 113,618, WR 122,455, WR 142,490, and pyrimethamine.

The difference in cross resistance patterns of CR *P. berghei* and CR *P. falciparum* can be explained either by a difference in the chemotherapeutic action of certain drugs in the two types of parasites or by a difference in the structures of their high-affinity drug receptors. In either case, predictions based on the behaviour of the *P. berghei* model in the mouse would not be valid for the *P. falciparum* model in the owl monkey. It is desirable therefore to evaluate the relationships between therapeutic doses, drug binding, and cross resistance patterns in *P. falciparum*. Such studies may be greatly facilitated in the future by using cell-free preparations of the drug receptor.

Acknowledgments

I am indebted to Drs. L. H. Schmidt and P. E. Thompson for the information about cross resistance of CR *P. falciparum* and CR *P. berghei* to the drugs used in this study, to Dr. D. M. Chevli for helpful discussions, to LTC. Craig J. Canfield and the Division of Medicinal Chemistry of the Walter Reed Army Institute of Research for supplying the test drugs, and to Mary Le Faivre, Yolanda Gonzalez, and Rekha Chevli for technical assistance.

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Pigment Changes in *Plasmodium berghei* as Indicators of Activity and Mode of Action of Antimalarial Drugs

D. C. WARHURST, C. A. HOMEWOOD, W. PETERS AND V. C. BAGGALEY

Department of Parasitology and MRC Research Group on the Chemotherapy of Protozoal Diseases and Drug Resistance, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, England.

ABSTRACT: Quinine inhibits chloroquine-induced clumping of haemozoin pigment (autophagic vacuole formation) in *P. berghei* *in vitro*. Relationships between quinine and chloroquine concentrations suggest that the two drugs are competing to bind to a common site, the "clumping site" (probably associated with the membrane of the digestive vacuole). Affinity of chloroquine for the site is approximately 10 times that of quinine. The chloroquine-induced clumping system has been used *in vitro* and *in vivo* to study quinine-like drugs; our results indicate that WR 33063 and WR 30090 each act *in vivo* by means of a metabolite. There are structural similarities between drugs which bind at the clumping site. Drugs which induce autophagic vacuole formation in *P. berghei* apparently bind to the clumping site as do quinine-like drugs but are also able to become doubly protonated at physiological pH.

Autophagic vacuole formation (clumping of haemozoin) which takes place after exposure of intraerythrocytic *P. berghei* to chloroquine (Figure 1) *in vivo* or *in vitro* requires a source of energy, RNA synthesis and protein synthesis, (Warhurst et al., 1971; Warhurst and Baggageley, 1972). Inhibition of the pigment clumping process by actinomycin D or by cycloheximide shows non-competitive characteristics demonstrating that neither of these agents acts at a site identical to the site acted on by chloroquine in the production of pigment clumping (Warhurst et al., in preparation).

In an earlier paper (Homewood et al., 1972) we suggested that the concentration of chloroquine by intraerythrocytic *P. berghei*

was due to accumulation of the basic drug in the acid digestive vacuoles, where it raised the pH and halted digestion. Resistance was considered to be linked to a loss of the ability of the digestive vacuoles to secrete acid. This would explain cross resistance to other basic drugs such as quinine. The concept of loss of acidity, whilst explaining the cross resistance of highly chloroquine-resistant strains such as RC of *P. berghei* does not explain how certain chloroquine-resistant strains (in *P. falciparum* and *P. berghei*) are still relatively sensitive to amopyroquine, amodiaquine and quinine (for example, see Reickmann, 1971).

With a view to elucidating the cross resistance and mode of action problems, we have explored further the way in which quinine (Figure 1) is able to inhibit chloroquine-induced autophagic vacuole formation in *P. berghei* (Warhurst et al., 1971; Warhurst and Baggageley, 1972). We have also studied the mode of action of quinine-like, chloroquine-like and other drugs.

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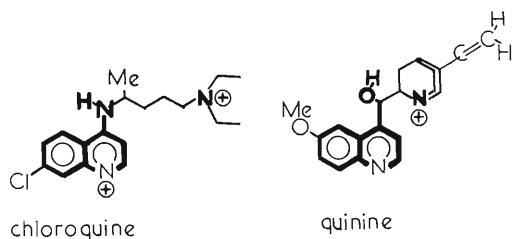


Figure 1. Structural formulae of chloroquine and quinine showing points of similarity (heavy line) and nitrogen atoms protonated at pH 7 (\oplus).

Materials and Methods

In vivo and *in vitro* techniques using N strain (K173) of *P. berghei* are described fully elsewhere (Warhurst et al., 1971; Homewood et al., 1972; Warhurst et al., in preparation). The incubations *in vitro* were carried out on a roller tube apparatus in 199 medium with 10% calf serum at 37°C. An atmosphere of 5% CO₂ : 95% air was used. Donor mice were exsanguinated 1 to 3 days after inoculation when less than 15% of the red blood cells were infected.

Duplicate tubes were used and experiments were repeated at least once. All tubes were in-

cubated for the same length of time. Drugs to be tested for inhibitory effects on clumping were added to the roller tubes 15 minutes before chloroquine which was allowed to act for 80 minutes before the cells were centrifuged down and thin films prepared from the pellets. In interpreting the slides the criteria for "fine", "granular" and "clumped" pigment were as described earlier (Warhurst and Robinson, 1971). Controls were run without chloroquine, without test drugs, and with saline only. Whilst sterile precautions were unnecessary absolute cleanliness was essential and glassware was acid-washed.

Insoluble drugs were made acid or alkaline and/or dissolved in ethanol before dilution. Up to 0.04 ml of ethanol could be added to 4.0 ml of medium without affecting granulation or clumping.

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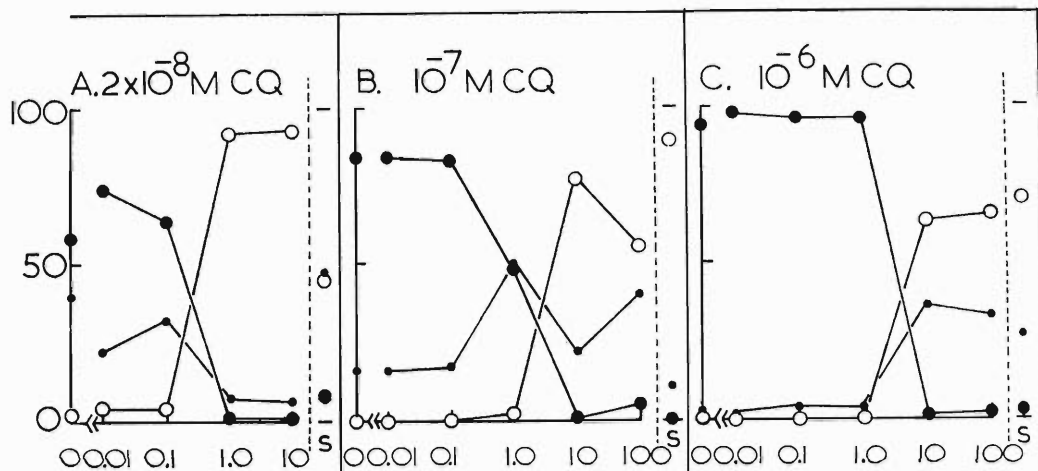


Figure 2. Effect of varying concentrations of quinine upon pigment clumping produced by 2×10^{-8} M (A), 10^{-7} M (B) or 10^{-6} M (C) chloroquine. Abscissa: Percentage of infected erythrocytes showing fine \circ —, granular \bullet —, or clumped \bullet — pigment after incubation for 80 minutes with chloroquine, in the presence of varied concentrations of quinine. Ordinate: Quinine concentration ($\times 10^{-6}$ M) (Log scale). "S" = control with saline alone.

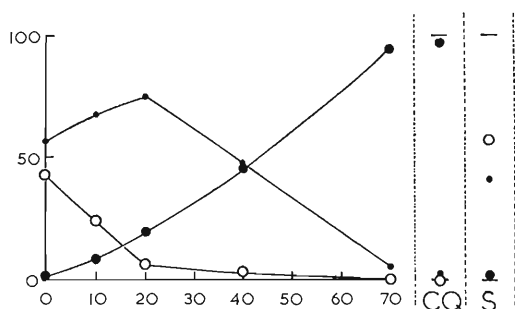


Figure 3. Time-course of inhibition of chloroquine-induced pigment clumping by 10^{-5} M quinine. *Abscissa*: Percentage of erythrocytes showing fine granular pigment, or clumped pigment after incubation for 80 minutes in 10^{-6} M chloroquine. *Ordinate*: Time in minutes during incubation at which 10^{-5} M quinine was added to the tubes. "CQ" = result of experiment where no quinine was added. "S" = control with saline alone.

Results

Quinine

It had been noted *in vivo* that quinine had a rapid and marked inhibitory effect on chloroquine-induced pigment clumping in *P. berghei* (Warhurst et al., 1971). The effect of pre-incubation with varying concentrations of quinine for 15 minutes was first examined *in vitro*, using three different chloroquine concentrations, 10^{-6} M, 10^{-7} M and 2×10^{-8} M (Figure 2). The inhibitory concentration of quinine appeared to be proportional to the concentration of chloroquine used. The time-course of action of 10^{-5} M quinine against clumping produced by 10^{-6} M chloroquine was also examined, adding quinine simultaneously with or at intervals after chloroquine. This concentration of quinine had a rapid effect on the clumping process, stopping it at whatever stage it had reached even after 40 minutes (Figure 3). Change from fine to granular pigment was clearly prevented as well as the change from granular to clumped. This inhibition of granulation is also seen in the dose-response curves in Figure 2. After further dose-response experiments, the concentration of quinine needed to inhibit clumping by 50%, [Q], was plotted (log/log) against chloroquine concentrations, [CQ] (Figure 4). The regression was significant (correlation coef-

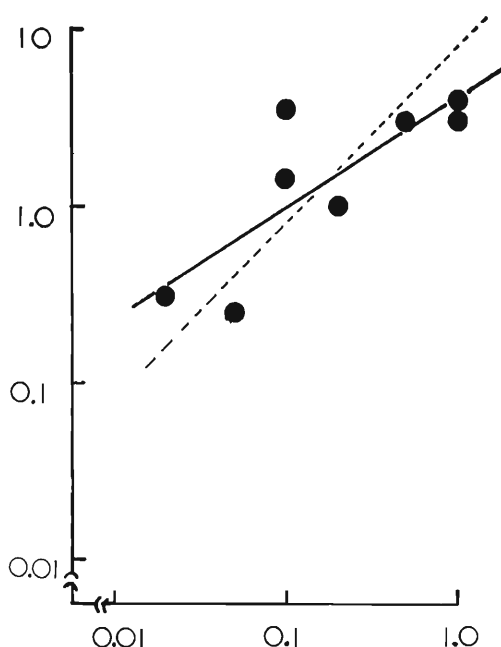


Figure 4. Inhibition of clumping by quinine at varying concentrations of chloroquine. *Abscissa*: Concentration of quinine giving 50% inhibition of clumping ($\times 10^{-5}$ M) (Log scale). *Ordinate*: Chloroquine concentration ($\times 10^{-6}$ M) (Log scale). The continuous line shows the calculated regression for the points. The theoretical slope of 1.0, for competitive inhibition is illustrated by the interrupted line.

ficient 0.8, $P < .02$) with a slope of 0.63. The slope expected for simple competitive inhibition is 1.0.

When the logarithm of the ratio of [Q] to [CQ] was plotted against the logarithm of [CQ] (Figure 5), the values fell within the 95% limits of the geometric mean of the ratios, 7.8.

Quinine-like drugs

Various quinoline methanols and phenanthrene methanols also inhibit chloroquine-induced clumping of pigment. We used a standardised technique where the drug to be tested is added in varying concentrations to the culture tube, 15 minutes before chloroquine at a final concentration of 10^{-6} M and incubation is continued for a further 80 minutes. It was

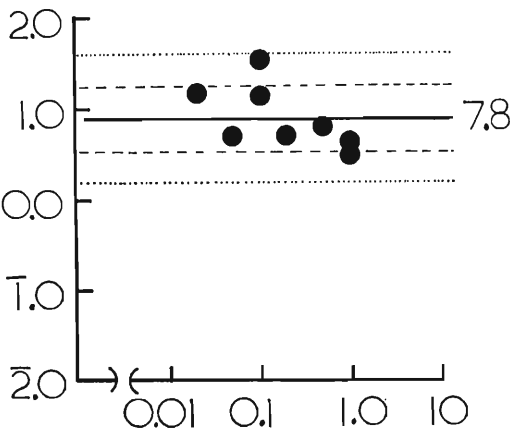


Figure 5. Quinine/chloroquine ratio for 50% inhibition of clumping at varying chloroquine concentrations. *Abscissa*: Logarithm of the ratio [Q]/[CQ]. *Ordinate*: Chloroquine concentration ($\times 10^{-6}$ M) (Log scale). The continuous line gives the geometric mean of the points. Dashed lines indicate standard deviation, dotted lines indicate 95% limits ($1.96 \times S.D.$).

possible to estimate the concentration of the test drug giving 50% inhibition of clumping, giving a direct comparison with quinine where this concentration was about 4×10^{-6} M.

In vitro WR 33063 and WR 30090 are noticeably less effective than WR 142490, WR 166391 and WR 122455 (Figure 6) although all five drugs are antimalarially active *in vivo*. On investigating the action of WR 33063 and WR 30090 against chloroquine-induced pigment clumping *in vivo* in the mouse we found that 30 mg/kg of either drug given one or two hours before chloroquine would inhibit clumping by up to 100%, (method of Warhurst et al., 1971).

Table 1. Inhibition of chloroquine-induced clumping *in vitro* by quinine-like drugs.

Drug	Type	Concn. giving 50% inhibition of clumping (10^{-6} M chloroquine)
Quinine	quinoline methanol	4×10^{-6} M
WR 142490	quinoline methanol	3.6×10^{-7} M
WR 166391	quinoline methanol	2.1×10^{-6} M
WR 30090	quinoline methanol	4.6×10^{-5} M
WR 122455	phenanthrene methanol	3.3×10^{-7} M
WR 33063	phenanthrene methanol	2.6×10^{-4} M

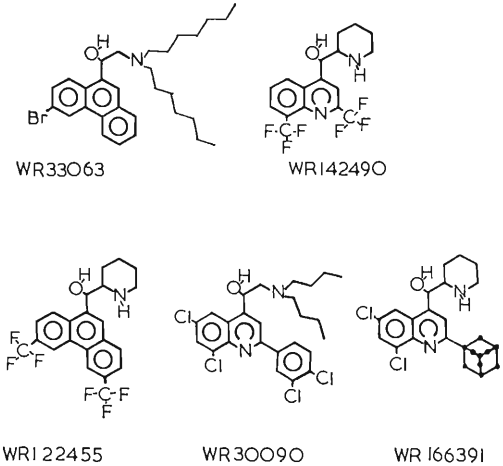


Figure 6. Quinine-like drugs: phenanthrene and quinoline methanols.

Effects of other drugs *in vitro*

A. Other cinchona alkaloids

Quinidine, cinchonine and cinchonidine will inhibit completely at 10^{-5} M the clumping effect of 10^{-6} M chloroquine and presumably achieve their effect in the same way as quinine. The cinchona alkaloids tested inhibit granulation of fine pigment, which many other inhibitors of pigment clumping do not affect.

B. 8-aminoquinolines (Figure 7)

Pamaquine, primaquine and pentaquine have no inhibitory effect on chloroquine-induced clumping at 10^{-5} M. Pamaquine and primaquine will inhibit at 10^{-3} M, pentaquine is lytic at this concentration. The 50% inhibitory concentration of primaquine against 10^{-6} M chloroquine was found to be 3×10^{-4} M.

C. "Anti-metabolite" drugs

It was noted earlier (Warhurst et al., 1971) that pyrimethamine and sulphadiazine had no effect on chloroquine-induced pigment clumping *in vivo*. This is also the case *in vitro*. Sulphadiazine at 10^{-4} M and pyrimethamine at 10^{-5} M did not prevent chloroquine-induced clumping of pigment. Cycloguanil likewise had no effect at concentrations as high as 10^{-3} M.

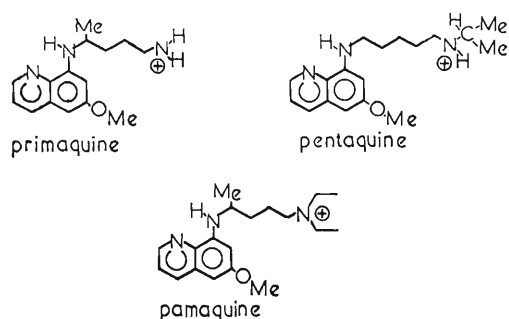


Figure 7. 8-aminoquinolines: Note proximity of aromatic nitrogen atom to >NH group. Nitrogen atoms protonated at pH 7 marked thus \oplus .

D. Other 4-aminoquinolines (Figure 8)

The other 4-aminoquinolines tested (sontoquine, amodiaquine and amopyroquine) caused clumping of pigment (in the absence of chloroquine) at concentrations down to 10^{-8} M. The changes following addition of these drugs are the same as those following addition of chloroquine. Pigment becomes granular and then clumps together. For amopyroquine the concentration of drug giving 50% clumping was 3×10^{-8} M essentially identical to that noted for chloroquine (Warhurst et al., in preparation). The concentration causing 50% inhibition of clumping was 2×10^{-5} M, again practically the same as noted for chloroquine.

E. Other drugs causing clumping of pigment *in vitro* (Figure 8)

The 9-amino acridine, mepacrine, (10^{-6} M, 10^{-7} M) has similar clumping effects to chloroquine. Another antimalarial drug, BW 377C54, also caused pigment clumping at concentrations down to 10^{-8} M (50% clumping at 3×10^{-8} M).

F. Antibiotics

The inhibitor of RNA polymerase, rifampicin, has been reported to be active against *P. berghei* *in vivo* (Alger et al., 1970). *In vitro* rifampicin at concentrations between 10^{-4} M and 3×10^{-5} M would completely inhibit chloroquine-induced pigment clumping. In the plasma of man concentrations of up to 3×10^{-5} M are reported (Rifadin handbook,

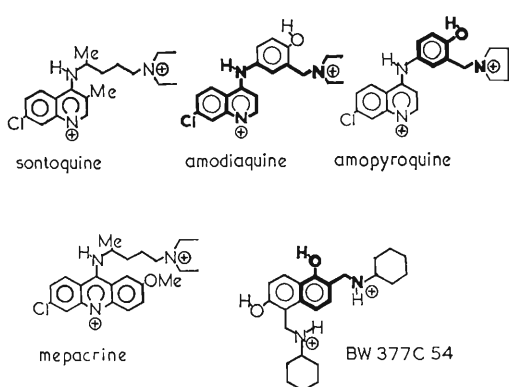


Figure 8. Drugs causing autophagic vacuole formation: Nitrogen atoms capable of protonation at pH 7 are marked thus \oplus (this is only an assumption in the case of BW377C54). In the formula for amodiaquine the heavy lines emphasize the close similarity of this drug and amopyroquine to chloroquine. In the formula for amopyroquine the resemblance to quinine is emphasized. The similarity of half of the molecule of BW377C54 to quinine is also emphasized by heavy lines.

Lepetit Ltd.). 10^{-5} M rifampicin did not inhibit clumping.

It was observed during *in vitro* studies that erythromycin, a lipid-soluble base with pKa 8.8, was the only inhibitor of bacterial protein synthesis causing some clumping inhibition at 10^{-4} M (21% inhibition). Tests in this laboratory (Robinson and Warhurst, in press) have shown that erythromycin has antimalarial activity against *P. berghei* in mice. (Other antibiotics are discussed in another paper, Warhurst et al., in preparation.)

Discussion

In another publication we report that the concentration of chloroquine causing 50% clumping at 37°C in our system is $\sim 4.5 \times 10^{-8}$ M (Warhurst et al., in preparation). Fitch (1969) obtained 50% saturation of his high affinity site at concentrations close to this (22°C). He took the 50% saturation concentration to be equivalent to the reciprocal of an affinity constant. Using the same argument we can calculate an affinity constant, measured by our technique, of $\sim 2.2 \times 10^7$ Moles $^{-1}$, comparing with $\sim 10^8$ Moles $^{-1}$ as found by Fitch. Kramer and Matusik (1971) obtained

the same value as Fitch, and were able to relate this affinity constant to the parasite membrane fraction.

We also note that chloroquine at concentrations above 10^{-6} M inhibits the clumping it normally induces, presumably acting directly on cellular synthesis. 50% inhibition of clumping is seen at $\sim 3.2 \times 10^{-5}$ M. By a similar argument this gives us a second affinity constant for chloroquine's action on cellular synthesis of $\sim 3.1 \times 10^4$ Moles $^{-1}$. This is close to the second affinity constant observed by Fitch of 10^5 Moles $^{-1}$ and attributed by Kramer and Matusik to the cytoplasmic fraction of the parasite.

The dose-response relationships observed when quinine inhibits chloroquine-induced autophagic vacuole formation suggest that the two drugs are competing for a common site in the cell—the "clumping site" (Figures 2, 4 and 5).

From our present results it would appear that the affinity constant for quinine at the clumping site is about one order of magnitude lower than that of chloroquine, i.e. $\sim 2.2 \times 10^6$ Moles $^{-1}$. It is interesting to note that Polet and Barr (1969) were able to wash out bound radioactive chloroquine from *P. knowlesi* using ten times the concentration of dihydroquinine.

It is reasonable to expect quinine-like drugs such as quinoline methanols and phenanthrene methanols to behave in the same way as quinine. The examples tested were found to be highly active in inhibiting pigment clumping *in vitro* except for WR 33063 and WR 30090, which were however active *in vivo*. These two drugs have long alkyl chains attached to the tertiary amino nitrogen atom of the side chain. These alkyl chains might be expected to be shortened during metabolism *in vivo*. It is significant that the more active *in vitro* of the two drugs, WR 30090, has shorter alkyl chains. We suggest that WR 33063 and WR 30090 act by means of a metabolite, whilst in WR 142490 and WR 122455, and possibly WR 166391, the parent drug is active. In our *in vitro* tests WR 142490 and WR 122455 are at least ten times as effective in clumping inhibition as quinine.

The 8-aminoquinoline pamaquine is considered to act *via* a quinoline quinone metabolite. Primaquine is suspected to act *via* a similar metabolite (see Peters, 1970). Al-

though Warhurst et al. (1971) found that primaquine would inhibit chloroquine-induced clumping of pigment *in vivo* by 24%, *in vitro* we find primaquine is only 1/100 as effective as quinine. A result such as this would be expected if an active metabolite were produced *in vivo*.

The inability of drugs such as sulphadiazine and pyrimethamine to affect chloroquine-induced pigment clumping *in vitro* or *in vivo* indicates that, in the short term, the pathways they affect are not necessary for development of the autophagic vacuole. This is the only information about such drugs which can be obtained using our system.

All 4-aminoquinolines tested caused clumping of pigment. 8-aminoquinolines, quinine and the other cinchona alkaloids did not. An essential feature of a drug capable of causing clumping appears to be, as described by Homewood et al. (1972) for chloroquine, the ability to pass through a biological membrane in a monoprotonated form and to pick up another proton on the other side, thus depleting areas of the digestive vacuole of hydrogen ions. Mepacrine shares this characteristic (see Table 2) and, judging by its structure, so does BW 377C54. [This is interesting, as these two drugs are ineffective against chloroquine-resistant plasmodia (see Peters, 1970).]

It now remains to discuss the nature of the "clumping site". Since the first detectable changes in the malaria parasite after chloroquine treatment take place at the digestive vacuoles it seems very likely that they incorporate the high affinity sites of Fitch (1969) as suggested by Homewood et al. (1972), and also our clumping site. Further to localise the site is more difficult.

When we proposed earlier that monoprotonated chloroquine on passing into the digestive vacuoles became doubly protonated and was effectively trapped within them causing their internal pH to rise and digestion to cease (Homewood et al., 1972), resistance was explained as a loss of the ability to secrete acid into the digestive vacuole leading to a reduction in concentrative ability for the drug and cessation of haemoglobin digestion. It is however very difficult to explain the activity of amodiaquine and amopyroquine on chloroquine-resistant strains on this basis. Assuming that the pKa values we have for

Table 2. Protonation and pigment-clumping.

Drug	Group	pKa ₁ (aromatic nitrogen)	pKa ₂ (side chain nitrogen)	% doubly protonated at pH 7.0*	Pigment clumping
pamaquine	8 amino- quinolines	3.5	10.2	0.03	NO
pentaquine		—	8.3	0	NO
primaquine		?	?	?	NO
quinine	cinchona alkaloids	4.3	8.4	0.19	NO
quinidine		4.2	8.8	0.16	NO
cinchonine		4.3	8.4	0.19	NO
cinchonidine		4.2	8.4	0.15	NO
chloroquine	4 amino- quinolines	8.1	10.2	93	YES
sontoquine		7.3	10.2	67	YES
amodiaquine		6.4*	8.2*	19	YES
amopyroquine		6.3*	7.9*	15	YES
mepacrine	9 amino acridine	7.7	10.2	83	YES

Except where stated, pKa values in this table are taken from Perrin (1965).
* From data of Albert (1968).
* pKa values in 50% methanol kindly supplied by Dr. A. J. Glazko.

amodiaquine and amopyroquine (see Table 2) do refer to the ring and side chain nitrogen atoms, the percentage of the singly-protonated membrane-passing species present at physiological pH must be very much higher than is the case for chloroquine. This might give these two drugs an advantage over chloroquine in resistant strains. There should also be a similar advantage against sensitive strains which is apparently not the case, so that this explanation does not seem at present very satisfactory. Kramer and Matusik (1971) have also given evidence which, whilst it supports our belief that the site is not DNA, indicates that the high affinity site is associated with parasite membrane. This seems to argue against a simple concentration of the drug by reaction with hydrogen ions. Our observations on the time-course of clumping inhibition by quinine (Figure 3) give the rather surprising result that even after the parasites have been exposed to chloroquine for 40 minutes and, on the acid theory, had their digestive vacuoles neutralised, quinine will still halt clumping. None of these points is sufficient in itself to rule out the idea that the clumping site and hydrogen ions inside the digestive vacuole are identical. Taken together they would seem to indicate that a “receptor” of classical type, associated with digestive vacuole membrane, is involved. Drugs which cause or competitively inhibit pigment clumping could be considered to bind at this site. Such drugs have three

structural features in common (Figures 1, 6 and 8).

1. A lipophilic ring system
2. A group (—OH or >NH), apparently capable of participating in a hydrogen bond, close to the ring but not adjacent to the ring nitrogen.
3. A protonated positively charged nitrogen atom spatially related to 1 and 2

Amodiaquine and amopyroquine, being both 4-aminoquinolines and α amino orthocresols, have two examples of 1 and 2 related to the same protonated nitrogen atom. They could be capable of binding to their site in two different orientations.

Acknowledgments

We thank the Medical Research Council for financial support. Dr. R. E. Howells and Dr. M. L. Chance offered constructive criticism on these studies. We wish to thank Miss P. Mallory for technical assistance, Miss S. Shaw for secretarial help and the Department of Medical Illustration, Liverpool School of Tropical Medicine, for photographic work.

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Comments on Host-Parasite-Drug Inter-Relationships

ERNEST BUEDING

The Johns Hopkins University, Baltimore, Maryland 21205

Every report in this group of papers is related to the uptake by, or the transport into, the malarial parasite of precursors of cell constituents or of drugs. Three of these six papers deal directly with the uptake of either amino acids, nucleic acid precursors or of chloroquine. Two of the other three reports are concerned with the phosphorylation of adenosine. These observations of Carter *et al.* should be of considerable interest because plasmodia share with other blood-dwelling parasites a dependence on exogenous adenine or adenosine, i.e., on the so-called "salvage pathway." In contrast to their hosts and to most other organisms, they are unable to synthesize adenine *de novo*. The observed absence of inhibition by chloroquine of adenosine incorporation into nucleic acids contrasts with the effects of this drug on DNA replication observed by other investigators.

The findings of Warhurst *et al.* suggest that chloroquine and quinine are bound to a site common to both these drugs. The findings of these authors of the clumping of malarial pigment are ascribed by them to an inhibition of

acid secretion in the digestive vacuole, resulting in a cessation of hemoglobin digestion. These observations raise the question whether amodiaquine or aminopyraquine cause clumping in chloroquine-resistant strains and whether this effect of chloroquine is observed in plasmodia other than *P. berghei*, and in particular, in *P. falciparum*.

In studying the uptake of amino acids by *P. lophurae*, Sherman and Tanigoshi found that only a limited number of amino acids, namely arginine, lysine, and glutamic acid are taken up by energy-dependent transport mechanisms. Again, it would be of considerable interest to determine whether this applies to plasmodia invading man. These investigators also observed that, while amino acids are taken up in uninfected red cells by a saturable energy-dependent mechanism, amino acids enter the parasitized erythrocyte by a simple diffusion process. This is associated with a loss of ATP by the red cell. Diffusion, as contrasted by a saturable transport mechanism, may be of physiological advantage to the parasite, as this may result in a greater amino acid concentra-

tion in the plasmodium. Effects of known anti-malarials on amino acid transport mechanisms do not appear to be related to their mode of action because they were observed only with concentrations far above those which are chemotherapeutically active. By contrast, the possibility that primaquine might exert its anti-malarial action by interfering with the uptake of thymidine and uridine deserves examination in view of the observations of Conklin and Chou with *Tetrahymena pyriformis*. Experiments with plasmodia, both *in vitro* and *in vivo*, should provide information on this problem.

Differences in the uptake and binding of chloroquine by susceptible and resistant *P. berghei* observed by Fitch could supply the basis for an understanding of the mechanism responsible for this type of drug resistance. However, in this species amodiaquine and aminopyraquine were found to compete with the binding site for chloroquine. Yet, as will be reported this afternoon, there is little cross-resistance of *P. falciparum* to these two compounds. Therefore, Dr. Fitch would clarify this problem by extending his studies to *Plasmodium falciparum*.

V

CHEMOTHERAPY

B. Lower Animal Models

A Screening Procedure, Based on Mortality, with Sporozoite-induced *Plasmodium gallinaceum* Malaria in Chicks*

L. RANE AND D. S. RANE

University of Miami School of Medicine, Medical Research Annex,
5750 N.W. 32nd Avenue, Miami, Florida 33142

ABSTRACT: A drug screening method has been devised for the determination of prophylactic activity against avian malaria, using *Aedes aegypti* mosquitos infected with the 8-A strain of *Plasmodium gallinaceum*.

Special techniques in the rearing and handling of infected and noninfected mosquitos were developed. A donor line, maintained in white Leghorn cockerels weighing 45–47 grams by bite-to-bite passes (from parasitized chick to mosquito to chick), was the source of the single blood meal by which mosquitos were infected.

A standard inoculum was prepared with suspensions of sporozoites obtained by grinding whole infected mosquitos on the 9th–11th day after a completed blood meal. Intrajugular inoculations into white Leghorn cockerels weighing 57–59 grams produced infections that resulted in the death of 100 per cent of all untreated birds. In 152 groups of negative controls, representing 1520 untreated chicks, the mean survival time was 8.34 days.

Treatment consisted of a single dose of a candidate compound, given subcutaneously immediately after infection. Prophylactic activity was assessed by comparing the maximum survival time of treated sporozoite-infected chicks and the survival time of untreated sporozoite-infected controls. A compound was regarded as active if it produced an increase of at least 100% in the survival time of the untreated controls.

Over 100,000 chicks were used in the developmental stage and the period introducing routine testing.

The success of this test may be attributed to: 1) the infected blood meals provided for mosquitos in egg production cages; 2) the bite-to-bite passes of our donor line; and 3) the intrajugular inoculation of a sporozoite suspension prepared by grinding whole *Aedes aegypti* mosquitos infected with *Plasmodium gallinaceum*.

It is generally recognized that a well-controlled test system based on sporozoite-induced *Plasmodium gallinaceum* infections in chicks would be effective: 1) as a primary screen of therapeutic and/or prophylactic activity; and 2) as a confirmatory test of antimalarial activity assessed in other systems.

Our main objective was the development of a clearly defined method for determining potential prophylactics. The test described in this paper has produced infections resulting in the death of 100 per cent of negative controls. Thus, our sporozoite-induced screen with *Aedes aegypti* mosquitos and *Plasmodium gallinaceum* infections in chicks like our blood-induced screen with *Plasmodium berghei* in mice and our blood-induced *Plasmodium gal-*

linaceum screen in chicks is based on mortality and not on morbidity.

In an operation testing large numbers of candidate compounds, simplicity of techniques and a high incidence of infection are essential. Bearing this in mind, modifications were made in accepted forms of procedure. However, in this test changes were also made in known methods of rearing and handling non-infected and infected mosquitos to permit the regulation of infections developed in order to achieve the desired end, the death of all negative controls. These carefully planned changes involved:

- (a) methods of handling eggs, larvae and pupae;
- (b) the nutritional requirements of larvae and pupae;
- (c) new and more satisfactory methods of feeding and watering adult mosquitos before and after blood meals;

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- (d) types of blood meals;
- (e) methods of preparing a standard inoculum;
- (f) avenues of drug administration and drug regimens.

The results of studies with more than 100,000 chicks suggest that our sporozoite-induced screening procedure has approximated and ultimately will match the standards of our older blood-induced screens. Mortality statistics of the negative controls prove that we have successfully produced a disease fatal to 100 per cent of untreated controls within periods with a mean survival time of 8.34 days.

In our test prophylactic value is assessed by comparing the maximum survival time of treated sporozoite-infected chicks and the survival time of untreated sporozoite-infected controls. A compound is considered to have potential prophylactic value if it produces a minimum increase of 100 per cent in the survival time of untreated controls.

Materials and Methods

Parasite

The 8-A strain of *Plasmodium gallinaceum* maintained for several years in this laboratory for our blood-induced screening procedure has been used for this test.

Mosquito Vector

The "Rock" strain of *Aedes aegypti* obtained from Dr. George B. Craig, Jr. of the University of Notre Dame has been used throughout this investigation.

Every step in the rearing process and the pre-test handling of our *Aedes aegypti* mosquitos (egg production, egg hatching, larval counts and distribution, larval feeding, the separating of male and female pupae, blood meals, preparation of the infecting inoculum as well as the biting of chicks by infected mosquitos for the bite-to-bite passage of our donor line) starts and is completed in a room in which the temperature is maintained at 80°F. \pm 1°F. and the relative humidity at 80% \pm 1%. The room is illuminated daily for 12 hours by automatic controls.

Since female pupae are conspicuously larger than male pupae, separation is accomplished on the basis of size with the help of a special aluminum screen similar to the one described by McCray (1961).

Approximately 2400 female pupae and 600 male pupae are used to start an egg production cage. The ratio of 4:1 has given us excellent yields. Although the total number of pupae may vary occasionally, the proportion remains constant.

Approximately 3000 female pupae are put into each test cage. Our test cage is a modified form of the egg production cage. In the test cage the wooden floor of the egg production cage is replaced by screening to allow increased ventilation.

In anticipation of the bite-to-bite passage of our donor line we place about 400 female pupae into a round one-gallon plastic bucket the sides of which have been lightly scored to provide roosts for the emerged mosquitos. The top of the bucket is first covered with nylon screening that is kept taut with two wide rubber bands. The bucket's lid has been cut so that only a narrow border and the gripping sides remain. This is then fitted over the nylon screening forming a secure closure, yet leaving an open area for feeding and watering the mosquitos and later an area for biting chicks.

We have designed a new method for feeding and watering mosquitos that has many advantages. Not only is it simpler and cleaner than the currently accepted methods but it provides food and water in a way that appears to be more easily accessible.

Our emerged mosquitos are fed for three days with a 10% sucrose solution from a plastic bellows bottle having a capacity of 30 ml. The neck of the bottle fits snugly into the hole drilled in the center of a plastic square that was made specifically to support the bottle when upended on the screen of a cage or bucket.* Water is provided in a similar manner.

The mosquitos in an egg production cage and a test cage seem to require at least three bottles of the sucrose solution and two bottles of water during this three-day period. The fewer mosquitos in a round bucket require only one bottle of each.

After three days the sucrose solution and water are removed from the egg production cage, oviposition jars are placed inside the

* The entire assembly, consisting of bellows bottle and support, is available from Wheaton Scientific, 1000 North Tenth Street, Millville, N.J. 08332.

cage and the mosquitos are given their first blood meal.

Our egg production cages are started for two different purposes: 1) for a reserve of normal eggs, and 2) for a supply of eggs from which will emerge the mosquitos used in our sporozoite-induced screening procedure. With one exception, the kind of blood meal provided, both are treated similarly. In both the mosquitos are first blood fed on the fourth day after emergence. In each case blood meals last ninety minutes. After a blood meal each receives a fresh supply of water and the sucrose solution. Five days later, and thereafter at five-day intervals, eggs laid are harvested, freshly prepared oviposition jars are put inside the cages, food and water are taken away and another blood meal is provided. After six harvests we destroy the mosquitos that are still alive.

The one distinguishing and critical factor is the blood meal. To obtain a supply of normal eggs, we follow the common procedure and use normal uninfected chicks as the source of a blood meal. For eggs that will give us the mosquitos largely responsible for the success of our test, we deviate from common practice and blood feed with infected chicks from our donor line. Eggs laid after an infected blood meal are also used to start other egg production cages in which emerged mosquitos will receive infected blood meals.

For the mosquitos in our test cages and round buckets there is a five day interval between emergence and their single infected blood meal. During the first three days, they are given an adequate supply of water and the 10% sucrose solution; for the last two days they receive water only.

The mosquitos in the test cages and in the round buckets are blood fed with chicks from our donor line. Immediately after the completion of their blood meal they receive fresh supplies of the sucrose solution and water, and for the next nine days they are undisturbed. The bellows bottles containing the sucrose solution and the water are changed several times during this period to ensure an adequate supply and to avoid spoilage in the warm humid atmosphere.

Our donor line is kept up entirely with bite-to-bite passes on Day 9 and Day 10 after a completed blood meal. White Leghorn

cockerels weighing 45–47 grams are clipped under the wing and fixed on the screened open top of a bucket in a position exposing the wing vein. Each bird is bitten for three minutes. Ten to fifteen birds are bitten by the mosquitos in a bucket. After being bitten, the birds are transferred to a room that is maintained at 76°–78°F. and 60% RH.

The infected mosquitos in a test cage are harvested on Day 9, 10 or 11 after a completed blood meal. To prepare a standard inoculum of sporozoites we proceed as follows:

- (a) dead mosquitos, if any, on the floor of a cage are removed with a small household vacuum cleaner;
- (b) the cage is then put into a –20°F. freezer for fifteen minutes. Exact timing is extremely important since a fifteen-minute exposure to this temperature merely stuns the mosquitos sufficiently to prevent a mass escape while they are being collected by vacuum and put into a polyester plastic envelope which is immediately heat sealed;
- (c) the envelope containing the mosquitos is then weighed on an analytical balance to determine the weight of the mosquitos and thus permit the calculation of the dilution required for the test;
- (d) the mosquitos, still in the sealed envelope, are laid on a glass plate on the top of a special 12°F. freezing table and ground. Grinding continues until an homogenous mass has been formed;
- (e) the seal of the envelope is then cut to allow the addition of cold saline in the amount calculated on the basis of the weight of the mosquitos and the dilution desired;
- (f) the uniform suspension is filtered through a nylon filter of the type normally used in the filtration of whole blood for human transfusions;
- (g) the filtered suspension is transferred to serum bottles, stoppered with multiple puncture rubber stoppers and sealed with aluminum caps.

All procedures following the grinding of the mosquitos on the freezing table are carried out in an ice bath. Since inoculations into chicks are made in the room maintained at 76° -78°F. and 60% RH, the birds are inoculated as rapidly as possible.

This method invalidates the grinding of mosquitos in mortars and centrifugation of the pulpy residue. Thus it minimizes possible losses of sporozoites through elevations in temperature.

Test Animal

White Leghorn cockerels weighing 57-59 grams are preferred. The birds are purchased from local hatcheries and are of fairly uniform stock. When delivered to the laboratory, they are one day old. They are then maintained under standard conditions, including a non-medicated diet, until they reach the weight used in our test.

Test Procedure

Chicks on test receive an intrajugular injection of 0.5 ml. of the standard inoculum prepared with *P. gallinaceum* infected *Aedes aegypti* mosquitos, ground whole.

To check factors such as variations in the infectivity of the sporozoites of the *P. gallinaceum* strain, changes in the susceptibility of the host or simply to detect technical errors, a given number of the infected chicks are treated with sulfadiazine (at a dose level known to produce definite increases in survival time) and included in each test as a positive control.

Drug Administration

In this test treatment consists of a single dose, administered subcutaneously immediately after infection. Graded doses of the candidate compound are dissolved or suspended in peanut oil and put on test. We have found that increases in the dose levels of highly active compounds are generally followed by increases in the survival time of the treated chicks.

Results

Routine testing for prophylactic activity began October 1, 1971. Of the 1130 selected compounds screened during the next five

months, 203 demonstrated a degree of activity that produced a minimum increase of 100 per cent in the survival time of sporozoite-induced *Plasmodium gallinaceum* infections in chicks.

In 152 untreated control groups, each of 10 chicks, the mortality of the 1520 chicks involved was 100 per cent. The mean survival time of these untreated groups was 8.34 days with the deaths distributed as follows:

Day									
6	7	8	9	10	11	12	13	14	
18	482	390	330	218	76	4	1	1	

Compounds

Examples of the classes of compounds that demonstrated activity in the sporozoite screen are listed below:

- (WR 14997) 1-Aminocyclopentanecarboxylic Acid
- (WR 151136) O-Methyl-L-Threonine
- (WR 179215) 6-Methoxy-8/1-Methyl-4/1-Ethyl-3-Piperidyl/Butylaminoquinoline
- (WR 179305) 4/1/4-Chlorophenyl/1-Ethoxyethyl/Piperidine
- (WR 61112) 3,5-Dichloro-2,6-Dimethyl-4-Pyridinol
- (WR 2978) 2,4-Diamino-5-(P-Chlorophenyl)-6-Ethyl-Pyrimidine
- (WR 5949) 2,4-Diamino-5-(3,4,5-Trimethoxybenzyl)Pyrimidine
- (WR 3090) 2,4,7-Triamino-6-O-Tolyl-Pteridine
- (WR 181063) 2,4,7-Triamino-6-Phenylpteridine
- (WR 7557) 2-Sulfanilamidopyrimidine
- (WR 4629) N'-(3-Methoxy-2-Pyrazinyl)Sulfanilamide
- (WR 4873) N'-(5,6-Dimethoxy-4-Pyrimidyl)Sulfanilamide
- (WR 26052) N'-2,6-Dimethoxy-4-Pyrimidinyl)Sulfanilamide
- (WR 49808) 3-/8-Cyclohexyloctyl/4-Hydroxy-1,4-Naphthoquinone
- (WR 25175) 2-Hydroxy-3-/3-Cyclohexylpropyl-1,4-Naphthoquinone
- (WR 174179) 7-N-Octadecylmercapto-6-Hydroxy-5,8-Quinolinequinone
- (WR 176204) 7-N-Tetradecylmercapto-6-Hydroxy-5,8-Quinolinequinone
- (WR 158122) 2,4-Diamino-6/2-Naphthylsulfonfyl/Quinazoline

- (WR 159407) 2,4-Diamino-6/0-Chlorophenyl/Thio/Quinazoline
 (WR 180872) 2,4-Diamino-6/2-Naphthylsulfonyl/5,6,7,8-Tetrahydroquinazoline
 (WR 181178) 2,4-Diamino-6/3-Trifluoromethylphenylsulfonyl/5,6,7,8-Tetrahydroquinazoline
 (WR 6527) Tetracycline
 (WR 87781) Minocycline

Compounds such as primaquine, chloroquine, amodiaquine, quinoline methanols, phenanthrene methanols, diaminodiphenylsulfone, the diformyl derivative of diaminodiphenylsulfone which are known to have antimalarial activity either in the blood-induced mouse screen or the blood-induced chick screen or in humans failed to show prophylactic activity in the sporozoite test.

Summary

A test has been developed with *Aedes aegypti* mosquitos and sporozoite-induced *Plasmodium gallinaceum* infections in chicks that allows the routine testing of candidate compounds for prophylactic activity. The success of this procedure may be attributed to: 1) the infected blood meals provided for the *Aedes aegypti* mosquitos in egg production cages; 2) the bite-to-bite passes of our donor line; and 3) the intrajugular inoculation of a sporozoite suspension prepared by grinding whole *Aedes aegypti* mosquitos infected with

Plasmodium gallinaceum by the method described in this paper.

We concede that the quantity of sporozoites introduced with an injection can only be approximate since the number of sporozoites per mosquito may vary considerably from lot to lot. Nevertheless, the mortality rate of the negative controls of our tests continues to be 100 per cent, proving the adequacy of our sporozoite injection. Since the overall mean survival time of the negative controls of 152 tests was no more than 8.34, it would seem that the variations in numbers of sporozoites produced by the different lots of mosquitos reared in this laboratory are not extensive.

Over 100,000 chicks were used during the development of our procedure and the introduction of routine testing.

During the developmental period improvements were made in the design of cages, methods of feeding and watering mosquitos, methods of grinding whole mosquitos and methods of filtering suspensions of ground mosquitos. Changes such as these simplified techniques, increased the efficiency of our operation and now enable us to proceed with the screening of candidate compounds on a large scale.

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Utilization of a Sporozoite Induced Rodent Malaria System for Assessment of Drug Activity

M. E. KING, A. M. SHEFNER AND M. D. SCHNEIDER

IIT Research Institute, Life Sciences Division,
10 West 35th Street, Chicago, Illinois 60616

ABSTRACT: A laboratory model employing the cyclical transmission of *P. berghei yoelii* in *A. stephensi* and mice is used for determining antimalarial action of drugs. By varying the time at which drug treatment of sporozoite infected mice is initiated, prophylactic or schizonticidal activity can be determined. Confirmation of prophylactic activity for those compounds which exhibit both prophylactic and therapeutic activity is obtained by subinoculation techniques.

Methodology for assessing the antimalarial activity of drugs against blood-passaged *P. berghei* infections in rodents has been described by Peters (1967). Such tests can provide valuable information but only concerning drug activity against blood stages of the parasite. A laboratory model useful for assessing both prophylactic and schizonticidal or gametocidal activity necessitates an abundant supply of infective sporozoites, which until recently could not be obtained from laboratory bred mosquitoes and the *P. berghei* strains that were in use.

The demonstration by Yoeli et al. (1964) of cyclical transmission of *P. berghei berghei* by the bite of experimentally infected *A. quadrimaculatus* and the subsequent characterization of sporogonic and pre-erythrocytic development of *P. berghei berghei* by Yoeli and Most (1965) led to the development of a system for repetitive sporozoite induction of rodent malaria. Vanderberg et al. (1968) have been able to develop such a system consisting of *P. berghei berghei* and *A. stephensi* for chemotherapeutic studies despite reported variability of sporozoite infectivity.

Landau and Killick-Kendrick (1966) later isolated *P. berghei yoelii* and found it to be infective for laboratory rodents and to produce infective sporozoites in laboratory bred *A. stephensi*. A system incorporating Landau and Killick-Kendrick's findings has been established in our laboratories and has been used for evaluating the antimalarial activity of

drugs for the past three years. By varying the time at which drug treatment of sporozoite infected mice is initiated, prophylactic or schizonticidal activity can be determined.

However, certain problems are inherent in the interpretation of results of drug activity in this system which are common to other drug evaluation systems as well. Particular drugs may have a relatively long residence time in the body either in unchanged form or as active metabolites. The apparent prophylactic activity may result from the presence of low but effective levels of drugs which are active against blood stages of the parasites. This problem of definition does not arise for those drugs which show activity on prophylactic schedules but which are inactive on therapeutic schedules.

In addition the assessment of drug activity based on an absence of parasitemia upon examination of blood smears has an obvious statistical meaning, i.e. no parasites are detected in that number of cells examined. A positive statement concerning the absence of infectious particles after drug therapy can only be verified by the use of subinoculation techniques.

This report describes the test system employed and the procedures that were followed in order to resolve some of the difficulties in interpretation of drug activity.

Materials and Methods

Weanling female Ha/ICR mice were purchased commercially and maintained on a standard pelleted diet and water ad libitum. All mice were quarantined for one week before experimental use.

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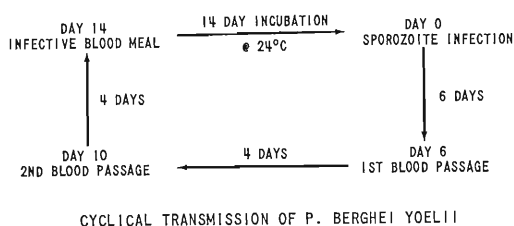


Figure 1

The *P. berghei yoelii* and *A. stephensi* utilized in this study were initially obtained from the Walter Reed Army Institute of Research. Parasitized blood from infected mice was cryopreserved at -190°C by the method of Schneider et al. (1968) and was retrieved after six months for use in our initial studies of cyclical transmission. The mosquito colony is maintained in an insectary at 26°C and 65% relative humidity. Fluorescent lighting is used for the 12-hour light cycle and there is stepwise switching to provide intensities corresponding to sunrise and sunset.

Steps in the cyclical transmission follow the suggestions of Wery (1966) and are shown in Figure 1. Mice are infected by intraperitoneal injection of sporozoites on day 0. Six days after infection a blood passage is made to fresh mice from which a second blood passage is carried out on the fourth day of their infection. Four days later, mice from the second passage are used as the infective blood meal for *A. stephensi*. The infected mosquitoes are placed in a 24°C incubator and fed daily on 10% sucrose. The mosquitoes are used for sporozoite infection after 14 days, thus 28 days are necessary to complete the cycle.

Infected mosquitoes, in lots of 60 are stunned by placing in a freezer for 3 minutes and then transferred to glass mortars chilled in ice. Each lot is triturated in 4 ml of 10% inactivated rabbit serum in saline and the mixture clarified by centrifuging for 5 minutes at 1000 RPM. Mice are then intraperitoneally injected with 0.2 ml of the supernatant liquid containing the sporozoites equivalent to 3 mosquitoes.

Drug doses of 160, 40, and 10 mg/kg are given subcutaneously to groups of 5 infected mice for 3 consecutive days. In testing for

prophylactic activity, mice are treated on the day before, the day of, and the day after sporozoite infection. In order to test for therapeutic activity, mice are treated on days 3, 4, and 5 post infection. Parasite counts are made in Giemsa stained thin films of tail blood.

Results

The effect of selected drugs on parasite growth in mice is shown in Table 1. Parasitemia values for the untreated control group reach a peak at 14 days post infection and decrease to zero by day 28. The strain is not lethal and 100% recovery is usually observed. Chloroquine has no effect when treatments were initiated on the day preceding sporozoite infection. However suppression, but not complete elimination, was observed when treatments were begun 3 days after infection. This is in agreement with the innate chloroquine resistance of the strain observed by Warhurst and Killick-Kendrick (1967). Similar results were obtained for quinacrine, to which the strain is also refractory. Primaquine, on the other hand, at a total dose of 90 mg/kg was completely effective in eliminating parasitemia when the mice were treated on the early prophylactic schedule but was ineffective when used for treatment on the later therapeutic schedule. Pyrimethamine, cycloguanil pamoate, 4,4-diaminodiphenyl sulfone (DDS), and trimethoprim eliminated parasitemia when used for treatment on either schedule.

When a drug exhibits both prophylactic and therapeutic activity it must be determined whether the prophylactic activity is real or apparent. A drug which acts only on blood stages may appear to have causal prophylactic activity when in reality it is slowly metabolized or eliminated and an effective dose may still be present when blood stages appear. In order to resolve this problem an additional test protocol is utilized for all drugs showing both prophylactic and therapeutic activity. Sporozoite infected mice are treated on the prophylactic schedule with the lowest drug dose that was found to be active in the initial prophylactic test. The treated mice are sacrificed at 72 hours post infection and fresh mice are subinoculated with pooled blood and pooled

Table 1. Activity of antimalarial drugs in sporozoite-mouse test system.

Group ^a	Daily dose mg/kg	Mean parasitemia %									
		Day on which treatment was initiated									
		-1					3				
		Parasitemia, day					Parasitemia, day				
		6	10	14	21	28	6	10	14	21	28
Controls		7.1	12.6	25.7	0.8	0					
Chloroquine	30	3.9	13.5	38.9	13.3	0	0	3.8	6.1	8.1	0
	15	1.6	10.1	32.8	3.5	0	0	5.5	10.8	12.4	0
Quinacrine	30	0.1	4.8	11.3	5.6	0	0.5	1.7	6.9	0	0
	15	0.8	5.5	17.9	0.2	0	0.1	1.9	5.6	0.1	0
Cycloguanil pamoate	30	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
DDS	30	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
Pyrimethamine	30	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
Primaquine	30	0	0	0	0	0	0	4.2	12.2	21.9	0
	15	1.5	9.7	14.8	13.1	0	1.4	8.4	21.2	30.5	0
Trimethoprim	30	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0

^a 10 mice per group.

liver homogenates from the treated donors. A true prophylactic drug eliminates all sporozoites and/or tissue schizonts in the donor mice and no infections should be obtained in the recipient mice.

Table 2 shows the results for the same group of antimalarials listed in Table 1 when they were used in the differentiation test. Except in one case these data parallel that previously obtained. As was expected patent infections were obtained in recipient mice injected with either blood or liver homogenates from donor mice treated with chloroquine or quinacrine. Patent infections were not obtained from blood or liver from donor mice treated with either primaquine, pyrimethamine, DDS or cycloguanil pamoate. On the other hand, trimethoprim which appeared to act both as a prophy-

lactic and as a therapeutic agent in the initial sporozoite test did not prevent infections from either blood or liver homogenates. The differentiation test thus confirmed the lack of prophylactic activity of chloroquine and quinacrine, and the prophylactic activity of primaquine, cycloguanil pamoate, DDS, and pyrimethamine. However trimethoprim which originally appeared to exhibit both types of activities, would be classed as a long acting therapeutic agent.

The sporozoite test has been used in screening over 700 drugs during the past 3 years. The initial screen not only eliminated those compounds that are completely inactive but also pointed out those compounds that are only effective against blood stages of the parasite. Approximately 60 of the compounds that passed the initial screen have been reevaluated in the differentiation test for confirmation of activity against sporozoites and/or tissue stages. Some 35 compounds showed prophylactic activity as verified by the subinoculation technique.

The use of this retesting procedure for all active compounds provides other useful information in that it distinguishes *between* those compounds which produce cures and those which reduce parasite levels below the limit of detection. Titration studies with *P. berghei yoelii* have shown that blood from infected control mice with parasite burdens of less than

Table 2. Differentiation test.

Group	Total dose mg/kg	Donor blood patency at 72 hours	Recipient patency infection source	
			Blood	Liver
Control		0/5	10/10	10/10
Chloroquine	30	0/5	10/10	10/10
Quinacrine	30	0/5	10/10	10/10
Cycloguanil pamoate	30	0/5	0/10	0/10
DDS	30	0/5	0/10	0/10
Pyrimethamine	30	0/5	0/10	0/10
Primaquine	30	0/5	0/10	0/10
Trimethoprim	30	0/5	8/10	9/10

10% can be diluted over 100,000-fold and still produce 100% infections in subinoculated recipient mice. Any drug capable of reducing or maintaining blood parasites at this level of one per 10^6 red cells would normally be judged to be curative if such decisions were only based in examination of blood smears. The probability of detecting parasites under these conditions would be extremely low unless an inordinately large number of red cells were examined. Thus, a conclusive demonstration of a true curative effect necessitates the use of subinoculation procedures. That infective blood can readily be judged to show an absence of parasites is also seen in Table 2 where no parasites were detected in blood smears of donors although both blood and liver homogenates were highly infective at this time.

An experiment currently underway may yield information relating to this question of cure. Mice prophylactically treated with trimethoprim show no evidence of blood parasites when examined on days 6, 10, 14, 21 and 28 of infection. Yet at three days post infection these mice have infective parasites in blood and liver. We plan to carry out subinoculations of trimethoprim treated mice at various time periods to determine whether infective cells are present over a long period of time without leading to a rise in parasitemia to detectable levels.

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In vitro Evaluation of Antimalarial Drug Combinations

G. J. MCCORMICK AND C. J. CANFIELD

Division of Medicine, Walter Reed Army Institute of Research, Washington, D. C. 20012

ABSTRACT: Synergism in the treatment of malaria has been found *in vivo* when drugs which are competitors of p-aminobenzoic acid are combined with drugs which are inhibitors of dihydrofolic acid reductase. Other drug combinations have given additive effects. The *P. knowlesi* system for antimalarial drug evaluation *in vitro* has demonstrated synergism between sulfalene (a p-aminobenzoic acid competitor) and trimethoprim (a dihydrofolic acid reductase inhibitor) and additive results in combinations of quinine with pyrimethamine or chloroquine. These results correlate well with *in vivo* observations. Combinations of trimethoprim and 5-fluoro-orotic acid, not studied previously for antimalarial activity, had additive effects *in vitro*. The *in vitro* system may be used as a simple technique for demonstration of synergistic combinations of drugs or classes of drugs for treatment of malaria.

Enhanced therapeutic effects have been observed with various combinations of drugs in the treatment of malaria. Historically, enhancement has been seen when each drug represents one of two classes of chemicals: compounds which compete with p-aminobenzoic acid in the biosynthesis of folic acid, and compounds which inhibit the action of the enzyme dihydrofolic acid reductase. Both actions result in inhibition of parasite growth by interruption of biosynthesis of thymidylic acid and thereby of DNA. The enhanced therapeutic effect is an example of drug synergism. Synergism, in pharmacological usage, describes the circumstance when the effect of drugs used in combination is greater than the sum of the effects produced by the drugs individually. If the enhancement is obtained by combination of two active compounds, the synergism is said to be due to potentiation; if by combination of an active and an inactive compound, to sensitization. Generally, the terms "synergism" and "potentiation" are used interchangeably, since studies commonly have been done with combinations of active drugs. Synergism of action generally is ascribed to sequential occurrence of affected sites in the same metabolic pathway. This is the case with the p-aminobenzoic acid competitors and the dihydrofolic reductase inhibitors which are synergistic against malaria. They are effective at different sites in the folic acid metabolic pathway. If the drugs acted on different path-

ways or at the same site, synergism would not be expected, but rather additive or perhaps inhibitory action (Rollo, 1955).

In the treatment of *Plasmodium falciparum* in humans, potentiation between pyrimethamine (an inhibitor of dihydrofolic acid reductase) and sulphadiazine (a p-aminobenzoic acid competitor) has been reported in several studies (Hurly, 1959; McGregor *et al.*, 1963; Chin *et al.*, 1966).

Similar potentiation was seen between pyrimethamine and diamino-diphenyl-sulfone (DDS) by Basu *et al.* (1962). The combination of sulfalene, a long-acting sulfonamide, and trimethoprim, a dihydrofolic acid reductase inhibitor, was synergistic (Martin and Arnold, 1968).

Potentiating effects of drug combinations have been demonstrated in experimental malaria studies in laboratory animals. Greenberg *et al.* (1948) reported synergism between proguanil and sulfadiazine against *P. gallinaceum* in chicks. Later, Greenberg (1949) noted that in the same parasite, proguanil was potentiated by p-aminobenzoic acid competitors which were themselves active antimalarial drugs. Proguanil was not potentiated by quinine, pamaquine, chloroquine, or quinacrine nor by sulfonamides or metanilamides which are not p-aminobenzoic acid competitors. Reilly *et al.* (1950) reported quinine and pamaquine to be synergistic in suppressive effect against *P. lophurae* in chicks. Quinine and quinacrine, and pamaquine and quinacrine were additive in combination, while quinine and pentaquine were considered possibly synergistic. However,

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the combination of quinine and pamaquine was found not to be synergistic against *P. gallinaceum* in chicks (Bishop and McConnachie, 1952). Rollo (1955) found pyrimethamine and sulphadiazine were mutually potentiating when combined against *P. gallinaceum*. No potentiation was seen in the combination of pyrimethamine and proguanil. Bishop (1956) saw no potentiation of effect between pyrimethamine and quinine.

Jacobs *et al.* (1963) found no evidence of synergism in combinations of chloroquine, amodiaquine, primaquine and pyrimethamine against *P. berghei* in mice. Pyrimethamine and diaphenylsulfone were synergistic when used in combination against *P. gallinaceum* in chicks and *P. cynomolgi bastianellii* in rhesus monkeys by Basu *et al.* (1964). Richards (1966) tested combinations of pyrimethamine with various sulphonamides and a sulfone in normal and drug-resistant strains of *P. gallinaceum* in chicks and *P. berghei* in mice. Marked potentiation was found not only against the normal strains, but also against the drug-resistant strains. The combinations sulphomethoxine-pyrimethamine, sulphadimethoxine-proguanil, and sulphadimethoxine-cycloguanil were all found to be synergistic against *P. berghei* in mice by Peters (1968).

Thus, various combinations of drugs have been established as being synergistic in anti-malarial effects *in vivo*, both clinically and in the laboratory. In the interest of developing simpler alternative techniques for identification of other synergistic combinations, the *in vitro* cultivation system with simian malaria, *P. knowlesi*, (Canfield *et al.*, 1970; McCormick *et al.*, 1971) has been used with several combinations of drugs. To date, synergism has been demonstrated in the combination of trimethoprim and sulfalene, while additive effects have been found with quinine-chloroquine, quinine-pyrimethamine, and trimethoprim with 5-fluoro-orotic acid.

Materials and Methods

The techniques and materials were as previously described (Canfield *et al.*, 1970; McCormick *et al.*, 1971). Cultivations were done in minimum essential medium containing no folic acid, but with added fetal bovine serum. Drug effect was measured as inhibition of

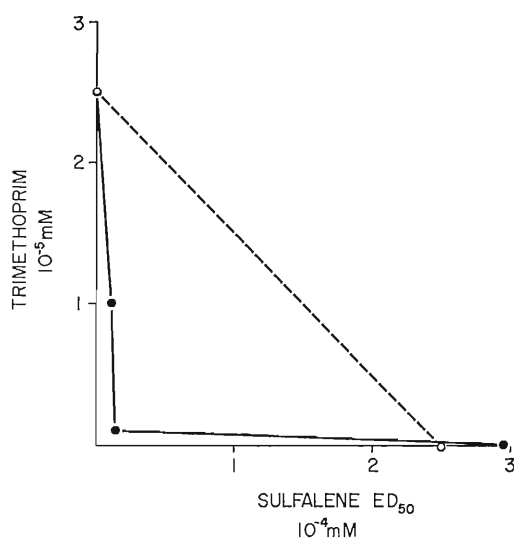


Figure 1. Synergism of sulfalene and trimethoprim in inhibition of incorporation of ^{14}C -orotic acid into *P. knowlesi* DNA *in vitro*. Dose response studies of sulfalene were done at different concentrations of trimethoprim. Values of ED_{50} for sulfalene are plotted on the abscissa versus concentration of trimethoprim on the ordinate. The open circles show the ED_{50} of each drug in the absence of the other, the broken line joining them is the theoretical locus of points if effects are additive. Points below this line indicate synergism, points above, antagonism.

the incorporation of radioactivity from ^{14}C -labeled orotic acid into DNA of *P. knowlesi* in blood from infected rhesus monkeys (*Macacca mulatta*). The effects of combination of two drugs were established by performing a series of dose response studies of one drug with different levels of the second drug. The dose response curves were plotted and the 50%-effective dose (ED_{50}) for each was determined graphically.

In order to visually demonstrate synergism in combined drug effect, the results were plotted as bolograms (Rollo, 1955). The individual ED_{50} 's of each of the drug pair were plotted on the graph and connected by a broken line. A simple additive effect would result in the experimental points falling along or near the broken line. Synergism would be indicated if the experimental points fell between the line and the origin. If the drugs

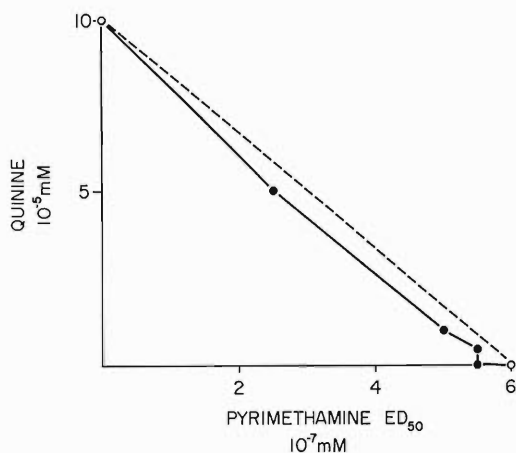


Figure 2. Additive inhibition by quinine-pyrimethamine combinations. Methods and construction as in Figure 1.

were mutually antagonistic, the points would fall outside the line.

Results

When the effect of trimethoprim on the ED_{50} of sulfalene was plotted (Figure 1), potentiation was apparent. The point nearest the origin indicates that 50% inhibition was achieved by a combination of sulfalene and trimethoprim at levels approximately one twenty-fifth of the individual ED_{50} 's for each

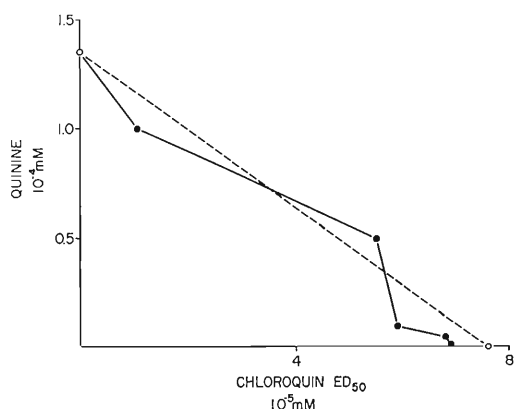


Figure 3. Additive inhibition by quinine-chloroquine combinations. Methods and construction as in Figure 1.

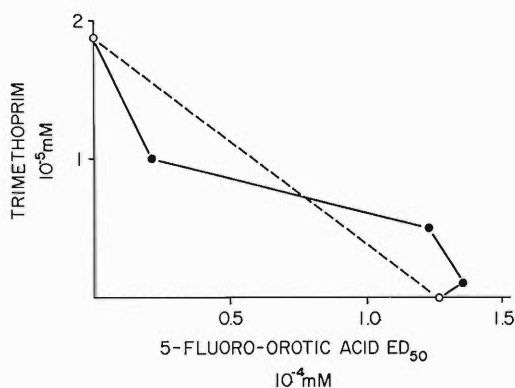


Figure 4. Additive inhibition by 5-fluoro-orotic acid-trimethoprim combinations. Methods and construction as in Figure 1.

drug. The ratio of the drugs at this point is 14 to one, sulfalene to trimethoprim.

Combination of quinine with pyrimethamine or chloroquine gave additive effects (Figures 2 and 3).

Combination of trimethoprim and 5-fluoro-orotic acid also gave additive results (Figure 4).

Discussion

Synergism of action has been observed with many combinations of antimalarial drugs when one drug competes with p-aminobenzoic acid and the other drug inhibits dihydrofolic acid reductase. It is generally accepted that the observed synergism results from the sequential nature of the two drug actions. Such sequential effectiveness has not, however, resulted in synergism in every case. Rubin *et al.* (1964) described lack of potentiation by successive inhibitors in the orotic acid-uridylic acid-metabolic sequence in an isolated system from rat liver and in intact dog leucocytes (in fact, the data reflected antagonism). This supported the view of Webb (1963) that theoretically in multiple enzyme sequences, the rate of formation of the end product can never be slower than the rate of the one slowest reaction in the sequence. More recently Werkheiser (1971) presented a mathematical model for DNA synthesis which did allow theoretical synergism calculations in a multiple enzyme system. This

same model predicts that potentiation may be found when one enzyme is acted upon by combinations of two inhibitors, one competitive, the other noncompetitive. This situation previously would be predicted to allow only additive or antagonistic results (Rollo, 1955).

Whether predictable or not, synergism requires a method for its demonstration. In the case of malaria, all demonstrations have been accomplished *in vivo* to date. The *P. knowlesi* system for evaluation of antimalarial drugs *in vitro* was applied in drug combination studies and has demonstrated the presence of synergism and given approximations of effective drug levels and optimum drug ratios.

Initial studies to establish a positive correlation with *in vivo* findings have been done with the combinations sulfalene and trimethoprim, pyrimethamine and quinine, and chloroquine and quinine.

The first combination is an example of the classic synergistic case. Sulfalene is a competitor of p-aminobenzoic acid in the synthesis of folic acid. Trimethoprim is an inhibitor of dihydrofolic acid reductase, the enzyme essential for reduction of the dihydrofolic acid formed during the methylation step yielding thymidylate. This reduction is necessary as a recycling step in metabolic synthesis of DNA. Inhibition results in cessation of DNA production. Both sulfalene and trimethoprim are effective antimalarials *in vivo* and *in vitro* in this system (McCormick *et al.*, 1971). The combination gave the expected synergistic results (Figure 1). Fifty percent inhibition was achieved by a combination of the drugs at one twenty-fifth of the required levels of the individual drugs. The ratio of the drug concentrations in this combination was 14 to one, sulfalene to trimethoprim. It is suggested that this approximates the optimum drug ratio which should be effective in therapeutic use *in vivo* at the site of action. It is recognized that actual administered dosages are governed by processes such as absorption, metabolic alteration, excretion, etc. These drugs were used successfully against *P. falciparum* by Martin and Arnold (1968) with a dose ratio of two to one, sulfalene to trimethoprim, at one-tenth and one-thirtieth of their respective individual required doses.

The combination of pyrimethamine and qui-

nine gave additive effects *in vivo* (Bishop, 1956; Bartelloni *et al.*, 1967). The results obtained *in vitro* in this study are also additive; the points lie very close to the line denoting addition (Figure 2).

Studies of chloroquine-quinine therapy against *P. falciparum* from Vietnam revealed that chloroquine apparently lowered the efficacy of quinine when used in combination with it. This finding is complicated by the resistance of the organisms to chloroquine; 89% of the patients treated with chloroquine alone relapsed within 30 days after treatment (Reed *et al.*, 1968). The combination of chloroquine and quinine gave no evidence of synergism *in vivo*. The results which were obtained *in vitro* also show no synergism; the experimental points are near the theoretical additive line (Figure 3).

On the basis of the results with these three combinations, the *in vitro* system seems to correlate well with the corresponding *in vivo* findings. Apparently both synergistic and additive effects are demonstrable in this system.

The first application to a previously untried combination was trimethoprim coupled with 5-fluoro-orotic acid (5 FO). 5 FO is effective against malaria in the *in vitro* system. This compound competes with orotic acid as a substrate for orotidylic acid phosphorylase (Bono *et al.*, 1964). The subsequent metabolic processes leading to the various pyrimidine nucleotides and thence to nucleic acids produce 5-fluoro-substituted analogs of the normal metabolites, including 5-fluoro-deoxyuridylic acid. This compound has a high affinity for thymidylic acid synthetase and competes with the normal substrate, deoxyuridylic acid. Thymidylic acid synthesis and subsequent DNA synthesis is inhibited (Welch, 1961; Bosch *et al.*, 1958). Since the action of trimethoprim results in lowered availability of the second substrate (5, 10-methylenetetrahydrofolic acid) for this same enzyme, the possibility of synergistic action existed (Dunlap *et al.*, 1971). The *in vitro* results, however, did not indicate that such action takes place. The experimental values do not describe a straight line, but absence of potentiation is obvious. Analogous combinations have given evidence of potentiation in leukemia *in vivo*, according to Goldin (1971). Methotrexate, like trimethoprim, an inhibitor of dihydrofolic acid reductase, was

combined with 5-fluoro-uracil or 5-fluoro-deoxyuridine. Prolongation of life in the test mice was reported to be greater than attributable to the individual drug effects. This exact experiment cannot be accomplished in the case of malaria because the parasites do not take up pyrimidines or ribosyl- or deoxyribosyl-pyrimidines; these compounds are not effective against malaria.

Additional studies are underway or planned which include other combinations which have been shown to be potentiating or additive *in vivo* in order to further establish the apparent correlation. The study of combinations of drugs or classes of drugs with possible synergistic activities which have not been investigated previously is an application which may yield much useful information for the treatment of malaria.

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The technical assistance of Gloria P. Willet, Robert D. Geer and Eugene C. Madonia is greatly appreciated.

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Studies on a Quinolinemethanol (WR 30,090) and on a Phenanthrenemethanol (WR 33,063) Against Drug-Resistant *Plasmodium berghei* in Mice¹

P. E. THOMPSON

Department of Parasitology, College of Veterinary Medicine,
University of Georgia, Athens, Georgia 30601

ABSTRACT: Studies were conducted in mice to characterize the activities of the quinolinemethanol WR 30,090 and of the phenanthrenemethanol WR 33,063 by use of sensitive *P. berghei* and lines that were resistant respectively to cycloguanil (T), pyrimethamine (Pyr), dapsone (S), and chloroquine (C). Quinine was included as a reference drug for the assessment of suppressive potency.

WR 30,090 and WR 33,063 were respectively about 60 and 5 times as potent as quinine, retained full activity against the C, Pyr, and S lines, were not antagonized by para-aminobenzoic acid (PABA), and acted rapidly. Both compounds exhibited diminished activity against chloroquine-resistant *P. berghei* proportional to the degree of chloroquine resistance. WR 30,090 acted for several days when given in one oral dose; WR 33,063 had less persistent action.

Brief comments are included on the structural relationships of WR 30,090 and WR 33,063 to quinine, their morphological effects on parasites, and interpretation of the results.

The synthesis and evaluation of quinoline-methanols and phenanthrenemethanols have been pursued for many years in the search for better antimalarial drugs (cf. review by Thompson and Werbel, 1972). Recently, certain members of the series, such as WR 30,090 and WR 33,063, have received special attention in the U. S. Army Malaria Program as potential agents for use in drug-resistant malaria.

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The quinolinemethanol WR 30,090 was first synthesized by Lutz *et al.* (1946). It was reported by Wiselogle (1946), under the designation SN 15,068, to be approximately 20 times as active as quinine against *Plasmodium lophurae* in ducks. Daily doses of 25 mg/kg, either orally or subcutaneously, were found to be highly effective against *P. berghei* in mice (Aviado and Belej, 1970). In contrast to the related quinolinemethanol SN 10,275 which is highly phototoxic in man (Wiselogle, 1946) and mice (Rothe and Jacobus, 1968), WR 30,090 has been found

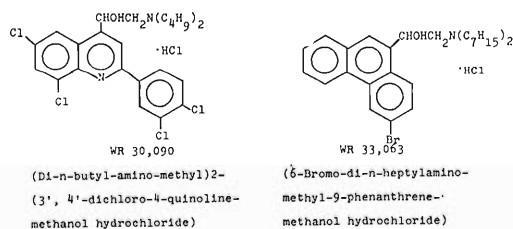
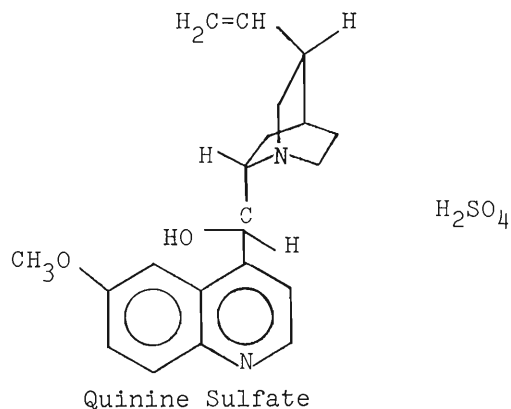
by the latter authors to have only low phototoxic potency in mice.

The phenanthrenemethanol WR 33,063 has been reported by Sweeney and Jacobus (1970) to be relatively free in mice of the phototoxic liability of the quinolinemethanols. They also reported that WR 33,063 was active in man against *P. vivax* and against both sensitive and moderately chloroquine-resistant strains of *P. falciparum*. It had, however, only suppressive action against a highly chloroquine-resistant strain of *P. falciparum* in man.

The present report deals with the characterization of the activities of WR 30,090 and WR 33,063 orally against *P. berghei* in mice. Aspects studied included potency relative to quinine, efficacy against lines that are resistant respectively to chloroquine, cycloguanil, pyrimethamine, and dapsone, rate and duration of action by a single oral dose, morphological effects of the drug on the parasites, and antagonism by para-aminobenzoic acid (PABA).

Materials and Methods

The drugs studied and their structural relationships to quinine are indicated below.



The mice used in the study were CF-1 females (Carworth Farms, Portage, Michigan) of approximately 18–24 gm weight range. They were fed Purina mouse chow. The parasites used included the sensitive KBG-173 strain of *Plasmodium berghei* (designated P) and 4 drug-resistant lines derived from it. The drug-resistant derivatives were lines C, T, S, and Pyr, which had been rendered resistant respectively to chloroquine, the triazine cycloguanil, the sulfone dapsone, and pyrimethamine. The history of these lines has been described by Thompson *et al.* (1967) and Thompson and Bayles (1968). The degree of resistance of the C line to chloroquine was moderate (30 to 40-fold) in early phases of the work but essentially complete (> 170-fold, resistant to maximum tolerated doses of the drug) during latter phases of the work. The T, S, and Pyr lines were highly resistant (> 100-fold) to the homologous drugs throughout the study. All of the resistant lines were kept under continuous drug pressure while maintained in mice except for the passage through donors serving as a source of blood for the tests. All infections were induced by injecting parasitized cells intraperitoneally.

Details concerning the various test procedures used are outlined below.

Parasites		Day Rx started	Day smears read
Lines	Inoculum		
Treatment by drug diet for 6 days:			
P,C,T,S	15×10^6	—1	+5
Treatment twice daily by gavage for 3 days:			
P	2.5×10^6	+3	+6
C,S	15×10^6	+3	+6
T	5×10^6	+3	+6
Treatment with single dose by gavage:			
P	15×10^6	—2 or —3	Daily

Drugs were given by the diet method by mixing them in powdered Purina mouse chow. In treatment by gavage, each dose was given in 10 ml/kg of aqueous 1% hydroxyethylcellulose-0.1 Tween 80 following dispersal by ultrasonication. All doses are expressed in terms of the free base.

Suppressive effects were assessed from Giemsa-stained blood smears as described by Thompson *et al.* (1969). In the graphs portraying the suppressive effects solid points are

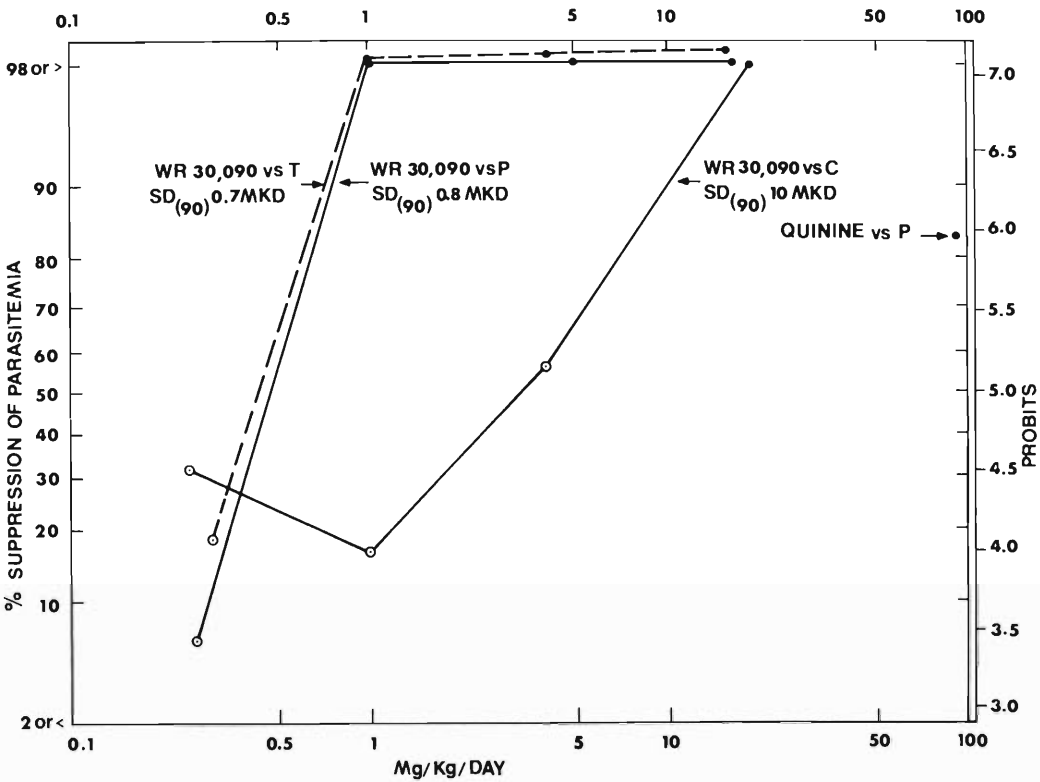


Figure 1. Comparative suppressive effects of WR 30,090 in the diet against the P, T, and C lines when the latter was moderately resistant to chloroquine; effects of quinine in the diet against the P line.

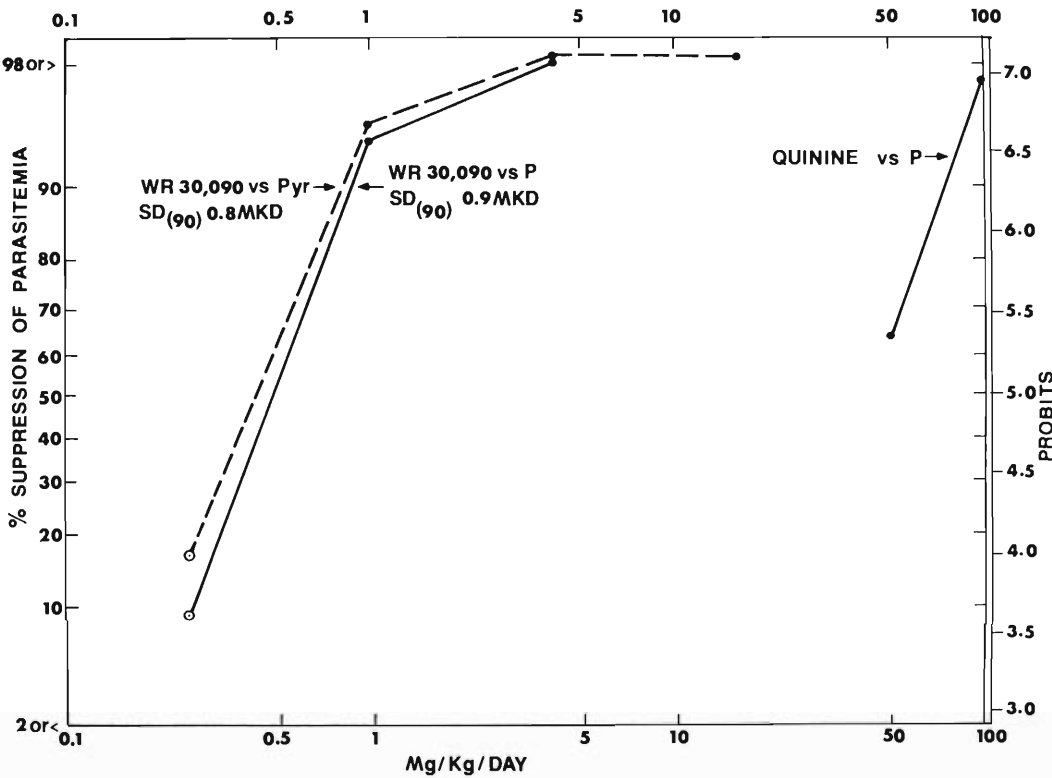
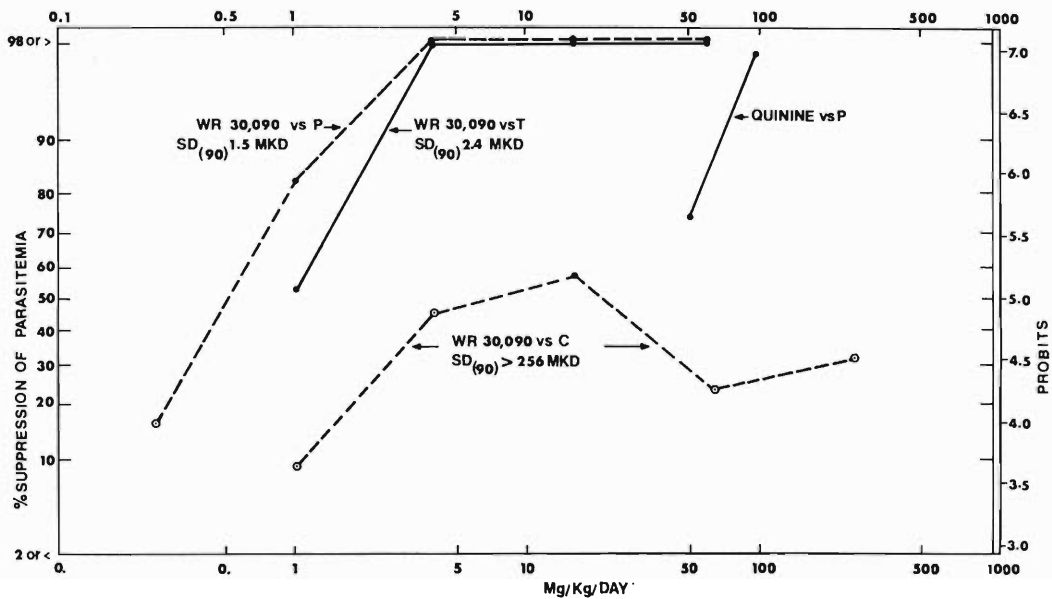
used when the effects were statistically significant ($\alpha = < 0.05$) by "t" tests and open circles are used to indicate values that were not statistically significant (i.e. $\alpha = > 0.05$). Quinine was usually included as a reference drug in tests for suppressive action against the P line.

Tests for antagonism of the action of a drug by PABA were conducted with the P line as in previous work (Thompson *et al.*, 1970). Briefly, mice were infected with 2.5×10^6 parasitized cells and the test for antagonism was done by adding 0.025% supplementary PABA to the ration used to maintain the mice throughout the 6 days of an experiment. This diet level results in the ingestion of 20 to 50 mg of supplementary PABA/kg/day. The test drug was given by gavage twice daily during days 3, 4, and 5. The re-

sults were read on day 6 by comparing the action of the drug at several dose levels in mice maintained with and without supplementary PABA.

To assess the rate and duration of action of the drugs, one oral dose was given in early patency (2 days after the injection of parasites) and effects were assessed by daily counts of the parasitemia. The duration of action was determined from the number of days elapsing between the onset of marked inhibition of the developing parasitemia and the resumption of a logarithmic increase in the parasitemia.

Seven or occasionally 10 mice were used for each dose level of drug tested and 10 mice were used as controls; the latter were sham-dosed in experiments based upon treatment by gavage.



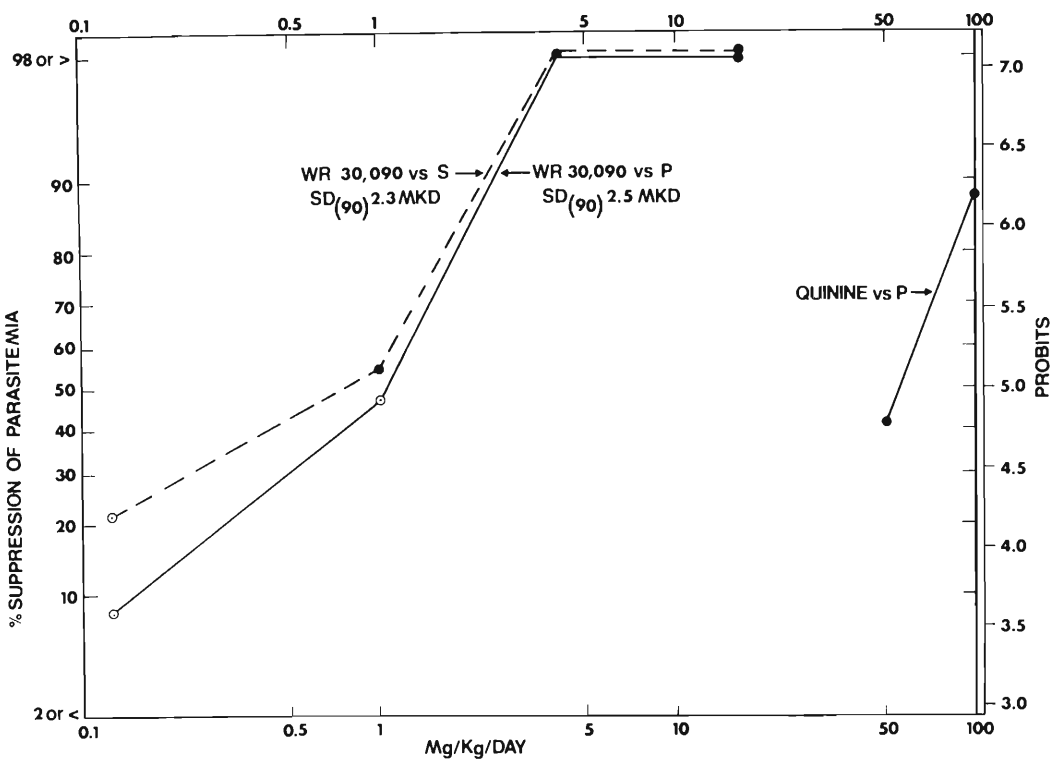


Figure 4. Comparative suppressive effects of WR 30,090 by gavage against the P and S lines; effects of quinine by gavage against the P line.

Results

(A) Quinolinemethanol WR 30,090

WR 30,090 was examined by the drug diet method concurrently against the P, C, and T lines when the C line was moderately resistant to chloroquine. The diet concentrations studied were 0.0125, 0.0031, 0.0008, and 0.0002%. Quinine was tested at a diet level of 0.06%. The results are summarized in Figure 1. The respective $SD_{90's}$ (dose required for 90% suppression) in mg/kg/day were as follows: 0.8 for line P, 10 for line C, and 0.7 for line T. The C line thus showed approximately 13-fold

resistance to WR 30,090, but the T line was fully sensitive to it. A quinine dose of 95 mg/kg conferred against the P line 84% suppression. This was 135-times as much quinine as the amount of WR 30,090 required for comparable suppression.

Later when the C line had become essentially fully resistant to chloroquine, WR 30,090 was tested by gavage at 4 to 5 dose levels against the P, C, and T lines and quinine was included at 2 dose levels against the P line. The results are shown in Figure 2. The $SD_{90's}$ were 1.5 for the P line, > 256 for the C line and 2.4 for the T line. The SD_{90} for

Figure 2. Comparative suppressive effects of WR 30,090 by gavage against the P, T, and C lines when the latter was extremely resistant to chloroquine; effects of quinine by gavage against the P line.

Figure 3. Comparative suppressive effects of WR 30,090 by gavage against the P and Pyr lines; effects of quinine by gavage against the P line.

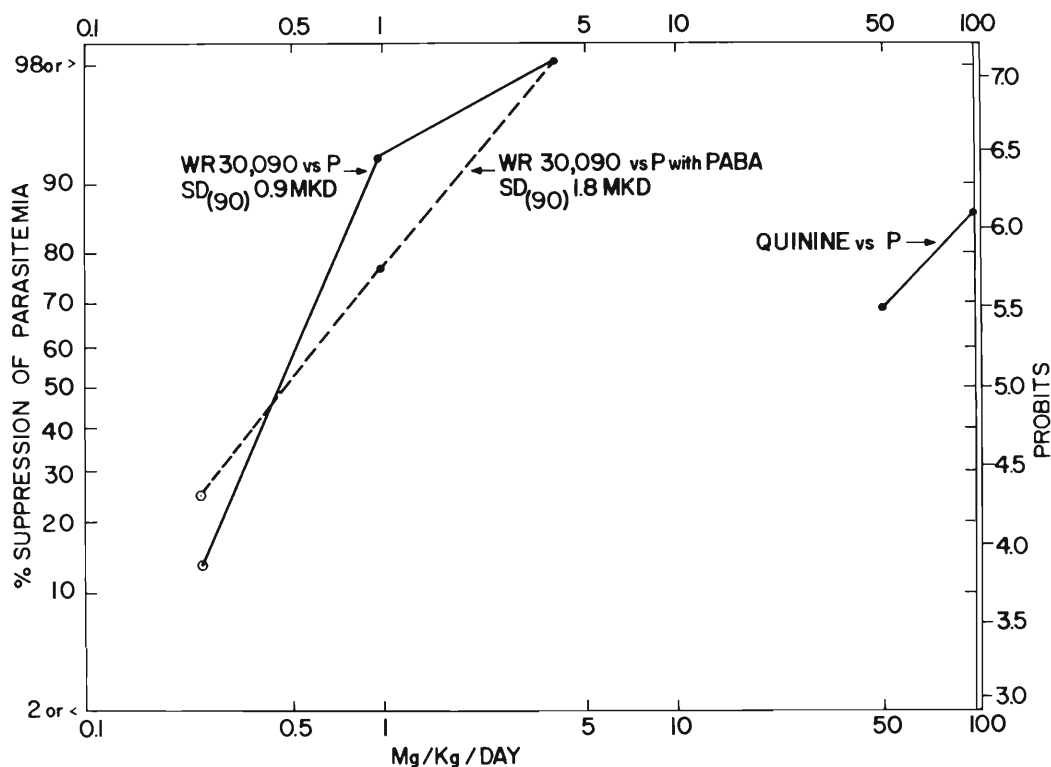


Figure 5. Comparative suppressive effects of WR 30,090 by gavage against the P line in the absence and presence of supplementary PABA in the diet; effects of quinine by gavage against the P line.

quinine was 70 mg/kg/day. The C line thus showed > 165-fold resistance to WR 30,090. The small difference between the responses of the P and T lines was probably within the experimental error of the work. The quinine equivalent of WR 30,090 in this experiment was 46 (based on the ratio of the $SD_{90's}$).

The comparative effects of WR 30,090 against the P and Pyr lines along with an additional assessment of the quinine equivalent of the drug against the P line were determined in another experiment (Fig. 3). The P and Pyr lines responded similarly with respective $SD_{90's}$ of 0.9 and 0.8 mg/kg/day. The SD_{90} of quinine against the P line was 72 mg/kg/day. Thus, WR 30,090 exhibited in this experiment a quinine equivalent of 81.

The effects of WR 30,090 against the P and S lines also were similar: the respective $SD_{90's}$ were 2.5 and 2.3 mg/kg/day (Fig.

4). The quinine equivalent of WR 30,090 against the P line was determined at the same time and, at the SD_{80} level of comparison, was found to be 45. Confirmation of full activity by WR 30,090 against the S line was observed in 2 less complete preliminary experiments.

The capacity of PABA to antagonize the action of WR 30,090 was determined by use of the P line. Quinine also was tested against the P line in mice on the regular diet. Groups of mice maintained with and without supplementary PABA were given WR 30,090 by gavage, in doses of 4, 1, and 0.25 mg/kg/day. The PABA intakes in the treated and control groups ranged from 29 to 50 mg/kg/day. The PABA controls had similar mean parasitemias to the controls not given supplementary PABA; actually 56% of the cells were parasitized in each group. The suppres-

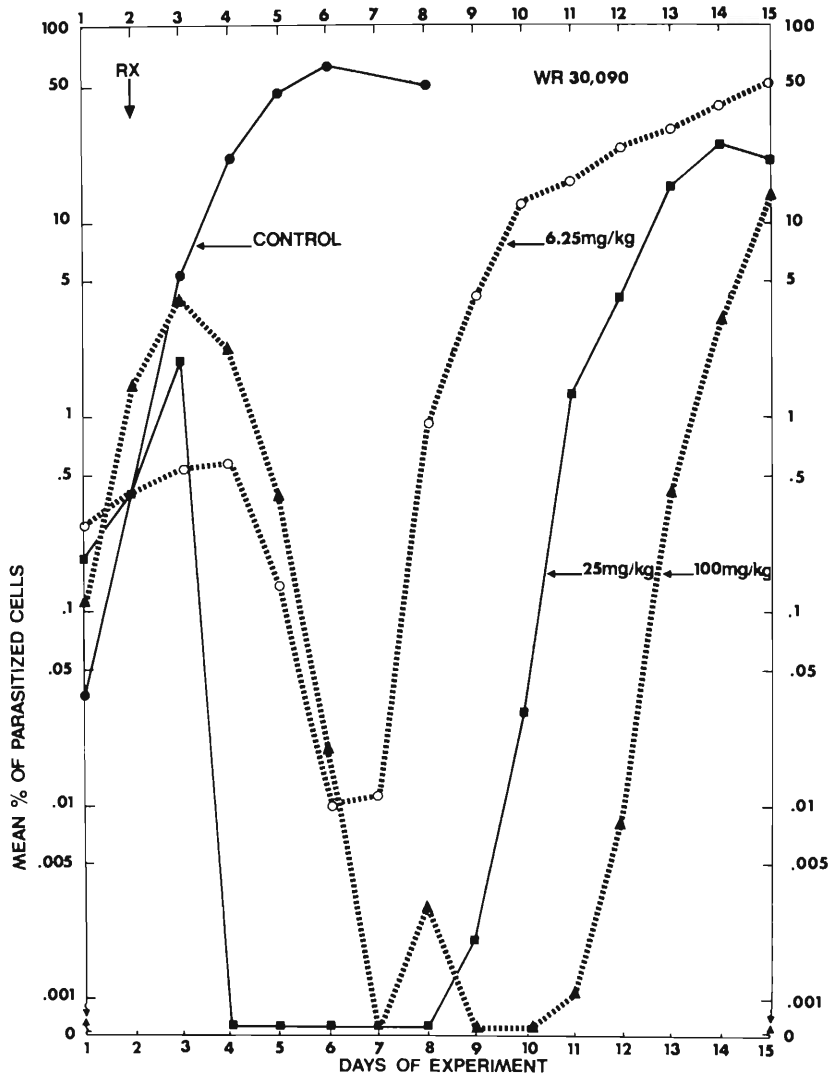


Figure 6. Rate and duration of suppressive action by single oral doses of WR 30,090 against patent P line infections.

sive effects of the drugs are shown in Figure 5. The $SD_{90\%}$ of WR 30,090 (in mg/kg/day) were 1.8 with supplementary PABA and 0.9 without it. This small difference probably is within the experimental error of the work. Such results, even though very high ratios of antagonist to drug were tested, made it evident that the action of WR 30,090 is not appreciably affected by PABA. The quinine

equivalent of WR 30,090 (at the SD_{80} level of comparison) was 80.

Data depicting the rate and duration of action of WR 30,090 against the P line when given in one oral dose of 100, 25, or 6.25 mg/kg are shown in Figure 6. A dose of 100 mg/kg inhibited parasite multiplication within one day and exhibited activity for 9 days. A 25 mg/kg dose was suppressive within 2 days

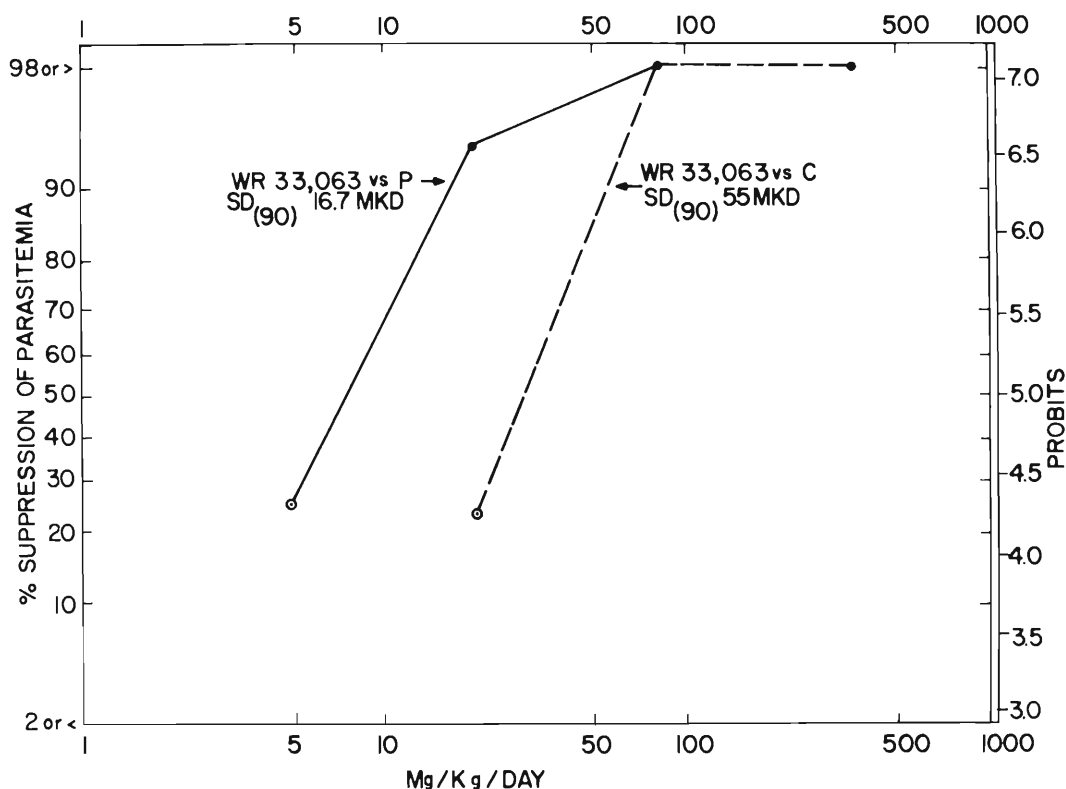


Figure 7. Comparative suppressive effects of WR 33,063 in the diet against the P and C lines when the latter was moderately resistant to chloroquine.

and was effective for 5 days. The 6.25 mg/kg dose was suppressive within 2 days and it continued to exhibit suppressive action for 4 days. Parasites were examined from treated mice on the second day after drug administration for evidence of drug-induced changes in morphology and staining reactions. Both the cytoplasm and to a less extent the chromatin appeared to be affected. The cytoplasm stained pink rather than blue and appeared more diffuse than normally. The changes in the chromatin were primarily in the direction of fragmentation, with a suggestion of alteration in the normal pattern of division; such changes in the chromatin were noted, however, in only a low percentage of the parasites.

(B) Phenanthrenemethanol WR 33,063

The comparative suppressive effects of WR 33,063 by the drug diet method against the

P line and against the C line when it was moderately resistant to chloroquine are depicted in Figure 7. The diet concentrations tested were 0.05, 0.0125, and 0.031% against the P line and 0.2, 0.05, and 0.0125% against the C line. Respective $SD_{90's}$ (in mg/kg/day) of 16.7 for the P line and 55 for the C line indicated that the latter was approximately 4-fold less sensitive to the drug than the P line.

In the second experiment, WR 33,063 was tested against the P, T, S, and C lines when the latter was fully resistant to chloroquine. Quinine also was tested at 2 dose levels against the P line. The results are summarized in Figure 8. The $SD_{90's}$ of WR 33,063 in mg/kg/day were 14.5 for line P, 19.5 for line T, 14.1 for line S, and > 320 for line C. The SD_{90} of quinine against line P was 75 mg/kg/day. These results indicated that WR 33,063

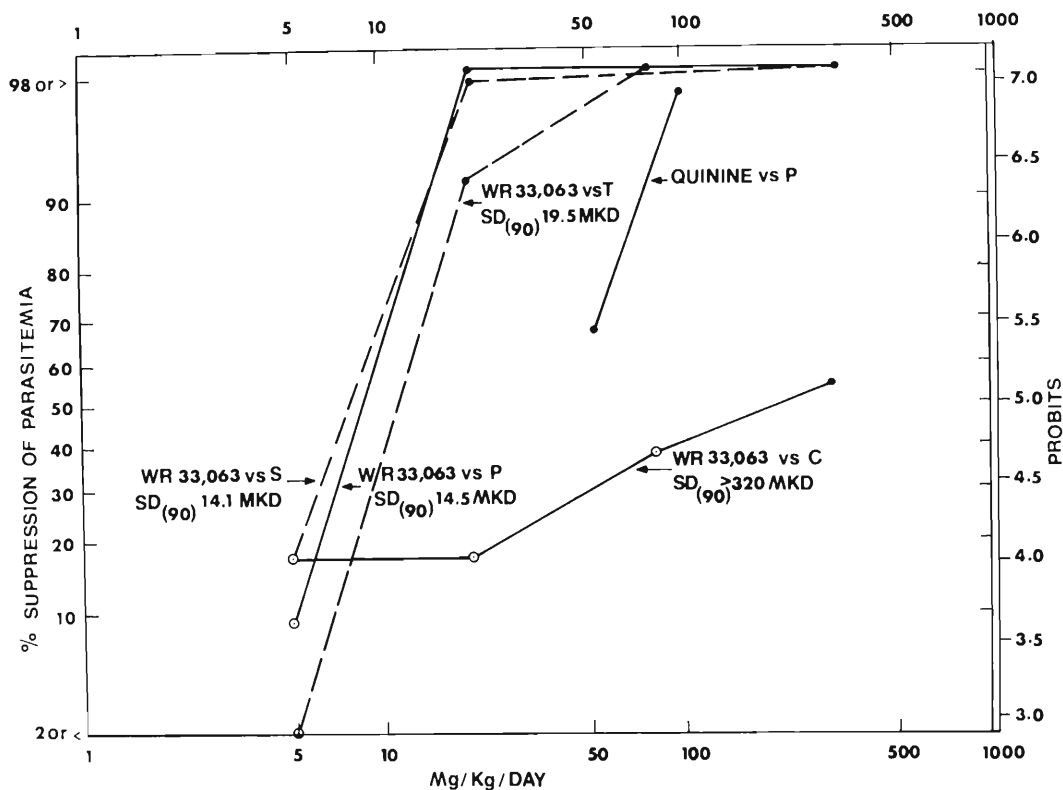


Figure 8. Comparative suppressive effects of WR 33,063 by gavage against the P, T, S, and C lines when the latter was extremely resistant to chloroquine; effects of quinine by gavage against the P line.

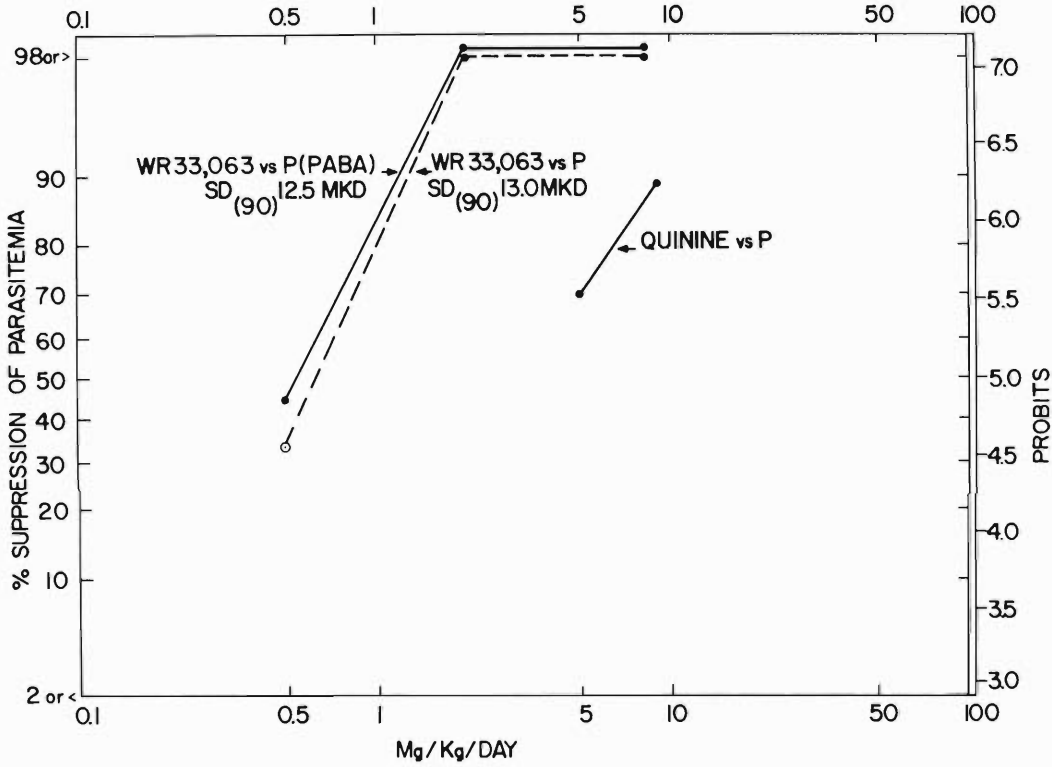
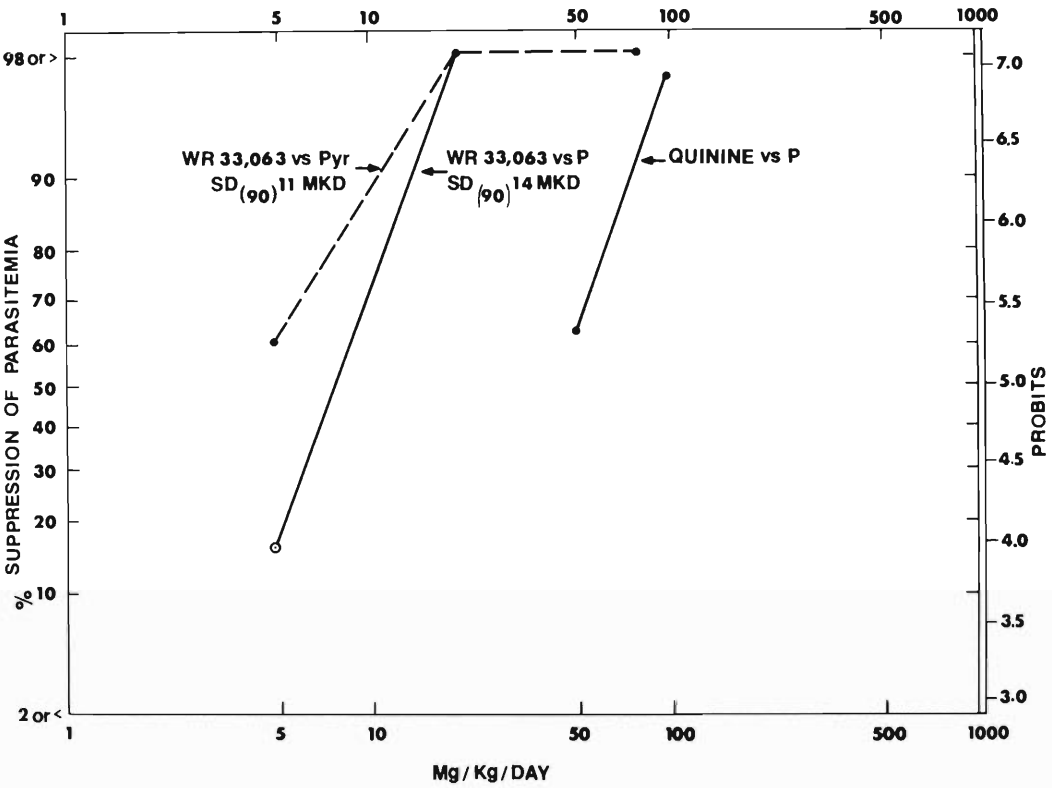
was approximately 5 times as active as quinine, exhibited essentially full activity against lines that were highly resistant to cycloguanil or dapsone, but had > 27 -fold reduction in activity against the line that was highly resistant to chloroquine.

The comparative suppressive effects of WR 33,063 by gavage against the P and Pyr lines along with the further comparison with quinine are shown in Figure 9. These data yielded the following values: SD_{90} versus P and Pyr, 14 and 11 mg/kg/day, respectively, with a quinine equivalent of 5 against P.

Tests for the capacity of supplementary PABA (0.025% in the diet) to antagonize the action of WR 33,063 followed the same pattern as has been presented for WR 30,090. The ratios of antagonist to drug were lower, however, owing to the lower potency of WR 33,063. The PABA/WR 33,063 ratios actually

tested were 36/80, 30/20, and 56/5. The results, summarized in Figure 10, were as follows: SD_{90} 's with and without supplementary PABA 12.5 and 13 mg/kg/day, respectively, and quinine equivalent of 6 (based on comparison at the SD_{80}). There was thus no indication that PABA could antagonize the action of WR 33,063.

Data dealing with the rate and duration of action by single doses of WR 33,063 are shown in Figure 11. A 200 mg/kg dose promptly halted parasite growth and acted for 2 days, a 50 mg/kg dose acted at the same rate but suppressed growth for only one day, a 12.5 mg/kg dose had only slight inhibitory effect. Parasite damage was only temporary and not severe; it was seen only in mice treated with 200 mg/kg and 50 mg/kg. On the second day after treatment, a few schizonts appeared to have less cytoplasm



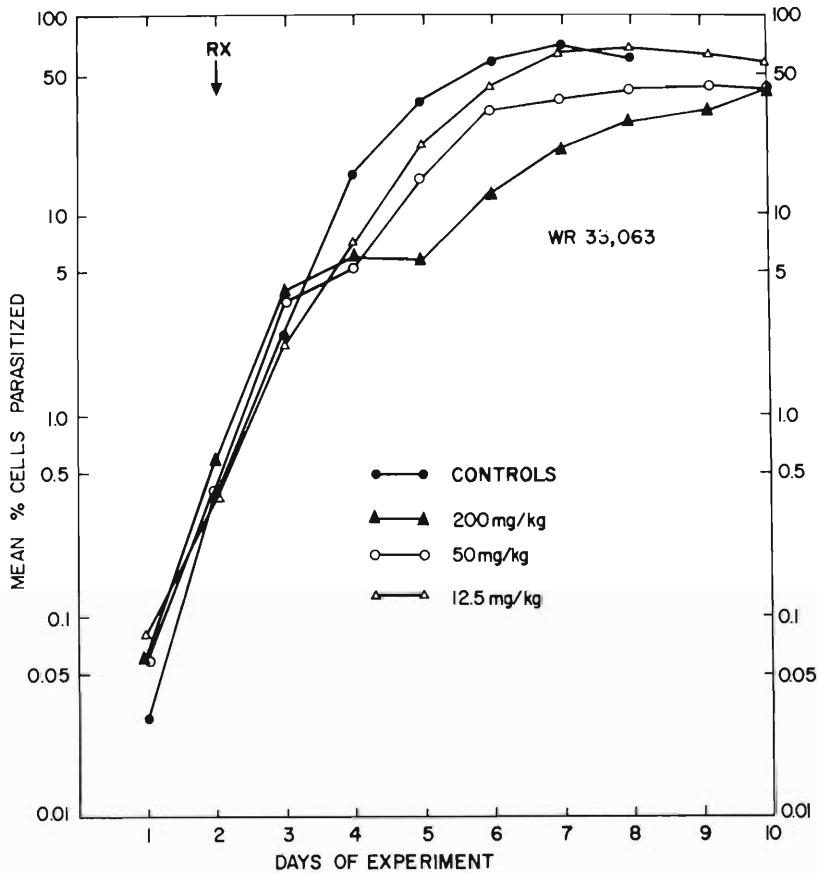


Figure 11. Rate and duration of suppressive action by single oral doses of WR 33,063 against patent P line infections.

than usual; in these parasites some of the chromatin appeared to be less well-defined than normally and somewhat blurred. The mean survival times in days of the mice were as follows: controls, 7; 200 mg/kg, 10; 50 mg/kg, 9; and 12.5 mg/kg, 8.

Discussion

The results obtained in this work offer both some encouragement and some discouragement

regarding WR 30,090 and WR 33,063 as potential drugs.

The quinolinemethanol WR 30,090 was approximately 60-fold more active than quinine. It acted rapidly and for a relatively prolonged period. It also was fully active against lines that are highly resistant to cycloguanil, pyrimethamine, or dapsone. Full activity against the dapsone-resistant line was consistent with the finding that its action was

Figure 9. Comparative suppressive effects of WR 33,063 by gavage against the P and Pyr lines; effects of quinine by gavage against the P line.

Figure 10. Comparative suppressive effects of WR 33,063 by gavage against the P line in the absence and presence of supplementary PABA in the diet; effects of quinine by gavage against the P line.

not appreciably antagonized by PABA. WR 30,090 exhibited, however, a disturbing degree of cross resistance with chloroquine; this ranged from 13-fold with moderately chloroquine-resistant parasites to > 165-fold with those that were extremely resistant.

The phenanthrenemethanol WR 33,063 was about 5 times as active as quinine. It acted rapidly but its effects were of relatively short duration. It also exhibited full activity against parasites that were resistant to cycloguanil, pyrimethamine, or dapsone, and its action was not antagonized by PABA. It showed, however, cross-resistance with chloroquine, which ranged from 4-fold with moderately chloroquine-resistant parasites to > 27-fold with those that were extremely resistant. The present indication with *P. berghei* of an inverse relationship between the degrees of efficacy by WR 33,063 and the degrees of parasite resistance to chloroquine is in agreement with the suggestion of Sweeney and Jacobus (1970) that the performance of the drug against *P. falciparum* in man depends upon its degree of resistance to chloroquine.

It is of interest to note that both WR 30,090 and WR 33,063 share with quinine a methanol side chain. Various investigators (cf. review by Thompson and Werbel, 1972) have found cross resistance with *P. berghei* between quinine and chloroquine. Some indications in the same direction have been noted also with *P. falciparum* in man (McNamara *et al.*, 1967). It should be emphasized, however, that the lines of *P. berghei* used have a much higher degree of resistance to chloroquine than is known to occur in *P. falciparum*. Consequently, the *P. berghei* results if interpreted too rigidly could lead to an underestimation of the potential value of a compound in dealing with chloroquine-resistant *P. falciparum*.

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The Relation of Polychromatophilia to Parasitemia and Mortality in CF₁ Mice with Drug-Resistant and Parent Lines of *Plasmodium berghei*¹

W. L. HANSON AND P. E. THOMPSON

Department of Parasitology, College of Veterinary Medicine,
University of Georgia, Athens, Georgia 30601

ABSTRACT: Mice inoculated with 15×10^6 RBC parasitized with the parent line of *Plasmodium berghei* develop mean parasitemias of 80% or greater 6–7 days after the injection of parasites and all such animals are dead by day 9. Mice injected with an equivalent number of *P. berghei* which are resistant to chloroquine or dapsone do not develop comparable parasitemias until 15–22 days after injection of parasites. The course of parasitemia in mice inoculated with an equivalent number of parasites resistant to triazine or pyrimethamine was similar to that seen in mice inoculated with the parent line.

In an effort to determine the reasons for the decline in virulence observed in lines of *Plasmodium berghei* that have acquired resistance to chloroquine and sulfone (dapsone), a number of possible factors were considered and systematically studied until a satisfactory explanation was found. It was found that the decline in virulence could not be accounted for by changes in number of progeny per schizont or sequestration of parasites in the blood of internal organs since both were similar in both chloroquine- or sulfone-resistant lines and the parent line. The use of inbred mice or mice of different ages resulted in no significant change in virulence. No evidence of antibody transfer in the inoculum of these lines was found. However, when mice were treated with phenylhydrazine resulting in 70–80% polychromatophils in the circulation, parasitemia produced by the chloroquine- or sulfone-resistant lines of *P. berghei* was not markedly different from that produced by parasites of the parent line. The increased virulence of the sulfone- and chloroquine-resistant lines in mice with polychromatophilia and the observations that these lines are restricted to polychromatophils indicate that virulence of these two drug-resistant lines of *P. berghei* is a function of the number of available polychromatophils.

A decline in virulence and restriction to polychromatophils are characteristic of lines of *Plasmodium berghei* that have acquired resistance to chloroquine (Peters, 1964, 1968; Thompson *et al.*, 1967) or dapsone (Thompson *et al.*, 1967). Chloroquine-resistant parasite lines as well as those resistant to dapsone lose the capacity of normal *P. berghei* to develop in mature erythrocytes and consequently are restricted to polychromatophil erythroblasts (cf. Peters, 1968; Thompson *et al.*, 1967).

The decline in virulence could be accounted for by a number of factors including lower numbers of progeny per schizont, lengthening of generation time, alteration of the capacity of the host to destroy parasites, disproportionate number of parasites in the blood in in-

ternal organs versus peripheral circulation, genetics and age of host, antibody transfer in the inoculum, or a direct function of the number of available polychromatophils. The present study was designed to investigate systematically these factors until a satisfactory explanation was found to account for the diminished virulence. As a prelude to these studies the delineation of the course of the parasitemia and mortality of a number of drug resistant lines of *P. berghei* was necessary.

Materials and Methods

Female albino mice (22–28 gram weight range, CF₁ strain, Carworth Farms, Portage, Michigan) were used in most of these experiments. Male CF₁ mice and females of an inbred strain (A/Jax, the Jackson Laboratory, Bar Harbor, Maine) were used for certain of the experiments designed to study the effect of sex or genetic makeup on *P.*

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berghei. The KBG-173 strain of *Plasmodium berghei* served as the parent line and the source of the drug resistant lines used in these studies. The resistant lines had previously acquired high levels of resistance to chloroquine, sulfone (dapson), triazine (cycloguanil) or pyrimethamine by the repeated treatment of infected mice with partially suppressive doses (Thompson *et al.*, 1967). These lines have been maintained subsequently by these investigators on fixed levels of the appropriate drug. For the present studies, the resistant lines were passaged once in untreated mice just prior to use in an experiment. To induce infections, heparinized parasitized blood was taken by cardiac puncture, the appropriate dilution prepared in 0.85% saline plus 2% deactivated calf serum, and a 0.1 ml aliquot containing 15×10^6 parasitized red blood cells was inoculated by use of a 1 ml syringe equipped with a 27-gauge needle into each recipient mouse via a tail vein. A minimum of three repetitions of each experiment was done. Thin smears of the blood were made 10–15 minutes subsequent to inoculation and at 24-hour intervals thereafter from the clipped tails of the mice. (Previous trials disclosed that an inoculum of 15×10^6 parasitized RBC was adequate to allow reliable estimates of the number of parasites immediately after injection.) The blood smears were fixed in methanol, stained with Giemsa's staining solution, and parasite and polychromatophil counts as well as limited morphological studies were made from these slides. The percentage of parasitized cells was estimated according to the method used by Thompson *et al.* (1970). Other smears were prepared from blood obtained from liver, spleen, and lungs as well as tail blood from certain mice, stained as described above, and the parasitemia, percentage of schizonts, and the number of merozoites per schizont determined. Parasite numbers were obtained from these preparations by counting the number of parasitized RBC per 10^3 RBC. Polychromatophilia was estimated by counting the number of polychromatophils per one hundred red blood cells. Polychromatophilia was induced in certain experiments by the intraperitoneal administration of phenylhydrazine hydrochloride suspended in distilled water two days prior to injection with *P. berghei* in a manner similar

to that of Ott (1968). The mice were weighed individually and phenylhydrazine hydrochloride was given in doses of 160 mg/kg.

Results

I. Parasitemia and Mortality in CF₁ Mice

The course of parasitemia of each line of *P. berghei* was studied in at least three separate experiments. The results of a representative experiment comparing the parent, chloroquine, and sulfone-resistant lines is illustrated in Figure 1. High levels of parasitemia and subsequent death of the host occurred relatively rapidly in mice inoculated with the parent line. Mice inoculated with the parent line had mean parasitemias of 80% or greater 6 or 7 days after the injection of parasites, at which time most of the animals died. No mice inoculated with 15×10^6 RBC parasitized with the parent line of *P. berghei* survived longer than 9 days and the majority died within 5 to 7 days.

High levels of parasitemia developed much less rapidly in mice inoculated with either the chloroquine- or sulfone-resistant lines of *P. berghei*. During the first two days following initiation of the infection with these lines, the increase in parasitemia was generally comparable to that observed in mice with the parent line (Fig. 1). Two through four days post-infection a decrease in numbers of parasites in the peripheral blood occurred in mice infected with the chloroquine or sulfone-resistant lines, while mice inoculated with the parent line had a continued increase in numbers of parasites. This decrease in numbers of circulating parasites seen in mice with the sulfone or chloroquine-resistant lines was followed by an increase in the degree of parasitemia. Some animals had parasitemias of 50% and above by day 12 and many of them had parasitemias of 80% or above 15–22 days post-infection. After developing parasitemias of 50–100%, some mice survived with this level of parasites for more than two weeks. An occasional mouse with either of these drug-resistant lines exhibited a rapid decrease in parasitemia 26–36 days post-infection and survived for prolonged periods thereafter.

The decrease in virulence of the chloroquine- and sulfone-resistant lines is emphasized by the data shown in Figure 2. The

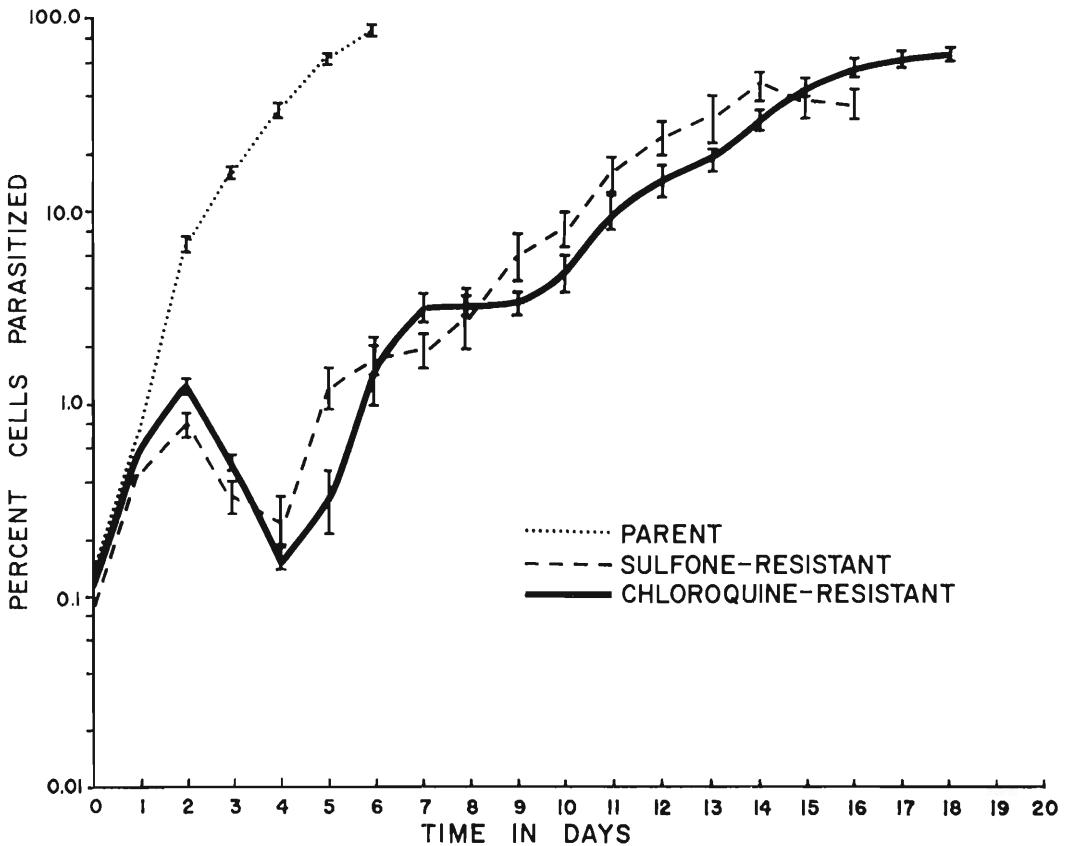


Figure 1. Comparison of the course of parasitemia in groups of mice following intravenous inoculation with 15×10^6 red blood cells parasitized with the parent, sulfone-, or chloroquine-resistant lines of *P. berghei*.

mortality is delayed significantly in the mice with these drug resistant lines since 100% mortality is observed around 20–24 days as compared to 9 days in those mice with the parent line.

The course of parasitemia in mice following inoculation with the lines resistant to triazine or pyrimethamine was similar to that seen in animals with the parent line. Thus parasitemias of 50% or greater occurred in mice inoculated with 15×10^6 RBC parasitized with the pyrimethamine-resistant line by days 5 to 7, during which time most of the animals died. No animals inoculated with 15×10^6 RBC parasitized with the pyrimethamine-resistant line lived longer than 10 days. Parasitemias of 50% or greater were seen in mice inoculated with an equivalent number of the

triazine-resistant line by days 6 to 10, during which time most of the animals died. No mice inoculated with 15×10^6 RBC parasitized with the triazine-resistant line survived longer than 13 days.

II. Types of Blood Cells Invaded and Pigment Formation

The parent line of *P. berghei* was observed to invade both mature and immature red blood cells and pigment is readily seen in the parasites. Parasites of the triazine and pyrimethamine-resistant lines were also observed in both mature and immature red blood cells, and malarial pigment could be observed readily in these organisms. In contrast, the chloroquine- and dapson-resistant lines were ob-

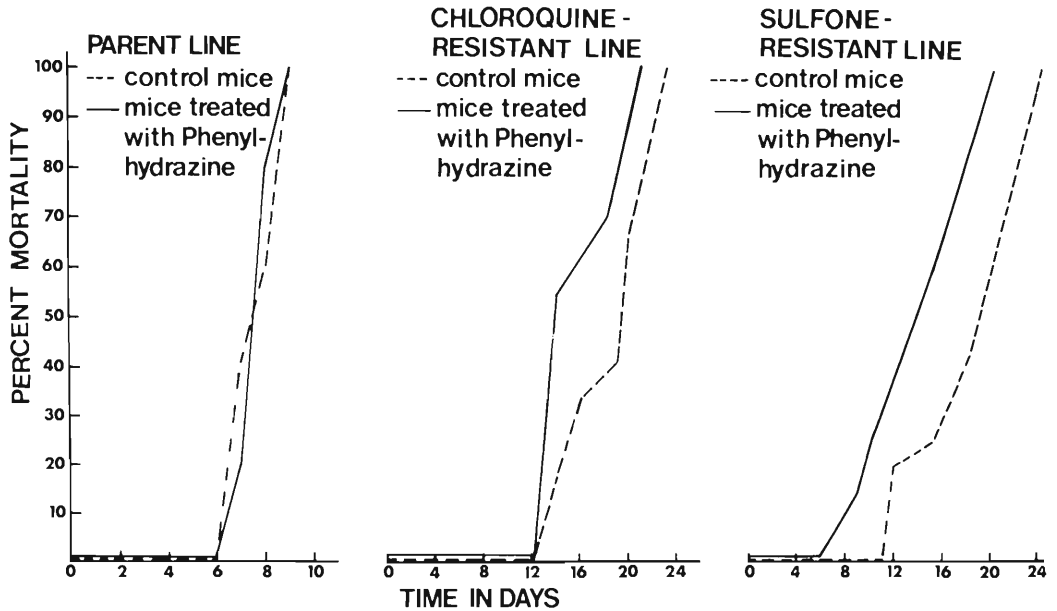


Figure 2. Cumulative mortality of mice following intravenous inoculation with 15×10^6 red blood cells parasitized with the parent, sulfone-, or chloroquine-resistant lines of *P. berghei*.

served only in polychromatophils. No pigment could be seen in organisms of the chloroquine-resistant line. Generally, pigment could not be seen in the sulfone-resistant parasites, but an occasional parasite had a few small refractile granules that possibly may have been pigment.

III. Factors Affecting the Course of Parasitemia and Mortality

The first experiments dealt with the possibility that free antibody might be transferred in the inoculum and thus inhibit the development of high levels of parasitemia in the recipient mice following inoculation with the sulfone- or chloroquine-resistant lines of *P. berghei*. The possibility that substantial amounts of anti-plasmodial antibody might be transferred with the parasite arose from the fact that 0.1 ml of whole or slightly diluted blood was required to yield an inoculum of 15×10^6 parasites. This was true because infections with these lines were routinely initiated from donor mice which had been inoculated 7 days previously and had para-

sitemia levels of only 3 to 5%. Experiments were conducted in which mice were inoculated with parasitized RBC which had been washed three times with saline by centrifugation and subsequently reconstituted to the original volume either in saline, serum from uninfected mice, or in the original serum. Parasites of the parent line were treated similarly. Such treatment was not found to alter the course of parasitemia or mortality in mice with either line of *P. berghei* tested. These results indicated that the low levels of parasitemia observed in the drug-resistant lines were not attributable to the passage of free antibody or any other inhibitory substance in the plasma contained in the inoculum.

Subsequently, the effect of age of the host (4 weeks versus 10 weeks old), sex of host, genetics of the host (inbred mice versus random bred mice), and stage of the infection in the donor animal (1 week infection with 3–5% parasitemia versus 2 weeks infection with 40–50% parasitemia) were considered. None of these was found to effect markedly the course of parasitemia of the chloroquine- or sulfone-resistant lines of *P. berghei*.

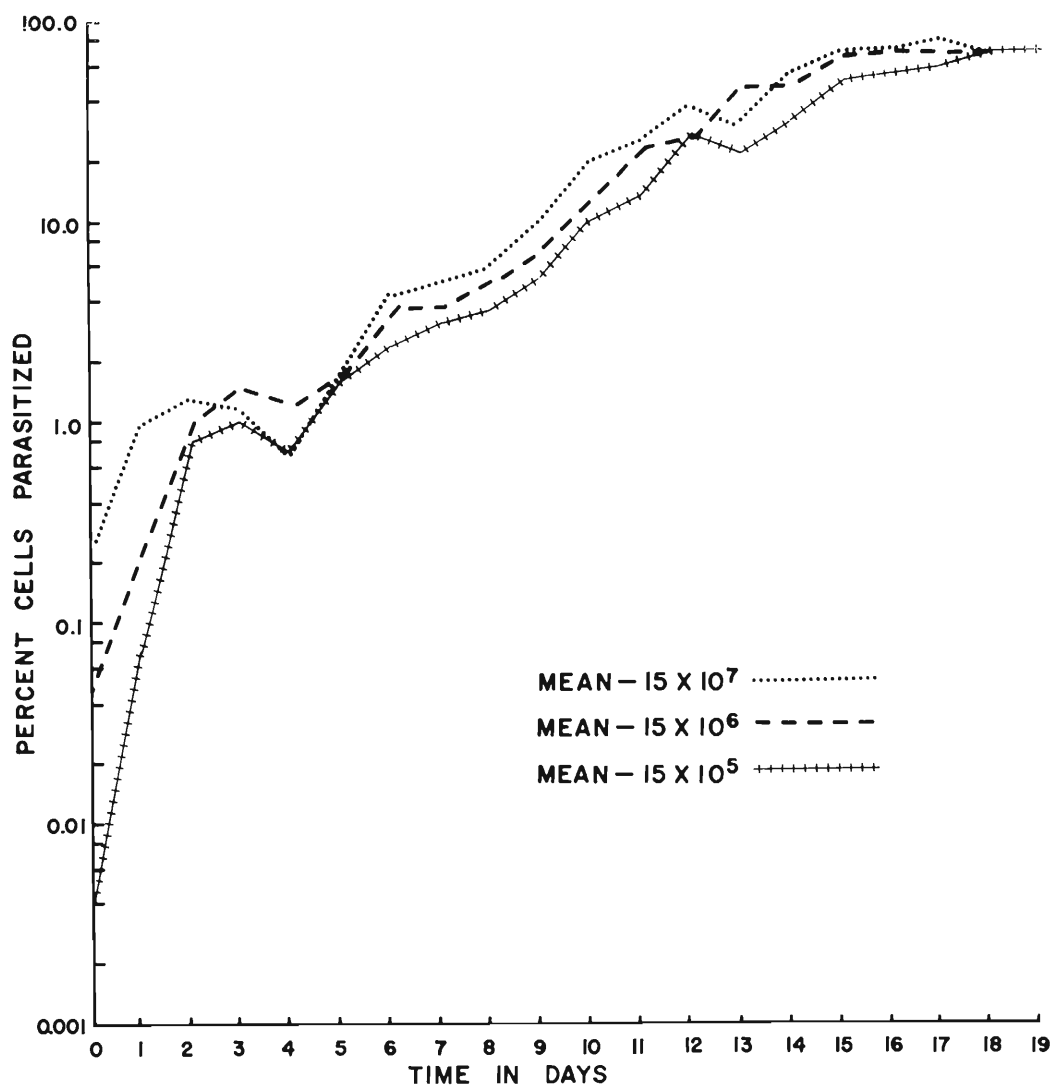


Figure 3. Comparison of the course of parasitemia in groups of mice following intravenous inoculation with various numbers of sulfone-resistant *P. berghei*.

Subsequently, the question of sequestration of parasites in the internal organs was considered. Studies were conducted to determine the stage of development of the parasites in blood from liver, spleen, or lung as compared to peripheral blood in mice with parasitemias of 3–5%, 10%, and 20–25% resulting from the chloroquine- and sulfone-resistant and parent lines of *P. berghei* and the percentage tropho-

zoites, bi-nucleated parasites, and parasites with 3 or more nuclei were recorded. Also the numbers of merozoites per schizont in the chloroquine- and dapsone-resistant lines were compared to that seen in the parent line. In these studies the percentage of the various developmental stages as determined from smears prepared from peripheral blood versus that obtained from smears prepared from blood from

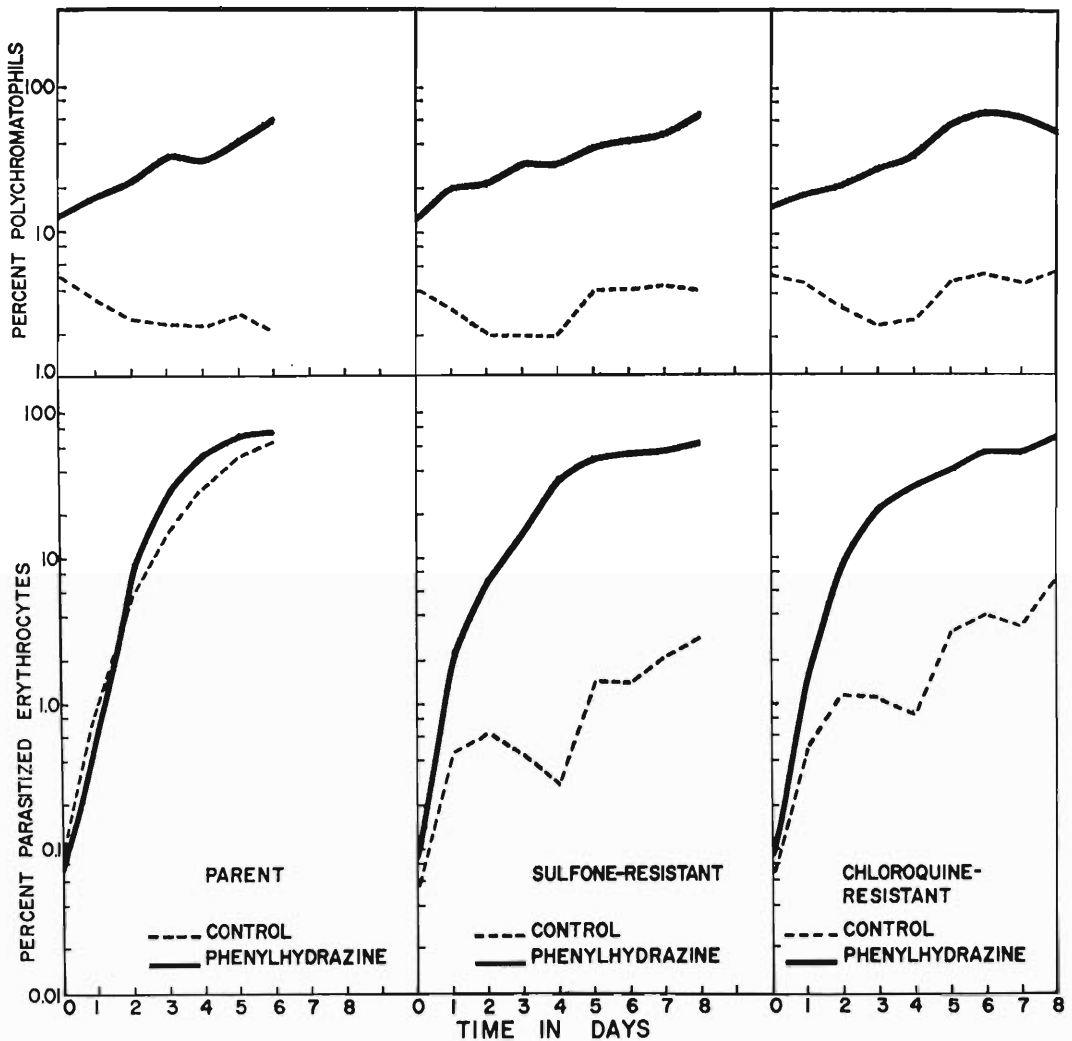


Figure 4. The effect of polychromatophilia on the course of parasitemia in groups of mice following intravenous inoculation with 15×10^6 red blood cells parasitized with the parent, sulfone-, or chloroquine-resistant lines of *P. berghei*.

liver, lungs, and spleen in animals inoculated with the drug resistant lines were generally similar to that determined from blood taken from those same sources in mice inoculated with the parent line. Further, no significant difference in number of merozoites per schizont was observed with the various lines of parasites used. These observations indicated that the slow attainment of high levels of parasitemias in mice with the chloroquine- or

sulfone-resistant lines of *P. berghei* could not be accounted for by sequestration of parasites in organs or production of lowered numbers of merozoites per schizont.

The next experiments were concerned with the effect of varying the size of the inoculum on the course of parasitemia in mice following inoculation with 15×10^5 , 15×10^6 , or 15×10^7 parasitized RBC of either the chloroquine- or sulfone-resistant lines. A representa-

tive experiment with parasites of the sulfone-resistant line is shown in Figure 3. Blood smears made immediately after the injection of parasites revealed differences in the mean parasitemia in the three groups, roughly commensurate with the number of parasitized cells injected. By day 3, however, the mean parasitemias of all groups had reached comparable levels and developed similarly thereafter. This experiment indicated that increasing the size of the inoculum did not significantly alter the course of parasitemia of sulfone-resistant lines and similar results were obtained with parasites of the chloroquine-resistant lines. In contrast, the course of parasitemia observed in animals with parent line parasites has been found to be roughly proportional to the number of parasites injected (Thompson *et al.*, unpublished).

These observations plus those mentioned previously concerning the restriction of the chloroquine- and sulfone-resistant lines to development in polychromatophils suggested that the course of parasitemia of these lines may be dependent directly upon the number of available suitable host cells (i.e. polychromatophils). Experiments were subsequently designed to test this suggestion. Polychromatophilia and the course of parasitemia of the chloroquine- and sulfone-resistant lines as well as the parent line in control mice and mice treated with phenylhydrazine were studied in three different experiments. Similar results were obtained from the three replicate experiments. A representative experiment is illustrated in Figure 4. In control mice the numbers of polychromatophils remained below five percent. Parasitemia levels of 60–80% were observed in untreated mice inoculated with 15×10^6 RBC parasitized with the parent line by days 5 or 6, while mice inoculated with an equivalent number of the sulfone-resistant parasites was less than 2% and the parasitemia in those mice inoculated with a equivalent number of the chloroquine-resistant parasites was around 4%. Thus the course of parasitemia of the parent, chloroquine- or sulfone-resistant lines in mice not given phenylhydrazine confirms former observations presented in Figure 1. In contrast, the mice previously treated with phenylhydrazine had polychromatophil levels exceeding 10% on the day they were inoculated with *P. berghei* and

they subsequently exhibited further increases in the numbers of polychromatophils which eventually reached levels of around 70% (Fig. 4). Mice with high polychromatophilias and undergoing infections with either the chloroquine- or sulfone-resistant lines developed parasitemias that resembled that seen in control mice infected with the parent line. Although similar high levels of parasitemias were observed in mice treated with phenylhydrazine and inoculated with either the chloroquine-resistant, sulfone-resistant, or parent lines of *P. berghei*, the mortality produced by the various parasite lines was different. All mice inoculated with the parent line were dead by day 9 while most of the mice with either the chloroquine- or sulfone-resistant lines were still alive at this time (Fig. 2). A significant number of these mice survived one to two weeks after developing mean parasitemia levels greater than 50 to 70%.

Discussion

Although a number of investigators have noted that chloroquine- or sulfone-resistant lines of *Plasmodium berghei* develop high levels of parasitemia considerably slower than the parent lines (Peters, 1968; Thompson *et al.*, 1967), no one has dealt with the reasons for this decline in virulence of our lines. Jacobs and Warren (1967) reported the sequestration of schizonts in the liver of mice infected with a chloroquine-resistant line of *P. berghei* and Miller and Fremount (1969) noted sequestration of schizonts of a chloroquine-resistant line of *P. berghei* in the liver, spleen, and lung of mice. These reports suggested the possibility that the low levels of parasitemia seen in our chloroquine- and sulfone-resistant lines might be related to sequestration of parasites of these lines in the blood of certain organs. However, this possibility was discounted since in our experiments the distribution of schizonts and other stages of the drug resistant lines in blood from liver, spleen, and lungs of infected mice was similar to that of the parent line.

Also considered as possible factors involved in the lowering of the virulence of the drug resistant lines of *P. berghei* were changes in the length of the asexual cycle or change in the number of merozoites per schizont. Since the parasites studied in this work are asynchronous, we have not ruled out the possibility

of a change in the length of the asexual cycle. However, we found no evidence of any significant change in the number of merozoites per schizont. Thus it appeared worthwhile to search for an explanation for the change in virulence elsewhere.

Subsequently, the additional factors systematically considered as contributing to the loss of virulence in the chloroquine- and sulfone-resistant lines of *P. berghei* were genetics and age of the mice, antibody transfer in the inoculum, or availability of suitable host cells, namely, polychromatophils. The former two were not found to contribute to the lowered virulence of the chloroquine- and sulfone-resistant parasites. However, since the course of parasitemia of the chloroquine- and sulfone-resistant lines in mice with marked reticulocytosis is very similar to that of the parent line in normal mice, it appears that the limiting factor in the development of high parasitemias in the drug resistant lines is the availability of suitable host cells. In the absence of an adequate number of polychromatophils, many of the parasites are lost after schizogony. An increase in polychromatophils by treatment with phenylhydrazine resulted in markedly higher numbers of appropriate host cells and hence more parasites survive. These results are similar to observations made by others studying strains of malaria which have a preference for immature red blood cells. In all such studies an elevation in the number of young red cells results in enhanced parasitemia (Schwink, 1960; Singer, 1954; Ott *et al.*, 1967; Ott, 1968). On the other hand, when the numbers of polychromatophils are reduced, the parasitemias are usually less severe (Garnham, 1966; Ladda and Lalli, 1966).

The question might arise concerning the effect of phenylhydrazine on the course of *P. berghei*. Other investigators (cf. Ott, 1968) saw no indication that this compound had any detrimental effect on *P. berghei* when administered to the animal prior to injection of parasites. In our experiments the parent line of *P. berghei* developed as well in mice pretreated with phenylhydrazine as in untreated controls.

The observations that the chloroquine- and sulfone-resistant lines occur only in polychromatophils along with the close relationship between the number of these red cells

in the circulation and parasitemia is evidence that these parasites either cannot invade mature red blood cells or cannot survive in the mature cells. It appears that the former may be true since no parasites of these lines have ever been seen in our laboratory in a mature cell. We have no experimental evidence for reasons for restriction of the chloroquine- and sulfone-resistant lines of *P. berghei* to polychromatophils.

The loss of ability to form pigment which is visible with light microscopy concomitant with the acquisition of resistance to chloroquine or sulfone is a phenomenon of considerable interest that deserves additional study. The possibility exists that pigment is formed but is stored in such small granules that it is not visible with the light microscope. We have no experimental information, however, concerning reasons for what appears to be a lack of pigment in the lines of *P. berghei* resistant to chloroquine or sulfone.

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Plasmodium gallinaceum: Survival of Resistant Parasites at Subpatent Levels Following Challenge of Immune Hosts

R. L. BEAUDOIN AND J. E. APPLGATE¹

Experimental Parasitology Division, Naval Medical Research Institute,
National Naval Medical Center, Bethesda, Maryland 20014

ABSTRACT: Birds immunized with normal *Plasmodium gallinaceum* were challenged with pyrimethamine resistant parasites of the same species. The animals rapidly cleared the challenge parasites from the blood, and the infections returned to a state of latency. By subinoculation, it was shown that pyrimethamine resistant parasites survived the immune response and were incorporated into the latent infections for at least 103 days after challenge.

It is concluded that semi-immune hosts may act to conserve rather than compromise drug resistant strains in endemic areas.

In avian malaria infections, circulating parasites persist indefinitely following the initial crisis, yet chronically infected birds are capable of rapidly clearing large numbers of challenge parasites during this "latent" phase, indicating that a strong immune competence against superinfection exists. This type of immunity is called premunition (Sergeant, Parrot and Donatien, 1924, 1925). As part of a

study on the dynamics of latency in malaria, we were interested in the ultimate fate of challenge parasites in hosts with demonstrable premunition. If individuals from a challenge infection survive within the immune host, they would enrich the subpatent malaria genome within that host by adding to its genetic variability. Semi-immune hosts then might eventually become the reservoirs of parasite populations with enormous genetic plasticity. This phenomenon would have significant epidemiological implications.

Materials and Methods

A pyrimethamine sensitive strain of *Plasmodium gallinaceum* was used for the primary, immunizing infections. Since it was essential that a recognizable genetic marker should persist in the progeny of challenge parasites, a pyrimethamine resistant strain of

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The experiments reported herein were conducted according to the principles set forth in "Guide for Laboratory Animal Facilities and Care," prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences—National Research Council.

¹ Present Address: Rutgers University, The State University of New Jersey, Forestry Section, New Brunswick, New Jersey 08903.

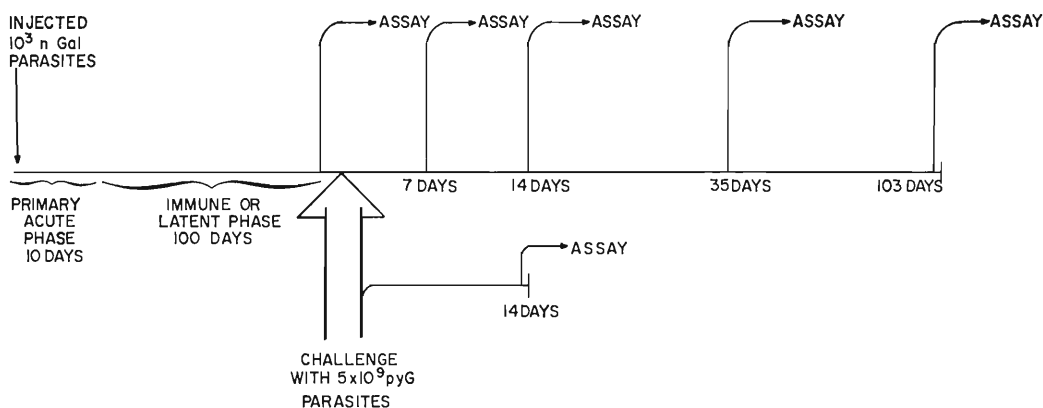


Figure 1. Diagram of the experimental protocol.

P. gallinaceum was used for the challenge infections.

Briefly, the experimental design was to infect chickens with normal, drug-sensitive parasites of *P. gallinaceum*, allow the birds to become immune, and to challenge them with the Bishop pyrimethamine-resistant strain. Assays for pyrimethamine-resistance were then made upon isolates from birds with latent infections at various intervals following challenge (Fig. 1). Controls were included to verify that pyrimethamine resistant parasites did not spontaneously appear in the immunized birds prior to challenge, and that the pyrimethamine-resistant trait persisted into the latent phase of an infection.

Specifically, immune birds were obtained by infecting 24 three-week-old white rock chickens with 10^3 infected erythrocytes of our original drug-susceptible 8A strain of *P. gallinaceum* (herein designated the nGal strain). Six of these birds survived the primary attack and were used in this study. One hundred and ten days after the primary infection, the six birds were challenged by intravenous inoculation of heparinized blood containing 5×10^9 parasites and monitored for persistence of pyrimethamine resistance. The parasites used in the challenge infection were of a strain developed by Bishop (1962) and maintained in this laboratory since 1963. The strain (herein designated pyG) is resistant to 5 mg/kg pyrimethamine base. Resistance to pyrimethamine has persisted in this strain even in the absence of the drug since 1963.

Assays for pyG parasites were made in the following way: 1 ml amounts of heparinized blood were subinoculated from each immunized bird into uninfected three-week-old chicks just prior to challenge and at 7, 14, 35 and 103 days after challenge with the pyG parasites (Fig. 1). When parasitemias exceeded 1% in the assay chicks, pyrimethamine was administered by intragastric intubation in daily doses of 5 mg/kg per chick for 10 days or until death. Daily parasite counts were made during this period to monitor the density of the infections. Pyrimethamine resistance was considered to have been demonstrated when the parasitemias in assay birds continued to increase under drug pressure. Six control birds infected with 5×10^9 pyG parasites were assayed by subinoculation in the same manner.

Results

Pyrimethamine-resistant parasites were not detected in any of the immunized birds prior to challenge as evidenced by the rapid clearance of parasites by pyrimethamine in subinoculated assay chicks (Fig. 2). Gametocytes were seen in blood films from test birds for several days after the disappearance of asexual stages, but this does not imply drug resistance as pyrimethamine does not reduce the number of gametocytes.

Following challenge (Fig. 1), one of the six test birds failed to clear the challenge parasites and died after six days with a para-

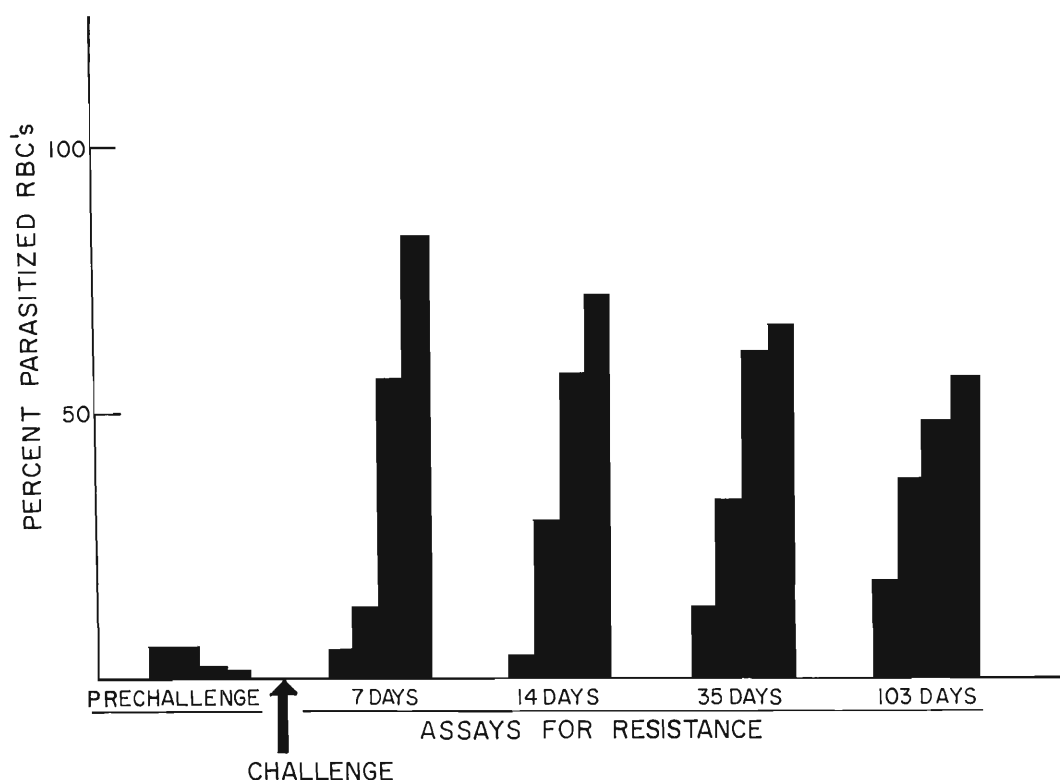


Figure 2. Histograms of the average parasitemia in test birds on each of four days following administration of pyrimethamine. Each histogram represents a single assay for presence of the trait for pyrimethamine resistance. Test birds in each assay were infected by subinoculation of blood from birds with latent infections. Assays for drug resistance were made before challenge and at four intervals following challenge as indicated.

sitemia of 73%. The surviving five birds demonstrated a strong immunity by rapidly clearing parasites from the peripheral blood and by maintaining this state of latency for the remainder of the 103-day test period. However, when blood was subinoculated from these immune birds into susceptible chicks at 7, 14, 35 and 103 days after challenge, all recipients developed patent infections. Further, in each instance the pyrimethamine-resistant characteristic was retained as parasites continued to multiply during pyrimethamine therapy (Fig. 2). These data conclusively demonstrate that subpatent populations of the challenge strain were able to survive in immune hosts for at least 103 days and that the strain retained its pyrimethamine-resistant characteristic during this period.

Discussion

It has long been recognized that, following the natural termination of acute malaria infections, the parasites frequently become difficult to find by microscopic examination of blood films (Wasielewski, 1902), whereas the subinoculation of blood at this stage may often produce acute infections in non-immune recipients (Hewitt, 1940; Lourie, 1934). This phase in the untreated course of a malaria infection is commonly called the latent phase and is characterized by the host's possession of a strong immunity to challenge by immunogenically similar parasites (Boyd and Coggeshall, 1938; Sinton, 1939; Taliaferro and Taliaferro, 1929). The phenomenon of immunity to superinfection was recognized by the

early workers in malaria and given the name premunition to differentiate it from the sterile immunity which persists after eradication of the infectious agent from the host (Sergeant, Parrot and Donatien, 1924, 1925).

Maier and Coggeshall (1944), on the other hand, have demonstrated that a degree of residual immunity is retained for varying lengths of time following radical cure of infections with monkey malaria. More recently, Brown (1969) has questioned the validity of distinguishing between sterile and non-sterile immunity on immunological grounds pointing out that the latter phenomenon may be explicable on the basis of an adjuvanted immune response which wanes following elimination of the parasite. At any rate, in the existing vernacular, the immunity which occurs following a normal course of infection with most malarías studied thus far, including human, primate and avian species, is primarily of the non-sterile or premunition type (Boyd and Coggeshall, 1968; Brown *et al.*, 1970; Hewitt, 1940; Krishnan *et al.*, 1934; Maier and Coggeshall, 1944; Redmond, 1939; Sinton, 1939; Taliaferro and Taliaferro, 1929).

The concept of non-sterile immunity carries important implications to the ecology and epidemiology of malaria. If challenge parasites are quickly and totally eradicated by immune hosts, these hosts would effectively limit the dissemination of introduced parasite genotypes. If, on the other hand, new genotypes are able to enter an endemic area and become established in semi-immune hosts without causing death, these hosts would preserve the new parasites by acting as a reservoir for them.

In a study of soluble antigens in the blood of Gambian subjects with falciparum malaria, McGregor *et al.*, (1968) found antibodies to antigens from separate localities in the serum of some adults. Since the *P. falciparum* antigens and antibodies were shown to be transient, both disappearing with or shortly after the parasitemias, it may be concluded that these adults were simultaneously infected with at least two genotypes acquired from two or more localities. The sequential development of antigenic variants of a single strain within a single host has already been described by Brown and Brown (1965). In the present report, and in that of McGregor *et*

al. (1968), evidence from the laboratory and field combine to show that different genotypes of the same species, acquired separately, may cohabit the same host not merely during the acute stage but well into the latent phase of infection.

Boyd and Coggeshall (1938) showed that a mosquito could simultaneously transmit more than one species of malaria. In view of their results, it is not difficult to imagine simultaneous transmission of more than one strain of the same species. Further, the possibility of intraspecific hybridization within the mosquito as an additional factor in increasing the variability of the *Plasmodium* gene pool should not be overlooked.

It would appear that these mechanisms are sufficient to account for the immense, perhaps even infinite plasticity of the malaria parasite in nature.

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Comments on Lower-Animal Models

L. H. MILLER

National Institute of Allergy and Infectious Diseases,
Laboratory of Parasitic Diseases, Bethesda, Maryland 20014

Of all areas of malaria research in animal models, chemotherapeutic studies have the most immediate application to man. I will comment on the papers from the viewpoint of how the information potentially could relate to malaria in man.

The introduction of a major drug screening program against the exoerythrocytic stages of malaria by the U. S. Army hopefully will introduce new and more effective drugs for causal prophylaxis of all malarias and for radical cure of *Plasmodium vivax* and *P. ovale*. The Ranes are to be congratulated on introducing a reproducible method for screening large numbers of drugs for prophylactic activity against *P. gallinaceum*. This test system should lead to the discovery of new groups of anti-malarials. Since drugs such as primaquine had no activity, no drug should be eliminated as ineffective on the basis of *P. gallinaceum* test system, and for this reason it is of value to have a parallel test system in *P. berghei* for large numbers of drugs. Because of the short exoerythrocytic cycle in rodent malaria (48 hours) it is difficult in some instances to differentiate activity against exoerythrocytic forms from blood schizonticidal

activity. King and Shefner attempted to differentiate these effects by subinoculation of liver tissue and blood 72 hours after sporozoite inoculation. Their approach has certain short-comings which make interpretation of the results extremely difficult. First, it has never been possible to transmit rodent malaria by inoculation of hepatic schizonts. Infected red cells within the liver were responsible for transmission of infection in their studies. Second, drugs may have activity against exoerythrocytic stages without completely eliminating the infection. Finally, while failure to transmit the infection by blood inoculation at 72 hours could be presumptive evidence for activity against exoerythrocytic forms, it also could indicate a highly effective schizonticidal drug which persists in the blood. The fact that trimethoprim did not have exoerythrocytic activity by their criteria would have to be confirmed. Alternative methods for differentiating drug effect on exoerythrocytic stages from blood schizonts have been published by Most *et al.* (1967) and Gregory and Peters (1970).

In primate malarias where drug studies in large numbers of animals are not feasible, in

vitro methods may be of great value. The use of *in vitro* methods for the study of drug interaction by McCormick and Canfield could potentially discover valuable combinations which might be missed in the *P. gallinaceum*-chicken model or the *P. berghei*-mouse model. Since the aim of this screening method is the discovery of new drugs against multidrug-resistant *P. falciparum*, it might be of greater interest to use isolates of drug-resistant *P. falciparum* rather than *P. knowlesi* in the *in vitro* test system and to compare the results of drugs against these same isolates in clinical trials.

Thompson demonstrated activity of quinine methanol and phenanthrenemethanol against various drug-resistant strains of *P. berghei*. Although these studies are of importance in demonstrating potential patterns of cross-resistance (chloroquine in this case), the author's warning warrants reemphasis. *P. berghei* results, if interpreted too rigidly could lead to an underestimation of the potential value of a compound, and perhaps to this should be added, drugs may be ineffective against *P. falciparum* despite cure of resistant strains of *P. berghei*.

Alterations in the characteristics of resistant lines of *P. berghei*, such as loss of pigment and restriction of growth to immature cells, are not observed in drug-resistant *P. falciparum* (Powers and Jacobs, 1972). It is not known whether resistant *P. berghei* is unable to invade mature red cells or is unable to develop within these cells. I have observed degenerating parasites within mature red cells in chloroquine-resistant *P. berghei*. Hanson and Thompson have increased the virulence of chloroquine-resistant lines by inducing phenylhydrazine hemolysis to increase the numbers of polychromatophilic red cells. In any malaria where the parasite is limited to a small percentage of the total red cell population, the reproductive potential and thus the pathogenicity will

be greater as the number of susceptible red cells is increased. It is of interest that the resistant strain studied by Hanson and Thompson did not demonstrate deep vascular schizogony. The characteristics of deep vascular schizogony in other lines of chloroquine-resistant *P. berghei* differ from *P. falciparum* in organ preference of schizont-infected red cells and in the absence of ultrastructural abnormalities on the red cell membrane (Luse and Miller, 1971). These differences between chloroquine-resistant *P. berghei* and drug-resistant *P. falciparum* make extrapolation of data from this model to malaria in man difficult.

Beaudoin and Applegate have demonstrated that chickens immune to one line of *P. galinaceum* can become infected by blood inoculation with another line. Since premunition implies the ability of infection to persist in an immune animal, it would be expected that new parasites could enter the pool by blood inoculation or from exoerythrocytic schizonts. In endemic areas repeated infection of immune adults is known to occur.

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V

CHEMOTHERAPY

C. Clinical Studies

Investigations of the *in vitro* Chloroquine Sensitivity of *Plasmodium falciparum* in Thailand

E. J. COLWELL, R. L. HICKMAN AND P. PHINTUYOTHIN*

U.S. Army Medical Component, SEATO, APO San Francisco 96346

ABSTRACT: Investigations of the *in vitro* chloroquine sensitivity of *P. falciparum* were conducted in four provinces in Thailand. The objectives were to evaluate the adaptability under field conditions of the *in vitro* technique employed and to accumulate additional information of the distribution and prevalence of chloroquine resistant *falciparum* malaria in this country.

Successful *in vitro* cultures were observed in 66 percent of 253 attempts. The proportions of *in vitro* chloroquine resistance among the culture successes ranged from 85 percent in the southeastern province Trad to 100 percent in the central province of Saraburi. The major causes for culture failures were inadequate trophozoite maturity of the inoculum, excessively high parasite densities and electrical power failures.

The determination of the sensitivity of *Plasmodium falciparum* strains to a standard regimen of chloroquine demands administration of this drug over a 3 day period and a follow-up examination period of 28 days (WHO Tech. Rep. Ser. 375, 1967). It is apparent that estimates of the prevalence of drug resistant strains based on an *in vivo* procedure have several limitations. The supervision of drug intake is often impossible. The requirement of a prolonged follow-up period introduces the problems of patient accessibility and reinfection. Finally, the varying degree of immunity among the population examined may effect interpretation of the clinical response to therapy.

A rapid and simplified technique for detection of chloroquine resistant *falciparum* malaria in experimental volunteers has been reported by Rieckmann and associates (1968). This technique has been utilized under field conditions and has proved reliable for detection of resistant strains in patients with naturally-acquired *P. falciparum* infections (Rieckmann and Lopez-Antunano, 1971; Colwell et al., 1972). This report describes our investigations of chloroquine resistant *falciparum* malaria in Thailand, employing the *in vitro* procedure described by Rieckmann et al. (1968). Our objectives were to evaluate the adaptability of the Rieckmann test under field conditions and to accumulate additional information on the distribution and prevalence of

chloroquine resistant *falciparum* malaria in Thailand.

Materials and Methods

Investigations were conducted at a base laboratory established in the provincial hospital of four provinces in Thailand, located in the central, southern, southeastern and northeastern regions (Fig. 1). Both *falciparum* and *vivax* malaria are endemic in these regions.

The majority of subjects selected for study at Phrabuddhabat, Yala and Trad were referred from the hospital or from the local Malaria Eradication Center. A few subjects residing in these areas reported directly to the base laboratory or sought medical assistance from our technicians during home visits. At Nong Khai, the number of referred patients from the hospital and the Malaria Eradication Center was low, presumably reflecting a subsiding transmission of infections with the onset of the dry season. In an effort to increase the number of experimental subjects at this northeastern study site, a field team visited several villages located approximately 60 km east of Nong Khai City where potential subjects were selected on the basis of clinical signs and symptoms.

Patients less than six years of age and those with a history of anti-malarial therapy within the previous 72 hours were excluded. In all other cases, blood smears were obtained to confirm the diagnosis, identify the parasite, determine the degree of asexual parasitemia

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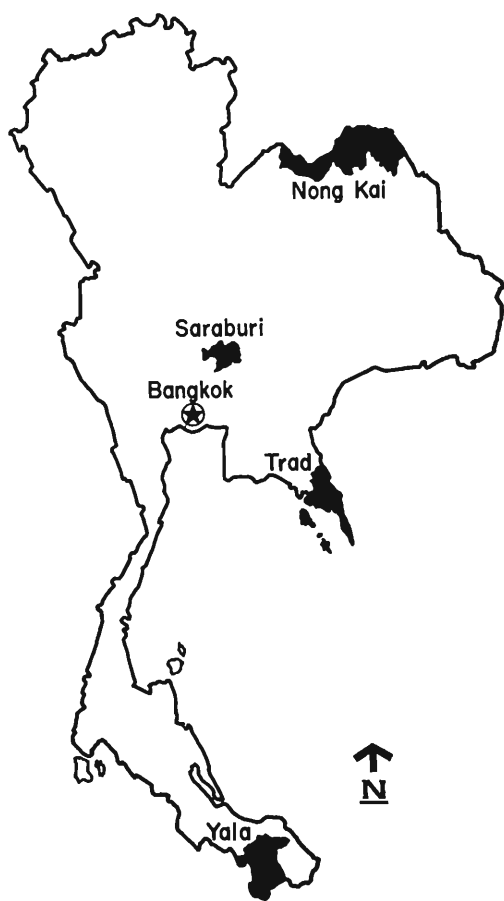


Figure 1. Map of Thailand showing the four study sites.

(Earle and Perez, 1932), and evaluate the stage of trophozoite maturity.

The *in vitro* test for chloroquine sensitivity was performed essentially as described by Rieckmann et al. with the exception that slightly different concentrations of chloroquine were used. Venous blood was transferred to a sterile flask containing glass beads and defibrinated by gentle swirling for ten minutes. When asexual parasitemias exceeded 20,000 per cu mm, the defibrinated blood was usually diluted with normal saline to adjust the concentration to 8,000 to 12,000 per cu mm. One ml aliquots were added to screw-cap glass vials containing five mg of glucose and either zero (i.e., control) or graded concen-

trations of chloroquine. The concentrations employed were 0.4, 0.6, 0.9, 1.35, 2.03 and 2.04 millimicromoles per ml of added blood (one millimicromole being equivalent to 0.32 ug base per ml). After 24 hours of incubation at 37.5–39.0°C, thick blood smears from the contents of each vial were prepared and Giemsa-stained. Each smear was coded and examined by technicians who had no knowledge of the corresponding concentration of chloroquine. The degree of maturation was determined by counting the number of schizonts per 200 consecutive asexual parasites and expressed as a percentage.

In order to determine the effect of cold storage, 46 undiluted specimens were recultured in control vials after storage in the refrigerator for eight or 24 hours. In addition, eight specimens were collected at a remote community in Nong Khai and maintained on wet ice for six hours while in transit to the base laboratory.

The success or failure of each test was defined arbitrarily according to the degree of schizogony that occurred in the control vial. If five percent or more of trophozoites mature to schizonts containing more than two nuclei, the test was considered successful. The test was considered a failure if less than five percent maturation occurred. Based on reported observations (Rieckmann et al., 1968; Rieckmann and Lopez-Antunano, 1971; Colwell et al., 1972; Peters and Seaton, 1971), *P. falciparum* parasites were considered to be chloroquine resistant *in vitro* when trophozoites matured to schizonts in vials containing 0.6 millimicromoles of chloroquine or more.

Results

Specimens from 253 subjects residing at the four study sites were examined for the *in vitro* chloroquine sensitivity of *P. falciparum*. Of 179 tests conducted with undiluted blood, 128 (72 percent) exhibited an adequate maturation response in the control vial (i.e., at least five percent schizont maturation). Diluted specimens were cultured 74 times resulting in 38 (51 percent) successful tests. In order of descending frequency, the causes of culture failures were inadequate trophozoite maturity of the inoculum, excessively high parasite den-

Table 1. Prevalence of chloroquine resistant falciparum malaria *in vitro* in Thailand.

Location	No. examined	No. culture successes ^a	Chloroquine resistant ^b	
			#	%
Phrabuddhabat	91	66	66	100.0
Yala	59	42	41	97.4
Nong Khai	33	18	16	88.8
Trat	70	40	34	85.0
Total	253	166	157	94.6

^a At least 5% schizonts in control vial.
^b Schizont maturation in a vial containing 0.6 millimicro-moles or more.

sity, electrical power failure and bacterial contamination.

Table 1 shows the percentage of chloroquine resistant parasite responses *in vitro* observed in the successful cultures. Maturation responses were considered resistant when trophozoites matured to schizonts in vials containing 0.6 millimicro-moles or more of chloroquine. Rates of *in vitro* chloroquine resistance of *P. falciparum* ranged from 85 percent at Trad to 100 percent at Phrabuddhabat.

The effects of chloroquine upon the *in vitro* maturation responses of *P. falciparum* in specimens obtained from the Nong Khai residents are shown in Table 2. Asexual parasitemias of specimens prior to incubation ranged from 520 to 46,000 per cu mm. The range of trophozoites that matured to schizonts in the

control vials after incubation was six to 72 percent. In vials containing 0.6 millimicro-moles or more of chloroquine, 16 or 18 sets exhibited *in vitro* schizogony. A dose-response relationship is suggested in which the degree of *in vitro* schizogony decreases as a function of increasing drug concentration. Complete arrest of schizont maturation in vials containing 0.4 millimicro-moles was observed in the remaining two specimens.

The frequency, range and median values of the *in vitro* maturation responses in vials containing the graded chloroquine concentrations for specimens obtained from subjects residing in the four study sites are summarized in Table 3. Similar to the individual maturation responses of *P. falciparum* in the culture sets shown in Table 2, the number (i.e., frequency) of vials exhibiting trophozoite maturation to schizonts at a given drug level decreases as the concentration of chloroquine increases.

In Tables 4 and 5, the degree of *in vitro* schizogony in control vials observed with unstored specimens is compared to that observed in control vials containing specimens of the same blood after eight or 24 hours storage in the refrigerator at 4°C. There was very little difference in the degree of schizogony noted after eight hours cold storage. After 24 hours, however, the degree of schizogony was less than five percent in 15 of 40 vials signifying

Table 2. Maturation responses *in vitro* of *P. falciparum* from Nong Khai.

Patient	Parasites/mm ³	Chloroquine concentration (millimicro-moles per ml inoculum)						
		0.0	0.4	0.6	0.9	1.35	2.02	3.04
1	3,020	72*	61	29	25	15	10	0
2	1,460	66	26	20	41	12	5	2
3	6,340	57	0	0	0	0	0	0
4	880	37	18	14	6	1	0	1
5	540	32	28	15	4	0	0	0
6	25,250*	27	24	1	15	7	4	3
7	520	26	28	20	0	0	0	0
8	21,900*	25	22	18	24	17	7	6
9	1,520*	25	20	13	0	0	0	0
10	6,200*	23	25	20	14	14	5	0
11	46,000**	23	19	10	7	7	6	1
12	17,520*	20	15	15	12	13	5	0
13	560	19	20	12	6	5	5	2
14	1,400	17	25	21	10	14	9	1
15	25,480	8	0	0	0	0	0	0
16	760	8	5	2	2	0	0	0
17	16,940	7	3	2	4	4	2	1
18	880	6	4	3	1	2	0	0

* Per cent schizonts.
* Test delayed 6 hours during transport on wet ice.
** Before saline dilution.

Table 3. Frequency, range and median values of *in vitro* maturation responses of *P. falciparum* from Thailand.

Location	Chloroquine concentration						
	0.0	0.4	0.6	0.9	1.35	2.02	3.04
Nong Khai	18* 5-72+ 24‡	16 0-61 19.5	16 0.29 13.5	15 0-41 6.5	12 0-17 4.5	10 0-10 2.0	9 0-6 0.5
Phrabuddhabat	66 5-80 29.5	66 3-79 20	66 2-75 15.5	66 1-59 11.5	59 0-46 6	46 0-36 2	33 0-35 0.5
Trat	40 5-93 19	39 0-88 13	34 0-78 9.5	34 0-78 5	27 0-62 3	16 0-58 0	13 0-40 0
Yala	42 5-89 25.5	40 0-80 17	41 0-85 15.5	39 0-74 8	35 0-47 3	30 0-57 2	18 0-5 0

* No. of vials with *in vitro* schizogony at a given drug level.

+‡ Range and median values, respectively, of % schizont maturation at a given drug level.

a failure of 37.5 percent. The mean decreases in the degree of schizogony was greater than 50 percent. Eight specimens were obtained from subjects residing in a remote geographical community and maintained on wet ice while in transit to the base laboratory. Five were cultured successfully and demonstrated parasites which were resistant to chloroquine *in vitro* (Table 2).

Previous investigations have demonstrated that the *in vitro* procedure employed in this study is a reliable method for detection of chloroquine resistant strains of *P. falciparum* in experimental volunteers (Rieckmann et al., 1968; Rieckmann, 1971). In the latter studies, specimens from volunteers infected with a known chloroquine sensitive strain from Uganda exhibited complete arrest of schizont development in vials containing 0.5 millimicro-moles of chloroquine. In contrast, this concentration was not completely inhibitory for parasite maturation in any specimens obtained from volunteers infected with known chloro-

quine resistant strains from Malaya and Vietnam.

The reliability of the Rieckmann test for determination of the chloroquine sensitivity of *P. falciparum* in patients with naturally acquired infections is supported by several investigations (Rieckmann and Lopez-Antunano, 1971; Colwell et al., 1972; Peters and Seaton, 1971). Peters and Seaton examined both the *in vitro* and *in vivo* responses of *P. falciparum* to chloroquine in two patients who acquired their infections in West Africa (Peters and Seaton, 1971). Specimens from both subjects demonstrated complete arrest of schizont development at the same inhibitory concentration for the Uganda strain and radical cures were produced in both subjects with conventional chloroquine (i.e., 25 mg base per kg) therapy.

Several field investigations in malarious areas have recently been conducted in which the Rieckmann technique has been employed in conjunction with standard *in vivo* procedures for detecting chloroquine resistant strains of *P. falciparum*. In Central Brazil, Rieckmann and Lopez-Antunano reported chloroquine resistance of *P. falciparum in vitro* in 30 patients of whom 28 (93 percent) experienced treatment failures following conventional doses of chloroquine (Rieckmann and Lopez-Antunano, 1971). In Central Thailand, 57 infected subjects demonstrated parasites resistant to chloroquine *in vitro* and 55 (96 percent) of these 57 failed to exhibit a radical cure following conventional chloroquine therapy (Colwell et al., 1972). In the present re-

Table 4. Maturation responses *in vitro* of *P. falciparum* before and after storage at 4°C for 8 hours.

Patient #	% Schizonts	
	Before	After
1	76	90
2	51	44
3	44	35
4	32	28
5	9	9
6	8	18

Table 5. Maturation responses *in vitro* of *P. falciparum* before and after storage at 4°C for 24 hours.

No. examined	% Schizonts ^a					Mean % decrease	No. of failures after storage
	Mean	Before Range	Mean	After Range			
25	43.4	20-80	22.2	0-80		54.4	5
15	12.2	6-19	4.1	0-15		66.2	10

^a In control vial.

port, 25 of the Yala residents in whom successful *in vitro* tests were accomplished were also administered a conventional course of chloroquine and followed for a 21 day period. All had demonstrated parasites resistant to chloroquine *in vitro* and none exhibited a radical cure after chloroquine administration (Hickman et al., in press). The excellent degree of agreement between the *in vitro* and *in vivo* parasite responses observed in these field studies indicates that the Rieckmann test is a reliable technique for detection of chloroquine resistant falciparum malaria.

The proportions of chloroquine resistant falciparum malaria detected by the *in vitro* technique were extremely high ranging from 85 to 100 percent at the four study locations. Similar high prevalences of resistance have been reported in Thailand employing standard *in vivo* chloroquine sensitivity procedures. Harinasuta and colleagues administered a conventional course of chloroquine to 107 patients in a Bangkok University Hospital under conditions in which natural transmission was unlikely and prolonged follow-up examinations were possible. Radical cures were produced in only two of these patients (Harinasuta et al., 1965; Harinasuta et al., 1967). Field investigations of the prevalence of chloroquine resistant falciparum malaria have recently been completed in Northeast and Southern Thailand (Hickman et al., in press; Segal et al., in press). Infected residents of these areas were given a conventional course of chloroquine and followed for a 21-28 day period. Over 85 percent of subjects at both locations exhibited *in vivo* chloroquine resistance; however, the delayed reappearance of asexual parasites in some of these patients could have reflected reinfection rather than recrudescence.

Many of the difficulties encountered during *in vivo* drug sensitivity investigations, particularly in rural areas, can be eliminated by re-

liable *in vitro* procedures. The simplicity and rapidity of the Rieckmann technique are obvious advantages which can considerably reduce cost and obviate a prolonged follow-up period. The latter is particularly problematical during *in vivo* surveys because of patient accessibility and the possibility of reinfection. Another problem which can interfere with interpretations of *in vivo* drug testing and which is excluded by the Rieckmann test is the immune status of the population sampled. It is conceivable that the interaction of partial immunity and drug effect can be sufficient to produce a radical cure even though the host is infected with relatively resistant parasites. However, factors probably do not affect the *in vitro* intracellular maturation responses of *P. falciparum* in the Rieckmann test. Investigations by Cohen and associates have demonstrated that circulating malaria antibodies exert a protective effect, not by inhibiting intracellular schizogony, but by interfering with merozoite re-invasion of erythrocytes (Cohen et al., 1969; Cohen and Butcher, 1970). Under the conditions of the more simplified Rieckmann test, a complete cycle with erythrocytic re-invasion does not occur.

The major technical problems of the Rieckmann test encountered during our field investigations were trophozoite immaturity of the inoculum, high parasite density of the inoculum and electrical power failures. The requirement for an adequate stage of *in vivo* trophozoite maturity before culture inoculation has been noted by Rieckmann et al. (1968). If cultures are initiated with a specimen consisting predominately of immature trophozoites, the control vial often exhibits sparse or no schizont development. It is necessary to wait several hours until the predominant trophozoite population has passed the intermediate developmental stage. This delay before obtaining blood specimens for culture is often impracti-

cal because of other patient commitments and the potential hazard of withholding treatment in subjects with moderate to severe degrees of illness.

Inoculation of cultures with heavily parasitized blood specimens is frequently followed by little or no *in vitro* schizogony. We attempted to resolve this problem by adjusting the parasite density of the inoculum with a normal saline dilution to a range of 8,000–12,000 per cu mm. Successful cultures were achieved with this modification in approximately 50 percent of 74 attempts. In general, the degree of *in vitro* schizogony in specimens with adjusted parasite densities was considerably less than that observed in undiluted specimens with comparable densities. Conceivably, the use of a more physiologic diluent could significantly improve the yield of culture successes.

Despite the technical limitations of the *in vitro* chloroquine sensitivity test of Rieckmann et al. (1968), its simplicity, rapidity and reliability represent major advantages for potential utilization in field surveys. In addition, this technique can be modified for incorporation of other antimalarial agents such as pyrimethamine, proguanil and cycloguanil. Examination *in vitro* of single specimens of blood from a representative sample population can contribute immensely to a background knowledge of the multi-drug sensitivity of *P. falciparum* in a given area.

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Quinine: Side-Effects and Plasma Levels¹

R. D. POWELL² AND J. V. MCNAMARA³

Difficulties with strains of *Plasmodium falciparum* that are resistant to chloroquine and to other widely-used synthetic antimalarial agents have made it necessary to resort to quinine for treatment of patients who are or may be infected with such parasites. Considerable attention has been given to the antimalarial effects of quinine against such strains. Relatively little recent attention has been focused on the untoward effects of quinine. The purpose of this report is to present information about side-effects and plasma levels of quinine. The data presented in this report were obtained during studies carried out primarily to assess the antimalarial effects of quinine and of other agents against experimentally-induced infections with chloroquine-resistant *P. falciparum*.

Materials and Methods

The observations that form the substance of this report were made between July 1, 1961, and July 1, 1969 during investigations performed at the University of Chicago-Army Medical Research Project at Stateville Penitentiary, Joliet, Illinois. General methods of study at this project and information pertinent to volunteers (healthy, American men from 21 to 45 years old) who participated in studies involving blood-induced or mosquito-induced infection with *P. falciparum* have been described in detail previously (Alving et al., 1948; Powell et al., 1964; Powell and McNamara, 1970; Powell et al., 1972).

A 10-day course of quinine (10 grains of

quinine sulfate—equivalent to 650 mg of quinine sulfate or to 540 mg of quinine base—orally every 8 hours) represents a key aspect of one therapeutic regimen that has been used widely for treatment of patients who are or may be infected with chloroquine-resistant *P. falciparum*. Data in this report pertain to all instances between mid-1961 and mid-1969 in which volunteers at this project received such 10-day courses of quinine and to 8 other instances in which administration of a 10-day course of quinine was planned and initiated but not completed because of adverse effects of quinine. Quinine sulfate, in 5-grain capsules (not in tablets or in enteric-coated preparations), was given orally at 8-hour intervals and under very close supervision.

Plasma quinine levels were measured with the fluorometric method of Brodie and Udenfriend (1943). Plasma quinine levels noted in this report in almost all instances reflect determinations carried out with samples of blood obtained prior to the morning dose of quinine. In a few instances (when marked cinchonism occurred, for example), plasma levels noted reflect determinations performed at other times of the day. That the capsules utilized contained the stated amount of quinine was checked periodically by quinine assays.

Volunteers treated with 10-day courses of quinine were considered in 4 groups (A, B, C, and D). Those in Group A were asymptomatic and had no patent parasitemia at the outset of treatment. Quinine was given to men in this group primarily as a precautionary measure. Many of these men had not developed patent parasitemia after exposure or had previously received treatment that had apparently effected radical cure. They received quinine as part of an effort to eliminate even slight chances of a persistence of possible subpatent parasitemia (Powell et al., 1972). Volunteers in Groups B, C, or D had patent asexual parasitemia at the outset of treatment with quinine. Those in Group B had a maximal detected temperature under 100° F., those in Group C had a maximal detected temperature

¹ From the Army Medical Research Project of the Department of Medicine, The University of Chicago, Chicago, Illinois, and the Department of Internal Medicine, The University of Iowa and the Iowa City Veterans Administration Hospital, Iowa City, Iowa. This work was supported by the U. S. Army Medical Research and Development Command under Contract No. DA-49-193-MD-2413 and is Contribution No. 1130 to the Army Research Program on Malaria. The investigations presented in this report were made possible through the cooperation of the inmates and administrative staff of the Illinois State Penitentiary, Stateville Branch, Joliet, Illinois.

² Present address: Department of Internal Medicine, The University of Iowa, and Veterans Administration Hospital, Iowa City, Iowa 52240.

³ Present address: Sansum Medical Clinic, Inc., 317 W. Pueblo St., Santa Barbara, California 93102.

Table 1. Symptoms in volunteers treated with 10 grains of quinine sulfate orally every 8 hours for 10 days.*

Group	A	B	A and B (%)	C	D	C and D (%)
Number of men	125	57	182 (100)	33	64	97 (100)
No symptoms	43	14	57 (31.3)	—	—	—
Headaches	26	17	43 (23.6)	16	46	62 (63.9)
Tinnitus	47	28	75 (41.2)	18	31	49 (50.5)
Vertigo	3	3	6 (3.3)	3	5	8 (8.2)
Nausea	7	10	17 (9.3)	6	14	20 (20.6)
Vomiting	5	3	8 (4.4)	4	14	18 (18.6)
Abdominal pain	12	5	17 (9.3)	5	10	15 (15.5)
Diarrhea	13	2	15 (8.2)	2	8	10 (10.3)
Constipation	8	2	10 (5.5)	1	0	1 (1.0)
Pruritus	6	4	10 (5.5)	2	3	5 (5.2)
Urticaria	2	0	2 (1.1)	1	2	3 (3.1)
Nervousness	2	1	3 (1.6)	1	1	2 (2.1)

* Volunteers in Group A had neither fever nor patent parasitemia at the outset of treatment. Volunteers in Groups B, C, and D had patent parasitemia with no fever (Group B), fever between 100 and 102° F. (Group C), or fever exceeding 102° F. (Group D) at the outset of treatment. As indicated in the text, some doses of quinine were omitted because of marked cinchonism in 7 volunteers. In 8 other volunteers (not included in this table) administration of quinine was discontinued before completion of a planned 10-day course because of adverse effects of quinine.

between 99.9 and 102° F., and those in Group D had a maximal detected temperature of 102° F. or higher on the day of initiation of treatment. Volunteers in Groups C or D were treated with quinine during initial acute attacks or during recrudescences. In most instances, administration of quinine to these men was instituted within 1 to 3 days after the onset of fever during initial attacks or during recrudescences.

Observations

Side-Effects of Quinine

Table 1 summarizes the symptoms noted in volunteers in Groups A, B, C, and D. Volunteers in Groups C or D often had, in addition to fever, malaise, myalgia, slight anorexia, and headaches that were associated with malaria and that preceded administration of quinine. Only 31.3% of the volunteers in Groups A or B remained asymptomatic during administration of quinine.

Headaches were by far the most bothersome symptom in volunteers in Groups C or D. Headaches were noted also in almost one-fourth of the men in Groups A or B. In all 4 groups, the most common side-effect ascribable to quinine was tinnitus. Tinnitus, which was noted more often in men in Groups C or D than in men in Groups A or B, was usually not severe. In 3% of men in Groups A or B and in 8% of men in Groups C or D, however, tinnitus was accompanied by vertigo. In 2 volunteers (1 in Group C and 1 in Group D)

postural vertigo represented a prominent symptom and was associated with postural hypotension. Transient nystagmus occurred in 1 volunteer.

Tinnitus occurred mainly on the second to fifth days of treatment and was in some instances associated with decreased auditory acuity or with a sensation of "fullness" or "stiffness" involving the ears. In men in Groups A or B, tinnitus on the 1st, 2nd, and 6th through 10th days of treatment was comparable in frequency to that in men in Groups C or D. Tinnitus on the 3rd, 4th, and 5 days of treatment, however, was more prominent in men in Groups C or D than in men in Groups A or B (Figure 1). Marked tinnitus, as discussed below, occurred in some men and was at times associated with slight and transiently blurred vision. Marked or persisting visual disturbances were not observed.

About 1% of the volunteers complained of unusual nervousness, about 5% noted pruritus without associated urticaria, and about 2% developed urticaria. Pruritus and urticaria were noted chiefly on the eighth to eleventh days after initiation of administration of quinine, were transient, and responded well to treatment with diphenhydramine. One volunteer developed a generalized erythematous rash on the 10th day of treatment with quinine. Four volunteers developed low grade fever (101 to 102° F.) that began on the eighth to tenth days after initiation of administration of quinine, that persisted for 2 to 3 days, and that

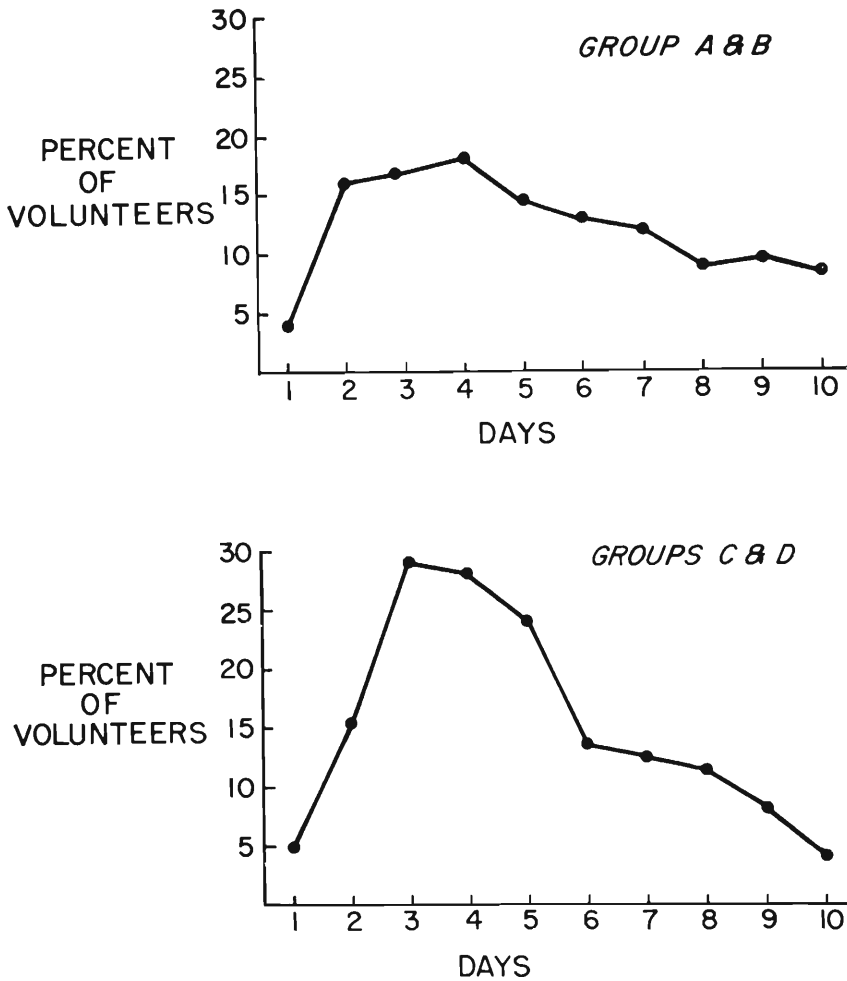


Figure 1. This figure indicates the per cent of volunteers in Groups A or B (top) or in Groups C or D (bottom) in whom tinnitus was noted on a particular day or days during administration of a 10-day course of quinine (10 grains of quinine sulfate every 8 hours).

subsided after treatment with quinine was completed.

Symptoms, coupled with information about plasma quinine levels, led to omission of some doses of quinine during treatment of 7 volunteers and resulted in the discontinuation of administration of quinine prior to completion of a 10-day course in 8 other volunteers. Such instances, which reflect more serious problems with respect to adverse effects of quinine, warrant special consideration.

Five volunteers in whom treatment with qui-

nine was discontinued before completion of a planned 10-day course were in Group A. All 5 men were asymptomatic and had no patent parasitemia at the outset of treatment. One man was the volunteer noted above who developed a generalized erythematous rash early on the 10th day of treatment. The plasma quinine level at the time was 6.6 mg/L. The last 2 planned doses of quinine were omitted, and the rash in this man gradually subsided over 3 to 4 days. In the second volunteer in this group, administration of quinine was

Table 2. Plasma quinine levels.*

	Days 3 or 4		Days 6 or 7		Days 9 or 10	
	No.	Mean	No.	Mean	No.	Mean
Group A	100	4.89	97	5.20	94	5.22
Group B	44	6.48	43	6.09	44	5.57
Group C	25	7.44	21	6.49	21	5.57
Group D	50	9.65	49	7.84	46	6.35

* Data presented indicate the number of volunteers in Groups A, B, C, and D in whom plasma quinine levels were determined on the 3rd or 4th, 6th or 7th, and 9th or 10th days of treatment and the mean levels detected (expressed in mg/L). About half of the determinations were performed on days 3, 6, and 9, and about half were performed on days 4, 6, and 10 of treatment.

stopped after 7 days of treatment because of severe gastrointestinal symptoms. The plasma quinine level at the time was 5.2 mg/L. Symptoms abated over the next 2 to 3 days. In the other 3 men in this group, after quinine had been given relatively uneventfully for 6 to 7 days, fever occurred and was followed by increasing cinchonism with marked tinnitus and vertigo. Plasma quinine levels in these 3 men, which had been 6.4, 9.4, and 9.6 mg/L on the 4th day of treatment, increased to 10.6, 12.5, and 12.3 mg/L, respectively, on the 8th day, at which point treatment with quinine was stopped. Fever persisted for 3 to 4 days and then subsided. Only low-grade fever occurred in 1 man. Fever reached 104 to 105° F. in the other 2 men. In all 3 men, symptoms of cinchonism abated within 2 to 3 days as fever declined following discontinuation of treatment with quinine.

One volunteer in whom several doses of quinine were omitted was in Group B. He was asymptomatic, and had a parasite count of 10 per cu mm, at the outset of treatment. Blood smears proved negative after the second day of treatment. He developed fever (103° F.), with tinnitus, vertigo, nausea, and vomiting, on the 4th to 6th days of treatment. Plasma quinine levels increased from 10.1 on the 4th day to 14.6 mg/L on the 6th day of treatment. The dosage of quinine was reduced, to maintain plasma quinine levels between 5 and 10 mg/L, and the 10-day course of quinine was completed. Fever subsided on the 7th day of treatment and symptoms abated over the next 2 to 3 days. It appeared clinically that this volunteer had developed an intercurrent viral infection associated with fever, increasing plasma quinine levels, and increasing cincho-

nism. Symptoms subsided in association with an abatement of fever and a reduction in the dosage of quinine.

The other 9 volunteers in whom doses of quinine were omitted or quinine was discontinued were in Group D. Each of these men had patent parasitemia and spiking fever at the outset of treatment. Quinine resulted in control of the acute attacks and in clearance of patent parasitemia in each instance. One man, who had fever of 105° F. at the outset of treatment, developed marked tinnitus, severe abdominal pain, and urticaria on the 3rd day of treatment, at which point plasma quinine levels reached 18.6 mg/L. He had previously received small doses of quinine as a part of studies at this project. Administration of quinine was stopped on the 3rd day of treatment. Symptoms subsided over the next 2 to 3 days. A sulfone was then administered to prevent a possible recrudescence.

Another volunteer in Group D developed severe cinchonism, with marked tinnitus, vertigo, nausea, and vomiting, on the 3rd day of treatment, at which time the plasma quinine level was found to be 13.1 mg/L. Treatment with quinine was stopped, symptoms abated over the next 2 to 3 days, and a combination of sulfadiazine and pyrimethamine was then given to prevent a possible recrudescence.

Six men in Group D developed severe cinchonism, with plasma quinine levels between 14 and 16 mg/L, on the 2nd to 4th days of treatment. In each instance the dosage of quinine was reduced, so as to maintain plasma quinine levels between 5 and 10 mg/L, symptoms of cinchonism largely subsided, and administration of a 10-day course of quinine was then completed without further untoward events.

One volunteer in Group D developed vertigo on the 2nd to 3rd days of treatment, at which point the plasma quinine level was 10.6 mg/L. Differential leukocyte counts that day disclosed marked granulocytopenia. Treatment with quinine was stopped. Agranulocytosis ensued. The volunteer was placed in protective isolation, and a 10-day course of tetracycline was given to prevent a possible recrudescence. Recovery from agranulocytosis gradually took place, without further complications, over a period of about 3 weeks following discontinuation of treatment with quinine.

Table 3. Statistical significance of difference between corresponding mean plasma quinine levels.*

Groups	Days 3 or 4			Days 6 or 7			Days 9 or 10		
	Difference between means	t	p	Difference between means	t	p	Difference between means	t	p
D-A	4.76	12.674	p < 0.001	2.64	7.960	p < 0.01	1.13	3.396	p < 0.001
D-B	3.17	7.239	p < 0.001	1.76	4.141	p < 0.001	0.77	1.990	0.05 < p < 0.1
D-C	2.21	4.248	p < 0.001	1.35	2.585	0.01 < p < 0.02	0.78	1.536	0.1 < p < 0.2
C-A	2.55	6.082	p < 0.001	1.29	2.817	0.001 < p < 0.01	0.35	0.781	0.4 < p < 0.5
C-B	0.96	2.019	0.02 < p < 0.05	0.40	0.775	0.4 < p < 0.5	0.00	0.012	p > 0.9
B-A	1.59	5.105	p < 0.001	0.88	2.614	0.01 < p < 0.02	0.35	1.165	0.2 < p < 0.3

* The statistical significance of differences between corresponding mean plasma quinine levels (data in Table II), expressed in mg/L, was determined with the t-test for small samples as described by Bancroft (6).

Plasma Quinine Levels

Plasma quinine levels were determined in 100 men in Group A, in 44 men in Group B, in 25 men in Group C, and in 50 men in Group D. Plasma quinine levels were usually measured on the 3rd, 6th, and 9th or 4th, 7th, and 10th days of treatment. Table 2 presents the mean values detected on the 3rd or 4th, 6th or 7th, and 9th or 10th days of treatment. Table 3 presents information about the statistical significance of differences between corresponding mean values.

The mean level detected on the 3rd or 4th days of treatment in men in Group D exceeded the corresponding mean level in Group C, which exceeded that in Group B, which in turn exceeded that in Group A. Each difference between corresponding mean levels proved to be statistically significant.

The mean level detected on the 6th or 7th days of treatment in men in Group D exceeded that in Group C, which exceeded that in Group B, which in turn exceeded that in Group A. The difference between mean values noted on the 6th or 7th day of treatment in Groups B and C was not statistically significant. Each of the other differences between corresponding mean levels was statistically significant. These differences were less marked than those noted on the 3rd or 4th days of treatment. Mean levels detected on the 6th or 7th days of treatment in Groups B, C, and D, but not that in Group A, were decreased compared to the mean levels noted in these men on the 3rd or 4th days of treatment.

On the 9th or 10th days of treatment, mean plasma quinine levels in Groups B, C, and D, but not that in Group A, were further reduced compared to those noted on days 3 or 4. Dif-

ferences between corresponding mean levels on the 9th or 10th days of treatment were less than those detected earlier during treatment. The mean level in men in Group D was significantly higher than that in Group A. Other differences between corresponding mean values on the 9th or 10th days of treatment were not statistically significant.

In essence, on the 3rd or 4th days of treatment, mean plasma quinine levels in Groups B, C, and D exceeded that in Group A by 33, 50, and 97%, respectively. During the next 6 days, mean levels in Groups B, C, and D decreased while mean levels in Group A underwent relatively little change.

Discussion

These observations serve as a reminder that quinine, given in conventional therapeutic antimalarial doses, is not exactly innocuous. The volunteers who participated in these studies, in general, did not tend to voice or emphasize minor complaints. It is likely that symptoms that were minimally bothersome at times went unmentioned. The data noted in Table 1 probably do not include, for example, all instances in which mild headaches, slight tinnitus, or minor gastrointestinal symptoms occurred.

Administration of quinine to men in Groups C or D was usually instituted within 1 to 3 days after the onset of fever, before parasitemia reached or exceeded 10,000 per cu mm. Some men in Group 4 experienced rather severe acute clinical attacks of malaria. In most instances, however, the acute attacks in volunteers in Groups C or D were mild to moderate in severity. The mean maximal temperature on the 1st day of treatment in men in Group

D was 103.5° F. Our observations, therefore, do not pertain to situations in which quinine is administered to patients who have very severe acute attacks of falciparum malaria with high levels of parasitemia and more protracted periods of high, spiking fever prior to treatment.

In patients who have acute attacks of malaria and who are treated with quinine, it is often difficult to determine the extent to which certain symptoms, such as nausea and vomiting, reflect malaria, side-effects of quinine, or a combination of both. The data pertinent to volunteers in Groups A and B (Table 1) provide insight into side-effects of quinine observed under circumstances in which malaria either is not present or is of minimal importance with respect to symptoms. The role of quinine in contributing to certain of the symptoms noted during the administration of this agent to patients with malaria is clearly of more than minor significance.

The symptoms detected in men in Groups A or B include most of the common side-effects often ascribed to quinine. Visual difficulties, although not prominent among the symptoms noted by the volunteers who participated in these studies, have represented a considerably more conspicuous side-effect of quinine in some previous studies of other workers (Coatney et al., 1948; Rollo, 1965; Meyler, 1952). Permanent visual impairment has at times been reported (Meyler, 1952). We were alert to this possibility but in no instance noted more than transient visual difficulties.

The observations pertinent to plasma quinine levels provide insight into some of the circumstances under which marked cinchonism may occur. It is well recognized that in acute renal failure, with diminished excretion of quinine via the kidneys, severe cinchonism with high plasma quinine levels may develop unless the dosage of quinine is reduced. Serial determinations of plasma quinine levels may provide very useful information upon which to gauge the dosage of quinine in such patients. Our findings provide insight into situations in which marked cinchonism is apt to occur in the absence of acute renal failure.

We encountered severe cinchonism almost exclusively in volunteers who had spiking fever at the outset of treatment or who became febrile during treatment. Fever in some of the latter volunteers may have reflected intercur-

rent viral or other infections. However, in several volunteers who did not have patent infections and who developed fever on the 7th to 10th days of treatment with quinine, it appeared likely that a febrile reaction to quinine had occurred. In association with fever in these men, plasma levels of quinine increased, in sharp contrast to the usual situation in which plasma levels either remained stable or decreased slightly during the last half of a 10-day course of quinine.

The data presented in Table 2 demonstrate that a relationship exists between fever at the outset of treatment and plasma quinine levels. In the absence of an intercurrent febrile illness or reaction, volunteers who were afebrile at the outset of treatment seldom developed plasma quinine levels exceeding 10 mg/L. Volunteers who were febrile at the outset of treatment, particularly those in whom fever exceeded 102° F., often developed plasma levels greater than 10 mg/L, especially on the 3rd to 4th days of treatment. Marked cinchonism, necessitating discontinuation of treatment with quinine or a reduction in dosage, occurred mainly on the 3rd to 4th days of treatment in men who had had spiking fever initially and who developed plasma quinine levels greater than 10 mg/L.

These findings suggest that patients who have spiking fever are more apt to develop unusually high plasma quinine levels with marked cinchonism than are patients who have relatively mild attacks with little or no fever. Plasma quinine determinations carried out on the second to fourth days of treatment may be of value in identifying instances in which unusually high plasma quinine levels are developing and may provide a useful basis for making appropriate downward adjustments in the dosage of quinine. A reduction in the dosage of quinine in such instances may serve to minimize the risk of severe cinchonism.

Studies presented recently by Brooks and co-workers, relating to treatment of men who had naturally acquired falciparum malaria and who received 10 grains of quinine sulfate orally every 8 hours for 14 days, disclosed mean plasma quinine levels between 11 and 12 mg/L on the 2nd through 5th days of treatment and decreasing mean plasma quinine levels subsequently during treatment (Brooks et al., 1969). A decline in plasma quinine levels

during treatment has been noted also by other investigators (Coatney et al., 1948). The mean maximal temperature on the 1st day of treatment in the men studied by Brooks and co-workers was 105° F. (Brooks et al., 1967). Vertigo, nausea, vomiting, abdominal pain, and diarrhea were more frequent in their patients than in volunteers in Groups C or D. In addition, detectable splenomegaly and symptomatic orthostatic hypotension were considerably more frequent in their patients than in volunteers in Groups C or D. It is likely that, in general, volunteers in Groups C or D received treatment earlier during the clinical course, and had less severe acute attacks of falciparum malaria, than did their patients.

Brooks and co-workers attributed most of the symptoms and signs they observed primarily to falciparum malaria and not to quinine. Mean plasma quinine levels on the 3rd or 4th days of treatment in their patients exceeded those detected in volunteers in Groups C or D. It is possible that the higher incidence of vertigo, nausea, vomiting, abdominal pain, and diarrhea that they detected reflect both a greater severity of the acute attacks of falciparum malaria in their patients and more marked side-effects of quinine. They noted that these particular symptoms were more conspicuous during the 1st half of treatment with quinine than during the 2nd half; in contrast, orthostatic hypotension was detected considerably more frequently during the 2nd half of treatment than during the 1st half (Brooks et al., 1967). As they indicate, both quinine and malaria may have contributed to the orthostatic hypotension observed in their patients.

Brooks and co-workers point out that decreases in plasma quinine levels during treatment with quinine coincided with clinical improvement. They suggest that transient hepatic dysfunction, rather than changes in urinary excretion, may account for this decline in plasma quinine levels (Brooks et al., 1969). Our data do not provide insight into the mechanisms of this decline. However, our findings, when considered together with those of Brooks and co-workers, are consistent with the notion that plasma quinine levels achieved on the 3rd to 4th days of treatment are related to the severity of the acute attacks and that such levels in patients with severe attacks are apt to be considerably higher than in patients

with mild attacks. Although there may be many exceptions in which unusually high plasma quinine levels and marked cinchonism do not go hand in hand with severe attacks, it appears that, in general, one should be especially alert to the possibility of unusually high plasma levels with marked cinchonism in those patients who experience severe acute attacks of malaria.

There was marked individual-to-individual variation in relationships between tinnitus and plasma quinine levels. In general, tinnitus was more common and more bothersome in men who had relatively high plasma quinine levels. In some instances, however, tinnitus was associated with relatively low plasma quinine levels (such as 2 to 3 mg/L), while in other instances volunteers noted no tinnitus whatsoever even though plasma quinine levels were relatively high (9 to 10 mg/L, for example). As noted previously by other workers, tinnitus often decreased as treatment with quinine was continued. Decreasing tinnitus in volunteers in Groups C or D coincided with clinical improvement and a decline in plasma quinine levels. But tinnitus also often decreased during treatment in volunteers in Groups A or B in the face of plasma quinine levels that remained stable. It appears that in the latter volunteers, some factor other than a decline in plasma quinine levels was responsible for the decrease in tinnitus that took place as treatment with quinine was continued.

Instances in which adverse effects of quinine necessitate discontinuation of treatment with the drug pose a thorny clinical problem. The administration of a 3- or 4-day course of quinine, although consistently effective in terminating acute clinical attacks caused by chloroquine-resistant *P. falciparum* in volunteers at this project, proved consistently ineffective in achieving radical cure. Unless other antimalarial agents were given, recrudescences of patent parasitemia and of symptoms occurred, usually about 10 to 12 days after completion of the 3- or 4-day course of quinine. To prevent such recrudescences, we usually employed either a 10-day course of tetracycline (Rieckmann et al., 1971) or a combination of pyrimethamine and sulfadiazine (Powell et al., 1967). The limitations of quinine as an antimalarial agent, coupled with the adverse side-effects of this compound, underscore

the on-going need for development of newer, more effective, and safer antimalarial drugs.

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Effects of Tetracycline Against Drug-Resistant *Falciparum* Malaria*

K. H. RIECKMANN, W. D. WILLERSON, JR., P. E. CARSON AND H. FRISCHER

ABSTRACT: The effects of tetracycline against the Vietnam (Marks) and the Cambodia (Buchanan) strains of *Plasmodium falciparum* were determined in 37 non-immune volunteers. In 31 volunteers, tetracycline was given, in conjunction with a 3-day course of amodiaquine or quinine, during an acute attack of malaria. Administration of tetracycline for 10 days cured 21 out of 22 infections and administration of the drug for 7 days cured 6 out of 9 infections. In 6 volunteers, the first dose of a 4-day course of tetracycline was given a few hours after the men had been bitten by infective mosquitoes. They were all protected against malaria. The findings indicate that tetracycline has a marked blood schizontocidal and causal prophylactic activity against 2 chloroquine-resistant strains of *P. falciparum*.

In a recent report we described the effects of tetracycline against chloroquine-resistant strains of *Plasmodium falciparum* (Rieckmann et al., 1971). Infected persons were treated only after they had developed partial immunity to their infections. Although tetracycline effected a radical cure in a high proportion of cases, remission of symptoms and clearance of asexual parasitemia was slow. The studies clearly indicated that tetracycline should not be used alone for controlling an acute attack of malaria in non-immune persons. Other anti-malarials often terminate an acute attack of malaria rapidly, but they may not achieve radical cure of the infection. The combination of tetracycline with such drugs might result both in rapid improvement in the clinical condition of a patient and in the cure of his infection. In September 1970, we initiated therapeutic studies to determine the effectiveness of tetracycline, given in conjunction with either amodiaquine or quinine, in treating non-immune persons infected with one of 2 chloroquine-resistant strains of *P. falciparum*. Additional investigations were undertaken to determine the causal prophylactic activity of tetracycline in preventing such infections.

Methods

All subjects who participated in these studies were male inmates of the Illinois State Penitentiary, Stateville Branch, located at Joliet, Illinois, U.S.A. Volunteers were screened prior to clinical examination by chest X-ray, electrocardiography, and various laboratory examinations. Special attention was given to the detection of any abnormality of the hepatic, renal or erythropoietic systems. After a review of the preliminary findings, each volunteer was interviewed and examined by one of the authors. Only men in excellent health were allowed to participate in the investigations. The purpose and procedures of the study were explained in detail to each man and special emphasis was placed upon the voluntary nature of his involvement. In accordance with previous procedures at this Center, the men were told that they could terminate their participation at any time during the course of the study and that continued participation or withdrawal from it would not effect the terms of their sentence. Ethical and medical aspects of the study were performed according to established procedures of the Committee on Human Investigation.

Participants in these studies included 18 Negro and 21 Caucasian volunteers. They were 21 to 45 years old and weighed between 58 and 116 kg (mean: 83 kg). The volunteers had not had falciparum malaria previously. Infections were initiated either by the bites of infective mosquitoes or by intravenous

* From the Laboratory for Tropical Diseases of the Department of Internal Medicine, Rush-Presbyterian-St. Luke's Medical Center, P. O. Box 112, Joliet, Illinois 60434.

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Table 1. Effects of a 3-day course of quinine, amodiaquine, and chloroquine against asexual erythrocytic parasites of *P. falciparum*.

Drug	Total dose (g)	Most common parasitologic response to treatment	
		Vietnam (Marks) strain	Cambodia (Buchanan) strain
Quinine	4.8	RI	RI
Amodiaquine	1.5	RI	RI
Chloroquine	1.5	RIII	RI

RI = Clearance of parasites with subsequent recrudescence.

RIII = No marked reduction in parasites.

inoculation of small samples of parasitized blood containing approximately 500,000 asexual parasites per mm³. To minimize the possibility of transmitting serum hepatitis to recipients of blood inoculations, potential donors of parasitized blood were questioned carefully concerning drug addiction and no blood was accepted from any volunteer who gave a history of having used drugs intravenously. In addition, recipients received immune serum globulin injections 10 and 40 days after inoculation of parasitized blood from donors with normal liver function tests. Currently, blood from donors is also examined to ensure the absence of Australia antigen and it is washed in normal saline before its inoculation into recipients. None of the volunteers participating in these studies developed serum hepatitis.

During the course of studies with 2 chloroquine-resistant strains of *P. falciparum*, 29 men were infected with the Vietnam (Marks) strain and 10 men were infected with the Cambodia (Buchanan) strain. Previous studies had shown that both strains are resistant to chloroquine and amodiaquine, but the Marks strain is much more resistant to chloroquine than it is to amodiaquine (Rieckmann, 1971). The Marks strain is also resistant to a 14-day course of quinine and standard doses of pyrimethamine, proguanil and trimethoprim (Willerson et al., 1972a). The response of the Buchanan strain to antimalarial drugs has not yet been fully characterized. Table 1 shows the response of non-immune volunteers infected with either the Marks or Buchanan strains to 3-day courses of quinine, chloroquine or amodiaquine.

Volunteers were hospitalized on the first day of patent parasitemia. Parasite counts were performed daily according to the method of Earle and Perez (1932) and were continued for at least 60 days after drug administration. During the period of study, hematocrit, total leukocyte, differential leukocyte and platelet counts were performed twice a week and SGOT, SGPT, blood urea nitrogen and alkaline phosphatase determinations were performed once a week.

Amodiaquine, quinine and tetracycline were administered orally under close supervision and any symptoms reported by the volunteers were carefully investigated. Doses of amodiaquine and quinine are expressed in terms of base (391 mg of amodiaquine hydrochloride is equivalent to 300 mg of amodiaquine base; 10 grains of quinine sulfate is equivalent to 540 mg of quinine base). The presence of amodiaquine in the urine was determined qualitatively by the Dill-Glazko eosin test (Lelijveld and Kortmann, 1970) and quinine levels in the plasma were determined by the fluorometric method of Brodie and Udenfriend (1943). Plasma levels of tetracycline were measured at Lederle Laboratories, American Cyanamid Co., Pearl River, New York.† As conditions of the study precluded reinfection, any reappearance of parasites after drug administration indicated a recrudescence of the original infection.

Therapeutic studies

Thirty-one volunteers received amodiaquine and tetracycline or quinine and tetracycline to determine the effects of these drug combinations against asexual erythrocytic forms of *P. falciparum*. They were treated during their first attack of malaria or, in 6 volunteers, during their second acute attack of malaria.

Amodiaquine and tetracycline were given to 27 volunteers. Amodiaquine was administered over a period of 3 days: 600 mg initially, 300 mg 6 hours later, and 300 mg on each of the next 2 days (total dose: 1500 mg of amodiaquine base). Tetracycline was administered over a period of 7 or 10 days: 250 mg every 6 hours (total dose: 7 or 10 g). Initially, patients were treated with amo-

† Through the courtesy of Dr. H. MacDonald.

diaquine for 3 days and, on the fourth day, treatment with tetracycline was started. In subsequent studies, administration of both drugs was started concurrently.

Quinine and tetracycline were given consecutively to an additional 4 volunteers. They were treated with 540 mg of quinine base every 8 hours for 3 days (total dose: 4.86 g) and this course of therapy was followed immediately, on the fourth day, by a course of 250 mg of tetracycline every 6 hours for a period of 10 days.

Causal prophylactic studies

Eight volunteers in 2 groups were each bitten by at least 10 heavily infected mosquitoes to determine the effects of tetracycline against preerythrocytic forms of *P. falciparum*. One group of 4 volunteers was bitten by mosquitoes infected with the Vietnam (Marks) strain and the other group was bitten by mosquitoes infected with the Cambodia (Buchanan) strain. Administration of 250 mg of tetracycline every 6 hours for 4 days was started 4 hours after volunteers were exposed to infection (Day 0). One member of each group of 4 volunteers did not receive any medication and served as a control subject.

Results

Therapeutic studies

Table 2 shows pertinent data concerning the effects of amodiaquine and tetracycline in 27 non-immune volunteers infected with a chloroquine-resistant strain of *P. falciparum*. Volunteers in Groups 1 to 4 were infected with the Vietnam (Marks) strain and volunteers in Group 5 were infected with the Cambodia (Buchanan) strain.

In the first group of 6 volunteers, a 3-day course of 1.5 g of amodiaquine base was started on the first day of patent infection when patients had only 5 to 40 asexual parasites per mm³ of blood. On the fourth day of patency, administration of tetracycline was started and the drug was given in divided doses of 250 mg every 6 hours over a period of 7 days. Fever and parasitemia subsided within 1 to 3 days from the start of amodiaquine therapy, but a recrudescence of parasitemia occurred in one of the 6 patients.

In the second group of 3 volunteers, treat-

Table 2. Effects of tetracycline, in combination with amodiaquine or quinine, against asexual erythrocytic parasites of *P. falciparum*.

Group	Strain of <i>P. falciparum</i>	Duration of treatment with tetra- cycline (days)	Treatment with amodiaquine	No. of persons treated	Conditions at start of treatment				Clearance time (days)		No. of persons cured
					No. of days with patent parasitemia	No. of asexual parasites per mm ³ ‡	Maximum rectal temp. (°C)	Fever*	Asexual parasites		
1.	Vietnam (Marks)	7	Consecutive§	6	1.0	14 (5-40)	39.1 (39.0-39.3)	1.3 (1-3)	1.5 (1-3)	5	
2.	"	7	Concurrent	3	3.0 (2-4)	1530 (160-3110)	40.4 (39.8-41.3)	4.3 (3-6)	3.7 (3-4)	1	
3.	"	10	Consecutive§	8	2.9 (2-6)	825 (10-3330)	40.5 (39.6-41.1)	3.8 (1-5)	5.0 (3-9)	7	
4.	"	10	Concurrent	4	9.0 (8-10)	2500 (680-5100)	40.3 (40.1-40.6)	3.5 (3-5)	4.7 (4-6)	4	
5.	Cambodia (Buchanan)	10	Concurrent	6	5.3 (2-10)	1488 (130-5280)	40.2 (39.0-40.6)	3.8 (1-7)	4.8 (3-7)	6	
6.	Vietnam (Marks)	10	Treatment with quinine Consecutive§	4	2.5 (1-4)	1775 (40-6580)	40.7 (40.1-41.2)	6.7 (5-9)	5.7 (3-7)	4	

‡ Maximum parasite count during the first 48 hours of treatment.
* Interval between onset of treatment and the first day of a 48-hour period during which rectal temperature remained below 38° C.
§ Administration of amodiaquine or quinine preceded that of tetracycline (see text).

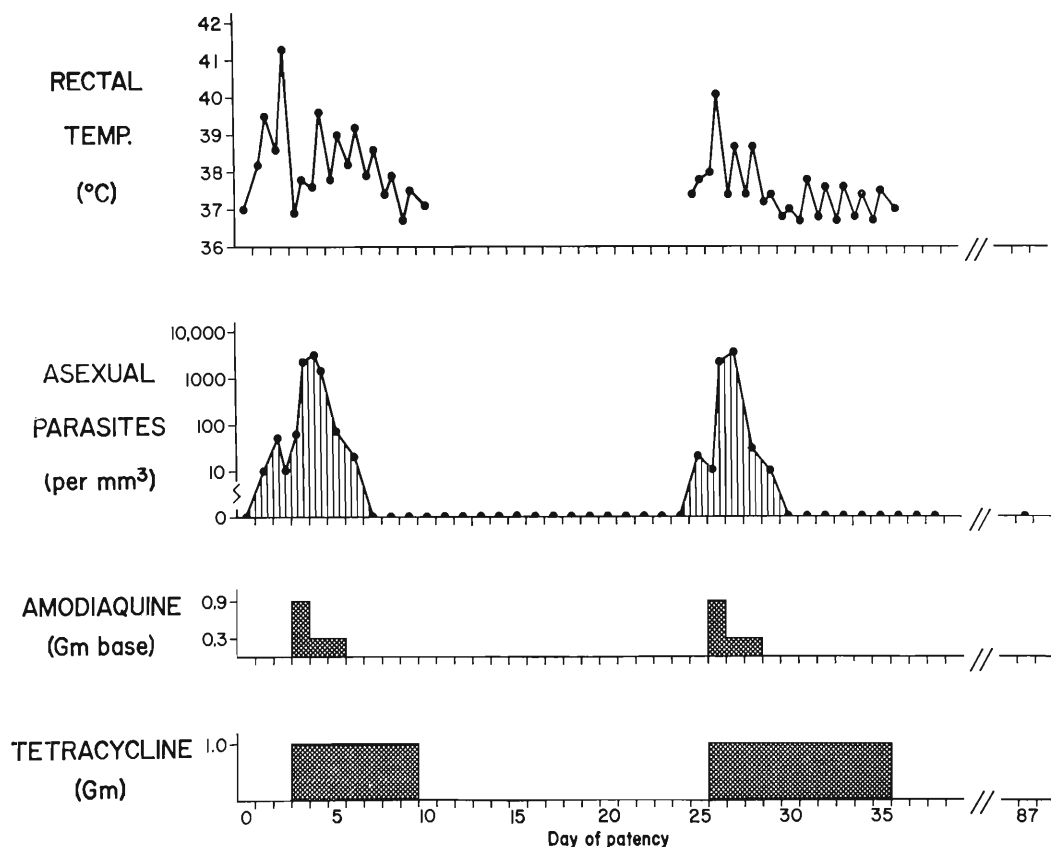


Figure 1. Slow response of fever to first course of treatment in a volunteer in Group 2.

ment was started after 2 to 4 days of patent parasitemia. By this time, the patients had developed a higher level of parasitemia and fever than was observed with patients in Group 1. Instead of the consecutive administration of amodiaquine and tetracycline, treatment with both drugs was started concurrently in this group of patients. Parasitemia cleared within 3 to 4 days and fever subsided completely within 3 to 6 days after the start of treatment. The course of the only volunteer whose temperature did not return to normal for 6 days is shown in Fig. 1. After initial clearance, patent parasitemia in this patient recurred 22 days after onset of therapy. Further treatment with amodiaquine for 3 days and tetracycline for 10 days cured the infection. Another volunteer also showed a

recrudescence of parasites 23 days after the beginning of drug administration. Only one of this group of 3 patients showed no recrudescence of parasitemia after treatment.

In the third group of 8 volunteers treatment was started during an acute attack of malaria with levels of parasitemia ranging from 10 to 3,330 asexual parasites per mm³. Volunteers were treated with amodiaquine for 3 days after which 250 mg of tetracycline was given every 6 hours for 10 days. Fever disappeared within 1 to 5 days after onset of therapy and a typical response is illustrated in Fig. 2. Clearance of parasites was observed within 3 to 9 days after the start of treatment. In the patient whose parasitemia persisted for 9 days after treatment, parasites reappeared 21 days after the first dose of

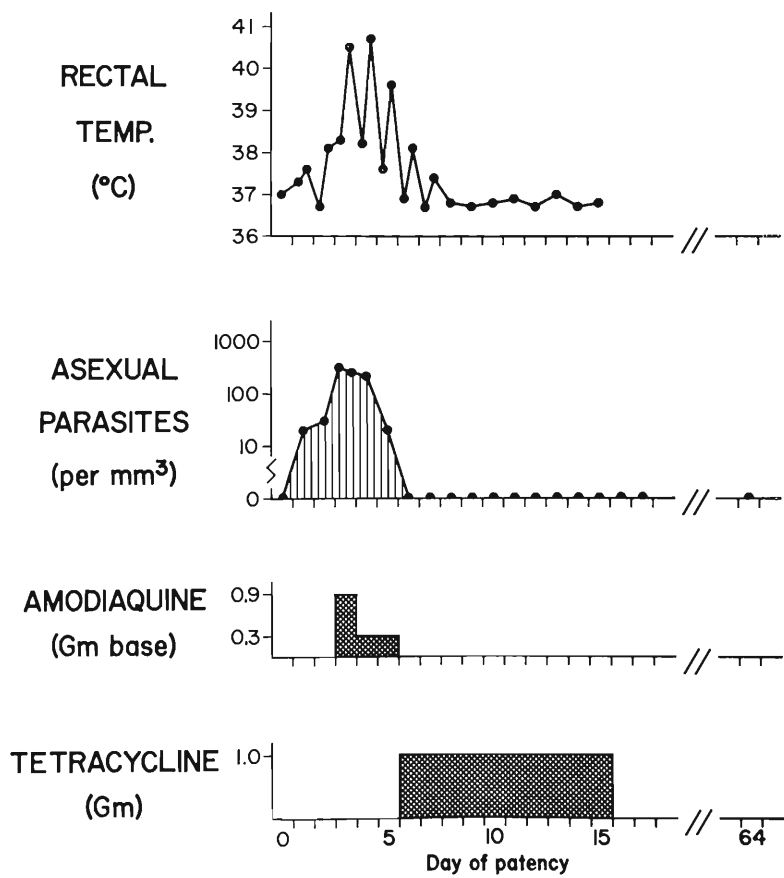


Figure 2. Typical response of fever to treatment in a volunteer in Group 3.

amodiaquine. The other 7 volunteers in this group were cured of their infection.

In the fourth group of 4 volunteers, administration of tetracycline for 10 days was started simultaneously with the first dose of amodiaquine. Volunteers were treated during their second acute attack of malaria, their first attack having been suppressed with a 3-day course of quinine or amodiaquine 12 to 23 days earlier. During the intervening period, no parasites had been detected in the peripheral blood and volunteers had shown patent parasitemia for only 8 to 10 days since the onset of their infection. The mean initial level of parasitemia was higher in this group than that observed in any of the other groups in this study. Acute clinical symptoms re-

sponded to treatment, fever subsided completely 3 to 5 days after initiation of therapy, and radical cure was achieved in all 4 volunteers of this group.

In the fifth group, 6 volunteers infected with the Cambodia (Buchanan) strain received a 3-day course of amodiaquine and a 10-day course of tetracycline. Medication with both drugs was started simultaneously. Four patients were treated during their first acute attack of malaria and 2 patients were treated during their second acute attack of malaria. Fever subsided completely within 1 to 5 days, but one patient continued to run a low-grade fever until the seventh day after the start of treatment (Fig. 3). The level of parasitemia in this patient, unlike the others in

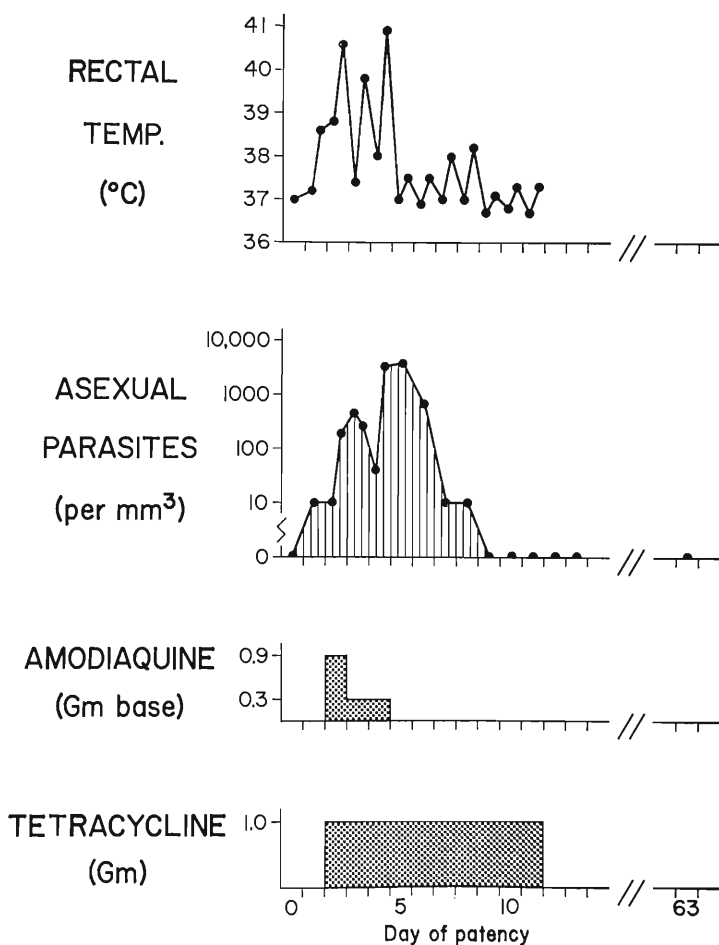


Figure 3. Slow response of fever to treatment in a volunteer in Group 5.

the study, continued to rise during the third and fourth days of treatment and asexual parasitemia did not disappear until the seventh day of drug administration. Nevertheless, his infection and those of the other volunteers in the group were cured by this drug regimen.

Among the 27 volunteers in the 5 groups, 4 were not cured by the administration of amodiaquine and tetracycline. Recrudescence of parasitemia was observed in 2 Caucasian and 2 Negro volunteers. They weighed between 67 and 90 kg (mean: 79 kg). Plasma levels of tetracycline were not determined in these 4 men, but urinary excretion of amodiaquine was confirmed in all volunteers of these 5 groups.

Table 2 shows the results obtained in the sixth group of 4 volunteers who had been infected with the Vietnam (Marks) strain and were then given 540 mg of quinine base every 8 hours for 3 days followed by 250 mg of tetracycline every 6 hours for 10 days. All patients had temperatures above 40° C and their parasite counts varied from 40 to 6,580 per mm³ of blood at the start of treatment. Although this regimen controlled acute clinical symptoms, low-grade fever persisted for 5 to 9 days. Asexual parasites disappeared from the peripheral blood 3 to 7 days after initiation of therapy and none of the volunteers showed a recrudescence of parasitemia. On the second day of treatment, volunteers had levels of

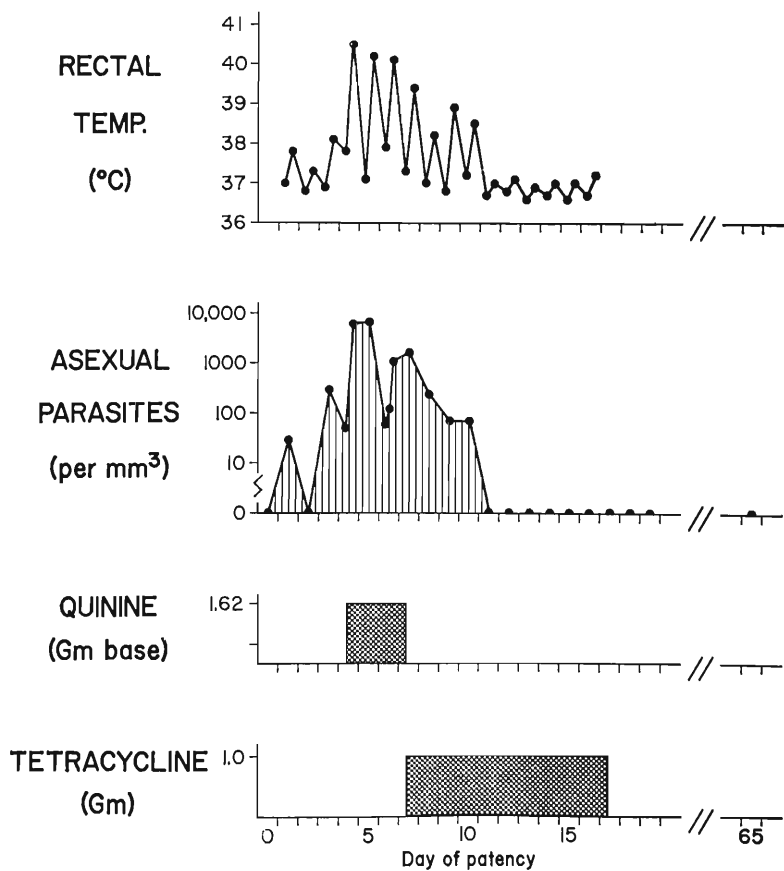


Figure 4. Fairly typical response of fever to treatment in a volunteer in Group 6

quinine which ranged from 8.0 to 11.4 mg per liter of plasma. Fig. 4 depicts the typical course of an infection after administration of quinine and tetracycline.

Causal prophylactic studies

Table 3 shows pertinent data concerning the casual prophylactic activity of tetracycline in 6 non-immune volunteers exposed to a chloroquine-resistant strain of *P. falciparum*. Volunteers in each group received an adequate sporozoite inoculum before they were given tetracycline. This was shown by the heavy infection of salivary glands during dissection of blood-fed mosquitoes and by the essentially normal prepatent period of the volunteers who were not given any medication. All 6 volunteers who received tetracycline were protected

against infection with either the Vietnam (Marks) strain or the Cambodia (Buchanan) strain of *P. falciparum*.

Levels of tetracycline in the plasma during and after administration of 250 mg of tetracycline every 6 hours for 4 days are shown in

Table 3. Effects of tetracycline against pre-erythrocytic parasites of *P. falciparum*.

Strain of <i>P. falciparum</i>	No. of volunteers	Days of administration of tetracycline*	Day of patency after sporozoite inoculation
Vietnam (Marks)	1	—	12
	3	0, 1, 2, 3	—
Cambodia (Buchanan)	1	—	11
	3	0, 1, 2, 3	—

* Administration of tetracycline was started on Day 0, 4 hours after inoculation of sporozoites.

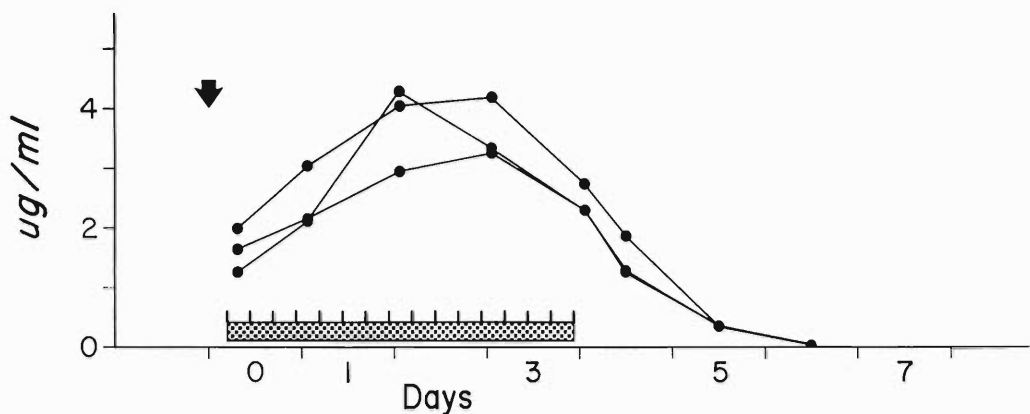


Figure 5. Levels of tetracycline in plasma of 3 volunteers who received 250 mg of tetracycline every 6 hours for 4 days. Arrow indicates time at which volunteers were bitten by mosquitoes infected with the Cambodia (Buchanan) strain of *P. falciparum*.

Fig. 5. These determinations were performed on venous samples of blood from the 3 volunteers who were bitten by mosquitoes infected with the Cambodia (Buchanan) strain. During drug administration the concentration of tetracycline reached a level of 3.3 to 4.3 μg (mean: 3.9 μg) per milliliter of plasma. No tetracycline was detected in any blood samples collected from the volunteers 3 days after the end of drug administration; this was 6 days after they had been bitten by mosquitoes.

Discussion

Studies conducted by us since 1967 have demonstrated that tetracycline cures chloroquine-resistant infections in persons who have become partially immune to malaria (Rieckmann et al., 1971). Subsequent studies with semi-synthetic tetracyclines have shown that these compounds also exert an effect against drug-resistant infections of *P. falciparum* (Clyde et al., 1971; Colwell et al., 1972a; Willerson et al., 1972b). These drugs, however, exert a slow activity against asexual erythrocytic parasites and, in non-immune persons, dangerously high levels of parasitemia might develop before an infection could be brought under effective control.

In our current investigations, administration of amodiaquine and tetracycline to non-immune volunteers infected with the Marks strain cured 11 of the 12 men who received tetra-

cycline for 10 days and 6 of the 9 men who received the drug for 7 days. In 6 volunteers infected with the Buchanan strain, administration of tetracycline for 10 days, in combination with amodiaquine, cured all the infections. Radical cure was also observed in all 4 volunteers infected with the Vietnam (Marks) strain who were treated with quinine for 3 days and tetracycline for 10 days. Low-grade fever, however, seemed to persist longer in these 4 patients than was usually observed in patients treated with quinine, amodiaquine, or amodiaquine and tetracycline. Recently Colwell and his coworkers (1972a, 1972b) described the antimalarial effects to tetracycline against chloroquine-resistant strains of *P. falciparum* in an endemic area of Thailand. In one of their studies, 29 patients received a 3-day course of quinine during an acute attack of malaria and they were then given a 10-day course of tetracycline. Clearance of asexual parasites was observed in all patients within 1 to 6 days (mean: 2.9 days) and none of the patients showed a recrudescence of parasitemia. The somewhat slower clearance of parasites observed in our study may simply reflect a difference in the immune status of persons in the 2 studies and, possibly, a difference in the drug susceptibility of the strains of *P. falciparum*.

Our studies showed that the combined administration of a rapidly-acting, but non-cura-

tive, blood schizontocidal agent and tetracycline adequately controlled an acute clinical episode of malaria in most patients who had been infected with one of 2 chloroquine-resistant strains of *P. falciparum*. In addition, most infections were cured by such a drug combination. The results of these investigations suggest that tetracycline, given in conjunction with certain non-curative blood schizontocidal drugs could be of value in the treatment of non-immune persons infected with chloroquine-resistant falciparum malaria.

Our studies also demonstrated the marked activity of tetracycline against the pre-erythrocytic tissue forms of 2 chloroquine-resistant strains of *P. falciparum*. Tetracycline was given for only 4 days after volunteers were bitten by infective mosquitoes. Three days later, when the first asexual parasites might have been released into the peripheral blood stream, no tetracycline was detected in any of the blood samples collected at this time. The observed causal prophylactic activity of tetracycline is due to the effects of the drug against primary tissue schizonts (not against sporozoites) because drug administration was started 4 hours after inoculation of sporozoites. The practical significance of these findings remains to be determined. In any case, tetracycline or its analogues should not be used as prophylactic agents against drug-resistant falciparum malaria until all the implications of such a step have been carefully considered.

Acknowledgments

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Comments on Clinical Studies

L. J. BRUCE-CHWATT

Ross Institute of Tropical Hygiene, London School of Hygiene and Tropical Medicine, London, WC1E 7HT, England

We learned yesterday from Dr. Sadun that according to Sebastiano Badi "the quotidian, tertian and quartan fevers are eliminated by an occult quality of the Bark. . . which must be included among the mirabilia." We were also told that Francesco Torti absolved the cinchona powder from causing blood dyscrasias, intestinal irritation, vomiting, excessive sweating or copious urination but that it may produce transient deafness. We know now that it destroys the febrile fermentations "because it possesses a specific power as an antidote against the pyrogenic substance present in the blood." Today, listening to Dr. Powell our thought spans the 3 centuries going back to these two Italian pioneers who together with Sydenham laid down the rational basis for the treatment of malaria.

Dr. Powell and Dr. MacNamara's observations on the present regimen of therapeutic administration of quinine are distinguished by their clarity and attention to detail. But before discussing their results, it would be pertinent to emphasize once again that most of our present knowledge of the effect of drugs on human malaria and of the behaviour of drug resistant strains of plasmodia comes from carefully conducted studies carried out in non-immune human volunteers in the U.S.A., though the Australian pioneering work of Hamilton Fairley and his colleagues must not be forgotten. It is good to know that the value of such human malaria research centers has been recognized by the W.H.O. even though the Organization could not sponsor this type of research for many reasons. Five years ago when this problem created a heated controversy in the columns of the *Scientific American* I took a personal stand on this point. (*Scientific American*, 31 March 1967).

Having visited many human malaria research centers in the U.S.A., I said then and still maintain that these studies carried out on selected healthy prisoner volunteers with their

full and free consent, under careful supervision are professionally and ethically more justified than many experiments or trials done in hospitals surreptitiously without any consent of the patients and without their knowledge. No one will deny that the results of this work are of incalculable benefit to medical research because no comparable animal model can be substituted for it. The importance of Dr. Powell's paper lies in making us aware of the fact that quinine is a powerful drug, that it may affect some individuals more than others and that the toxic effects may vary from person to person.

It would be interesting to know whether these individual or group differences in response to apparently the same dosage of drug were due to differences of absorption or excretion. Are they related to dissimilarity in metabolizing the quinoline nucleus thus forming a new example of pharmacogenetics as seen in slow or fast acetylators of isoniazid?

The plasma quinine levels show that in spite of the same dosage of the drug the group of subjects with mild or no symptoms of infection had lower quinine levels during the first week than one group with more severe symptoms. Could it be that these higher levels of the drug in plasma reflect an impairment of one hepatic or renal function due to severe infection?

The fact is that some side effects (such as headache, vertigo, nausea, pruritus, blurring of vision) noted with quinine are also seen when chloroquine is administered.

Many observations on the toxic effects of quinine were made in the past but few of them combined clinical observation with pharmacological studies. Pharmacodynamics of antimalarial drugs are rarely investigated in the way shown in this paper and more studies of this kind are needed.

One can only agree with the authors that the limitations of quinine stress the need for newer more effective and safer antimalarial

drugs. But this, as Alice said, is another story.

The paper by Colwell *et al.*, based on an impressive number of *in vitro* tests carried out in a field condition showed that in 67% of cases the results of the test could be satisfactorily interpreted and that the prevalence of chloroquine resistance in Thailand was high. The fact that Colwell like Peters and Seaton (1971) confirmed the parallelism of results *in vivo* and *in vitro* is an additional proof of the reliability of Rieckmann's test.

In Colwell's series of tests the range of concentrations of chloroquine was only slightly different from that used by other authors. However justified this might be it would be useful to standardize the conditions of the test so as to make it fully comparable with the method applied by other authors. The dose response relationship of the degree of schizogony versus drug concentration is important and the comparison of results from four areas of Thailand very instructive.

Colwell's discussion of some technical limitations encountered with Rieckmann's test is of particular value and should stimulate the ingenuity of workers to overcome some of them. One cannot always obtain trophozoites with a required degree of maturity unless they have a regular pattern of periodicity.

It may be of some interest to recall that the action of antimalarial drugs *in vitro* on the asexual erythrocytic stages of plasmodia was investigated some 40 years ago by Muhlen and Kirschbaum (1925) and then by Lourie (1934) and a few other authors. It appears that quinine added at 1:5000 dilution for 5 hours does not interfere with the infectivity of *P. vivax* while mepacrine has a more pronounced effect. Today the *in vitro* test for detection of strains of *P. falciparum* resistant to 4-aminoquinolines has gained acceptance as a field method in Brazil and in Thailand. It is of particular value in conditions when the W.H.O. test requiring a selected group of subjects and at least one week's time cannot be carried out for a number of reasons. It is hoped that this test will be more widely used.

The paper by Rieckmann and his colleagues represents a further step in our slow advance to face the serious threat of resistance of *P. falciparum* to 4-aminoquinolines. In fact, the attempt to use antibiotics for treatment of

malaria is not new. Over 25 years ago Taliaferro *et al.*, (1944) investigated the action of tyrothricin (gramicidin) against *P. gallinaceum* and Coatney *et al.*, (1949) evaluated the effect of aureomycin not only on avian malaria, but also on the Chesson strain of *P. vivax*. A number of other workers and especially Ruiz-Sanchez in Mexico tried to use penicillin, chloramphenicol, streptomycin and terramycin with variable success. Coatney and Greenberg (1952) collected all the then available information on the activity of antibiotics against plasmodia of birds and rodents and came to the general conclusion that a series of products derived from *Streptomyces* had some activity. Chloramphenicol, aureomycin and terramycin assessed in *P. gallinaceum*, and *P. cathemerium* infections of chicks and canaries and in *P. berghei* infection of mice had a range of quinine-equivalent between 0.1 and 1.0: aureomycin scored the highest marks, with terramycin a close second. The activity of aureomycin was more obvious in *P. cathemerium* than in *P. gallinaceum*. These two compounds also acted as casual prophylactics against the two avian parasites.

Of the 30 antibiotics tested on animal plasmodia, five were tried against human malaria and once again, chloramphenicol, aureomycin, and terramycin showed some activity in *P. vivax* and *P. falciparum*. The two latter drugs were more effective but the dosage was of the order of 1.4–2.5–5.0 grams daily for 3–7 days. The general conclusion was that therapeutic effects have been achieved but even with high dosage the effects of these compounds were slow and they offered little advantage over existing drugs.

Strictly speaking, this statement would still be true if it had not been for the problem of chloroquine resistance which proved how narrow the range of our reliable drugs is for treatment of *P. falciparum* infections. In view of the fact the aureomycin belongs to the tetracycline series of antibiotics, it seems that the recent work confirms the earlier results and provides some valuable new data.

The tetracyclines are a family of at least 7 antibiotics of which 3 have been in use for over 10 years. Discovered in 1945 by Duggar in the American Cyanamide Laboratories from the soil inhabiting *Streptomyces aureofaciens* the first antibiotic of this group was called

Aureomycin (chlor-tetracycline); there followed Terramycin (oxytetracycline) from *S. rimosus* and in 1953 tetracycline *sensu proprio* which can be obtained from chlortetracycline.

More recently several new tetracyclines have been discovered (demethylchlorotetracyclin, pyrrolidinomethyl tetracycline and others). They are "broad spectrum" compounds acting not only on gram-positive but also on many gram-negative bacteria; they are also anti-treponemal but not anti-tuberculous. Although resistance to these compounds is relatively rare it has recently become more common in staphylococci, haemolytic streptococci and pneumococci. Tetracyclines are bacteristatic by interference with protein synthesis through the formation of peptide linkages. The usual route of administration is by mouth but the absorption depends on the acidity in the gastrointestinal canal and shows much individual variation. The blood level curve shows a slow rise and a slow fall. The persistence is due to biliary excretion and re-absorption as well as to protein-binding. Parenteral administration speeds up the action. A unique feature is the deposition of tetracyclines in bone and teeth of children.

The value of the study by Rieckmann and his colleagues lies in the proof of a synergistic action of amodiaquine and tetracycline against two strains of *P. falciparum* from Vietnam. This extends Colwell's recent work in Thailand where similar good effects of quinine and tetracycline were described, though the comparison between 10 days treatment by tetracycline and 3 days "standard" treatment by chloroquine could be fairer for the latter drug.

The causal prophylactic effect of tetra-

cycline is of particular interest and indicates that further studies of analogues of this drug are justified, even if the compounds now available cannot be used as prophylactic agents because of their possible side effects.

This brings me to the consideration of toxicity of tetracyclines. It appears that fears of teratogenicity have been exaggerated, but tetracyclines are very powerful drugs and may cause liver damage, gastrointestinal complications due to the replacement of normal intestinal flora by *Proteus*, *Pseudomonas*, *Candida* and *Staphylococcus aureus* besides such relatively harmless effects as staining of teeth.

Tetracycline, because of its systemic anti-anabolic effects, will often increase azotaemia in any patient with renal impairment. Kanamycin and gentamycin have the same effect. There is some recent evidence (Stott, 1971) that tetracyclines may inhibit the inactivation of endotoxins. I would suggest that this compound should not be used for treatment of malaria in children or pregnant woman until further data become available.

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VI

PHYSIOLOGY AND MOLECULAR BIOLOGY

The "Time Signal" for Control of Growth and Division Synchrony of Mouse Malaria

J. D. ARNOLD, A. E. BERGER AND A. R. FLESHER

Harry S. Truman Medical Research Laboratory, 2322 Holmes, Kansas City, Missouri 64108.

ABSTRACT: In our previous studies in male mice, it became apparent that the control of parasite growth and division synchrony was a very complex problem and that the female responds in a way different from the male. It appears that only one of the five steps of estrus was partly correlated with parasite synchrony. The study of the ovariectomized female provides us with what seems to be a clue about the mechanism of control of parasite growth and division by the mammalian host. The actual signal for a continuation of parasite growth is unknown to us, but we have been able to eliminate a number of possible mechanisms. We can suggest that the signal for cell division comes from some kind of solar phenomenon which can be effectively screened out by large masses of earth but not by the usual materials of our laboratory.

In our previous studies in male mice, it became apparent that the control of parasite growth and division synchrony was a very complex problem and that the female responds in a way different from the male. This work established that the duration, quality, phasing and intensity of light and diet, and the endocrine state of the animal are all critical to the production of growth and division synchrony of the parasite population (Arnold, et al., 1969c & 1969d).

The estrus cycle of mice is somewhat different from that of some other mammals. For one thing, it is under very close control by the photoperiodic rhythm. The time of ovulation apparently can be controlled by the on-off cycle of light (Snell, et al., 1940). In addition, the estrus cycle is short (four to five days). Thus, the corpora lutea do not produce a decidual response to uterine trauma and there is little chance of extreme depression of the ovary during anestrus. In turn, anestrus hypophyseal depression is unlikely. The short survival of follicles suggests a brief and abortive rise of estrogen.

In our preliminary experiments, it appeared that only one of the five steps of estrus was partly correlated with parasite synchrony. Although this was a limited correlation, the observation did, however, suggest that a study

of female animals after ablation of several endocrine glands would be useful. The study of the ovariectomized female provided us with what appears to be a clue about the mechanism of control of parasite growth and division by the mammalian host.

Materials and Methods

In general our procedure is similar to the procedure stated in previous reports (Arnold, et al., 1969d). In summary, this involved acclimatization of the host mouse to a standard environment of light, temperature and diet. After two weeks, the animals were infected with *Plasmodium berghei* malaria. Then the population distribution of the several growth stages of the parasite was studied by blood smears made from the peripheral blood.

The effect of estrus was correlated with the changes in the parasite population and this was studied by removing the appropriate endocrine gland or by hormone replacement.

Parasites

The KGB-173 strain of *P. berghei* was used throughout. Identification of six growth stages follows the criteria cited previously (Arnold, et al., 1969d). This strain has been long adapted to the laboratory mouse and no longer undergoes sexual reproduction. The peripheral blood contains all growth stages but occasionally the dividing parasites may sequester in the blood vessels.

The growth stages can easily be identified by Giemsa's staining of the thin blood smear.

From Harry S. Truman Medical Research Laboratory, 2322 Holmes, Kansas City, Missouri 64108.

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Table 1. Summary of association between steps of vaginal smears and existence of augmented synchrony of *Plasmodium berghei* infection in Swiss mice.

Steps of estrus from vaginal smear	I	II	III	IV	V
No. of animals that showed synchrony	5	11	7	2	9
No. of animals that showed asynchrony	11	8	25	8	18

The sequestered blood vessel forms were studied by tissue sections, chiefly of the liver and spleen, either after quick fixation of the tissue or after perfusion of the organ with unparasitized blood followed by fixation.

Tissues were examined by light microscopy after paraffin embedding and staining with hematoxylin and eosin in a modified Tomlinson stain (Tomlinson and Grocott, 1944).

Parasite synchrony was determined by changes in the ratio of different growth stages in successive blood smears. For convenience, we chose an indicator stage that was rapidly changing during daylight. A doubling of this indicator growth stage (Stage III) was required for us to call the effect "augmented growth and division synchrony."

The parasites were inoculated by an intra-peritoneal injection of 0.1 cc of nearly 100 percent infected blood.

Blood smears were usually studied on day four of the infection. When the infection became terminal, the population of parasites was distorted.

Animals

The host animal was the CFW Swiss mouse. Almost all animals were from four to six weeks of age. The normal host was probably *Thamnomys surdaster* of the Belgian Congo. In the Swiss mouse, the infection occurs chiefly in mature cells.

Environment

All animals were housed in clear plastic cages and were exposed to a controlled light from the Sylvania metalarc lamp at 300 foot-candles (fc.) for 18 hours per day (6 AM to 12 PM). This cycle was most effective in inducing growth and division synchrony in male mice. It is also a light cycle that has not been studied as intensively as some other cycles (i.e., 12 hours of light and 12 hours of darkness) for its effect on estrus. Thus, we do not know for sure that ovulation is occurring in the dark phase, as was reported in a 12-hour light and 12-hour dark rhythm (Snell, et al., 1940). Even so, the mean cycling time of estrus on 18:6 photoperiodic rhythm was approximately that of other studies (Quay, 1963).

The temperature of the room was 78°F. Female animals were conditioned to the odor of male urine in order to utilize the effect of pheromone for shortening and reducing the variation in length on the estrus cycle of females (Dominic, 1966). This seems to have the sole effect of reducing the variation normally encountered in the length of the estrus cycle.

In order to look for the "Zeitgeber" that sets off the train of growth from the elastic period (Prescott, 1956), we have examined intact male CFW Swiss mice maintained underground in a limestone cave and stimulated only by the electromagnetic spectrum of the Sylvania metalarc lamp. The underground location in the Bethany falls limestone layer is 40 feet underground. This effectively eliminates long wave radiation from the sun. The average temperature in the cave is 58°F. There are no perceptible diurnal variations in noise, temperature or electromagnetic radiation.

Table 2. The role of the ovaries in affecting augmentation of growth and division synchrony of *Plasmodium berghei* in Swiss mice (18:6 in surface lab).

No. of animals	Time	Mean in % of stage III parasites	Standard deviation	% difference needed between the two time intervals to be called augmented synchrony	Observed % difference	Conclusion
18	9 AM 3 PM	9.9 20.8	0.96 4.50	> 9.9	10.9	Augmented Synchrony

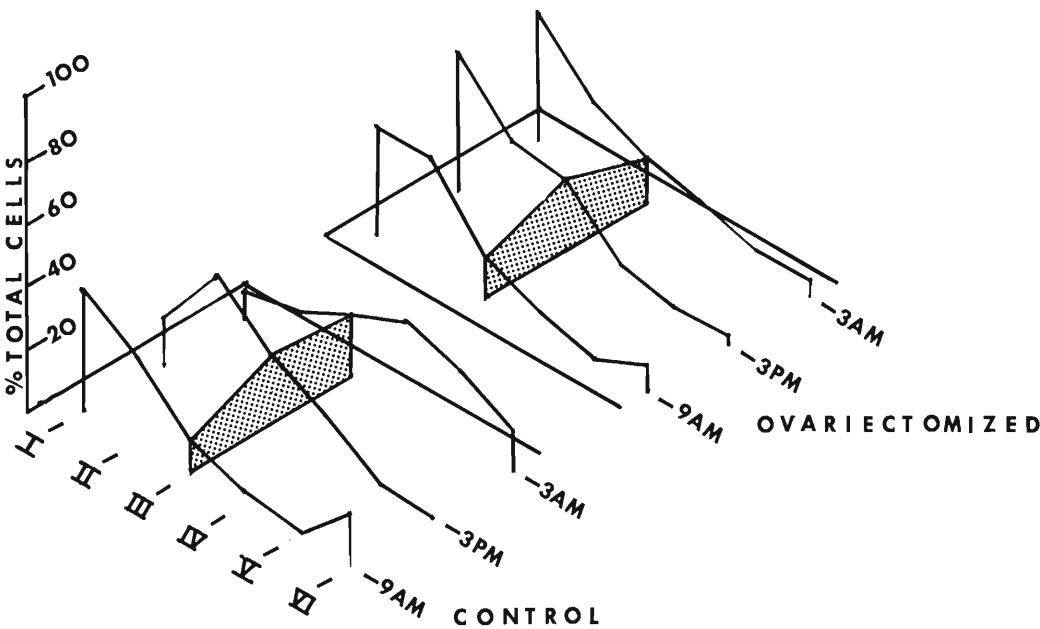


Figure 1. A comparison of normal vs. ovariectomized mice on growth and division synchrony.

Diet

There were two groups of animals for each experiment. One group was fed Purina Lab Chow; the smaller group of animals was fed a natural diet with minimal estrogen content.

Determination of estrus cycle

A degree of uncertainty exists with respect to the time of ovulation in relation to the

Table 3. The apparent hold-up of early growth changes along with augmented synchrony of *Plasmodium berghei* in Swiss mice after ovarian ablation.

Control						
Stage	I	II	III	IV	V	VI
9 AM	39	28	10	5	1	17
3 PM	13	39	33	14	1	0
3 AM	7	10	20	30	21	12

Ovariectomized Animal						
Stage	I	II	III	IV	V	VI
9 AM	35	36	11	5	1	9
3 PM	46	25	21	3	2	3
3 AM	43	23	16	10	4	5

time of the vaginal smear. The vaginal smears were taken only once for each animal during the study. This smear was made at the same time the peripheral blood smear was made for parasite synchrony. The vaginal smears were graded into five steps by the following criteria of Long and Evans (1922):

- a. Step One (Proestrus): A smear is made up of uniform size epithelial cells. Heat is manifest toward the end of this stage.
- b. Step Two (Oestrus): A smear of cornified cells plus epithelial cells. Heat is manifest mostly during this stage and ovulation probably occurs.
- c. Step Three (Metoestrus 1): The smear

Table 4. The role of 20 γ of 17 β estradiol on augmentation of growth and division synchrony of *Plasmodium berghei* in ovariectomized Swiss mice.*

Stage	I	II	III	IV	V	VI
9 AM	24	28	34	12	2	0
3 PM	25	19	25	16	5	10
3 AM	27	26	26	14	3	4

* A close examination of growth stage III shows lack of augmented growth and division synchrony.

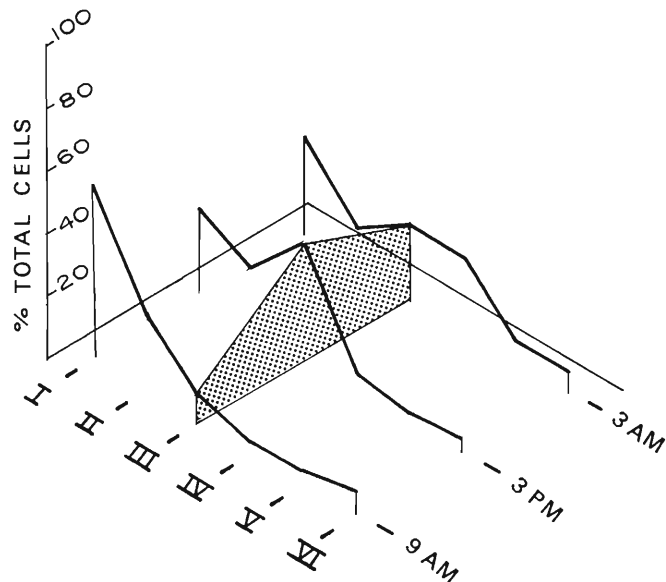


Figure 2. The effect of ubiquinone with pinealectomized and ovariectomized mice on growth and division synchrony.

- shows cornified cells only. The animal is not in heat.
- d. Step Four (Metoestrus 2): Cornified cells, leukocytes, and corpora lutea are present in the smear.
 - e. Step Five (Diestrus): Leukocytes and epithelial cells are present.

In addition to judging the estrus cycle by the vaginal smear, we also determined the time of heat by the appearance of mating behavior and the lordotic posture, according to the technique of Wang (1923).

Endocrine ablation

The ovaries were removed by the general technique of Ingle and Griffith (1963).

Table 5. The role of combined ablation of pineal and ovary on ring stage hold-up and augmented growth and division synchrony of *Plasmodium berghei* in female mice.*

Stage	I	II	III	IV	V	VI
9 AM	29	28	30	11	0	0
3 PM	34	24	25	14	3	2
3 AM	31	24	26	13	3	4

* A close examination of growth stage III shows lack of augmented growth and division synchrony.

Hormone and chemical supplements

All drugs and hormones were administered subcutaneously. Estradiol-17 β , progesterone and ubiquinone were dissolved in alcohol and then diluted in water.

Results

Correlation between growth and division synchrony and changes in the vaginal smear due to estrus

A total of 104 female Swiss mice were conditioned for ten days to two weeks on the standard photoperiodic rhythm (18.6) at 300 fc. In male mice, this photoperiodic rhythm would normally have induced a growth and

Table 6. The effect of 25 γ ubiquinone on combined ablation of pineal and ovary and on ring stage hold-up and augmented growth and division synchrony of *Plasmodium berghei* in female mice.*

Stage	I	II	III	IV	V	VI
9 AM	58	22	9	1	3	7
3 PM	30	20	38	5	3	4
3 AM	31	11	26	21	5	6

* A close examination of growth stage III shows the presence of augmented growth and division synchrony.

Table 7. The effect of the metalarc lamp in the cave during the solar day (18:6) on growth and division synchrony of *Plasmodium berghei*.

No. of animals	Time	Mean in % of stage III parasites	Standard deviation	% difference needed between the two time intervals to be called augmented synchrony	Observed % difference	Conclusion
5	9 AM 1 PM	7.2 17.8	2.4 3.4	> 7.2	10.6	Synchrony

division synchrony of the parasite population (Arnold, et al., 1969a). The results of the study with female mice indicates that the appearance of growth and division synchrony of the parasite clusters around a specific time period of the estrus cycle (Step 2). The association of these two events is not absolute, as can be seen by the data in Table 1. However, the best ratio of synchronous to asynchronous infections occurs during estrus (Step 2).

Correlation between ovariectomy and growth and division synchrony of the parasite

After ovariectomizing and conditioning the host mouse to the synchronizing light rhythm, the animals were inoculated and studied for growth and division synchrony. Table 2 shows that ovariectomy produces a striking change in the growth and division pattern of the parasites. For one thing, a majority of parasites (70 percent) seem to be in the earliest growth stage. This stage is the small ring form or early trophozoite. In order to further define the effect of ovarian ablation on parasite growth, a group of eight animals was studied as controls over a 24-hour period. The data from this study are shown in Table 3. Figure 1 shows the isometric representation of this data.

Those parasites which are not held in an early growth stage (ring or trophozoite) develop in growth and division synchrony. The

progression can be determined by examining the growth stages older than growth stage II.

This development is of great interest for it suggests that the mechanism of host control of the parasite growth pattern comes from a signal that recurs on a daily basis. At the same time each day, a certain proportion of the parasites from the early (or elastic) growth stage proceed to further growth and development in a synchronous pattern.

To be quite certain that this unusual accumulation of parasites in the early growth stages is not due to some sort of population selection by sequestration in the tissues, we also examined most of the viscera with particular attention to the liver and spleen.

From the results of the study of the liver and spleen, it appears very unlikely that the older parasites are lost on the lining of the visceral blood vessels during the early part of each day. Instead, it suggests that old growth forms do not exist in significant number at this time of day any place in the vascular system.

A hypothesis is suggested by this data; this hypothesis proposes that the length of the early growth phase or α phase of Prescott, is the variable by which all cells are brought to division at approximately the same time. In the estrogen depleted female, parasites would remain in this early growth stage (parasite hold-up) for two, three, or four days before being released for further division.

Table 8. The effect of continuous darkness in the cave on growth and division synchrony of *Plasmodium berghei*.

No. of animals	Time	Mean in % of stage III parasites	Standard deviation	% difference needed between the two time intervals to be called augmented synchrony	Observed % difference	Conclusion
5	9 AM 5 PM	6.6 9.6	2.3 3.8	> 6.6	3.0	Synchrony

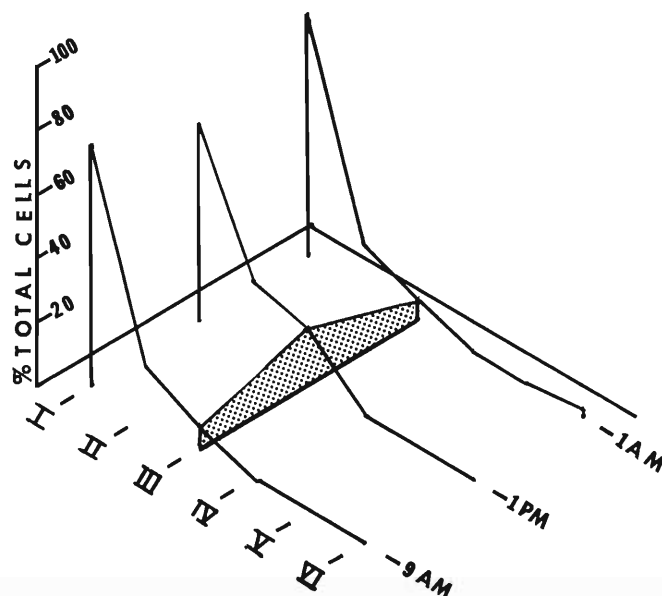


Figure 3. The effect of a metalarc lamp in a cave using a cycle of 18:6 (on at 6 AM, off at 12 midnight) on growth and division synchrony.

Effect of estrogen on parasite hold-up

Since the low point of the estrogen cycle in estrus was the apparent period of the most frequent parasite growth synchrony, and since the removal of the ovaries revealed a synchronous pattern for a proportion of parasites, we gave Estradiol 17 β to the ovariectomized mice. The results (documented in Table 4) confirm the impression that estrogen destroys the synchrony. This Table also shows that the hold-up of growth between Stages II and III is abolished by estrogen.

It should be noted that the estrogen supplement to ovariectomized animals reproduces conditions during only part of the estrus cycle. The parasite growth synchrony at Step

2 of estrus has not been precisely reproduced in the ovariectomized female.

Effect of pinealectomy on the growth and division synchrony of parasites in ovariectomized mice

In our earlier experiments on male animals, it was found that the pineal exerted a permissive effect on growth and division synchrony (Arnold, et al., 1969c). This effect was considered a permissive one because the pineal was necessary for growth and division synchrony, but it did not establish the time relationships of this process.

Since ovariectomy permitted a synchronous subpopulation to develop, it was possible to test the role of the pineal in females with a synchronous parasite population. In Table

Table 9. The effect of a 12-hour reversal of the 18:6 light cycle in the surface lab on growth and division synchrony of *Plasmodium berghei*

No. of animals	Time	Mean in % of stage III parasites	Standard deviation	% difference needed between the two time intervals to be called augmented synchrony	Observed % difference	Conclusion
5	9 AM 5 PM	3.4 14.4	2.4 2.6	> 3.4	11.0	Synchrony

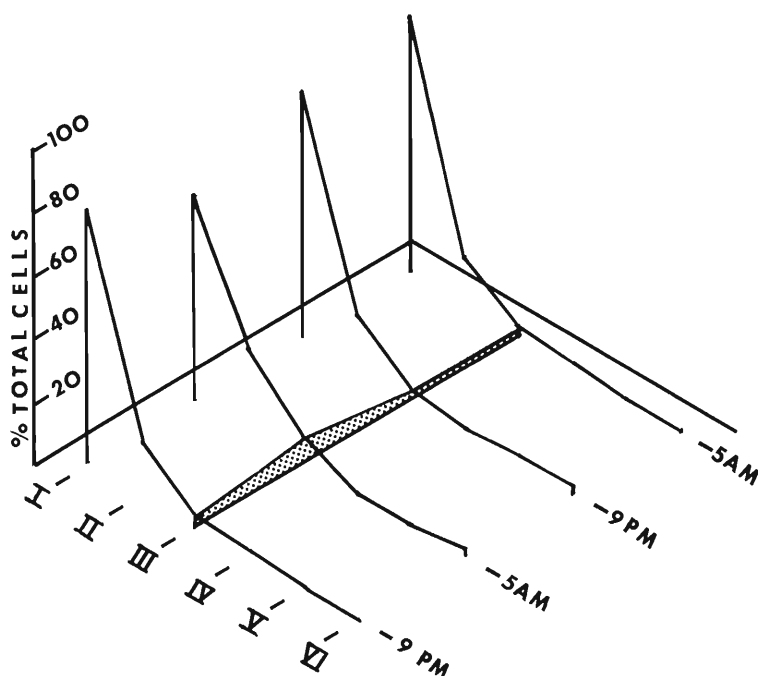


Figure 4. The effect of reversing the light cycle in a cave by 12 hours (on at 12 midnight and off at 6 PM) on growth and division synchrony.

5 the data from an experiment on pineal ablation in the ovariectomized female is given. This data strongly suggests that the pineal is also necessary for the expression of growth and division synchrony in the female, but there does not appear to be a continued hold-up of early growth stages.

In male mice we found that the pineal could be replaced by ubiquinone (Arnold, et al., 1969b). In Table 6 (Figure 2) data is displayed that shows this is also true in females. This does not, however, restore the unique conditions that lead to growth delay or stoppage in the early stages.

Effect of screening off all solar electromagnetic radiation

From the present data we were still unable to account for the timing of the growth and division cycle. When we repeated our experiments in the limestone cave it became apparent that the signal to which all growth and division originated was from the metalarc lamp. By employing the same 18:6 daytime light cycle as we used on the surface, the parasite population was synchronous (Table 7 and Figure 3). When the light was removed, resulting in continuous darkness in the cave,

Table 10. The effect of a 12-hour reversal of the 18:6 light cycle in the cave on growth and division synchrony of *Plasmodium berghei*

No. of animals	Time	Mean in % of stage III parasites	Standard deviation	% difference needed between the two time intervals to be called augmented synchrony	Observed % difference	Conclusion
7	9 AM	3.1	1.1			
	5 PM	4.1	1.5	> 3.1	1.0	Asynchrony
	9 PM	2.6	1.4			
	5 AM	7.5	3.2	> 2.6	4.9	Synchrony

the population of parasites was random or asynchronous (Table 8). When the light cycle was altered by twelve hours (on at 12 AM and off at 6 PM) the results from the surface laboratory showed synchrony during the solar day (Table 9). In the cave the parasite population showed synchrony during the induced solar day (Table 10 and Figure 4).

Above ground the timing of growth and division synchrony was always dominated by the natural solar day. The materials used in our surface laboratory were only able to screen out visible radiation and short electromagnetic waves. When long electromagnetic radiation of the sun was eliminated by using an underground cave, we could then impose our own timing signal on the phenomenon.

Discussion

There are a number of demonstrable effects produced by the host endocrine system in the host-parasite relationship. From these studies, it would appear that the malaria infection of rodents with *P. berghei* (which has had a long acclimatization in laboratory mice) is very sensitive to estrogens.

During the low point of the estrogen cycle, as well as in the absence of the ovary, *P. berghei* will be entrained to a degree of growth and division synchrony by some natural rhythm of the host.

The machinery which controls such a natural rhythm is still obscure but the effect can be abolished by estrogen and by pinealectomy.

When the ovaries are removed, we see a new phenomenon. This is the rapid accumulation of small growth stages so that in the environment of the ovariectomized female, the parasites seem to hold up in the early growth stage.

Prescott has made the point that the control of generation times in cells of widely different origin is largely a matter of the control of the α phase, or as he has phrased it, the "elastic phase." In other words, the S, G₁, and dividing phases of cell growth occur in a remarkably similar time span from cell to cell. Some signal is apparently required to set the growth machinery moving during the α phase. This hold-up of parasite growth in the α phase gives us a good explanation of the mechanism by which the host entrains the

parasite population to a synchronous growth pattern.

The actual signal for a continuation of parasite growth is unknown to us, but we have been able to eliminate a number of possible mechanisms. Thus, certain systems are indicated to play a permissive role. It can be shown that the ovaries and pineal can influence growth and division synchrony but they do not activate the timing mechanism.

We can suggest that the signal for cell division comes from some kind of solar phenomenon which can be effectively screened out by large masses of earth but not by the usual materials of our laboratory. This solar-related signal dominates all the other photoperiodic signals when the animals are in a surface laboratory. When we screen out the solar signal, we can successfully replace it by our metalarc lamp.

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The Arrangement of DNA in the Nucleus of Rodent Malaria Parasites

G. F. BAHR AND U. MIKEL

Biophysics Branch, Armed Forces Institute of Pathology, Washington, D.C. 20305

ABSTRACT: The DNA of rodent plasmodia is associated with four times its own mass in protein. This nucleoprotein is structurally organized into bumpy, tortuous, sometimes doublet fibers of an average diameter of 150Å, and an average dry mass of 4.66×10^{-16} g/ μ . There are over 15,000 μ of DNA double helix in a merozoite nucleus and about 460 μ of chromatin fibers, which necessitates that on the average 32 lengths of DNA are to be accommodated in one length of fiber.

Replication forks were observed in which the two new strands show comparable patterns of chromatin condensation. From 20 to 25 nuclear pores occur in a trophozoite nucleus serving both as anchor-point for spindle fibers and as anchor-points for the chromatin fibers. There would be a nuclear pore for each 20 μ of fiber.

No chromosomes could be discerned as distinct organizations of chromatin fibrils. Excess genetic information in parasite DNA suggests that a major fraction is not transcribed and may serve structural purposes.

Similarity of structure and composition of host and parasite chromatin suggest, that the parasite nucleus and specifically its chromatin is an unlikely target for antimalarials, but that the largely selective action of antimalarials is aimed at a unique feature of the parasite-host relationship. A candidate for the latter is the "metabolic window" recently described by Bodammer and Bahr (1972).

Because of the inaccessibility and the small size of the nucleus of plasmodium little has become known about its qualitative and less so its quantitative composition. Attempts at isolating pure nuclear fractions have not been convincingly successful, since appreciable quantities of parasite cell membranes and host cell membranes remain associated with the nuclear fraction, Bahr (1966), Cook et al. (1969). Additional difficulties arise from the mixture of complex developmental stages which one encounters in most experimental hosts from which quantities of infected blood can be drawn.

Alternatively one is obliged to deal with individual parasites in situ in the host cell, i.e., the methods of histo- and cytochemistry are employed, preferably in a quantitative manner.

The present report deals with the application of quantitative cytochemistry to intra-erythrocytic parasites.

Materials and Methods

Infections of *Plasmodium berghei*, *Plasmodium chabaudi* and *Plasmodium vinckei* were transferred by blood samples in white mice.

Highly infected blood was used (40-50% parasitaemia). It was obtained by heart punc-

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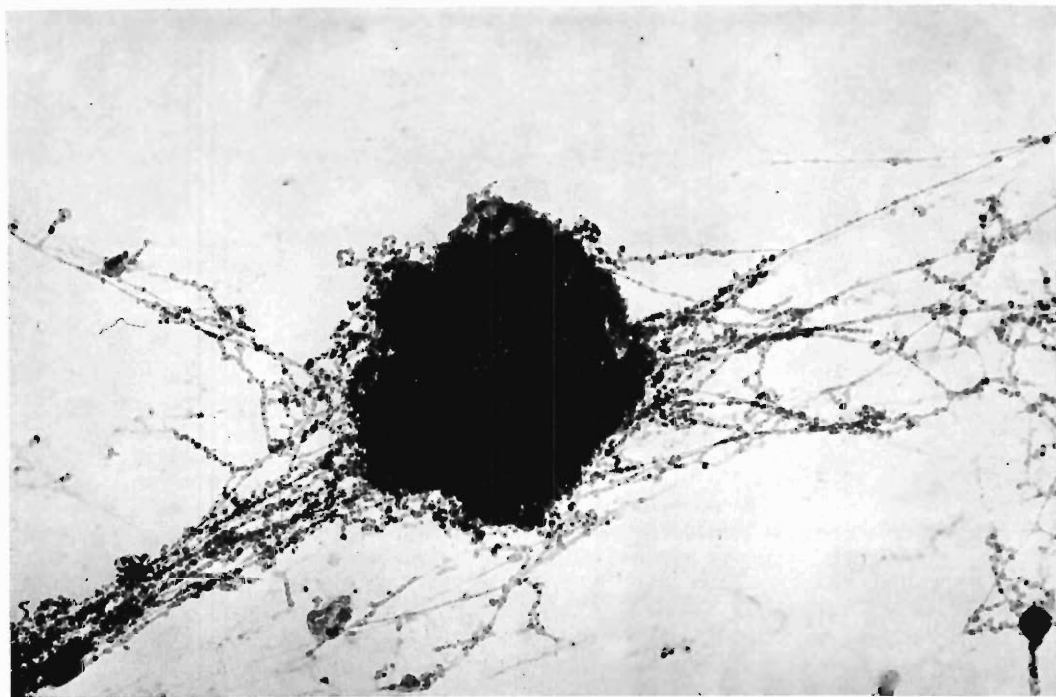


Figure 1. Critical-point dried, unstained nucleus of mature trophozoite from which chromatin fibrils emerge in small loops and in long, knobby strands of varying diameter, often rendering the impression of beads-on-a-string. Fibers further away from the nucleus show greater variability than those close by. This is analogous to what has been observed for human chromosomes (Bahr, 1970). Mag: X 45,000 *P. berghiei*.

ture and was washed in saline, spread on glass slides for staining or released onto the surface of distilled water for subsequent pickup by carbon-coated, or Formvar coated grids for electron microscopy. Some such grids were regularly dehydrated by the critical point drying method of Anderson (1951), others were air-dried.

A preparation of sporozoites was kindly made for us by Dr. Jerome Vanderberg, New York University School of Medicine. The cells were directly transferred as a suspension to Formvar coated grids and air-dried. These grids were subsequently washed in distilled water in order to remove soluble inorganic salts of the suspension medium.

The dry mass of whole parasites and selected parts of it was determined by quantitative electron microscopy. In this method the electron scattering properties of the object are quantitatively compared to the scattering

of polystyrene spheres of known mass. The photographic emulsion in the electron microscope is the recorder of the intensity modulation in the electron beam produced by scattering in the object. The emulsion also serves as an analog converter of electron intensity in such a way that the dry mass of the object becomes proportional to the optical transmission in the recording plate. Details of the technique and treatments of theory can be found in papers by Bahr and Zeitler (1965) and Zeitler and Bahr (1965).

Fluorescence measurements were made with a specially constructed unit which is described in some detail with the results.

Blood smears were for this purpose stained one minute in Acridine orange or Euchrysine 1:10,000 diluted in 0.1M citrate-phosphate buffer at pH 3.8 to which 0.2% Tween 80 and 2% of a 1 molar magnesium sulfate solution had been added (Dart and Turner 1959).

After three one-minute buffer rinses, the slides were mounted in buffer, sealed with wax and measured without delay.

Results

Dry mass

Merozoites and young trophozoites, as free as possible from accompanying host cell membranes were selected for dry mass determinations from unfixed, whole mounted electron microscope preparations of hypotonic lysates. Fifty measurements rendered a mean of $0.72 \times 10^{-12}g$. In some favorable instances also the nucleus could be measured by optically dissecting its transmission (masking) out of the image field. Twenty-one measurements could be made which set the nuclear dry mass at $0.25 \times 10^{-12}g$, S.D. = 0.05. That is close to one-third of the parasite mass. In another experiment the dry mass of 91 sporozoites was determined to $1.08 \times 10^{-12}g$, S.D. = 0.125. A sporozoite thus appears to have more than one-third larger mass than a merozoite. In some preparations a few oblong cells were found which grossly resembled gametocytes. Three measurements on these suggest that gametocytes have a mass close to $1.8 \times 10^{-12}g$. This figure, then, puts merozoite and young trophozoite at the lowest end of the weight scale of malaria parasites.

If infected erythrocytes are hypotonically lysed, spread on distilled water, and critical point dried one observes in favorable instances bumpy fibers of about 150Å diameter emerging from the parasite area. The fibers are, in analogy to human chromatin fibers considered to represent the nucleoprotein of the parasite (Figs. 1 and 2).

At suitable electron microscopic magnifications a determination of fiber diameter and weight per unit length can be made. For this assessment, a densitometric trace is put across the diameter of the fiber. The area under the scan is subsequently compared to a scan over the image of a polystyrene latex sphere of known mass. The comparison renders a fiber mass of $4.66 \times 10^{-16}g/\mu$, S.D. = 1.4 ($n = 32$).

The average nucleus of a trophozoite measures about 1.4μ in diameter and is surrounded by the nuclear double membrane of about 250Å thickness, i.e., the nuclear membrane



Figure 2. Apparent network of chromatin fibrils from a nucleus of *P. berghei*. Notice many thin fibers exhibiting thickenings to varying degrees. This electron micrograph shows a replication fork (arrow) characterized by a larger mass at the replication site and thickenings in the daughter strands in comparable positions. Mag: $\times 72,000$.

contributes roughly 10% of the nuclear dry mass. Nuclear content thus amounts to $0.225 \times 10^{-12}g$.

Taking this dry content of the nucleus and the weight per μ for a chromatin fiber of $4.66 \times 10^{-16}g/\mu$ we find

$$\frac{0.225 \times 10^{-12}g}{4.66 \times 10^{-16}g/\mu} = 482.8\mu$$

of 150Å fiber packed into the volume of the nucleus. From other experiments the insight was gained that less than five (5) percent dry mass is lost in the spreading procedure, a fraction most likely consisting of smaller molecules present in the nuclear sap. Thus we will accept a chromatin fiber length of 460μ for the young trophozoite.

DNA determination

Technical prerequisites: In biochemical procedures for determining the amount of DNA per cell the number of cells in the sample has to be known. No such approach can be taken to malaria parasites since enumeration is difficult if not impossible. Synchronously dividing populations containing exclusively ring stages or other defined stages of the growth cycle cannot easily be obtained in

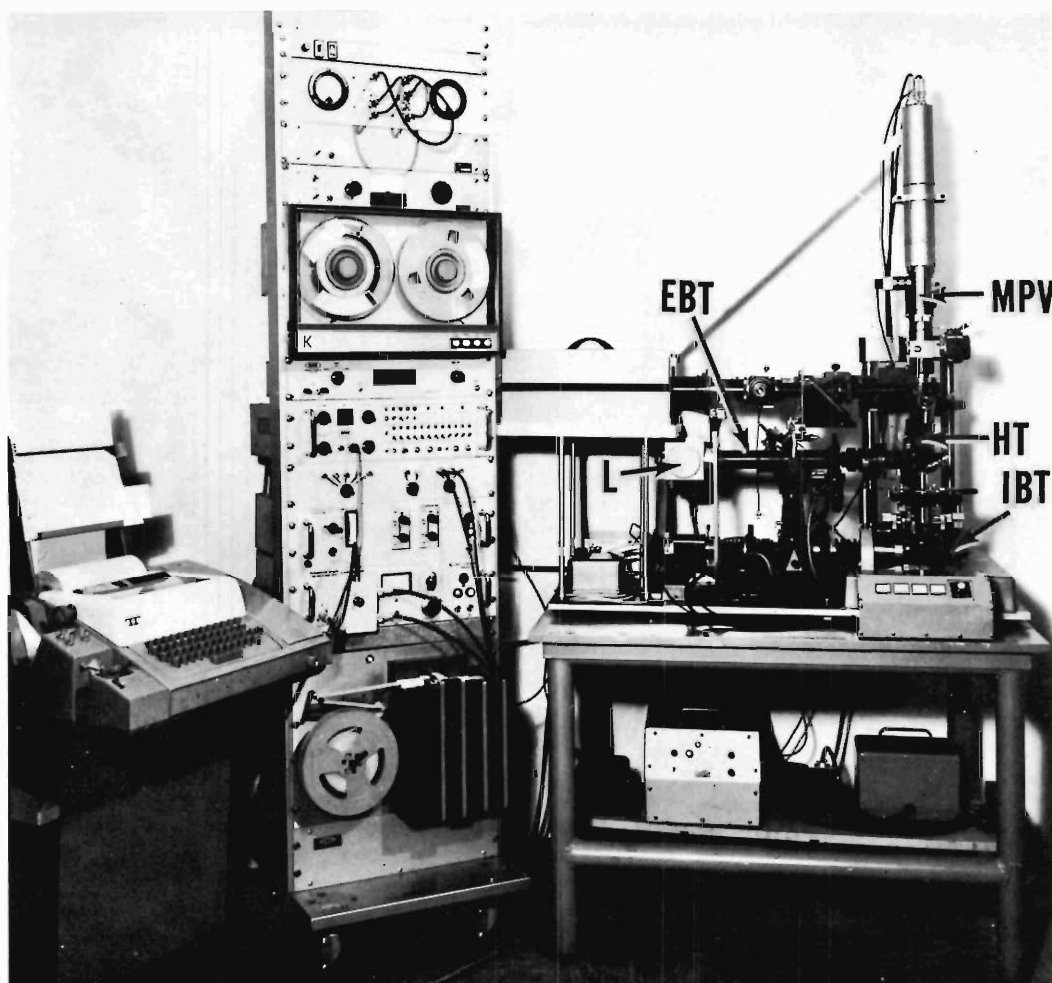


Figure 3. Illustration of the instrument for continuous monitoring of low fluorescence intensity in two wavelength (horizontal light path) and total fluorescence (vertical path). A Xenon 150-watt lamp (L) serves as the source of short wavelength excitation radiation. The light beam passes a 90° prism into the excitation beam tube (EBT). The latter is intersected at 45° by a rotating disk with two large openings passing the beam unchanged, and also with a uranium bar radially mounted so that at its short and distal end a light pipe collects pulses of by-passing intensity conducting it to a projection lens which throws this monitor light onto the photomultiplier. In a fourth position on the disk a mirror is mounted reflecting the full intensity downward into the incandescent beam tube (IBT). In the EBT provisions for high speed electric shutters, for filter holders and a laterally and rotationally adjustable field diaphragm have been made. In the EBT there is further an adjustable lens for focusing the field diaphragm into the object plane. The excitation beam enters the microscope sideways (from the left) and is reflected downward by a dichroic mirror through the objective onto the specimen. Excited fluorescence passes the *dichroic mirror* and ascends in the microscope tube to be measured by the vertically mounted photomultiplier. Between microscope and photomultiplier a Leitz MPV head is mounted which allows the simultaneous observation of object and measuring diaphragm. The photomultiplier is a specially selected IPM-9558 with increased red sensitivity. A variable measuring diaphragm is mounted in the MPV. A prism in the microscope tube allows one to divert the fluorescent light into the horizontal, uppermost vertical measuring tube (MT). This tube contains in addition to a variable and adjustable measuring diaphragm and associated lenses a

quantities required for the strict separation of parasites from white cells. Asynchronously growing infections of e.g., *P. berghei* cannot be defined with respect to the momentary state of synthesis of DNA, which is particularly difficult for young schizonts in which synthesis of DNA proceeds in a unknown manner. We decided therefore on a quantitative cytochemical approach, which in principle allows the selection of a visually identified object to be measured regardless of the composition of the rest of the sample. Cytochemical techniques are well established for cells and tissues, but are not directly applicable to objects as small as malaria parasites without increased sensitivity. Thus it was found that the well understood quantitative stains for DNA, namely, Feulgen and Gallocyanine chromalum, produced insufficient chromophore in the parasite nucleus for making rational absorption measurements.

Only fluorescent microscopy possessed the required sensitivity but suffered from other drawbacks, chiefly fading. Sensitivity of measurement is at least two orders of magnitude better than in absorption measurements (Ruch, 1959). Also, the proportionality of emitted fluorescence to the amount of fluorochrome present obviates the requirement for scanning or the approximations of two-wavelength techniques. Still, the amounts of DNA per parasite are so small that a special instrument for the assessment of low fluorescence intensity had to be built (Fig. 3).

Incident illumination with appropriately filtered ultraviolet light from a Xenon source is admitted to the specimen through an adjust-

able field diaphragm. The diaphragm can be given any desired rectangular shape and orientation. The field of illumination can be strictly limited to the object, whereby irradiation of the object's surrounding with attendant fading and nonspecific light scatter is avoided. Emitted fluorescence is collected by the same objective, which also serves as condenser. Fluorescent light passes a dichroic mirror, an interference band-pass filter, and the adjustable measuring diaphragm which is usually kept slightly smaller than the image of the field diaphragm. Search and adjustment of the object is accomplished in the phase microscopy mode of the instrument, for which purpose an auxiliary light source and substage phase condenser are used. The detector is a specially selected PMI-936A head-on photomultiplier with highly stabilized power supply, cathode follower, and low temperature (dry ice) housing for optimal signal to noise conditions. The instrument is equipped with fast shutters for the control of excitation light and search light. The latter is turned-off before UV is admitted to the specimen. An amplifier transmits the chopped photocurrent to an incremental tape recorder. In this way the first and usually the strongest fluorescence is recorded within 200 microseconds. Subsequent flow of current samplings constitute a fading curve, the extrapolation of which renders fluorescence levels at zero time. Moving the measuring field off the object renders background levels. These are automatically used for correction of fluorescence in a computer subroutine. It is only through this arrangement that we have been

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slit into which the rim of a rotating trapezoid cup is rotating and intersecting the beam. This rim carries two interference filters for selectable wavelengths, one blank and one open space. Cup and rotating disk in MT and EBT are mounted on opposite axes of the same motor, thus rotating synchronously. An open space in the disk corresponds to a filter position in the cup. The mirror on the disk is synchronous with the open hole in the cup. Mirror surfaces on the rim of the disk reflect light into diodes serving as trigger signals for the recording system. Vertically a IPM 9558 is mounted in a cooling box (dry ice) for abatement of electric noise. IBT carries white light from a regular microscope lamp or the downward reflected light from the rotating disk to the microscope's phase condenser to be used for searching and centering the object in phase microscopy. Upon opening of respective shutters (and closing the shutter for incandescent light a fraction of time earlier) fluorescence is produced in the object and the first value recorded digitally (magnetic tape) during the first 200 μ seconds and at selectable intervals thereafter in order to establish a fading curve for the two wavelengths selected by the filters in the rapidly (but variably) rotating cup.

The instrument was conceived and constructed by G. F. Bahr and William Wuertenberg of this laboratory.

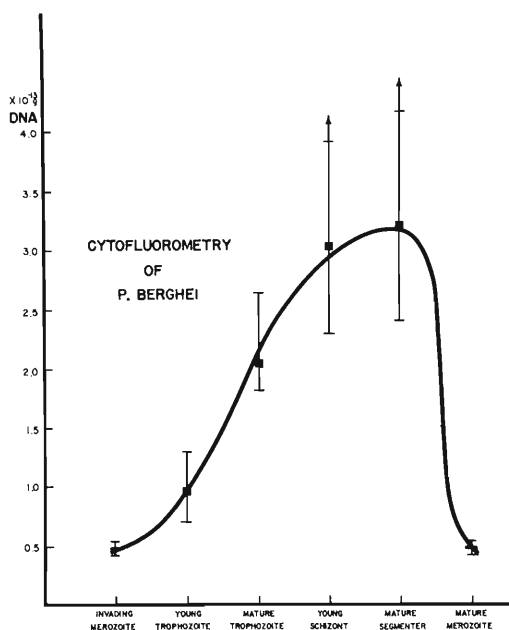


Figure 4. Change of DNA content in units of 10^{-13} g for *P. berghei*. The choice of developmental stages follows the classification of Drs. Nussenzweig and Yoeli. Standard deviations increase rapidly for populations of roughly 150 individual measurements each. At the mature segmenter stage all merozoites, complete and incomplete, plus the same fluorescence from the residual body is measured. Mature merozoites are those seen directly outside disrupted red cells, but separate enough to allow individual measurement. Some fluorescent values for segmenters were considerably larger than 5.0×10^{-13} g DNA. It is doubtful that these reflect true DNA content.

Measurements were made with Acridine orange after RNAase treatment or directly at 590 nm and were compared to the fluorescence of chick erythrocytic nuclei known to contain 2.5×10^{-12} g DNA. The fluorescence of platelets was low, at an equivalent of around 0.1×10^{-13} g DNA. Acid mucopolysaccharides are likely to be responsible for this unspecific dye-binding.

able to procure acceptable measurements from both standards and parasites.

Since fluorescence is strongly dependent on the optical conditions of illumination and light collection a standard has to be measured along with the unknown if absolute values are desired.

Mature merozoites or late stages of schizogony were chosen for parasite DNA determination

under the assumption that these nuclei were not actively synthesize DNA. The chromatin would be Solution I. condensed and inactivated Solution II. by the addition of protein. Thymus lymphocytes (from the same animal), and duck or chicken erythrocyte nuclei were chosen as standards because of states of low genetic expression (Gledhill, 1970) assumed to be comparable to that of a merozoite.

Measurements of individual nuclei in schizonts render a value of 0.49×10^{-13} g DNA per nucleus, S.D. = 0.072 for 112 measurements. Since 1μ of DNA double helix in B-configuration has a mass of 3.24×10^{-18} g, DuPraw and Bahr (1969), Bahr (1970), or

$$\frac{0.49 \times 10^{-13} \text{ g DNA}}{3.24 \times 10^{-18} \text{ g DNA}/\mu} = 15,123\mu$$

DNA double helix per parasite.

Serial measurements of DNA content in *P. chabaudi* infected mouse erythrocytes indicate a brief period in which the DNA remains constant, namely through the transition from invading merozoite to young trophozoite (Fig. 4). DNA rises continuously (S-phase) and falls off at late segmenter stages until the individual mature merozoite (late segmenter) nucleus has the same amount of DNA as the invading merozoite.

In fluorometric measurements only the sum total of fluorescence from the area of single erythrocyte is seen. This amount was divided by the number of visible nuclei. At mid-schizogony levels of DNA are higher, because some of the DNA for a new nucleus has already been synthesized without visible formation of a nucleus.

Computation of DNA Packing Ratio

With only about 460μ of chromatin fiber available in the parasite nucleus (vide supra) into which $15,000\mu$ of DNA has to be packed, a ratio of at least 1:32 results, or 32 lengths of DNA double helix have to be accommodated in one length of fiber.

Morphology

In no instance has anything comparable to a metaphase chromosome been seen. Some chromatin clumps consist of more tightly

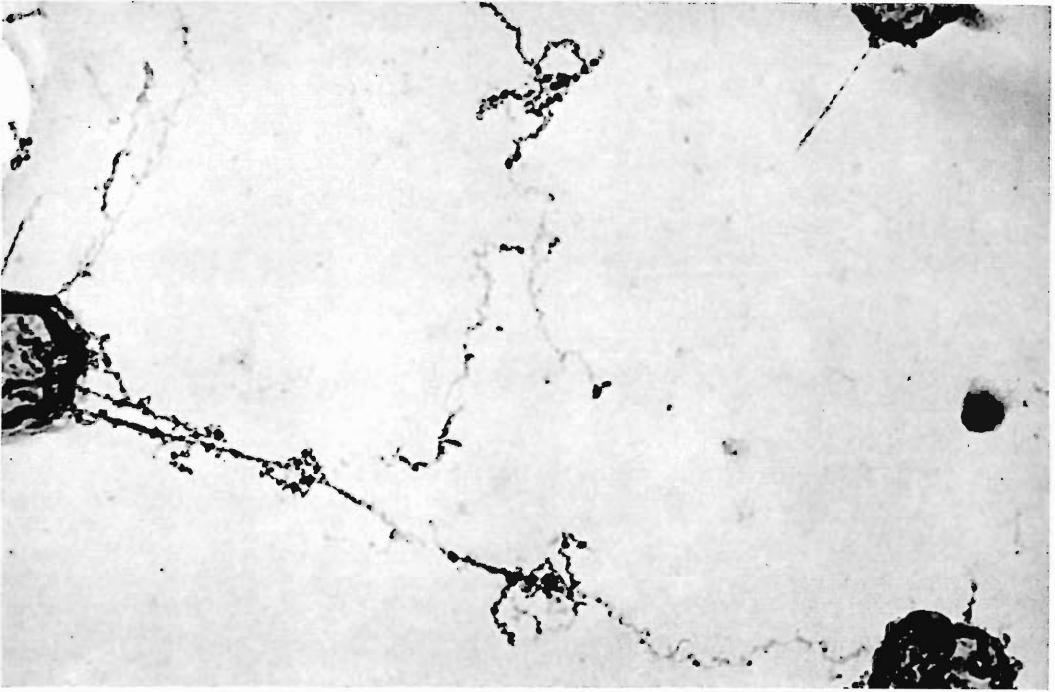


Figure 5.

Figures 5, 6. There are sections along chromatin fibers at which the normal fiber conformation appears to be loosened and a twisting of two fibers around each other and a parting for short stretches is very suggestive (arrows). This feature was only discovered while writing this article and higher power electron micrographs at better resolution will be substituted for those submitted with the working paper. Mags: Fig. 5, X 60,000; Fig. 6, X 72,000.

folded fibers, but their significance is unknown at this time.

Parasitic chromatin fibrils are much more kinky and irregular than human chromatin fibers. Not only is there more variation in cross-section over short stretches, but parasite fibers have typically lateral protrusions of the type described by Ris (1969), but more varied still, than that these protrusions could give rise to the X-ray diffraction values observed by Bram and Ris (1971).

In preparations of normal and infected mouse blood that were treated for 10 minutes with a 5 percent glycerin solution little change is seen in chromatin fibrils from leukocytes, but parasitic fibrils exhibit their characteristics

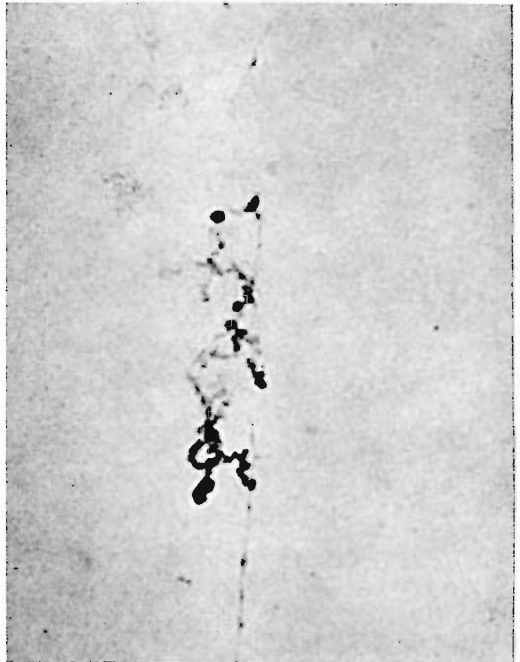


Figure 6.

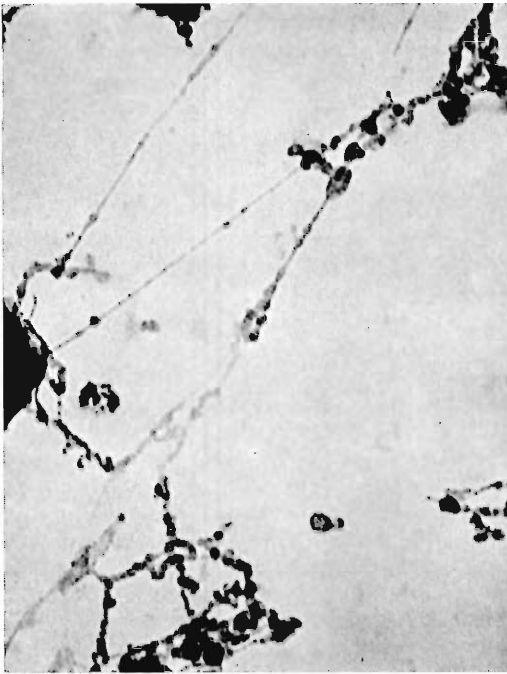


Figure 7.

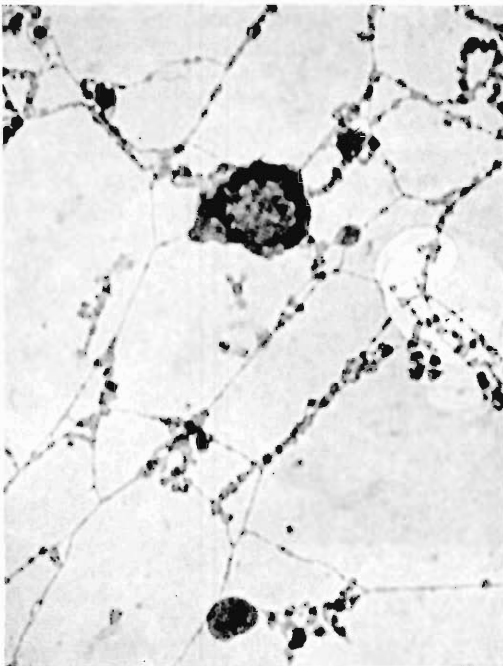


Figure 8.

much more clearly. There is ample evidence that many fibers are doublets as observed by Ris (1969) after sodium citrate treatment, (Figs. 5 and 6).

In addition many instances can be clearly identified which must be replication forks of parasitic DNA. In difference to replication forks of naked DNA, the DNA of plasmodium (and implicitly of other eukaryotic cells) appears to be immediately coiled into higher orders of supercoiling which is evident from the thickness of the fiber itself, 40Å as well as bead-like accumulations of material in corresponding positions of the replicated strands. At the site of the fork proper a small mass of chromatin is usually found, probably providing the enzymatic machinery for uncoiling, replication and recoiling (Figs. 7 and 8).

In addition a type of banding pattern within the individual parasite chromatin fibril was observed in some preparations especially after glycerin treatment. This banding pattern obviously corresponds to the accumulations of chromatin seen on parallel strands after DNA replication. Eventually the tedious task of charting these bands throughout the parasite genome will have to be undertaken.

There is other structural detail defying explanation. Ringlike structure along the fibril can be seen together with prominent thickenings of the fiber. Patches of homogeneous material occur into which fibers (usually parallel ones) enter and emerge again (Figs. 9, 10).

The parasite appears to have only a limited number of nuclear pores. Some can be identi-

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Figures 7, 8. Chromatin fibers are emerging or released from nuclei of *P. berghei* at stages of active DNA synthesis. In these two illustrations, as in Fig. 2, structures are presented which can only be interpreted as synthesis forks from which the newly synthesized strands are emerging. The criterion for considering these forks as sites of DNA synthesis is the rather obvious similarity of the newly synthesized strands, which in conformance to the laws of strand replication have like patterns of supercoiling and/or protein association. Note that these images exclude the appearance of "naked" DNA strands in the synthesis phase. The synthesis site itself is marked by a more or less prominent accumulation of matter (arrows). Mags: Fig. 7, X 72,000; Fig. 8, X 72,000.

fied, but none as easily as annuli in a human nuclear membrane. Estimates from freeze-etching work (Dr. Charles Meszoely) and from thin sections suggest that a parasite nucleus possesses between 20–25 nuclear pores, i.e., about a pore for 20μ of chromatin fibril. Scalzi and Bahr (1968) have shown that nuclear pores are focal points for the insertion of spindle fibers in schizogony. They are playing a significant role also in the organization of chromatin (DuPraw and Bahr, 1969).

Corroborating Data

We have employed the fluorochrome Brilliant Sulfaflavin at pH 2.8 for the determination of total protein according to Ruch and Leeman (personal communication) and dansyl chloride for lysine-rich structures, according to Ringertz (1968). The general nature of binding of this fluorochrome made it difficult to isolate nuclear fluorescence from that of surrounding cytoplasm. In disrupted cells containing mature merozoites some measurements were possible. Total protein was estimated in comparative measurements using whole human erythrocytes, to $0.30 \times 10^{-12}g$, S.D. = 0.8. Obviously this is a high value and must be due to non-pertinent fluorescence. The general magnitude, however, is in good agreement with electron microscopic values of dry mass.

Discussion

It is obvious from the results presented numerically, but also from studying the accompanying electron micrographs, that the malaria parasite is governed by a rather complex genetic machinery, typical for eukaryots. Only a little less than 20% of dry matter of the parasite nucleus is DNA, the remaining 80% are proteins of the basic histone and non-histone types, all of which is organized into fibers of about 150Å diameter. Thirty-two lengths of DNA are packed into one length of fiber. Details of the packing arrangement are not known, but orders of increasing supercoiling of DNA are most likely. Histones are unspecific suppressors of gene activity and appear to serve chiefly structural functions (Pardon et al. 1967, DuPraw and Bahr 1969, Bahr 1970).

We have some illustrations suggesting un-

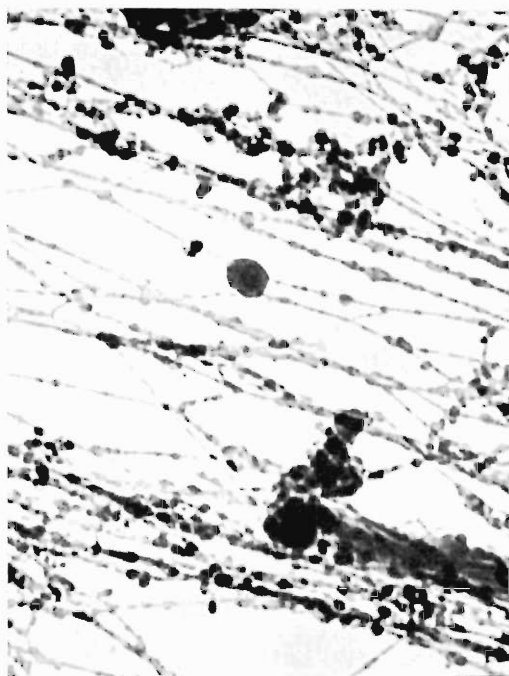


Figure 9.

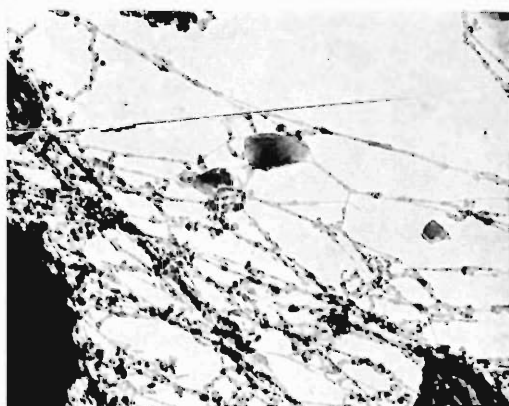


Figure 10.

Figures 9 and 10. Often patches of a homogeneous material (arrows) is seen among chromatin fibers. The latter enter and transect the material. An explanation for these patches cannot be given at this time. Mags: Fig. 9, $\times 72,000$; Fig. 10, $\times 53,000$.

coiling, replication, and recoiling of fibers, but no knowledge of chromosome movements or the sequence and rates of replication of the genome. The insertion of the spindle (cen-

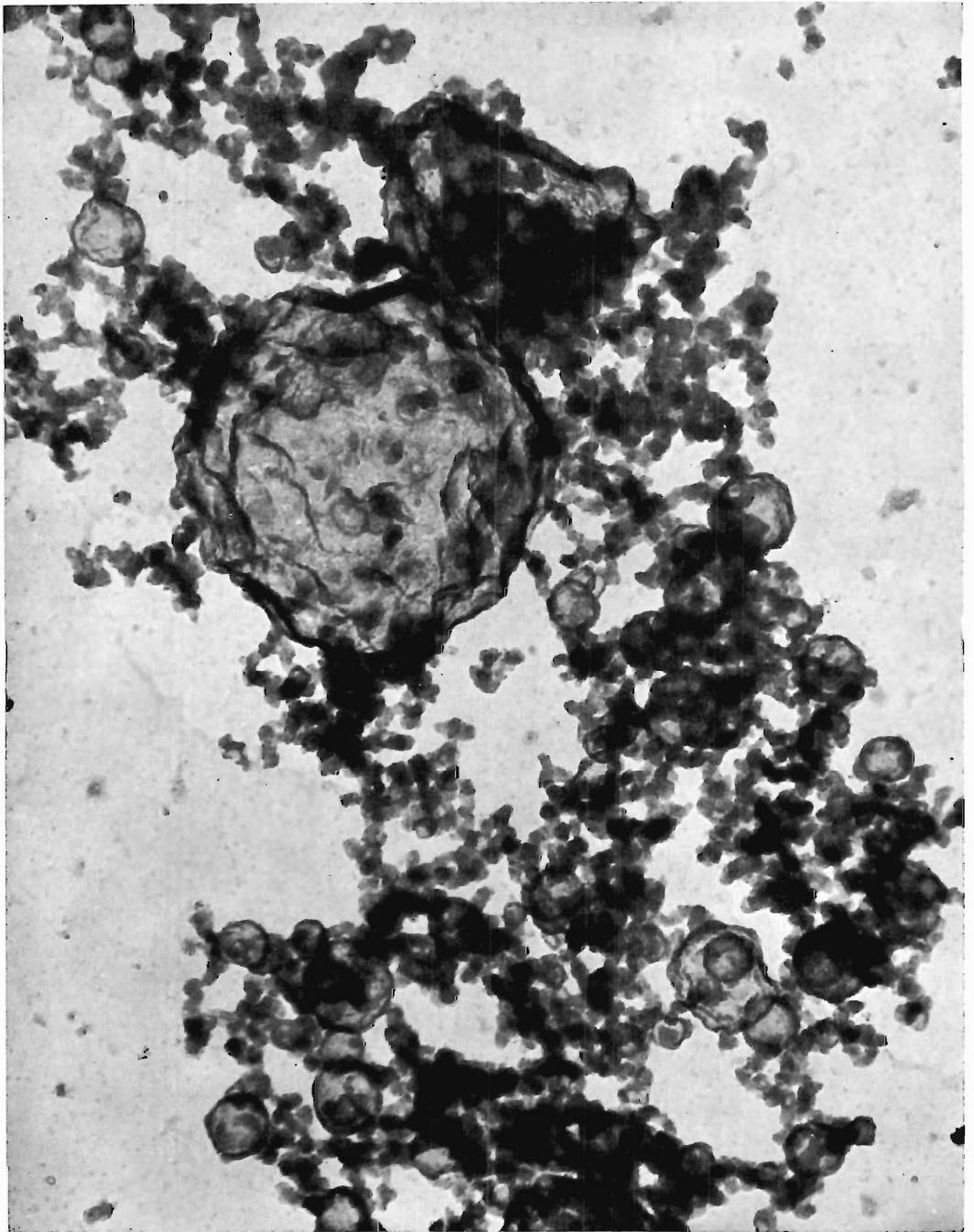


Figure 11. A critical-point dried preparation of mouse erythrocytes in which 40 percent reticulocytosis has been produced by phenylhydrazine. Exclusively thick 720Å fibers from the remainders of pyknotic nuclei are seen. Such fibers are short and very tortuous. No confusion with parasite material is likely, which consists of 150Å fibers. Mag: X 27,000.

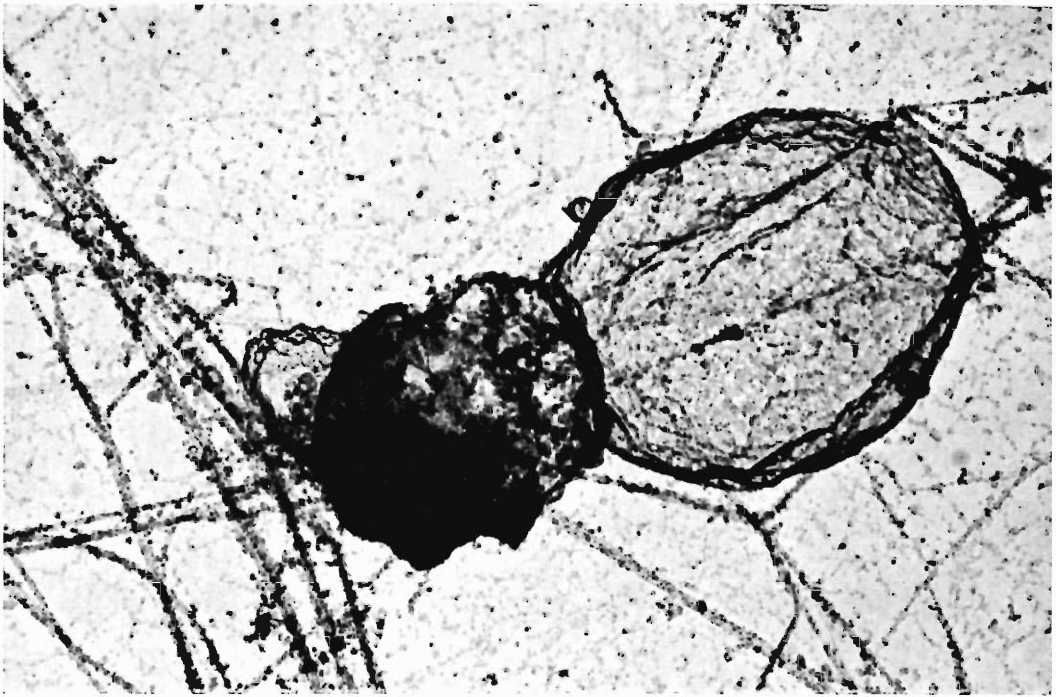


Figure 12. The red cell membrane has flipped over the parasite and bared some features of its body including fibrous structures (chromatin fibers) inside, in this preparation of non-heparinized blood. The slender, rather straight fibers are fibrin covered with some precipitated plasma proteins. These fibers are presented here for reasons of comparison with chromatin fibrils. Mag: X 27,000.

triole) in nuclear pores (Scalzi and Bahr 1968, Aikawa and colleagues, this conference) points to the major role of the nuclear membrane and its about 30 pores in regulating the orderly course of genome replication. The fact that even and odd numbers of merozoites are produced at schizogony and the off center location of the mitotic spindle, indicates a nonsynchronous progress of replication in part of the nucleus. Bianchi et al. (1969) observed intranuclear spindle fibers with fully retained nuclear membrane also in the leptomonad of *Leishmania tropica*. Of course, endomitosis or mesomitosis in protozoa has long been described by classical protozoology using the light microscope. Cells of higher forms retain during metaphase only pieces of nuclear membrane attached to chromatin or chromosome, at a time when genetic activity (RNA synthesis) is at a minimum or nil. In the parasite both DNA

and RNA synthesis is a continuous process from the invasion of the erythrocyte to late schizogony with the nuclear membrane providing compartmentalization, comparable to the interphase nucleus.

Except for maintaining its membrane throughout the phases of erythrocytic growth and schizogony, the essential constituents of the parasite nucleus are the same as for host cell nuclei. It appears therefore unlikely that antimalarials act at the level of the nucleus. In fact the contributions to this workshop strongly suggest that the action is on transport, possibly recognition of selected metabolites. A candidate feature for this process is the "metabolic window", described by Bodammer and Bahr (1970). At this site the parasite is closely applied to the erythrocyte membrane which is markedly modified. The parasite nucleus lays in direct apposition to the modified area. Membranous material from

the parasite cytoplasm is discharged at this site.

Over 15,000 μ of DNA double helix are constituting the genome of *P. berghei*. The coding capacity of this amount of DNA is for about 75,000 different peptides of an average molecular weight of 20,000, or 1,500 different proteins of a million molecular weight each. This is a quantity far in excess of what the parasite would require. In analogy to other eukaryotic DNA, it appears that a large portion of the genome is non-coding and serves structural functions.

While measuring the mass and diameter of chromatin fibers from infected red cells, the question arose if such fibers were not simply derived from the increasing number of reticulocytes. In an experiment, phenylhydrazine was used in normal mice to induce reticulocytosis. When the resulting material was prepared for electron microscopy only rather thick and short pieces of fibers were observed, the average diameter of which was 710Å, clearly distinguishing them from parasitic chromatin fibrils.

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In Vitro and *In Vivo* Studies on a Lytic Factor Isolated from *Plasmodium knowlesi*

E. H. FIFE, JR., A. E. VON DOENHOFF, JR., AND L. E. D'ANTONIO¹

Department of Serology, Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: A lytic factor (LF) capable of lysing normal erythrocytes has been isolated from *Plasmodium knowlesi* parasites that had been effectively separated from host erythrocyte components. Results of preliminary studies on the functional activity and chemical nature of the LF are reported. Physicochemical analyses revealed that the principal component of the LF was lipid in character and contained relatively large amounts of cholesterol, cholesterol esters, free fatty acids and phospholipids; its molecular weight was less than 500. Studies on the kinetics of LF-induced hemolysis revealed that the time and temperature of incubation and the concentration of LF significantly influenced the velocity and magnitude of the reaction. Exposure of erythrocytes to LF markedly increased their osmotic fragility both *in vitro* and *in vivo*. Additionally, erythrocytes from different animal species varied considerably in their susceptibility to LF-induced lysis and osmotic fragility changes, and the older cells of a given population were more susceptible to lysis than were the younger cells. Finally, i.v. injection of LF into hamsters produced a severe hyperthermia, and the magnitude of the temperature increased with each successive administration of the LF. However, the temperatures returned to normal within 24 hours after each injection. In view of the capacity of LF to induce certain clinical and hematological changes (e.g. fever, increased erythrocyte osmotic fragility and hemolysis) characteristically observed in malaria, it is suggested that this plasmodial component may play a role in the pathogenesis of anemia and fever of acute malaria infection.

During the course of studies on the isolation of specific complement fixing antigens from *Plasmodium knowlesi*, it was observed that certain effluent fractions from the Sephadex G-200 gel column possessed the capacity to lyse erythrocytes. The present report summarizes the results of preliminary investigations on the functional activity and chemical nature of this lytic factor.

Materials and Methods

Preparation of lytic factor

Collection of parasites: The procedures developed by D'Antonio et al. (1966) were used to free the malaria parasites from the host erythrocytes. In brief, adult Rhesus monkeys were infected by intravenous injection with *P. knowlesi*-infected blood. When parasitemias reached 25–30% or greater, and exhibited a preponderance of mature schizonts, the animals were tranquilized with phencyclidine HCl (0.1 mg/kg IM) and exsanguinated by cardiac puncture. The blood was collected in heparin, immediately centrifuged in

the cold (3°C) at 3500 rcf for 10 min to sediment the erythrocytes, and the packed red cells washed four times in 0.15 M NaCl solution to assure removal of the plasma. A 20% suspension of the washed erythrocytes then was prepared in the saline solution, placed in a cooled (3°C) French pressure cell, and slowly (dropwise) forced through the needle valve at a pressure of 1500 ± 50 psi. The first 2–3 ml were discarded to avoid contamination with the few unruptured erythrocytes that passed through the valve with the initial effluent. The effluent obtained by this procedure first was centrifuged in the cold (3°C) for 10 min at 50 rcf to remove gross debris, and the supernate containing the intact malaria parasites then was centrifuged at 3500 rcf for 5 min. The parasite sediment was resuspended in saline and washed 3 times to remove the hemoglobin and erythrocyte fragments. Examination of Giemsa-stained smears revealed that the parasite harvest was essentially free from identifiable erythrocyte contaminants; no intact red cells were noted and only an occasional red cell ghost was observed.

Isolation of lytic factor: The washed parasites were suspended in 7 volumes of saline, placed in the cooled French pressure cell, and

¹ Present address: 1000 Clifton Ave., Collingdale, PA 19023.

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fragmented by slowly passing the suspension through the needle valve at $20,000 \pm 50$ psi. The effluent then was centrifuged in the cold (3°C) at 10,000 rcf for 30 min to sediment the pigment granules and extraneous debris. The supernate containing the solubilized parasite components was fractionated by passage through a Sephadex G-200 gel column (20 cm height, 2.5 cm dia) using 0.15 M NaCl solution. An 11 ml portion of the solubilized parasite material was passed through the column and the effluent was collected in 36 four-ml aliquants. The lytic factor resided in collection tubes 24–32.

Isolation of lytic factor from acute-phase plasma

Blood from a heavily parasitized monkey (60–80% parasitemia) was collected in heparin and immediately centrifuged in the cold (3°C) for 10 min at 3500 rcf to sediment the erythrocytes. A 2-ml volume of the plasma then was fractionated by filtration through a Sephadex G-200 gel column (20 cm height, 2.5 cm dia) using 0.15 M NaCl solution. The effluent fractions were collected in 70 two-ml aliquants. Hemolytic assays of the various fractions revealed that effluents in collection tubes 25–33 possessed lytic activity. Another portion of the acute-phase plasma was treated with CaCl_2 (10 mg%), the precipitated fibrin sedimented by centrifugation, and the supernate filtered through the Sephadex G-200 gel column. The effluent fractions were assayed for hemolytic activity.

Hemolytic assay of lytic factor

Although sheep erythrocytes initially were used as the indicator system for determining the hemolytic capacity of a given lytic factor (LF) preparation, ancillary studies revealed that hamster erythrocytes were considerably more susceptible to LF-induced lysis than were sheep red cells. In continued investigations, it was observed that hamster erythrocytes provided a more sensitive, reproducible measure of LF activity than did sheep cells. The methodology accordingly was modified, and the procedure that ultimately evolved for the quantitative assays of LF activity in the present studies was as follows: First, a 40% suspension of washed hamster erythrocytes

was prepared in 0.15 M NaCl solution. The cells suspension then was standardized spectrophotometrically by adjusting the concentration of the suspension so that the lysate of a 1:200 dilution in distilled water gave an optical density of 0.500 ± 0.010 at wavelength $540 \text{ m}\mu$ in a Beckman DU Spectrophotometer using a standard 1-cm cuvette. Direct hemocytometer counts revealed that suspensions standardized in this manner contained *ca.* 1.8×10^9 erythrocytes per 0.4 ml. The standardized cells suspension was used to construct a hemoglobin curve to facilitate conversion of optical densities to the number of cells lysed in the assay procedure. LF assays were performed by combining 0.4 ml of the standardized cells suspension with an equal volume of LF and incubating in a 37°C waterbath for 24 hrs in stoppered tubes with periodic agitation. After incubation, the tubes were centrifuged at 1500 rcf for 10 min and the supernatant hemoglobin solutions were transferred to another set of tubes. Each supernate then was diluted 1:100 in the 0.15 M NaCl solution and the optical density determined spectrophotometrically at wavelength $540 \text{ m}\mu$ after adjusting the spectrophotometer to zero with a blank in which saline was substituted for LF. The optical densities of the various tubes finally were compared with the standard hemoglobin curve and the number of cells lysed was estimated. In studies on the kinetics of LF activity, the time and/or temperature of incubation and the concentration of LF were varied to fit the requirements of a given experiment.

Erythrocyte osmotic fragility

The effects of LF on erythrocyte osmotic fragility were studied both *in vitro* and *in vivo*. Fragility measurements were made by the method of Allen and Shields (1966) which provided for continuous recording of the osmotic fragility. Osmograms were prepared and the points representing 50% hemolysis were used to compare the erythrocyte fragilities of the test and control animals. For the *in vitro* studies, erythrocytes from hamsters, chimpanzees, monkeys, humans, sheep, or chickens were incubated with various concentrations of LF for 3 hours at 37°C and the osmotic fragilities then immediately determined.

Hamsters weighing 105–124 g were employed in *in vivo* studies of the effects of LF on erythrocyte osmotic fragility. For these studies, 36 hamsters were divided in 3 groups. Group I contained 28 animals, and Groups II and III contained 4 animals each. All hamsters in Group I received an intravenous injection of 2.0 ml of LF. The animals of Group II each received three 2.0-ml injections of LF administered i.v. at 24 hour intervals. Group III served as the control with each animal receiving a sham injection of 2.0 ml of isotonic saline administered i.v. Four animals of Group I were exsanguinated at each of the following periods: $\frac{1}{2}$, 1, 2, 3, 4, 5, and 6 hours after injection with the LF. The hamsters of Group II who received the multiple injections of LF were exsanguinated 4 hours after the last injection. The controls (Group III) were sacrificed 4 hrs after receiving the saline. Immediately after collection in heparin, the erythrocytes from each animal were washed in saline to remove the plasma and the osmotic fragilities determined.

Febrile response to LF

To investigate the pyrogenic properties of the LF, 4 hamsters each were given three 2.0-ml injections of LF administered i.v. at 24 hr intervals. Four hamsters receiving sham injections of saline served as controls. Rectal temperatures were taken with a thermocouple probe immediately preceding each injection, and then $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, and 4 hours later.

Radioisotope labeling of young and old erythrocytes

Ten hamsters first were inoculated intraperitoneally with ^{55}Fe . The animals then were reinoculated 28 days later with ^{59}Fe . Three days after the last injection, the animals were exsanguinated and the erythrocytes used in the standard LF assay procedure. (Under these conditions, the older cells will bear the ^{55}Fe label and the newly formed cells will be tagged with the ^{59}Fe .) After incubation for 24 hours, the tests were centrifuged and the supernates examined in a scintillating counter to determine the relative levels of ^{55}Fe and ^{59}Fe . Finally, the cells that remained intact at the end of the incubation period were lysed

in distilled water and the ^{55}Fe and ^{59}Fe levels of the lysates determined.

Physical and chemical analysis of the LF

Quantitative tests: Quantitative protein determinations were made according to the procedure of Lowry et al. (1951). The method of Shetlar and Masters (1957) was used to estimate the carbohydrate content of the LF.

Thin layer chromatography (TLC): Preparation of thin-layer plates and the identification of spots were carried out by the methods described by Mangold (1965) and Randerath (1966). The LF first was extracted three times with chloroform-methanol (2:1, v/v) and the trace amounts of water present in the lipid-containing fraction were removed by treatment with anhydrous CaCl_2 . The solvents then were removed from the extract by treatment with anhydrous N_2 . The residue consisted of a light brown oil-like substance and a fine white crystalline powder. The residue was redissolved in chloroform just prior to application to the Silica Gel G TLC plate. Although several solvent systems initially were employed for chromatographic separation of the LF, hexane-ether-acetic acid (90:10:1, v/v/v) provided the best separation. The lipid components were made visible by exposing the plate to iodine fumes, then spraying with sulfuric acid and charring by heating to 110°C for 2 hours.

Stability of LF: To evaluate the stability of the LF, portions of the factor were incubated for 1 hour at 37° , 45° , 50° , and 56°C respectively, and the hemolytic activity of each was determined. An additional sample of LF was rapidly heated to 100°C and portions removed at 0, 5, 10, and 15 min intervals, immediately cooled, and assayed for hemolytic activity. Finally, aliquants of LF were stored at 3°C or -70°C and assayed at monthly intervals to determine its stability under these conditions of storage.

Results

In vitro studies

In studies on the kinetics of the hemolytic activity of the plasmodial LF on hamster erythrocytes, the effects of time and temperature of incubation first were investigated. The

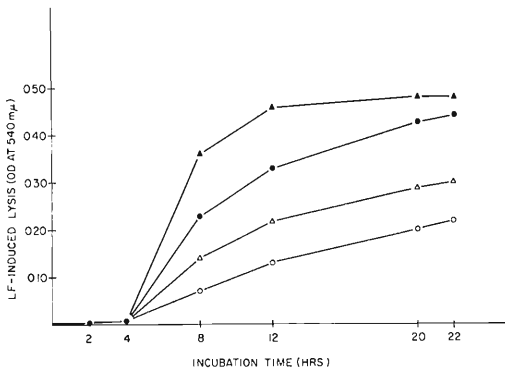


Figure 1. Effect of time and temperature on the hemolytic activity of the LF. ○ = 5° C; △ = 15° C; ● = 25° C; ▲ = 37° C. All tests conducted with undiluted LF and read on spectrophotometer zeroed with corresponding blanks.

results of these studies are summarized in Fig. 1. It was observed that regardless of the temperature, little or no hemolysis occurred during the first 4 hrs of incubation. Hemolysis then began and progressed rapidly through hour 12, at which time the velocity of the reaction diminished to hour 20. Temperature obviously influenced the velocity of the reaction. In tests incubated at 5°C, less than 50% of the red cells were lysed during incubation for 20 hours. However, as the temperature was raised, the amount of hemolysis at hour 20 progressively increased, and greater than 90% hemolysis was observed in the tests incubated at 37°C.

Further analysis of the tests incubated at 37°C revealed that the reaction spontaneously ceased after incubation for 20 hrs, even though intact red cells remained in the test mixture. Initially, it was believed that this was due to depletion of the LF. However, this proved not to be the case; addition of fresh LF failed to induce further hemolysis of these cells. In subsequent studies in which the older cells were labeled with ^{55}Fe and the younger cells were tagged with ^{59}Fe , it was observed that the older cells were more susceptible to the lytic action of the LF. After incubation for 20 hrs at 37°C, the relative level of ^{55}Fe in the hemolysate was significantly greater than that of the ^{59}Fe . Moreover, the majority of cells that remained intact at the end of the incubation period bore the ^{59}Fe label.

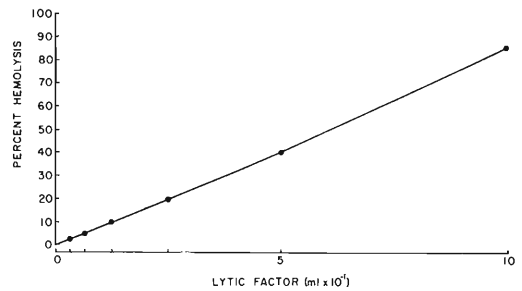


Figure 2. Influence of lytic factor concentration on LF-induced hemolysis of hamster erythrocytes. Graph indicates per cent hemolysis after incubation at 37° C for 20 hours.

The concentration of LF also influenced the magnitude of LF-induced hemolysis. This was illustrated by the results presented in Fig. 2. The per cent hemolysis obtained after incubation at 37°C for 20 hours increased in a linear fashion with progressive increases of LF concentration.

Variations of pH in the range pH 6–8 had no significant effect on the hemolytic activity of the LF. Attempts to evaluate the effects of higher or lower hydrogen ion concentrations were unsuccessful because of the deleterious effects on the erythrocytes of the assay system.

In *in vitro* studies conducted during the pre-lytic phase (*i.e.* after incubation at 37°C for 3 hrs), it was observed that relatively small amounts of LF significantly increased the osmotic fragility of hamster erythrocytes (see Fig. 3). However, it was noted that the LF-induced osmotic fragility varied considerably among different animal species. The hamster and chimpanzee erythrocytes showed the greatest increase of osmotic fragility when incubated with LF. On the other hand, monkey, human, sheep, and chicken red cells progressively in this order were considerably more refractory. In fact, chicken erythrocytes showed essentially no change of osmotic fragility following incubation for 3 hrs at 37°C with the highest obtainable concentration of LF. In ancillary studies of LF-induced hemolysis, a similar variation among animal species was noted; the hamster cells were most susceptible and the chicken cells most resistant to lysis.

Spectrophotometric analysis of the various effluent fractions obtained from the Sephadex

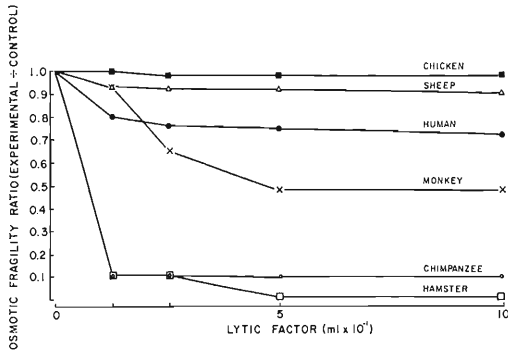


Figure 3. Effect of lytic factor concentration on osmotic fragility of erythrocytes from various donor species. Osmotic fragility determinations made after incubation at 37° C for 3 hours (*i.e.*, during the pre-lytic period).

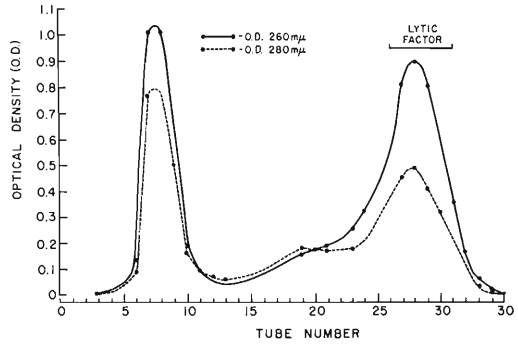


Figure 4. Sephadex G-200 gel filtration pattern of solubilized *P. knowlesi* constituents. The lytic factor is associated with the second major absorption peak.

G-200 gel column revealed two major absorption peaks, the first showing maximum absorbance with effluent tubes 7 & 8, and the second showing maximum absorbance with tube 28 (Fig. 4). Assays of the various effluent fractions revealed that tubes 26–31 possessed hemolytic activity. These were combined to provide the LF used in the present studies.

Physical and chemical analyses were performed on the LF and the findings are summarized as follows: Based on the filtration rates of markers through the Sephadex G-200 gel column, the LF appeared to have relatively low molecular weight. Further evidence of the low molecular weight of the LF was obtained during efforts to concentrate the factor by filtration through an ultrafilter. This filter was equipped with a membrane having a porosity designed to retain all components with molecular weights of 500 or greater. No concentration of LF was effected by this filtration; the hemolytic activities of the filtrate and the unfiltered material were identical, indicating that the LF passed through the membrane and that its molecular weight was less than 500. Although relatively large amounts of calcium (200 mg/100 ml) inhibited the hemolytic activity of the LF, addition of calcium equivalent to that of the normal blood level (10 mg/100 ml) showed no inhibitory properties.

The absorption peak obtained by spectro-

photometric analysis of the LF at wavelength 280 mμ (Fig. 4) suggested the presence of protein or other amino acid components. Quantitative Lowry determinations revealed that these components, if protein, were present at a concentration of 35 μg/ml. However, in view of the low molecular weight of the LF, it is believed that these components consisted of amino acids and/or small peptides rather than proteins *per se*. A small amount of carbohydrate (12.5 μg/ml) also was present in the LF.

The principal component of the LF-containing material appeared to be lipid in character, with a high cholesterol content. Using the appropriate standards alone and in combination, thin-layer chromatography revealed the presence of relatively large amounts of cholesterol and cholesterol esters, free fatty acids, and phospholipids. Additionally, the LP contained trace amounts of tryglycerides and straight-chain hydrocarbons. It was observed that some material did not migrate in the solvent system and remained at the point of origin. This material gave a positive ninhydrin reaction and was presumed to consist of amino acid residues. Several solvent systems were employed in attempts to effectively separate the free fatty acids. Although the results were somewhat inconclusive, the major fatty acid component(s) exhibited a R_f comparable to that of oleic acid. None of the employed solvent systems, however, effectively separated oleic and *cis*-vaccinic acids, both of

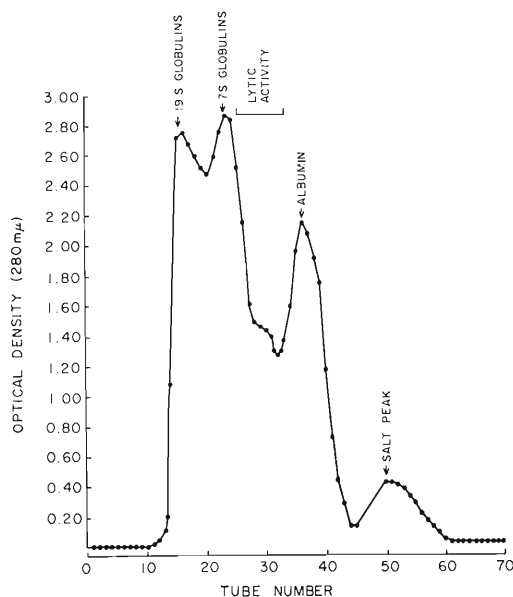


Figure 5. Sephadex G-200 filtration pattern of acute-phase plasma from a *P. knowlesi*-infected Rhesus monkey. Lytic activity observed in effluent fractions between the 7S globulins and albumin peaks, but not in the "salt" peak.

which are mono-unsaturated, mono-carboxylic fatty acids with the chain length C_{18} .

The LF proved to be quite stable when exposed to various conditions of temperature and storage. Incubation for 1 hr at temperatures to 56°C caused no reduction of the hemolytic activity of the product. Furthermore, the LF could be rapidly heated to 100°C and immediately cooled without loss of activity. However, hemolytic activity was destroyed when the temperature was maintained at 100°C for 5 min or longer. Finally, the LF could be stored at 3°C for at least 3 mos or at -70°C for more than 1 yr without evidence of deterioration.

Although unfractionated plasma from a heavily parasitized monkey exhibited no hemolytic properties, Sephadex G-200 gel filtration of the plasma revealed the presence of a relatively high molecular weight component possessing hemolytic properties. This component was present only in effluent tubes collected between the 7S globulins and the albumin fractions (see Fig. 5); the greatest

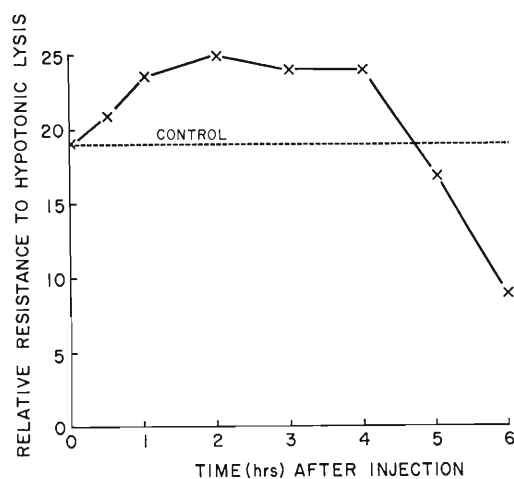


Figure 6. *In vivo* effects of lytic factor on hamster erythrocyte osmotic fragility. Each animal received a 2-ml i.v. injection of undiluted LF. The points are based on the average osmotic fragility of four hamsters sacrificed at the indicated times.

hemolytic activity was obtained with effluent tubes 26 & 27. No hemolytic activity was detected in the effluents from the "salt peak". Thus, the hemolytic component from the plasma appeared to have a molecular weight of less than 2,000,000 but greater than 150,000. It is noteworthy, however, that no fraction with hemolytic activity could be obtained from the same plasma if Ca^{++} had been added and the fibrin precipitated prior to filtration through the Sephadex column. Finally, repeated efforts to isolate a hemolytic component from the serum of a heavily parasitized monkey uniformly were unsuccessful.

In vivo studies

The *in vivo* effects of LF on erythrocyte osmotic fragility were investigated in hamsters receiving an i.v. injection of the factor. The results are summarized in Fig. 6. Examination of the osmograms revealed that the individual 50% hemolysis points for any given group of animals did not vary more than ± 1 cm. Curiously, the erythrocytes became considerably more resistant to hypotonic lysis during the first 2 hrs after injection of the LF. This increased resistance persisted at a relatively high level until 4 hrs after injection.

Table 1. Rectal temperatures of hamsters after receiving 1, 2, and 3 intravenous injections of plasmodial lytic factor administered at 24-hour intervals.

Injection no.	Average temperature (°C)* at indicated time after injection					ΔT†
	0 hr.**	½ hr.	1½ hr.	2½ hr.	4 hr.	
I (initial)	33 (33–33.5)	32.5 (32–33)	34 (34–34.5)	35.5 (35–36)	35.5 (35–37)	2.5 (4.5° F)
II (24-hour)	33 (32–34)	32.5 (31–33)	35 (34.5–35)	36 (35.5–36)	36.5 (36–37)	3.5 (6.3° F)
III (48-hour)	33 (32.5–33)	33 (32.5–33)	35 (34–35)	36.5 (36–37)	37.5 (37–37.5)	4.5 (8.1° F)
Saline control	32.5 (32–33)	31 (31–31.5)	31 (31–31.5)	32 (32–32.5)	32 (31.5–32.5)	

* Average temperature for 4 animals. Figures in parentheses indicate range.

** Temperature immediately before injection.

† Rise in temperature in 0–4 hour period.

The osmotic fragility then rapidly increased, at least through hour 6. Animals receiving multiple injections of the LF showed a similar but not greater increase of osmotic fragility 5 hrs after the last injection.

The febrile response of hamsters to i.v. injection of the LF also was investigated. The results of these experiments are summarized in Table 1. In animals receiving repeated injections of LF administered at 24-hr intervals, a significant increase of temperature was observed within 4 hrs after each injection. Furthermore, it was noted that the 4-hr temperature progressively increased with each successive injection of LF. Nevertheless, in each instance the temperature returned to normal within 24 hrs after injection.

Discussion

It is well established that in acute malaria, destruction of the erythrocytes involves not only direct rupture of the red cells by emerging merozoites, but also severe damage to the nonparasitized red cells (Cox et al., 1966; Kreier et al., 1966, 1972; Zuckerman, 1964, 1966). This leads to anemias that are inconsistent with the levels of parasitemia. Numerous investigators have postulated that the extensive destruction of nonparasitized red cells is the result of an immune process of some nature and have presented a considerable body of evidence to support this concept (see Zuckerman, 1964, 1966 for refs.). Other investigators have suggested that factors other than an immune process could be responsible for the anemia in acute malaria. Maegraith and his colleagues (Maegraith et al., 1943, a, b, c; Devakul and Maegraith, 1959) demonstrated the presence of a species-specific lytic agent, presumably an enzyme, in normal ani-

mal tissues, and the presence of a nonspecific inhibitor in the tissues and blood serum of homologous and heterologous species. Although this lytic agent readily lysed homologous normal erythrocytes, hemolysis was effectively inhibited by the presence of normal serum. However, it was observed that the inhibitory activity of sera from individuals with blackwater fever was significantly less than that of normal serum. In view of these findings, the authors suggested that hemolysis associated with blackwater fever is due to reduction in the activity of the inhibitory factor which disturbed the balance of the lytic factor and the inhibitor to favor lysis. The authors further postulated that there is no abnormal lysis involved in blackwater fever; rather that "the lysis simply is a manifestation of excessive uninhibited activity of a normal lytic process".

Other investigators have isolated a very potent hemolytic substance from the blood and tissues of normal animals and studied its activity *in vitro* (Laser and Friedmann, 1945; Laser, 1946, 1948, 1950). This substance was found in varying amounts in a large number of animal tissues and was present in small amounts in normal erythrocytes. However, the concentration was increased 25–75% in the red cells of monkeys heavily parasitized with *P. knowlesi*. It was reported that the hemolytic substance isolated in crystalline form from the parasitized red cells could not be distinguished from that obtained from normal red cells and tissues. These observations led to the postulate that the malaria parasites produce, or cause to appear inside the erythrocytes, a hemolytic substance, possibly identical with the naturally occurring hemolytic substance, which destroys the erythrocytes when its concentration is sufficiently high, and is then released into the plasma. Chemical

analysis revealed that this lytic component was a mono-unsaturated, mono-carboxylic fatty acid with the chain length C_{18} which subsequently was identified as *cis*-11-12 octadecanoic acid (*cis*-vaccinic acid). The hemolytic activity of this fatty acid was shown to be inhibited by a number of substances that normally occur in the body. Among the most effective inhibitors were proteins, cholesterol, lecithin, and calcium.

The present report describes *in vitro* and *in vivo* studies on a lytic factor (LF) of plasmodial origin. Although the major constituents of the LF were lipid in character with molecular weights less than 500, the kinetics of LF-induced hemolysis did not appear to reflect a simple chemical reaction on the red cell; the curve of hemolysis was that of a biphasic reaction (Fig. 1). There was an initial 4-hr lag phase during which little or no hemolysis occurred. This suggested that a "Preconditioning" action of some nature was necessary before the hemolytic event could take place (*i.e.* a multiset reaction). Following the lag phase, hemolysis progressed rapidly through hour 12, and then at a slower rate through hour 20 at which time the reaction spontaneously ceased. Temperature markedly influenced the velocity of the reaction and there was a significant difference in the susceptibility of erythrocytes from various animal species. There was also variation of susceptibility to LF-induced hemolysis within the red cell population of a given animal; the older cells were more susceptible than the younger cells.

Fogel et al. (1966) have shown that the osmotic fragility of nonparasitized as well as parasitized red cells was significantly increased in acute *P. berghei*, *P. knowlesi*, *P. galinaceum*, and *P. falciparum* infections. The present studies revealed that a similar increase of osmotic fragility occurred when normal hamster erythrocytes were exposed to the plasmodial LF. In *in vitro* studies, it was observed that relatively small amounts of LF significantly increased the osmotic fragility of the hamster red cells. However, red cells from different animal species varied considerably in their susceptibility to LF-induced changes of osmotic fragility (Fig. 3). Additionally, it was shown that increased erythrocyte osmotic fragility could be induced *in vivo* by intra-

venous injection of LF into normal hamsters. In these latter studies (Fig. 6), the red cells curiously showed an initial increase of *resistance* to osmotic lysis. This persisted for 4 hrs after injection. Whether coincidental or not, this corresponded in time to the lag phase observed in the *in vitro* studies on hemolysis. However, 4 hrs after injection, the osmotic fragility of the cells rapidly increased, and by hour 6 the osmotic fragility was significantly greater than that of the controls.

In continued *in vivo* studies, it was observed that the plasmodial LF could induce a febrile response in normal hamsters (Table 1). Hamsters receiving an i.v. injection of the factor showed a significant rise of rectal temperature within 4 hrs after injection. In animals receiving repeated injections of the LF administered at 24-hr intervals, the maximum temperature increased with each successive exposure to the factor. The hamsters receiving 3 daily injections showed severe hyperthermia with an average temperature increase of 4.5°C (8.1°F). This degree of fever corresponded to a temperature of greater than 106°F in the human. Nevertheless, in each instance the temperature returned to normal within 24 hrs. Maegraith (1954) in discussing the etiology of the febrile response and paroxysm in malaria noted: "The obvious time relation between parasitic sporulation and the appearance of the clinical paroxysm has not yet been explained. There is little evidence to connect the initiation of this reaction with the liberation into the circulation of merozoites, erythrocyte debris, proteins, hemoglobin or malaria pigment. The clinical features of the paroxysm are similar to those resulting from intravenous injection of pyrogenic agents and the humoral effects to those of anaphylactic shock. Most of the latter, however, can be explained in terms of circulating hormones." He concluded with the statement: "It has been suggested that the unknown initiating factor may be some non-specific pyrogenic agent liberated by the parasite." Results of the present studies suggest that the plasmodial LF-containing material may function in this capacity and thus play an important role in the clinical events of acute malaria.

Although the presence of *cis*-vaccinic acid could not be excluded in the plasmodial LF

preparations employed in these studies, the functional activity of the LF appeared to differ in certain respects from that reported for Laser's crystalline *cis*-vaccinic acid. Laser noted that the hemolytic activity of the *cis*-vaccinic acid was effectively inhibited by lecithin, cholesterol and calcium. This did not appear to be the case with the plasmodial LF since the product used in the present studies contained relatively high levels of both lecithin and cholesterol. However, the possibility that higher concentrations of these substances might be inhibitory cannot be overlooked. The addition of 10 mg% calcium had no effect on the hemolytic activity of the LF preparation. The biphasic nature of the hemolytic curve suggested a sequence of reactions more complex than that of a single chemical reaction. Finally, modest variations of LF concentration or temperature of incubation markedly influenced the velocity of the hemolytic reaction.

A component with hemolytic properties also was isolated from the plasma of a heavily parasitized monkey. In contrast to the low molecular weight of the plasmodial LF (MW = < 500), the molecular weight of the plasma component was quite high (MW = < 2,000,000 but > 150,000). Although it is possible that the active component from the plasma could be plasmodial LF bound to plasma proteins, this is considered unlikely since the plasma component was inhibited by small amounts of Ca⁺⁺ whereas the plasmodial LF was not. Curiously, a hemolytic component could not be isolated from the serum of a heavily parasitized monkey. This suggested that the plasma hemolytic component may have been inactivated or physically removed during the clotting process. Furthermore, an active component could not be obtained from the plasma or serum from a noninfected animal. Unfractionated plasma from a heavily parasitized monkey also was devoid of hemolytic activity. Although the hemolytic component isolated from the plasma obviously was associated in some manner with the malaria infection, its character and possible role in the clinical events of acute malaria are not known at this time.

In ancillary investigations, hemolytic components similar to those obtained from *P.*

knowlesi were isolated from *P. falciparum* parasites.

The present studies have shown that certain components of the malaria parasite possess the capacity to induce some of the clinical and hematological changes (e.g. fever, increased erythrocyte osmotic fragility, and hemolysis) characteristically observed in malaria infections. It is suggested that these plasmodial components may play a role in the pathogenesis of anemia and fever of acute malaria infections.

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Electron Transport in Intraerythrocytic *Plasmodium berghei*

C. A. HOMEWOOD, D. C. WARHURST, W. PETERS AND V. C. BAGGALEY

Department of Parasitology and MRC Research Group on the Chemotherapy of Protozoal Diseases and Drug Resistance Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, England.

ABSTRACT: When *P. berghei* is treated with inhibitors of electron transport, the effects on oxygen uptake and on clumping of pigment in response to chloroquine (an energy-dependent process) strongly suggest that these parasites require electron transport for the production of energy. Antimycin A inhibits both oxygen uptake and clumping; rotenone inhibits only clumping. Clumping is almost unaffected by cyanide or removal of oxygen, but even anaerobically, is still inhibited by rotenone. These observations are consistent with the presence in *P. berghei* of an unusual electron transport chain which can function in the absence of oxygen.

It has been reported that *P. berghei* degrades glucose by glycolysis and does not possess a functional citric acid cycle (Bowman et al., 1961; Bryant et al., 1964; Scheibel and Miller, 1969). Nevertheless, oxygen is utilised (Fulton and Spooner, 1956) and cytochrome oxidase has been demonstrated in the parasite (Scheibel and Miller, 1969; Howells et al., 1969), although it has been

suggested that in these circumstances, cytochrome oxidase probably has little physiological significance and that the rest of the electron transport chain may not be present in the intraerythrocytic stage of mammalian malarial parasites (Scheibel and Pflaum, 1970). The role of electron transport in energy production in *P. berghei* therefore remains uncertain.

The impossibility of preparing "free" parasites uncontaminated by host material has been demonstrated (Killby and Silverman, 1969) and parasites so prepared are not physiologically normal, as indicated by their

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consumption of much smaller amounts of oxygen and glucose than when in the erythrocyte (Bowman et al., 1960). It would therefore be preferable to investigate initially the metabolism of the undamaged parasite-erythrocyte complex. The main difficulty in this approach has been the lack of a suitable measure of energy production by the parasite. The rate of growth *in vitro* cannot be used as this is at best only a fraction of the rate *in vivo*.

It has recently been shown that clumping of malarial pigment after treatment with chloroquine *in vivo* (Warhurst and Robinson, 1971; Warhurst et al., 1971) or *in vitro* (Warhurst and Baggaley, 1972) is a synthetic process which requires energy. The extent of clumping after chloroquine treatment of parasitised red cells *in vitro* can therefore be used as a measure of the parasites' ability to produce energy, and the effects of specific inhibitors, combined with measurements of oxygen utilisation, can therefore provide information on the nature of the energy source.

Materials and Methods

The "N" strain of *P. b. berghei*, maintenance and strain of albino mice were as described previously (Warhurst and Robinson, 1971). Infections of not more than three days' duration were preferred. For convenience in counting, blood from mice with fewer than 15% of erythrocytes infected was used in all clumping experiments; blood with 40–70% infected erythrocytes was used for oxygen uptake measurements.

The incubation medium [modified from Trigg, 1969, and Polet and Barr, 1968] and its use in clumping experiments was described previously in part (Homewood et al., 1972) and will be described in further detail elsewhere (Warhurst et al., in preparation). 10 ml of concentrated Medium 199 (Bio-Cult Laboratories Ltd., BCL 166) were diluted to 100 ml with distilled water. 0.2 g glucose, 0.148 g NaHCO_3 , and 11 ml foetal bovine serum (Bio-Cult, BCL 005C) were added. After mixing and equilibration at 37°C for 30 minutes with 95% air : 5% CO_2 the medium (at pH 7.2) was distributed in 3.8 ml quantities into 10 × 1 cm neutral glass test tubes fitted with silicone stoppers. Tubes were gassed where necessary for final pH adjust-

ment and equilibrated for a further 15 minutes at 37°C. To each tube was then added 0.04 ml parasitised blood collected in a heparinised syringe from an infected mouse bled by axillary puncture. The tubes were rotated at 12 revolutions per hour at 37°C in a roller tube apparatus (Gallenkamp, CX 804) for a further 10 minutes before addition of inhibitor in 0.1 ml 0.85% sodium chloride (w/v) at a concentration 40 times the desired final concentration. Contents of tubes were mixed by inversion and replaced in the incubator on the roller tube apparatus. Saline was added to control tubes. After a further 15 minutes incubation at 37°C, chloroquine diphosphate (kindly supplied by I.C.I. Ltd.) was added in 0.1 ml saline to give a final concentration of 10^{-6}M . Saline was again added to control tubes.

After 80 minutes incubation with chloroquine, the tubes were centrifuged at $500 \times g$ for 5 minutes. The medium was decanted off and its pH recorded. One drop of foetal bovine serum was mixed with each pellet of parasitised blood cells and a thin smear made. The dried smears were viewed under oil-immersion and the percentage of pigmented cells in which pigment was completely clumped was determined.

Sterile precautions were not found to be necessary as incubation times were short. All glassware was acid-washed.

Oxygen uptake was measured with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambs.). The oxygen content of 1.5 ml of Medium 199 (with added glucose and bicarbonate as above, but without the serum) was recorded for several minutes; 0.5 ml of well oxygenated parasitised blood from which white cells had been removed (Baggaley and Atkinson, 1972) was then added and the rate of oxygen uptake recorded for about 5 minutes; inhibitor was then added in 0.1 ml Medium 199 and oxygen uptake recorded for at least a further 10 minutes. Control recordings of the oxygen uptake of parasitised blood without inhibitor were made before and after the experimental recording.

Results and Discussion

It has previously been reported (Warhurst and Baggaley, 1972) that inhibitors of electron transport and uncouplers inhibit clump-

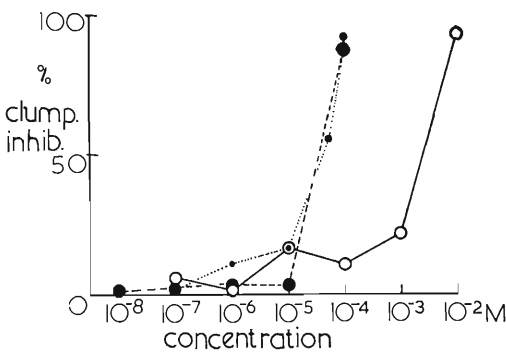


Figure 1. Effects of inhibitors of electron transport on clumping *in vitro*. Dose-response curves of cyanide ○—○, antimycin A ●---●, and rotenone ●.....●.

ing. Dose-response curves of rotenone and antimycin A show that both these drugs inhibit at a concentration of 10⁻⁴M (Figure 1). These concentrations are very much higher than those needed for inhibition of electron transport by isolated mammalian mitochondria, but it has been shown that 10⁻⁴M antimycin A is necessary for inhibition of oxygen uptake by the well-characterised isolated mitochondria of *Crithidia fasciculata* (Hill et al., 1968). It may well be that the sensitivity of protozoa in general to many antibiotics lies between that of mammals and that of bacteria. The inhibition shown in Figure 1 therefore indicates that *P. berghei* needs a functional electron transport chain to produce enough energy for clumping. Cyanide, however, does not inhibit clumping significantly until a concentration of 10⁻²M is reached; at this concentra-

Table 1. Inhibition of oxygen uptake of *P. berghei*-parasitised erythrocytes.

Day of infection	Parasitaemia	Drug	Concentration	Inhibition
3	40	KCN	10 ⁻³ M	100%
3	40	"	"	100%
3	30	"	"	100%
3	55	Rotenone	10 ⁻⁴ M	26%
3	60	"	"	26%
3	30	"	"	27%
4	70	Antimycin A	10 ⁻⁴ M	90%
3	60	"	"	100%
3	50	*Malonate	10 ⁻² M	9%
3	50	*Malonate	"	12%

* Cells sonicated for 5 seconds and treated with 10⁻⁴ M rotenone.

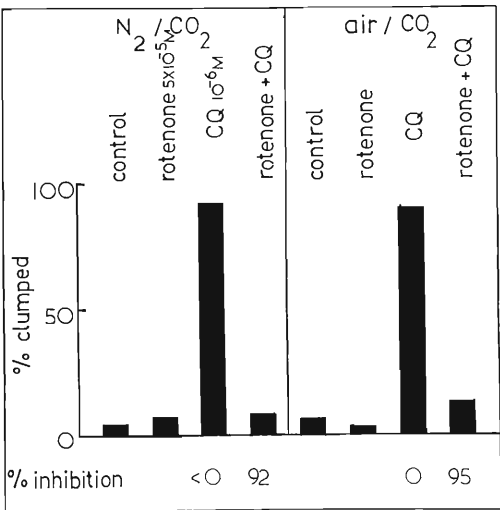


Figure 2. Effect of rotenone on clumping in the absence of oxygen.

tion, enzymes other than cytochrome oxidase will be affected. At the more usual inhibitory concentration of 10⁻³M, clumping was only about 20% inhibited (Figure 1). This suggests either that the cytochrome oxidase of *P. berghei* is insensitive to cyanide and continues to function even in the presence of the extremely high concentration of 10⁻³M cyanide, or that the cytochrome oxidase is in fact inhibited by cyanide and that *P. berghei* can produce enough energy for clumping in the absence of a functional cytochrome oxidase system.

Measurements of oxygen uptake indicated that the *P. berghei*-erythrocyte complex has a normal cyanide-sensitive cytochrome oxidase. 10⁻³M cyanide, which reduces clumping by only about 20%, completely stopped oxygen uptake (Table 1); the parasites must therefore be able to produce energy anaerobically. It would seem however that glycolysis alone cannot provide an adequate supply of energy, as interruption of the electron transport chain by antimycin A almost abolishes clumping, even though glycolysis is presumably continuing. Even when no oxygen is being used (i.e. in the presence of 10⁻³M cyanide), the electron transport chain must therefore be supplying energy. This is confirmed by the

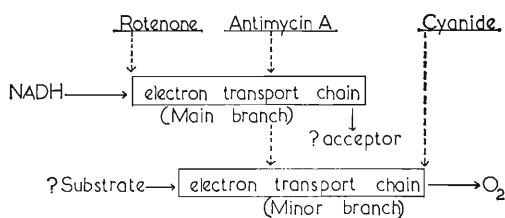


Figure 3. Possible electron transport chains of intraerythrocytic *P. berghei*.

experiment shown in Figure 2. This shows that clumping still occurs in oxygen-free nitrogen, but that this clumping is abolished by rotenone, demonstrating that the electron transport chain is still involved in energy production for clumping even in the absence of oxygen. Table 1 also shows the effects of rotenone and antimycin A respectively on oxygen uptake. Rotenone at 10^{-4} M inhibits oxygen uptake by only about 25%, although it inhibits clumping by more than 90%. Antimycin A, on the other hand, almost completely abolishes both oxygen uptake and clumping.

The simplest explanation for the apparently inconsistent results described above is that the *P. berghei*-erythrocyte complex possesses two electron transport chains (Figure 3). The main branch, with an acceptor which is not oxygen, has not been described for mammalian tissue and must belong to the parasite. The minor branch, using oxygen as acceptor, could be of reticulocyte or platelet origin. If this were so, its oxygen uptake would probably be reduced by rotenone plus malonate. Fulton and Spooner (1956) showed that the oxygen uptake of *P. berghei*-parasitised rat reticulocytes was less sensitive to malonate than was the oxygen uptake of reticulocytes alone, suggesting a malonate-insensitive component of respiration in the parasite. In the present experiments, to ensure that malonate was not prevented from reaching its site of action, parasitised erythrocytes were sonicated sufficiently to disrupt red cell and parasite membranes. No intact parasites or red cells could be seen in Giemsa-stained preparations. Table 1 shows the effect of malonate on parasitised erythrocytes treated with rotenone. It can be seen that malonate had almost no effect on the parasite respiration. Both electron transport

chains may therefore be associated with the parasite.

They may be physically separate or may be linked to form a branched electron transport chain, similar to those described for bacteria (White and Sinclair, 1971) and for nematodes (Moon and Schofield, 1968) and cestodes (Cheah and Bryant, 1966). Both branches are assumed to contain the central part of the normal electron transport chain, including the antimycin A-sensitive site. The minor branch presumably accepts electrons from flavoproteins and passes them finally to oxygen via a cyanide-sensitive cytochrome oxidase. The major branch accepts electrons from NADH and passes them to a final acceptor without the intervention of cytochrome oxidase. This branch is therefore cyanide-insensitive and can function in the absence of oxygen. The nature of the final electron acceptor in these conditions is not known.

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Plasmodium gallinaceum: Erythrocyte Membrane Alterations and Associated Plasma Changes Induced by Experimental Infections*

T. M. SEED AND J. P. KREIER

Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

ABSTRACT: This study correlates changes in erythrocyte morphology during *Plasmodium gallinaceum* infection with physiological and biochemical alterations of the host cells. Gross morphologic abnormalities of red cells from infected chickens stem from loss in osmotic control, which is in part the result of inactivation of membrane-bound ATPase, cation imbalance and change in the concentration of erythrocyte lipids. Early in the patent period morphologic alterations are limited to parasitized erythrocytes, whereas physiologic abnormalities are not. Red cell ion imbalance and depressed ATPase activity precede morphologic change and parallel changes in the plasma pH, and in plasma lipid, cation and protein concentrations. The magnitude of abnormalities in erythrocytes, and the suspending plasma, increase as the infection progresses. The implications of the abnormal red cell function and changes in plasma constituents for the mechanisms of erythrocyte destruction are discussed. Late in the acute phase of infection and during the recovery period morphologic abnormalities occur in non-parasitized erythrocytes. Many of the abnormally shaped, nonparasitized cells are basophiles. The influx into the diseased bird's circulation of many basophilic erythrocytes greatly influences the physiologic as well as the morphologic characteristics of the red cell population and obscures the cellular pathology associated directly with the infectious process. The nature of the changes in cell physiology, biochemistry and morphology associated with the shift in age composition of the erythrocyte population following parasitic destruction are described.

Many workers have studied one or another facet of pathological change in erythrocytes of animals with malaria, however, few have attempted an integrated study of these changes and their correlation with the physiological changes which occur in the erythrocyte population during infection. The erythrocyte population in a healthy animal is in dynamic equilibrium, there is a constant and steady input of newly produced erythrocytes and an equal and also steady removal of senescent ones. The population as a whole consists of erythrocytes of all possible ages in equal numbers. As infection begins the new subclass of parasitized erythrocytes appears in the population, these cells increase in number, reach a peak and then disappear. Parasitized cells also change with the growth and development of the parasites. As the infection progresses and erythrocytes are destroyed by the parasite's actions, the hematological equilibrium is upset. To compensate for cell loss input rates increase, and as a result young erythrocytes increase their representation in the population.

In the later stages of infection the class of "crisis reticulocytes" appears in the population. These cells are large, commonly irregularly shaped and basophilic (Kreier *et al.*, 1972). They have a wide range of life spans some being very short lived and some having an almost normal life span. The animal whose erythrocyte mass has been replaced by reticulocytes of this type mimic hematologic equilibrium (Mohan 1971). The data obtained from studies of erythrocytes of animals with malaria must be interpreted with a full consciousness of the complex changes in the erythrocyte population or the interpretation is meaningless. The present paper reports an attempt at an integrated study of the changes in the erythrocyte population set in motion by plasmodial infection.

Materials and Methods

Experimental animals and parasites: In all experiments White leghorn chickens were the host for the malaria parasite, *Plasmodium gallinaceum*. The birds were housed, fed and handled according to the standards set by the National Society for Medical Research.

The strain of *Plasmodium gallinaceum* used in this study was originally obtained from Dr.

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Geoffrey M. Jeffery, Institute of Malaria Chemotherapy, National Institutes of Health, Bethesda, Maryland. The infection in chickens was maintained by weekly syringe passage of infected blood.

Hematological procedures: On each blood sample collected for any experiment, packed red cell volumes (pcv) were determined by conventional methods, and percentage of red cells infected and percentage of basophilic erythrocytes present were determined by direct microscopic examination of Giemsa-stained thin blood films.

Course of infection: For purposes of experimentation and discussion the course of the avian malarial infection was defined on the basis of the animals' hematological picture as having six phases. They were:

(1) "Normal." Preinfection state.

(2) Prepatent state. This phase occurs approximately 5–7 days after infection. There are no infected cells found upon examination of thin blood films and no basophilic erythrocytes occur in the blood.

(3) Early patent period. In this stage there is rising parasitemia, but less than 10% of the cells are infected and no basophilic cells are seen. This phase occurs approximately 6–9 days after infection.

(4) Middle patent period. This phase of infection is characterized by high parasitemia and low basophilia (less than 10%). This phase occurs approximately 10–12 days after infection.

(5) Late patent period. During this stage there is falling parasitemia and rising basophilia. It occurs approximately 11–14 days after infection.

(6) Recovery state. This stage is characterized by very low or no parasitemia and falling basophilia. It occurs approximately 13–20 days after infection.

Induction of anemia by phenylhydrazine injection: Anemia was induced in chickens by three intramuscular injections of phenylhydrazine hydrochloride. On day one, 5 mg were injected, on day three, 10 mg, and on day five, an additional 10 mg.

Morphology: Electron microscopy was performed by the techniques used by Kreier *et al.*, 1972.

Osmotic fragility: The osmotic properties of the red cells in each of the samples were tested by a method described by Herman (1969). The optical density of erythrocyte samples lysed in distilled water was the standard for 100% hemolysis.

For visual demonstration of differences in the osmotic resistance of normal and infected red cells, washed erythrocytes were suspended to a 1% (v/v) concentration in hypotonic (0.45% NaCl) salt solutions and incubated at room temperature for 30 minutes. Droplets were placed on microscope slides, covered with glass slips and examined by bright field microscopy with an oil immersion lens.

Red cell volume measurement: The blood was fixed in 1% (v/v) glutaraldehyde: phosphate buffered saline (pH 7.3) for 10 minutes. The fixed red cells were diluted 1:20,000 in filtered physiological saline. The red cell suspensions were placed in the Celloscope particle counter and counts were taken at 5 unit intervals starting with 10 and ending with 80. The 100 μ m orifice was used for all the counts. Trigger numbers, at which counting efficiency was 50%, were correlated to specific volumes obtained from a standard curve constructed by plotting threshold readings of reference spheres (1–15 μ m diameters) against their known volumes.

To determine red cell size and approximate cell volume by direct microscopic examination, whole blood was treated as for electronic counting. Fixed erythrocytes were diluted to a 1% concentration in physiological saline. Droplets were placed on glass microscope slides and allowed to air dry. The slides were rinsed in distilled water and then stained with Giemsa reagent and examined by bright field microscopy. Individual cells were measured in length, width and depth with a calibrated ocular micrometer. Measured cells were grouped according to stage of infection and maturity.

Intraerythrocytic sodium and potassium ion concentration determinations: Alterations in intraerythrocytic ion concentrations were determined by atomic absorption spectrophotometry. A Perkin-Elmer AA spectrophotometer, model 403 was used. Sodium concentrations were determined at 295 nm. Potassium concentrations were analyzed at 383 nm.

Whole blood was collected in heparinized syringes. The blood was centrifuged at $770 \times g$ for 5 minutes. The plasma and the buffy coats were removed. The packed cells were washed, centrifuged and resuspended to the volume of the original blood sample in Ringer's solution. Packed red cell volumes were determined on the suspensions as were red cell numbers in the suspensions. One tenth milliliter aliquots of the red cell suspensions were pipetted into 9.9 ml and 19.9 ml of double distilled, deionized water for Na^+ and K^+ determinations respectively. The red cells were allowed to lyse completely after which the red cell ghosts were removed by centrifugation ($7,500 \times g$ for 10 minutes). The supernatant fluids were analyzed for sodium and potassium. Intraerythrocytic ion concentrations were adjusted for the ions of the suspending fluid. The volume of the suspending fluid was determined by subtracting the total red cell volume, estimated by multiplying individual mean cell volume by the number of rbc's per aliquot, from the total volume of the aliquots.

Cytochemical localization of ATPase: Whole blood collected in heparinized syringes was fixed for 30 minutes in 1% glutaraldehyde-4% sucrose, phosphate buffer, pH 7.3. The fixed cells were then washed five times in a modified Ringer's solution which contained 10.0 mM MgCl_2 . The red cells were then incubated at 41°C for 2 hours in the basic medium which contained the ATP substrate (5.0 mM) plus $\text{Pb}(\text{NO}_3)_2$ (0.5 mM). Controls lacked the substrate ATP. After incubation the samples were washed twice in the basic medium, fixed in 1% osmium for one hour, dehydrated, and embedded in Epon 812. Unstained sections were examined by electron microscopy.

Quantitative biochemical analysis of red cell ATPase activity: Erythrocytes washed once in Ringer's solution, pH 7.3 were resuspended in Ringer's solution to give a final cell concentration of 1.00×10^9 rbc's/ml. One tenth milliliter quantities of these suspensions were pipetted into 1.9 ml of a modified Ringer's solution (pH 7.3) which contained 10.0 mM MgCl_2 and 2.5 mM ATP. Controls lacked either ATP or the whole cells. Ouabain (10^{-4}M) was added to some samples in order to determine the sensitivity of the enzyme system to the drug. After incubation

at 41°C for 50 min, the enzymatic reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The tubes were centrifuged at $1,800 \times g$ for 10 minutes after which 1 ml of the supernatant fluid was pipetted off. Concentrations of inorganic phosphate were determined by methods outlined by Clark (1964). Enzymatic activity was computed in terms of μM of phosphate cleaved from the ATP substrate per minute per red cell.

Concentration of red cell and parasite cholesterol and phospholipids: Red cells were washed twice in 12 times their volume of Ringer's solution, pH 7.3. The washed red cells were resuspended to a 33% (v/v) concentration in Ringer's solution. Red cell concentrations were determined on aliquots of the suspensions. Hemoglobin free erythrocyte ghosts were prepared by lysing 0.1 ml (for phospholipid or 0.5 ml for cholesterol) of the 33% rbc suspensions in 100 times their volume of 20% physiological saline followed by distilled water lysis. The erythrocyte ghosts were collected by centrifugation at $1,500 \times g$ for 10 minutes. Ten times their volume of a 2:1 chloroform:methanol solution was added to the packed ghosts. The suspensions were vigorously shaken and allowed to incubate at 25°C for 2 hours, then incubated for 48 hours at 4°C . Gross particulates were removed by passing the suspensions through compressed cotton filters 0.5 centimeters thick. The samples were then washed twice with distilled water. The organic solvents were driven off by placing the samples in a 90°C water bath for several hours. The amount of organic lipid phosphorus per aliquot of red cell ghosts was determined by a modified Fiske-Subbarow method (1925) described by Dodge and Phillips (1967). Cholesterol was determined by the method of Webster (1964). Red cell concentrations were reported in picograms per red cell.

To calculate the contribution of the intracellular parasites to red cell lipids, parasite phospholipid and cholesterol concentrations were estimated. Nonparasitized blood and infected blood with more than 50% parasitized erythrocytes were collected. The red cells were washed twice in Ringer's solution. After centrifuging the suspensions at $770 \times g$ for 5 minutes the packed cells were diluted with Ringer's solution to a 10% concentration. The erythro-

cytes were then passed through a continuous flow ultrasound apparatus at a flow rate of approximately 30 ml/minute at maximum ultrasonic intensity. The samples were centrifuged at $30 \times g$ for 10 minutes. The top two thirds of the supernatant fluid, which contained mainly free nuclei and membrane contaminants, in the nonparasitized erythrocyte samples, or free nuclei, parasites and residue membrane contaminants in the parasitized erythrocyte samples, were pipetted off and centrifuged at $10,000 \times g$ for 10 minutes. After centrifugation the supernatants were aspirated off and discarded while the pellets were resuspended in 3 ml of physiological saline. Nuclei, or nuclei and parasites were counted using a hemocytometer. The phospholipid and cholesterol concentrations of the preparations were determined by methods previously described. After adjustment for numbers of nuclei in the various preparations, the difference between lipid content of nuclei derived from control, non-infected red cells and the lipid content of nuclei in the mixed nuclei-parasite samples was assumed to be the lipid content of the parasites.

Osmotic properties of normal avian erythrocytes treated in vitro with plasma and plasma ultrafiltrates from healthy chickens or chickens with malaria: Normal, noninfected avian erythrocytes were washed and suspended in 10 times their volume of either normal or infected blood plasma and then incubated for 30 minutes at 37°C . The treated cells were then spun down, washed twice in physiological saline and resuspended to a 2% (v/v) concentration. The osmotic properties of the *in vitro* treated red cells were examined by a multiple tube osmotic fragility test (Herman 1969).

Normal and late-patent phase blood plasma were filtered through membranes having pore sizes small enough to retain plasma constituents larger than 50,000 (xm-10, Diaflow membranes) in molecular weight. Ultrafiltrates of normal (ncp-low) and infected plasma (icp-low) were tested for their fragility inducing capacity. The sodium and potassium cation concentrations in the various plasma fractions were determined by atomic absorption spectrophotometric methods. The pH of each of the fractions was also measured.

In vivo survival of Chromium⁵¹ labeled,

normal erythrocytes pretreated in vitro with normal and infected plasma and plasma ultrafiltrates: The survival of the plasma and plasma filtrate treated chicken rbc's was evaluated by the Cr^{51} labeling technique previously described (Kreier and Leste, 1967). The red cells to be tested were washed twice in Ringer's solution, pH 7.3 and incubated for 30 minutes at 37°C in ten times their volume of pooled blood plasma from healthy chickens or chickens in the late patent phase of malaria or with osmotic fragility inducing, low molecular weight ultrafiltrates of the normal and infected plasma. Following incubation the treated cells were washed twice in Ringer's solution, labeled and then injected back into the chickens from which they were withdrawn.

The chickens were bled on the 1st, 18th, 43rd, 88th, 135th, 182nd and the 279th hour after infusion. One milliliter venous blood samples were taken at each bleeding period. The weight of each sample was determined. The samples were then diluted to 5 ml with double distilled water and placed in a Hewlett-Packard dry well gamma counter to determine their radioactivity.

The effect on red cell ATPase activity of incubation of erythrocytes in blood plasma or low molecular weight plasma ultrafiltrates. Normal, washed erythrocytes were incubated for 30 minutes at 41°C with pooled blood plasma or plasma ultrafiltrates from healthy chickens and chickens in the middle and late patent periods of *Plasmodium* infection. Incubating fluids were aspirated off and red cell concentrations were adjusted to 1.00×10^9 rbc's/ml. in Ringer's solution. Each of the red cell samples were then assayed for ATPase by procedures described in the section on red cell ATPase activity.

Blood plasma chemistry prior to and during P. gallinaceum infection: Sodium and potassium cation concentrations in blood plasma were determined by methods described by Kerber (1971) in the Perkin-Elmer model 403 A. A. spectrophotometer methods manual.

Plasma chloride concentrations were determined by electronic titration with a Cotlove Chloridometer according to standard methods (Cotlove *et al.*, 1958).

Blood plasma concentrations of acid soluble, inorganic phosphate were determined by as-

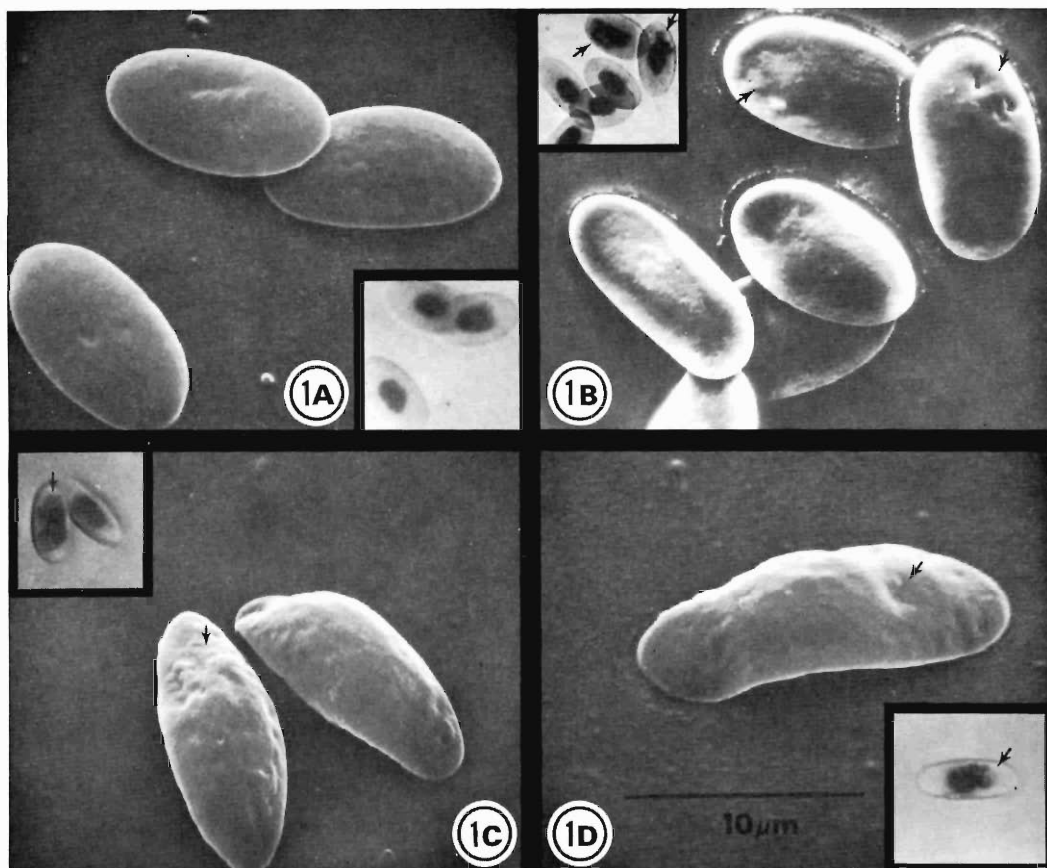


Figure 1. Scanning electron micrographs of erythrocytes from a healthy chicken (A) and from infected chickens (B, C, D). Red cells shown in B were obtained from a malarious chicken during peak parasitemia. Cells shown in C and D are from animals in the late patent phase of infection. Red cells from the middle patent phase of infection (Fig. B) have surface depressions where the larger intracellular parasites are located (arrows-a). Abnormal cell shape occurs in both infected and non-infected erythrocytes from infected chickens in the late patent period (Figs. C, D). Arrows indicate location of intracellular plasmodia. Line marker equals 10 μ m.

saying deproteinized samples for inorganic phosphate by methods outlined by Clark (1964).

The pH of blood plasma was determined on blood samples obtained by venous puncture. Blood samples were immediately chilled to 4° C and centrifuged to separate plasma from red cells. Plasma supernatants were promptly pipetted off and stored at -20° C in small volume vials capped with rubber stoppers. At the time of testing, the vials were removed from the refrigerator and thawed in

such a manner as to maintain the plasma in a chilled state.

Lipids were extracted from one tenth ml quantities of plasma by the same methods used to extract lipid from red cells. Lipid phosphorus was assayed by the modified method of Fiske and Subbarow (1925) described by Dodge and Phillips (1967).

Blood plasma cholesterol concentrations were determined by Webster's method (1964).

Plasma samples were assayed for total protein by standard Biuret methods (Clark, 1964).

Table 1. Osmotic resistance of red cells obtained from chickens prior to and during the course of *Plasmodium gallinaceum* infection.

Day	Percentage of hemolysis (average)						Hematology			No. sampled
	In: 50% saline/d-H ₂ O		40%		30%		PCV	% IRBC	% BRBC	
Pre-infection	* 38.7 ± 6.3	—	77.7 ± 4.0	—	93.3 ± 1.4	—	33.9	0	0	18
7th day	29.3 ± 10.8	ns	61.0 ± 5.8	s	88.6 ± 2.3	s	30.8	0	0	11
9th day	35.6 ± 5.9	ns	79.0 ± 4.3	ns	94.4 ± 1.7	ns	29.2	1.1	0	12
11th day	72.1 ± 8.4	s	91.1 ± 3.7	s	98.2 ± 1.1	s	28.9	25.2	0	10
13th day	63.5 ± 6.5	s	88.2 ± 3.5	s	100 ± 0.0	s	17.8	37.1	22.8	7
15th day	56.7 ± 6.2	s	80.0 ± 4.9	ns	91.1 ± 5.9	ns	18.3	8.2	48.7	8
17th day	50.5 ± 8.2	s	80.1 ± 6.5	ns	88.3 ± 4.5	ns	28.2	1.6	15.0	8
19th day	35.2 ± 7.7	ns	70.2 ± 4.1	s	81.8 ± 2.9	s	30.1	.03	0	7

ns = not statistically significant at the 95% confidence level. Student's "t" test.

s = statistically significant at the 95% confidence level. Student's "t" test.

* = mean value ± standard error.

UV absorption properties of plasma diluted 200 fold in distilled water were determined spectrophotometrically in the UV range at wave lengths of 260 nm and 280 nm. Ratios of the absorbancies at 280 nm and 260 nm were calculated.

Urea nitrogen (BUN) concentrations in pooled plasma samples were assayed by the methods described by Crocker (1967).

Pooled plasma samples were assayed for blood sugar by the Harleco micro glucose, o-toluidine method (Relander, 1963).

The ATPase activity of plasma was determined by the procedure used in determination of red cell ATPase activity, excepting that 0.1 ml of plasma replaced 0.1 ml of rbc's as the "enzyme preparation."

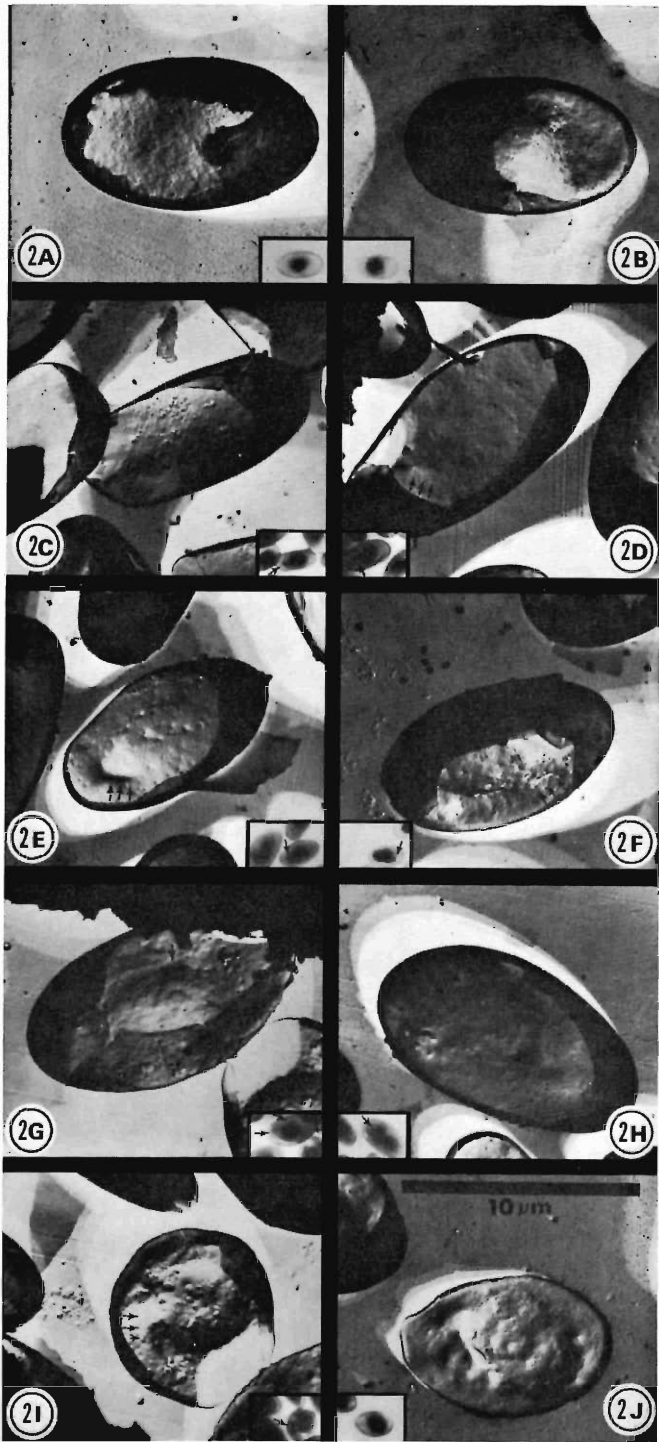
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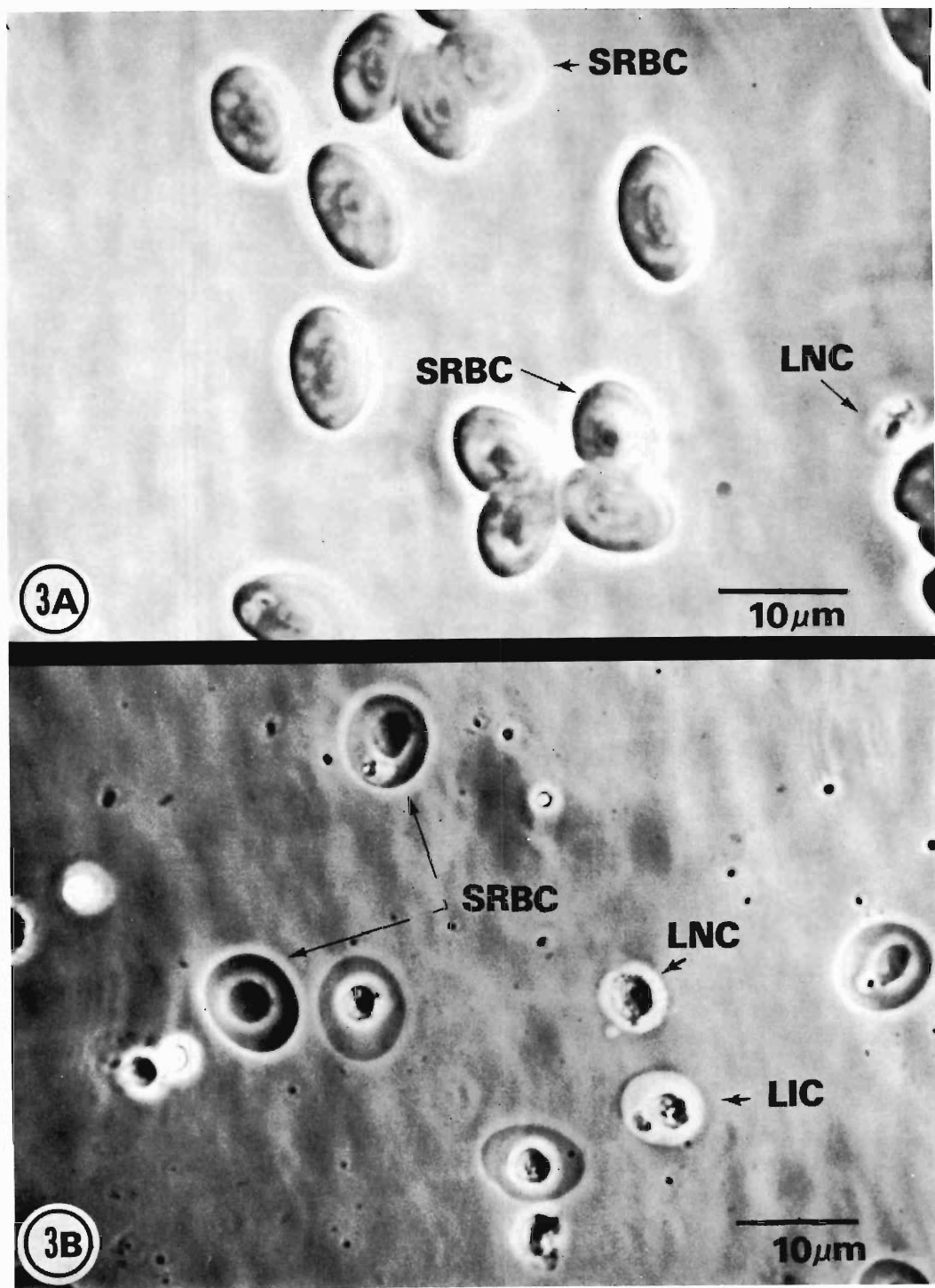
Morphology: Figure 1 is a sequence of scanning electron micrographs of erythrocytes from normal (1A) and *Plasmodium*-infected chickens. The erythrocytes from the infected chickens were collected during the middle (1B) and late (1C,D) patent periods in the infection. Middle patent phase erythrocytes

had nearly normal shape unless they contained large parasites (1B). Gross surface defects were noted only on those red cells which were parasitized (1B, arrows). Similarly, carbon replicas (Fig. 2A,B) of nonparasitized red cells collected during the early and middle patent period had almost normal morphology, except that some had a slightly collapsed appearance. The only readily noticeable abnormality in erythrocytes containing small parasites was a slit-like lesion occasionally present near the intracellular parasite (Fig. 2C,D; arrows). Carbon replicas of erythrocytes containing large parasites had abnormal morphology. Cell membranes were often collapsed in the area where the parasites resided and the erythrocytes nuclei protruded (Fig. 2F). While the surfaces of only some normally shaped red cells were abnormal (Fig. 2E), all the rounded and enlarged cells had abnormal surfaces (Fig. 2G,H,I). In the late patent phase of infection nonparasitized erythrocytes (Fig. 1C; Fig. 2J) as well as parasitized ones (Fig. 1C, D) were abnormally shaped. Many but not all of these abnormally shaped cells were basophiles (Fig. 2J).

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Figure 2. Electron micrographs of carbon replicas of erythrocytes from *P. gallinaceum*-infected chickens showing the relationship of parasitization to erythrocyte pathology. Electron micrographs (A) and (B) are of nonparasitized erythrocytes from an infected chicken. Photographs (C) through (H) show the relationship between red cell alteration and the size of the intracellular parasite. Arrows indicate the location of the intracellular parasite in the light (inserts) and electron micrographs. The red cells in Figs. (C) and (D) have small parasites. Note the slit-like lesions in the area of the intraerythrocytic plasmodia (C, D; arrows). Except for the latter, these cells appear quite normal and have little surface alteration. In contrast, the red cells in Figs. (G, H, and I) contain large parasites or have multiple infections. These cells are swollen and have considerable surface alteration. Electron micrograph (J) is of an abnormally shaped, non-infected red cell from a late patent phase blood sample. Line marker equals 10 μ m.





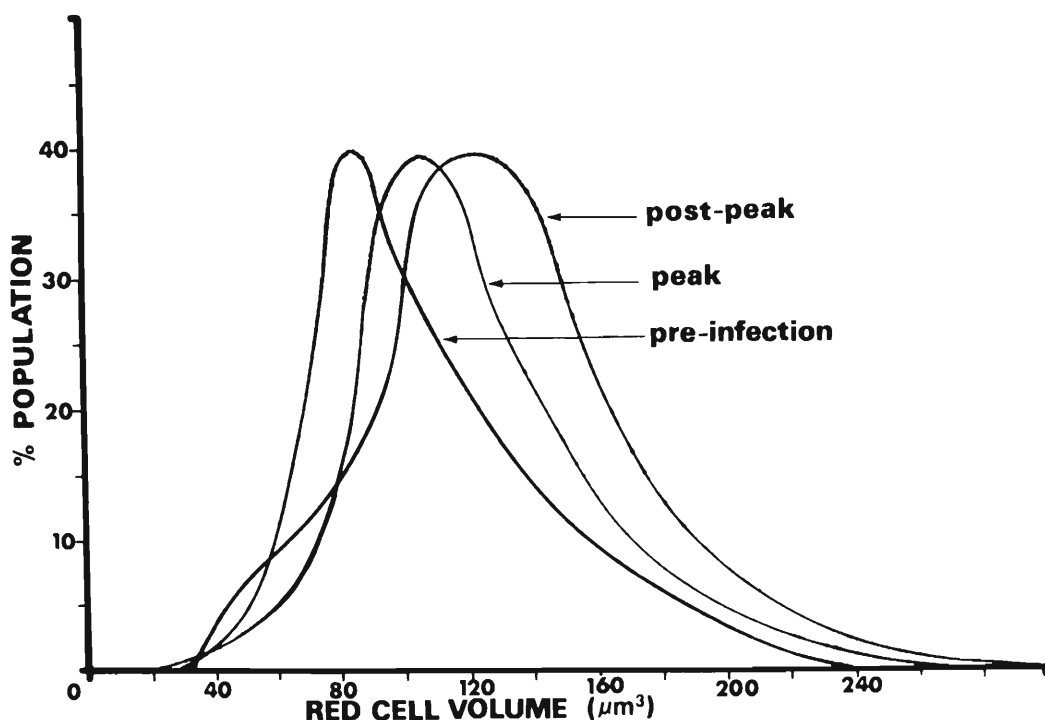


Figure 4. Red cell volumes determined by an electronic particle counting and sizing device, of erythrocytes in samples from normal chickens and chickens in the middle and late patent phases of malaria. Mean cell volumes increase and cell volume distribution about the mean spreads with the onset of patent infection.

Osmotic fragility (Table 1): Erythrocytes taken during the patent phase of infection from *Plasmodium*-infected chickens were quite fragile and did not withstand as much osmotic stress as normal erythrocytes from healthy chickens. The fragility increased in proportion to the increase in parasitemia during the early stages of infection but later in the infection some nonparasitized erythrocytes were less osmotically resistant than normal cells. As the birds began to recover red cell fragility decreased from the levels which occurred during late patency, but fragility was still

elevated above normal. During the late recovery period when the hematological picture of the birds was normal erythrocytes exhibited the same or even greater osmotic resistance than red cells from healthy chickens. Figure 3A shows normal red cells stressed by incubation in a hypotonic salt solution. The cells were slightly spherocytic. A low percentage of the erythrocytes were lysed. In contrast, similarly treated erythrocytes from a chicken in the late patent period (Fig. 3B) of infection were all spherocytic. As many non-

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Figure 3. Phase-contrast photomicrographs of normal erythrocytes (a) and erythrocytes from a *Plasmodium gallinaceum* infected chicken in the late-patent phase of infection (b) suspended in hypotonic salt solution. The red cells from the infected chicken (b) are more fragile than the red cells from a healthy chicken (a). Abbreviations: srbc, spherocytic red blood cell; lic, lysed infected cell; lnc, lysed non-infected cell. Line marker equals 10 μm .

Table 2. Mean red cell volumes in chickens with *Plasmodium gallinaceum* infection.

Stage of infection	Mean red cell volume (μm^3)		Hematology			No. sampled (n)
			pvc	% irbc	% brbc	
Normal (pre-infection)	* 88.7 ± 1.20	—	30.2	0.0	0.0	6
Pre-patent	94.8 ± 3.80	ns	27.5	0.0	0.0	6
Pre-peak	97.3 ± 3.32	s	26.3	4.2	0.0	4
Peak	109.4 ± 3.91	s	20.6	29.2	4.4	5
Post-peak-1	122.8 ± 6.65	s	15.2	14.6	37.4	5
Post-peak-2	123.7 ± 4.54	s	19.9	0.0	37.7	7
Recovery	99.4 ± 2.96	s	26.4	0.0	3.1	9

* = mean value \pm standard error.
ns = not statistically different from normal sample at 95% confidence level. Student's "t" test.
s = statistically different from normal sample at 95% confidence level. Student's "t" test.

parasitized erythrocytes as parasitized ones were swollen or lysed.

Erythrocyte volume: The changes in osmotic fragility of erythrocytes from chickens with *Plasmodium gallinaceum* infection correlated with erythrocyte volume changes. The most fragile erythrocytes had large cell volumes. Figure 4 shows the volumes of red cells obtained from birds prior to *P. gallinaceum* infection and during the middle and late patent phases of the disease as determined by electronic cell sizing. A high percentage of the normal, preinfected red cell population fell within a narrow range of volumes. With initiation of infection and the onset of patency, there developed a wide distribution of erythrocyte volumes about the mean. The mean red cell volume (mcv) also increased, from approximately $90 \mu\text{m}^3$ prior to infection to $110 \mu\text{m}^3$ at peak parasitemia. The greatest average red cell volume ($125 \mu\text{m}^3$) occurred during the late patent phase of infection. Erythrocyte vol-

umes were also large in birds which recently recovered from plasmodial infection in which parasitemia was negligible (Table 2). The mean red cell volumes determined by electronic cell sizing and by direct microscopic examination were very similar (Table 3). By direct microscopic examination the average normal red cell from a healthy chicken measured about $10.3 \mu\text{m} \times 5.6 \mu\text{m} \times 1.9 \mu\text{m}$ and had a volume of about $90 \mu\text{m}^3$. During the late patent period of infection the large basophilic erythrocyte was the cell type which contributed the most to the increase in red cell volume. This cell type had a volume of about $148 \mu\text{m}^3$ and was considerably larger than the other cells found in blood of chickens in the late patent phase of infection. Red cells of all types, however, including noninfected, nonbasophilic erythrocytes within the late patent phase blood samples had larger volumes than those from healthy chickens. Parasitized erythrocytes collected at any stage of infection were considerably longer (10.7

Table 3. The relationship of parasitization to red cell size and volume as determined by direct microscopic examination in chickens with *Plasmodium gallinaceum* infection.

Sample type	Size-volume relationships		n	Hematology		
	*Size (μ)	Estimated volume (μ^3)		pvc	% irbc	% brbc
Normal (preinfection)	$10.3 \times 5.6 \times 1.9$	90.5	70	30.2	0.0	0.0
Prepatent	$9.9 \times 5.7 \times 1.9$	94.0	100	32.1	0.0	0.0
Prepeak	$10.0 \times 5.5 \times 1.9$	92.5	50	32.7	2.5	0.0
Peak	$10.4 \times 5.2 \times 2.2$	96.5	150	25.2	31.0	0.0
Post-peak	$10.0 \times 5.7 \times 2.5$	125.0	125	18.4	17.0	32.4
Cell type:						
1) Small parasite	$10.7 \times 5.3 \times 2.2$	112.0	62			
2) Medium	$10.6 \times 5.3 \times 2.2$	111.0	32			
3) Large	$10.9 \times 5.1 \times 2.3$	115.0	25			
4) Basophile (5-6+)	$9.3 \times 5.6 \times 2.4$	109.0	22			
5) Basophile (1-2+)	$10.4 \times 7.0 \times 2.4$	148.0	38			
6) Non-infected	$10.6 \times 5.7 \times 2.2$	118.0	38			
Recovery	$10.3 \times 5.7 \times 2.2$	114.0	70	28.7	0.0	6.7

* Mean value at n number determinations.

Table 4. Sodium and potassium concentrations determined by atomic absorption spectrophotometry of erythrocytes of chickens with avian malaria.

Stage of infection	Na ⁺ per red cell (× 10 ⁻¹³ gr Na ⁺ /rbc)	K ⁺ per red cell (× 10 ⁻¹³ gr K ⁺ /rbc)	Hematology		
			pvc	% irbc	% brbc
Normal (preinfection)	* 0.916 ± 0.088 (22) -	5.590 ± 0.120 (28) -	32.9	0.0	0.0
Pre-patent	2.082 ± 0.142 (21) s	4.960 ± 0.156 (21) s	32.0	0.0	0.0
Early patent	2.323 ± 0.230 (14) s	5.070 ± 0.201 (16) s	29.0	2.3	0.6
Middle patent	2.606 ± 0.350 (9) s	5.570 ± 0.302 (11) ns	23.1	30.4	2.0
Late patent	4.624 ± 0.762 (10) s	6.710 ± 0.150 (17) s	18.1	9.3	34.8
Recovery	2.790 ± 0.211 (14) s	6.250 ± 0.293 (16) s	27.2	0.1	4.8

* = mean value ± standard error.
s = significantly different value from normal at 95% confidence level.
ns = not significantly different value from normal at 95% confidence level. Student's t test.
(n) = number sampled.

μm-10.9 μm) and narrower (5.1 μm-5.3 μm) with greater thickness than normal erythrocytes. The degree of distortion was greater in erythrocytes with larger parasites than in erythrocytes with smaller ones (Fig. 2). During the late recovery period the red cells again had normal sizes.

Intraerythrocytic sodium and potassium concentrations (Table 4): Red cells from healthy chickens prior to infection contained high concentrations of potassium (5.59 × 10⁻¹³ g K⁺/rbc or about 80 meq/ml packed rbc's) and low concentrations of sodium (0.916 × 10⁻¹³ g Na⁺/rbc or about 20 meq/l packed rbc's). During the prepatent and early patent period of infection potassium levels decreased whereas sodium concentrations increased. Intraerythrocytic sodium concentrations reached a maximal level during the late patent phase of infection at which time there was approximately a five fold increase over normal. Cell sodium concentration decreased during the recovery phase, however, the sodium levels at this time were still higher than in normal erythrocytes. Following the drop in red cell potassium concentration during the early

phases of infection, potassium concentrations increased. The increase started when basophilic erythrocytes appeared in the circulation. Red cell samples with high potassium levels from chickens recovering from malaria had a high percentage of basophilic erythrocytes. The preinfection K⁺/Na⁺ ratio (6:1) dropped to 2.4:1 during the prepatent period and continued to fall throughout the patent period. The lowest cation ratio 1.6:1 occurred following peak parasitemia. In the recovery period cation ratios slowly returned to normal.

The alterations in concentration of intracellular sodium and potassium during avian malaria were mimicked in birds injected with phenylhydrazine (Table 5). On the day following drug administration a two and one half fold increase in cell sodium occurred, but potassium concentration remained at near normal levels. When drug injection was continued the potassium concentration increased in parallel to the number of basophilic erythrocytes in the blood.

Erythrocyte ATPase activity: Cytochemical studies indicated that the major sites of ATPase activity were in the limiting plasma mem-

Table 5. Sodium and potassium concentrations determined by atomic absorption spectrophotometry of erythrocytes of chickens injected with phenylhydrazine.

Day post-injection	Amt. drug given phenylhydrazine	Na ⁺ per red cell (× 10 ⁻¹³ gr Na ⁺ /rbc)	K ⁺ per red cell (× 10 ⁻¹³ gr K ⁺ /rbc)	Hematology	
				pvc	% brbc
0	-	* 1.003 ± 0.096 (25)	5.468 ± 0.042 (31)	32.9	0.0
1	5 mg	2.507 ± 0.797 (3)	5.643 ± 1.800 (3)	22.3	10.3
2	-	2.707 ± 0.408 (3)	7.490 ± 0.172 (3)	26.8	23.0
3	10 mg	1.533 ± 0.861 (3)	7.293 ± 0.487 (3)	27.1	29.7
4	-				
5	10 mg				
6	-				

* = mean value ± standard error.
(n) = number sampled.

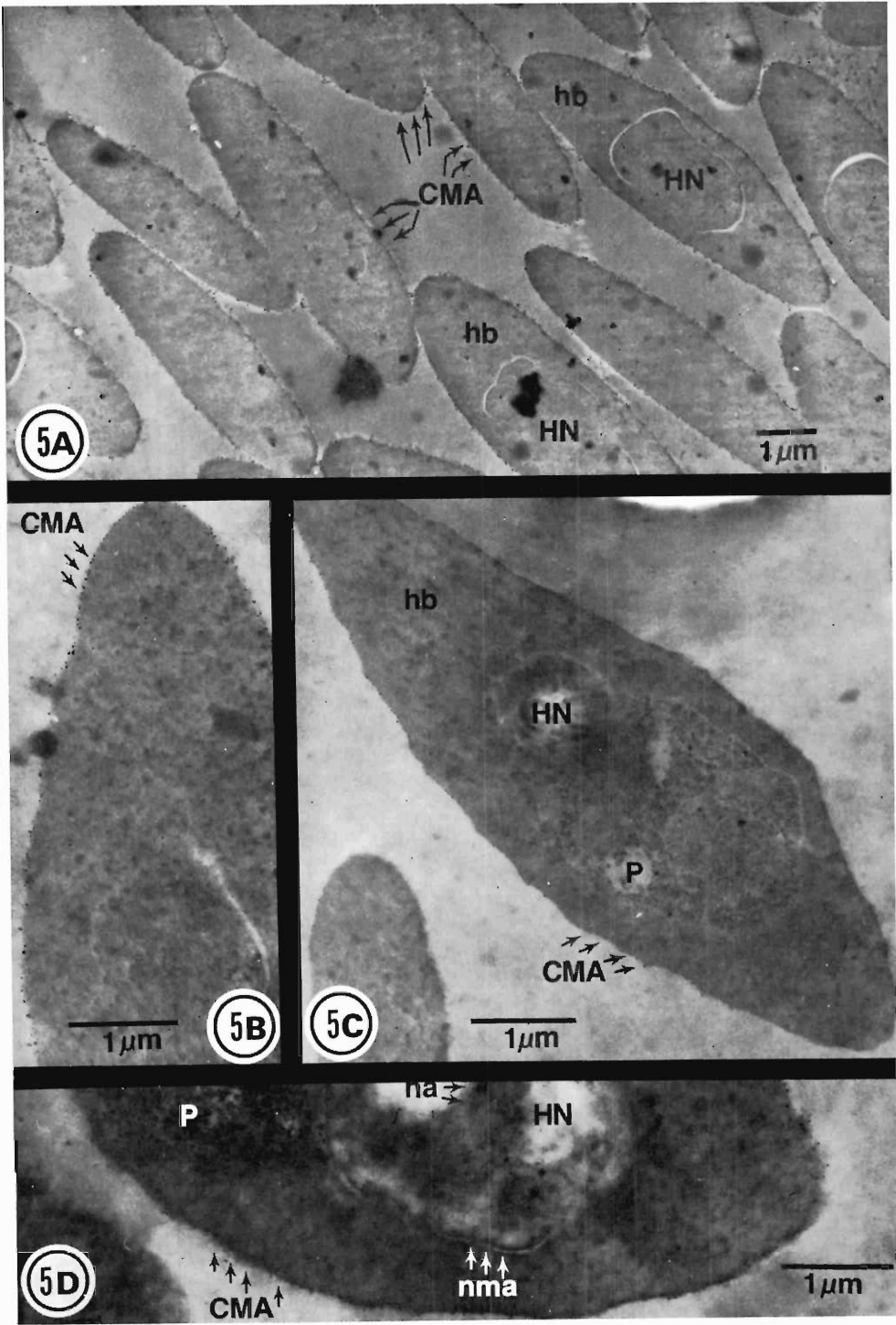


Table 6. Red cell ATPase activity in erythrocytes of chickens with *Plasmodium gallinaceum* infection.

Stage	Day	ATPase activity of whole red cells ($\times 10^{-10}$ μ M Pi/min./rbc)*	Hematology			No. sampled
			pvc	% irbc	% brbc	
Normal	0	1.311 \pm .06874	33.5	0	0	66
Pre-patent	4	1.155 \pm .10810**	33.6	0	0	20
Pre-peak	6	0.753 \pm .07656***	32.1	1.6	0	18
Peak	8	0.771 \pm .13110***	22.1	30.5	0	17
Post-peak ₁	10	0.630 \pm .06732***	15.5	22.6	43.2	12
Post-peak ₂	12	1.565 \pm .24900**	21.5	7.6	41.6	20
Recovery	14	1.634 \pm .32990**	27.8	0	4.6	15

* Mean \pm S.E.M.
** Not significantly different at 5% level.
*** Significantly different at 5% level.

brane (Fig. 5A, CMA). Activity was greater in general in membranes of normal chickens than of infected chickens. Nuclear membranes had low enzymatic activity (Fig. 5D, NMA). Some ATP hydrolysis also occurred in the chromatin (Fig. 5B & D; na). Red cell cytoplasm generally lacked activity except for occasional active “spherical body inclusions” which lay close to the nucleus. The ATPase activity of intraerythrocytic plasmodia varied depending on the stage of maturation (Fig. 5C & D).

By quantitative biochemical methods it was determined that normal erythrocytes from healthy chickens had ATP hydrolytic activity equivalent to 1.311×10^{-10} μ M Pi/min/rbc. The enzymatic activity was partly sensitive to ouabain (g-strophanthin). About a 10% decrease in activity was noted when red cells were incubated with the cardioglycoside at 10^{-4} M concentration.

In chickens with malaria (Table 6) erythrocyte ATPase activity decreased slightly during the prepatent period. Enzymatic activity was depressed at the time of onset of patent blood infection and remained depressed well into the late patent phase of the disease. The lowest level of cell enzyme activity, 0.630×10^{-10} μ M Pi/min/rbc, occurred shortly after

peak parasitemia. Erythrocytes collected from chickens in the late patent and in the recovery periods, when the major portion of the cell population was immature erythrocytes and young normacytes had enzyme activity greater than that of normal red cells from healthy chickens.

Some insight of the relationship of red cell ATPase activity to the stress of hemolysis induced by malaria was given by study of ATPase activity in red cells of chickens made anemic by injection on phenylhydrazine (Table 7). Following the initial injection of phenylhydrazine, when birds were anemic and had not yet compensated for extensive red cell loss, their red cells had significantly lower ATPase activity than normal cells. Later as the birds adjusted to the hemolytic stress by increasing cell production and releasing immature red cells from hemopoietic tissues ATPase activity increased. During this period red cell ATPase activity increased to normal levels or above (1.100×10^{-10} μ M Pi/min/rbc to 3.160×10^{-10} μ M Pi/min/rbc).

Erythrocyte lipids: Total erythrocyte phospholipid concentrations increased with the onset of active blood infection. Normal erythrocytes had 0.550 picograms of phospholipid per erythrocyte. At peak parasitemia phos-

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Figure 5. Localization of ATPase activity by cytochemical methods. Fig. 3a is an electronmicrograph of a thin section of normal chicken erythrocytes incubated prior to embedding with ATP substrate and lead nitrate. At sites of ATPase activity, electron dense lead has been deposited. Limiting plasma membranes have high ATPase activity whereas nuclear membranes have little. Figs. b, c, and d are micrographs of erythrocytes obtained from a chicken with malaria. The plasma membranes of erythrocytes from infected birds have less enzyme activity than have plasma membranes of erythrocytes from healthy chickens. Differences in ATPase activity within various plasmodia occur. Abbreviations: HN, host cell nucleus; hb, hemoglobin; P, intraerythrocytic plasmodia; na, nuclear ATPase activity; nma, ATPase activity in nuclear membranes; CMA, cytoplasmic membrane activity. Line marker equals one μ m.

Table 7. The relationship of hemolytic crisis and recovery to ATPase activity in erythrocytes of chickens injected with phenylhydrazine.

Day post-injection	Amt. drug given phenylhydrazine (IM)	ATPase activity ($\times 10^{-10}$ μ M Pi/min./rbc)*	Hematology		No. sampled
			pvc	% brbc	
0	—	1.100 \pm .099	34.3	0.0	12
1	10 mg	1.195 \pm .094**	29.4	6.3	12
2	—	—	—	—	—
3	20 mg	0.665 \pm .046**	25.2	39.0	12
4	—	—	—	—	—
5	30 mg	1.008 \pm .082**	24.5	47.5	12
6	—	—	—	—	—
7	40 mg	2.006 \pm .059**	26.6	38.3	12
8	—	—	—	—	—
9	0 mg	—	—	—	—
10	—	3.164 \pm .265**	31.5	1.8	12

* Mean \pm S.E.M.

** Value significantly different from normal at 5% level.

pholipid concentrations were greatly elevated (1.167 picograms per cell). High levels of phospholipid also occurred in erythrocytes from chickens in the late patent phase of infection when few parasitized cells were present but when basophilic erythrocytes were plentiful. During the recovery phase, red cell phospholipid concentrations approached normal levels again (0.726 picograms phospholipid per cell). The changes in cell cholesterol were much less dramatic than the changes in phospholipid. Concentrations remained relatively constant throughout the earlier phases of infection but rose progressively during the latter stages of the disease (Table 8).

Total phospholipid to cholesterol ratios were constant prior to initiation of infection and during the prepatent and recovery periods, with values of 2.8:1; 2.9:1; and 2.7:1 respectively. The patent period of infection was characterized by high total phospholipid to cholesterol ratios.

The phospholipid concentration of a sample

of red cells varied with the percentage of infected red cells and the percentage of basophilic erythrocytes in the sample. In chickens made anemic by phenylhydrazine injection, erythrocyte phospholipid and cholesterol concentrations increased in proportion to the percentage of basophilic cells in the sample (Table 9).

Individual malarial parasites contained about 0.68 picograms of phospholipid and 0.40 picograms of cholesterol. After adjustments were made in total red cell phospholipid concentration for the contributions of parasite phospholipids and phospholipids of young, immature cells, concentrations were in the normal range. Before infection there were 0.550 picograms of phospholipid per mature cell; at peak parasitemia after adjustment for parasite phospholipid there were 0.792 picograms; and during the late patent period of infection there were 0.559 picograms in each uninfected mature red cell. Cholesterol levels, in contrast, dropped progressively as the severity of infec-

Table 8. Phospholipid and cholesterol composition of erythrocytes of normal and *Plasmodium gallinaceum* infected chickens.

Stage of infection	Phospholipid per red cell ($\times 10^{-12}$ gr per rbc)	Cholesterol per red cell ($\times 10^{-12}$ gr per rbc)	Hematology		
			pvc	% irbc	% brbc
Normal (preinfection)	* 0.550 \pm 0.058 (12) —	0.196 \pm 0.008 (21) —	28.0	0.0	0.0
Pre-patent	0.631 \pm 0.045 (18) ns	0.218 \pm 0.005 (9) s	27.0	0.0	0.5
Early patent	0.791 \pm 0.058 (15) s	0.196 \pm 0.009 (12) ns	25.0	3.4	0.6
Middle patent	1.166 \pm 0.076 (11) s	0.225 \pm 0.013 (12) s	20.0	39.0	3.8
Late patent	1.198 \pm 0.096 (12) s	0.248 \pm 0.021 (10) s	17.0	16.8	48.6
Recovery	0.726 \pm 0.068 (6) ns	0.268 \pm 0.011 (6) s	25.0	8.4	5.1

* = mean value \pm standard error.

s = significantly different value from normal at 95% confidence level.

ns = not significantly different value from normal at 95% confidence level. Student's "t" test.

(n) = number sampled.

Table 9. The effect upon red cell phospholipid and cholesterol concentrations of injecting chickens with phenylhydrazine.

Day post-infection	Amt. drug given phenylhydrazine (IM)	Phospholipid per red cell (× 10 ⁻¹² gr per cell)	Cholesterol per red cell (× 10 ⁻¹² gr per cell)	Hematology	
				pvc	% brbc
0	—	* 0.649 ± 0.038 (14)	0.181 ± 0.009 (27)	27.0	0.0
1	5 mg	—	—	—	—
2	—	0.626 ± 0.040 (6)	0.171 ± 0.016 (6)	24.4	8.1
3	10 mg	—	—	—	—
4	—	0.924 ± 0.038 (4)	0.291 ± 0.024 (6)	26.8	23.0
5	10 mg	—	—	—	—
6	—	nd	0.292 ± 0.010 (6)	27.1	29.7

* Mean value ± standard error.
nd = no determination made.

tion increased. Adjusted red cell cholesterol values were 0.196 picograms per cell prior to infection; 0.089 picograms per cell at peak parasitemia; and finally 0.051 picograms/cell during the late patent phase of the infection.

Plasma chemistry: Blood plasma chemistry was significantly altered as a result of *P. gal-linaceum* infection (Table 10). Early in the infection plasma sodium levels fell and potas-sium concentrations increased. The plasma cation concentrations changed in a reciprocal relationship to the changes in intraerythrocytic sodium and potassium. During the late patent and recovery periods plasma potassium levels were below normal. The low plasma potas-sium levels were coincident to the elevated

red cell potassium levels which occurred at this time. Plasma sodium concentrations were depressed throughout the entire course of in-fection and only returned to preinfection levels during the late recovery period. The mean pH of plasma remained constant prior to the onset of patent blood infection. During the middle and late patent phases of infec-tion the pH of the blood plasma dropped. Dur-ing the patent phase of infection a wider range of pH values than normal occurred. Concen-tration of plasma lipids was higher than nor-mal. Phospholipids increased from preinfec-tion values of 1.65 mg/ml early in the patent phase of infection and decreased thereafter until nearly normal values were reached in the recovery period. Plasma cholesterol levels

Table 10. Changes in plasma of chickens infected with *P. gallinaceum*.

Determination	Sample period					
	Preinfection	Prepatent	Early patent	Middle patent	Late patent	Recovery
<i>Hematology</i>						
Hematocrit	30	32	32	25	19	28
% irbc	0	0	3	35	17	0
% brbc	0	0	0	3	32	7
<i>Plasma</i>						
Sodium (mg/ml)	3.79 ± .037**	3.40 ± .045	3.39 ± .077	3.60 ± .084	3.34 ± .181	3.53 ± .072
Potassium (mg/ml)	.155 ± .003	.166 ± .009	.171 ± .006	.182 ± .007	.148 ± .004	.142 ± .009
Chloride* (mg/ml)	4.33	nd	3.80	4.15	3.98	4.02
Phosphate (mg/ml)	.199 ± .022	.143 ± .003	.143 ± .007	.128 ± .002	.129 ± .006	.118 ± .009
pH	7.64 ± .042	7.67 ± .049	7.72 ± .031	7.38 ± .100	7.50 ± .083	7.78 ± .037
Phospholipid (mg/ml)	1.65 ± .048	2.17 ± .125	2.40 ± .214	2.22 ± .195	2.12 ± .212	1.52 ± .093
Cholesterol (mg/ml)	1.01 ± .064	1.17 ± .072	1.16 ± .038	1.21 ± .048	1.39 ± .080	1.18 ± .010
Protein (total) (mg/ml)	43.2 ± 1.43	44.3 ± 1.76	48.1 ± 1.83	53.5 ± 2.21	58.9 ± 2.87	55.1 ± 2.19
<i>UV absorption*</i>						
280 nm	.358	.318	.386	.455	.540	.559
260 nm	.234	.202	.236	.292	.364	.363
280/260	1.52	1.58	1.63	1.56	1.48	1.54
Blood urea*	2.0	nd	2.0	3.0	3.0	4.0
Nitrogen (BUN) (mg%)						
Blood sugar* (mg%)	222.0	nd	205.0	202.0	202.0	214.0
ATPase (μm Pi/min/ml)	.0213	.0196	.0180	.0210	.0201	.0166

* = pooled plasma from six animals.
** = mean value ± S.E.M.
nd = no determination made.

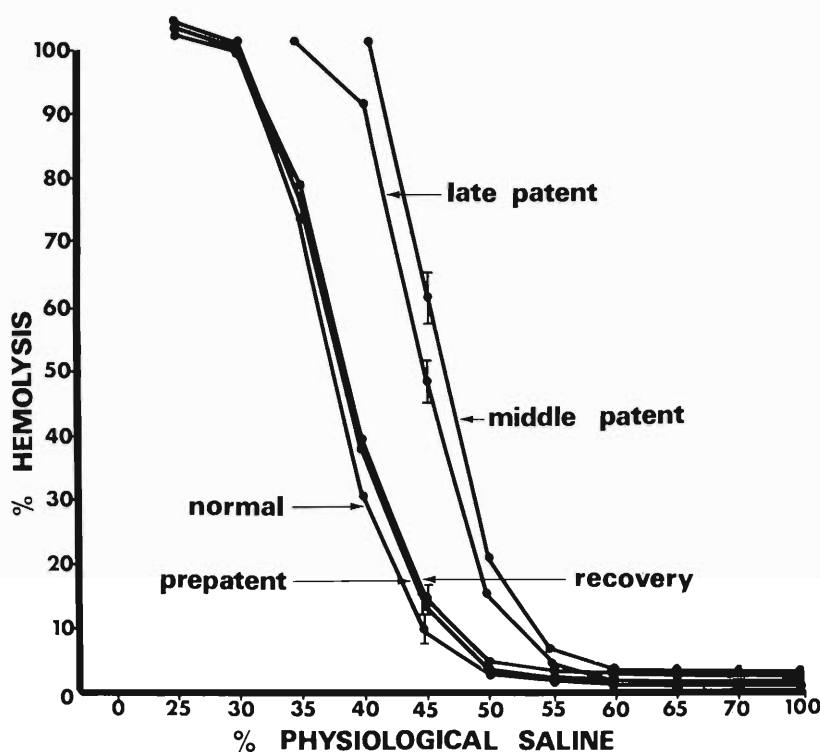


Figure 6. An osmogram of a multiple tube fragility test showing the osmotic fragility of normal chicken erythrocytes treated with plasma from chickens in the middle and late patent phase of *P. gallinaceum* infection. Osmotic properties of red cells treated with blood plasma of chickens recovered from malaria or in the prepatent phase of infection are similar to those of red cells incubated with normal, preinfection plasma. Vertical lines indicate range of values.

were elevated throughout the infection but were highest during the late patent phase. Total plasma proteins increased during the patent phase of infection and remained elevated in the recovery period. UV absorption at 280 nm by plasma collected during the patent phases of infection increased. There was a decrease in 280:260 UV absorption ratio during the late patent period of *P. gallinaceum* infection. Elevated urea nitrogen levels occurred during the late patent and recovery periods. Blood sugar decreased during the patent phase and then increased in the recovery period.

The most profound changes in blood plasma occurred during the middle and the late part of the patent period. Normal red cells incubated in plasma samples collected during these stages of infection were changed so

that they physiologically resembled red cells from infected chickens in the acute phases of infection (Fig. 6). The late patent phase plasma samples, which caused an increase in osmotic fragility of normal red cells, had low pH's. When the pH of late patent phase plasma samples was adjusted to values close to the pH of preinfection plasma, the osmotic fragility inducing capacity was reduced but not entirely lost.

ATPase activity of normal erythrocytes incubated with patent phase plasma was also reduced. The ATPase activity of whole red cells treated with normal plasma was 1.14×10^{-10} $\mu\text{M Pi/min/rbc}$. Red cells incubated with late patent phase plasma had an average activity of 0.90×10^{-10} $\mu\text{M Pi/min/rbc}$. If the pH of these plasma samples was adjusted to the pH of preinfection phase plasma before

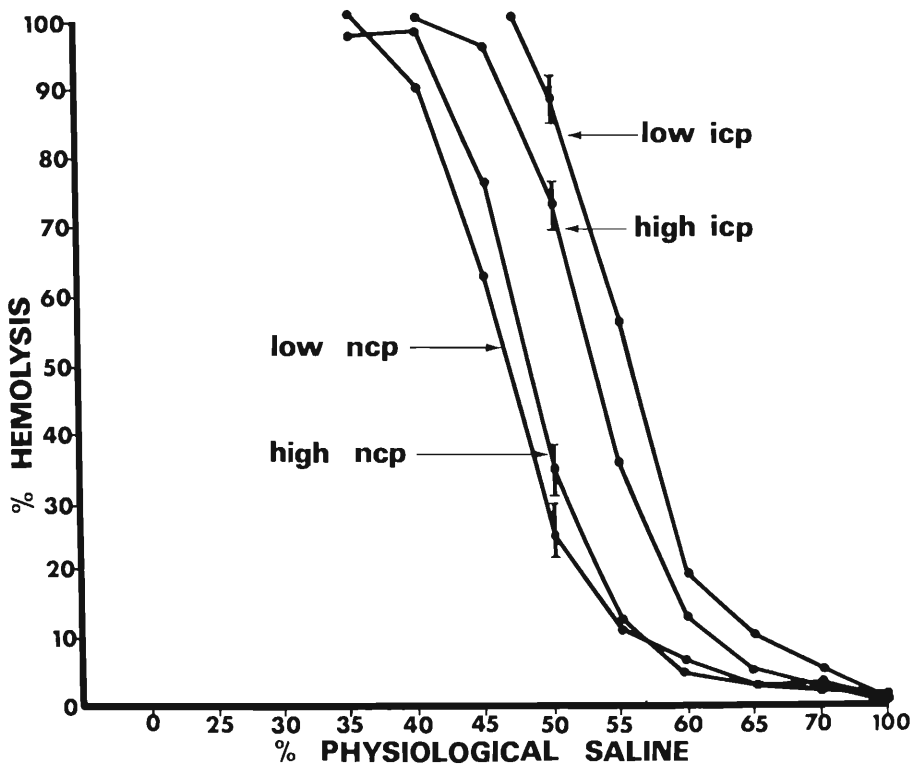


Figure 7. An osmogram of a multiple tube fragility test indicating the effects of treating red cells with fractions of normal and late patent phase plasma. Fractions of plasma were obtained by passing samples through membranes having molecular weight exclusion limits of 50,000, collecting the filtrate (low molecular weight fraction) and reconstituting the filtered plasma residue (high molecular weight fraction). Both the high and the low molecular weight fractions of patent phase plasma (icp) are more active in increasing osmotic fragility of normal red cells than are the control, preinfection plasma fractions (ncp). Vertical lines indicate the range of values.

the erythrocytes were incubated then the inhibitory effects were reduced but still clearly evident.

Both high ($> 50,000$) and low ($< 50,000$) molecular weight fractions of patent phase plasma had osmotic fragility inducing properties (Fig. 7). However the patent phase low molecular weight filtrate was considerably more active in inducing fragility than the high molecular weight plasma fraction. Red cells incubated with low molecular weight fractions of pooled normal plasma were more osmotically resistant than red cells incubated in the reconstituted, high molecular weight ($> 50,000$) filtered normal plasma residue. The low molecular weight fractions of patent

phase plasma had slightly lower pH's than normal plasma fractions and had elevated K^+ and decreased Na^+ concentrations. Incubation of normal red cells in patent phase low molecular weight plasma fractions (icp-low) reduced their ATPase activity 28%. Incubation of normal red cells with the equivalent, low molecular weight fractions of pooled normal plasma (ncp-low) also reduced red cell ATPase activity but to a lesser extent.

The red cells incubated in late patent phase plasma and low molecular weight plasma ultrafiltrates, despite their greater fragility and decreased ATPase activity did not have significantly shorter survival times than normal erythrocytes incubated in preinfection phase

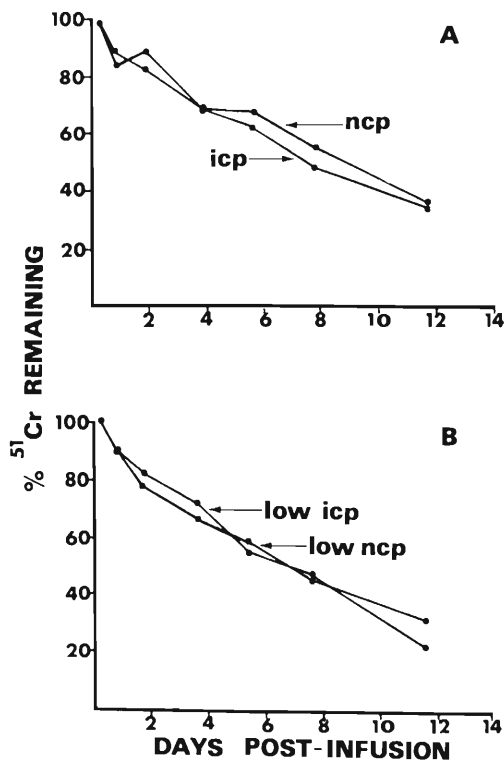


Figure 8. Effect of incubation in whole plasma and plasma ultrafiltrates on survival of Chromium⁵¹ labeled erythrocytes. Fig. 6a shows survival curves of erythrocytes incubated with plasma from healthy chickens and chickens in the late patent phase of infection. Little difference is noted between the two curves. Fig. 6b shows the survival curves of erythrocytes treated with low molecular weight ultrafiltrates of normal and late patent phase plasma. The red cells incubated in fractions of plasma from acutely infected chickens were not more rapidly destroyed than those incubated in normal plasma filtrates.

plasma when tested by the Chromium⁵¹ technique (Fig. 8).

Discussion

A variety of interrelated changes occur in the morphology, physiology and biochemistry of red cells of chickens undergoing *P. galinaceum* infection. The changes are induced by the infection and develop progressively as a consequence of it. Some of the changes are directly caused by the action of the parasites

and some are indirect consequences. The magnitude of red cell alteration increases as a direct result of increasing parasitemia during early stages of the infection and is greatly influenced late in the infection by the basophilia which occurs as an indirect consequence of the infection.

Morphology studies utilizing various light and electron microscopic techniques (Kreier *et al.*, 1972) and survival studies (Kreier *et al.*, 1967, 1968) have indicated that early in the infection when parasitemias are increasing nonparasitized red cells have nearly normal morphology and survive normally. Red cells containing small parasites also are almost normal in size and shape indicating that the merozoites produce little permanent morphologic damage by the act of penetration (Kreier *et al.*, 1972). The hypothesis (Ladda, 1969; Ladda *et al.*, 1969) that the merozoite enters the erythrocyte by a phagocytic type process with the membrane pinching closed over the intracellular parasite is compatible with observations made previously in this laboratory (Kreier *et al.*, 1972). The pinching off of the parasitic vacuole by the host cell membrane after entry would most probably be the cause of slit-like lesions (Fig. 2) which occur in red cell membranes near intracellular merozoites (Rudzinska and Vickerman, 1968; Trager *et al.*, 1966; Aikawa and Thompson, 1971). The suggestion that merozoite penetration results in little change in cell morphology is contrary to the observation of Arnold *et al.*, (1969, 1971) who have suggested that penetration results in gross erythrocyte lesions and open holes in the cell surface through which parts of the parasite may protrude. Arnold *et al.*, (1969, 1971) probably observed red cells which were caught by fixation while being penetrated by merozoites or erythrocytes containing large parasites which were undergoing lysis. Rinehart *et al.*, (1971), used scanning electron microscopy in combination with a latex fixation procedure specific for erythrocyte membranes, to demonstrate that erythrocyte membranes over intracellular parasites were distorted but still intact. This observation also supports the assertion that merozoite penetration leaves an intact erythrocyte membrane. Workers other than Kreier *et al.*, (1972) concluded that in early phases of infection intracellular parasite

growth is the major cause of gross erythrocyte alteration. Lewis and his coworkers (1969) concluded from their ion-etching and scanning EM studies that red cell morphology becomes more abnormal as the parasite matures. These workers reported not only progressive distortion of gross morphology of the erythrocyte as the parasite developed but also modifications of the limiting plasma membrane.

While the extent of morphologic alteration to the blood cells increases in proportion to the level of parasitemia during the early stages of patent parasitemia, late in the patent period of infection morphology deviating from that seen in normal animals is not limited to parasitized cells. A significant determining factor in the character of the blood cells during the late patent period of infection is the influx of new red blood cells produced under the stress of anemia. While the characteristics of these newly produced cells differ from the characteristics of cells in animals that have not undergone the stress of erythrocyte destruction their characteristics should not, except in the most critical way, be considered as part of the pathology of malaria.

The changes in morphology of erythrocytes from chickens with malaria just discussed parallel the changes in parameters of osmotic regulation reported in this study. Increases in red cell fragility are proportional to increases in parasitemia during the early phases of infection just as during this period morphologic abnormality is largely limited to parasitized erythrocytes. Fogel *et al.*, (1966) utilizing a recording fragiligraph reported that increased osmotic fragility was characteristic of animals in the patent phase of *Plasmodium gallinaceum*, *P. berghei*, *P. knowlesi* and *P. falciparum* infections and that a linear relationship existed between osmotic fragility and the percentage of parasitized red cells in blood samples from *P. berghei* infected hamsters. These workers also reported that nonparasitized erythrocytes separated from infected blood were more resistant to osmotic lysis than parasitized cells, but were less resistant than normal erythrocytes from healthy animals. Shen and his collaborators (1946) in reporting the results of testing the osmotic and mechanical properties of *Plasmodium knowlesi* infected red cell populations, suggested that

only those erythrocytes containing the larger and more fully developed parasites are abnormally fragile. This is in contrast to the observations made by various other investigators (Dannon and Gunders, 1962; Bahr, 1969) which indicate that both nonparasitized and parasitized erythrocytes are abnormally fragile. While these reports seem to be in conflict with one another, the conflict may be a result of failure of the workers to consider that not only is parasitemia changing during infection but the entire age composition of the erythrocyte population is changing also. If one tests the osmotic fragility of erythrocytes in blood samples taken from *P. gallinaceum* infected chickens during the early patent period, one finds a direct relationship between the parasitemia and cell fragility. If one tests fragility of erythrocytes in blood samples from infected chickens collected during the late patent period of infection a direct relationship between fragility and parasitemia is not observed. The stress of the anemia induced by the parasites induces production of defective reticulocytes which are fragile. Healthy chickens can be induced to produce similar fragile cells by stressing by extensive bleeding or by injection of hemolytic drugs.

Even though nonparasitized erythrocytes collected during the latter part of the patent period are quite fragile, still they are more osmotically resistant to lysis than parasitized cells. This is the factor which is responsible for the overall decrease in fragility which follows peak parasitemia. The entry of some physiologically normal young erythrocytes into the blood probably contributes to the decrease in osmotic fragility which occurs during the late patent period. In the late recovery period when the cell population is made up of predominantly physiologically normal, young erythrocytes, the erythrocytes are significantly more resistant to osmotic lysis than erythrocytes in pre-infection samples.

Alterations in volume occur as changes in red cell fragility take place. Mean cell volume increase during the early patent period of infection is a result of volume change in parasitized erythrocytes. Volume changes in nonparasitized red cells do not contribute much to the total volume change. The extent of red cell volume change induced by parasitization depends on the parasite and host system.

Mouse erythrocytes infected with *P. berghei* are reported to have mean cell volumes three times greater than normal. In contrast, heavily parasitized duck erythrocytes show little change in volume (Bahr, 1966). Volume increase may result from either increase in cellular water due to influx or increase in cytoplasmic substance. Weiss (1967) in our laboratory analyzed the buoyant densities of normal and *P. gallinaceum* infected erythrocyte samples by the copper sulfate, pycnometer and hydrometer float methods. By all three methods, parasitized cell samples had lower specific gravities (1.0404) than red cells from healthy chickens (1.0420). An influx of water into the cell was surely the cause of the volume increase which occurred in erythrocytes collected during the early patent period of infection. In the late patent period mean cell volumes were large due not only to the uptake of water by erythrocytes damaged by the infection but also due to the large numbers of large volume basophilic erythrocytes within the blood samples.

The alterations in fragility and volume which occur during infection roughly parallel changes in intraerythrocytic cation balance. Changes occur in red cell sodium and potassium somewhat before changes are detected in fragility, volume or gross cell morphology. Even in the prepatent period cell sodium increases and potassium levels fall. The magnitude of intraerythrocytic cation imbalance increases as the infection progresses. Other investigators have noted similar changes in intracellular ion concentration in animals with a variety of experimental plasmodial infections e.g. *P. berghei*, *P. coatneyi*, *P. falciparum* (Dunn, 1969a); *P. knowlesi* (Overman, 1948; Dunn, 1969a); and the avian malarial, *P. gallinaceum* (Overman *et al.*, 1950) and *P. lophurae* (Sherman and Tanigoshi, 1971). Kruszynski (1951) on the other hand reported increases in both cell sodium and potassium during *P. gallinaceum* infections. This result could have been obtained if the latter investigators' measurements were made on late patent phase blood samples in which erythrocyte potassium levels are high because of high basophilia. This explanation is suggested by observations on chickens stressed by phenylhydrazine injection which first undergo a hemolytic crisis and develop high cell sodium

and low cell potassium levels and then when basophil production occurs, develop high cell potassium levels. The cell sodium levels remain high because damaged erythrocytes with high sodium levels are still present. The effect on cell cation concentration of the presence in the samples of damaged red cells and of cells of various physiological types complicates the interpretation of measurements of cation concentration in erythrocytes of the sample.

The abnormality of cation concentrations in red cells of infected animals may result from either an increased permeability of the membrane to these cations or to change in the activity of active transport mechanisms. Dunn (1969a, b) suggests that elevated erythrocyte sodium concentrations during plasmodial infection result from impaired active transport mechanisms. This is a reasonable suggestion since ATPase activity is depressed when parasitemias are high. The suppressed ATPase activity includes the ouabain sensitive Na^+/K^+ ATPase transport system because total ATPase activity of uninfected erythrocytes is inhibited more by $\times 10^{-4}\text{M}$ ouabain than is the ATPase activity of infected cell populations. Incubating normal erythrocytes with plasma from acutely infected chickens depresses the total ATPase activity. This observation complements Dunn's (1969a) observation that there is a less active Na^+ flux from normal erythrocytes treated with blood plasma of monkeys in the acute phase of *P. knowlesi* infection than from normal erythrocytes incubated in normal plasma. Cell alterations induced *in vitro* by incubation in plasma are reversible while alterations resulting from parasitization are not. Red cells from *P. gallinaceum* infected chickens transfused into normal, healthy chickens are recognized as defective by healthy birds and removed at an accelerated rate (Schacter, 1969), whereas normal red cells which have increased osmotic fragility and decreased ATPase activity as a result of *in vitro* treatment have survival times *in vivo* similar to the survival times of normal cells treated with pre-infection plasma. It is probable that duration of exposure to the deleterious environment determines the reversibility of the damage.

A number of investigators have reported that red cells are altered antigenically during plasmodial infection and that these changes are

similar to those induced in normal cells by *in vitro* treatment with trypsin (Kreier *et al.*, 1966; Seed and Kreier, 1969; Gautum *et al.*, 1970). It is therefore probable that red cells are altered by circulating proteolytic enzymes during the acute phases of the disease. Normal red cells which have been trypsinized, like red cells from animals with acute malaria have a reduced electrophoretic mobility (Seed, 1969), are osmotically fragile and a large portion of their total ATPase activity is lost (Seed, unpublished observations). Trypsinization induces, as does infection, irreversible membrane damage as evidenced by shortened survival time after transfusion into healthy animals (Kreier, 1969). No one has looked for proteolytic enzymes in plasma of malarious animals. If they occur there and act on erythrocytes their action would explain much of the pathology of malaria including ATPase activity reduction, fragility increase, and ion imbalance, as well as antigenic modification.

Various investigators have suggested that the red cell destruction in animals with malaria is due in part to a circulating toxin (Overman, 1948; Zuckerman, 1964; Bahr, 1969; Dunn, 1969a, b; Herman, 1969). Evidence for such a toxin is based on demonstration, by *in vitro* techniques, of the ability of infected plasma to alter normal red cell physiology and bring about increased osmotic fragility (Bahr, 1969; Herman, 1969) and change cation transport (Dunn, 1969a). The work presented here substantiates that plasma from acutely infected chickens affects normal erythrocyte function. However the effect on erythrocytes of incubating them in plasma from animals with acute malaria was reduced by adjusting the pH of infected plasma to the pH of plasma prior to infection. Dunn (1969a) also reports that effects of infected plasma on cation transport are reduced greatly by adding certain metabolites and buffers to the plasma. These findings do not at all rule out the possibility that circulating toxins may contribute to cell pathology, but do confirm the belief that the physical state of the plasma during infection is a critical factor in production of cell pathology and premature erythrocyte senescence.

The increase in total plasma protein particularly of the strongly acid α M (Abele *et al.*, 1965) together with the reduction in pH which occur during the acute phases of infec-

tion suppress the ionization of acidic groups on the erythrocyte and reduce its net negative charge and increase the viscosity of the cell and of its suspending fluids. The sludging and capillary blockage resulting in erythrocyte damage and phagocytosis commonly noted in malaria probably result from the high plasma viscosity and the low surface charge of the erythrocytes (Brown, 1933; Findlay and Brown, 1934; Krishnar *et al.*, 1935; Nirady, 1969). A direct relationship between high plasma viscosity and short red cell life span has been experimentally demonstrated (Berlin, 1964). Murphy (1967) demonstrated that human red cells from healthy individuals and from patients with hereditary spherocytosis became more rigid and less filterable as the suspending fluids became more acid. The spherocytic cells were affected by pH change to a greater extent than normal erythrocytes. The pH values used by Murphy to demonstrate these effects were within the range of plasma pH values which occur in birds with *P. galinaceum* infections. Murphy suggested that red cell destruction in people with hereditary spherocytosis was a result of increased blood viscosity and increased rigidity of spherocytes brought about by the low pH of the plasma. Similar pH affects probably occur in animals with malaria.

The reduction in plasma pH during the patent phases of malaria also has the adverse consequence of causing reduction in the oxygen binding capacity of hemoglobin (Rigdon and Rostorfer, 1946) and this in turn may be in part responsible for the anoxic state which develops during infection (Palecek *et al.*, 1967).

The consequences of a reduction in the activity of the red cells cation pumps and the associated decrease in ATPase activity are far reaching. They include not only alteration in the intra and extracellular cation concentrations, water balance and osmotic fragility, but also alterations in the overall metabolic rate of the red cells because of affects on the intraerythrocytic ATP (Neerhout, 1968; Zarkowsky *et al.*, 1968). It has been proposed that the intraerythrocytic level of ATP is a prime factor in determining the severity of malarial infection (Powell *et al.*, 1966; Brewer and Coan, 1969). A positive correlation between red cell ATP levels prior to infection

and the rate of parasite growth and multiplication has been reported (Brewer and Powell, 1965). One might postulate that a transitory increase in red cell ATP resulting from an inhibition of erythrocyte ATPase might be a critical factor in accelerating parasite buildup. A transitory increase in red cell ATP early in the patent period of *P. berghei* infection has been demonstrated (Brewer and Coan, 1969). The increase occurs just as the decline in red cell ATPase we have observed begins. During the acute phases of *P. berghei* infection there is a significant decrease in red cell ATP (50%) far in excess of that which may be accounted for by parasite utilization (Brewer and Coan, 1969). The low ATP levels which occur as the disease progresses probably result primarily from inactivation of ATP synthesizing systems in the membrane but may result in part from a feed back repression mechanism which would be the ultimate consequence of depressed erythrocyte ATPase activity.

In addition to partial control of cellular metabolism the cation pump regulates the rate of turnover of red cell lipids (Jacob and Karnovsky, 1967). During the acute phase of *P. gallinaceum* infection when red cell ATPase activity is markedly depressed, the concentration of erythrocyte lipids is altered. Our studies indicated that in *P. gallinaceum* infected chickens, the intracellular plasmodia account for nearly all the increase in phospholipids in the erythrocytes during the middle patent phase of infection. Similar results were reported for *P. berghei* infected erythrocytes (Lawrence and Cenedella, 1969; Cenedella *et al.*, 1969) and *P. knowlesi* infected red cells (Rock *et al.*, 1971). Erythrocyte phospholipid concentrations are slightly higher than normal during the early and middle patent phases of infection, but for all practical purposes the phospholipid concentration remains nearly constant throughout the course of infection. The dramatic increase in host cell phospholipid, reported by Rao *et al.* (1970) to occur in rats with *P. berghei* infection may have resulted from these authors insufficient adjustment for the contribution of parasite lipids and in the late patent phase of infection for the high phospholipid content of basophilic erythrocytes. While there is only minimal change from normal in host cell

phospholipids during the patent period, if one excludes parasite and basophil phospholipid, there is a great decrease in erythrocyte cholesterol. The low erythrocyte cholesterol content might result from failure of plasma cholesterol to replace membrane cholesterol which is lost. The fact that high plasma cholesterol levels occur during the acute phase of infection suggests that such a process is occurring. That this is the case is also supported by the work of Cenedella *et al.* (1969) which indicates that normal erythrocytes incorporate labeled plasma cholesterol at a much greater rate than parasitized erythrocytes.

Murphy (1965) has shown that the shape and osmotic fragility of normal mammalian erythrocytes can be altered by changing red cell cholesterol concentrations or modifying cholesterol distribution in the membrane. Erythrocytes depleted of cholesterol are fragile. If the cholesterol is replaced the osmotic fragility decreases. In certain hepatic diseases such as Zieve's syndrome, the patients red cells have increased amounts of cholesterol and are often more resistant to osmotic stress than red cells of healthy individuals with normal lipid content (Westerman, 1968). As Neerhout (1968) points out, in a review of biochemical pathology in hematological disorders, in patients with hereditary spherocytosis there is a positive correlation between red cell lipid composition, altered cell permeability, activity of cation pumps and metabolic activity.

Pathology in animals with malaria is initiated by the plasmodial parasites which set in motion a chain of events not only in the physico-chemical condition of the red cells already present in the circulation but also initiate pathological and physiological changes in other systems of the animal. Changes first occur primarily in parasitized cells. Later in the course of infection, when more parasites and parasite products and more products of cellular destruction are present, changes also occur in nonparasitized cells. These changes are similar to, but develop more rapidly than the alterations in normal red cells which occur with aging and senescence (Dannon, 1967). Modification of erythrocyte surfaces by toxic products which inactivate functional enzymes of active cation transport systems might be the initial process in a

sequence of cellular events characterized by abnormal cation and water flux, lipid loss, and increased osmotic fragility.

Cell damage resulting in premature senescence of a large portion of the infected cell population initiates a response in the hemopoietic system which results in extensive change in the age distribution of the erythrocyte population and in the production of a population of crisis reticulocytes. The biochemical, physiological and morphological characteristics of the newly produced erythrocytes dominate the hematological picture from the late patent period through recovery. A population of these cells is not a normal equilibrium erythrocyte population in shape, size, membrane characteristics, or in general physiology. The characteristics of these newly produced cells are not the characteristics of cells made abnormal by the infection per se but are rather the characteristics of the cells contributing to the recuperative responses of the host.

Hypersplenism and autoimmunity are the two physiological responses most frequently suggested as causes of pathology in malarious animals. Both are the direct result of the introduction into the circulation of large amounts of modified autologous, and heterologous antigens. Hypersplenism and autoimmunity develop as the parasites grow and destroy erythrocytes and regress rapidly when parasites actions end. The parasite is the cause of the disease, and all pathology is a consequence either direct or indirect of its actions. The change associated with recuperative mechanisms is not pathology. The reversibility of the course of the disease following specific chemotherapy should be enough to make us reject hypotheses in which consequences of parasitization, direct or indirect, are elevated to independently causal roles. The occasional occurrence of an anomalous situation, such as blackwater fever following falciparum malaria, in which deranged physiological response becomes a prime cause of pathology should not confuse us as to the normal course of events and the role of the parasite in production of pathology.

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Composition of Phospholipids in *Plasmodium knowlesi* Membranes and in Host Rhesus Erythrocyte Membranes

R. A. DE ZEEUW*, AND J. WIJSBEEK

Laboratory for Pharmaceutical and Analytical Chemistry, State University, Groningen, 8004, The Netherlands

R. C. ROCK

Department of Laboratory Medicine, The Johns Hopkins Hospital, Baltimore, Maryland 21205

G. J. MCCORMICK

Division of Medicine, Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT. A study of phospholipid composition of membranes in *Plasmodium knowlesi* and a comparison with phospholipids in normal host Rhesus red blood cells and in *P. knowlesi*-infected red blood cells has been started. Such a study is necessary for investigations of lipid synthesis in the parasite and its possible relationship to that of the host erythrocyte.

A new two-dimensional thin-layer chromatographic system has been designed, with one development carried out as vapor-programmed TLC. The method provided complete separation of all phospholipid classes and is combined with a colorimetric phosphorus assay for precise quantitative determinations. Accuracy of the analytical procedure was shown to be good, with standard deviations for the major components in the 1–3% range.

Phosphatidylcholine and phosphatidylethanolamine are the major two phospholipid classes in the three types of membranes, but differences occurred particularly in the minor phospholipid classes. Sphingomyelin represents higher percentages in normal and infected erythrocytes than in parasites. Phosphatidylserine can be in the order of 10 per cent in normal and infected erythrocytes, but is virtually absent in parasites. Parasites, however, show higher percentages of phosphatidylinositol, which is very low in normal erythrocytes. This finding may represent a possibility to selectively interfere with parasite phosphatidylinositol metabolism without damaging the host.

Differences between normal erythrocyte membranes and those from infected animals could not yet be detected, except for a slightly decreased percentage of phosphatidylethanolamine in infected animals.

Further studies are necessary on an animal-to-animal basis as the present results on pooled samples indicate considerable differences between animals.

Phospholipids comprise a major portion of the cell membrane lipids of the malarial parasite, *Plasmodium knowlesi*. Parasite membranes were found to have significantly higher amounts of phospholipids than normal monkey host erythrocytes (Rock et al., 1971; Angus et al., 1972).

Initial work (Rock et al., 1971) on the composition of these lipids and those in the host erythrocyte membranes of Rhesus monkeys (*Maccaca mulatta*) has revealed a number of differences between these membranes. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the two major components in both classes, but parasites contained less phosphatidylserine (PS) and

sphingomyelin (SM) than the red cell and more phosphatidylinositol (PI).

However, complete separation and exact quantitation of PS and PI posed a major problem as their spots overlapped in two-dimensional thin-layer chromatography. Although various systems have been described that show adequate separation between these two components (Broekhuysse, 1968; Rouser et al., 1969; Turner and Rouser, 1970), none of these were completely satisfactory in our hands. This is most probably due to the composition of our samples, of which large quantities have to be applied to the plate in order to detect and quantitate the minor phospholipid classes of the parasite and the red cell.

The above mentioned differences in phospholipid composition between the parasite and the host erythrocyte clearly indicate that they

* Address after 1 September 1972: Department of Chemistry, University of Maryland, College Park, Maryland 20742.

occur particularly among the minor phospholipid classes PS, PI and SM. Therefore, precise data on these classes are a prerequisite for an intelligent study of lipid synthesis in the parasite and its possible relationship to lipid metabolism pathways in the host erythrocyte.

This report describes an improved thin-layer chromatographic system for complete separation of all phospholipid classes encountered, combined with a colorimetric phosphorus assay that allows precise quantitative determinations. Phospholipid composition values are reported for normal Rhesus red blood cell membranes, membranes of *P. knowlesi* infected red cells after removal of all parasites from the host cell, and washed membranes of whole lysed parasites.

Materials and Methods

All chemicals used were of reagent grade.

Phospholipids

Phospholipids of normal Rhesus erythrocyte membranes, of *P. knowlesi* infected erythrocyte membranes and of whole lysed parasite membranes were isolated at the Walter Reed Army Institute of Research as described previously (Rock et al., 1971). Lipid extracts, containing pooled material of 2–7 animals, were concentrated by evaporation under nitrogen and stored in chloroform at -20°C . 2,6-di(tert.)-butyl-p-cresol (Eastman Kodak, Rochester, New York) was used as an antioxidant in a concentration of about 0.5 per cent. Transportation to the Netherlands was done in dry ice in a Dewar flask and required less than 24 hours. Samples were stored at -20°C until time of analysis, in tightly sealed tubes flushed with nitrogen and were stable for at least one year under these conditions, even when tubes were repeatedly opened for analysis.

Phospholipid standards were used as reference materials. PC, PS, SM and LPC were obtained from Sigma, St. Louis, Missouri; PE and LPE were from British Drug Houses, Poole, England.

Thin-layer chromatography

Silica gel 60 HR (Merck, Darmstadt, W. Germany) was used as sorbent, 40 g/90 ml of demineralized water to prepare 5 plates, 20

$\times 20$ cm, for a layer thickness of 0.4 mm. After spreading, the plates were air-dried for 15 min., heated for 30 min. at 110°C in an oven with a fan, then cooled and stored in a desiccator. Samples, usually containing 1–2 μg of lipid phosphorus per μl , were applied to the plate in the lower left hand corner, 2.5 cm from the side edges, by means of a Hamilton syringe 70IN. Sample size was usually 10 μl and spotting was done under nitrogen.

Two-dimensional development was carried out as follows. Plates were developed in the first direction with chloroform-methanol-25% ammonia (55+35+7, v/v) as solvent in a saturated chamber to a height of 17 cm. Saturation time was 30 min. and the chamber was lined with filter paper. After completion of the first run plates were dried for 15 min. under a stream of nitrogen.

Development in the second direction was carried out under vapor-programmed conditions (De Zeeuw, 1968) in a VP-chamber (Desaga, Heidelberg, W. Germany).

For this purpose, strips of gel, 0.5 cm wide at the side edges and the bottom edge are removed from the plate. The developing solvent was chloroform-methanol-water (55+40+5, v/v) and the liquid composition in the troughs was (C = chloroform, M = methanol, W = water): trough 1: C-M-W (55+45+5), trough 2: C-M-W (40+55+5), trough 4: C-M-W (30+65+5), trough 7: C-M-W (20+75+5), trough 10: M-W (95+5), trough 13: M-W (95+5), trough 16: M-W (95+5), troughs 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 19, 20, 21: chloroform. Spacers were 1 mm thick, saturation time was 10 min., development was to a height of 18 cm, cooling temperature was 20°C . All experiments were done at $21 \pm 0.5^{\circ}\text{C}$ and at a relative humidity of $50 \pm 2\%$.

Detection of the separated phospholipids was performed by iodine vapor or by using the molybdenum spray reagent of Dittmer and Lester (1964). This reagent is specific for phospholipids, which show up as blue spots. In some instances the ninhydrin spray reagent of Skipski et al. (1962) was used prior to the molybdenum blue spray to detect the amino-phospholipids PE, LPE and PS.

Color development was done at 110°C for 20 min. Identity of separated phospholipids

was confirmed by means of comparing infrared spectra and chromatographic behavior with those of phospholipid reference standards.

Phospholipid phosphorus determination

Following detection and marking out of the spots, fractions were scraped into small sintered glass funnels with a razor blade and eluted 4 times with 2-ml portions of a mixture of chloroform-methanol-acetic acid-water (50+39+1+10, v/v). A blank area of average spot size was also collected and carried through the procedure. To each eluate 4 ml of 4 M ammonia was added and the mixtures were shaken and centrifuged (Renkonen, 1971). The upper phase was carefully removed by suction and discarded. The lower phase was transferred to a micro Kjeldahl flask and evaporated to dryness under nitrogen. 0.2 ml of 96 per cent sulfuric acid was added and the mixture digested in a sand bath for 60 min. at 200°C. After addition of 2 drops of 30 per cent hydrogen peroxide and reheating for 10 min. at 200°C, samples were colorless and were cooled to room temperature. Phosphorus was then measured by the method of Chen et al. (1956). The sample was transferred to a 10 ml Pyrex tube with Teflon screwcap with the aid of 4.0 ml of demineralized water. Then 4.0 ml of reagent C was added (reagent C consists of 1 volume 6 N sulfuric acid, 2 volumes of demineralized water, 1 volume of 2.5 per cent ammonium molybdate and 1 volume of 10 per cent ascorbic acid). After mixing, the sample was incubated for 90 minutes in a water bath at 37°C. After cooling, absorbance was measured against the sorbent blank at 830 nm in a Zeiss PMQ II spectrophotometer (Zeiss, Oberkochen, W. Germany). The sensitivity of the method was 0.05–0.1 μ g of lipid phosphorus, representing about 1.25–2.5 μ g phospholipid. Absorbance was linear up to at least 18 μ g lipid phosphorus ($A=1.72$). Total recovery was measured:

- a. against a total sample spotted on a TLC plate that was not developed with solvent but otherwise treated in the same way as described above;
- b. against direct digestion of a total sample followed by phosphorus analysis.

Both methods gave identical recoveries of $96 \pm 2\%$.

Results and Discussion

The two major separation problems were to obtain complete separation between PS and PI, and to prevent major spots (PC and PE) from overlapping minor ones (PS, PI, SM). The latter holds in particular for parasite membrane samples, in which PC and PE comprise 85–90% of the total phospholipids. Hence, in order to obtain acceptable data on the quantitation of the minor components in such samples, rather large amounts had to be applied on the plate, which, in turn, resulted in large spots of PC and PE, due to diffusion and overloading. With the two-dimensional thin layer chromatography system complete separation of all phospholipid classes encountered could be obtained.

The availability of a constant temperature and humidity room was an important factor in improving the reproducibility of the separation. Variations in the relative humidity had a marked effect on the migration rates of PS and PI in particular. At low relative humidities (<40 per cent) some spots lost their compactness due to tailing which was most pronounced with PS. At high relative humidities (>80 per cent) spot compactness decreased due to too much diffusion, especially with PE and PI.

The molybdenum blue spray reagent allowed for a specific and sensitive detection of the phospholipids. The limit of detection is in the order of 0.05 μ g lipid phosphorus, depending on the compactness of the spot. By using the less sensitive iodine vapor detection we found various spots present between PE and the 2nd solvent front in almost every sample. In this area less polar phospholipids such as cerebrosides and diphosphatidylglycerol may be expected (Broekhuysse, 1968), but a negative reaction was obtained with the molybdenum blue reagent. Detection of the amino-phospholipids PE, LPE and PS by using the ninhydrin spray can be done prior to spraying with molybdenum blue. The latter two spray reagents did not affect the phosphorus assay, provided the plates were sprayed evenly and under reproducible conditions (amount of spray reagent

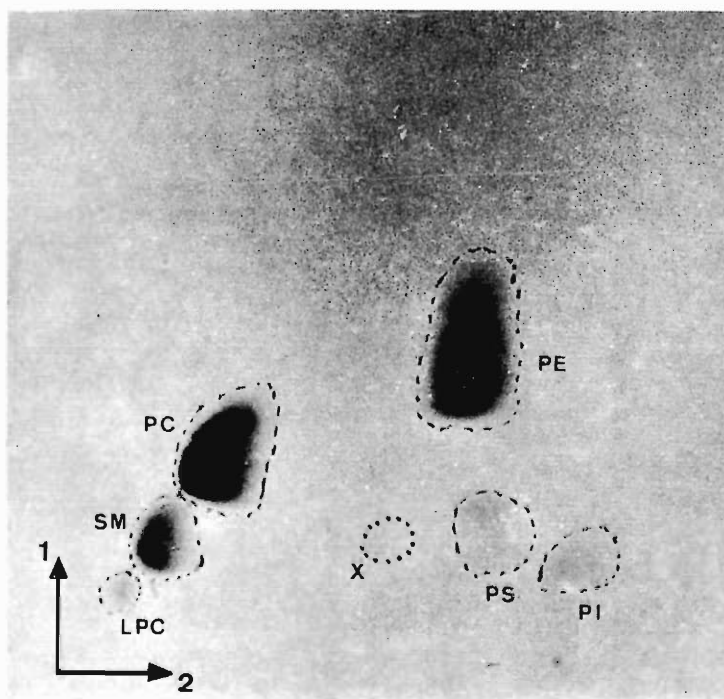


Figure 1. Two-dimensional thin-layer chromatogram of host erythrocyte membrane phospholipids (sample N₂, spotted quantity 12 μ g of lipid phosphorus). Direction of development indicated by arrows. Detection with the molybdenum blue spray reagent. Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; X, tentatively identified as phosphatidic acid; Y, unidentified phospholipid. X and Y indicate positions in the chromatograms; the components were not detected in the chromatograms shown (see text).

used, distance of spray bottle to the plate, pressure on spray bottle, etc.).

The colorimetric phosphorus determination is based on the reaction of phosphate ions with ammonium molybdate, to form ammonium phosphomolybdate $(\text{NH}_4)_3\text{P}(\text{Mo}_3\text{O}_{10})_4$, which is then reduced by means of ascorbic acid to yield the intensely colored molybdenum blue. This is not a stoichiometrically well-defined component, in which both molybdenum (IV) and molybdenum (V) occur (Fischer and Peters, 1968). Therefore, the reaction must be carried out under standardized conditions. It was also found that substances present in the silica gel can be eluted and then influence the degree of color development. These substances, the character of which is unknown, cannot be removed by predeveloping the plate before use. More successful was washing of

the spot extracts with diluted ammonia. This step has been recommended by Renkonen (1971) to remove silver ions and dichlorofluorescein from lipid extracts in silver nitrate chromatography, but proved to be equally useful for our impurities.

Figures 1 and 2 show typical two-dimensional chromatograms of normal red cell membranes and parasite membranes, respectively. The respective positions of two less frequently encountered phosphorus-containing spots have also been indicated. These are an unknown spot in sample P₄ and a spot tentatively identified as phosphatidic acid (PA) in sample I₂. Table 1 lists the phospholipid composition of membrane samples analyzed. Values found by Rock et al. (1971) are given for comparison. Standard deviations are given in parentheses. It can be seen that the present

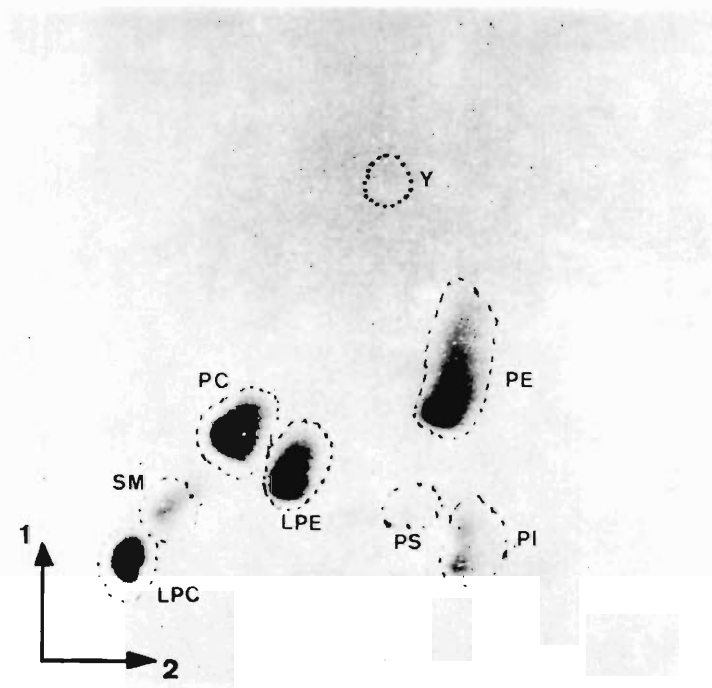


Figure 2. Two-dimensional thin-layer chromatogram of *P. knowlesi* membrane phospholipids (sample P_2 , spotted quantity 17 μg of lipid phosphorus). Further details as in Fig. 1.

methodology offers smaller standard deviations which is especially important for a more precise evaluation of the minor components.

The results on the three types of membranes are essentially in agreement with the findings of Rock et al. (1971). PC and PE represent the major phospholipid classes in all types, whereas PS, PI and SM are minor components. Normal erythrocyte membranes and erythrocyte membranes of infected animals were previously found to have similar phospholipid compositions, but the present data suggest one small but interesting difference to exist: PE is slightly lower in infected cells than in normal erythrocytes. The values obtained for the minor components show considerable variations, preventing more definite conclusions except that LPC seems to be a decomposition product rather than an intrinsic membrane component. It should be remembered that the membranes of infected animals were freed of parasite material and that they represent a mixture of parasitized and non-

parasitized cells as samples were obtained after development of 40–60 per cent parasitemias. Thus, one could only expect to detect major changes in phospholipid composition. The present results indicate that analysis of additional samples is necessary, in which a differentiation is made between non-infected and infected red cell membranes of parasitized samples. Such differentiation would allow for an easier detection of minor changes between infected erythrocytes of parasitized animals, non-infected erythrocytes of parasitized animals, and normal erythrocytes of healthy animals, respectively.

Parasite membranes differ in a number of ways from normal red cell membranes and infected red cell membranes, the general differences being as follows:

1. PC and PE comprise 85–90 per cent of total phospholipids in parasites, against 70–80 per cent in normal and infected cells.
2. PS is very low in parasites, whereas it

Table 1. Phospholipid composition of membrane lipid extracts.

Membrane	Percentage of total phospholipid						
	PC	PE	PI	PS	SM	LPC	LPE
Normal red blood cell membranes							
Sample N ₁ n = 10	42.8(0.88)	33.2(0.90)	5.4(0.41)	9.0(0.35)	7.2(0.38)	2.2(0.22)	—
Sample N ₂ n = 10	43.8(1.30)	36.5(0.95)	1.2(0.14)	2.7(0.22)	14.2(0.50)	1.6(0.17)	—
Sample N ₃ n = 4	39.5(0.70)	32.0(0.90)	0.8(0.15)	6.9(0.32)	19.5(0.40)	1.3(0.16)	—
Rock et al. (1971) n = 28	36.2(3.2)	31.1(2.6)	2.9(1.5)	11.3(1.8)	14.8(3.4)	3.3(1.7)	—
<i>P. knowlesi</i> infected host membranes							
Sample I ₁ n = 4	44.2(1.39)	25.7(0.61)	2.8(0.49)	10.1(0.47)	16.5(0.42)	<0.5	—
Sample I ₂ * n = 4	45.4(1.15)	29.4(1.05)	2.9(0.73)	7.0(0.47)	12.5(0.55)	—	—
Rock et al. (1971) n = 12	40.2(6.7)	28.2(4.4)	2.3(1.2)	12.5(4.8)	12.5(3.9)	4.3(2.1)	—
Whole <i>P. knowlesi</i> membranes							
Sample P ₁ n = 10	49.1(0.58)	42.5(0.80)	6.1(0.37)	<0.5	2.3(0.33)	<0.5	—
Sample P ₂ n = 7	18.1(0.78)	20.9(0.66)	6.0(0.55)	<0.5	4.3(0.55)	29.4(0.66)	21.3(0.54)
Sample P ₃ n = 8	18.4(0.53)	21.0(0.62)	5.0(0.47)	<0.5	2.7(0.40)	30.4(0.61)	22.5(0.62)
Sample P ₄ ** n = 4	46.7(0.67)	41.8(1.43)	5.8(0.50)	2.3(0.34)	2.3(0.37)	—	—
Rock et al. (1971) n = 16	44.7(6.4)	40.9(5.8)	8.1(4.9)	1.7(1.3)	3.2(3.9)	1.4(2.4)	—

Results are expressed as percentage of total lipid phosphorus, with the value of one standard deviation in parentheses.
n = the number of individual determinations.
* In sample I₂ a component tentatively identified as phosphatidic acid (PA) was found, comprising 2.7% (0.41) of total lipid phosphorus.
** In sample P₄ an unidentified phospholipid was found which comprised 1.2% (0.28) of total lipid phosphorus.

- comprises up to about 10 per cent in normal and infected cells.
3. PI in parasites is higher (6–8 per cent) than in normal and infected cells (1–5 per cent).
 4. SM is lower in parasites (2–3 per cent) than in normal and infected cells (7–20 per cent).
 5. The ratio PS/PI in parasites is <1 whereas in normal and infected cells this ratio is >1.

The findings suggest that PS is not an intrinsic membrane component in parasites and that its presence in parasite samples is a marker for host red cell membrane contamination (Rock et al., 1971). The results for PI indicate the component to be a minor but important constituent in parasites, in particular if we remember that parasites have higher amounts of phospholipids than host erythrocytes. On the other hand, the low PI values for the latter membranes, especially in sample N₃, may suggest that PI is not an essential phospholipid in red cell membranes. This finding could be useful in the search for therapies that will interfere with the parasite but which do not, or not in the same degree, influence the host. SM seems to be an intrinsic membrane component in parasites.

However, it can also be seen from Table 1 that significant differences occurred from sample to sample within a particular membrane

class. This makes interpretation of the present data more complicated.

As all samples consisted of pooled material it is obvious that data on an animal-to-animal basis are required as pooled samples may hide important information. Work on an animal-to-animal basis should be done on normal red blood cells, on infected and non-infected red blood cells of parasitized animals and on parasites, all obtained from one and the same animal. In addition to this it may be useful to study the phospholipid composition of normal host plasma and infected host plasma as well.

Two samples of parasite membranes (P₂ and P₃) showed a rather unusual composition in that they contained large amounts of lysophospholipids, i.e. lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC). No other lyso-components could be detected. However, if we consider the sums of LPE + PE and LPC + PC, respectively, it can be seen that these sums in P₂ and P₃ are in fairly good agreement with the values for PE and PC alone in the samples P₁ and P₄. No explanation for these high amounts of lysophospholipids can be given. It may well be that a phospholipase has played a role. Moreover, these unusual samples P₂ and P₃ were characterized by a reddish-brown color, whereas “normal” samples were slightly yellowish. The reddish-brown colored substance migrates on the plate between SM and

PC, but does not contain detectable amounts of phosphorus. Values for LPC in the other samples indicate this component to be a decomposition product instead of an intrinsic membrane component.

In parasite sample P₁ an unidentified phospholipid was found, comprising about one per cent of total lipid phosphorus, whereas a spot, tentatively identified as phosphatidic acid, was encountered in sample I₂, which may be a decomposition product. No other phospholipids were detected with the present method, so that their possible concentration must be less than 0.2 per cent. Detection studies were carried out by spotting at least 25 µg of lipid phosphorus. No phosphorus containing material could be found in the solvent from or at the origin.

It becomes evident that for an adequate evaluation and interpretation of the various differences in phospholipid composition a larger number of samples should be screened to obtain more reliable data and a better insight in "general" phospholipid distributions and in extremes. This is now being carried out on an animal-to-animal basis.

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Comments on Physiology and Molecular Biology

DONALD FAIRBAIRN

Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003

At the risk of uttering platitudes, I am going to comment generally rather than specifically on the biochemistry and physiology of the malarial parasite. One reason is that I do not myself work in this field but instead have been concerned with the biochemistry and host-parasite relations of helminths. It would be presumptuous for me to pose as an expert on *Plasmodium*. I do feel, however, that those who study *Plasmodium* may not be fully aware of lessons we have learned in the study of helminths, some of which may be worth mentioning.

No eucaryotic parasites are available in quantities that permit their use on the scale, for example, that bacteriophage is used in molecular biology. Our task is made more difficult by this limitation of biological resources, and our experiments and hypotheses must be highly selective. Nevertheless, we all know that some parasites in a given group are more available than others. I think it is fair to claim that in the field of helminthology the many thorough-going studies that have been made on two relatively abundant species, *Ascaris* and *Hymenolepis*, have already established a useful basis for the critical and necessarily more selective study of species that are of greater importance in medicine and public health. *Ascaris* and *Hymenolepis*, in short, have begun to serve as models for the host-parasite relationship upon which we are in a position to base our working hypotheses concerning other parasitic helminths. In any group of organisms it pays to have a model with which other organisms can be compared. Perhaps it is important for malariologists to search consciously for one or two model species among the plasmodia. In doing so, the lesson states that availability must be the primary criterion for selection. Perhaps some would argue that this is all very well for biochemistry but is not necessary for physiological studies. This argument has little validity because physiological findings lead naturally to biochemical investigations. In the unlikely case that availability and economic

or medical importance are combined in a single species, one should, I suppose, count one's blessings. I might add that models in one group of organisms may serve as a source of ideas for other groups. The unconventional electron transport system discussed by Dr. Homewood and associates in *P. berghei* is similar to that first described in *Ascaris* by Bueding and others, and since discovered to be present, with some variations, in a number of other helminths. The helminth literature on electron transport also suggests that there are additional, critical experiments that might substantially extend the understanding of the *P. berghei* system.

Biochemists accept as a truism the statement that the study of metabolism may be self-defeating unless a lot is known about the chemical composition of an organ or organism. Chemical composition is the foundation upon which we build. Biologists, I fear, have been less willing to perform these detailed, necessary, and tedious analyses whose ultimate worth has been proved over and over again. The paper by de Zeeuw and associates on membrane phospholipids in *P. knowlesi* doesn't, at this moment, tell us anything to which we can attach great significance. But eventually this sort of work has to be done. I think it illustrates the lesson that our understanding of the metabolism of eucaryote parasites is of primary interest when it is compared and related to that of the host. In this particular example, we do know a lot about the chemical composition of the host membranes, but don't really understand why the proportions of the various lipids are fixed. This being so, we are hardly able to gauge the significance of somewhat different proportions that are characteristic of the parasite.

Those who study the biochemistry and physiology of parasites are committed to the study of particular organisms. For this reason they cannot hope to confine themselves to narrow technical specialties, but must be prepared to become generalists who are able and unafraid to extend their experimental

activities as knowledge of their organisms grows. This takes courage, humility, and a great deal of care. No one needs to be told, for example, that the metabolic rhythms of the malaria parasite are extraordinarily important. Nor can anyone who looks over the literature on circadian rhythms fail to be impressed by the complexity of the stimulus-response patterns. An organism may be sensitive to a stimulus for only a few moments of its life, and experts distinguish with difficulty between primary and secondary stimuli. Literally, the world is too much with us when we study biological rhythms. All this is made apparent from the work of Arnold, Berger, and Flesher on the rhythms governing growth and division of *P. berghei*. Although it is a brave beginning, I suspect, and feel sure the authors are aware, that it is only a beginning. There can be no simple answers to questions concerning biological rhythms, for they encompass the entire history of a host-parasite relationship.

Finally, we are beginning to learn the lesson that parasites are seldom 'degenerate' organisms, and we should be prepared to resist this age-old myth that has arisen only because of their relative structural simplicity. In terms of metabolic control and regulation, and responsiveness to external signals, any organism with as complicated a life history as the average parasite can scarcely be thought of as being simple, let alone degenerate. In helminth parasites we are beginning to learn a little about the bewildering complexity of metabolic controls, and Searcy has provided

evidence that the genome size of a free-living flatworm is actually smaller than that of a parasitic flatworm such as a trematode. It should therefore not surprise us to learn in the paper of Bahr and Mikel that the nucleus of *P. berghei* contains a lot of DNA. Granted that, as in other eucaryotes, much of this DNA may not be transcribed, we should nevertheless bear in mind that plasmodia not only lead a reasonably complicated life in their secondary hosts, but quite possibly have an even more complicated existence in the insect hosts. Genes operating at one point in the life cycle will be repressed at another point. We know that this is true for helminths, and may be sure that it is also true for *Plasmodium*. We must take care not to perpetuate the ancient error that when we look for some biological function and do not find it, we can justifiably conclude that that property has been 'lost'. Such a claim cannot possibly be made until the entire life cycle has been analyzed. *P. knowlesi*, for example, is an aerobic fermenter in the erythrocytic stage and has an unconventional electron transfer chain. Although nothing seems to be known about the metabolism of this or other plasmodia in the insect host, there is a distinct possibility that such metabolism is aerobic and thoroughly conventional. This is already known to be true in trypanosomes and some of the helminth parasites. I believe we have no choice but to recognize the genetic complexity of all such parasites, and to include it in all our working hypotheses.

VII

ENTOMOLOGY

Comparative Susceptibility of *Anopheles balabacensis* and *Anopheles minimus* to Naturally Occurring *Plasmodium falciparum* in Central Thailand

R. N. WILKINSON, D. J. GOULD, AND A. BOONYAKANIST

Department of Medical Entomology, U.S. Army Medical Component, SEATO,
APO San Francisco 96346

ABSTRACT. *Anopheles balabacensis* and *Anopheles minimus* were fed simultaneously on 36 subjects with natural infections of *Plasmodium falciparum*. *A. balabacensis* was more susceptible to infection than was *A. minimus*. *A. balabacensis* was infected more often and the proportions infected and oocyst indices were higher than in *A. minimus*. Little association was apparent between gametocyte densities and infection in either mosquito species. The development of oocysts did not influence mosquito mortality in this study.

Anopheles balabacensis and *Anopheles minimus* are recognized as the most important vectors of human malaria in Thailand today. *A. minimus* was first established as a vector of human malaria in Thailand in 1933 by Payung Vejjasatra and was considered the only important malaria vector until the early 1960's when *A. balabacensis* became recognized as a highly efficient vector in the forested areas of the Kingdom (Scanlon and Sandhinand, 1965). A list of the important references concerning these two vectors in Thailand up to 1968 has been provided by Scanlon and associates (1968).

Today, *A. balabacensis* is considered to be the primary malaria vector in Thailand. The once important role of *A. minimus* in transmission of human malaria has been reduced, primarily because malaria control programs utilizing DDT residual house spray have been very effective against this species.

Several investigators have noted that *A. balabacensis* was the vector in areas of S.E. Asia where chloroquine resistant strains of *Plasmodium falciparum* were detected (Scanlon and Sandhinand, 1965; Montgomery and Eyles, 1963; Rutledge et al., 1969), and the question has been posed as to the role of *A. balabacensis* in the transmission of these strains of *P. falciparum*. The present study was designed to determine if there is a difference in the susceptibility of colonized *A. balabacensis* and *A. minimus* to infection with naturally occurring *P. falciparum* in an area

where chloroquine resistant *falciparum* malaria is endemic.

Materials and Methods

This study was conducted in the town of Phra Phutthabat, Saraburi Province, which is located in Central Thailand approximately 130 Km north of Bangkok. This area has been described in more detail by Rutledge et al. (1969), and it has been shown to be highly endemic for chloroquine resistant strains of *P. falciparum* (Colwell et al., 1972). The subjects utilized in this study were selected without regard to prior schizonticidal therapy from persons presenting at the local Malaria Eradication Center in Phra Phutthabat or at the Phra Phutthabat District Hospital for treatment of acute malaria. Blood smears from these patients were stained with Giemsa's stain and examined for parasites. Parasite densities were determined by the Earle-Perez (1932) thick film method as modified by Powell and co-workers (1964). Areas of the thick film near the edge were avoided when counting parasites in this study. Adult or adolescent subjects of both sexes with single infections of *P. falciparum* and gametocyte densities of at least 100 per cmm were selected for study. Severely ill subjects or those with renal and/or cerebral complications were excluded. The subjects came from such a wide geographic area that follow-up blood smears for determining the status of chloroquine susceptibility could not be obtained.

The *A. balabacensis* and *A. minimus* used in this study were colonized strains maintained

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Table 1. The median and range for the number of engorged and dissected mosquitoes (groups of 50) for 36 attempted infections.

		<i>A. balabacensis</i>		<i>A. minimus</i>	
		In-fected	Not in-fected	In-fected	Not in-fected
No. groups		24	12	16	20
No. fed	Median	41.5	38.0	25.5	22.5
	Range	31-49	18-48	7-42	7-44
No. dissected (survived)	Median	37.5	32	20.0	17.5
	Range	30-47	15-47	6-38	6-33
Percent fed dissected		90.3	84.2	78.4	77.7

in an insectary by the forced mating methods used by Esah and Scanlon (1966). The insectary was kept at approximately 28°C and 85 percent relative humidity and had a natural photoperiod. The *A. balabacensis* colony was originally established in 1964 from mosquitoes collected at Khao Mai Kaeo, Chon Buri Province (Scanlon and Sandhinand, 1965; Esah and Scanlon, 1966). The *A. minimus* colony was established in 1968 from collections in Saraburi Province. Both colonies had been maintained at a relatively low numerical level until the numbers were increased during 1971 for the purpose of this investigation. Eggs from wild females collected in Saraburi and Nakhon Ratchasima Provinces have been added periodically to both colonies during the period of this study in an attempt to maintain the natural susceptibilities of these two species to infection with *P. falciparum*.

Simultaneous feeding of *A. balabacensis* and *A. minimus* was accomplished by placing a group of 50, three to five day old unmated females of each species in separate cylindrical plastic feeding cages and securing one cage to each forearm of the subject for approximately one hour. Species were alternated between left and right forearms of consecutive patients. After feeding, the cages were removed from the subjects forearms, and the engorged individuals were separated, counted and returned to the insectary until dissected. All surviving mosquitoes were dissected on days eight through ten and, if infected, the oocysts counted. Dissections and oocyst counts were made by a single senior technician and the results spot-checked by the senior investi-

Table 2. Results of dissections of 16 groups of *A. balabacensis* and *A. minimus* when both groups were oocyst positive.

		<i>A. balabacensis</i>	<i>A. minimus</i>
Percent positive	Median	83.3	33.2
	Range	6.5-100	3.9-69
Oocyst index	Median	13.4	3
	Range	1.4-248	2-42

gator. The proportions of dissected mosquitoes which had oocysts (percent positive) and the mean number of oocysts per infected female (oocyst index) were used to compare the susceptibility of the two mosquito species.

Because of marked skewness of the data for both percent positive and oocyst index, the range and medians were used to describe the distributions of values obtained. Where appropriate, Spearman's rank correlation coefficients (Walker and Lev, 1953) were calculated and used to assess the degree of association between these distributions.

Results

A. balabacensis and *A. minimus* were fed simultaneously on 36 subjects with naturally occurring *P. falciparum* malaria. The medians and ranges for the number of mosquitoes engorged and dissected (survived) for the groups of mosquitoes in the infected and the non-infected categories are shown in Table 1. More *A. balabacensis* fed and were dissected than *A. minimus*. However, within each species the median numbers fed and proportions of those fed dissected were similar regardless of the subsequent presence or absence of oocysts.

Sixteen of the 36 subjects infected *A. minimus*. *A. balabacensis* was infected by these same subjects and also by an additional eight subjects which did not infect *A. minimus*. No infections resulted after feeding on 12 of the subjects. The dissection results of the 16 paired *A. balabacensis* and *A. minimus* feedings where both groups contained infected mosquitoes are presented in Table 2. For both indicators of infection the medians were higher and the ranges larger for *A. balabacensis* than for *A. minimus*. The medians of percent positive and of oocyst indices were 2.5 and 4.5 times greater, respectively, for

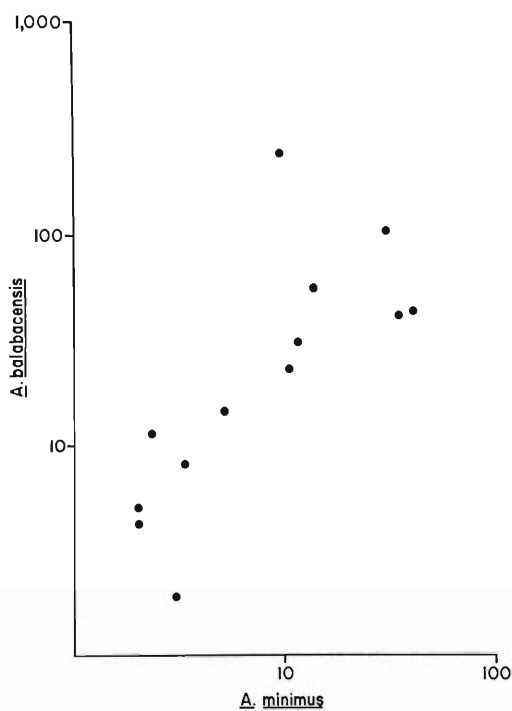


Figure 1. Oocyst index for *A. balabacensis* and *A. minimus* by subject.

A. balabacensis than for the corresponding *A. minimus*. The results of dissections from eight subjects which infected only *A. balabacensis* are presented in Table 3. The medians of percent positive and oocyst indices for these *A. balabacensis* were 0.4 and 0.1 of those for *A. balabacensis* when both species were infected. There were no noticeable differences in size or morphology of the oocysts which developed in the two species.

The *A. balabacensis* and *A. minimus* oocyst indices from 13 subjects are shown in Fig. 1. The results were highly correlated ($r = .80$,

Table 3. Results of dissections of eight groups of *A. balabacensis* when the corresponding *A. minimus* group negative.

Percent positive	Median	— 36
	Range	— 2.3–100
Oocyst index	Median	— 1.5
	Range	— 1–34.8

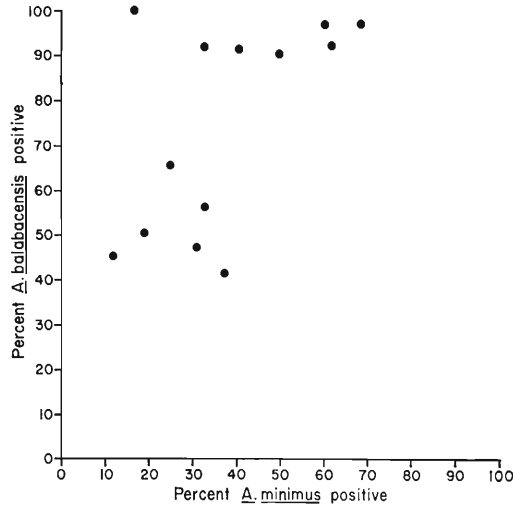


Figure 2. Percent positive *A. balabacensis* and *A. minimus* by subject.

$p < .01$). In figure 2 the percent positive results are presented for these 13 subjects; association was less marked ($r = .39$, $p < .15$). The data from the additional three subjects which infected both species are not included in Figs. 1 and 2 because in each case less than ten *A. minimus* were dissected and, of these, only one was infected.

The percents positive for both mosquito species were also compared with the gametocyte densities in the subjects. When mosquitoes were infected, larger proportions of *A. balabacensis* were infected at each gametocyte density. However, the association between gametocytemia and proportion infected for the above mentioned 13 subjects was not appreciable for either *A. balabacensis* ($r = 0.36$, $p < .15$) or *A. minimus* ($r = .15$, $p < .50$). Similar results were obtained for both mosquitoes when oocyst indices were compared with levels of gametocytemia. The gametocyte densities and percentages of mosquitoes infected for all 36 subjects are shown in Fig. 3.

Discussion

The results of earlier studies conducted at SEATO Medical Research Laboratory in Bangkok suggested that colonized *A. balabacensis* were more inclined to feed in the laboratory and were more susceptible to

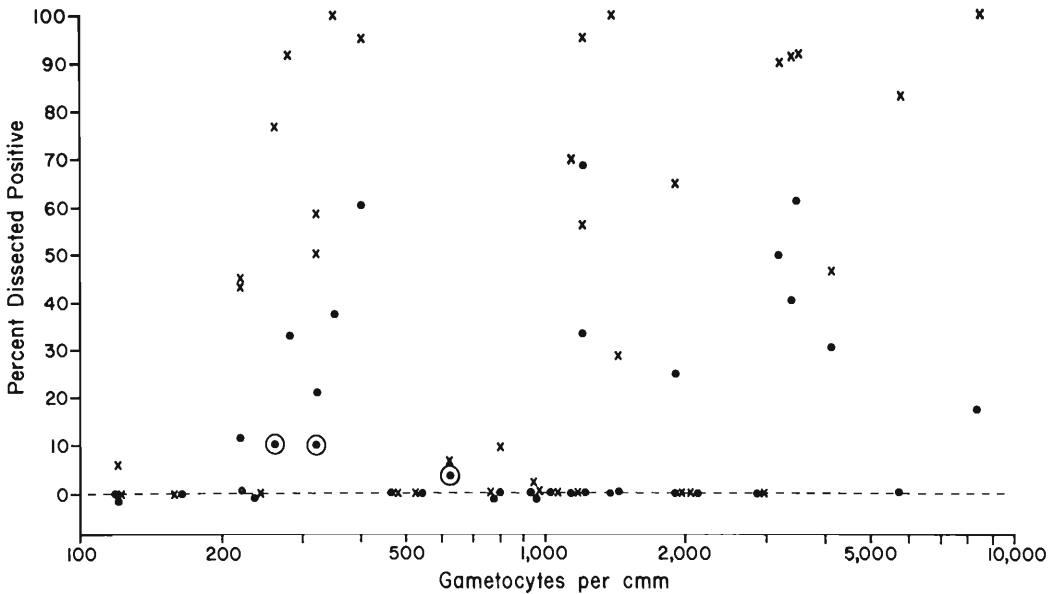


Figure 3. Percent of *A. balabacensis* and *A. minimus* infected by feeding simultaneously on 36 subjects with *P. falciparum*, as related to gametocyte density. (X = *A. balabacensis*, ● = *A. minimus*, ○ = *A. minimus*, less than 10 dissected.)

infection with *P. falciparum* than wild *A. minimus*, but because small numbers of *A. minimus* were involved in these experiments the results were not considered conclusive (Annual Progress Report, 1966; Annual Progress Report, 1968). The present studies have shown a marked difference between the relative susceptibilities of *A. balabacensis* and *A. minimus* to infection with naturally occurring Thai strains of *P. falciparum*.

Although the oocyst indices were larger for *A. balabacensis* than *A. minimus*, the distribution of values for the oocyst indices by human subject for the two species was highly correlated ($r = .80$, $p < .01$). This indicates that the observed differences in oocyst indices between the mosquito species were not due to the human subject per se but were reflecting actual differences in the susceptibility of *A. balabacensis* and *A. minimus* to infection with *P. falciparum*. The poorer correlation ($r = .39$, $p < .5$) between the distribution of values for the percent positive probably suggests that this indicator is a less reliable variable than the oocyst index for comparing the two species. The lack of a strong association

between gametocytemia levels and the degree of mosquito infectivity in this study agrees with the findings of others (Rutledge et al., 1969; Jeffery and Eyles, 1955). A comparison of the infected and non-infected groups of mosquitoes indicated that the subsequent development of oocysts did not influence the mortality of engorged mosquitoes of either species (Table 1).

The strain of *A. balabacensis* used in this study thus appeared to be susceptible to infection with *P. falciparum* at a lower threshold than was the *A. minimus* strain, and even above this threshold *P. falciparum* was more successful in infecting *A. balabacensis* than *A. minimus*. All of these findings suggest that *A. balabacensis* is a better vector of *P. falciparum* than is *A. minimus*.

The results observed in our colonized mosquitoes may not be representative of the susceptibilities of wild populations of these two mosquito species. However, since wild-caught individuals were periodically added to these colonies, we believe that our colonized strains probably do not appreciably differ in this respect from wild populations. In Thai-

land, higher infection rates have been reported for wild-caught *A. balabacensis* than *A. minimus* (Scanlon and Sandhinand, 1965; Powell et al., 1969), although this is probably a result of factors in addition to those investigated in this study.

Chloroquine resistant strains of *P. falciparum* are widespread in Thailand and in areas of intensive study have been shown to be the predominant strains (Colwell, in manuscript). *A. balabacensis* has been shown to be the probable vector of chloroquine resistant strains of falciparum malaria in Malaysia (Scanlon et al., 1969), Cambodia (Montgomery and Eyles, 1963) and in Thailand (Scanlon and Sandhinand, 1965). This observed relationship could be coincidental and, in Thailand, could have been further accentuated because the role of *A. minimus* as a vector had been drastically reduced as a result of control measures directed principally against this species. However, the hypothesis that the dramatic rise in the incidence of chloroquine resistant falciparum malaria was at least partially due to *A. balabacensis* being a more efficient vector of resistant than of susceptible strains is an attractive one.

The status of chloroquine sensitivity of the strains of *P. falciparum* employed in this study was not determined; however, over 90 percent of the *P. falciparum* infections from the study area are known to be resistant to chloroquine. Although no definite conclusions can be drawn as to the relative susceptibilities of *A. balabacensis* and *A. minimus* to infection with known chloroquine resistant strains of *P. falciparum*, the results presented here and the conditions of the investigation strongly suggest that *A. balabacensis* is a better vector of chloroquine resistant strains of *P. falciparum* than is *A. minimus*. Further studies comparing the susceptibilities of both mosquito species to known chloroquine resistant and susceptible strains of *P. falciparum* are in progress.

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Nosematosis: Its Effect on *Anopheles albimanus* Wiedemann, and a Population Model of its Relation to Malaria Transmission¹

D. W. ANTHONY, K. E. SAVAGE, AND D. E. WEIDHAAS

Entomology Research Division, Agr. Res. Serv., USDA, Gainesville, Fla. 32601

ABSTRACT. The effects of infection by *Nosema stegomyiae* Marchoux, Salimbeni and Simond on the fecundity and longevity of *Anopheles albimanus* Wiedemann were studied. Infection produced by continuous exposure of larval stages to dosages of 5×10^2 , 5×10^3 , and 5×10^4 *Nosema* spores per milliliter of rearing water significantly reduced the number of eggs laid by infected females, and a level of 5×10^4 spores/ml sharply reduced the ability of *A. albimanus* females to produce eggs beyond the 2nd gonotrophic cycle. Uninfected females regularly oviposited through the 4th and 5th gonotrophic cycles. Longevity of infected and uninfected adults was compared by computing LT-50's and LT-90's at 3 dosage levels of *Nosema* spores. At the highest dosage level (5×10^4 spores/ml), the longevity of females was reduced by one half (from 32 to 16 days) at the LT-90 level. A hypothetical population model for *A. albimanus* is proposed to illustrate the theoretical effects of *Nosema* infection on a population of infective (capable of transmitting malaria) females. This model shows that a *Nosema* infection which reduces the female LT-90 by one half would cause a 85–97% reduction in the number of infective females in a hypothetical population.

The use of pathogens for the control of mosquitoes or as a supplement to other control measures has been suggested from time to time since the turn of the century. Microsporidian (Protozoa, Cnidospora) pathogens of anophelines have been known since Hesse (1904) described *Thelohania legeri* from *Anopheles maculipennis* Meigen. At least 12 species of Microsporidia of the genera *Nosema*, *Pleistophora* and *Thelohania* have now been described from anophelines, and several more species are known but are yet to be described. Chapman et al. (1970) tabulated the *Anopheles* species, 23 in all, that are known hosts for these parasites.

Three species of *Nosema* have been reported from anopheline mosquitoes: *Nosema stegomyiae* Marchoux, Salimbeni and Simond, *Nosema anophelis* Kudo, and *Nosema algerae* Vavra and Undeen. However, after reviewing the present knowledge of the history and origin of several *Nosema* strains that were involved in colony epizootics and subjected to further experimentation, Hazard and Lofgren (1971) suggested that *N. stegomyiae* and *N. algerae* may be synonymous.

Nosema stegomyiae has been studied as a pathogen of mosquitoes at the Insects Affecting Man and Animals Research Laboratory,

ARS, USDA, Gainesville, Fla., since 1966. The results of these studies (Hazard 1970; Savage and Lowe 1970; Hazard and Lofgren 1971) indicated that *N. stegomyiae* is quite pathogenic to anophelines and that it may have a potential use in an integrated program for the control of these mosquitoes. Also, a number of workers have found *N. stegomyiae* to be a serious pathogen of colonized mosquitoes. Fox and Weiser (1959) reported an epizootic caused by *N. stegomyiae* in colonies of *Anopheles gambiae* Giles and *Anopheles melas* (Theobald) in Liberia, West Africa. Vavra and Undeen (1970) reported a *Nosema* (which they described as a new species, *N. algerae*) infecting a colony of *Anopheles stephensi* Liston at the University of Illinois, Urbana. Canning and Hulls (1970) described an epizootic, which they attributed to *N. algerae*, in a colony of *A. gambiae* established from mosquitoes originating in Tanzania. Hazard (1970) documented in detail two additional epizootics of *Nosema* in colonized anophelines, one at the Walter Reed Army Institute of Research, Washington, D. C., and the other at the School of Medicine, University of Maryland, Baltimore, and discussed the probable origin of the parasites in these colonies.

It is apparent that nosematosis in anopheline colonies seriously restricts the use of such colonies for malaria transmission studies.

¹ Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U.S. Department of Agriculture.

Colony vigor, reproduction, and longevity are markedly reduced, and the *Nosema* parasite may inhibit or retard the development of *Plasmodium*. Fox and Weiser (1959) reported that *Plasmodium falciparum* (Welch) did not complete its development in females of *A. gambiae* that were heavily infected with *N. stegomyiae*, and concluded that heavy infections of *N. stegomyiae* so disintegrated the cells of the midgut that the *Plasmodium* ookinetes could not find a suitable place for development. Recent studies by Savage et al. (1972) showed that when *Anopheles quadrimaculatus* Say infected with a *Nosema* sp. (*N. stegomyiae*) were fed on chicks infected with *P. gallinaceum* Brumpt, only 15.5% of the mosquitoes developed sporozoites. In contrast, 61.5% of the control mosquitoes (which did not contain *Nosema* infections) developed sporozoites. These workers also noted that the combined infection of *N. stegomyiae* and *P. gallinaceum* severely reduced the longevity of *A. quadrimaculatus* as they had difficulty obtaining sufficient numbers of mosquitoes for their tests.

In view of the earlier observations and the results obtained by different workers, we initiated research in 1972 at the Insects Affecting Man and Animals Research Laboratory to determine the effects of *N. stegomyiae* on *Anopheles albimanus* Wiedemann which is a vector of human malaria in Mexico and throughout much of Central America. The present paper reports data concerning the effects of this parasite on the longevity and fecundity of *A. albimanus*. We have also proposed a hypothetical population model showing the effects of the pathogen on the survival of adult females and on the transmission of malaria.

Materials and Methods

Eggs of *A. albimanus* obtained from the U.S. Public Health Service Laboratory, Savannah, Ga., were used to start a colony of these mosquitoes at the Gainesville laboratory. All tests reported herein were conducted with mosquitoes from this colony. The strain of *N. stegomyiae* used in these studies was obtained from infected *Anopheles* colonies at the Walter Reed Army Institute of Research. Hazard (1970) found that this strain readily

invaded most of the tissues of *A. albimanus* and that it was highly pathogenic to other anophelines. This strain was passed through our laboratory colony of *A. quadrimaculatus* many times before its use in the tests with *A. albimanus*.

The spores used to infect the mosquitoes were obtained by macerating previously infected mosquitoes (larvae or adults) in distilled water. These crude spore suspensions were partially cleaned by centrifugation and filtration through cheesecloth. The resulting spore suspension was then calibrated with a Petroff-Hausser bacterial counter, and dilutions were made to obtain the proper dosages for exposure. First-instar larvae of *A. albimanus* were infected by continuous exposure at dosages of 5×10^2 , 5×10^3 , and 5×10^4 *N. stegomyiae* spores/ml of rearing water throughout their development. (These dosages were previously determined by Savage during investigations, pertaining to the mass production of *Nosema* spores.) Control groups of *A. albimanus* were reared for all tests, and were handled identically to the test mosquitoes except that they were not exposed to spores of *N. stegomyiae*.

Adult mosquitoes were maintained at a laboratory temperature of 29.4°C and a relative humidity of 70–80%. Fifty virgin males and 50 virgin females from each exposure dosage were held in cages measuring 15 × 20 × 25 cm and provided with a 25% sugar solution (soaked in cotton) and a blood meal every Monday, Wednesday, and Friday. A cup containing water was placed in each cage for egg deposition. Dead adults were removed daily, counted, and examined for *N. stegomyiae* spores. When fecundity was studied, eggs were removed from the cages within 12–14 hr after deposition, held for 4–5 days, and counted, and the percent hatch was determined. Eggs laid on the sugar-soaked cotton were counted but were not included in determining percent hatch. In tests to determine the effects of *N. stegomyiae* on successive gonotrophic cycles of individual females, the mosquitoes were initially held in cages for mating, a blood meal was provided when they were 4–5 days old, and the engorged females were then removed from the cage and placed individually in 3-dr plastic vials containing a small amount of water for oviposition. Raisins

Table 1. Fecundity of adult *Anopheles albimanus* exposed to 3 concentrations of *Nosema stegomyiae* spores throughout their larval development (50 males and 50 females/test group).

Dosage (spores/ml)	No. eggs laid	% Hatch	% Reductions of no. 1st instar larvae
0 ^a	15,447	84	
5 × 10 ²	13,246	78	21
5 × 10 ³	7,377	68	61
5 × 10 ⁴	5,272	63	75

^a Control. Average of 2 replicates.

were provided as a source of carbohydrates, and blood meals were offered after each oviposition. Records were made on the number of ovipositions, the number of eggs, and the percent hatch/female until oviposition ceased or until the female died.

Results

As previously determined, continuous exposure of larvae to dosages of 5 × 10², 5 × 10³, and 5 × 10⁴ spores/ml of rearing water resulted in negligible mortality of larvae and pupae, but the adults averaged 30–40, 80–90, and 95–100% infection, respectively. The results of the studies of fecundity are presented in Table 1. The number of eggs produced by adults exposed to 5 × 10², 5 × 10³, and 5 × 10⁴ spores/ml were reduced by 14, 52, and 66%, respectively; the percent of viable eggs decreased from 84% for the control group to 78, 68, and 63%. Thus, the data indicate that even at very low dosages of *Nosema* spores, egg production, viability, and, consequently,

production of F₁ generation larvae were significantly reduced.

The results of the tests to determine the effects of infection by *N. stegomyiae* on egg production and viability in successive gonotrophic cycles are given in Table 2. These data show a significant reduction of the number of infected females ovipositing and the number of eggs laid in the first gonotrophic cycle. Also the numbers of ovipositing infected females and the number of eggs they laid were markedly reduced in the following gonotrophic cycles. However, in this series of experiments, infection by *N. stegomyiae* appeared to have little or no effect on the viability of the eggs.

Longevity of infected and uninfected adults was compared by computing LT-50's and LT-90's at 3 dosage levels of *Nosema* spores. The results for both males and females are presented in Table 3. Obviously, *N. stegomyiae* infections had little if any effect on the longevity of the males; however, significant reductions in female longevity were evident at both the LT-50 and LT-90 levels in the groups exposed to 5 × 10³ and 5 × 10⁴ spores/ml of rearing water. At these dosages the female LT-50's were reduced by 5.5 and 7.5 days, respectively, while the LT-90's were reduced by 12 and 16 days. Thus, at the highest dosage level (5 × 10⁴ spores/ml), the longevity of females was reduced by ½ at the LT-90 level.

Therefore, from the preliminary data and from the research of the previously cited workers it appears that the introduction of

Table 2. Egg production and viability in successive gonotrophic cycles of *Anopheles albimanus* free of and infected with *Nosema stegomyiae*. (Infected mosquitoes exposed to 5 × 10⁴ spores/ml throughout larval development, (50 females/test).)

Test no.	1st cycle			2nd cycle			3rd cycle			4th cycle		
	No. females ovi- positing	Total no. eggs	Avg. hatch (%)	No. females ovi- positing	Total no. eggs	Avg. hatch (%)	No. females ovi- positing	Total no. eggs	Avg. hatch (%)	No. females ovi- positing	Total no. eggs	Avg. hatch (%)
<i>Uninfected females</i>												
1	37	4,052	85	13	1,431	88	7	675	84	1	106	71
2	38	5,636	89	21	2,876	89	11	1,093	82	2	184	82
Average	37.5	4,844	87	17	2,153	88.5	9	884	83	1.5	95	76.5
<i>Infected females</i>												
1	29	2,698	87	7	520	88	1	119	87	1	14	93
2	32	3,354	90	10	972	74	0	0		0	0	
Average	31	3,026	88.5	8.5	746	81	0.5	59.5		0.5	7	
Average reduction (%)	16	38	0	50	70	11	95	94		33	93	

Table 3. Longevity of adult *Anopheles albimanus* exposed to 3 concentrations of *Nosema stegomyiae* spores throughout their larval development (50 males and 50 females/test group).

Test group	Larvae exposed to (spores/ml)			
	0 ^a	5 × 10 ²	5 × 10 ³	5 × 10 ⁴
Female LT-50 (days)	21.5	22	16	12
Male LT-50 (days)	13.5	11	14	12
Female LT-90 (days)	32	35	18	16
Male LT-90 (days)	19.5	20	18	17

^a Control. Average of 2 replicates each.

N. stegomyiae into natural populations of anophelines such as *A. albimanus*, if it could be accomplished, could dramatically limit the capability of a population to transmit malaria. For example, a reduction in the survival time of adult females would reduce both the number and percentage of infective females capable of transmission, and reductions in the survival and fecundity of females could cause reductions in density over time which would further limit transmission.

However, at present, we do not know to what degree transmission of malaria might be reduced by the presence of such a pathogen in a field population or how easily it could be established. Consequently, we decided to construct a model of an anopheline population to determine theoretically what effect a change in the survival of adult females caused by *Nosema* infection in these females might have on the number and percentage of infective (malaria transmitting) females present in that population. The assumptions we used in our model are based on those outlined by MacDonald (1952). (We did not expand the model to cover the effects of changes in density over time which might be caused by *Nosema* infections.) In developing our model we had 2 general objectives: (1) to construct a population model that appeared reasonable biologically and (2) to determine the effect of survival on the number and percentage of infective females in the population.

First, we constructed a population with (1) varying rates of mortality for adult females, (2) varying rates of survival for the immature stages, and (3) varying gonotrophic cycles. Our purpose was to establish that this hypothetical population was capable of existing in the field with reasonable rates of increase

Table 4. Densities and LT-50's and LT-90's for a hypothetical population of mosquitoes into which 1,000,000 adult females are emerging per day (assumed 7 mortality rates of adult females per day).

Assumed % mortality of adult females per day	Absolute density of females	LT-50 (days)	LT-90 (days)
5	2 × 10 ⁷	13–14	44–45
10	1 × 10 ⁷	6–7	21–22
15	6.7 × 10 ⁶	4–5	14–15
20	5.0 × 10 ⁶	3–4	10–11
25	4.0 × 10 ⁶	3–4	9–10
30	3.3 × 10 ⁶	1–2	6–7
35	2.9 × 10 ⁶	1–2	5–6
40	2.5 × 10 ⁶	1–2	4–5

and decrease. For this part of the model, we used the following assumptions:

1. *Mortality rates for adult females*

The rate of mortality of adult females can vary from time to time, species to species, and place to place. Consequently, we selected 8 rates which would represent values that could be expected to occur. These rates were 5, 10, 15, 20, 25, 30, 35 and 40% mortality/day of surviving females.

2. *Survival rates for immature forms*

Immature stages are represented by the egg, larval, and pupal stages, but rather than deal separately with each stage, they were dealt with as 1 group. Since survival in the immature stages is quite variable, 3 levels of survival (5.0, 10.0 and 50.0%) were chosen.

3. *Gonotrophic cycles*

To determine the number of egg batches expected for a given population, we assumed 3 different gonotrophic cycles—4, 3, and 2 days. Also, we assumed that all egg rafts from the 1st gonotrophic cycle were laid when the females were 6 days old and that subsequent egg batches were all laid at intervals of 4, 3, or 2 days depending on the gonotrophic cycle.

4. *Number of eggs per batch*

Since our primary interest in this study was *A. albimanus*, we used a value of 120 eggs/batch, which was considered an average number for this species.

To construct the biological part of our model, we assumed a stable population into which 1,000,000 females were emerging per

Table 5. Reproductive success (rates of increase) of a hypothetical population of *Anopheles albimanus* into which 1,000,000 females are emerging per day (assumed 7 levels of mortality of adult females, 3 levels of survival of the immature stages, and 3 gonotrophic cycles).

Survival of immature forms (percent)	Rates of increase for indicated percent mortalities of adult females per day							
	5	10	15	20	25	30	35	40
4-day gonotrophic cycle								
50.0	118 ×	46.0 ×	24.0 ×	13.0 ×	7.8 ×	4.6 ×	2.8 ×	1.6 ×
10.0	24 ×	9.0 ×	4.7 ×	2.7 ×	1.6 ×	.9 ×	.6 ×	.3 ×
5.0	12 ×	4.6 ×	2.4 ×	1.3 ×	.8 ×	.5 ×	.3 ×	.2 ×
3-day gonotrophic cycle								
50.0	155 ×	59.0 ×	29.0 ×	16.0 ×	9.0 ×	5.4 ×	3.1 ×	1.8 ×
10.0	31 ×	12.0 ×	5.9 ×	3.2 ×	1.8 ×	1.1 ×	.6 ×	.4 ×
5.0	15 ×	5.9 ×	2.9 ×	1.6 ×	.9 ×	.5 ×	.3 ×	.2 ×
2-day gonotrophic cycle								
50.0	226 ×	84.0 ×	41.0 ×	22.0 ×	12.0 ×	6.9 ×	3.9 ×	2.2 ×
10.0	45 ×	17.0 ×	8.0 ×	4.4 ×	2.4 ×	1.4 ×	.8 ×	.4 ×
5.0	23 ×	8.0 ×	4.1 ×	2.2 ×	1.2 ×	.7 ×	.4 ×	.2 ×

day. We calculated the absolute densities, the LT-50's and LT-90's, and the rates of increase for populations with the variables we assigned when these populations attained a stable equilibrium. The results of these calculations are given in Tables 4 and 5. In our model, the absolute density of females is dependent only upon survival in the adult female stage, and we assumed that the population would remain stable after reaching equilibrium at any mortality rate. The model seemed reasonable to us since the rates of increase fell within acceptable values below or above 1× with only a few rates that were exceptionally high (15× to 226×).

Next, we used our model to calculate the number and percentage of infective females that would exist in stable, hypothetical populations characterized by the 8 mortality rates for adult females that we had selected. To correspond with our beginning model we assumed the following:

1. The mortality rates of the adult females were 5, 10, 15, 20, 25, 30, 35, and 40% per day of surviving females.
2. Adult females bite to take a blood meal only once each gonotrophic cycle. All adult females bite first when they are 3 days old and then at 4-, 3-, or 2-day intervals.
3. At any biting time, only 10% of the females feed on humans, and only 10% of the humans upon which they feed have mature *Plasmodium* gametocytes

circulating in their blood, and all of these females become infective.

4. Development time for the malaria parasite in the mosquito is 9 days.

Using these assumptions, we calculated (as shown in Table 6) the number and percentage of infective females that would be found in populations characterized by gonotrophic or biting cycles and the selected mortality rates.

An increase in the daily mortality rate of 5% per day for a given biting cycle reduced the number of infective females present by 64.8–85.7%. A decrease in biting cycles from 4-day to 2-day intervals increased the number of infective females present from 1.8- to 3.5-fold.

We then compared the number of infective females that would be present in a population at 1 mortality rate with the number of infective females present in a population with a mortality rate that reduced the LT-90 by ½. For example, Table 4 shows that the LT-90 for adult females would be halved in populations changing from 5 to 10%, from 10 to 20%, from 15 to 30%, from 20 to 35% and from 25 to 40% mortality per day. Also, our laboratory data indicated that the LT-90 for *Nosema*-infected females was only about one half the LT-90 of uninfected females. From Table 6, we can see that a change from 5 to 10%, 10 to 20%, 15 to 30%, 20 to 35% and 25 to 40% resulted in a dramatic reduction in the number of infective females as follows:

Table 6. Number and percentage of infective females in a hypothetical population of mosquitoes (assumed 8 levels of adult mortality with females biting at 4-day, 3-day, and 2-day intervals).

% mortality females per day	4-day biting interval			3-day biting interval			2-day biting interval		
	No. infective females	% infective females	% reduction females	No. infective females	% infective females	% reduction females	No. infective females	% infective females	% reduction females
5	152,587	0.763		255,545	1.28		536,225	2.68	
10	23,648	.236	84.5	37,967	.38	85.1	76,767	.77	85.7
15	6,193	.093	73.8	9,475	.14	75.0	18,286	.27	76.2
20	1,961	.039	68.3	2,861	.057	69.8	5,248	.11	71.3
25	674	.017	65.6	936	.023	67.3	1,632	.041	68.9
30	236	.0071	65.0	314	.0094	66.5	515	.015	68.4
35	83	.0029	64.8	106	.0037	66.2	162	.0057	68.5
40	27	.0011	67.5	34	.0014	67.9	50	.0020	69.1

Percent decrease with indicated biting cycle			
	4-Day	3-Day	2-Day
5–10%	85	85	86
10–20%	92	93	93
15–30%	96	97	97
20–35%	96	96	97
25–40%	96	96	97

Therefore, if reductions in the survival of adult females in stable populations were similar to that observed in the laboratory experiments, this *Nosema* species could be an effective agent in reducing the transmission of malaria in the field simply by reducing the number of infective females present. Moreover, this particular *Nosema* species has additional adverse effects on anopheline mosquitoes, such as reduced fecundity of females and reduced transmission rates when infected with both *Nosema* and malaria. This pathogen appears particularly promising for further research.

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Effects of Microsporidian Parasites upon Anopheline Mosquitoes and Malarial Infection

R. A. WARD¹ AND K. E. SAVAGE²

ABSTRACT. Infection of *Anopheles stephensi* mosquitoes with a microsporidian parasite, *Nosema algerae*, reduced their susceptibility to infection with the simian malarial parasite, *Plasmodium cynomolgi*, as measured by malarial oocyst counts 6 days after an infective feed. Mortality from nosematosis was so great that 90–95% of the exposed mosquitoes died before sporozoite levels could be assessed (day 14). It is concluded that the low incidence of *Nosema* in adult anopheline populations in the field is related to the virulence of this pathogen.

In the course of rearing anopheline mosquitoes for malaria transmission experiments it has been observed that the presence of microsporidian parasites can either decimate laboratory colonies to such an extent that transmission studies cannot be conducted or if the mosquitoes survive, the results of malaria transmission experiments are extremely variable, frequently with marked decreases in oocyst and sporozoite counts (Bano, 1958; Fox and Weiser, 1959; Garnham, 1956; Hazard, 1970; Vavra and Undeen, 1970 and Vandenberg, J., personal communication).

These studies started when it was observed that the WRAIR *Anopheles stephensi* colonies exhibited excessive larval and adult mortality during July 1968. The cause of the mortality was not known until dissection of adult mosquitoes infected with the simian malarial parasite, *Plasmodium cynomolgi*, revealed the presence of numerous spore-like structures in the fat body surrounding the midgut. They were tentatively identified as *Nosema* and further study by E. I. Hazard confirmed the diagnosis (Hazard, 1970). Records of the infection were kept and following elimination of the pathogen from the insectary, collaborative experiments were conducted with the U.S. Department of Agriculture Insects Affecting Man and Animals Laboratory, Gainesville, Florida. Their purpose was to determine whether a concurrent malaria and microsporidian infection in an anopheline vector could reduce the vector potential of an anopheline species or even prevent the

species from transmitting malaria to a rhesus monkey. This information would be of value in designing strategies for the control of vector species with this pathogen.

Materials and Methods

*Nosema*¹ (Walter Reed isolate) spore suspensions were prepared at the Gainesville, Florida laboratory, sent to WRAIR by air mail and stored at 5°C until used. The spores used for infecting anophelines were obtained from adult *Anopheles albimanus*. The larvae of *A. albimanus* were continuously exposed from the 1st stage to pupation at a spore concentration sufficient to produce 95–100% infection. The adults were maintained on 25% sugar water for 3–7 days at 30°C and 70% relative humidity. These adults were immobilized, placed in distilled water and macerated in a tissue grinder. The resulting suspension was strained through paper toweling and concentrated by centrifugation. The resulting spore suspension was calibrated with a Petroff-Hausser bacterial counter.

Procedures for infecting *Anopheles stephensi* and *A. quadrimaculatus* on *Macacca mulatta* monkeys² infected with *Plasmodium cynomolgi* (RO/PM and *bastianellii* strains) were identical with those previously described (Ward and

¹ A difference of opinion exists concerning the identity of this parasite. Hazard and Lofgren (1971) consider it to be *N. stegomyiae* Marchoux, Salimbeni and Simond, 1903 while Cairning and Hulls (1970) have identified this microsporidian as *N. algerae* Vavra and Undeen, 1970. The authors of the current paper have different opinions on the correct nomenclature; the senior author accepts *N. algerae* Vavra and Undeen as the appropriate name.

² In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

¹ Department of Entomology, Walter Reed Army Institute of Research, Washington, D.C. 20012.

² Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Florida 32601.

Table 1. Susceptibility of *Nosema*-infected *Anopheles stephensi* colony at the Walter Reed Army Institute of Research to infection by *Plasmodium cynomolgi*.

Date	Rhesus number	<i>Nosema</i> present Mean no. oocysts	Number dissected	<i>Nosema</i> absent Mean no. oocysts	Number dissected	Percent mosquitoes with <i>Nosema</i>
2 Oct 68	E-868	10.6	7	16.5	23	23
3 Oct 68	E-868	2.9	8	4.7	12	40
4 Oct 68	E-868	10.0	6	12.4	15	29
7 Oct 68	E-868	10.0	8	9.4	12	40
8 Oct 68	E-868	1.9	10	4.0	10	50
9 Oct 68	E-868	6.2	5	8.5	15	25
21 Oct 68	E-869	60.5	4	138.6	8	33
23 Oct 68	E-869	23.5	4	49.8	8	33
29 Oct 68	E-869	47.8	5	68.4	7	42
30 Oct 68	E-869	1.9	7	17.0	5	58
5 Nov 68	E-845	4.1	6	3.9	5	55
7 Nov 68	E-845	57.5	8	153.6	4	67
19 Nov 68	E-845	168.4	5	141.3	7	42
20 Nov 68	E-845	90.8	5	133.4	7	42
25 Nov 68	E-845	64.7	7	125.2	5	58
27 Nov 68	E-845	4.2	4	7.8	8	33

Hayes, 1972). The degree of *Nosema* infection in the mosquito was assayed on day 8 when specimens were dissected for malarial oocyst counts. Four levels of nosematosis were considered: 0—no infection visible, 1+—moderate (1–100 spores per microscope field), 2+—heavy (100–1000) and 3+—very heavy (1000+).

Results

Some aspects of the *Nosema* epizootic in the WRAIR mosquito colony were reviewed by Hazard (1970). The infection was first detected on 14 August 1968 among *Anopheles stephensi* which were being dissected for *Plasmodium cynomolgi* oocysts. Initially, no excessive mosquito mortality was observed. Seven days after feeding on rhesus monkeys, one-half of the anophelines were dead. Between 28 August and 26 September, 54 feeds were made on *P. cynomolgi*-infected monkeys. Of these only 25 or 46% survived beyond one week. Ninety-five percent of the remaining mosquitoes were dead by day 14. Dissection and histological examination of 4 *Anopheles stephensi* strains disclosed *Nosema* infection rates of 75–100% in the colonies. At this point measures were taken to eliminate the pathogen from the insectary. Surface sterilization of anopheline eggs with 70% ethanol reduced the level of infection so sufficient mosquitoes were available for transmission experiments.

The presence of a *Nosema* infection was noted when samples of *A. stephensi* (India

strain) were dissected for malarial oocyst counts between 2 October–27 November 1968. During this period, 23–67% of day 8 mosquitoes exhibited microsporidian infections. Mean malarial oocyst counts were calculated for female anophelines with and without *Nosema* infections from each cage exposed to the *P. cynomolgi*-infected rhesus monkeys (Table 1). 13/16 or 81.3% of the mean oocyst counts from *A. stephensi* females with microsporidian infections were lower than the counts from uninfected mosquitoes. The mean oocyst counts for the various feeding dates were compared by equating the combined counts of both groups to a base of 100. The combined mean oocyst count of *Nosema*-infected groups was 37.78 ± 2.70 while that of the non-infected groups was 62.18 ± 2.67 . Mean oocyst values of *Nosema*-infected anophelines were approximately half that of uninfected females. This difference was highly significant ($t = 6.42$, $p = 0.001$).

Since the *Nosema* infection could not be eliminated from the insectary by surface sterilization of anopheline eggs, all *A. stephensi* and *A. quadrimaculatus* mosquitoes were discarded in January 1969, rearing facilities were sanitized and new laboratory colonies introduced.

In the first series of experiments, first stage *A. quadrimaculatus* larvae were exposed to *Nosema* spore suspensions in the Gainesville laboratory, reared to adults and shipped by air to WRAIR for experimental malaria studies with two strains of *P. cynomolgi* (RO/PM and *P. cynomolgi bastianellii*). Control lots of

Table 2. Effect of *Nosema* infection of *Anopheles quadrimaculatus* on susceptibility to *Plasmodium cynomolgi* (RO/PM) and *bastianellii* strains).

Date	Parasite strain	Mean number oocysts	
		<i>Nosema</i> -infected	Non-infected
12 Mar 70	RO/PM	7.3	1.1
12 Mar 70	RO/PM	4.5	0.9
13 Mar 70	<i>bastianellii</i>	0	10.8
26 Mar 70	RO/PM	2.5	6.6
4 May 70	<i>bastianellii</i>	2.0	4.6
7 May 70	<i>bastianellii</i>	0.3	0.1

mosquitoes were reared in Gainesville and simultaneously sent to WRAIR.

The level of *Nosema*-infection ranged between 50–95% in material received at WRAIR. After receipt, the adults were kept in the insectary for 1–4 days and then fed on infected monkeys. In some instances, the microsporidan infection was so severe that only a small sample or no mosquitoes were available on day 8 for dissection. In a series of 6 experiments with the 2 *P. cynomolgi* strains no significant differences were observed in mean malarial oocyst counts among groups of *Nosema*-infected and control lots of *A. quadrimaculatus* simultaneously fed on infected monkeys (Table 2). Sporozoite dissections could not be made on day 12 as less than 5% of the microsporidan-infected mosquitoes survived until this date.

In the second series of experiments, *Nosema* spore suspensions were prepared in Gainesville and first stage *Anopheles stephensi* larvae were infected with the spores at WRAIR. Between April and July 1971 300 late 1st-stage and early 2nd-stage larvae were placed in pans containing a *Nosema* spore concentration

of 5.6×10^3 spores/ml solution. They remained in these pans until pupation occurred. From August onward, 300 1st-stage larvae were exposed to spore concentrations (2.4×10^4 spores/ml solution) in small beakers for 5–21 hours and then transferred to larval rearing pans. Control pans of *A. stephensi* larvae were reared at similar densities adjacent to groups of treated larvae.

The results of dissections made 8 days after feeding on *P. cynomolgi*-infected rhesus monkeys indicated that there was a reduction in mean malarial oocyst counts in the majority of feedings from *Nosema*-infected pans (Table 3). 8/11 or 72.7% of the mean oocyst counts from female *A. stephensi* reared in infected larval pans were lower than the counts from uninfected controls. A similar comparison of mean oocyst counts between both groups showed a count of 43.49 ± 3.56 oocysts from *Nosema*-infected pans while the controls averaged 56.60 ± 3.59 oocysts. The anophelines from infected larval pans had a reduction in oocyst count of approximately 25% when compared to untreated controls. This difference was significant ($t = 2.59$, $p = 0.05$). During the 8 day post-emergence period, mortality in the controls ranged between 10–20% while that in the *Nosema*-infected cages varied from 25–75%.

Salivary gland dissections were made 6 days later from *Nosema*-treated cages which contained at least 6 live anophelines. There was a marked reduction in the incidence of *Nosema* in cages from the treated pans in the intervening period (Table 4). Four cages which initially had a *Nosema* incidence between 8–75% were negative for the parasite on the

Table 3. Effect of *Nosema* infection of *Anopheles stephensi* (India strain) on susceptibility to *Plasmodium cynomolgi* *bastianellii* oocyst development.

Date	Rhesus number	Percent mosquitoes with <i>Nosema</i>	Oocysts: <i>Nosema</i>		Oocysts: Control	
			No.	Mean \pm SE	No.	Mean \pm SE
14 Apr 71	H-776	50.0*	30	16.7 ± 3.1	25	23.8 ± 2.9
14 Apr 71	H-776	92.3*	13	11.6 ± 4.7	25	25.4 ± 2.5
6 May 71	H-711	83.3	12	215.3 ± 42.2	12	103.8 ± 37.2
12 Oct 71	K-822	50.0	10	0.9 ± 0.6	6	1.3 ± 1.2
13 Oct 71	K-822	25.0	12	39.3 ± 8.9	12	87.7 ± 18.6
13 Oct 71	K-821	75.0*	12	178.4 ± 31.3	12	411.6 ± 30.6
13 Oct 71	K-821	58.3*	12	352.8 ± 49.2	10	384.1 ± 48.3
18 Oct 71	K-822	8.3	12	57.8 ± 10.9	12	46.7 ± 17.5
21 Oct 71	K-821	83.3*	12	48.0 ± 7.4	12	60.6 ± 9.9
21 Oct 71	K-821	45.5*	11	33.6 ± 9.1	10	61.1 ± 26.3
22 Oct 71	K-821	50.0	12	21.1 ± 2.5	12	18.3 ± 3.6

* Mosquito cages derived from different larval rearing trays.

Table 4. Effect of *Nosema* infection of *Anopheles stephensi* on *Plasmodium cynomolgi bastianellii* sporozoite development.

Date	Rhesus number	Percent mosquitoes with <i>Nosema</i>		Sporozoite level ¹	
		Day 8	Day 14	<i>Nosema</i> group	Control
13 Oct 71	K-821	75.0	0	2.3	3.1
13 Oct 71	K-821	58.3 ²	12.5	2.6	3.0
13 Oct 71	K-822	21.1	0	1.2	3.0
14 Oct 71	K-821	8.3	0	0	0.7
14 Oct 71	K-822	16.7	0	0.4	—
22 Oct 71	K-821	50.0	16.7	3.5	3.7
22 Oct 71	K-821	58.3 ²	33.3	3.0	—

¹ Mean of 6 dissected pairs of salivary glands; 1.00 = < 100 sporozoites, 2.00 = 100–1,000, etc.
² Mosquito cages derived from different larval rearing trays.

later date. Similarly, the other cages showed a marked reduction in parasite incidence. The sporozoite levels in the surviving anophelines from treated pans did not differ significantly from those observed in control groups. Among the cages which contained mosquitoes for dissection on the 14th day after the infective feed, mortality in the controls averaged 50% while that in treated *A. stephensi* ranged between 90 and 95%.

An attempt was made to determine whether a correlation existed between the level of *Nosema* infection in individual mosquitoes and the malarial oocyst count of that female. No significant differences were found between *A. stephensi* females which had *Nosema* infections scored as moderate (1+) as compared with those scored as very heavy (3+).

Discussion

The above studies indicated that the concurrent infection of female *Anopheles stephensi* mosquitoes with a microsporidian parasite, *Nosema algerae* Vavra and Undeen, produced a reduction in the mean number of oocysts of the simian malarial parasite, *Plasmodium cynomolgi*, which developed on the mosquito midgut. Although the reduction was statistically significant no appreciable difference was observed in the salivary gland infection rate 6 days later. This was undoubtedly related to the differential mortality of *Nosema*-infected females, the majority of which had died by day 14.

These observations closely paralleled those of Hulls (1971) who investigated the con-

current effect of *Nosema algerae* in *A. stephensi* infected with the rodent malarial parasite, *P. berghei* (Nigerian strain, N-67). He noticed a reduction in oocyst count on *Nosema*-infected midguts and in addition a reduction in sporozoite counts. Titration of sporozoites from *Nosema*-infected mosquitoes in mice showed a significant decrease of infectivity of sporozoites as compared to uninfected controls.

Fox and Weiser (1959) pointed out that there is no evidence that *Nosema* is physiologically antagonistic to *Plasmodium*, or that the former attacks the latter. In heavily infected mosquitoes, the midgut wall was so disintegrated that suitable sites were not available for *P. falciparum* oocyst development. These general observations are valid for the dual *Nosema-P. cynomolgi* infections in *Anopheles stephensi*. The only exception is that *A. stephensi* females with extremely heavy *Nosema*-infections which reached the point of midgut disintegration, did not survive sufficiently long to permit midgut oocyst counts on day 6.

There are few records of microsporidian parasites from wild-caught anophelines. During the period June 1955 through April 1956 R. M. Fox dissected more than 7,000 *Anopheles gambiae* in Liberia yet only encountered a few mosquitoes with microsporidian infections (Fox and Weiser, 1959). This may indicate that natural infections of *Nosema* exist at very low frequencies or that adult mortality is fairly high when this parasite is present in a population. In view of laboratory experiments with the pathogen, the latter interpretation is probably the more valid.

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A Comparative Study of the Development of the Mosquito Cycle of *Plasmodium cynomolgi* in Primary Cultures Versus an Established Cell Line of *Anopheles stephensi*

I. SCHNEIDER

Department of Entomology, Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT. *Plasmodium cynomolgi* oocysts of varying ages were placed in culture with cells from primary cultures as well as from an established cell line of *Anopheles stephensi*. Four day old oocysts showed the least favorable response to *in vitro* culture, whereas 6 day old oocysts responded primarily by growth and 8 day oocysts by differentiation. There were no striking differences in the response of *P. cynomolgi* oocysts to cells of the established line versus those from the primary cultures. Cells from adult tissue were no more effective than those from larval tissues in promoting development of the parasites *in vitro*. However, the oocysts invariably did better when cultured together with *A. stephensi* cells, regardless of origin, than in medium alone. Refinements in culturing techniques and the design of media will inevitably lead to the development of cell cultures, both primary and established, which more adequately reflect the *in vivo* milieu. More substantial results in culturing the insect cycle of malarial parasites should then be forthcoming.

An *in vitro* system, capable of supplying the physical, nutritional, hormonal and/or other factors necessary for the growth and differentiation of the insect cycle of plasmodia would serve many useful purposes. Studies involving the biochemistry, physiology and host specificity of the invertebrate phases could be readily undertaken. Conceivably, such a system could be utilized to collect large numbers of malaria sporozoites, free of host tissue, for immunological studies. Screening of possible anti-malarial drugs for their effectiveness as

sporontocides would also be feasible. Although this potential has long been recognized, attempts to devise an adequate *in vitro* system have met with limited success.

Considerable work in this field has been done by Ball and Chao (see Chao and Ball, 1964, for a general review of their work, also Ball, 1964; 1965). As a working model they chose an avian malaria, *Plasmodium relictum*, with *Culex tarsalis* and the canary as the invertebrate and vertebrate hosts, respectively. Although attempts were made to culture all stages of the sporogonic cycle, emphasis was placed on culturing oocysts of varying ages.

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Such oocysts, regardless of age, continued their development *in vitro* for an additional 4 to 5 days, but in no instance was it possible to obtain the entire cycle from zygote to mature sporozoites.

Schneider (1968a) obtained essentially similar results culturing *P. gallinaceum* oocysts alone or in the presence of various *Aedes aegypti* extracts or organs. Since the culture system employed was a more or less static one, it seemed plausible that more substantial results might be forthcoming if the various stages were cultured in the presence of actively growing mosquito cells. However, only modest improvement was noted in the development of *P. gallinaceum* oocysts when cultured in Grace's cell line of *A. aegypti* (Schneider, 1968b). It was suggested that the failure of the cell line to evoke a more promising response from the oocysts might be attributed to modifications in the cells' properties which inevitably accrue during prolonged culture *in vitro*.

Recently, however, Ball and Chao (1971) reported that the development of *P. relictum* oocysts was approximately twice as great when cultured in this same cell line as when cultured alone. Three day old oocysts underwent a 2–3 fold increase in diameter within 7 days and apparently mature sporozoites were obtained from 5 day old oocysts after 6 to 8 days *in vitro*.

In view of these encouraging results, it seemed advisable to make a more thorough assessment of the influence of actively growing mosquito cells on the sporogonic stages of malarial parasites. Within the past 5 years, techniques have been developed which make it possible, on a routine basis, to initiate and establish cell lines from many dipteran species (Schneider, 1971). The purpose of this paper was to compare the development of *P. cynomolgi* in primary cultures versus an established cell line of the anopheline host as well as to determine whether cultures initiated from adult tissues elicited a more favorable response on the part of the parasite than did those from immature stages.

Materials and Methods

The B strain of *Plasmodium cynomolgi* (*P. cynomolgi bastianellii*) was maintained by

both sporozoite and blood transfer in rhesus (*Macaca mulatta*) monkeys.* Adult female *Anopheles stephensi* (India strain) served as the invertebrate hosts.

The cell line of *A. stephensi* was initiated in 1968 and has since been maintained in continuous culture by weekly passage (Schneider, 1969). The medium utilized was a modification of Grace's original medium (Grace, 1962) supplemented with 1% abbreviated NCTC-135 medium and 15% inactivated fetal bovine serum. Penicillin and streptomycin were employed at the respective concentrations of 100 units and 100 mcg/ml of medium.

Primary cultures of *A. stephensi* were initiated from neonate larvae and from adult ovaries. The explants were cut into 2–3 pieces and treated with 0.2% trypsin (1:250, Difco) in Rinaldini's salt solution (Rinaldini, 1954) for 20–60 minutes at room temperature. After successive washes in fetal bovine serum and culture medium the pieces were transferred either to sitting drops or to individual wells of Microtest tissue culture plates containing 0.02–0.03 ml of complete medium. The primary cultures were allowed to develop to the point of active cell migration and division, usually 4–7 days, before the parasites were introduced.

In an attempt to obtain *P. cynomolgi* gametocytes free of the other blood stages as well as from uninfected erythrocytes, infected, heparinized or defibrinated blood was layered on the bottom of discontinuous bovine serum albumin gradients with specific gravities ranging between 1.068 and 1.108. The gradients were spun at 39,000 rpm for 60 min in a Spinco centrifuge with a SW-50.1 head. The uppermost fractions were removed, washed with medium and placed in culture.

Since it was not technically feasible to dissect out oocysts less than 20 μ in diameter, entire midguts containing the oocysts were placed in culture until the latter were 6 days old. From day 9 on, the oocysts could be excised from the midgut without any detectable extraneous material. Some midgut material was deliberately left attached to the

* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

Table 1. Response of *Plasmodium cynomolgi* oocysts to differing conditions of *in vitro* culture.

Age of oocysts in days	No. of cultures	Culture system	Observations
4*	10	Medium only	Degenerative changes apparent in 24 hrs
4*	10	<i>A. stephensi</i> cell line	Maintenance for 2-3 days; no growth or differentiation of oocysts
4*	10	Primary culture (adult)	Maintenance for 48 hrs; no growth or differentiation
4*	10	Primary culture (larvae)	Maintenance for 48 hrs; no growth or differentiation
6	3	Medium only	Maintenance for 24-48 hrs
6	4	<i>A. stephensi</i> cell line	Diameter of oocysts increases 4-10 μ within 72 hrs; internal changes not apparent
6	3	Primary culture (adult)	Diameter of oocysts increases 3-8 μ in 48-72 hrs; no internal changes
6	2	Primary culture (larvae)	Diameter increases 4-13 μ within 72 hrs; cytoplasmic cleavage in majority of oocysts of one culture
8	4	Medium only	Minimal growth; some cytoplasmic cleavage in almost all oocysts by day 5 or 6
8	4	<i>A. stephensi</i> cell line	Growth of oocysts; cytoplasmic cleavage; formation of immature sporozoites in 4-5 days
8	5	Primary culture (adult)	Growth and cytoplasmic cleavage of oocysts; immature and mature sporozoites but oocysts remain intact
8	5	Primary culture (larvae)	Same as above; sporozoites are not motile within oocyst but if released the mature forms are quite active
9	5	Medium only	Mature(?) sporozoites in oocysts in 72 hrs; no free sporozoites
9	5	<i>A. stephensi</i> cell line	Free sporozoites in 4 of 5 cultures within 24-48 hrs but many intact oocysts
9	5	Primary culture (adult)	Free sporozoites in 4 of 5 cultures within 24 hrs; some intact oocysts
9	5	Primary culture (larvae)	Free sporozoites in 5 of 5 cultures within 24 hrs; very few intact oocysts
10	5	Medium alone	Motile sporozoites within intact oocysts; some free sporozoites
10	5	<i>A. stephensi</i> cell line	Free sporozoites in 24 hrs or less
10	4	Primary culture (adult)	Free sporozoites in 24 hrs or less
10	5	Primary culture (larvae)	Free sporozoites in 24 hrs or less

* Oocysts were not dissected away from midgut.

6 and 8 day oocysts to avoid dissection injury. A single midgut or from 5-15 oocysts were placed in each culture.

The cultures were maintained at $25 \pm 1^\circ\text{C}$ with a gaseous phase of ambient air. Daily observations were made with an inverted phase contrast microscope.

Results

Separation of erythrocytes from leukocytes was readily achieved by passing the blood through packed, powdered filter paper columns (Cook *et al.*, 1969). However, attempts to separate the various blood forms of *P. cynomolgi* in different fractions of the serum albumin gradient were unsuccessful. The majority of gametocyte-infected cells were found in the two lightest fractions (1.068 and 1.078) but so were the majority of schizonts.

Although exflagellation may be seen within 10-15 minutes after freshly drawn blood is placed in culture, this process was not observed after aliquots of cells from the lightest fractions of the gradient were placed *in vitro*

with medium alone or with the *A. stephensi* cell line. After 24 and 48 hours, cells from both series were fixed with methanol and stained with Giemsa. There was no evidence of zygote formation or ookinete development in any of the 14 preparations examined.

Table 1 summarizes the results obtained when *P. cynomolgi* oocysts of varying ages were placed *in vitro*. In general, the oocysts responded more favorably when cultured together with *A. stephensi* cells, regardless of their origin, than in medium alone.

Four day old oocysts, still attached to the midgut, showed the least favorable response to *in vitro* culture. Such oocysts survived less than 24 hours when placed in medium alone and showed degenerative changes in both primary cultures and in the established cell line within 48 to 72 hours. Neither growth nor any visible signs of internal development took place within that span of time.

Based on measurements of 55 individual oocysts, the diameter of 6 day old oocysts when placed in culture averaged 18 μ . Al-

though a few oocysts showed no evidence of growth the majority of those cultured in the presence of cells increased in size. Within a period of 72 hours, these increases ranged from a modest 3–4 μ to a fairly substantial 13 μ . Further growth did not take place even though some of the oocysts showed no signs of deterioration for as long as a week thereafter. With the exception of one culture, there was no evidence that the oocysts responded more favorably to the cells of a primary culture than to those of the established line. This culture consisted of 12 oocysts and numerous cell colonies from a neonate larval explant. Cleavage of the cytoplasm took place in all but one of the oocysts and after 72 hours their appearance was similar to that of an 8 day oocyst although somewhat smaller than normal. At this point the medium was renewed but rather than stimulating further growth it had quite the reverse effect and the culture was soon discarded.

The growth of 8 day oocysts *in vitro* was quite limited, usually to 5 μ or less. However, cytoplasmic cleavage readily took place in most of the oocysts including those in medium alone. If the oocysts were cultured in the presence of *A. stephensi* cells, cleavage of the cytoplasm was followed by the appearance of immature sporozoites within 72 hours (Figs. 1, 2a-c). Most of the sporozoites did not attain a mature size or shape but rather were somewhat foreshortened and resembled broken signets (Fig. 2d). The oocysts did not rupture spontaneously but if external pressure was applied the liberated sporozoites made active flexing movements.

By day 9, immature sporozoites had already budded from the sporoblast cores and required only an additional 24–48 hours to mature. Freed sporozoites were present in almost all of the cultures containing cells, usually within 24 hours. Approximately 40 percent of the oocysts cultured with the cell line did not rupture whereas the figure for both primary cultures from the adult and larval stages was about 25 percent.

On day 10, most oocysts contained mature or virtually mature sporozoites and needed less than 24 hours to rupture and free the parasites. About 30 percent of the oocysts did not rupture in cultures containing only medium as compared to less than 5 percent in



Figure 1. Typical culture of 8 day old *P. cynomolgi* oocysts after 48 hours *in vitro*. Cells of the *A. stephensi* line are seen growing on the floor as well as on the wall (dark area) of a Microtest culture chamber. X 100.

cultures containing cells. There was no difference in this percentage between the established line and the primary cultures.

Discussion

In 1967, Rowley *et al.*, used a bovine serum albumin gradient in an attempt to separate the various stages of schizogony of *P. berghei* malaria. Quite adequate separation was achieved between the trophozoites and schizonts but not between the latter and the gametocytes. Using this technique with appropriate adjustments for osmotic pressure and specific gravities, essentially the same results were obtained with *P. cynomolgi*. In addition to the lack of substantial numbers of gametocytes for *in vitro* culture, the relatively long length of time on the gradient at 4°C may well have impaired the ability of these forms to develop further. Nor was it, of course, possible to duplicate *in vitro* the milieu of the mosquito midgut *in vivo*. Any one of the above factors would undoubtedly suffice to block the processes of exflagellation and fertilization. Hence, it is not surprising that

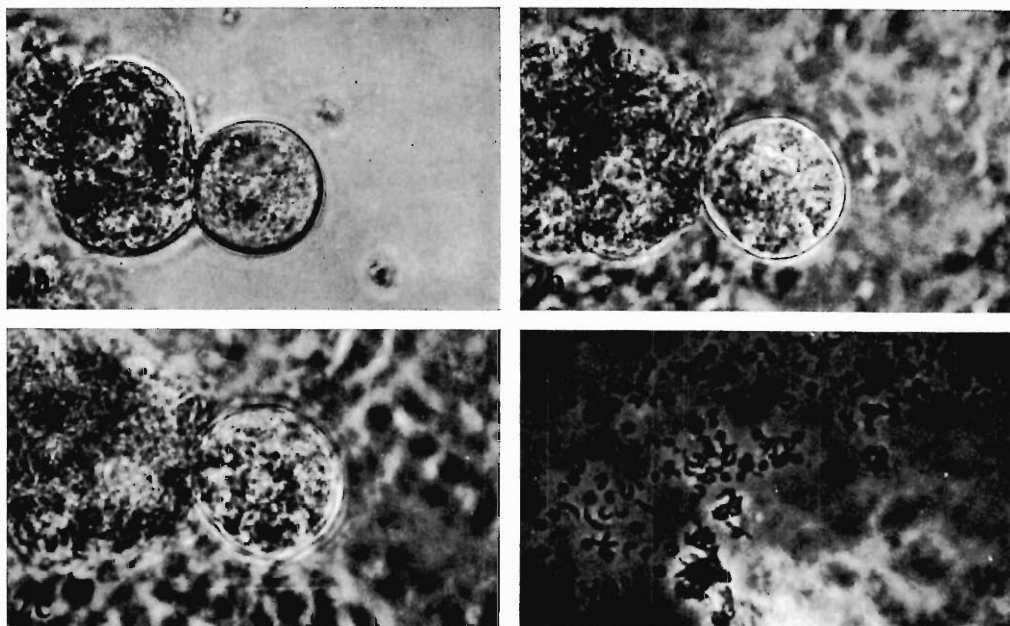


Figure 2a. Eight day old oocyst, still attached to a portion of midgut tissue, a few hours after being placed in culture. Small clumps of cells from the *A. stephensi* line may be seen above and to the right of the oocyst. 2b. Segmentation of oocyst cytoplasm is apparent after 48 hours in culture. The midgut tissue flattens out due to cell migration but the cells do not divide. 2c. Same oocyst after 72 hours in culture. Immature and mature sporozoites are present in the oocyst which is now completely surrounded by *A. stephensi* cells. 2d. Normal appearing and "broken signet" shaped sporozoites expressed from cultured oocyst. X 200.

neither zygotes or ookinetes were found in the preparations examined.

The test of the capacity of 4 day old oocysts to develop *in vitro* was perhaps not too valid. The inclusion of the entire midgut in the cultures necessitated using fungazone, in addition to penicillin and streptomycin, to control contaminants. Starving the mosquitoes for 24–48 hours prior to dissection reduced but did not eliminate the contamination. Stanley and Vaughn (1967) reported that some insect cells are markedly sensitive to this antibiotic and it may have had an adverse effect, albeit visually undetectable, on the midguts which in turn affected the oocysts.

The insect cycle of *P. cynomolgi* lasts approximately 11 days at 25–26°C. Until day 6 the oocysts increase in size at a fairly moderate pace. Between days 6 and 8 a considerably higher growth rate prevails.

Thereafter, it tapers off and the emphasis switches to differentiation. This same pattern was evident in the cultured oocysts in that day 6 oocysts responded primarily by growth whereas in 8 day oocysts cleavage of the cytoplasm took place followed by the appearance of sporozoites. Rarely, however, did the size of the oocysts *in vitro* attain that found *in vivo* on comparable days nor was the rate of development as fast. For example, the size of 6 day oocysts after 3 days *in vitro* (in primary cultures from neonate larvae) averaged 25 μ in diameter or an increase of 7 μ . *In vivo*, 9 day oocysts averaged 42 μ in diameter, an increase of 24 μ between days 6 and 9. And developmentally, 8 day oocysts *in vitro* invariably lagged 24 or more hours behind their counterparts *in vivo*.

In their most recent paper, Ball and Chao (1971) reported that 3 day *P. relictum* oocysts placed in culture with Grace's *A. aegypti* cells

grew from 8μ to 27μ in 6 days; 4 day oocysts from approximately 18μ to 37μ in 7 days and 5 day oocysts, 20–25 μ in diameter, developed apparently mature sporozoites within 6–8 days. The above growth rates approximate that found *in vivo*. Nothing comparable was obtained in the present study.

It is rather remarkable that such extensive development of *P. relictum* took place in Grace's cell line of *A. aegypti* but not in a line established from the same species by Singh (1967). Recently, Greene and Charney (1971) have shown by chromosome data, agar gel immunodiffusion and isoenzyme analysis that the cell line of Grace, designated as "*Aedes aegypti*" is, in reality, a line from the silkworm, *Antheraea eucalypti*. (It is not known when the original line of *A. aegypti* was contaminated by the moth cells or when a mis-labelling of flasks occurred. However, the evidence is fairly conclusive that all subcultures of this line which have been propagated in the United States and elsewhere since 1967 consist of moth rather than mosquito cells). Identical tests by the same investigators indicated that the cell line established by Singh is a *bona fide* *A. aegypti* line. *P. relictum* has one of the broadest host and vector distributions of any malarial parasite, encompassing many avian orders and mosquito genera. Avian plasmodia typically, and especially *P. relictum*, exhibit this pattern as contrasted to mammalian plasmodia of the subgenera *Plasmodium*, *Vinckeia* and *Laverania* which have extremely restricted host and vector associations—often limited to a single mammalian species and an anopheline species group. This relative lack of specificity on the part of *P. relictum* may account for the successful growth of these parasites in abnormal *in vitro* systems. Even so, it is noteworthy that cell cultures from a silkworm are seemingly more conducive to the development of *P. relictum* than are those from a mosquito.

In the present study, it is a little surprising that there was so little difference in the response of *P. cynomolgi* oocysts to cells of the established *A. stephensi* line versus those from either of the two types of primary cultures. It seems reasonable to assume that the cells of the primary cultures, having undergone much less selection or adaptation, would be much more likely to retain the characteristics

of the cells *in situ* than would those from an established line. It might also be expected that primary cultures from an adult stage would serve as a better supportive system than those from an immature stage. The contrary seemed to be the case in this study as somewhat better success was often obtained with cultures of neonate larvae than with those of adult ovaries. However, this may simply be a reflection of their relative growth rates since explants of the former invariably grew much faster than those of the latter.

In one sense, studies such as this one may be somewhat premature and misleading. Insect tissue culture is still an inexact science with many existing gaps in our knowledge. The composition of insect hemolymph may vary considerably in different stages of the same insect—shifts in the concentrations of amino acids, carbohydrates, peptides and proteins as well as in inorganic cation-anion ratios have been amply documented. With few exceptions these differences, when known, have not been incorporated into the design of culture media. Nutritional studies on the cell lines, once established, are also needed. And much more emphasis must be placed on determining which organs or tissues, particularly from the adult insect, can serve as sources of proliferating cells. Until such quantitative data are available, the adequacy and usefulness of any cell cultures obtained, whether primary or established, may well fall short of their potential.

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Comments on Entomology

J. E. SCANLON

The University of Texas, School of Public Health, Houston, Texas

The control of malaria has usually focused primarily on attack on the anopheline vectors. Whether "bonification" or Paris Green, or residual application of DDT, emphasis has been upon the lowering of the numbers or shortening of the lifespan of the mosquito vectors, supplemented at times by mass chemotherapeutic measures, such as distribution of treated table salt.

Thus, detailed knowledge of the relationships of the malaria parasites with the mosquitoes, and of the biology and ecology of the Anophelines are obviously of immense importance in the control of the disease. The papers in this section have addressed some of these relationships, but understandably cover only a tiny portion of the spectrum. In many places in the malarious world today we still do not know the answers to some of the most basic epidemiological questions—such as the relative importance of the various *Anopheles* species as vectors under varying ecological conditions.

The method of comparative *Anopheles* feeding on carriers and subsequent dissection or

transmission has a long history in malaria investigation (Boyd and Stratman-Thomas, 1934) and has provided much useful information. Some of the pitfalls are obvious (strain differences in mosquitoes and parasites, the selective pressures at work in colonization of mosquitoes, etc.) and are discussed in the paper by Wilkinson, *et al.*

Since 1962 most of the reports of chloroquine resistant strains of *Plasmodium falciparum* in Southeast Asia have come from areas where *Anopheles balabacensis* was the most likely vector, and the authors point out that this has led several observers to suggest that the relationship might be one governed by more than chance. In many cases it has been possible to trace the exact site of infection, under circumstances where *A. balabacensis* was almost certainly the vector. Recently, however, McKelvey, *et al.* (1971) have reported well documented cases of chloroquine resistant *P. falciparum* contracted in areas of Malaysia and Singapore where *A. balabacensis* is not known to occur, although the subspecies *introlatus* presumably is present.

It is unfortunate that the status of chloroquine sensitivity could not have been determined for the human subjects observed in the present study by Wilkinson, *et al.*, but the results reported certainly make it worthwhile to continue the work with human parasites of known drug status. The control of *Anopheles balabacensis* appears to be considerably more difficult than the control of *A. minimus*, although the precise contributions to this difficulty of such matters as exophagy, exophily and dispersal of human population in forested areas remains to be determined. If further work does confirm an advantage for *A. balabacensis* in the transmission of chloroquine resistant strains of *P. falciparum* it is obvious that an even greater effort will have to be expended in developing control measures for this species. It should be noted, however, that *A. minimus* was quite capable of supporting the development of many of the strains examined, and presumably this could have included some chloroquine insensitive strains.

The insecticide resistance problem has not as yet influenced the major campaigns greatly, but Garnham (1971) and other workers have held that the potential danger from this source may outweigh the drug resistance problem. One attractive research area for alternative anopheline control measures has been the field of biological control—particularly in the face of the growing outcry against pesticides in the environment.

Fish, particularly *Gambusia* have long been used against *Anopheles* larvae, but many vector species are found in aquatic environments unsuitable for these predators. The use of microbial agents against *Anopheles* was summarized in a symposium at the Walter Reed Army Institute of Research in 1969 by Chapman, *et al.* (1970) and by Roberts (1970). Little additional work appears to have been done since that time with any agents except the microsporidian parasites discussed here in the papers by Ward and Savage, and Anthony, *et al.* and a species of *Coelomomyces* fungus which shows some promise.

For a non-specialist, the taxonomic confusion surrounding the microsporidian species found in *Anopheles* mosquitoes makes a critical review of the literature a more than usually difficult problem. In recent years there has

been a virtual explosion of reports of microsporidian parasites in *Anopheles* colonies (Hazard, 1970; Canning and Hulls, 1970) and a number of names have been applied to the parasites. It would certainly seem worthwhile to support the work of an experienced protozoan taxonomist to clarify this problem—since the results of *Nosema* infestation in colonies of *Anopheles* have often been catastrophic, and they appear to be among the more promising microbial agents for *Anopheles* control.

The report by Anthony, *et al.* on reduction of longevity of *Anopheles albimanus* infected with *Nosema* offers a very interesting possibility for the use of *Nosema* in malaria control programs. The reductions in longevity at 50 and 95 percent levels reported certainly appear to be impressive. The various parameters examined in the model are listed in the paper but it would also have been useful to have the mathematical statement of the model employed for the hypothetical population. It appears that a method has been developed for production of reasonable numbers of spores, by maceration and filtration of infected adults or larvae. However, an even simpler method, possibly *in vitro*, will probably have to be developed if this parasite is to be considered for large scale field use. This is one problem which has heretofore presented a roadblock in microbial control of insects. There are a few exceptions, such as *Bacillus* species, which can be produced on a commercial scale. The information presented here on *Nosema* certainly seems to warrant intensified early work on methods of production of the parasite.

This is particularly true in view of the bonus effect referred to by Anthony, *et al.*, and amplified by Ward and Savage—namely, that infection with *Nosema* may somewhat reduce the ability of the surviving adults to transmit malaria. This effect has now been reported for the combinations—*Plasmodium berghei* : *Anopheles stephensi*; *P. gallinacium* : *A. quadrimaculatus* and *P. cynomolgi* : *A. stephensi*. The earliest report of this nature appears to have been that of Fox and Weiser (1959) who found in experimental feedings that *Anopheles gambiae* females which were heavily infested with *Nosema* would not support the development of *P. falciparum*. They

attributed this to the disintegration of the midgut wall in heavily infected females. It thus appears that this phenomenon may be fairly widespread and may play some role in nature in reducing the vector capacity of *Anopheles*. The whole topic of the interactions of protozoa, viruses and other microorganisms in mosquitoes is in need of much more extensive examination.

As Dr. Schneider notes in her paper the availability of an *in vitro* culture system for the arthropod phase of the malaria parasites—from zygote to sporozoite, would provide us with an extremely useful tool. Not only would such a system permit detailed and finely controlled biochemical, physiological and drug studies, it might also enable us to obtain large amounts of relatively pure sporozoite tissue for antigenic analysis and immunization trials. The latter element is the primary rationale upon which Dr. Schneider's studies were originally undertaken. It is therefore, somewhat disappointing to find that the goal is almost as elusive as ever, despite the progress which she and others have made in the field. The pioneering work in this field by Ball and Chao is now over fifteen years old, and the results presented here by Dr. Schneider represent the furthest advance which appears to have been made by any of the small group of workers publishing in this field. It has been possible to bring rather late oocysts of several species through to the sporozoite stage, or to bring early oocysts to a somewhat later stage but not to carry a single zygote or ookinete through the complete cycle. While some biochemical and other studies may be possible under such conditions it is obvious that progress will be slow until it is possible to complete the mosquito cycle in one step.

The fact that the establishment of primary cultures, as well as cell lines of mosquito tissue is now accomplished much more readily than heretofore, is a hopeful sign since mosquito cells appear to accelerate the growth of the oocysts. What is needed badly at the moment is a definitive study of the biochemistry of mosquito hemolymph at various stages in the mosquito life cycle. The possible interference in oocyst growth encountered when fungazone was employed might point up a need for working with axenically reared mosquitoes, but the small number of mosquitoes

which can usually be made available by that technique is a hinderance in itself.

Despite the present rather slow rate of progress in culturing of the invertebrate stages of malaria parasites it certainly seems to be worth continued, and perhaps expanded, support—particularly if *in vitro* methods for all stages of the vertebrate phase can also be developed. However, Trager (1969), in the last WRAIR Panel Workshop, remarked that it was clear that we were not very close to a method for continuous *in vitro* cultivation of the erythrocytic stages of any malaria species. Nevertheless, work with the mosquito stages should be continued in the hope of eventually coupling the two systems.

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VIII

IMMUNITY

A. Resistance Mechanisms

The Protective Effect of African Human Immunoglobulin G in *Aotus trivirgatus* Infected with Asian *Plasmodium falciparum*

C. L. DIGGS, B. T. WELDE, J. S. ANDERSON, R. M. WEBER, AND E. RODRIGUEZ, JR.

Department of Medical Zoology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT. Human immunoglobulin G, isolated from plasma obtained in Nigeria, where *Plasmodium falciparum* malaria is highly endemic, was studied for its protective effect in *Aotus trivirgatus* infected with a strain of *P. falciparum* (Camp) isolated in Malaysia. Control monkeys were treated with comparable immunoglobulin prepared with plasma obtained in the U.S.A. A delay in onset of patency and an increase in survival time was observed in monkeys that received IgG from the malaria endemic area. Three Nigerian IgG treated monkeys survived challenge; one of these did not become patent. The data indicate that protection is mediated by IgG and not by contaminating materials.

Modification of the course of malaria infections by passively transferred antibody is a familiar phenomenon. Among the most noteworthy contributions are those of Cohen, et al. (1961) who clearly demonstrated that the IgG fraction of plasma collected in Gambia suppressed falciparum malaria contracted locally. Activity was not observed in control IgG from plasma collected in the United Kingdom. Sadun, et al. (1966) also demonstrated the therapeutic effect of human West African gamma-globulin against homologous falciparum malaria, in this case in chimpanzees. In addition, it was shown that the material can be used prophylactically. McGregor, et al. (1963) demonstrated that West African gamma-globulin was also effective against East African *Plasmodium falciparum*, suggesting antigenic similarity between the geographically distinct lines of the parasite. Sadun, et al. (1966) using the chimpanzee model studied the effect of African globulin on the Southeast Asian strain of *P. falciparum*. Although they observed a trend toward a lower parasitemia, the results were not considered conclusive. The present experiments were designed to pursue the study of the effect of West African immunoglobulin on Southeast Asian *P. falciparum*, using *Aotus trivirgatus*, the owl monkey, as the experimental host.

Materials and Methods

The line of *P. falciparum* used in these experiments was derived from a Malaysian

isolate, designated as the Camp strain, which has been serially passaged in *Aotus* monkeys* for several years in this laboratory. A history of this strain and the course of the disease it produces have been described (Hickman, 1969; Welde, et al. 1972). Plasma was obtained from blood collected at the Blood Bank of the University College Hospital of the University of Ibadan in Nigeria where malaria due to *P. falciparum* is highly endemic and from the Blood Bank at the Walter Reed General Hospital in Washington, D.C. In a pilot experiment, control globulin consisted of Immune Serum Globulin, USP, obtained commercially (Courtland Laboratories, Los Angeles, California). In all other experiments, control and experimental preparations were processed in parallel by the same methods. Plasma was clotted with calcium chloride and the serum collected by centrifugation. Isolation of IgG was accomplished on DEAE cellulose (Campbell, et al. 1963) or DEAE Sephadex (Baumstark, et al. 1964) by batch methods. Protein concentrations were monitored by absorbance at 280 nm (McDuffie and Kabat, 1956). The composition of serum protein preparations was examined by electrophoresis on cellulose acetate, immunoelectrophoresis (Scheidegger, 1955) and two-dimensional immunodiffusion against a potent anti-whole human serum (Hyland Division Travenol Laboratories, Inc., Costa

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* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

Mesa, California, Lot #8030M002A1) using wells 2.5 mm in diameter and 1.5 mm apart (Hyland, pattern D). In addition, a commercially obtained anti-human transferrin (Hyland Lot #8215H001A1) was also used. Examinations of the preparations for immunoglobulins M and A were performed through the use of radial immunodiffusion plates obtained commercially (Hyland). Standard methods were used for determining the toxicity of IgG preparations for the ten day old chick embryo (Smith and Thomas, 1956).

Physical, hematological and blood parasitological examinations were performed on the *Aotus* monkeys and only apparently healthy animals were chosen for experimental use. The animals varied in weight from 500 to 1100 grams. Procedures for maintenance of the monkeys has been described (Wellde, et al. 1971). Proteins were administered intraperitoneally on a weight basis at the time of challenge by the intravenous injection of $1-3 \times 10^6$ parasitized erythrocytes obtained from a donor monkey. Thin blood films were obtained from the monkeys daily thereafter until death or until 30 days had elapsed. In spite of the efforts to exclude unhealthy animals from the experiments, microfilariae were seen in the blood of two animals after the experiments were begun. Since we have noted a decrease in susceptibility to *P. falciparum* in animals with filariasis (unpublished observations), these were excluded from the experiment. Postmortem examinations were performed routinely for evidence of inapparent disease. In three cases animals which died with low parasitemias were found to have intercurrent disease; one animal had massive peritonitis and two had severe bronchopneumonia with lung abscess. These animals were also excluded from further consideration.

In analyzing the data, two parameters were given major consideration; the prepatent period and the survival time. The prepatent period was defined as the time required for the first positive blood smear after injection of parasites. The day of death was taken as the day after the last blood film was obtained, since in a few cases the exact time of death (before or after midnight) was not recorded. Comparisons between groups of animals on the basis of these parameters were made by the Mann-Whitney U Test (Siegel, 1956) and

the 95% confidence level was chosen for significance testing.

The concentration of transfused human IgG in *Aotus* monkey serum was determined through the use of commercially obtained radial immunodiffusion plates (Hyland). Although some cross reaction with a material in *Aotus* serum (presumably *Aotus* IgG) was evident, this did not interfere with quantitative estimates when measurements of human IgG were made in the presence and in the absence of monkey serum.

Passive hemagglutination tests were performed on globulin preparations by the method of Wellde, et al. (1969) as modified by McAlister (1972).

Results

Experiment 1

The pilot experiment consisted of observations in two monkeys which were inoculated with 1×10^6 parasitized erythrocytes and treated with globulin within the hour. The first animal, which weighed approximately 1000 grams, was given 200 mg of American globulin (Immune Serum Globulin, USP); the second monkey, which weighed approximately 800 grams, was given an equal amount of Nigerian globulin which had been lyophilized and stored for six years. The control animal became patent on the third day after injection and succumbed to the infection on day 9. In contrast, the animal which received Nigerian globulin did not become patent until day 12 and death was deferred until day 21. These observations are included in Fig. 1. Based on this pilot trial, it was decided to examine the effect observed in detail.

Experiment 2

An experiment was performed in which serum prepared from plasma of Nigerian or American origin at a dose level of 30 ml per kilogram was given to monkeys immediately before challenge with 1×10^6 parasitized erythrocytes per kilogram body weight. The results of this experiment are summarized in Fig. 1. Control animals became patent 4 to 10 days later whereas the experimental monkeys became patent on days 13 and 18 respectively. One of the animals (#533)

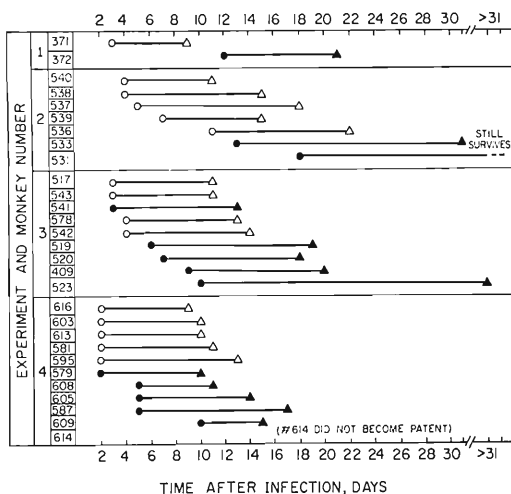


Figure 1. Prepatent periods (circles) and survival times (triangles) in 29 *Aotus* monkeys infected with the Camp strain of *P. falciparum*. Solid symbols refer to animals treated with serum or globulin obtained in Ibadan, Nigeria, whereas open symbols refer to the control animals. Controls were treated with serum or globulin obtained in Washington, D.C., U.S.A., except for monkey Nos. 616 and 603 which received saline.

which received immune serum exhibited a protracted course of parasitemia; on the day before death of the last control animal the parasitemia in this monkey (#533) had risen only to 5%. It increased gradually thereafter to 17% on the 28th day of infection, then rapidly rose to 31% on the 29th day and to 73% on the 30th, the day before death. Monkey #531 exhibited an even more dramatic course; although it became patent on day 20, the parasitemia remained below 1% until day 28 at which time it rose to 2%; the peak parasitemia at 8% occurred on the 29th day, after which parasites could not be detected. The animal is still alive, more than one year after infection.

Experiment 3

The effect of isolated immunoglobulin on the course of the infection was next studied. The material was obtained by the DEAE cellulose method and was administered at a dose level of 200 mg per kilogram body weight. The challenge infection (2×10^6 parasitized erythrocytes per kilogram) was given

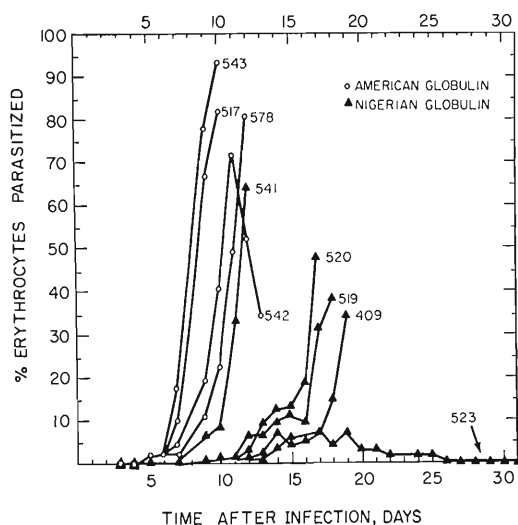


Figure 2. Parasitemia in *Aotus* monkeys treated with the crude IgG fraction of serum collected in Ibadan (triangles) or Washington, D.C. (circles) and infected with the Camp strain of *P. falciparum*. Monkey numbers follow the appropriate curves.

approximately one hour after the globulin treatment. These results are summarized in Fig. 1 and presented in greater detail in Fig. 2. Whereas one of the animals which received Nigerian globulin became patent on day 3, the prepatent period for the other four experimental monkeys varied from 6 to 10 days; in contrast, all control animals became patent on the third day after infection. The first death in the Nigerian globulin treated group was on day 13 and the last on day 42. Postmortem examination revealed acute renal cortical necrosis as the cause of death in this latter case (Monkey #523). The etiology of the renal pathology was not clear, but it seems likely that it was unrelated to the malaria infection *per se*. The peak parasitemia in this animal was 7% (Fig. 2). A uniformly progressive parasitemia resulted in the death of the control animals on days 11–14.

In spite of the fact that the prepatent period of only one experimental monkey was within the range of the controls, the group difference is not statistically significant at the 5% level. However, survival times were significantly different in the American and Nigerian IgG groups.

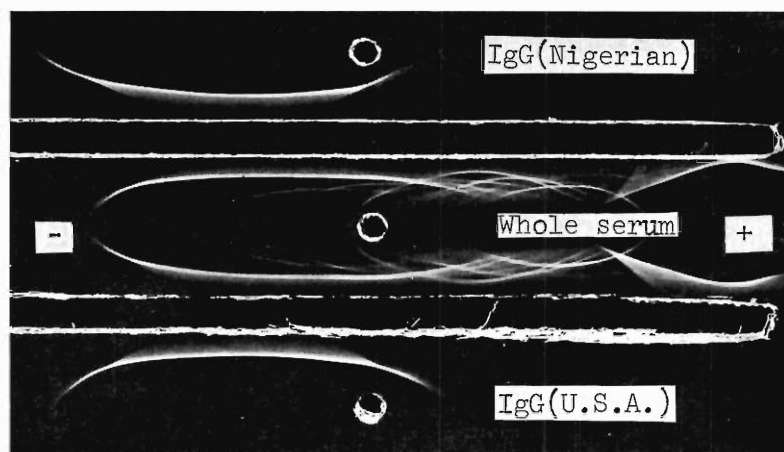


Figure 3. Immunoelectrophoretic analysis of human IgG preparations derived from blood obtained in Nigeria and the U.S.A. Patterns were developed with an antiserum to whole human serum.

Since the immunoglobulin preparations used in experiment 3 were relatively impure (an 8 and 18% beta globulin contaminant in the Nigerian and American gamma-globulin preparations respectively by cellulose acetate electrophoresis), the possibility that some material

other than IgG was responsible for the observed effects could not be lightly dismissed. We therefore performed an additional experiment using highly purified IgG in an attempt to minimize the possible effects of contaminating materials.

Experiment 4

The preparations were isolated from the plasma by the DEAE Sephadex method, and concentrated to 50 mg per ml. At this level, one of the preparations (American globulin) gave 2 lines on immunoelectrophoresis and in two-dimensional immunodiffusion against anti-whole human serum. On examination with anti-transferrin in two dimensional immunodiffusion experiments, a precipitin line could be detected. When the preparation was diluted with an equal volume of saline (i.e. at 25 mg of protein per ml) a single line was seen with anti-whole human serum and transferrin could no longer be detected. The experimental (Nigerian) IgG preparation revealed a single line in immunoelectrophoresis and two-dimensional immunodiffusion experiments at a concentration of 50 mg per ml, and the test for transferrin was negative. No contamination was observed in either preparation by cellulose acetate electrophoresis. Immunoelectrophoresis patterns of the proteins are illustrated in Fig. 3. No contaminant can be discerned in either IgG preparation in this photograph.

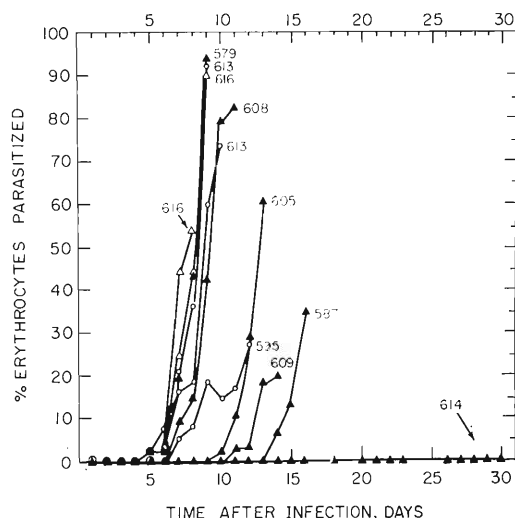


Figure 4. Parasitemia in *Aotus* monkeys treated with a purified IgG fraction of serum collected in Ibadan (solid triangles) or Washington, D.C. (circles) or with saline (open triangles) and infected with the Camp strain of *P. falciparum*. Numbers as in Fig. 2.

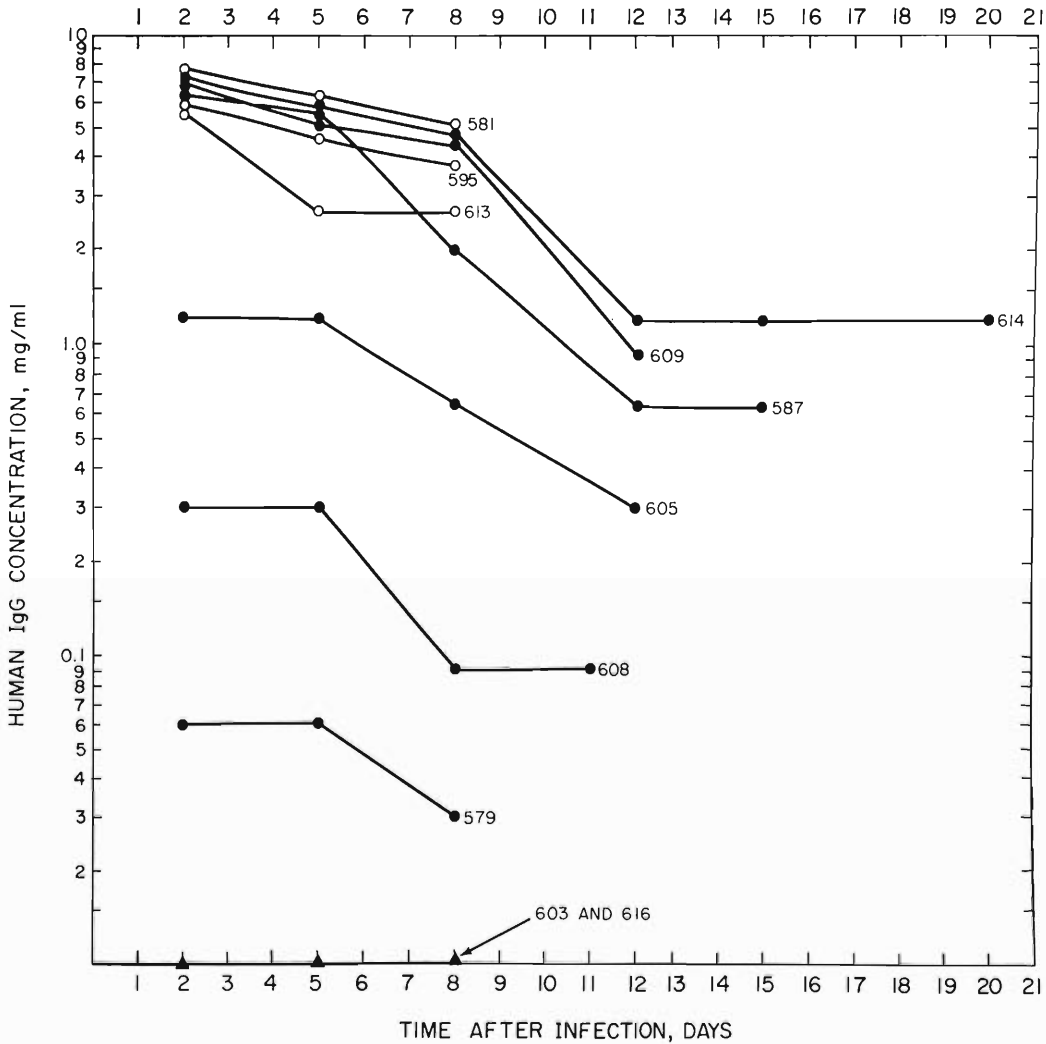


Figure 5. Human IgG levels in the serum of *Aotus* monkeys after the injection of saline (▲) or purified IgG prepared from serum obtained in Ibadan (●) or Washington (○). Numbers as in Figs. 2 and 3.

Hemagglutination tests for antimalarial antibody were performed on these preparations. Whereas the American IgG gave no hemagglutination at a dilution of 1:4, the Nigerian IgG preparation was reactive at a 1:512 dilution.

Both preparations were tested for possible endotoxin contamination in chick embryo toxicity tests; no evidence for toxicity was obtained with 1/10 ml volumes of the preparations at a concentration of 5 mg per ml.

Six monkeys were given the experimental Nigerian IgG and three the American preparation; two animals were given saline. IgG was administered at a dose level of 500 mg per kilogram body weight. The animals in this experiment ranged in weight from 510 to 760 grams. The globulin was administered intraperitoneally immediately prior to the intravenous injection of 2.5×10^6 parasitized erythrocytes per kilogram body weight. The results are included in Fig. 4. It can be seen

that the control animals were patent on the second day after infection and that all were dead by the 13th day of the experiment. Whereas one of the experimental monkeys was also patent on day 2, patency in the other monkeys was delayed; three until day 5, one until day 10, and one indefinitely. This latter animal had not shown parasites on the 35th day after initiation of the experiment. There was a tendency for animals receiving American immunoglobulin G to succumb somewhat more slowly to the parasitemia than animals receiving saline only (Fig. 4). In spite of the fact that there was a delay in onset of disease in four of the animals given Nigerian globulin, patency in two of the experimental animals was within the range of the controls. Nevertheless, the experimental group was significantly different from the controls with respect to both prepatent period and survival time.

Figure 5 illustrates the results of determinations of human IgG concentrations in the serum of the monkeys in the experimental (Nigerian IgG) group. Tests on serum taken from each monkey prior to injection of the human IgG or saline were all negative. It can readily be appreciated from these data that whereas high levels of human IgG were achieved in monkeys No. 605, 587, 609, and 614, monkeys No. 579 and 608 had lower serum concentrations of the human immunoglobulin. Monkeys which were given American IgG (Nos. 581, 595 and 613) also exhibited high serum IgG levels. All sera from monkeys injected with saline were negative.

Discussion

These studies demonstrate that human serum collected in West Africa, or the globulin fraction of such serum, extends the pre-patent period and survival time of *Aotus* monkeys infected with a Malaysian strain of *P. falciparum* (Fig. 1-3). In addition, some treated animals survived. In our experience, spontaneous cure is extremely rare (Wellde, et al. 1972). The results suggest that a degree of similarity exists between the protective antigens of these geographically remote strains of *P. falciparum*. This is the first convincing evidence, to our knowledge, of protection from a line of *P. falciparum* by antibody derived from a different continent. The results com-

plement findings of others who have found partial immunity to heterologous challenge in humans (Jeffrey, 1966) and *Aotus* monkeys (Voller and Richards, 1970) actively immune to *P. falciparum*.

The data indicate that the protection demonstrated is due to immunoglobulin G antibody. Although the presence of small amounts of other serum proteins cannot be ruled out, there is no reason to suspect that such contamination could be responsible for the effects observed. Small quantities of bacterial endotoxins have been shown to induce resistance to rodent malaria (Martin, et al. 1967) and could therefore conceivably be active in the present system. However, this is unlikely since no toxicity of the preparations for the chick embryo was detected. Most small molecular weight materials (viz. drugs) would be expected to have been removed by the extensive dialysis which was a part of the procedure for IgG preparation in Experiments 1, 3 and 4.

As observed in most studies of passive serum mediated immunity to malaria, the protection demonstrated was only transient in most of the animals. It can be speculated that parasites with an altered antigenic specificity might have emerged which were no longer reactive with the passively administered antibody. Antigenic variation of this type has been convincingly demonstrated for *P. knowlesi* (Brown and Brown, 1965) and *P. berghei* (Briggs, et al. 1968) infections. Alternatively, it might be considered that even with the relatively massive doses of protein used, the amount of protective antibody was too limited to destroy all parasites, and that the survivors replicated and ultimately killed the majority of the animals. Whether variant or species specific, it seems likely that the antigen(s) involved in the protective effects noted exist in at least two geographically separated strains of *P. falciparum*; namely, those responsible for the development of the antibody obtained in Africa and in the challenge population. However, the possibility that the protective effect is due to antibody elicited in response to cross reactive nonplasmodial antigens cannot be ruled out.

To refer to the Camp strain in *Aotus* monkeys as an Asian strain of *P. falciparum* is perhaps presumptive, since by now a more ac-

curate description might be "laboratory strain." The "Asian" designation is used only to indicate origin and not necessarily a similarity between the laboratory line and parasites to be found in the human population in Southeast Asia. The actual relationship between the antigenic structure of the parasites studied in these experiments and human pathogens can only be speculated upon. However, it is remarkable, in our view, that this laboratory parasite is sufficiently related to the pathogens in West Africa to allow the effects noted. Our results give more credence to the possibility that widespread geographic strain similarities may occur.

The examinations of the *Aotus* monkey sera for human IgG were informative (Fig. 4). These determinations offer an explanation for the failure of the antibody treatment to protect certain individual monkeys; i.e. high IgG levels were either not achieved or were only transient in these animals. Whether these differences are due to technical factors or to peculiarities of the response of the individual animals to the foreign protein has not been determined.

The question of the quantitative relationship between the "cross protection" demonstrated in this investigation and "homologous protection" is an important aspect of humoral immunity in *P. falciparum* infections which has not yet been approached. It would be important to learn whether or not the protection observed is inferior to homologous protection. Attempts are being made to obtain the necessary materials to make such trials feasible.

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Antibody Mediated Immunity to *Plasmodium berghei* Independent of the Third Component of Complement*

C. L. DIGGS, H. SHIN,† N. T. BRIGGS, K. LAUDENSLAYER AND R. WEBER

Department of Medical Zoology,

Walter Reed Army Institute of Research, Washington, D.C. 20012 and

The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT. The serum complement requirement for antibody mediated passive immunity to *Plasmodium berghei* malaria in rats was studied. An anti-complementary factor from Cobra venom (CVF) was used to destroy the third component of rat complement (C3) *in vivo*. Little or no direct effect of CVF on parasitemia was observed. Immune serum administered at the time of infection inhibited development of parasitemia. Depression of C3 levels by CVF did not diminish this antibody mediated protection. These results indicate that immune effector mechanisms in *P. berghei* malaria in the rat include a protective pathway which is antibody mediated but C3 independent.

Antibody has been shown to be an important determinant of immunity to *Plasmodium berghei* infections in rats (Diggs and Osler, 1969). To date, no information has been obtained which bears on the possible participation of complement components in this reaction. An anticomplementary factor (CVF) derived from cobra venom, first used successfully *in vivo* by Nelson (1966), provides an approach through which information relevant to this possibility can be obtained. This factor, which destroys the third component of complement (C3), has been used extensively to investigate immunopathologic mechanisms (Maillard, 1968; Cochrane et al., 1970).

It was considered that administration of CVF to rats infected with *P. berghei* but protected by passively supplied antibody would

allow a determination of whether or not C3 is required for the antibody mediated protection. The present study demonstrates a protective pathway which is antibody mediated but C3 independent.

Materials and Methods

A derivative of the NYU-2 isolate of *P. berghei* was used throughout. Forty to ninety gram inbred Fischer rats* were used for the experiments and for maintenance of the parasite. Methods of parasite maintenance have been previously described (Diggs and Osler, 1969). Hyperimmune serum (IS) was prepared by infection of one month old rats with 1×10^6 *P. berghei* parasitized erythrocytes followed by three additional injections of parasitized cells over a period of six months. A total of 3×10^7 parasitized cells and 1×10^9

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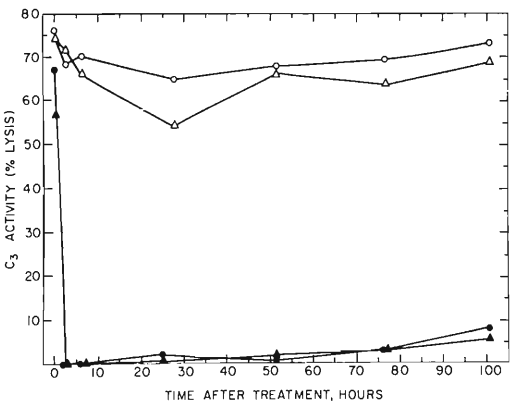


Figure 1. The effect of cobra venom factor (CVF) on serum C3 activity in individual rats. Solid symbols pertain to rats given 20 µgm CVF protein whereas the open symbols demonstrate control values. Serum was diluted 1/2000 prior to assay.

erythrocytes per animal was administered. Control serum (NS) was obtained by parallel injections of uninfected red cells at the time of injection of the immunized rats. All animals were bled one week after the last injection, the serum collected, pooled and stored at -20°C.

Experimental animals were infected with 2.5×10^6 parasitized erythrocytes in 0.05 M sodium phosphate, 0.01 M NaCl at pH 7.0 with 5% control rat serum per 100 gm body weight.

CVF was prepared from the crude venom of *Naja haje* as previously described by Shin et al. (1969). Parasitemia was monitored by examinations of thin blood films stained with Giemsa's stain; unless otherwise stated, fifty oil immersion fields were studied before a slide was considered negative, and 200 erythrocytes were counted for a quantitative estimate of the percentage of erythrocytes parasitized. Activity of C3 was estimated by a hemolytic assay using purified guinea pig components as described by Shin and Mayer (1968). Statistical analyses were by the non-parametric Mann-Whitney U test (Siegel, 1956); the 95% confidence level was chosen.

Results

In vivo de complementation by CVF was first explored. Fig. 1 shows the effect of CVF

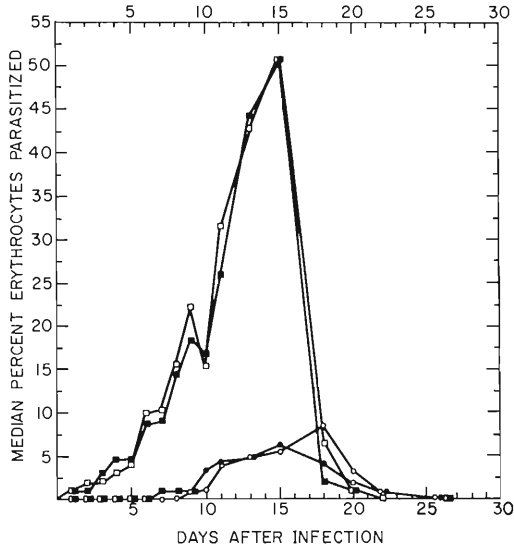


Figure 2. Median percentage of erythrocytes parasitized in rats infected with mixtures of parasitized erythrocytes, cobra venom factor, 30 µgm per 100 gm body weight (solid symbols) or saline (open symbols) and immune (circles) or control (squares) serum, 0.5 ml per 100 gm body weight (eight rats per group).

on C3 levels over a 4 day period. Whereas serum C3 levels varied but little during the observation period in control animals, experimental rats had no detectable C3 2.5 hours after injection of CVF and this depression was maintained during the 100 hours of sample collection; only a slight tendency towards a return to normal was noted and this was only apparent in the 75 and 100 hour samples. Experiments with durations of up to 4 days can therefore be performed with confidence that C3 levels will remain depressed.

Studies were then initiated to determine whether or not CVF can influence malaria due to *P. berghei*. Since the effect of CVF is known to be transient, it was important to design experiments in which possible effects would be expressed shortly after CVF treatment. Two kinds of effects of CVF might be anticipated; (a) a direct influence on parasites which might be detected by altered parasitemias when CVF is given at the time of infection and (b) an interference with com-

Table 1. Development of patent *P. berghei* parasitemia after treatment with immune serum and cobra venom factor.

Immune serum	Venom factor	No. animals patent on day				
		1-5	6	7	8	9-11
+	—	0	2	0	3	8
—	—	8	8	8	8	8
—	+	8	8	8	8	8
+	+	0	1	4	7	8

plement interaction with parasite-antibody complexes. It was considered that the probability of detection of this latter event could be increased by passive administration of antibody simultaneously with CVF and the infectious inoculum. An experiment was therefore conducted in which animals were injected intravenously with mixtures of parasitized erythrocytes, either CVF or saline, and IS or NS in the 4 possible combinations. The animals were examined for parasitemia over the next 26 days, during the first eleven days of which daily examinations were performed.

All NS treated animals were patent on the day after infection whether or not they received CVF. In contrast, all 16 rats receiving IS remained negative for parasites for five days and not until the ninth day were all positive. A slight contraction of the prepatent period by CVF in the IS treated groups is, however, suggested. The subsequent course of parasitemia (Fig. 2) also indicated a profound effect of IS but only marginal effects of CVF within serum groups.

Statistical comparisons between parasitemias in NS groups receiving either CVF or saline as observed on each of 17 days revealed one isolated significant difference (higher parasitemia in the CVF group) and this occurred on the fourth day after infection. The com-

Table 2a. Effect of preinfection de complementation on expression of antibody mediated immunity to *P. berghei*; C3 levels and 18-20 hour patency.

Immune serum	Venom factor	Serum C3 activity ^{1, 2}		No. animals patent ^{2, 3}
		2-5 hr Before infection	18-20 hr after infection	
+	—	57 (48-58)	59 (54-61)	1
—	—	55 (53-63)	60 (59-66)	5
—	+	0 (0-1)	1 (0-1)	5
+	+	1 (0-1)	1 (0-1)	2

¹ Per cent lysis, median (range). Serum dilution 1:3000.
² Five animals per group.
³ Smears were exhaustively studied (> 150 microscopic fields) before being declared negative.

parisons in levels of parasitemia in the IS groups revealed a significant difference in CVF vs saline categories only on day 7 (Table 1).

In order to study the system for complement dependence in more detail, it was necessary to obtain evidence of de complementation prior to introduction of the parasite and to then examine the animals for (a) persistent low activity of C3 and (b) parasitemia. This was achieved by an additional experiment in which 33 μ gm of CVF and 0.66 ml of IS or NS per 100 gm body weight were injected before infection with the standard inoculum of *P. berghei*.

Table 2a demonstrates that C3 activity was reduced to undetectable levels in the CVF treated animals and that no restoration of activity occurred over the next 18-20 hours. Nevertheless, there was an easily detectable effect of the immune serum on parasitemia during the same interval. Subsequent data on parasitemia are presented in Table 2b, in which it can be seen that depressed parasitemia persisted in both IS treated groups.

Table 2b. Effect of preinfection de complementation on expression of antibody mediated immunity to *P. berghei*; peripheral parasitemia on the fourth, seventh, and eleventh day after infection.

Immune serum	Venom factor	Per cent erythrocytes parasitized ¹		
		4 days	7 days	11 days
+	—	+ ²	7.5 (5.5-10.0)	17.0 (9.5-27.5)
—	—	6.0 (3.0-7.5)	18.8 (10.5-21.0) ³	36.0 (20.5-43.5) ³
—	+	6.0 (1.5-7.0)	16.0 (9.5-20.5)	40.5 (24.5-52.5)
+	+	+ ²	9.5 (6.5-14.0)	26.5 (5.5-33.0)

¹ Median (range); 5 animals per group.
² All animals positive, but parasites too scarce for quantitative estimates.
³ One animal dead in this group.

Later bleedings were less informative due to death of a number of the animals.

Discussion

These studies demonstrate that the third component of complement is not required for expression of the protective effect of immune serum in the *P. berghei*-rat system (Fig. 2, Tables 1 and 2). Although it is conceivable that amounts of C3 too small to be measured in the tests we employed could have persisted and participated in the observed immune effects, this possibility seems unlikely. Evidence for a minor enhancement of parasitemia by CVF in both the NS and IS treated rats was obtained but is less than compelling. Even if further studies should indicate that an effect does occur it is evidently a minor one. It should be pointed out that these experiments were performed in the presence of a relatively large amount of antibody, and that if both complement dependent and independent reactions occur, the former could be masked by the latter. To resolve this question, it will be necessary to perform experiments with limiting amounts of antibody so that partial protective effects can be observed.

These findings are in agreement with those of Cohen and Butcher (1970) who used *in vitro* assay of antiparasitic activity involving *P. knowlesi* and homologous antibody. These workers obtained two kinds of evidence for a complement independent pathway; (a) fresh serum is not required and (b) the pepsin digest of immune IgG ($F(Ab')_2$) is active. Although whole serum was used to induce the protective effects observed in this report, evidence for involvement of antibody in the reaction has been obtained previously (Diggs and Osler, 1969).

The results suggest that the antiparasitic effect of antibody on *P. berghei* may be analogous to a type of viral neutralization as has been previously suggested (Osler, 1969; Cohen and Butcher, 1969) in that complement appears to play a minor role in these reactions. If either a cytotoxic reaction or opsinization were involved, the participation of C3 might be expected. It can be speculated that once

"neutralized," malaria parasites are handled as inert debris, and phagocytized by the mononuclear phagocytes which act as scavengers. Alternatively, specific immune phagocytosis may occur by a complement independent pathway. Obviously multiple mechanisms may obtain and much more work will be required to elucidate the cellular and molecular mechanisms of the expression of humoral immunity in malaria.

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Enhanced Susceptibility of Bursectomized Chickens to *Plasmodium gallinaceum*: Comparison of Three Bursectomy Methods

D. R. STUTZ, D. H. FERRIS* AND E. W. VOSS, JR.**

Department of Medical Zoology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT. Four week old chickens bursectomized using three different methods; 1) *in ovo* treatment with testosterone (HBx), 2) surgical removal of the bursa from 18 day old embryos (EmSBx), and 3) surgical removal of the bursa from 1 day old chicks followed by sublethal irradiation from a Cobalt 60 source (SBxI), were injected with 5×10^5 erythrocytes infected with *Plasmodium gallinaceum*. All bursectomized birds died with the HBx and SBxI groups surviving 4 days. The EmSBx groups survived until the 5th day after infection. Intact birds reached a peak parasitemia (45%) on the 7th day and all survived. Parasitemias were much higher and parasites were observed earlier in the bursectomized chickens than in the intact control animals. The course of infection in the EmSBx chickens was slower than that observed in the other bursectomized groups, possibly demonstrating the influence of the cell-mediated immune system.

The relative roles of humoral and cellular mechanisms in immunity to malaria have not been elucidated. Serum mediated passive immunity has been well established (Coggeshall and Kumm, 1937; Manwell and Goldstein, 1940; Taliaferro and Taliaferro, 1940; Briggs et al., 1966) and cellular factors involved in malaria infections have also been extensively studied (Brown, 1969). Avian malaria infections provide a unique opportunity to study the relationships between these two commonly accepted types of mechanisms during such an infection. The bursa of Fabricius has been shown to mediate production of humoral antibody in the chicken (Cooper et al., 1966) at least in the early stages of life. When the bursa is removed surgically or its development impaired with hormone treatment during the incubation period, production of the known classes of antibody can be prevented. In addition, such chickens are unresponsive with respect to the production of humoral antibody in response to antigenic stimuli. The study of malaria infections in bursectomized chickens has, how-

ever, not been pursued to any great extent. Studies done with *Plasmodium lophurae* (Longnecker et al., 1966; Farmer and Brietenbach, 1968) indicate an increased susceptibility of the bursectomized birds to the infection. Studies involving *P. gallinaceum* in bursectomized birds have been limited in number (McGhee, 1968) whereas infections involving intact chickens have been extensively studied (Todorovic, 1967). The present studies were initiated to determine the effect of bursectomy, accomplished by several methods, on *P. gallinaceum* infections in the chicken.

Materials and Methods

Animals

Hybrid single comb white leghorn chickens* were used in all experiments. Eggs were obtained from the Poultry Research Farm, University of Illinois. Newly hatched chicks were provided by Honneggers Poultry Farm, Forrest, Illinois.

Bursectomy techniques

Hormonal bursectomy was accomplished as described by Warner et al. (1969). Surgical

* College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801.

** Department of Microbiology, School of Life Sciences, University of Illinois, Urbana, Illinois 61801.

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* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

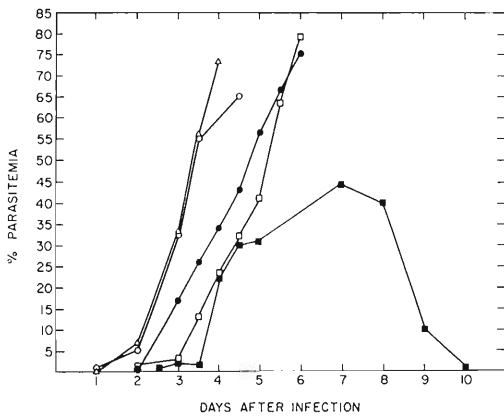


Figure 1. Mean percent parasitemia in hypogammaglobulinemic chickens infected with *Plasmodium gallinaceum*. Intact chickens —■—; surgically bursectomized at 1-day of age and irradiated with 700–800 R —○—; hormonally bursectomized —△—; embryonic bursectomy —□—; irradiated with 700–800 R —●—.

bursectomy of the embryo was performed using the technique of Van Alten et al. (1968). Surgical bursectomy of one day old chicks followed the basic procedure cited above and was followed by sublethal gamma irradiation from a Cobalt 60 source with an average dose of 700 to 800 R.

Measurement of immunoglobulin concentration

Serum immunoglobulin concentrations were measured by radial immunodiffusion (Fahey and McKelvey, 1965) using rabbit antichickens 7S gamma globulin produced as described by Stutz et al. (1972).

Experimental infections

For purposes of comparing effects of various bursectomy methods on the course of a *P. gallinaceum* infection, hypogammaglobulinemic birds were selected that had less than 100 µg/ml of serum gamma globulin as demonstrated by radial immunodiffusion. These chickens were infected with 5×10^5 parasitized cells and the course of the disease monitored daily for changes in weight, parasitemia, packed cell volume, erythrocyte count, and deep body temperature. Clinical signs of disease were

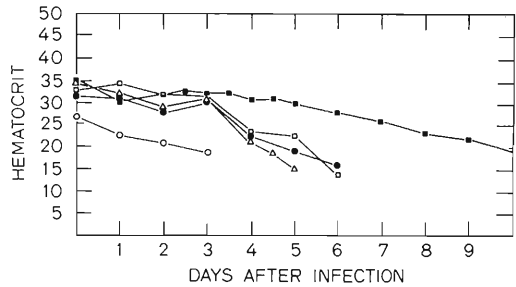


Figure 2. Mean percent packed cell volume in hypogammaglobulinemic chickens infected with *Plasmodium gallinaceum*. Intact chickens —■—; surgically bursectomized at 1-day of age and irradiated with 700–800 R —○—; hormonally bursectomized —△—; embryonic bursectomy —□—; irradiated with 700–800 R —●—.

also noted. Uninfected intact chickens and those which had been subjected to from 700 to 800 R of Cobalt radiation were used as controls. Each experimental group consisted of 6 birds and the experiments were repeated 3 times for a total of 18 birds per group and a total of 90 animals.

Results

P. gallinaceum infections were compared in chickens bursectomized by one of the following three methods; hormone treatment (HBx), surgical bursectomy of the embryo (EmSBx), and surgical bursectomy of 1 day old chicks followed by sublethal irradiation (SBxI). Controls consisted of intact and irradiated animals given an identical infective dose.

All intact chickens survived the infection with little apparent stress. The peak parasitemia, approximately 45%, usually occurred on the 7th day after infection (Fig. 1). The parasitemia declined at a rapid rate and parasites were rarely seen after the 10th day. Body temperature rose slightly during the peak of parasitemia but quickly returned to normal. Temperatures ranged from 42.8 C to 44.6 C with the highest temperature observed in the intact group at peak parasitemia (44.6 C). Prolonged anemia was observed in many of the intact chickens (Figs. 2 and 3). Packed cell volumes and erythrocyte counts did not return to normal values until approximately 2 weeks after the parasitemia had

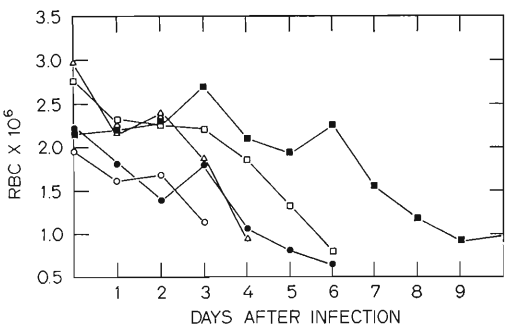


Figure 3. Mean erythrocyte count ($\times 10^6$) in hypogammaglobulinemic chickens infected with *Plasmodium gallinaceum*. Intact chickens —■—; surgically bursectomized at 1-day of age and irradiated with 700–800 R —○—; hormonally bursectomized —△—; embryonic bursectomy —□—; irradiated with 700–800 R —●—.

disappeared. The chickens began to lose weight just prior to the occurrence of the peak of parasitemia and continued for a week after parasitemia had disappeared. The period of time required for an intact chicken to recover from a *P. gallinaceum* infection was approximately 2 weeks. Antibodies to serum soluble antigens were first demonstrated by gel diffusion one day after peak parasitemia.

In contrast to these relatively mild signs of disease, dramatic changes occurred during the course of infection in the bursectomized chickens. All bursectomized chickens died from the infection. HBx and SBxI birds had similar parasitemias. Parasitemia was first observed on the second day after infection and rose rapidly to a peak between 65 to 75% by the fourth day (Fig. 1). No chickens in either of these groups survived past the fourth day after infection. In contrast, EmSBx birds

survived at least 24 hours longer and the percent parasitemia reached an average of 80% (Fig. 1). Control irradiated chickens also died on the fourth day after attaining a peak parasitemia of approximately 80%.

Packed cell volumes and erythrocyte counts declined slowly in all groups of bursectomized chickens until death (Figs. 2 and 3). Packed cell volumes as low as 15% were often observed in individual chickens just prior to death with erythrocyte counts of less than 1×10^6 per ml. Body temperature rose slightly in the SBxI group while a slight depression followed by a rise was observed in the other experimental groups. Body weight (Table 1) continued to increase in all groups until just prior to peak parasitemia at which time it began to decrease.

Discussion

Impairment of the humoral immune response of chickens by bursectomy presents an excellent opportunity for study of the importance of this response in *P. gallinaceum* infections. Several methods for bursectomy are used by workers investigating various aspects of the humoral antibody response. Three of the methods, which are representative of the many procedures now in common practice, were chosen for a comparative study. Two of these methods, hormone treatment (Szenberg and Warner, 1962; Warner et al., 1962) and surgical bursectomy followed by sublethal irradiation (Weber and Weidanz, 1969) are somewhat nonspecific in their action in that systems other than the bursa-mediated, or humoral system, are affected; on the other hand, available information indicates that ablation of the bursa in the embryo by surgery will not adversely affect the cell-mediated

Table 1. Body weight in grams of hypogammaglobulinemic chickens infected with *Plasmodium gallinaceum*.

Type ^a	Days after infection							
	0	1	2	3	4	5	6	7
N	1580	1712	1671	1663	1630	1636	1631	1558
HBx	1461	1527	1505	1468	1391	1391	1391	1391
EmSBx	1534	1606	1552	1575	1507	1465	1465	1465
SBxI	682	753	767	779	779	779	779	779
Irr	693	778	785	801	793	774	773	728

^a Normal chickens—N; hormonally bursectomized—HBx; embryonically surgically bursectomized—EmSBx; surgically bursectomized-irradiated—SBxI; irradiated—Irr.

immune system (Beamer et al., 1971). Therefore, comparison of this method with the other two provides an opportunity for interpretation relevant to the possible influence of the cell-mediated system.

The infection dose used in this study was such that chickens with an unimpaired immune system recovered from malaria. Those chickens which had been manipulated in such a way as to impair the humoral antibody response invariably succumbed to infections initiated with the same dose. An average survival time of 4 days was observed in those groups in which the total immune response had been affected; i.e., hormone treatment and surgical bursectomy followed by irradiation. This amount of time has been shown to be sufficient for a detectable antibody response in intact chickens to nonpathogenic antigens (Benedict, 1971; Stutz et al., 1972). Antibody against serum soluble malarial antigens has been demonstrable in the serum of intact chickens undergoing a *P. gallinaceum* infection as early as 3 days after the infective dose was administered (Smith et al., 1969). These antibodies were observed shortly after the peak parasitemia was observed. Lack of a functional immune system is undoubtedly responsible for the failure of these bursectomized chickens to survive the infection. Although there was no conclusive evidence in this study to indicate that a cell-mediated response is active, there was a significant delay in the peak parasitemia and death of the EmSBx group when compared with the other treated groups. This is the only group in which the cell-mediated system is thought to be unaffected and it is therefore reasonable to assume that cellular factors may play an active role in resistance to *P. gallinaceum* infections. It is also important to point out the apparently vital role which humoral antibody has in the defensive mechanisms of the chicken. None of the chickens in which only the humoral antibody production system was impaired were able to withstand the infection indicating that humoral antibody is necessary for the chicken to survive. Other factors, as yet unknown, may be involved in this resistance. This study, however, points up the relative importance of what are considered to be the two main divisions of the total immune response; in the experimental system used in this study the

humoral antibody response appears to be the more effective.

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Passive Serum Transfer Experiments in Bursectomized Chickens Infected with *Plasmodium gallinaceum*

D. R. STUTZ, D. H. FERRIS,* AND E. W. VOSS, JR.**

Department of Medical Zoology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: Four week old bursectomized (Bx) chickens were administered 2.5×10^7 erythrocytes parasitized with *P. gallinaceum*. The erythrocytes were suspended in preinfection or convalescent sera from a single donor which had experienced a *P. gallinaceum* infection; 12 of 16 birds receiving preinfection serum survived while only 4 of 16 receiving convalescent serum survived. All Bx controls died. Specific antibody provides a degree of protection but a factor(s) is present in the sera of certain chickens (donors) which limits this infection. This factor(s) may be depleted during infection and therefore not present in convalescent serum. Chicken anti-SRBC serum from the same donor failed to provide protection but did retard the progress of the infection.

Bursectomy has been shown to effectively abolish the ability of the chicken to raise an immune response to *Plasmodium gallinaceum* infections (Stutz et al., 1972a). Since this functional deficiency is best interpreted in terms of an inability to produce antibody, it is of interest to explore the feasibility of reconstitution of immunity with immune serum. Although such studies can be performed with intact experimental animals, the use of surgically bursectomized and irradiated chickens allows interpretation of protective effects in

terms of the serum administered, rather than actively acquired immunity on the part of the host. The present investigation, in which the effect of normal and convalescent serum on *P. gallinaceum* infections in bursectomized chickens is studied, was initiated as a result of these considerations.

Materials and Methods

Chickens used in these experiments were hybrid single comb white leghorns* and were obtained from Honnegger Poultry Farm, Forrest, Illinois as newly hatched chicks. Chicks

* College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801.

** Department of Microbiology, School of Life Sciences, University of Illinois, Urbana, Illinois 61801.

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* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

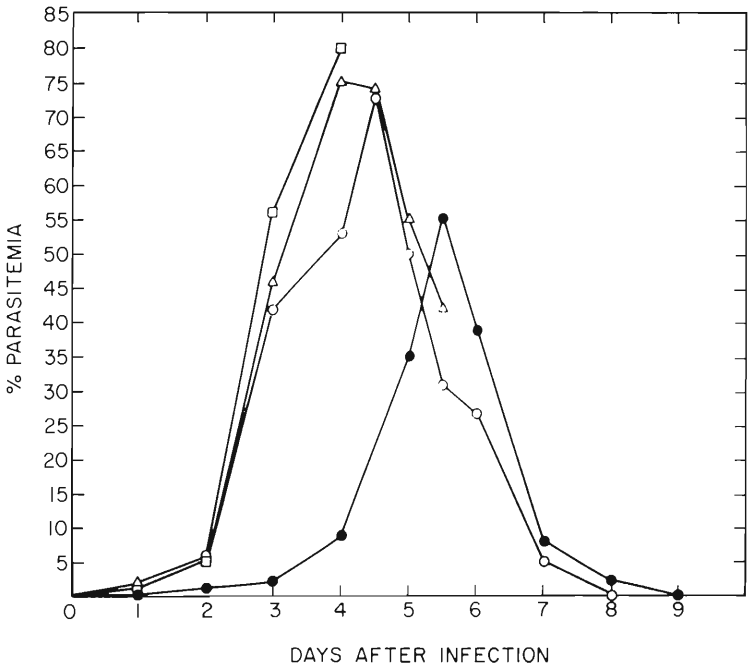


Figure 1. Parasitemia in hypogammaglobulinemic chickens passively administered normal chicken serum or convalescent serum and infected with *P. gallinaceum*. Group I, parasitized cells resuspended in normal chicken serum —○—; Group II, parasitized cells resuspended in chicken anti-malarial recovered serum —△—; Group III, parasitized cells resuspended in saline —□—; Group IV, normal chickens, parasitized cells resuspended in saline —●—.

were surgically bursectomized (Stutz et al., 1972b) and irradiated with an average dose of 700–800 R from a Cobalt 60 source. Effectiveness of the bursectomy procedure was determined using methods outlined previously (Stutz et al., 1972b). Only chickens with less than 100 μ g/ml of serum gamma globulin were selected for use in these experiments; previous investigations have indicated that the humoral immune response is severely depressed in chickens selected using this criterion (Stutz et al., 1972a, b). Bursectomized and intact animals were raised under the same environmental conditions including the isolation of the chickens to prevent intercurrent infections.

Intact chickens of the same age were selected as donors for serum to be used in passive transfer experiments. Each bird was bled by cardiac puncture of 20–25 ml of blood. The blood was allowed to clot and the serum removed, clarified and stored at –20 C. Two

weeks later all donors were infected intravenously with 1×10^6 parasitized cells. Those that survived this infection were reinfected 2 weeks after parasites were no longer observed in stained smears of peripheral blood. When there was no longer a detectable parasitemia after the second infection, each survivor was bled by cardiac puncture of 25 ml of blood. A clot was allowed to form and the serum removed and clarified. Both the pre-infection and convalescent sera from each donor were tested for serological activity against serum soluble antigens produced in white leghorn chickens infected with *P. gallinaceum*. Sera from only one donor were used in each experiment.

Infections were produced by passage of infected cells. Intact chickens were used as infected cell donors. Each experimental recipient was screened for isoantibodies before the donor was infected as follows: each donor

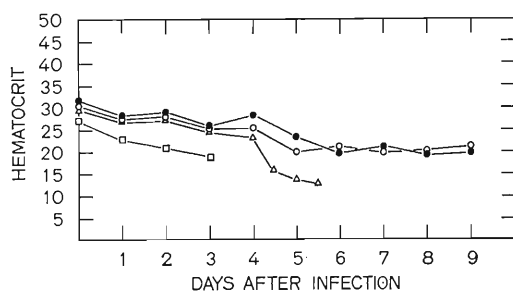


Figure 2. Packed cell volume in hypogammaglobulinemic chickens passively administered normal chicken serum or convalescent serum and infected with *P. gallinaceum*. Group I, parasitized cells resuspended in normal chicken serum —○—; Group II, parasitized cells resuspended in chicken anti-malarial recovered serum —△—; Group III, parasitized cells resuspended in saline —□—; Group IV, normal chickens, parasitized cells resuspended in saline —●—.

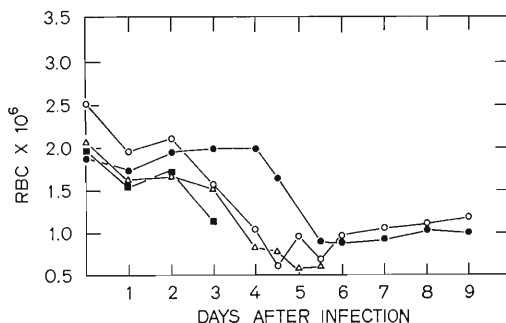


Figure 3. Erythrocyte counts ($\times 10^6$) in hypogammaglobulinemic chickens passively administered normal chicken serum or convalescent serum and infected with *P. gallinaceum*. Group I, parasitized cells resuspended in normal chicken serum —○—; Group II, parasitized cells resuspended in chicken anti-malarial recovered serum —△—; Group III, parasitized cells resuspended in saline —□—; Group IV, normal chickens, parasitized cells resuspended in saline —●—.

was bled of 20 ml of whole blood and the erythrocytes separated from the plasma and washed 3 times in saline; each bursectomized recipient was then injected intravenously with 1×10^6 washed normal erythrocytes from the cell donor. Bleedings were obtained 7 days later and the serum was assayed for isoantibodies by hemagglutination (Cain et al., 1969). Providing no isoantibodies were detected in the prospective experimental recipients, 3×10^6 parasitized cells were administered intravenously to the infected cell donor for each experiment. Parasitemia was monitored and when it reached 80–85% the donor was bled out by cardiac puncture.

Four groups of chickens were infected in each experiment with 8 chickens per group. The parasitized cells (2.5×10^5 per chicken) were suspended in 2 ml of (1) preinfection serum, (2) convalescent serum, or (3) saline. The inoculum was administered intravenously and the following parameters monitored: parasitemia, packed cell volume, erythrocyte count, deep body temperature and body weight.

Results

Experiments were designed to demonstrate the effect of serum from intact chickens recovering from a *P. gallinaceum* infection on the course of infection in bursectomized and

irradiated chickens. Parasitized cells from an intact donor were washed and separated into 4 portions. The following groups were established based on the medium used to suspend the cells for inoculation. Group I consisted of bursectomized chickens administered the parasitized cells in normal preinfection serum (NPB). Group II, also bursectomized chickens, received parasitized cells suspended in convalescent serum after malaria (CS) from the same donor that provided the preinfection serum for Group I. Group III consisted of bursectomized chickens which received the parasitized cells suspended in saline. Group IV was a control group and consisted of intact chickens which had been administered parasitized cells suspended in saline.

Infections in the normal (IV) and bursectomized (III) control groups were similar to those observed in this type of infection already described (Stutz et al., 1972b), whereas those of Groups I and II differed. During the early portion of the infection, parasitemias in the animals of Group I (Fig. 1) were similar to those of the bursectomized controls. However, 12 chickens (75%) survived the infection. Peak parasitemias (75%) were observed on the 4th day whereas the intact controls exhibited peak parasitemias on the 5th day (55%). Packed cell volumes (Fig. 2) and

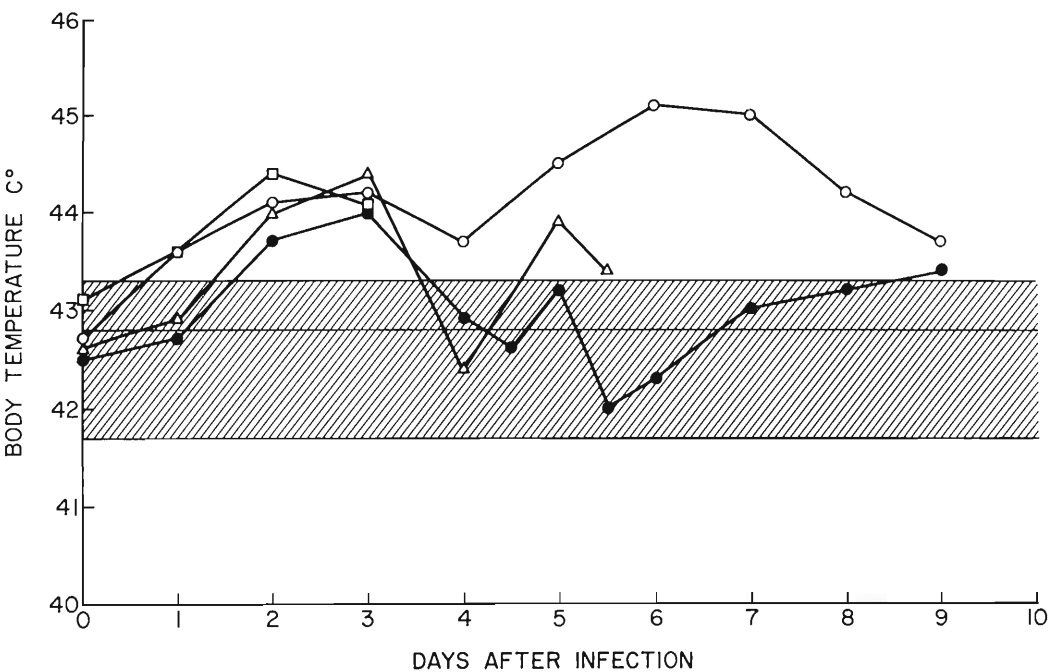


Figure 4. Deep body temperature (C°) in hypogammaglobulinemic chickens passively administered normal chicken serum or convalescent serum and infected with *P. gallinaceum*. Group I, parasitized cells resuspended in normal chicken serum —○—; Group II, parasitized cells resuspended in chicken anti-malarial recovered serum —△—; Group III, parasitized cells resuspended in saline —□—; Group IV, normal chickens, parasitized cells resuspended in saline —●—.

erythrocyte counts (Fig 3) followed patterns similar to those of the normal controls except for a more rapid decrease in the erythrocyte count in Group I. Body weights were also similar (Table 1) to those of control bursectomized animals. The body temperature curve differed markedly (Fig. 4) in hypogammaglobulinemic animals. In these chickens the temperature rose prior to the peak of parasitemia then decreased rapidly while in the intact chickens the temperature peaked at the same time of maximum parasitemia and then decreased slowly. Birds receiving convalescent serum (Group II) followed a course similar to those receiving preinfection serum (Group I) except that the progress of the infection lagged approximately 12 hours behind. Only 4 chickens survived the infection. Death occurred 24 to 36 hours after those in the hypogammaglobulinemic control group. In individual chickens the parasitemia was depressed

longer than in Groups I or III and rose rapidly just prior to death. A steady increase was observed in Group I chickens. Packed cell volume and erythrocyte counts were lower just before death in the chickens of Group II than at any time for Group I chickens. Body temperature curves were similar at Groups I

Table 1. Response of hypogammaglobulinemic chickens to a *Plasmodium gallinaceum* infection. Body weight in grams.

Group	Days after infection						
	0	1	2	3	4	5	6
I	649	682	722	735	741	708	696
II	594	631	647	659	698	670	660
III	693	778	785	801	793	733	728
IV	736	801	863	880	894	879	825

Group I, parasitized cells resuspended in normal chicken serum; Group II, parasitized cells resuspended in chicken anti-malarial recovered serum; Group III, parasitized cells resuspended in saline and injected into hypogammaglobulinemic chickens; Group IV, normal chickens, parasitized cells resuspended in saline.

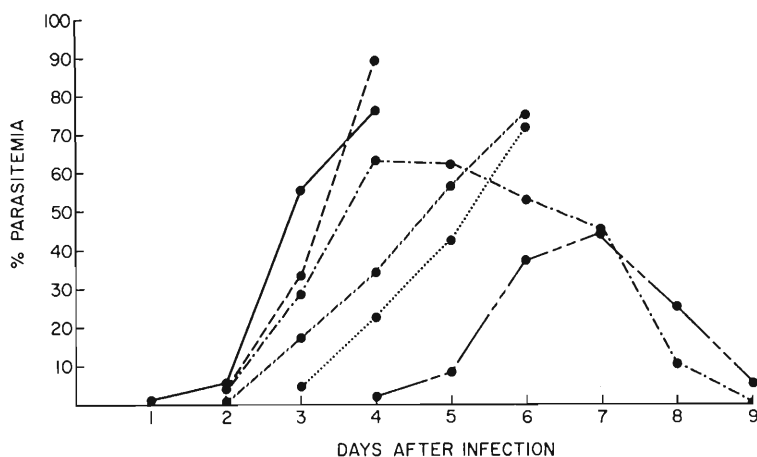


Figure 5. Parasitemia in hypogammaglobulinemic chickens passively administered normal, anti-SRBC, or convalescent chicken serum and infected with *P. gallinaceum*. — Bx chickens administered 1 ml convalescent serum; - - - Bx chickens administered 1 ml normal serum; . . . Bx chickens administered 1 ml anti-SRBC serum; — Bx control chickens (saline); - - - irradiate control (saline); - - - intact control (saline).

and II, however, chickens in Group II began to lose weight before those in Group I.

Two additional experiments, similar to those described above, were done with the following changes: (1) the chickens were two weeks younger; (2) the amount of all types of serum injected was 1 ml per chicken; and (3) the serum donor was stimulated twice with sheep red blood cells (SRBC) before infection. Serum from bleedings taken after the secondary response to SRBC was used as a preinfection control as well as serum obtained before the initial SRBC stimulation. Results obtained in these experiments were the same as those related above. Serum containing anti-SRBC antibodies, retarded the rise of the parasitemia but did not prevent death in any of the experimental chickens (Fig. 5). The groups which received the normal serum survived while those receiving convalescent serum did not. The groups receiving anti-SRBC serum survived approximately 24 hours longer than those in the convalescent serum groups. A rapid rise in parasitemia was observed just before death. All controls followed patterns described earlier (Stutz et al., 1972a). No detectable increase in serum gamma globulin was found after the peak of parasitemia, and antibodies to serum soluble antigens could

not be demonstrated in hypogammaglobulinemic chickens that survived the infection. By contrast all normal control chickens exhibited a detectable increase (50 to 100 μ g/ml) in their serum gamma globulin after infection. Antibodies to serum soluble antigen were demonstrated in serum from intact infected chickens no later than the day after peak parasitemia; in contrast, soluble antigens were demonstrated in the serum of hypogammaglobulinemic chickens as late as 2 weeks after the peak parasitemia had passed.

Discussion

Passive transfer of immunity to malaria by administration of serum from immune subjects to nonimmune subjects has been demonstrated for humans, animals and birds (Brown, 1969). Passive transfer of serum mediated immunity to *P. gallinaceum* has not been studied extensively (McGhee, 1970) and in particular, only limited work (Farmer and Breitenbach, 1968) has been done on avian malaria using birds in which the humoral immune response was suppressed. The importance of specific antibodies in protection or resistance to malaria has been demonstrated in monkeys against *P. cynomolgi bastianelli* (Cohen and McGregor, 1963), in man against *P. falciparum*

(Cohen et al., 1961) and in canaries to *P. circumflexum* (Manwell and Goldstein, 1938). Protection against *P. gallinaceum* has been difficult to achieve by passive administration of serum from convalescent birds (Todorovic et al., 1967).

In this study passive administration of preinfection serum provided sufficient humoral activity for the hypogammaglobulinemic chickens to survive an infection which would normally kill a bursectomized bird. Serum from the same donor after it had survived an infection did not provide the same degree of protection although a delay in the progress of the infection was observed. Our observations indicate that there is a component(s) present in the serum of certain donor chickens which is involved in immunity to malarial infection. This must be other than specific antimalarial antibodies. This component(s) may be present in the preinfection serum in sufficient quantity to limit the infection. Chickens used as donors for the sera were of the same hybrid line, hatched and reared under the same conditions as the hypogammaglobulinemic chickens. Since none of the donors had been exposed to *P. gallinaceum* before the preinfection serum was obtained, we can exclude the possibility of specific antimalarial antibodies being present. Moreover, individual variation among donors was not responsible since both the preinfection serum and convalescent serum used in each experiment were from the same individual. However, donor chickens were selected on the basis of survival and genetic differences may have played a role. The fact that few individuals, even with unimpaired immune responses, were able to survive the relatively massive challenges indicates that these birds had some factor(s) unique to them that allowed them to survive. One or more of these factors were present in the preinfection serum as evidenced by the ability to passively transfer the protection. This serum factor(s) was not present in the hypogammaglobulinemic chickens, or if present, was in insufficient quantity to allow survival, as demonstrated by the fact that no hypogammaglobulinemic chickens survived the infection when donor serum was not administered.

Administration of chicken anti-SRBC serum, which contained more immunoglobulin than

the normal serum, delayed the rise in parasitemia by 24 hours when compared to control hypogammaglobulinemic chickens. The convalescent serum, however, had a higher level of immunoglobulin and yet protection was not achieved, although a delay in parasitemia was observed. This would indicate that the presence of another serum component(s) may be necessary for protection or that the specific protective antibodies were not present in sufficient quantity to provide complete protection. Normal gammaglobulin may be involved in this non-specific activity although these experiments do not indicate so. Further studies of this non-specific activity of normal serum from individual chickens with an innate resistance to *P. gallinaceum* infection are highly desirable.

Convalescent serum did not provide protection to the degree exhibited by preinfection serum from the same donor. This may be due to depletion of the serum factor(s) present in the preinfection serum during the infection. At the same time, protective antibody may be present in small quantities and/or be bound to persisting antigen in the donor animal so that little was available in the serum. Experiments using serum from solidly immune chickens rather than convalescent chickens are planned to gain insight into these possibilities. The presence of both serum factor, or factors, and specific antimalarial antibody may be required for complete protection.

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The Effect of Protective Sera on the Course of *Plasmodium berghei* in Immunosuppressed Rats

S. H. LOURIE* AND M. A. DUNN**

Department of Medical Zoology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: All phases of the immune response play some role in concert to give protection against malaria. Predominant in killing the parasite and keeping the infection within controllable limits are the humoral factors contained in protective serum. However, suppression of the cell mediated system as done with ATG allows the malaria parasite to persist at higher levels and for longer periods of time. Thus, there appears to be a combination of cellular factors and humoral factors with the latter suppressing the infection, but requiring sensitization through cell mediated mechanisms.

The immunity to malaria has been looked at from a number of different views. All components of the immune response have been implicated in the immune clearance of malaria. In rodent malaria the protective effect of antibodies has been well documented, as in the work of Diggs and Osler (1969). The role of the reticuloendothelial system has also been implicated (Zuckerman, 1966) (Lucia & Nussenzweig, 1969). The role of cellular hypersensitivity has been implicated by studies on cell transfer (Phillips, 1970) (Stechshulte, 1969a) and neonatal thymectomy (Stechshulte, 1969b) and (Brown *et al.*, 1968).

These studies have not examined the comparative effect of the various components of immunity in the response. The studies with antibody alone cannot rule out an effect of cellular immunity. The studies with cell transfer cannot rule out transfer of antibody forming cells.

The present study is an attempt to explore in a comparative manner the humoral *vs.* cellular aspects of immunity to malaria when cell mediated immunity is suppressed using a potent inhibitor, Anti-thymocyte Globulin (ATG).

Materials and Methods

ANIMALS:

Rabbits: All anti-sera were produced in Albino New Zealand Rabbits bred at this institution. **Rats:** Lewis Rats (Microbiological

* Present address: Department of Medicine, State University of New York, Upstate Medical Center, 750 East Adams Street, Syracuse, New York 13210.

** Present address: Department of Medicine, Hospital of the Univ. of Pennsylvania, 3600 Spruce Street, Philadelphia, Pa. 19104.

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Assoc., Inc., Bethesda, Maryland) ranging in weight from 130–170 gms. were used in studies on the course of infection. Sprague-Dawley Rats (Walter Reed Strain) bred at this institution were used for *in vivo* assay of sera and maintenance of malaria parasites. All animals were maintained under conditions promulgated by the National Society for Medical Research. *Malaria Parasites: Plasmodium berghei* (NYU-2) had been maintained serially by weekly blood passage in Walter Reed Strain Rats 12–15 days old. All smears were stained with Giemsa Stain. Parasitemias were counted per 10^4 erythrocytes unless more than 10% were parasitized.

PREPARATION OF ANTI-SERA: Anti-thymocyte Serum (ATS) was prepared by two injections of approximately 10^7 washed thymocytes from Walter Reed Strain Rats emulsified in Complete Freund's Adjuvant (CFA), a week apart. Starting ten days later, the rabbits were bled by cardiac puncture bi-weekly. At the end of two weeks they were exsanguinated. The sera was separated and stored at 4°C, then pooled and stored at -20°C. The CFA is prepared from Incomplete Freund's Adjuvant (Difco) and 2 mg/ml *M. tuberculosis* H37Ra (Difco).

Anti-Macrophage Serum: The anti-serum was prepared by collecting peritoneal exudates from Walter Reed Strain rats 4 days after intraperitoneal injection of light mineral oil, NF (Halsy Drug Co., Brooklyn, New York). The cells were washed three times in Hanks Basic Salt Solution (HBSS) and cultured in glass petri dishes in Medium 199 enriched with 10% fresh homologous rat serum and 1% Penicillin/Streptomycin (5,000 units each/ml) for 3 days. The non-adherent cells were washed off with HBSS at 37°C. The adherent macrophages were then removed with a rubber policeman, washed once and emulsified in an equal volume of CFA and injected on the same schedule in preparation of the ATS. The sera were pooled and collected in the same manner also.

Normal Rabbit Serum: A group of rabbits were immunized on the same schedule as the above sera but with 10 mg of Bovine Plasma Albumin (Armour Pharmaceutical Co., Kansas, Illinois) emulsified in FCA.

Hyperimmune "Protective" Serum: (HPS). Two groups of 12 Lewis Rats were infected

with *P. berghei*. After the peak parasitemia, the rats were reinfected weekly with large but varying doses of *P. berghei* for 4 months. Sera were collected by retro-orbital venipuncture after that time. The rats were reinfected and bled at irregular intervals after that. Animals were always bled at least 2 weeks after the most recent reinfection. Parallel groups of rats were given an equivalent amount of normal Walter Reed Strain Rat erythrocytes on the same schedule to form a Control Rat Serum (CRS).

Preparation of the Globulin Fraction: The globulin fraction of the anticell sera and control sera were prepared by precipitation at 40% Saturation of Ammonium Sulfate (Am SO_4) at 4°C. Washed with 50% saturated Am SO_4 , then dialyzed vs. 0.01M phosphate buffer pH 7.2 and concentrated by lyophilization. The powder was redissolved in distilled water to give a final salt concentration of 0.1M phosphate buffer and the solutions were stored at -20°C. All preparations were sterilized by passage through Millipore filters prior to being given.

General Experimental Protocol: Lewis Rats were injected with (ATG) intraperitoneally in a dose equivalent to approximately 1 ml of the original serum on days -2, -1 and day zero. Day zero was designated as the day of infection with malaria (or sensitization in the case of other antigens). Three hours after the last injection a complete blood count was performed. One hour after this, the animals were infected intraperitoneally with *P. berghei* infected rat erythrocytes. The AMS was used in a similar protocol except that the entire dose was given at one time on day zero, followed three hours later by infection with the malaria parasites. The exact dose of malaria parasites given in each experiment varied but was in the range of 10^7 parasitized erythrocytes except where mentioned.

Assay of the Anti-sera: *In vitro* thymocyte agglutination was measured by microtitration as noted in Jasin *et al.* (1968). Macrophage cytotoxicity was measured by evaluation of cell viability after incubation for 30 minutes at 37°C with heat inactivated anti-sera in fresh guinea pig sera. The guinea pig sera was tested and diluted to a non-toxic level. Peritoneal macrophages were obtained three days after stimulation of rats with sterile min-

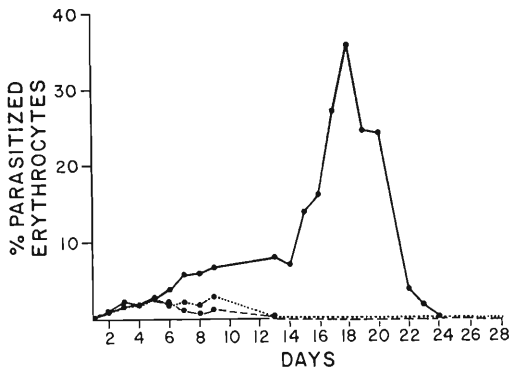


Figure 1. Effect of ATG on the course of malaria. Each line is the mean for a group of 6 rats given malaria on day 0; ● —● pretreated with ATG, ● ---● pretreated with NRG, ●● pretreated with saline on days -2, -1, and 0.

eral oil. The macrophages were washed three times prior to use, initial concentration of cells was about 2.5×10^6 /ml. Cell viability was measured by trypan blue dye exclusion, to an end point of 50% cell death.

In Vivo: The anti-cell sera were given to Walter Reed Strain Rats (120–140 gms.) on the schedule listed in the General Experimental Protocol noted above. The animals were sensitized with sheep erythrocytes (SRBC). At 7 and 14 days after sensitization the animals were tested for hemagglutinating antibody. The ATG blocked the formation of anti-SRBC antibody by an average of 5 doubling dilutions when compared to animals given NRG. Animals given the AMG were equal to or slightly higher in antibody titer to SRBC and the skin reaction to DNCB was not different from the control animals.

ATG and AMG were also tested *in vivo* for their ability to block clearance of malar by the reticuloendothelial system. The antisera were given as outlined in the materials and methods section above. Three hours after the last injection of anti-cell globulin, the rats were injected with colloidal carbon intravenously. Clearance of carbon was measured by the method of Halpern *et al.* (1953). No difference could be detected in the clearance rate of control animals or any of the treatment groups.

Table 1. Properties of ATG and AMG.

	<i>In vitro</i>		<i>In vivo</i>
	Thymocyte agglutination	Macrophage cytotoxicity	Anti-SRBC at day 11
ATG	1:1024	1:32	1:1.14
AMG	1:32	1:256	1:43
NRG	0	0	1:8

Results

The course of *P. berghei* malaria in Lewis Rats of this age and size is a low parasitemia which rapidly becomes subpatent. Generally, there is a peak parasitemia about the first week of about 4–5 percent, which levels off and this drops to a very low or subpatent levels during the second week. The effect of pretreatment with ATG on days -2, -1 and zero, as shown in Figure 1, has the effect of causing a minor increase toward the end of the first week of infection. This is followed by a short period (3–4 days) of a relatively stable level of 8–12 percent of erythrocytes parasitized. During the second week of infection, however, there is a marked increase in the parasitemia reaching a mean peak of 30% of the cells infected at the time the uninfected group had become negative on peripheral smear. There was no increase in the mortality of the animals in this treatment group. However, there was a greater anemia in ATG treated animals, on day 18 mean hematocrit being 17.5% compared to 44.8% in controls. Further they appeared ill with jaundice, weight loss, ruffled fur and decreased activity. None of these features occurred in infected rats treated with NRG or in uninfected rats given ATG.

ATG *in Vitro* and AMG *in Vitro*

The ATG and AMG were assayed for activity first using an *in vitro* assay. Thymocyte agglutination titer as shown in Table I was 1:1024 for ATG, but was only 1:32 for AMG, a difference of five doubling dilutions. Conversely, when assayed for cytotoxicity for macrophages, the AMG had a titer of 1:256 while the ATG was 1:32. This shows the AMG had three doubling dilutions, more activity in this system. Thus, while the *in vitro* assays show that the preparations are not completely specific, they are considerably

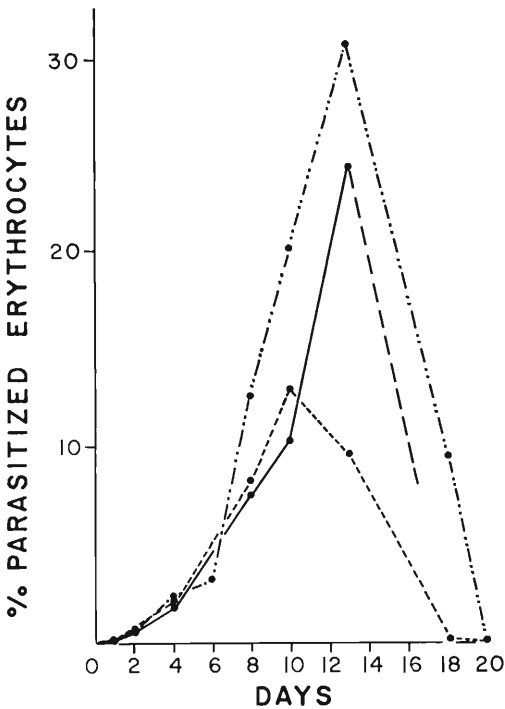


Figure 2. Effect of AMG on the course of malaria. Each line is the mean of 6 rats; ●—● pretreated with AMG, ●---● pretreated with AMG in 1/2 dose, ●- - -● pretreated with NRG. The dashed line after the AMG group represents the fact that one rat had a high parasitemia which could not be counted because of fragility of the cells, so no mean could be accurately made.

more effective against the cell for which they were designed than is the other preparation. It has been shown that ATG has an effect on macrophages (Maclaurin & Humm, 1970). To show that increased and prolonged parasitemia was not due to the effect of the ATG on macrophages, an AMG was studied in a similar system. As shown in Table 1 the ATG almost completely suppressed the antibody response to sheep RBC while NRG treated rats had a geometric mean titer of 1:8. Conversely, the AMG as shown caused an increase in the titer of antibody to SRBC to a titer of nearly 1:43.

The effect of the AMG on parasitemia is shown in Figure 2. The parasitemia in the control group is higher in this experiment than

Table 2. Effect of hyperimmune serum *in vivo*.

Group	Number	Antisera ^a		Route ^b	Mean percent parasitemia		
		HPS	NRS		Day		
					2	5	8
1	6	0	1.0	ip	0.10	0.32	1.9
2	6	1.0	0	ip	0	0.16	0.3
3	6	0.5	0.5	ip	0.17	1.7	1.5
4	6	0.25	0.75	ip	0.08	1.4	1.6
5	6	1.0	0	ip	0	0.80	0.75
6	6	0.5	0.5	ip	0	0.60	0.58

^a HPS = Hyperimmune rat serum; NRS = Normal rat serum.
^b ip = intraperitoneal. iv = intravenous.

in the studies, because the rats were slightly younger with a mean weight of 121 gm. There was also an increase in mortality with 4/7. However, the AMG did not cause any increase in the level of parasitemia or in mortality. A third group was given a dose of AMG 1/3 as great on the basis of an OD₂₈₀ reading. The peak of parasitemia was significantly lower in this group than in the controls. The mortality in this group was also lower. Another group which was given an amount of AMG equivalent in protein concentration to the ATG had approximately the same course of parasitemia as the NRG treated group and there was no increased mortality. Thus, it appears that when an equivalent amount of activity against macrophage is given, as assayed by *in vitro* techniques, there was no increase in parasitemia nor a prolongation of course. It appears that the lower dose may have even been somewhat protective, however, more work would have to be done to confirm this point. These data suggest that the activity of the ATG as used in these experiments is not primarily exerting its effect by acting on macrophages. Further evidence was obtained by showing that neither the ATG nor the AMG was able to alter the pattern of carbon clearance *in vivo*.

A hyperimmune anti-malarial serum (Hyperimmune Protective Serum) or (HPS) was prepared as noted in the methods section. This material was pooled and tested *in vivo* for its ability to suppress an infection with *P. berghei* malaria. The HPS was given in doses of 0.25, 0.5, 1.0, either i.p. or i.v. one hour after 1×10^6 parasites intraperitoneally one hour previously. The results obtained are shown in Table 2, where it will be seen

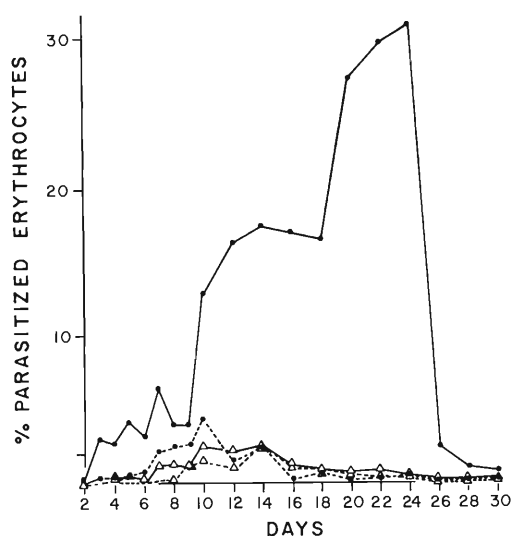


Figure 3. Effect of hyperimmune serum on ATG treated rats with malaria. Each line represents the mean of 7 rats. The groups marked with a solid line (—) were pretreated with ATG on days -2, -1, and 0. The groups marked with a broken line (----) were pretreated with NRG. All animals were infected with 4×10^6 *P. berghei* infected erythrocytes. One hour later they were given HPS (Δ) or CRS (\bullet).

that increasing doses of HPS had an increasing effect on blocking the parasitemia. For the first 4 days after giving the HPS, in all the groups there was suppression of the parasitemia to some degree. By the fifth and sixth days after treatment, the greatest difference between groups could be seen. By the eighth day all the rats had a significant parasitemia with greater than 1% of RBC's parasitized in all groups. The groups treated with 1 ml of the HPS either intravenously or intraperitoneally had a lower mean parasitemia than the other treatment groups, but were not different from each other. It was decided, therefore, to use HPS in doses of about 1 ml per animal and to administer it intraperitoneally.

To evaluate the relative role of humoral factors HPS was used in conjunction with the ATG. The ATG or NRG was given as before on day -2, -1 and day zero. Three hours later the rats were infected intraperitoneally with 4×10^6 *P. berghei*. One hour later,

half the rats in each group were given 1.24 ml of the HPS intraperitoneally. The rest of the rats in the groups were treated with CRS.

The results of this experiment are plotted in Figure 3. As can be seen, the group given ATG plus CRS had a course similar to that seen in the ATG treated group in the previous experiment. Those animals that were given the ATG plus HPS had a course which was similar to those treated with the NRG plus CRS, the rise in parasitemia seen with ATG alone was not found. On the other hand those animals which were given the NRG plus HPS were basically not different from untreated rats.

Unfortunately there was an increased mortality in both groups of ATG treated animals; 2/6 of the animals given ATG/HPS died and 3/6 those given ATG/CRS. However, most of the animals died in the first 24 hours and none had a high parasitemia. Gross pathology showed they died of massive respiratory infection. Therefore, the experiment was then repeated with the same protocol but the animals were given drinking water containing tetracycline to suppress the respiratory infection. To counteract the effect of the tetracycline on *P. berghei* the dose of parasites was increased to 2.1×10^8 given i.p. The results of this experiment are shown in Figure 4. The results parallel those seen in the groups not given antibiotic, however, the course was much shortened and the maximum parasitemia was reduced markedly. There was, however, no mortality in this group.

Discussion

The immune response to *P. berghei* malaria in rats was evaluated in this study by altering the immune response. The effect of immunosuppressive agents on the course of infection was measured by giving anti-thymocyte globulin (ATG) on the three days prior to infection. This caused an increase in the parasitemia, the animals appeared more severely ill by clinical evaluation and their hematocrit dropped markedly compared to the controls. Thus, it was seen that immunosuppression with a potent agent would cause a prolonged and more severe course of malaria in rats. These results are quite consistent with those of Barker and Powers (1970) in mice and Spira *et al.* (1970) in rats.

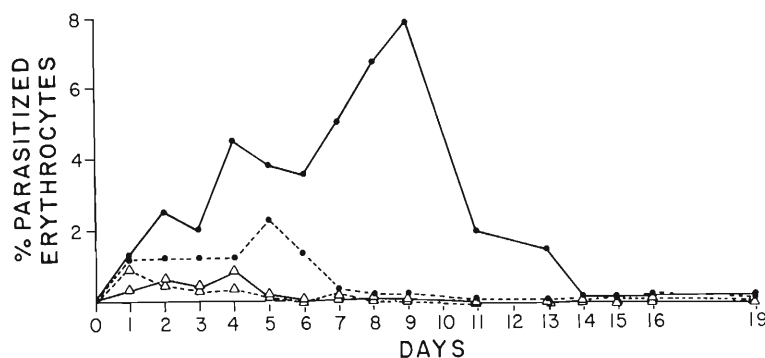


Figure 4. Effect of hyperimmune serum (HPS) on ATG treated rats with malaria. The key for this figure is the same as Fig. 3, except the dose of *P. berghei* was 2×10^8 infected erythrocytes and the animals had tetracycline in their drinking water.

The exact mechanism by which the ATG effects the response to malaria was not clear because the antibody is known to have several effects on the immune response. Firstly and most prominent is its suppression of cellular immunity. Second, in some instances it can suppress the ability to produce antibody and third, it has been implicated as having anti-macrophage activity in many cases. The last effect was examined first. A potent anti-macrophage globulin AMG was prepared against peritoneal macrophages. Tests *in vitro* showed that it had a considerably greater effect against macrophages than did the ATG while it had a minimal effect against lymphocytes. *In vivo* studies showed that where the ATG was markedly suppressive for antibody response to sheep erythrocytes the AMG did not lower the antibody level below the controls. Another parameter of macrophage function, phagocytosis, was measured by *in vivo* carbon clearance. It was shown that there was no difference in the ability of an animal to clear particular colloidal carbon after treatment with control globulin or with either of the anti-cell sera.

Further the AMG was evaluated for its effect on malaria infection. Animals treated with AMG had no increase in the level of parasitemia or the severity of clinical illness. In fact a low dose of AMG seemed to cause a slightly lower parasitemia. This latter effect may have been related to a stimulation of the reticuloendothelial system due to a submaximal dose. Thus, it would seem that the anti-

macrophage activity present in the ATG cannot be implicated as the reason for the increased parasitemia and the more severe course of malaria seen in immunosuppressed animals.

An effort was then made to determine the effect of antibody in these immunosuppressed animals. If in the face of severe immunosuppression humoral factors would block the rise in parasitemias it would be strong evidence of a predominating role for humoral immunity over cellular factors. A hyperimmune protective serum was prepared by frequent reinfection of rats with *P. berghei*. This material was shown to be a potent but temporary inhibitor to patent parasitemia. This effect was seen as marked delay before patency and slower initial rise of parasitemia over the course of five or six days after treatment with the protective material. Similar results were shown by Diggs & Osler (1969). The latter authors also show that the effect was mediated by 7S IgG.

The results of the experiments with ATG show that the protective serum completely blocked the increased morbidity. The parasitemia levels were no greater than controls. Further, the malaria did not persist although there is reason to suspect that the animals own defenses were still limited by the ATG. The first time this was studied there was a high mortality from intercurrent infection, probably related to the ATG induced immunosuppression but unrelated to the malaria. To overcome this problem, this experiment was repeated in animals protected with tetra-

cycline. This was an experiment in miniature since *P. berghei* is also effected by the drug. A very similar pattern of parasitemia was seen although absolute values are lower. The pattern of the response to ATG and protective serum during the course of malaria is quite consistent and shows that the effect is reproducible even in the face of an antibiotic treatment.

It has been apparent that complex mechanisms are involved in the immune response to malaria. Studies to test both cellular and humoral factors have both seemed to play a role in protection. Diggs and Osler (1969) showed by the use of hyperimmune serum that there was a marked decrease in the parasitemia in subjects or animals pretreated with the sera. But other work has shown that the protection afforded by antibody is not complete and that there are relapses of parasitemia, which in several cases has been shown to be due to antigenic variation (Phillips, 1970).

Other workers have shown the importance of cellular factors in this disease. Stechschulte (1969a) and Brown *et al.* (1968) found that neonatal thymectomy resulted in an increased and more prolonged parasitemia. Stechschulte (1969b) and Phillips (1970) showed that transfer of spleen cells was associated with decreased parasitemia and a more complete immunity as evidenced by sterile immunity in rats treated with cells as compared with those protected with serum.

The data presented here have shown that ATG, a potent suppressor of cellular hypersensitivity, gives a course similar to that seen with neonatal thymectomy. Barker and Powers (1971) and Spira *et al.* (1970) found similar results on the course of parasitemia after immunosuppression with ATG. However, there is a disparity between these two reports in their assessment of the role of antibody. The former group found that there was no recovery until they could detect antibody by the fluorescent antibody test. The latter group use a microprecipitation test and could detect no difference between the ATG treated and control rats, which led them to suggest that non-humoral aspects of the immune response were predominant. It has, however, been very difficult to correlate protective antibody with any of the *in vitro* tests for antibody level in malaria (Brown, 1969). In the data presented

in this paper, serum within proven ability to inhibit parasites *in vivo*, show that even when cellular hypersensitivity is suppressed the animals were able to survive the infection when given hyperimmune serum. This suggests that the primary means by which malaria is blocked is by antibody.

The data presented do not rule out a role of cell mediated factors. In the situation of a large complex antigen, it is known that the thymus, thus presumably cellular factors, are important in production of antibody by action of the so-called helper cell. Brown (1971) has suggested that immunity to malaria might be dominated by such thymic helper cells process. The data in this report are compatible with such a hypothesis, but do not specifically give information about it. Possibly the antibody production to malaria may require some cellularly mediated action to start sensitization, while the malaria parasites would succumb to the antibody rather than some cytotoxic factor from the cells *per se*.

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The Effect of *Plasmodium berghei* on the Immune Response in Rats

S. H. LOURIE*

Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: The effect of infection with *P. berghei* on the immune response in Lewis rats was studied. No difference was seen in antibody level to sheep erythrocytes. Nor was a difference noted in the response to inflammation caused by dinitrochlorobenzene. When cellular hypersensitivity was measured by adjuvant arthritis, no difference was seen if the malaria infection was started before the adjuvant was given or after the arthritis had begun. However, if the infection was started between time, the adjuvant was given and the onset of arthritis, there was a marked increase in the arthritis. A similar pattern was seen in the response to tuberculin, although not as marked. The results are discussed especially with regard to possible role of the macrophage.

This study was designed to examine the effect of malaria on some of the parameters of the immune response. Although much effort has gone into examining the effect of the immune response on malaria infection, little work has been done on what the infection does to the host's ability to respond to other antigens. Malaria is a potent "immunogen" and prolonged exposure leads to elevated levels of immunoglobulins (Cohen, 1961). Antibodies to malaria are found in high titer and "abnormal" antibodies are also seen. In chronic *P. malariae* infection deposition of antigen-antibody complexes may lead to a nephrotic syndrome (Ward & Kibukamusokoe, 1969). Studies on the effect of malaria infection on immunoglobulin production in intact germ free mice (Finerty, 1972) have shown an increased amount of immunoglobulin is formed. Studies by Barker (1971) showed

that in unsensitized mice there was an enhancement of anti-sheep cell antibodies as measured by the Jerne plaque technique. It has been suggested that in certain more complex systems that malaria infection has a suppressive effect on the ability of the host to respond. Studies with several murine leukemias suggest that the animals succumb to the leukemia at a much earlier time when they have been infected with *P. berghei yoeli* (Salaman *et al.*, 1969), (Wedderburn & Salaman, 1970). Conversely in NZB mice which have immune-complex deposition nephritis, Greenwood & Greenwood (1971) noted that the mice infected with malaria no longer succumbed to chronic glomerulonephritis. In another study (Greenwood *et al.*, 1970) it was suggested adjuvant disease of rats, a syndrome mediated by cellular hypersensitivity (Pearson, 1963), was suppressed in animals infected with malaria. The following studies were designed to determine the mechanism of these effects on the immune response and whether the findings in mice could also be documented in rats.

* Present address: Department of Medicine, State University of New York, Upstate Medical Center, Syracuse, New York 13210.

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Table 1. Reciprocal geometric mean titer of anti-SRBC by microagglutination on day 7 and 14 before and after treatment with 2-mercaptoethanol (2ME).

Group	Treatment		Number	Titer ⁻¹			
				Before 2ME		After 2ME	
	Day 0	Day 5		Day 7	Day 14	Day 7	Day 14
1	<i>P. berghei</i>	Sulfalene in oil	8	115.9	52.5	11.3	3.3
2	<i>P. berghei</i>	Oil only	8	64	37.3	5.6	3.4
3	Normal RBC	Sulfalene in oil	8	76.1	29.9	5.2	3.1
4	Normal RBC	Oil only	8	105	29.3	5.1	3.7

Materials and Methods

Animals: Lewis strain female rats (Microbiological Associates, Inc., Bethesda, Maryland) 100–130 gms were used in all of these experiments. Rats were divided into groups according to a set of random numbers. All animals were maintained under conditions meeting the standards promulgated by the National Society for Medical Research.

Malaria parasites: *P. berghei* (NYU-2) was maintained by weekly passage in 12–15 day old Sprague-Dawley rats by intraperitoneal injection of parasitized erythrocytes. All experimental animals were infected intraperitoneally with 2×10^7 malaria infected erythrocytes in Alsever's Solution. Parasitemias were counted in thin smears with Giemsa Stain; 1000 erythrocytes were counted unless there was greater than 10% parasitemia. At various intervals subpatent infection was tested by subinoculating mice with 1 ml of blood from the study animals.

Sensitizations: 0.2 ml of sheep erythrocytes (1×10^7 /ml) washed three times in normal saline, were injected intraperitoneally. Anti-sheep cell antibodies were measured by a microagglutination technique in the presence or absence of 2-mercaptoethanol to estimate both 19S and 7S antibody against sheep cells as described previously (Jasin *et al.*, 1968).

Adjuvant arthritis was produced in the rats by injection of 0.1 ml Complete Freund's Adjuvant (CFA) made of Incomplete Freund's Adjuvant (Difco) with 0.6 mg of *M. tuberculosis* (H37Ra/ml) subcutaneously at the base of the tail. The degree of arthritis was measured by estimating a joint score based on 0–2 small joints and 0–4 large joints according to the method of Currey and Ziff (1968). Scores were plotted for each animal and the area

under the curve measured with a planimeter. The area was used in all calculations of means and statistical evaluations.

Skin sensitivity to tuberculin was measured by intracutaneous injection of 0.2 ml of Purified Protein Derivating (PPD) ($50 \mu\text{gm/ml}$) (Merck, Sharp & Dohme, Detroit, Michigan) in 0.2 ml Hanks Basic Salt Solution (HBSS). Induration was measured in two diameters at 24, 48 and 72 hours. The product of the two diameters was recorded and used in the tabulations.

Inflammatory stimulus was assessed in animals pretreated with 0.1 ml 10% Dinitrochlorobenzene (DNCB) in acetone applied on the shaved abdomen. Two weeks later a test dose of 0.1 ml of 1%, 0.5% and 0.25% DNCB in acetone was applied to separate sites on the shaved backs of rats. At these doses the response to DNCB is non-specific inflammation with polymorphonuclear leukocytes being predominant. Reactions were graded as 0.5 = erythema, 1.0 = induration, 2.0 = marked induration and 3.0 = necrosis. Results were tabulated by adding all positive scores for each animal. Evaluation was by rank ordering with Mann-Whitney U Test.

Results

The first experiment was to determine the effect of malaria infection on the antibody response to sheep erythrocytes (SRBC). One hour after giving SRBC, half the rats were infected intraperitoneally with *P. berghei* parasitized erythrocytes. On the fourth day after injection, the rats were further subdivided into four groups. One group with malaria and one control group, were treated with 320 mg/Kg Sulfalene* subcutaneously in Peanut

* Kindly donated by COL Rothe, Department of Medical Chemistry, Walter Reed Army Institute of Research.

Table 2. Response to test doses of dinitrochlorobenzene (DNCB) as a measure of inflammation when infected with *P. berghei* before (Table A) or after (Table B) initial exposure to high base DNCB.

A.				DNCB			
Group	No. of animals	Injection on day zero with	Day Given		MTR ^b	P value	Parasitemia
			High dose	Low dose			
1a	7	<i>P. berghei</i> ^a	0	14	1.1 (0–2.5)	NS	30.0 ^c
1b	6	NRBC			1.0 (0.5–2.5)		0
2a	6	<i>P. berghei</i>	14	28	1.1 (0.5–2.0)	NS	23.8
2b	6	NRBC			0.6 (0–1.5)		0
3a	6	<i>P. berghei</i>	28	43	0.8 (0.5–1.5)	NS	21.8
3b	6	NRBC			1.0 (0.5–1.5)		0

B.							
Group	No. of animals	High dose day	DNCB <i>P. berghei</i> given day	DNCB Tested			
				Day	MTR ^a	P value	Parasitemia
1	7	0	None	15	1.9 (1.0–2.5)	NS	0 ^c
2	8	0	4	15 (11) ^d	1.3 (0–2.5)		6.5
3	7	0	8	15 (7)	1.3 (0.5–2.0)	NS	3.57

^a *P. berghei* = 2 × 10⁷ *Plasmodium berghei* infected erythrocytes. NRBC = normal rat erythrocytes.
^b MTR = mean total response at 48 hours.
^c Mean for the group per 10⁴ erythrocytes 7 days after infection.
^d () = numbers of days after malaria infection.
^e Mean for the group per 10⁴ erythrocytes on 11 days after infection.

Oil (Planter's). This dose is known to be highly effective in killing *P. berghei* in mice (Raines, 1971). Two similar groups were given Peanut Oil alone. As shown in Table 1, there is no difference in the anti-SRBC titers between any of the sensitized groups. This was true for both 2-mercaptoethanol sensitive and resistant antibody. Nor was there an appreciable change in the ratio of the two types of antibody.

The next set of experiments were designed to study the effect of malaria infection on a non-specific inflammatory response. Dinitrochlorobenzene (DNCB) was used to cause the inflammation. In experiment 2, the malaria was given simultaneously with or prior to DNCB stimulation. Three groups of rats were exposed to a high dose of DNCB on day 0, 14 or 28. Half of each group was infected with *P. berghei* on day zero, the other half was given normal rat erythrocytes at the same time. The response to the high dose of DNCB was too severe to quantitate in all animals. Each group was retested 14 days later with 1%, 0.5% and 0.25% DNCB. The reaction to this lower dose was quantitated as noted in the methods section. As shown in Table 2A there was no significant difference between treatment groups when DNCB exposure was after infection.

In experiment 3, the effect on inflammation when first DNCB exposure was prior to malaria was studied. The results are shown in Table 2B. All animals were exposed to high dose DNCB on day zero. Group 1 is a control

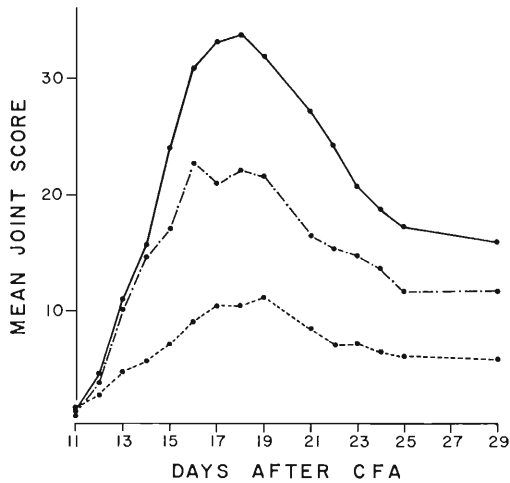


Figure 1. Effect of malaria on the course of adjuvant arthritis. CFA was given after infection with *P. berghei* on ●-----● 30 days or ●-----● 26 days. The other group ●- - - -● was not infected.

Table 3. Tuberculin reaction in animals given CFA a long interval after infection with malaria.

Group	CFA given ^a	Mean arth- ritis ^b	PPD ^c	
			Given ^a	Mean response ^d (range)
1	—	1.67	—	143.0 (88–156)
2	30	2.07	44	113.5 (70–195)
3	26	1.09	40	124.4 (35–176)

^a Days after malaria infection, group 1 was not infected.
^b As measured by the area under the curve of the individual joint score, in arbitrary units.
^c PPD = tuberculin, Purified Protein Derivative. Tested 15 days after CFA was given.
^d mm² of induration at 48 hours.

which was not infected. Groups 2 and 3 were infected 4 and 8 days after DNCB, respectively. No significant difference is seen between the groups.

The effect of malaria infection on cellular hypersensitivity was studied next. The effect on adjuvant arthritis and tuberculin sensitivity was measured at various times in relationship to infection. Adjuvant arthritis is induced by injecting Complete Freund's Adjuvant (CFA) and approximately 11 days later the rats develop a severe polyarthritis, balanitis, conjunctivitis and wasting (Pearson, 1963). In Figure 1 are results of giving CFA following malaria. This is the same group of rats used in experiment 3. The CFA was given 10 days after the last exposure to DNCB. This corresponds to day 26 and day 30 after infection with malaria in the respective groups. All the animals were negative on smear for parasites at that time, but most were still positive by mouse subinoculation. There was considerable

Table 4. Arthritis and tuberculin response in animals given CFA with or a few days after malaria infection.

Group	No. of animals	Infection ^a	CFA given day	Mean arthritis	PPD ^c response	Parasitemia ^d
1a	7	<i>P. berghei</i>	0	3.15 ^b	196.7 (11)	9.7
1b	7	NRBC	0	2.26	176.3 (11)	0
2a	6	<i>P. berghei</i>	11	1.07	123.0 (14)	22.0
2b	7	NRBC	11	1.29	123.7 (14)	0
3a	6	<i>P. berghei</i>	22	2.84	118.3 (11)	9.1
3b	7	NRBC	22	1.10	100.5 (11)	0

^a *P. berghei* = 2×10^7 *Plasmodium berghei* infected erythrocytes. NRBC = normal rate erythrocytes.

^b As measured by the area under the curve of the joint scores, in arbitrary units.

^c PPD—tuberculin, Purified Protein Derivative; in mm² of induration number in () days after CFA that PPD was tested.

^d Per 10^4 erythrocytes, mean for the group on day 12.

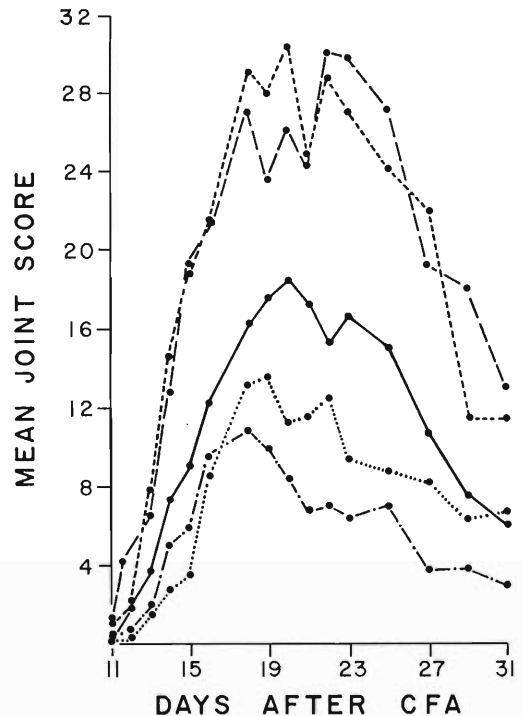


Figure 2. Effect of malaria on the course of adjuvant arthritis. *P. berghei* was given after the CFA on —○— day 4, —●— day 7, —●— day 11, —●— day 14. The lowest curve —●— was not infected. Arthritis was first seen on day 11 after CFA.

variation in the arthritis within each group and neither of the two infected groups was statistically different from the control. The figures are relatively unchanged if the animals, which were not positive on subinoculation are deleted. Tuberculin reactions were also measured in these animals 6 and 15 days after CFA. The results are shown in Table 3. On neither occasion there was a difference in the response to PPD in the three treatment groups.

The effect of giving the CFA simultaneously with or shortly after the malaria infection was tested in experiment 4. Here the animals were given CFA in three groups. One half of each group was infected with *P. berghei* on day zero, the other half given normal rat erythrocytes. Group 1 was given CFA on day zero, group 2 on day 11 and group 3 on day 22.

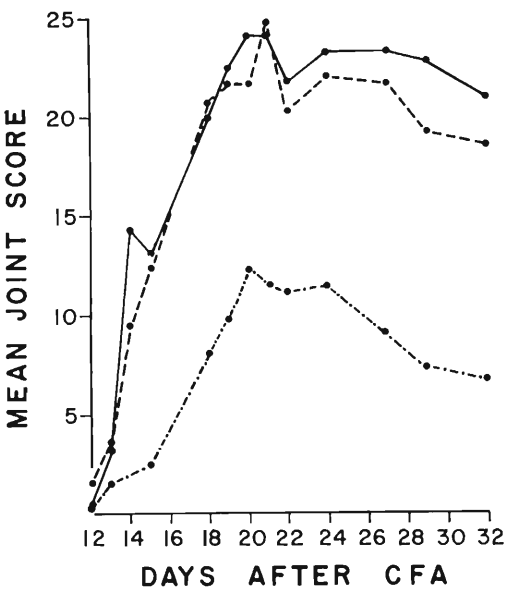


Figure 3. Effect of malaria on the course of adjuvant arthritis. *P. berghei* was given after CFA on ●---● day 4, on ●—● day 11. The lowest curve ●---● was not infected. Arthritis was first seen on day 11 after CFA.

The results are given in Table 4. As seen before, there is no statistically significant difference between treatment groups. The tuberculin reaction was measured on a number of occasions starting on day 14 and also showed no difference between the treatment groups.

In experiment 5 the CFA was given first and the malaria subsequently. In this study rats were first divided into groups, then given CFA by groups and finally they were infected with malaria 4, 7, 11 or 14 days later. One

control group was not infected but served as negative control for the repeated tuberculin testing. In Figure 2 are plotted the mean joint scores for this experiment. The onset of arthritis is approximately on day 11.

The animals infected day 4 and 7 after the CFA were given, but before the onset of arthritis, had a marked increase in the total joint disease. This increase was less dramatic but still evident when the malaria was given just about the time the arthritis developed on day 11. The difference from the control was statistically significant for the animals infected both 4 and 7 days after giving CFA. After the onset of arthritis the effect of infection with malaria was in the same direction but not as marked. Although joint score is elevated it is not statistically significant. The tuberculin response had a similar pattern although not as striking, as seen in Table 5, and was significant in the groups treated on day 4 after CFA. The other groups tended to be increased as was the arthritis but were not statistically different.

The results of the last experiment were repeated using larger groups of animals. In this study the animals were given CFA on day zero before being sorted into groups, to reduce any bias which might be introduced by injecting by groups. The rats were then divided into three groups. Group 1 was infected with malaria on the 4th day after CFA and group 2 infected on the 11th day. Group 3 was not infected. Parasitemias are approximately the same in the two infected groups. The results on the adjuvant arthritis are plotted in Figure 3. As can be seen, there again was a significant increase in the amount of arthritis when there has been infection with malaria. This difference was significant at $P < 0.01$ by Student's *t* Test. In this experi-

Table 5. Tuberculin response in animals given CFA before infection with malaria.

Group	No. of animals	CFA given day	Malaria given day ^a	Mean arthritis ^b	P. value ^c	PPD response ^d	P. value ^c
1	5	0	4	2.52	0.05	161	0.01
2	6	0	7	2.17	0.05	124	N. S.
3	6	0	11	1.45	N. S.	123	N. S.
4	6	0	14	0.98	N. S.	100	N. S.
5	6	0	none	0.75	—	103	—
6	6	none	none	—	—	0	—

^a Days after CFA animals were infected with 2×10^7 *P. berghei*.
^b As measured by the area under the curve of the individual joint scores, in arbitrary units.
^c Student's *t* test, increase in arthritis compared to the group 5.
^d In mm² of induration, tested 14 days after CFA; N. S. = not significant at $P < 0.05$.

ment animals infected as late as 11 days after sensitizations with CFA had a significant increase in the amount of arthritis.

Discussion

Infection with malaria parasites causes potent stimulation of the immune response. There are high titers of antibody to the parasite, and as well, high levels of non-specific immunoglobulins are found. Chronic infection with malaria has been associated in man with "abnormal" antibodies, such as biological false positive test for syphilis. Also, there is evidence for defects in the immune response during malaria infection. Salaman *et al.*, (1969) noted a sharp decline in the production of anti-sheep erythrocyte plaque forming cells in animals. Barker (1971) and Greenwood (1971a) had similar results, also using SRBC. Further studies by Salaman and Wedderburn (1970) showed that mice infected with viral leukemias would die more rapidly if infected with *P. berghei yoelli*. Greenwood (1968) suggested that certain human diseases known to have a strong immunological component have a lower incidence in Nigeria, an area holoendemic for *P. falciparum*. Studies on NZB mice, thought to be a model for systemic lupus erythematosus, show that malaria infected mice had a less severe course. Although the mice had Coomb's antibody and hemolytic anemia they did not get glomerulonephritis (Greenwood & Greenwood, 1971). This has been recently confirmed by Whelton (1972).

In this paper, the effect of plasmodium infection on the immune response in rats was studied by several methods. Antibody formation to SRBC was measured after infection with *P. berghei* by microhemagglutination. Both the mercaptoethanol sensitive and resistant antibody were measured, giving an estimation of 19S and 7S immunoglobulins. No difference was detectable between the infected and the control for both 7S and 19S antibody. Nor was there any apparent difference whether the malaria was treated or not. These results differ from the earlier studies done in mice (Salaman *et al.*, 1969), (Greenwood *et al.*, 1971a) and (Barker, 1971). The reason for the different results is probably due to the differences in measuring the anti-

body. The earlier workers used the plaque formation assay which measures the ability to make antibody at a given point in time. The microhemagglutination method used here tends to average these periods and gives an impression of the ability to make antibody over a course of time. SRBC is an antigen which persists over a period of time and a short period of deficient antibody formation might not be detected. Using mice, Greenwood *et al.* (1971a) used a similar method and did find a reduction if the SRBC were given at the peak of parasitemia. It is not clear whether this difference is due to timing of sensitization or strain differences.

In the next series of experiments non-specific inflammation was measured. DNCB is a severely caustic compound, which also has contact sensitizing reagent properties. In the doses used in this experiment (0.25% to 1%) the principal reaction is non-specific inflammation and the predominant cell seen in the lesion is polymorphonuclear leukocytes. The response to inflammation in the rats studied was not affected by malaria infection. It did not matter whether the malaria was given before or after DNCB.

A study was then made of systems which function largely through cellular hypersensitivity. The system used is arthritis induced in rats by injecting Complete Freund's Adjuvant (CFA) subcutaneously. The polyarthritis, which develops about 11 to 12 days later, is mediated by a cellular hypersensitivity mechanism. There is a systemic disease which is also associated with balanitis, conjunctivitis and wasting (Pearson, 1963). Many mechanisms of adjuvant arthritis are not known, however, it is transferred by lymphocytes but not by serum. It is blocked by the action of anti-lymphocyte globulin. Also, a prominent delayed hypersensitivity reaction to tuberculin develops.

If malaria was given after the CFA but before the onset of arthritis, there was a remarkable increase in the amount of the arthritis. This was less evident when the malaria was given after the arthritis started, but tended to be in the same direction. The tuberculin skin test results were parallel but the difference between the infected animals and control was not as great as the arthritis. It appears that the cellular hypersensitivity

was enhanced when malaria was given at a critical time. When the CFA was given a considerable time after the infection, when the malaria was subpatent although not cured, there was no longer a consistent pattern in the arthritis response and no significant difference from controls. In the groups given CFA simultaneously with, or only a few days after, *P. berghei* also showed no significant difference from the control animals.

The mechanisms of both delayed hypersensitivity reactions and adjuvant arthritis are not completely understood. There are a number of underlying steps relating first to sensitization and stimulation of lymphocytes. This is followed by the attraction of less specific inflammatory cells, the phagocytic cells. There is considerable information about what will suppress these reactions, but little is known what might enhance them. However, there is evidence that the arthritis has an inflammatory component which can be separately suppressed (Perper *et al.*, 1971). It is possible that certain stimuli which enhance the reticuloendothelial system (RES) at critical times will increase adjuvant arthritis, since a similar enhancement can be seen with the Fab fragment of ATG,* which is not immunosuppressive. Such a mechanism may be acting in malaria. It appears some aspects of cellular reactions may be increased by plasmodial infection. More work is planned on the study of this phenomenon.

The mechanisms involved in this altered response remain unclear. Greenwood *et al.* (1971b) suggest there might be a defect in dendritic macrophage processing of antigen by the RES in the mice infected. He does not, however, mention parasitemias in his animals. In fact the whole question of the level of parasitemia is very important in any discussion of immune phenomena in malaria. Lucia and Nussenzweig (1969) showed that carbon clearance was not increased unless the parasitemia was over 10%. Kitchen and Di Luzio (1971), however, found there is increased clearance of carbon in rats at all levels of parasitemia. However, using another agent they felt that phagocytosis *per se* was decreased, perhaps with increased absorption of particles. It is possible to speculate that some

stimulation of macrophages may occur at certain stages of a malarial infection. As yet there is little information on the function of the macrophage in the course of malaria infection, but it is possible that at some point the overall effect may be a stimulation of the reticulo-endothelial system. More work is needed in this area to elucidate the exact mechanism.

Note added in proof: A recent publication (Corradetti *et al.*, 1972) reported similar results in rats measuring anti-SRBC antibody, which was unchanged in infected rats. Further there was an increase in rosette forming cells. This suggests there may be significant differences in the effect of *P. berghei* on the immune response in rats and mice. Further it supports the concept of enhanced cellular hypersensitivity in rats with *P. berghei* infection.

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Malarial Immunosuppression—A Macrophage Mediated Defect

L. D. LOOSE, J. A. COOK AND N. R. DI LUZIO

Department of Physiology, Tulane University School of Medicine, New Orleans, Louisiana 70112

ABSTRACT: A state of immunosuppression has been demonstrated to occur in a malaria infection. The immunosuppression appears to be essentially humoral and not cellular in nature since classical lymphocyte-mediated cellular immune responses are normal. Since antigen uptake and processing by macrophages is an initial event in antibody response, studies were undertaken to define possible macrophage dysfunction as a contributory factor in malaria immunosuppression.

The intravascular clearance and tissue distribution of ^{51}Cr -sheep red blood cells (SRBC) in malaria-infected mice were comparable to that manifested in control groups. The normal fate of the particulate antigen was associated with a profound reduction in splenic plaque forming cells in malaria infected mice. Since SRBC must be processed by macrophages for induction of antibody formation, studies were undertaken to define the role of altered macrophage antigen processing as a mechanism of malaria-induced immunosuppression.

Peritoneal macrophages obtained from normal mice upon exposure to SRBC's were capable of evoking an immune response when transferred to either normal or malaria infected mice. The immune response in the latter group was, however, significantly reduced. A further reduction in the immune response was observed when antigen exposed peritoneal macrophages from malaria infected mice were transferred to either normal or malaria infected mice. These results indicate a malaria induced impairment in macrophage antigen processing as a contributing factor in malaria induced immunosuppression.

Previous studies have demonstrated that a relationship exists between the functional expression of the reticuloendothelial system (RES) and the response of animals to a variety of antigens. In general, activation of

the RES has been associated with an enhanced immune response to particulate antigens while a depression of RES activity results in a decreased immune response (Thorbecke and Benacerraf, 1962; Wooles and Di Luzio, 1963; Sharp, 1968; Schwartz *et al.*, 1970). However, in malaria infections, a pronounced increase in RES activity, as evaluated by colloidal carbon (Cox *et al.*, 1964; Lucia and

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Nussenzweig, 1969; MacGregor, *et al.*, 1969; Cantrell and Elko, 1970; Kitchen and Di Luzio, 1971) and ^{125}I -microaggregated albumin (Sheagren *et al.*, 1970), has been demonstrated to be associated with a significant immunosuppressive state (Kaye *et al.*, 1965; Salaman *et al.*, 1969; Barker, 1971; Greenwood *et al.*, 1971a and 1971b; Sengers *et al.*, 1971). The degree of immunosuppression appears to parallel the course of the parasitemia (Greenwood *et al.*, 1971a).

Malarial-induced immunosuppression has been demonstrated using such diverse antigens as heterologous erythrocytes (Salaman *et al.*, 1969; Greenwood *et al.*, 1971a; Barker, 1971; Sengers *et al.*, 1971), human gamma globulin (Greenwood *et al.*, 1971a), tetanus toxoid (McGregor and Barr, 1962), *Salmonella typhimurium* (Kaye *et al.*, 1965), murine sarcoma virus and urethane leukemia virus (Salaman *et al.*, 1969) as test antigens. However, a normal immune response to keyhole limpet hemocyanin (KLH) and a normal contact sensitivity reaction and phytohemagglutinin (PHA) transformation of lymphocytes have been demonstrated in malaria infected mice (Greenwood *et al.*, 1971a).

The normal contact sensitivity and PHA transformation of lymphocytes denote that malaria infected mice are capable of eliciting a delayed hypersensitivity type reaction. Since this reaction has been demonstrated to be thymic lymphocyte dependent (Miller *et al.*, 1962; Peterson *et al.*, 1966; Miller and Osoba, 1967), it appears that thymic lymphocyte cell-mediated immunity is not impaired significantly in malaria infected mice. A normal immune response of malaria infected mice to KLH, an antigen that does not require macrophage processing for immunogenicity (Unanue, 1969), suggests that humoral antibody production by lymphocytes and plasma cells is relatively unimpaired. These composite findings initiated the suggestion by Greenwood *et al.* (1971a) that the defect in the impaired immune response of malaria infected animals resides at the level of the afferent limb of the immune reflex arc or the macrophage.

Since normal peritoneal macrophages which are exposed to antigens have been demonstrated to be capable of eliciting antibody formation upon transfer to normal recipients

(Argyris, 1967; Cruchaud and Unanue, 1971), studies were undertaken to ascertain if macrophage dysfunction is a factor in malarial immunosuppression. Peritoneal cells obtained from malaria infected mice were tested, following antigen exposure, for their ability to induce antibody formation upon transfer into normal recipients. The comparative ability of antigen exposed peritoneal macrophages from normal mice to induce antibody formation upon transfer to either normal or malaria infected recipients was also evaluated. Additionally, the intravascular removal and tissue distribution of ^{51}Cr -labeled sheep red blood cells (SRBC) was determined to delineate the possible contribution of an alteration in antigen clearance or tissue distribution to the malaria-induced state of immunosuppression.

Materials and Methods

Animals and parasite

The NYU-2 strain of *Plasmodium berghei* was maintained by weekly transfer into Carworth Farms white male mice (CF_1) weighing 20–25 g. The inocula were obtained from the inferior vena cava of the mice maintained under light ether anesthesia and in general contained 1×10^6 infected erythrocytes. The erythrocytes were washed $3 \times$ in phosphate buffered saline (pH 7.20) and administered intraperitoneally. The presence of parasites in the inocula were determined from thin blood smears stained with Giemsa stain and the percent parasitemia was calculated using the technique of Gingrich (1932).

Phagocytic determinations

The phagocytic response of malaria infected mice to a particulate antigen was measured by the vascular clearance of ^{51}Cr -labeled sheep erythrocytes (SRBC). The vascular clearance and tissue distribution of ^{51}Cr -labeled heterologous erythrocytes have been previously used to measure RE phagocytic function (Halpern *et al.*, 1957; Wooles and Di Luzio, 1963; Morrow and Di Luzio, 1965; Schildt, 1970). In the studies of phagocytic status, CF_1 mice were inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes; control mice (non-infected) received isotonic saline isovolumetrically. Four days after inoculation with the parasite (or

Table 1. Vascular clearance and tissue distribution of ⁵¹Cr-labeled sheep red blood cells in *P. berghei* infected mice.¹

Treatment	Number of mice	t/2 ³ (min)	Tissue distribution ²					
			Liver		Lung		Spleen	
			% ID/g	% ID/TO	% ID/g	% ID/TO	% ID/g	% ID/TO
Saline	10	5.7 ± 0.6	23.9 ± 1.2	67.3 ± 3.3	5.4 ± 0.8	0.93 ± 0.14	16.6 ± 1.0	2.2 ± 0.2
Malaria	10	3.8* ± 0.3	19.9* ± 0.7	64.9 ± 2.4	7.5 ± 1.6	1.3 ± 0.3	17.9 ± 1.0	3.9* ± 0.2

¹ Mice inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes; controls received saline isovolumetrically. Four days after inoculation (or saline injection), all mice received 0.1 ml of 20% SRBC labeled with Na²⁵¹CrO₄ containing 7.05 μ Ci.

² Tissue distribution of the ⁵¹Cr-SRBC was measured at 11 min after injection and tissue radioactivity expressed as percentage of the injected dose (% ID) on a weight and total organ basis.

³ Vascular clearance determined by taking 0.01 ml aliquots of blood from the tail vein at 1, 3, 5, 7, and 9 min. The blood radioactivity, as percentage of the injected dose, was plotted semilogarithmically against time in minutes and the intravascular half-time (t/2) calculated.

⁴ Data expressed as mean \pm standard error of the mean; * indicates significance at $P < .05$.

injection with saline) both groups of mice (infected and controls) received an intravenous administration of 0.1 ml of 20% SRBC labeled with 7.1 μ Ci of Na²⁵¹CrO₄. The ⁵¹Cr-SRBC were prepared by the technique of Gray and Sterling (1950). After intravenous injection of the ⁵¹Cr-SRBC suspension, 0.01 ml aliquots of blood were collected from the tail vein at 1, 3, 5, 7, and 9 minutes and hemolyzed in 2.0 ml of distilled water. The blood radioactivity, as percentage of the injected dose, was plotted semilogarithmically against time in minutes and the intravascular half-time calculated. Tissue distribution of the ⁵¹Cr-SRBC suspension was measured at 11 minutes after its injection, and the radioactivity of liver, lung, and spleen expressed on a weight and total organ basis as a percentage of the injected dose (%ID).

Immunological procedures

CF₁ mice were inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes. Control mice (non-infected) received isotonic saline isovolumetrically. Four days after inoculation with the parasite (or saline injection) the mice received an intravenous injection of 0.1 ml of 10% SRBC. Splenic plaque-forming cells (PFC) were determined on days 3, 4, and 5 following immunization with the SRBC. The localized hemolysis in gel technique of Jerne and Nordin (1963) was used to detect spleen cells producing SRBC hemolytic antibody. The plaques formed in this technique

have been suggested to represent 19S or IgM antibody producing cells (Dresser and Wortis, 1965, and Plotz *et al.*, 1968).

Macrophage transfer studies

Male mice (CF₁) received intraperitoneally 1×10^6 *P. berghei* infected erythrocytes. Control mice received saline isovolumetrically. Four days later, both groups of mice received an intraperitoneal injection of 0.1 ml of 5% (2.5×10^7) SRBC. Three hours later peritoneal macrophages, with ingested SRBC, were removed by peritoneal lavage using 5 ml of 1 \times TC 199 (Microbiological Associates) (pH 7.40). The cells were then centrifuged at 1500 g for 10 min and resuspended in distilled water for 40 sec to lyse those SRBC which were not in the intracellular phase of the macrophage. A solution of 1.8% NaCl was then added isovolumetrically to the suspension in order to restore the cells to isotonicity. The cell suspension was then centrifuged and washed 3 \times with cold 1 \times TC 199. An aliquot of 1×10^7 macrophages, obtained from either malaria infected mice or non-infected controls and containing, on the basis of light microscopic examination, essentially equal numbers of SRBC was injected intravenously to normal mice. Splenic PFC were determined 4 days later. The identical procedure was performed using malaria-infected mice as recipients of antigen exposed normal macrophages or macrophages obtained from malaria infected mice.

Table 2. Immunosuppressive effect of *P. berghei* infection on splenic plaque forming cells in mice immunized with SRBC.¹

Group	Number of mice	SRBC	Plaque forming cells/spleen			
			Day			
			3	4	5	
Control	12	—	108 ± 12	73 ± 10	100 ± 13	
Control	12	+	36,000 ± 7,067	99,600 ± 12,639	15,933 ± 2,716	
Malaria infected	12	—	95 ± 12	105 ± 15	100 ± 40	
Malaria infected	12	+	4,404 ± 580	8,940 ± 588	6,750 ± 1,159	

¹ Mice inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes; controls received saline isovolumetrically. Four days after injection, mice were immunized intravenously with 0.1 ml of 10% SRBC. Splenic plaque forming cells (PFC) were determined 3, 4, and 5 days after immunization.
² Data expressed as mean ± standard error of mean.

Statistical analysis

The data were examined statistically using the Student “t” test with a 95% confidence level. All data are expressed as the mean ± standard error of the mean.

Results

The presence of a *P. berghei* NYU-2 infection in CF₁ mice was associated with increased phagocytic expression of the RES as indicated by a 47% enhancement in the intravascular removal of the ⁵¹Cr-SRBC (Table 1). The enhanced vascular clearance of ⁵¹Cr-SRBC was associated with an approximate 75% increase in uptake of the ⁵¹Cr-SRBC per spleen in the infected mice. Since this difference was not noted on a per gram basis, it reflects the increased splenic weight in the malaria-infected state. The increased uptake by spleen, which amounted to approximately 2% of the injected dose could not, however, account for the increased vascular clearance.

The predominant organ localization of the foreign red cells was liver. Hepatic uptake accounted for 96% of the total tissue radioactivity. The uptake of the SRBC by liver was decreased by 20% on a per gram basis in the infected mice as compared to non-infected controls. This minor alteration in hepatic uptake was not reflected on a total organ basis, again reflecting change in organ weight. Lung uptake of ⁵¹Cr-SRBC, either on a total organ or a per gram basis, was comparable in malaria infected mice to the non-infected control group and constituted approximately 1% of the injected dose.

The effect of a *P. berghei* NYU-2 infection

on the immune response to intravenously administered SRBC was studied concomitantly. Malaria infected CF₁ mice immunized with SRBC produced a peak splenic plaque-forming cell (PFC) response on day 4 following immunization (Table 2). A similar peak in splenic PFC was observed in normal non-infected controls. However, the PFC response of non-infected mice immunized with SRBC was increased 11-fold over the response observed in malaria infected mice. The PFC response was also significantly reduced in malaria infected mice at the 3rd and 5th day interval.

To determine if macrophage dysfunction was responsible for the observed decrease in PFC in the malaria infected mice, normal CF₁ mice received peritoneal macrophages obtained from malaria-infected or normal mice. The macrophages were either in their normal state or in an antigen exposed mode. The transfer of macrophages obtained from normal or malaria infected mice, which were not exposed

Table 3. Comparative influence of transferred antigen exposed macrophages obtained from normal and malaria-infected mice¹ on splenic plaque formation in normal mice.²

Donor	SRBC	Recipient	No.	PFC/spleen on day 4
Normal	—	Normal	10	105 ± 21
Normal	+	Normal	10	34,651 ± 1,477
Malaria	—	Normal	10	125 ± 21
Malaria	+	Normal	10	6,904 ± 1,359

¹ Peritoneal macrophages were obtained by peritoneal lavage using 5 ml TC 199. Prior to peritoneal lavage the mice were injected intraperitoneally with 0.1 ml of 5% SRBC or saline. Three hours later, the peritoneal macrophages were harvested. Recipients received 1×10^7 macrophages intravenously.
² Data expressed as mean ± standard error of the mean.

Table 4. Comparative influence of transferred antigen exposed macrophages obtained from normal and malaria-infected mice¹ on splenic plaque formation in *P. berghei* infected mice.²

Donor	SRBC	Recipient	No.	PFC/spleen on day 4
Normal	—	<i>P. berghei</i> infected	10	170 ± 41
Normal	+	<i>P. berghei</i> infected	10	13,740 ± 741
Malaria	—	<i>P. berghei</i> infected	10	135 ± 21
Malaria	+	<i>P. berghei</i> infected	10	5,080 ± 1,020

¹ Peritoneal macrophages were obtained by peritoneal lavage using 5 ml TC 199. Prior to peritoneal lavage mice were injected intraperitoneally with 0.1 ml of 5% SRBC or saline. Three hours later, the peritoneal macrophages were harvested. Recipients received an intravenous injection of 1×10^7 macrophages.

² Data expressed as mean ± standard error of the mean.

to SRBC, did not induce a splenic PFC response (Table 3). In marked contrast, the transfer of antigen exposed control macrophages to normal mice was associated with a profound enhancement in PFC. However, the transfer to normal mice of antigen-containing macrophages obtained from malaria-infected mice was associated with a significant reduction in PFC. The 5-fold greater enhancement in PFC with normal macrophages, containing equivalent antigen loads based upon red cell presence in the transferred macrophages, clearly denotes a significant reduction in the ability of malaria infected antigen-laden macrophages to effectively initiate an immune response.

A parallel experiment was conducted in which malaria infected mice received an intravenous injection of 1×10^7 peritoneal macrophages, obtained from either malaria infected mice or normal non-infected controls. The PFC in malaria-infected mice, which received an injection of normal or malaria macrophages not exposed to SRBC, was of background magnitude (Table 4).

The number of PFC per spleen was significantly increased over this background value when normal macrophages which were exposed to SRBC were administered to malaria-infected mice. The PFC response was reduced approximately 60% when macrophages derived from malaria-infected mice were administered, again denoting an inability of malaria macrophages to effectively enhance antibody formation in a manner equivalent to normal antigen exposed macrophages.

Discussion

A profound state of immunosuppression, which appears to parallel the course of the disease state, has been demonstrated in malaria infection (Kaye *et al.*, 1965; Salaman *et al.*, 1969; Barker, 1971; Greenwood *et al.*, 1971a and 1971b; Sengers *et al.*, 1971). Salaman *et al.* (1969) attributed the suppressed immune response to altered immune reactivity and speculated that this may be a causal factor in the etiology of Burkitt's lymphoma in areas of chronic malaria infection.

The mechanism whereby the malaria disease state induced immunosuppression has not as yet been delineated. The altered immune response appears primarily humoral in nature and not cellular since lymphocytic cell-mediated responses are normal (Greenwood *et al.*, 1971a). Since the depressed immune response appears to involve the afferent immune arc, Greenwood *et al.* (1971a) hypothesized that the malarial induced immunosuppression may be macrophage mediated.

The immunosuppression to SRBC, a macrophage processed antigen (Argyris, 1967; Hoffman, 1970; Feldman and Palmer, 1971), has been confirmed in this study using *P. berghei* NYU-2 infected mice. Our present results, in essence, confirm the observations of Salaman *et al.* (1969), Greenwood *et al.* (1971a), and Barker (1971) who used *P. berghei yoeli* and Sengers *et al.* (1971) who used *P. berghei* K-173. Since an immunosuppression to SRBC does occur in malaria infected mice, and since SRBC are a macrophage processed antigen, a macrophage functional alteration, either in the phagocytosis of the antigen or in antigen processing, can be predicated.

In an effort to evaluate the role of altered antigen fate in malarial immunosuppression, the vascular clearance and tissue distribution of ⁵¹Cr-SRBC were evaluated in malaria infected mice. In malaria infected mice, an enhanced vascular clearance rate of the particulate antigen was demonstrated. The enhanced removal rate was not associated with any pronounced alteration in tissue distribution of the ⁵¹Cr-SRBC. These results may be interpreted to indicate that along with normal phagocytic function, an adequate vascular supply to principal RE organs such as liver, lung, and spleen, which were previously re-

ported to be congested in malaria (Mae-graith, 1954; Singer, 1954), is maintained in the malaria infected mice.

Previous investigators (Greenwood *et al.*, 1971b) have demonstrated an approximate 8-fold enhancement in vascular clearance of ^{51}Cr -SRBC in malaria infected mice with a significant increase in the hepatic localization of the intravenously injected ^{51}Cr -SRBC. However, due to a difference in blood sampling time employed by Greenwood *et al.* (1971b) (1 and 30 min), and the late time interval at which tissue radioactivity was measured (24 hr) as well as a difference in the infected state, a comparison between their results and those presented in this study would not be appropriate. Indeed, the relatively rapid loss of radioactivity of liver following ^{51}Cr -SRBC injections has been observed in mice (Morrow and Di Luzio, 1965) rendering studies at late time intervals most difficult to interpret.

Since antigen clearance was enhanced and distribution essentially normal in the malaria infected mice, the phagocytic event *per se* cannot be the basis for the observed reduction in the immune response in malaria infected mice. These results suggest that an impairment in subsequent phases of macrophage participation in the immune response, namely, antigen digestion and/or antigen processing is defective in malaria-infected mice.

To test the hypothesis that macrophage antigen processing may be altered in a malarial infection, isolated peritoneal macrophages from either normal or malaria infected mice were allowed to phagocytize SRBC *in situ*. The capacity of the antigen exposed macrophages to evoke antibody formation upon transfer was ascertained. Previous investigators have demonstrated that mouse peritoneal macrophages are required for an *in vitro* primary and secondary immune response to SRBC (Hoffman, 1970; Feldman and Palmer, 1971) and may potentiate or enhance the antibody response of newborn mice to SRBC (Bendinelli *et al.*, 1971). Furthermore, studies by Argyris (1967) and Cruchaud and Unanue (1971) have demonstrated the ability of mouse peritoneal macrophages to phagocytize SRBC *in vitro* and to elicit antibody to the SRBC when transferred to syngeneic recipients. In agreement with these observations, macrophages

exposed to SRBC when transferred to normal mice clearly elicited a significant immune response.

Peritoneal macrophages obtained from normal mice induced a significantly greater number of splenic PFC in normal recipient mice than did the antigen exposed macrophages obtained from malaria infected mice. Indeed, an approximate 5-fold increase in PFC formation was observed in mice which received normal macrophages, with ingested SRBC, than in those mice which received malaria derived macrophages. Visual observation with light microscopy confirmed the presence of ingested SRBC, in essentially equal numbers, in both normal and malaria-derived peritoneal macrophages. The uptake of SRBC was ascertained to assure that a comparable antigenic exposure of normal and malaria infected macrophage occurred. The present experiments demonstrate that whereas SRBC-exposed peritoneal macrophages obtained from normal mice were capable of eliciting antibody synthesis upon transfer, a significant reduction in the number of PFC occurred when macrophages were derived from malaria infected mice. This finding suggests an inability of malaria derived macrophages to present an adequate immunogen to immunocompetent cells through a defect in digestion and/or processing of the antigen. A parallel experiment was conducted in which malaria infected mice received macrophages derived from either normal or malaria infected mice. The macrophages obtained from normal mice elicited an approximate 2-fold increase in splenic PFC as compared to malaria derived macrophages. The results of the macrophage transfer indicate that the impaired immune response in malaria infected mice is, in part, a manifestation of impaired antigen processing.

An additional macrophage alteration which has recently been observed in malaria infected mice is the inability to detoxify endotoxin. In a previous paper (Loose *et al.*, 1971), a 41-fold increase in endotoxin sensitivity in malaria infected mice was reported. This enhanced endotoxin sensitivity was demonstrated to be a result of the loss of hepatic endotoxin detoxification ability, an event demonstrated to be a function of hepatic Kupffer cells (Filkins, 1971). It was suggested (Loose *et al.*, 1971) that this Kupffer cell dysfunc-

tion may be a result of malaria induced lysosomal enzyme alterations since Kupffer cell lysosomes have been reported to be essential in endotoxin detoxification (Filkins, 1971). The postulated lysosomal alteration in malaria-infected mice had been predicated to be due to lysosomal enzyme inhibition or depletion due to the ingestion and/or accumulation of debris, either cell or parasite in nature (Loose *et al.*, 1971). Indeed, the accumulation of hemozoin in the phagolysosomes could well be a contributory factor (Maegraith, 1954; Jap and Jerusalem, 1971). Diversion of lysosomes to deal with the cellular debris or hemozoin resulting from parasitic infection may be an important event in reducing available lysosomes for not only endotoxin detoxification but also for antigen digestion and processing.

In addition to the recently reported impairment in macrophage endotoxin detoxification associated with malaria infection, the present study adds a new dimension to disturbances in macrophage function associated with a malaria infection, i.e., an apparent inability of the macrophage to process in a normal fashion a particulate antigen, resulting in the development of a state of immunosuppression. The contribution of this macrophage defect not only to the persistence of the parasite in the host but to other host defense alterations associated with malaria, such as increased sensitivity of malaria infected mice to bacterial and viral infections, is yet to be ascertained.

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Comments on Resistance Mechanisms

D. J. STECHSCHULTE

Robert B. Brigham Hospital, Boston, Massachusetts 02120

Acquired resistance to plasmodial infection is a characteristic response in all species including man. The mechanism of resistance is best explained by host immune responses stimulated by previous contact with parasite antigen or antigens. A better understanding of the nature of the host response associated with protective immunity is essential if immune prophylaxis is to become a reality. The immune response is classically divided into humoral and cellular events but it is becoming

increasingly clear that complete separation of these phenomena exists only in the *in vitro* situation, and most certainly the host in organizing its defenses against a plasmodial infection utilizes an integrated immune response. The ability of antibody to suppress a malaria infection has been demonstrated by both *in vivo* and *in vitro* experiments. The observation by Diggs and coworkers that the laboratory-maintained Camp strain of *P. falciparum* in *Aotus* monkeys is inhibited by IgG antibody

derived from an African population is very encouraging. This indicates that unique strains of the parasite have some common antigenic determinants and that interaction of these antigens with antibody adversely affect the parasite life cycle. The species and strain specificity of malarial immunity reported at previous Workshops suggests that the number of antigens that are both common and susceptible to immune suppression are very limited. Four subclasses of the IgG molecule have been identified on the basis of physicochemical and biologic properties (Cohen, 1971). It would be of interest to determine if protective antibodies to malaria infection resided in one of the subclasses of human IgG. The availability of monospecific reagents to these subclasses make such a study feasible. There is no evidence that malaria infection stimulates the production of IgE, IgA or IgD antibody and therefore it seems unlikely that these antibodies play any role in humoral immunity. IgM antibody, as mentioned earlier by Dr. Cohen, is capable of inhibiting parasite replication in the *in vitro* test system.

The mechanism by which antibody exerts its inhibitory effect on the parasite is not clear. One of the amplification or effector systems of antibody activity is complement activation. The second paper by Diggs and associates reporting studies in rats depleted of the third component of complement (C3) indicate that the protective pathway is antibody mediated but complement or C3 independent. This is consistent with the observations of Cohen and Butcher (1969) that *in vitro* antibody activity was not dependent on complement proteins. The *in vivo* studies do not exclude a role for complement but certainly indicate that it is not a critical component of passive humoral immunity as studied by these workers. However, as the authors point out, with limited amounts of antibody the complement system might be recruited in order to reinforce immune protection. In the C5661 treated animals it would be of interest to know if red blood cell destruction was less severe. This raises the unresolved question of autoimmune destruction of nonparasitized erythrocytes via complement activation.

The studies by Stutz, Ferris and Voss using *P. gallinaceum* infection in bursectomized chickens clearly implicates humoral immunity

in the control of this parasite. The delay in peak parasitemia and death in the embryonically bursectomized group when compared to the bursectomized-irradiated and hormonally bursectomized groups, all of which are hypogammaglobulinemic, is indirect evidence that host resistance is not simply dependent on antibody production. The complexity of host resistance in this model is demonstrated by the second paper. The protective effect of normal serum in bursectomized-infected chickens is evident particularly if one looks at parameters such as the hemocrit or mortality rate. The mechanism of this protection is not clear. Repeat experiments with various fractions of normal serum are indicated as well as *in vitro* assessment of specific antibody activity in these preparations. More puzzling than the protective effect noted with normal serum is the minimal protection observed with convalescent serum. The critical need in these experiments is again the identification of specific antibody in "convalescent serum;" if antibody is present and protective in the intact animal then a reinterpretation of the effect of bursectomy in this experimental model is indicated.

The study by Lourie and Dunn in rats immunosuppressed by the administration of anti-thymocyte globulin and then infected with *P. berghei* demonstrates an increased morbidity and mortality in these animals. That this effect can be reversed with hyperimmune serum excludes a critical role for effector systems of cellular immunity such as lymphocytotoxicity or the production of molecules such as chemotactic factor or migration inhibitory factor (Bloom, 1971). One might speculate that the thymic-dependent immune system plays a role at the level of antigen recognition or enhanced antibody production and that passive administration of antibody is capable of eliminating the need for an intact cellular immune response. The role of the macrophage in malaria immunity is difficult to assess in these studies in view of the failure to demonstrate *in vivo* activity of the anti-macrophage serum. The effect of *P. berghei* on the immune response of the rats as measured by antibody production to sheep erythrocytes or cutaneous sensitivity to purified protein derivatives is unremarkable. An enhancing effect of *P. berghei* infection on adjuvant

arthritis was observed but an explanation is not readily apparent and I can see no easy way to clarify this phenomenon.

The paper by Loose, Cook and Di Luzio nicely confirms enhanced clearance of particulate antigen in malaria infected mice and identifies impairment of antigen processing by macrophages as an important factor in malaria-induced immunosuppression. This could be due to a variety of parasite effects and this observation should stimulate some interesting investigations.

In summary, these papers have looked at a variety of host responses stimulated or inhibited by a very adaptive organism whose existence depends on the failure to elicit a brisk sterile immune response in the host.

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VIII

IMMUNITY

B. Vaccination Attempts

Increased Phagocytic Activity of the Reticulo-Endothelial System During Immunization with X-irradiated Sporozoites of *Plasmodium berghei* in Mice¹

C. RIVERA-ORTIZ* AND R. S. NUSSENZWEIG, M.D.

Department of Preventive Medicine, New York University Medical Center,
550 First Avenue, New York, New York 10016

ABSTRACT: Stimulation of the reticulo-endothelial system with X-irradiated sporozoites of *Plasmodium berghei* was investigated in A/J mice. The carbon clearance method was used to determine this activity. It was found that a) the injection of infected or uninfected salivary glands, which were subjected to X-irradiation, produced increased clearance rates and increased spleen weights; b) these changes are more pronounced after inoculation of sporozoite-infected salivary glands; c) increased clearance rates occur rapidly, reach a maximum two days after sporozoite injection, and return to slightly above normal values after approximately one week; and d) these effects are not enhanced by multiple sporozoite salivary gland injections. From these data we can conclude that the spleen appears to play an important role in these increases in clearance rate. It also seems reasonable to conclude that the stimulation of the RES does not play a major role in the enhancement of protection which results from a multiple course of immunization. This is evidenced by the fact that protective immunity and increased phagocytic activity do not show a temporal relationship.

The role played by macrophages of the reticulo-endothelial system as a mechanism of defense against infectious diseases has long been recognized. It was not until relatively recently, that their activity was investigated quantitatively by the use of colloidal suspensions which are avidly phagocytosed by components of the RES (Biozzi *et al.*, 1953, 1957). Phagocytosis of malarial parasites was first recorded by McCallum (1893) and subsequently by other investigators (Goble and Singer, 1960).

It was demonstrated by quantitative analysis that the phagocytic function of the RES is stimulated in chickens infected with *P. (H.) gallinaceum* (Cox, 1963), and in the rats and mice infected with *P. berghei* (Fabiani and Orfila, 1959). The importance of this function has also been evidenced by the stimulation of the RES system using *Corynebacterium parvum*, which causes an increase in resistance to infection with sporozoites of *P. berghei* in mice (Nussenzweig, 1967). Increased phagocytic activity in the rats infected with blood forms of *Plasmodium berghei* at the time of

onset of parasitemia was also reported by Biozzi *et al.* (1970).

In the rodent malaria system, total protection has been repeatedly obtained in mice immunized with several doses of X-irradiated sporozoites (Nussenzweig *et al.*, 1969). The possibility existed that this protection might be due to an enhanced activity of the macrophages of the RES. The present work was undertaken to investigate this possibility by determining the degree of increase of phagocytic activity in animals receiving either infected or uninfected salivary gland as antigenic material. We also investigated the duration of this phagocytic activity, the effect produced by an increasing number of boosters and the effect of splenectomies on this RES activity.

Materials and Methods

A. Procedure for immunization

All animals were A/J mice, obtained from Jackson Memorial Laboratories (Bar Harbor, Maine). These animals were females, varying in age from 2–4 months in the different experiments. All experimental animals received single or multiple injections of either sporozoite-infected or uninfected, irradiated salivary glands.

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* Predoctoral Trainee, grant A100361, National Institutes of Health.

Table 1. Comparison of carbon clearance rates in mice, determined at different time intervals, after a single i.v. injection of infected mosquito salivary glands.¹ (Carbon clearance rates expressed as phagocytic index K).²

Phagocytic index (K)	Time after sporozoite injection						
	Controls	1 hour	1 day	2 days	4 days	7 days	9 days
Exp. I ± SD ³	.020 ± .004	.018 ± .004	.059 ± .009	.096 ± .002	.066 ± .002	.024 ± .002	.022 ± .001
Exp. II ± SD ³	.020 ± .004	.021 ± .001	N.D.	.097 ± .003	.047 ± .003	.048 ± .003	.028 ± .004

¹ Injection 7.5×10^4 irradiated sporozoites.

² Phagocytic index $K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$

³ Standard deviation of the mean.
N.D. = Not Done.

Sporozoites of *P. berghei* (NK 65 strain) were harvested from the mosquito, *Anopheles stephensi*, during the time of optimal recovery of salivary gland sporozoites, i.e. 14–18 days after a blood meal on an infected hamster, *Cricetus auratus*. The method of recovery has been previously described (Vanderberg *et al.*, 1968).

Uninfected salivary glands were recovered from normal mosquitoes by dissecting and triturating the glands in the same manner as infected salivary glands. Individual mosquitoes were counted in order to equate, as closely as possible, the number of uninfected salivary glands to that of sporozoite containing material used for immunization.

The preparations were irradiated in the 280 KVp X-ray beam of a Picker Teletherapy Vanguard unit. All vials received 10,000 rads, a dosage which has previously been shown to attenuate these parasites and preserve their immunogenicity (Vanderberg *et al.*, 1968).

B. Schedule of immunization

Animals immunized with irradiated sporozoites received in all instances an initial injection of 7.5×10^4 parasites. For boosters, the animals received 2.0×10^4 parasites at intervals of one to two weeks.

Animals injected with uninfected irradiated salivary glands received initial injections of material obtained from 15 mosquitoes. Their boosters consisted of salivary gland material obtained from 5 mosquitoes, administered at similar time intervals. All injections were done intravenously.

C. Splenectomy

Mice which were splenectomized were operated on under Pentobarbital (Pentobarbital, Winthrop) anesthesia prior to immunization. After the animals had recovered from surgery they received an injection of 7.5×10^4 irradiated sporozoites followed by 4 boosters of 2.0×10^4 sporozoites each. Carbon clearance was then done as scheduled below.

D. Method of determination of carbon clearance rates

Carbon (C11/1431, Gunther Wagner Laboratories) was prepared following the procedure of Biozzi *et al.* (1953).

Animals received 1.6 mg. of colloidal carbon in gelatin per 10 gm. of body weight intravenously. The rate of clearance from the blood was investigated following the technique described by Biozzi *et al.* (1957). Several blood samples were drawn at different time intervals and the phagocytic index (K) was determined. This phagocytic index is defined as an exponential function of the rate of carbon clearance from the blood during a given time interval as given by the formula

$$K = \frac{\log C_1 - \log C_2}{t_2 - t_1},$$

where C_1 and C_2 are the concentrations of carbon in the blood, at times t_1 and t_2 after an i.v. injection of colloidal carbon.

Following determination of these clearance rates, animals were killed and body weight, liver, and spleen weights were recorded.

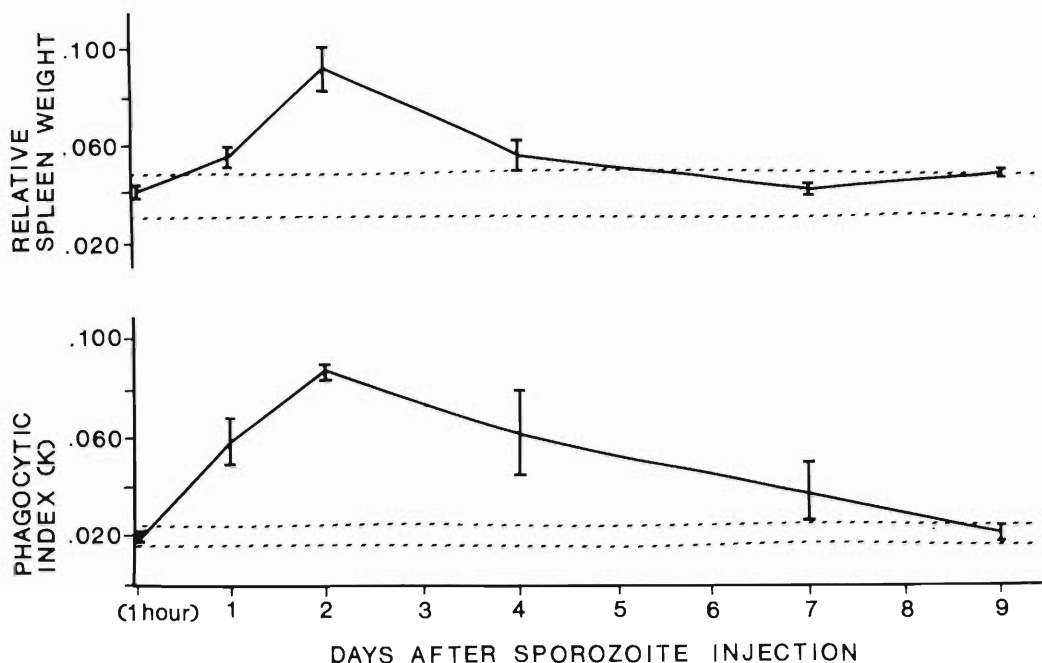


Figure 1. Comparison of carbon clearance rates and relative spleen weights, in mice, determined at different time intervals, after a single i.v. injection of infected irradiated mosquito salivary glands.

E. Determination of carbon clearance rates during the course of immunization

Control animals were chosen from the different batches of mice used for the experiments and received injections of 1.6 mg. colloidal carbon per 10 gm. of body weight.

A preliminary experiment was undertaken to determine optimal times for the determination of clearance rates. The mice received an initial injection of 7.5×10^4 irradiated sporozoites and were subsequently injected with colloidal carbon at different time intervals. The time intervals chosen were 1 hour, 1 day, 2 days, 4 days, 7 days and 9 days following the injection of the antigenic material. In all other experiments, clearance determinations were done two and nine days after antigenic injection.

Results

Preliminary experiments had indicated that a single intravenous injection of sporozoite-infected, irradiated salivary glands resulted in

what appeared to be significant changes of both the carbon clearance rate and the relative spleen weight of the animals.

In order to evaluate the significance of these results carbon clearance rates were determined on a series of 16 normal female A/J mice, of the same lot and age group as the experimental ones.

In normal A/J mice, the mean value of colloidal carbon clearance rates, expressed as phagocytic index (K) ± 1 standard deviation was found to be $0.020 \pm .004$. The relative weight of the spleen in these animals was $0.039 \pm .009$ g/10gms. of body weight. The average liver weights of these animals was $0.475 \pm .076$ g/10gms. weight.

A. Time course of the increase of phagocytic activity and relative spleen weight induced by the injection of *P. berghei* infected salivary glands

The main purpose of these experiments was to determine at which point in time a maximal

Table 2. Comparison of relative spleen weights¹ obtained from mice at different time intervals after a single i.v. injection of infected mosquito salivary glands.²

Relative spleen weights ¹	Controls	Time after sporozoite injection					
		1 hour	1 day	2 days	4 days	7 days	9 days
Exp. I ± S.D. ³	.039 ± .009	.042 ± .003	.056 ± .004	.097 ± .003	.059 ± .003	.042 ± .002	.048 ± .001
Exp. II ± S.D. ³	.039 ± .009	.040 ± .004	N.D.	.069 ± .002	.059 ± .003	.040 ± .005	.050 ± .002

¹ Weights of spleens recorded as gms/10 g. body weight of mice.
² Injection of 7.5×10^4 irradiated sporozoites.
³ Standard deviation of the mean.
N.D. = Not Done.

increase of both carbon clearance rate and spleen weight occurs as a result of the injection of sporozoite-infected salivary glands. Data thus obtained would also provide a sensitive parameter to permit the evaluation and quantitative comparison of the effects of various conditions of immunization on the phagocytic activity of mice.

In two experiments, a total of 37 A/J mice each received a single inoculum of 7.5×10^4 X-irradiated sporozoites i.v. These animals were divided into six groups in which carbon clearance rates as well as spleen and liver weights were determined at different times, from 1 hour up to 9 days after the initial sporozoite injection. Their carbon clearance rates, as measured by the phagocytic index (K) are shown in Table 1. Clearance rates were not altered 1 hour after the sporozoite injection, the period during which sporozoites can still be detected in the circulation (Nussenzweig *et al.*, 1972). The phagocytic index (K) was first observed to increase one day after sporozoite inoculation. Maximal clearance rates were observed two days after inoculation, the mean phagocytic index being 4.8 times greater than that of control animals

(Table 1 and Fig. 1). After this time period the phagocytic index (K) decreased gradually to about normal values by the 9th day after inoculation. This has been shown to be the time at which protective immunity is at its height (Spitalny and Nussenzweig, 1972).

Spleen and body weights were determined for this same group of animals and it was found that the relative spleen weight followed essentially the same pattern as the phagocytic index during the same period (Table 2 and Fig. 1). Relative spleen weights were first observed to be increased 1 day after sporozoite inoculation. Maximal values were also, in this instance, found to occur on the second day after injection, decreasing thereafter to values slightly above normal on the 7th and 9th day.

B. Effect of multiple injections of *P. berghei*-infected irradiated salivary glands on carbon clearance rates and spleen weights of mice

Since the degree of sporozoite-induced protective immunity increases considerably after several boosters, it became important to investigate the effect of multiple immunizing

Table 3. Carbon clearance rates obtained on the second and ninth day after either a single or multiple sporozoite injection.¹

	Controls	Number of boosters ¹							
		0 boosters		1 booster		2 boosters		4 boosters	
		2 days	9 days	2 days	9 days	2 days	9 days	2 days	9 days
Average phagocytic index (K) ± S.D. ²	.020 ± .004	.086 ± .021	.028 ± .005	.067 ± .020	.038 ± .014	.084 ± .011	.033 ± .066	.075 ± .020	.033 ± .001
No. of animals	16	10	10	23	8	13	10	14	10

¹ Initial injection of 7.5×10^4 irradiated sporozoites, followed by boosters of 2.0×10^4 irradiates sporozoites each.
² Standard deviation of the mean.

Table 4. Relative spleen weights¹ obtained on the second and ninth day after either a single or multiple sporozoite injection.²

	Controls	Number of boosters							
		0 boosters		1 booster		2 boosters		4 boosters	
		2 days	9 days	2 days	9 days	2 days	9 days	2 days	9 days
Relative spleen weights ¹ ± S.D. ³	.037 ± .011	.086 ± .017	.062 ± .014	.071 ± .016	.044 ± .009	.070 ± .016	.045 ± .005	.074 ± .025	.038 ± .005
No. of animals	10	10	9	22	8	14	10	12	9

¹ Spleen weights recorded as g/10 g body weight.
² Initial injection of 7.5×10^4 irradiated sporozoites followed by boosters of 2.0×10^4 irradiated sporozoites.
³ Standard deviation of the mean.

doses on the phagocytic activity and spleen weight of these animals.

Experimental animals received either a single i.v. immunizing dose, or an initial antigen administration followed by one, two or four injections. Clearance rates, as well as relative spleen and liver weights of these animals were determined on the second and ninth day after the final sporozoite injection, of each of the experimental groups. On the basis of the data summarized in Table 3, it can be concluded that an increasing number of boosters, did not result in increasingly higher clearance values. Multiple sporozoite injections did not result in a longer lasting RES stimulation, which would be reflected in the persistence of increased carbon clearance rates. In fact this clearly did not occur, since the phagocytic index was back to values slightly above normal on the 9th day after the 4th booster was given.

As for the relation of spleen weight to body weight of these same animals, the data have been summarized in Table 4. It was found that sporozoite-immunized animals undoubtedly present a significant increase of spleen

weight, subsequent to each sporozoite injection. However this increase is not cumulative after various boosters. On the contrary, spleen weight rapidly decreases and reaches close to normal values, approximately one week after each sporozoite injection, independent of the number of boosters.

Neither phagocytic index nor spleen weights are therefore boosted by multiple sporozoite injections. They respond essentially in the same manner to each single sporozoite injection, independent of the number of boosters which might have preceded that particular injection.

C. Effect of single and multiple doses of non-infected mosquito salivary glands on carbon clearance rates and spleen weights

Since every sporozoite inoculum always contains a considerable amount of contaminating salivary gland material it became important to evaluate the effect of non-infected salivary glands on carbon clearance rates.

Table 5. Carbon clearance rates obtained on the second and ninth day after either a single or multiple injection of uninfected, irradiated salivary glands.¹

	Controls	Number of boosters							
		0 boosters		1 booster		2 boosters		4 boosters	
		2 days	9 days	2 days	9 days	2 days	9 days	2 days	9 days
Phagocytic index (K) ² ± S.D. ³	.020 ± .004	.038 ± .005	.024 ± .003	.038 ± .009	.026 ± .005	.033 ± .010	.028 ± .003	.034 ± .007	.027 ± .004
No. of animals	16	13	11	26	10	11	8	28	9

¹ Initial injection of 15 uninfected, irradiated salivary glands, followed by boosters of 5 uninfected, irradiated salivary glands each.
² Phagocytic index $K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$
³ Standard deviation of the mean.

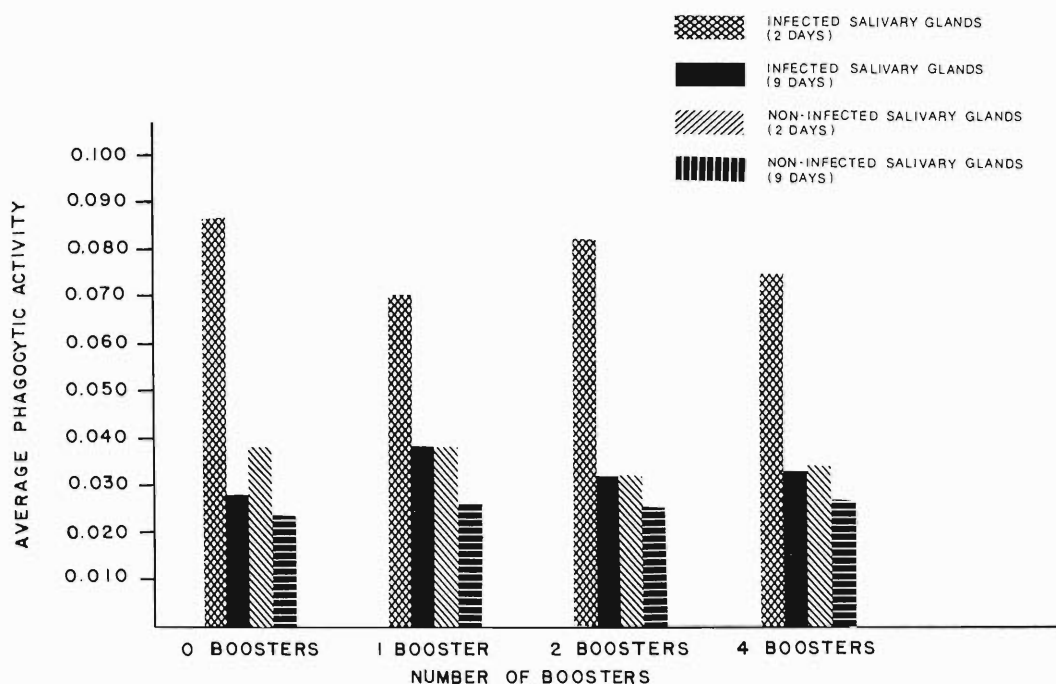


Figure 2. Comparison of carbon clearance rates obtained on the second and ninth day after either single or multiple, and either infected or uninfected irradiated salivary gland injections.

Experimental animals were injected with a number of non-infected salivary glands which corresponded closely to that used in the previous experiments with *P. berghei* infected irradiated salivary glands. Clearance rates were increased on the second day after each inoculation, reaching values slightly above normal on the 9th day after each injection (Table 5). It was noteworthy that maximal clearance values in these animals were consistently lower than those observed in mice injected with

sporozoite-infected irradiated salivary glands (Fig. 2). No enhancing effect on clearance values resulted from multiple salivary gland injections.

Similar conclusions can be drawn from the corresponding data obtained on spleen weights (Table 6). A) No 'booster' effect on spleen weights results from multiple injections of non-infected salivary glands, and B) increases of spleen weight in response to normal salivary gland injection are smaller than those induced

Table 6. Relative spleen weights¹ obtained on the second and ninth day after either a single or multiple injection of uninfected salivary glands.²

	Controls	Number of boosters							
		0 boosters		1 booster		2 boosters		4 boosters	
		2 days	9 days	2 days	9 days	2 days	9 days	2 days	9 days
Relative spleen weights ¹ ± S.D. ³	.037 .011	.054 .001	.052 .002	.057 .012	.046 .014	.058 .008	.042 .005	.042 .002	.032 .008
No. of animals	10	11	11	26	10	11	8	28	9

¹ Spleen weights recorded as g/10 g body weight.

² Initial injection of 15 uninfected irradiated salivary glands followed by boosters of 5 uninfected irradiated salivary glands.

³ Standard deviation of the mean.

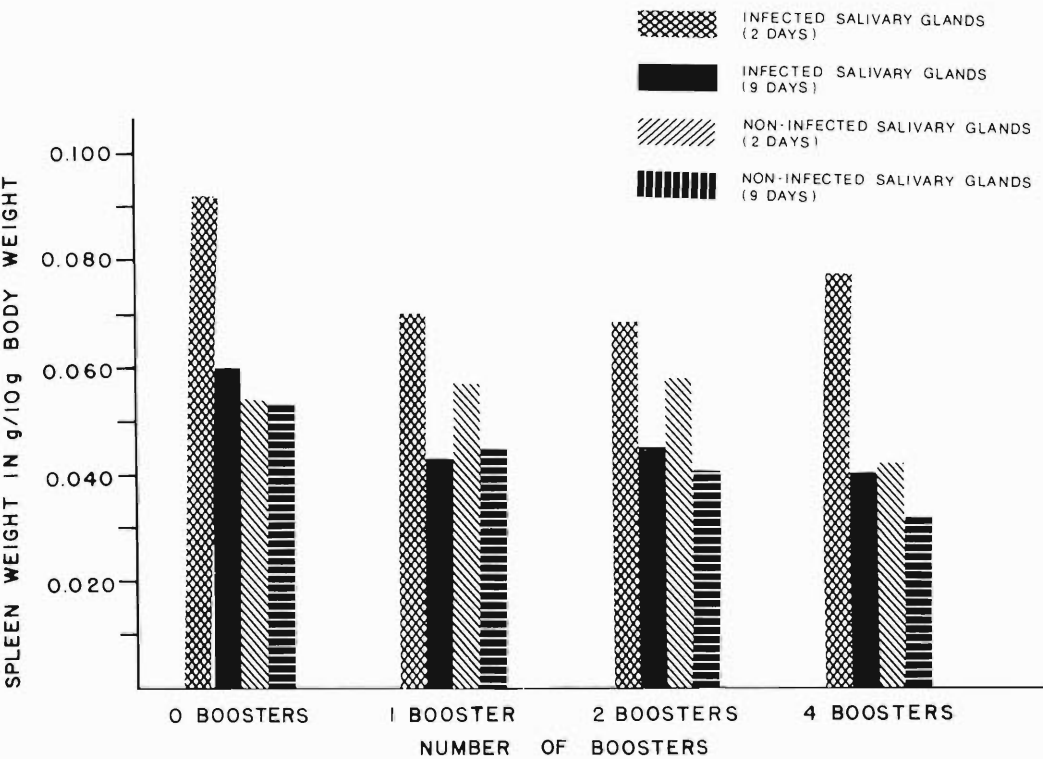


Figure 3. Comparison of relative spleen weights obtained on the second and ninth day after either single or multiple, and either infected or uninfected irradiated salivary gland injections.

by the injection of sporozoite-infected salivary glands, under otherwise similar experimental conditions (Fig. 3).

D. Effect of splenectomy on the carbon clearance rate of sporozoite-immunized animals

The preceding experiments had shown that changes in spleen weights closely follow the pattern of changes in carbon clearance rates, after injection of either infected or uninfected salivary glands. To further clarify the relationship between these two occurrences, carbon clearance rates and liver weights were determined on animals splenectomized prior to their immunization.

All data summarized in Table 7 were obtained on the second day after the fourth booster with infected salivary gland material. Under these conditions clearance rates were

still significantly higher than normal. However, the mean phagocytic index values of these animals were considerably lower than those obtained in non-splenectomized mice,

Table 7. Carbon clearance rates obtained in splenectomized¹ mice following multiple injections of irradiated sporozoites².

	Sporozoite injected splenectomized animals	Splenectomized controls	Intact controls
Phagocytic index (K) ³ ± S.D. ⁵	.046 ± .009	.021 ± .004	.020 ± .004
Relative liver weight ⁴ ± S.D. ⁵	.506 ± .054	.472 ± .045	.475 ± .076

¹ Experimental and control mice splenectomized before the immunization period.

² Initial injection of 7.5×10^4 irradiated sporozoites followed by 4 boosters of 2.0×10^4 irradiated sporozoites.

³ Phagocytic index $K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$

⁴ Relative liver weights recorded as g/10 g body weight.

⁵ Standard deviation of the mean.

under similar conditions (Table 3). An increase of the relative weight of the liver was observed in these splenectomized animals which had received multiple injections of infected irradiated salivary glands.

Discussion

The stimulation of the reticulo-endothelial system by X-irradiated sporozoites of *Plasmodium berghei* has enabled us to draw conclusions about phagocytic activity during the course of immunization with this antigenic material. The results of the experiments presented in this paper have clearly shown that there is no temporal correlation between the immunity afforded by these antigens and the stimulation of the phagocytic system. This has been evidenced by the fact that although there is an increased clearance rate after the injection of X-irradiated sporozoite, this increase in activity occurs rapidly, reaching a maximum after two days and then gradually falling off until it reaches normal values by the ninth day (Table 1). This occurrence differs from the timing of the process of immunization where protection is afforded approximately 1 week after the injection of the antigenic material (Spitalny and Nussenzweig, 1972). Another basic difference between the RES stimulation and the specific immune response is the failure of the RES to mount enhanced responses to multiple sporozoite injections (Table 3 and Fig. 2). In fact, the phagocytic index values obtained in response to the initial sporozoite inoculation was, in each instance, greater than the response to any of the booster immunizations (Fig. 2). This is probably due to the fact that our initial inoculum consisted of a considerably larger dose of antigenic material. In all instances, the RES response to initial immunizing doses, as well as boosters, was equally prompt, and returned to normal values after one week. This is not the case in specific sporozoite-induced immunity where a progressive resistance is afforded by an increasing number of boosters (Nussenzweig *et al.*, 1969).

It should also be stressed that multiple intravenous injections of uninfected salivary glands cause significantly increased clearance rates (Table 4 and Fig. 2). However, the use of this material as antigen has earlier been

shown to produce no protective immunity (Nussenzweig *et al.*, 1969, and Spitalny and Nussenzweig, 1972).

In trying to relate spleen and liver weight increases to RES activity, we found that the liver, although increased, remains so in all experimental groups showing no correlation with changes in carbon clearance rates. On the other hand, the spleen showed the same pattern of behavior as clearance rates in the different groups of animals (Fig. 3, Tables 4 and 6). Spleen weights gradually increase to a maximum 2 days after injection of the sporozoite material, with a decline to normal values by the ninth day (Table 2). In splenectomized mice, clearance rates were found to be increased, but their phagocytic index values never attained those of non-splenectomized immunized animals under the same experimental conditions. The last seems to indicate that the spleen is, to a considerable degree, responsible for increased clearance rates. This differs strikingly from the fact that the spleen does not have an essential role in the development of the protective immune mechanism. In fact, a considerable percent of mice splenectomized prior to their immunization became totally protected against challenge (Rivera-Ortiz and Nussenzweig, 1972).

Studies correlating parasitemia and phagocytic activity were reported by Cox *et al.* (1963) in chicks infected with *P. gallinaceum* and by Biozzi *et al.* (1970) in rats infected with *P. berghei*. These authors reported an enhancement of phagocytic activity which precedes the onset of parasitemia. Biozzi *et al.* (1970) also reported increased phagocytic activity in rats which received irradiated *P. berghei* infected erythrocytes. These authors found gradual increases in clearance rates up to the 20th day following vaccination. They stated that there is a chronological correlation between the protection conferred by their vaccination and RES stimulation and that the duration of this stimulation is transitory falling rapidly to normal values. These authors conclude that the protection against the malarial infection obtained by this vaccination can then be explained by two mechanisms of defense, a specific immunological response and a non-specific stimulation of phagocytic activity.

It is difficult to attempt to compare these results, obtained from vaccination using blood stage antigens with our results on sporozoite-induced immunity, not only because different rodents are used in these two sets of experiments, but mainly because protective immunity in these two systems seems to be based upon quite different mechanisms.

In the case of sporozoite-induced immunity, it is still quite possible that non-specific stimulation of the RES might be important predominantly during the early stages of the immune response. Under these conditions, serum mediated immune response cannot be demonstrated and it has been shown that animals can be protected prior to the appearance of ant sporozoite antibody (Spitalny and Nussenzweig, 1972).

In hyperimmune animals, Nussenzweig *et al.* (1972) have shown that infective sporozoites of *P. berghei* remain detectable for a much shorter period of time in the peripheral blood of immunized animals when compared to normal controls. This accelerated clearance seems to be largely due to the presence of specific ant sporozoite antibodies, since the passive transfer of serum also induces an accelerated sporozoite clearance rate. But, if the injection of immunizing antigen, besides inducing a specific immune response, also activates phagocytic cells, then this non-specific effect will collaborate with the specific one, thus amplifying the immune response.

Further clarification of the role of RES stimulation within the context of protection against sporozoite-induced malaria could possibly be obtained through the use of sporozoite preparations containing a minimum of contaminating material. This could be approached either through immunization produced by mosquito bite (Vanderberg *et al.*, 1970) or through the use of purified sporozoite preparations. This would permit us to verify if an accelerated clearance also occurs under these circumstances and also if an additional administration of uninfected salivary gland exercises an adjuvant effect.

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Effect of Various Routes of Immunization and Methods of Parasite Attenuation on the Development of Protection Against Sporozoite-Induced Rodent Malaria¹

G. L. SPITALNY* AND R. S. NUSSENZWEIG, M.D.

Department of Preventive Medicine, New York University Medical Center,
550 First Avenue, New York, New York 10016

ABSTRACT: The development of protective immunity and CSP antibody were studied in A/J mice immunized by different routes of inoculation (intravenous, intramuscular, intraperitoneal, intracutaneous, and per os) with X-irradiated sporozoites of *Plasmodium berghei*. Various methods of parasite attenuation: X-irradiation, heat inactivation, freeze-thawing, sonication, and homogenization were also tested to determine if the altered sporozoites retained their immunogenicity i.e. if the mice injected with the different preparations could develop CSP antibody as well as an effective protective immune response.

Data obtained from experiments using different routes of immunization showed that i.v. inoculation results in consistently high protection and CSP titers. Immunization of mice by the other routes produced much lower levels of protection. CSP antibody was present in these groups, but in most cases at a lower titer.

Animals immunized i.v. with X-irradiated or heat inactivated sporozoites were completely protected whereas mice immunized with sonicated or homogenized sporozoites showed little if any protection. Animals injected with freeze-thawed parasites developed a level of protection intermediate to the above preparations.

Our results indicate that the development of protection may depend upon the injection of intact attenuated sporozoites. It was further observed that no direct correlation was evident between levels of CSP antibody and degree of protection.

Previous work has shown that mice immunized i.v. with X-irradiated sporozoites of *P. berghei* become completely protected against an otherwise lethal sporozoite challenge (Nussenzweig et al., 1969). Our knowledge of the mechanism of this protective immunity is still very fragmentary. We have found that serum mediates part, but not all of this immune response, and that the passive transfer

of immune serum increases the rate of sporozoite removal or inactivation (Nussenzweig et al., 1972). It has also been shown that immune serum produces a microscopically detectable reaction when incubated with sporozoites (CSP reaction) (Vanderberg, Nussenzweig and Most, 1969) and that a considerable decrease of sporozoite infectivity results from such in vitro incubation (Nussenzweig et al., 1969).

Knowledge of the antigen(s) involved in the immune response is extremely scant. However it has been found that sporozoites need

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to undergo a certain degree of maturation, possibly connected to their migration to the salivary glands, in order to induce protective immunity (Vanderberg, Nussenzweig and Most, 1972).

In the present experiments, we aimed to obtain information on a) the type of antigen preparation which is able to induce protection and or CSP antibodies, b) whether disrupted parasites and parasite fractions would produce an immune response and c) the effectiveness of various routes of immunization.

Such knowledge should improve the efficacy of the methods of immunization and should also shed some light on the nature of the antigens involved in inducing protection. It hopefully would also indicate the way in which the sporozoite antigen is handled when introduced into the host.

Materials and Methods

Animals—All experiments were done with A/J, female mice (Jackson Memorial Laboratories). The animals were at least 8-weeks old at the start of each experiment.

Maintenance and Dissection of Mosquitoes for Sporozoites—In all experiments sporozoites of the NK 65 strain of *Plasmodium berghei* were used. This strain has been kept by weekly blood passages in hamsters, alternating with frequent cyclical transfers through *Anopheles stephensi*. The techniques relating to maintenance, infection of mosquitoes and harvesting of sporozoites from the infected mosquito salivary gland have been previously described (Vanderberg, Nussenzweig and Most, 1968).

After dissection of the infected salivary glands, they were maintained in cold tissue culture medium 199 (M 199), without the addition of plasma. After trituration of the infected salivary glands, the released sporozoites were counted in a hemocytometer.

Antigen Preparation and Immunization—The sporozoites were diluted with M 199 to 1.0×10^6 or 7.5×10^5 /ml. At these concentrations, the parasites were subjected to X-irradiation, heat inactivation, or freeze-thawing. The details for these procedures are outlined below.

A. X-irradiation: In all these experiments 10 Krads were used to attenuate the parasite. The details concerning the procedures used

for X-irradiation have been previously published (Vanderberg et al., 1968).

B. Heat inactivation: In a preliminary experiment, we determined that sporozoites incubated in a water bath at 40–42°C for 45 minutes were unable to produce a patent infection. This procedure was then followed for the purpose of immunization.

C. Freeze-thawing: The parasites, placed in small glass vials, were lowered into a liquid nitrogen tank, removed after 15 minutes and allowed to thaw at room temperature. This procedure was repeated three times after which none of the animals injected intravenously (i.v.) with this preparation ever showed any parasitemia. This method was then used for the purpose of immunization.

Following attenuation by any of the above methods, sporozoites were diluted to the desired concentration so that the number of parasites to be injected would be delivered in 0.2 ml to each animal. Unless otherwise stated, the first injection consisted of 7.5×10^4 sporozoites and each booster of 2.0×10^4 parasites.

In one of the experiments, groups of mice were injected with uninfected salivary glands. We injected these mice with a number of salivary glands equivalent to that injected into animals receiving sporozoites infected glands.

These uninfected salivary glands were processed using the same methods as outlined above and then injected into groups of mice at the same dilution as the infected glands.

D. Sonication: Sonic disruption was carried out in a MSE ultrasonicator. The energy output was raised to 1.75 amperes and maintained at this level for the full period of disruption.

The sporozoite suspension was placed in a heavy walled, glass vial. During sonication the vial was immersed in an ice bath to diminish any denaturation caused by the heat of disruption.

The shortest sonication time for inactivation was found to be four minutes. Under these conditions, none of the animals injected with these sporozoites ever developed a patent infection. Frequently, however, animals inoculated with sporozoites sonicated for a shorter time interval (two or three minutes) could be found to develop parasitemia.

The sonication period was carried out in two, 2-minute periods with a five minute interval between each sonication. After disruption, a sample was checked under phase contrast microscopy to assure that no intact sporozoites remained.

The resulting suspension was diluted to 7.5×10^4 sonicated sporozoites/0.2 ml. Groups of mice were injected with the following fractions:

Group 1: Both soluble and insoluble sporozoite fractions.

Group 2: The pellet obtained by centrifugation at $700 \times g$ for 10 minutes was resuspended to the same volume as the supernatant and injected into this group.

Group 3: The animals were injected with the final supernatant.

Boosters were administered every two weeks and the mice challenged 10–14 days after the final booster.

E. Homogenization: For homogenization 1.0×10^6 sporozoites were suspended in 5 ml of isotonic (0.25 M) sucrose. They were placed in a tight-fitting teflon tissue homogenizer (Potter-Elvehjem) with a clearance between pestle and tube of 0.004"–0.006" (Kontes Glass Co.). The apparatus was surrounded by an ice bath.

We found the sporozoites quite resistant to our attempts to homogenize them. However, with a variable speed drill at 3,200 rpm we were able to disrupt the parasites after 25 to 30 minutes. Homogenization was carried out in five minute periods with a short break in-between. The resulting suspension was checked under phase contrast microscopy. If any intact sporozoites were detected, the homogenization was continued for one minute periods until no intact parasites could be found.

Differential centrifugation was done to separate various components. Groups of mice were injected with the following fraction:

Group A: Centrifugation at $700 \times g$ for 10 minutes, mice injected with pellet #1.

Group B: Centrifugation at $3,000 \times g$ for 30 minutes, mice injected with pellet #2.

Group C: Centrifugation at $100,000 \times g$ for 60 minutes, mice injected with pellet #3.

Group D: Injected with the resulting supernatant.

Each mouse in groups, A, B and C received

the equivalent of 5.0×10^5 sporozoites. Group D received the equivalent of 2.0×10^5 sporozoites. Boosters were given at approximately two-week intervals. The animals were challenged 14 days after the last booster.

CSP Assay—Vanderberg et al. (1969) described the circumsporozoite precipitate reaction (CSP). The same procedures were followed in order to detect this circulating antibody shown to be specific for the sporozoites. For the purposes of the present paper only strongly reacting sporozoites were considered to be positive.

Criterion for Protection—Unless otherwise stated, mice were challenged i.v. with 5.0×10^3 viable sporozoites. This inoculum of viable parasites usually produces close to 100% infection in unimmunized controls.

Daily smears were taken of the challenged animals starting on the 3rd day and continued until the 11th day, post challenge. Animals were never observed to develop parasitemia later than 9 days after the challenge. Mice which have acquired a partial immunity manifest a delayed pre-patent period when compared to non-immunized controls. However, as soon as parasites are detected in the blood of the partially protected animals, the course of the infection is not different from the controls.

Animals which never develop a detectable parasitemia are considered to be completely protected from sporozoite challenge. Nussenzweig et al. (1972) have shown that the size of challenging inoculum can be increased to 3.0×10^5 without the animals developing a patent infection. Nussenzweig et al. (1969) have also shown that subinoculation of their blood does not produce any detectable parasites in the recipient animals. Even if the immune animals are splenectomized, after challenge, they remain protected.

Results

Comparison of different routes of sporozoite inoculation on protective immunity

Previous experiments using X-irradiated sporozoites given i.v. have consistently resulted in a high degree of protection. The best results were obtained when an initial large immunizing dose was given (7.5×10^4

Table 1. Protection produced in animals repeatedly immunized with X-irradiated sporozoites introduced by various routes of immunization.

Exp. #	Route of sporozoite immunization†											
	Intravenous		Intramuscular		Intraperitoneal		Intracutaneous		Per os*		Controls	
	Ani- mals in- fected	Mean day of patency	Ani- mals in- fected	Mean day of patency	Ani- mals in- fected	Mean day of patency	Ani- mals in- fected	Mean day of patency	Ani- mals in- fected	Mean day of patency	Ani- mals in- fected	Mean day of patency
I	1/6	6.0	N.D.		1/6	5.0	4/7	5.6	N.D.		5/5	5.2
II	0/3	—	4/6	5.6	6/6	5.5	3/4	5.7	N.D.		6/6	3.5
III	0/7	—	2/7	7.0	7/7	5.4	6/6	6.0	6/8	5.0	5/5	4.6
IV	0/5	—	9/9	5.4	N.D.		N.D.		10/10	3.5	5/5	3.2
Total No. of animals	1/21	6.0	15/22	5.6	14/19	5.4	13/17	5.0	16/18	4.1	21/21	4.0
Overall % protected	95.2%		31.8%		26.3%		23.6%		11.2%		0.0%	

* Animals receiving inocula per os were administered five doses of 2.0×10^5 X-irradiated sporozoites.
† All animals challenged i.v. with 5.0×10^3 sporozoites 10–14 days after the last booster.
N.D. = Not Done.

sporozoites) followed by 4 boosters with a relatively small concentration of antigen (2.0×10^4 sporozoites), (Nussenzweig, Vanderberg and Most, 1969). In the present series of experiments we continued to follow this scheme of antigen dosage in order to determine the efficacy of i.v. injections when compared to three other parenteral (intraperitoneal i.p., intramuscular i.m., and intracutaneous i.c.) and one non-parenteral (per os) route of immunization. The results of these experiments (Table 1) show clearly that animals inoculated i.v. are able to mount an effective immune response and develop a high degree of protection. Protection acquired by i.v. immunization was consistently produced not only in the present series of experiments, but has also been obtained in very many previous experiments.

These findings contrast quite sharply with

those obtained when antigen is administered by any other route. Experiments in which mice were immunized with sporozoites, either i.m. or i.p., had a considerable variation in the number of totally protected animals. It ranged from 83.4% to 0.0% in i.p. and 70.4% to 0.0% in i.m. immunized mice. Minimal protection was obtained when the sporozoites were administered i.c. Animals administered sporozoites per os showed practically no protection at all, even though they received in the five injections, nearly seven times as many sporozoites (1.0×10^6) as any other group of animals (1.5×10^5).

The delay in the pre-patent period of the partially protected animals also reflects the efficacy of each route of inoculation. The overall results show that as the percentage of animals protected increases, the onset of patency also increases.

Table 2. Antisporozoite antibody production (CSP) in groups of animals repeatedly immunized with X-irradiated sporozoites introduced by various routes of immunization. Titer expressed as the highest serum dilution giving a positive CSP reaction.*

Exp. #	Route of sporozoite immunization				
	Intravenous	Intramuscular	Intraperitoneal	Intracutaneous	Per os
I	1:20	N.D.	1:10	Undiluted	N.D.
II	1:20	1:20	1:10	Undiluted	N.D.
III	1:10	1:10	Undiluted	1:5	Undiluted
IV	1:20	Undiluted	N.D.	N.D.	Negative

* Animals were bled one week after the last booster and the sera from each group was pooled.
N.D. = Not Done.

Table 3. Protective immunity produced by sporozoites subjected to various methods of attenuation.

Antigen	Route of* immunization	X-irradiation	Heat inactivation	Freeze-thawing
		Animals infected	Animals infected	Animals infected
Sporozoite infected salivary glands	I.V.	0/5	8/13†	3/5
	I.P.	4/5	4/5	8/8**
Non-infected salivary glands	I.V.	5/5	5/5	5/5
	I.P.	5/5	4/4	10/10***
None (Controls)	—	7/8	7/8	7/8

* All animals challenged i.v. with 5.0×10^3 sporozoites.
** A subgroup was immunized five times with 4.5×10^5 sporozoites.
*** A subgroup was immunized five times with non-infected salivary glands in number equivalent to those containing 4.5×10^6 sporozoites.
† In two other experiments only one of 17 animals given five injections of heat inactivated sporozoites was protected.

Effects of different routes of sporozoite injection on the production of CSP antibody

Mice injected i.v. not only develop a high degree of protection but also produce CSP antibody in response to the antigen (Vanderberg, Nussenzweig and Most, 1969). Table 2 shows the antibody response of animals injected with sporozoites using various routes of inoculation. Animals inoculated i.v. consistently produced as high or higher antibody titers as mice injected by another route, although the results show variations in the antibody titers from experiment to experiment. Generally, groups inoculated i.c. or per os, which gave the poorest protection, also showed the lowest antibody titer. However, a direct correlation between antibody titers and protective immunity, could not be established.

Effect of various methods of sporozoite attenuation on the development of protection and the production of CSP antibodies

The only method previously used in our laboratory to attenuate sporozoites had been X-irradiation. In the present series of experiments we wanted to determine if some other procedure such as heat inactivation or freeze thawing could also render the sporozoites non infective while still maintaining their immunogenicity. These methods had previously been used by Alger et al. (1972) in rodent malaria, immunizing and challenging the animals i.p. and using not only infected but also uninfected salivary glands. In order to determine how these results could be affected

by different routes of immunization and challenge, infected and uninfected salivary glands attenuated by three methods (X-irradiation, heat inactivation and freeze thawing) were injected either i.v. or intraperitoneally.

Unless otherwise stated these mice received a single injection of 7.5×10^4 sporozoites or a dose containing a similar quantity of uninfected salivary glands and six boosters (2.0×10^4 sporozoites or the corresponding number of uninfected glands). All groups were challenged intravenously.

Animals immunized i.v. with X-irradiated sporozoites were able to completely neutralize the challenging inoculum, failing to present a patent infection. Mice immunized i.v. with heat inactivated or freeze-thawed sporozoites exhibited a lower degree of protection than the previous group (Table 3). Intraperitoneal immunization with any of the antigen preparations resulted in a minimal, if any, degree of protection. One sub-group which was given a ten times larger dose of freeze-thawed sporozoites i.p. was still unprotected against the i.v. challenge.

When uninfected salivary glands were subjected to the identical methods of attenuation and the mice immunized by the same routes, the results were even more striking, since none of these animals were protected from a sporozoite challenge given i.v. The pre-patent period of most of these animals showed little if any, delay when compared to that of the unimmunized controls.

In order to determine the effect of these various antigen preparations and routes of immunization on the animals ability to produce CSP antibodies mice of each group were bled repeatedly to obtain individual serum

Table 4. Course of antisporozyte antibody formation (CSP) in animals repeatedly immunized with parasites. Attenuated by various methods.

Route of antigen administration	Sporozoite preparation					
	X-irradiation		Heat-inactivated		Freeze-thawed	
	Positive CSP reactions		Positive CSP reactions		Positive CSP reactions	
	After booster No.	Proportion of animals	After booster No.	Proportion of animals	After booster No.	Proportion of animals
I.V.	2	5/5	2	5/5	4	5/5
I.P.	6	1/5	4	1/5	6	5/8*

* One subgroup was given 5 injections of 4.5×10^5 freeze-thawed sporozoites.

samples after the 2nd, 4th and 6th booster. Reactions were done on undiluted serum samples, the results being shown in Table 4.

The two antigen preparations (X-irradiated and heat inactivated) and the route of immunization (i.v.) which had given the best results for protection (Table 3) also showed the best results for CSP antibody production. Thus all of the animals injected i.v. with these two preparations had circulating antibody after the second booster. All of the animals injected i.v. with freeze-thawed sporozoites also showed CSP reactivity, but this was not evident until after the fourth booster.

Interestingly, almost half of the total number of mice injected i.p. with the different antigen preparations elicited CSP antibody formation (7 out of 18 mice (Table 4)). However, none of these animals were protected from sporozoite challenge. In the two mice which did not become patent in these groups (Table 3) CSP antibodies were consistently undetectable.

None of the animals injected with any of the uninfected salivary gland preparations ever gave the slightest indication of producing CSP antibodies.

In another series of experiments we attempted to determine the immunogenicity of sporozoite preparations in which the parasites had been disrupted. Parasites were subjected to sonication or homogenization and the different components were injected i.v. Pooled serum samples were collected from each group one week after each booster and the animals were challenged two weeks after the last booster. The results of protection and CSP antibody determinations are shown in Table 5.

All the groups of mice immunized with any of the fractions obtained after sonication de-

veloped CSP antibody. The antibody was detectable only after several boosters and even then the titers were low. However, even though these animals responded to the antigen by producing antibodies they were unable to neutralize the sporozoite challenge and all mice developed a patent infection.

Mice injected with homogenized sporozoites gave similar results. Group A injected with material from the first sediment (nuclear fraction, parasite fragments, etc.) was able to produce CSP antibodies. The antibody was not detectable till after several boosters and the titer was also low. None of the animals from this group were protected from challenge.

Group B injected with material from the second sediment (mitochondrial fraction) did not produce any antibody but one animal did not become patent upon challenge. No protection or CSP antibody was detected in any of the remaining groups.

Discussion

The only criterion available to determine protection in these and other experiments on sporozoite-induced immunity has been the capacity of immunized animals to neutralize, *in vivo*, a challenging inoculum of viable sporo-

Table 5. Protective immunity and antisporozyte antibodies (CSP) in mice immunized with disrupted sporozoites.

	Sporozoite preparation			
	Sonicated		Homogenized	
	Animals infected	CSP reaction	Animals infected	CSP reaction
Immunized*	48/48	Positive	8/9	Positive
Controls	17/17	Negative	5/5	Negative

* All animals challenged with 5.0×10^5 sporozoites.

zoites. Results of the experiments presented in this paper clearly demonstrate that animals injected i.v. with X-irradiated sporozoites of *P. berghei* consistently produce a high degree of protection. This observation corroborates previous reports (Nussenzweig, Vanderberg, and Most, 1967 and 1969) and extends their findings. Groups of mice immunized by other parenteral routes (i.m., i.p., and i.c.) exhibited an overall level of protection much lower than the i.v. immunized mice. These groups also had lower CSP titers especially those inoculated intracutaneously.

The elaboration of a protective immune response is contingent upon the hosts ability to establish systemic immunity. It is plausible that animals immunized by any route other than i.v. respond to the sporozoites and may process the antigen to produce a localized immune response which is unable to arrest an i.v. challenge. This hypothesis might clarify the protection which Alger et al. (1972) reported when mice were immunized i.p. and challenged intraperitoneally. It would also explain the lack of protection when animals were immunized i.p. and challenged intravenously.

The fact that in a few experiments i.p. or i.m. immunization resulted in high levels of protection, may be related to the sporozoites capacity to enter the circulation by avoiding the local response. That this depends on a chance occurrence is reflected by the wide variation of protection which occurred from experiment to experiment.

Yoeli and Most (1971) recently reported successful sporozoite-induced infection by intubating animals per os. We expanded this observation by administering X-irradiated sporozoites per os in order to determine the effectiveness of this route of immunization. These animals were given nearly seven times as many sporozoites as any other group over the immunization period.

It is evident from the experiments done that protective immunity is minimal in these animals. The mostly negative CSP determination may indicate the inability of many of the X-irradiated parasites to reach the circulation when introduced per os.

Methods for parasite attenuation are quite numerous but have only, in relatively few instances, been applied to malaria research.

Recently, Richards (1966) subjected sporozoites of *P. gallinaceum* to a variety of conditions such as drying, formalin fixation and U.V. irradiation. Under these circumstances, he showed that the parasites retained their immunogenic properties and that birds immunized with these preparations recovered from an otherwise lethal sporozoite challenge. D'Antonio et al. (1970 and 1972) used heat inactivated and French Pressure cell treated erythrocytic parasites to immunize animals. He found these immune animals able to control the infection resulting from the challenge with blood stages. The immunizing capability of X-irradiated erythrocytic stages has been shown with *P. gallinaceum* (Ceithmal and Evans, 1946), and with *P. berghei* (Corradetti, Verolini and Bucci, 1966; and Welde and Sadun, 1967). In our previous experiments, X-irradiation of sporozoites has proven to be an effective means of antigen attenuation. The sporozoites subjected to 10 Krads of irradiation completely lose their ability to produce patency, but still retain the properties which stimulate an immune response. We have also found that gamma irradiation from a Cs¹³⁷ source (12 Krads) is just as effective as X-irradiation.

The present results show that heat inactivation like X-irradiation does not alter extensively the parasite, and possibly for this reason mice injected with this preparation were protected. Freeze-thawed and heat inactivated sporozoites are less immunogenic, since the mice inoculated with this preparation were unable to completely arrest sporozoite challenge. Microscopic examination of the three sporozoite preparations revealed that X-irradiated and heat inactivated parasites appeared quite normal, whereas in the freeze-thawed suspension we found many broken, obviously altered sporozoites. Intraperitoneal immunization with any of these preparations resulted, as in earlier experiments, in considerably fewer protected animals.

Fractionation and purification of the sporozoite antigen if feasible would greatly advance malaria vaccination. Sporozoites were therefore subjected to sonication and homogenization. However, even though sonicated sporozoite fractions were able to induce CSP antibody formation, they did not stimulate the animals to elaborate a protective immune

response. Only one group immunized with homogenized fractions produced CSP antibody and a single animal was protected.

Considering the various methods of attenuation used in these experiments, and comparing the effects that X-irradiation, heat inactivation, freeze-thawing, sonication and homogenization had on the integrity of the sporozoites, it seems plausible to conclude, that under our conditions of immunization, sporozoites must be intact in order to stimulate protection. One possible explanation for this might be that intact attenuated sporozoites may retain the capacity to undergo some degree of development in the liver or elsewhere, development which might be essential for the induction of an immune response. This hypothesis is supported by data obtained on CSP antibody production in adult rats, inoculated with either irradiated or non-irradiated viable sporozoites. Rats injected with irradiated parasites produce antibody detectable after 7 days and persisting for greater than 4 months after which the titers decrease slightly. Rats injected with the viable sporozoites also have detectable antibody after 7 days but by the 5th week antibody is undetectable in any of the animals (Spitalny and Nussenzweig, 1972).

Prior to these observations, CSP reactivity appeared to be closely related to protective immunity. Furthermore, Mulligan, Russell and Mohan (1947) had shown good correlation between sporozoite agglutination titers and protection in birds immunized with *P. gallinaceum*. From the present experiments, it is quite evident that CSP reactivity is not necessarily related to protection under certain circumstances. Furthermore, in another series of experiments, we found that during the early sages of immunization, protection can occur without the presence of CSP antibody (Spitalny and Nussenzweig, 1972). The presence of other serum mediated mechanism(s) of protection during the stage of the immune response are presently being investigated.

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Stage Specificity of Anti-Sporozoite Antibodies in Rodent Malaria and its Relationship to Protective Immunity

J. P. VANDERBERG, R. S. NUSSENZWEIG, Y. SANABRIA, R. NAWROT, AND H. MOST

Department of Preventive Medicine, New York University Medical Center,
550 First Avenue, New York, New York 10016

ABSTRACT: The stage specificity of ant sporozoite antibodies in *Plasmodium berghei* malaria was studied. Both protective immunity, and antibodies inducing alterations in parasite morphology (CSP) were investigated. Attempts were made to determine where in development the antigens responsible for these antibodies first arise, and what cross reactivity exists between the various stages.

The results indicated that:

1. Two distinct CSP antigens can be demonstrated in sporozoites: an "immature" sporozoite antigen primarily associated with oocyst sporozoites, and a mature sporozoite antigen first demonstrable in oocyst sporozoites, but persisting and increasing in salivary gland sporozoites. These antigens may possibly be involved in sporozoite penetration mechanisms.

2. The capacity to induce protective immunity is demonstrable primarily after the sporozoites have moved from the oocyst to the salivary gland.

3. Antibodies formed after immunization of mice with mature salivary gland sporozoites have no apparent effect on the morphology or development of erythrocytic parasites, gametocytes, or ookinetes.

In previous studies, we have shown that mice can be immunized against rodent malaria by repeated intravenous injections of X-irradiated sporozoites (Nussenzweig, Vanderberg, and Most, 1969). Such immunized mice do not develop parasitemia after a challenge inoculum of sporozoites that produces parasitemia and death in close to 100% of non-protected controls. This protection is specifically restricted to sporozoite challenges. The immunized animals are completely susceptible to challenge with blood parasites (Nussenzweig et al., 1969).

We have demonstrated the presence of anti-sporozoite antibodies in blood serum from immunized animals in 2 ways: first, by the

effects of immune serum on sporozoite infectivity; and secondly, by a morphological alteration of sporozoites incubated in immune serum in vitro:

1. A partial loss of sporozoite infectivity occurs after in vitro incubation in immune serum (Nussenzweig, Vanderberg, and Most, 1969). In addition, infective sporozoites are more rapidly cleared from the blood of animals which have passively received immune serum (Nussenzweig et al., 1972) than from animals which have received normal serum.

2. Sporozoites incubated in vitro in immune serum develop a long thread-like precipitation at one end of the parasite (Vanderberg, Nussenzweig, and Most, 1969). We have termed this morphological alteration, the circumsporozoite precipitation reaction (CSP).

We decided to investigate the stage spec-

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ificity of these anti-sporozoite antibodies by testing them against other life cycle stages of the malaria parasite, and by attempting to determine at what point in development the antigens responsible for these antibodies first arise. Such information might 1) give us a better understanding of the more fundamental aspects of immunity to sporozoites, 2) from the practical standpoint, would better enable us to select the most immunogenic stage for protective immunization, 3) and finally, a knowledge of when these antigens are first seen during the course of development might give us a clue as to their functional significance in the physiology of the parasite.

The study was carried out by immunizing mice with a series of different stages of sporogonic development of the rodent malarial parasite, *Plasmodium berghei*. The immune status of these mice was then tested by challenge with mature viable sporozoites, thereby determining the capacity of the different developmental stages to induce protective immunity against sporozoites. Sera from these immunized mice were tested for their ability to reduce the infectivity and developmental capacity of various stages of the parasite, and for their ability to induce morphological alterations of the various parasite stages.

Materials and Methods

The techniques used by us to maintain the *Plasmodium berghei*-*Anopheles stephensi*-rodent system of mammalian malaria have previously been described (Vanderberg, Nussenzweig and Most, 1968, and Vanderberg and Nawrot, 1968) with the addition that we now use Tissue Culture Medium 199 as the dissecting and diluting fluid for sporozoites. In view of the striking effect that temperature has on the rate of sporogonic development (Vanderberg and Yoeli, 1966), infected mosquitoes were maintained in a Precision Scientific Incubator (Model 805) at $21 \pm 1^\circ\text{C}$ to more precisely standardize the development time of sporozoites.

Sporogonic stages used for immunization

A/J mice were immunized with five intravenous injections of the appropriate stage of sporogonic development irradiated at 10,000

rads under conditions previously described (Vanderberg et al., 1968). For each set of initial or booster immunizations, a single batch of infected mosquitoes was used, with mosquitoes being removed and dissected at various stages during the course of development. Thus, within each experiment the source of infected mosquitoes remained constant, and the only variable was the age of the oocysts or sporozoites utilized. Immunizations were generally at intervals of 10–14 days. Sporozoite doses chosen for immunization were several times larger than in earlier experiments (Nussenzweig, Vanderberg, and Most, 1969), since it was assumed that antigen found in the early stages of development might be present in relatively small quantities.

The following stages of development, representing arbitrarily defined landmarks in sporogonic development, were used. The days cited indicate the number of days after the mosquitoes' infective blood meals.

(a). *7-day oocysts*. Differentiated sporozoites are almost never found at this stage, though most of the cytoplasmic components which will eventually go into the sporozoites are already present within the sporoblastoid body, which is well differentiated (Vanderberg, Rhodin and Yoeli, 1967, Vanderberg and Rhodin, 1967). The initial immunization dose was 15 infected midguts per mouse, triturated and then irradiated, followed by 4 similar booster immunizations.

(b). *10-day sporozoites*. Oocyst sporozoites are well differentiated at this stage, and sporozoites are almost never seen in salivary glands. The initial immunization per mouse was 100,000 sporozoites from triturated infected midguts, followed by 4 boosters of 25,000 sporozoites each.

(c & d). *14-day sporozoites from oocysts or salivary glands*. At this time in development, heavy infections of sporozoites can be found both in oocysts and in salivary glands. Infected mosquitoes were cut in half at the junction of the thorax and abdomen, and then separated into different batches to avoid cross contamination of sporozoites from the two halves. Midguts were dissected out of the abdominal half, and triturated for recovery of oocyst sporozoites, while salivary glands were dissected out of the anterior half of the mosquito, and triturated for recovery of sali-

vary gland sporozoites. Thus, the very same infected mosquitoes were used for each immunization comparing 14-day oocyst sporozoites with 14-day salivary gland sporozoites. The initial immunization per mouse in each case was 100,000 sporozoites, followed by 4 boosters of 25,000 sporozoites each.

(e). *18-day sporozoites*. By this stage of development, most of the oocysts have matured and ruptured, and salivary gland infections are at their peak. The initial immunization per mouse was 100,000 sporozoites, followed by 4 boosters of 25,000 sporozoites each.

(f). *18-day sporozoites injected by X-irradiated mosquitoes*. Only sporozoites from the lumen of the salivary gland are injected. These represent a stage of development presumably beyond that of the sporozoites found within the secretory cells of the gland. Initial immunization was approximately 30 infected irradiated mosquitoes feeding on each mouse, followed by 4 booster immunizations with about the same number of infected irradiated mosquitoes allowed to feed. The technique was as described by Vanderberg, Nussenzweig and Most (1970).

Effects of immunization with various stages of sporogonic development

(a). *Morphological Alteration of Sporozoites by Immune Serum*

Blood was collected from the retro-orbital plexus of immunized mice 7 days after the 4th booster immunization in each experiment. Sera from each experimental group were pooled and stored at -12°C . Determinations of anti-sporozoite antibodies were made by the circumsporozoite precipitation (CSP) reaction as previously described (Vanderberg, Nussenzweig and Most, 1969). All groups of sera from a given experiment were tested at the same time against suspensions of both 10-day sporozoites from oocysts and 18-day sporozoites from salivary glands. All series of reactions included known positive and negative antisera controls.

Observations for data collection were made with the phase contrast microscope. To study the reaction more carefully histologically, glutaraldehyde fixation was also used. Suspensions of sporozoites were allowed to react

for about 15 minutes with immune serum which had been diluted to avoid artifacts caused by fixed concentrated serum. The reacted sporozoites were then mixed with about 15 volumes of a solution of 1.25% glutaraldehyde, 0.05 M phosphate buffer and 4% sucrose (pH 7.3), and fixed for 1 hour at room temperature. The preparation was then centrifuged at 750g for 20 minutes to sediment the sporozoites. The pellet was resuspended in a small volume of the solution, smeared on slides, and allowed to dry. Staining was done with Giemsa stain, as well as with PAS, Mercury Bromphenol Blue, and Sudan black b (Pearse, 1968).

Inasmuch as 10-day sporozoites are contaminated with mosquito midgut material, and 18-day sporozoites with mosquito salivary gland material, extracts of these mosquito tissues were cross tested to determine whether they might have either a potentiating or inhibitory effect on the CSP reaction. Anti-midgut antiserum and anti-salivary gland antiserum, prepared by repeated immunization of mice with these non-infected mosquito tissues, were also tested as control sera against sporozoites of all ages.

(b). *Protective Immunity*

Immunized mice were challenged intravenously with 1,000 to 10,000 18-day salivary gland sporozoites, approximately 2 weeks after their 4th booster immunization. Because of the great variation in viability between different batches of sporozoites (Vanderberg, Nussenzweig, and Most, 1968), an attempt was made in some experiments to assess the viability of a given batch prior to challenge. A sampling of sporozoites was injected intravenously into young rats, which were killed 42 hours later for a determination of the numbers of exoerythrocytic forms present. Cryostat microtome sections of the livers allowed a rapid determination of approximately sporozoite viability, and the challenge dose from the same source of infected mosquitoes was then given accordingly to the experimental mice.

All mice within a given experiment were challenged at the same time from a single pool of sporozoites. Control mice for each group were from the same original batch as

the experimentals. Controls were generally untreated, but in 2 experiments they were immunized with uninfected mosquito midguts exactly as described above for midguts with 7-day oocyst infections. Daily blood smears were taken of all animals for the first 10 days after challenge to determine the first day of patency in each case.

Effects of sporozoite induced immunity on erythrocytic stages

(a). *Morphological Alteration of Parasites by Immune Serum*

Anti 18-day sporozoite immune serum was incubated at 37°C in vitro with heparinized blood from immunized A/J mice infected with *P. berghei*. In addition, mice hyperimmunized with 18-day sporozoites were challenged with erythrocytic parasites, and blood samples were taken when the parasitemia had gone above 10 percent. In both cases the parasitized erythrocytes were examined under phase contrast microscopy to detect any possible morphological alteration.

In order to test the possible morphological effects of this immune serum on free erythrocytic parasites, the parasites were obtained in 3 different ways. 1). Blood samples were taken from hamsters with high parasitemias. These animals have a relatively large number of extracellular merozoites in their blood. 2). Free parasites were prepared from infected mouse blood utilizing the saponin lysis technique as done by Zuckerman, Spira, and Hamburger (1967). 3). Free parasites were prepared from infected mouse blood utilizing the French Pressure Cell method as done by D'Antonio, Von Doenhoff, and Fife (1966). The free erythrocytic parasites thus obtained were incubated in vitro at 37°C with the anti-sporozoite immune serum, and then observed under phase contrast microscopy. The presence of anti-sporozoite antibodies had been previously demonstrated in all sera used by appropriate testing with viable 18-day sporozoites. All animals from which serum had been obtained had been shown to be completely immune to sporozoite challenge.

(b). *Infectivity of Parasites*

The effects of anti-sporozoite immune serum on the infectivity of erythrocytic parasites

were studied by us by challenging 18-day sporozoite-immunized mice, with parasitized erythrocytes as described in a previous paper (Nuzzenzweig et al., 1969).

Effects of sporozoite induced immunity on sexual stages

(a). *Morphological Alteration of Parasites by Immune Serum*

Anti 18-day sporozoite immune serum was incubated at 37°C with heparinized blood from non-immunized *P. berghei* infected mice having macrogametocytes and microgametocytes in various stages of development. In addition, 4 mice hyperimmunized with 18-day sporozoites were challenged with erythrocytic parasites, and blood samples were taken when the gametocytemia became high. In both cases the gametocytes were studied with the aid of phase contrast microscopy to detect any possible morphological alterations. Thin blood smears, fixed with glutaraldehyde and stained with Giemsa stain were also examined.

To detect the effects of this anti-sporozoite immune serum on ookinetes, wet mount squash preparations of mosquito midguts were made 20 to 24 hours after an infective blood meal, and incubated in vitro at 37°C with immune serum. Preparations were observed unfixed with the phase contrast microscope, as well as after glutaraldehyde fixation and Giemsa staining.

(b). *Development and Infectivity of Parasites*

Mosquitoes were allowed to feed on the mice previously hyperimmunized with 18-day sporozoites, having gametocytemia. Squash preparations of mosquito midguts were taken from 15 to 60 minutes after feeding, then fixed with glutaraldehyde and stained with Giemsa stain, and examined for exflagellation of microgametocytes, while some preparations were examined unfixed under the phase contrast microscope for observation of active exflagellation. Squash preparations taken 20 to 24 hours after feeding were similarly examined for ookinetes. The mosquito midguts were then dissected out at regular intervals up to 18 days after feeding, and examined in an unfixed condition under the phase contrast microscope to follow the development of the

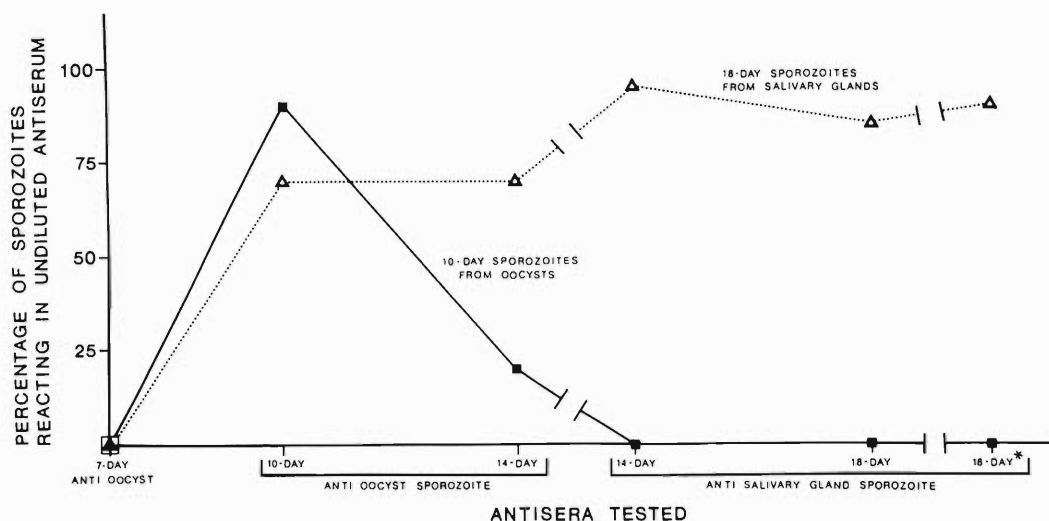


Figure 1. CSP reactions of 10-day and 18-day sporozoites in antisera to various stages of sporogonic development (Exp't 7). (*Injected by X-irradiated mosquitoes.)

oocysts, and subsequent infection of the salivary glands with sporozoites. The viability of these salivary gland sporozoites was tested by intravenous injection into susceptible A/J mice.

Results

Sporogonic stages used for immunization

Even under the well-controlled conditions for infection and maintenance of infected mosquitoes in these experiments, sporogonic development was generally found to proceed asynchronously. Any given mosquito dissected 14 days after an infective blood meal, for instance, could be found to harbor a wide range of developmental stages of the parasite from undifferentiated oocysts in the midgut to infective sporozoites in the lumen of the salivary gland. In addition, development proceeded more rapidly in some mosquitoes in a batch than in others. Finally, the rate of development appeared to vary somewhat among different batches of mosquitoes. For example, sporozoite development was considerably accelerated in mosquitoes used for the initial immunization in one experiment (exp. 8). Differentiation of fully formed sporozoites within oocysts had begun to occur by day 7. Ordinarily, there is little development beyond the solid sporoblastoid stage of the oocyst at this

time. Thus, each immunization in these experiments involved a somewhat heterogeneous population of sporozoites, and should be regarded as representing a statistical average for a given stage of development rather than a clear and specific time point in development.

Effects of immunization with various stages of sporogonic development

(a). Morphological Alteration of Sporozoites by Immune Serum

Sporozoites 18-days of age from salivary glands were found to react strongly with antisera against salivary gland sporozoites of all ages, and somewhat less so against antisera to developed sporozoites from the oocysts. The results are shown graphically for experiments 7 and 8 in Figs. 1 and 2, respectively. The general shape of the plot was roughly similar in all other experiments that were run. In only 1 out of 4 experiments, (exp. 8) did 18-day sporozoites react in anti 7-day antiserum. (It is perhaps significant that this experiment was unusual for the accelerated development of oocyst sporozoites used for the initial immunization as indicated above.) In all other experiments, these sporozoites did not react until they were incubated in antisera to sporo-

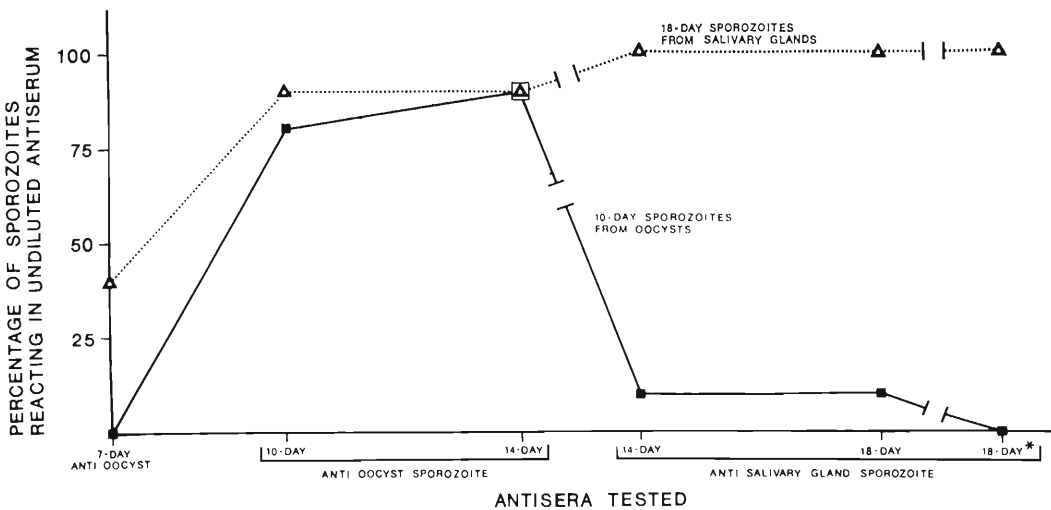


Figure 2. CSP reactions of 10-day and 18-day sporozoites in antisera to various stages of sporogonic development (Exp't 8). (*Injected by X-irradiated mosquitoes.)

gonic stages beyond the 7th day. This precipitation reaction of mature salivary gland sporozoites is the one which we first described in our previous study (Vanderberg, Nussenzweig, and Most, 1969). The antigen responsible can first be recognized somewhere between the 7th and 10th day of oocyst development. This antigen is then responsible for the production of higher titers of antibodies in the sporozoites moving to the salivary gland, and persists thereafter in these mature sporozoites.

Sporozoites 10 days of age from oocysts were found to react most strongly with antisera against oocyst sporozoites (Figs. 1 and 2). Serum titers above 1:5 were rarely observed, in contrast to the much higher titers that we have repeatedly observed for salivary gland sporozoites reacting with their homologous antiserum. These immature sporozoites never reacted with antisera against 7-day oocyst material. In about half of our experiments these sporozoites did not react at all against antisera to salivary gland sporozoites

Table 1. Immunization of mice with irradiated 7-day oocysts: results of challenges with 18-day sporozoites.

Exp't	Group	Sporozoite challenge	Animals infected	Prepatent period in days*		
				Mean	S.D.	p
4	Immunized	1,000	8/8	5.0	0	>.2
	Midgut controls**	1,000	8/8	5.1	0.5	
	Untreated controls	1,000	8/8	5.0	0	
6	Immunized	5,000	10/10	4.2	0.4	<.1
	Midgut controls**	5,000	10/10	3.7	0.5	
	Untreated controls	5,000	10/10	3.8	0.4	
7	Immunized	5,000	5/5	4.6	0.5	>.2
	Untreated controls	5,000	6/6	4.3	0.5	
8	Immunized	10,000	5/5	4.4	0.2	>.2
	Untreated controls	10,000	10/10	4.1	0.3	
Overall	Immunized	—	28/28	4.5	—	—
	Midgut controls**	—	18/18	4.3	—	—
	Untreated controls	—	34/34	4.3	—	—

* S.D. = Standard deviation, "p" as measured by Student's t test for differences between means for immunized and untreated control groups. "p" of <.05 regarded as significant.

** Immunized with uninfected mosquito midguts.

Table 2. Immunization of mice with irradiated 10-day sporozoites from oocysts: results of challenges with 18-day sporozoites.

Exp't	Group	Sporozoite challenge	Animals infected	Prepatent period in days*		
				Mean	S.D.	p
1	Immunized	1,000	5/6	4.4	0.8	<.2
	Controls	1,000	4/4	3.5	0.5	
2	Immunized	1,000	6/8	5.5	0.5	<.01
	Controls	1,000	8/8	4.5	0.5	
3	Immunized	1,000	8/8	5.4	0.5	<.1
	Controls	1,000	8/8	5.0	0.5	
6	Immunized	5,000	8/10	4.5	0.5	<.01
	Controls	5,000	10/10	3.8	0.4	
7	Immunized	5,000	4/4	5.0	0	<.05
	Controls	5,000	6/6	4.3	0.5	
8	Immunized	10,000	5/5	4.8	0.7	<.1
	Controls	10,000	10/10	4.1	0.5	
Overall	Immunized	—	36/41	5.0	—	—
	Controls	—	46/46	4.2	—	—

* S.D. = Standard deviation, "p" as measured by Student's t test for differences between means for immunized and control groups. "p" of <.05 regarded as significant.

(e.g., Fig. 1); in the remainder of our experiments only about 5 to 10 percent of these immature sporozoites reacted with undiluted antisalivary gland sporozoite antiserum (e.g., Fig. 2). These oocyst sporozoites clearly did not lack the basic capacity to react, since almost all of them from the very same pools would react strongly against their homologous antisera. The precipitation reaction involved thus appears to be different from the one previously described. The antigen responsible can first be recognized at about the 10th day of sporogonic development. These oocyst sporozoites have the ability to induce the formation of antisera causing strong CSP reactions with 10-day sporozoites. This ability drops precipitously after the sporozoites move from the oocyst to the salivary glands.

Reactions of oocyst sporozoites with their homologous antisera were found to be difficult

to read because of the intense clumping that occurs during the reaction. This presumably is due to the agglutination of mosquito midgut material by the homologous antiserum.

Control studies with uninfected mosquito midgut and salivary gland material and with antisera to these tissues indicated no inhibitory or potentiating effects. The precipitated CSP sporozoite material, after glutaraldehyde fixation, was found to stain clearly with PAS, Mercury bromphenol blue, and Sudan black b.

(b). *Protective Immunity*

The results of the challenges of immunized mice, with 18-day sporozoites are shown in Tables 1–5, and summarized in Table 6.

Mice immunized with 7-day oocyst material were not protected at all against challenge (Table 1). In each of 4 experiments, 100 per

Table 3. Immunization of mice with irradiated 14-day sporozoites from oocysts: results of challenges with 18-day sporozoites.

Exp't	Group	Sporozoite challenge	Animals infected	Prepatent period in days*		
				Mean	S.D.	p
7	Immunized	5,000	5/5	5.0	0	<.02
	Controls	5,000	6/6	4.3	0.5	
8	Immunized	10,000	9/10	5.0	0.5	<.01
	Controls	10,000	10/10	4.1	0.5	
Overall	Immunized	—	14/15	5.0	—	—
	Controls	—	16/16	4.2	—	—

* S.D. = Standard deviation, "p" as measured by Student's t test for differences between means for immunized and control groups. "p" of <.05 regarded as significant.

Table 4. Immunization of mice with irradiated 14-day sporozoites from salivary glands: results of challenges with 18-day sporozoites.

Exp't	Group	Sporozoite challenge	Animals infected	Mean day of patency
6	Immunized	5,000	1/9	5.0
	Controls	5,000	10/10	3.8
7	Immunized	5,000	2/4	5.0
	Controls	5,000	6/6	4.3
8	Immunized	10,000	0/9	None
	Controls	10,000	10/10	4.1
Overall	Immunized	—	3/22	5.0
	Controls	—	26/26	4.0

cent of immunized as well as control mice developed parasitemia upon challenge. In 3 of the experiments there was a slight delay in patency on the part of the immunized mice. However, these mean delays in patency were not found to be significant when compared with mean days of patency for control mice, as measured by Student's t test for statistical significance.

Mice immunized with 10-day oocyst sporozoites were found to be slightly protected upon sporozoite challenge (Table 2). The overall results of 6 experiments indicated that 88 per cent of the immunized mice became patent, compared to 100 per cent of the non-immunized controls. There was invariably a delay in mean patency of those immunized mice that did develop parasitemia. These delays were found to be statistically significant in half of the experiments ($p < .05$) as determined by the t test.

Mice immunized with 14-day oocyst sporozoites were also found to be slightly protected upon sporozoite challenge (Table 3). Total protection was very slight or absent in that 93 per cent of the challenged mice became patent, compared to 100 per cent of the controls. However, the delayed patency of the immunized mice was found to be highly significant ($p < .02$) as determined by the t test.

Mice immunized with 14-day salivary gland sporozoites were found to be highly immune to sporozoite challenge (Table 4). Overall, only 14 per cent of the immunized mice developed parasitemia, compared with 100 per cent of the controls. The few immunized mice that did develop parasitemia after challenge had delayed patencies.

Table 5. Immunization of mice with irradiated 18-day sporozoites from salivary glands: results of challenges with 18-day sporozoites.

Exp't	Group	Sporozoite challenge	Animals infected	Mean day of patency
7	Immunized	5,000	0/5	None
	Controls	5,000	6/6	4.3
8	Immunized	10,000	0/10	None
	Controls	10,000	10/10	4.1
Overall	Immunized	—	0/15	None
	Controls	—	16/16	4.2

Mice immunized with 18-day sporozoites, either in the usual manner or by mosquito bite, were found to be totally protected upon sporozoite challenge (Tables 5 and 6). None of these mice ever developed a patent infection.

The overall results as shown in Table 6 indicate an increasing level of protective immunity with increasing development of the sporozoites used for immunization. Salivary gland sporozoites were far more immunogenic than oocyst sporozoites from the very same mosquitoes. All immunized mice that developed parasitemia after challenge eventually died after a normal course of infection.

Effects of sporozoite induced immunity on erythrocytic stages

(a). Morphological Alteration of Parasites by Immune Serum

No evidence of morphological alteration of extracellular or intracellular parasites was observed under any of the experimental conditions. All samples of immune sera tested had high titers of CSP antibodies.

Table 6. Immunization of mice with various irradiated stages of sporogonic development: overall results of challenges with 18-day sporozoites.

Group of mice	Animals infected	Mean day of patency
Untreated controls	54/54	4.3
Mosquito midgut controls	18/18	4.3
7-day oocyst immunized	28/28	4.5
10-day oocyst sporozoite immunized	36/41	5.0
14-day oocyst sporozoite immunized	14/15	5.0
14-day salivary sporozoite immunized	3/22	5.0
18-day salivary sporozoite immunized	0/15	None
Mosquito-injected sporozoites	0/10	None

(b). *Infectivity of Parasites*

As was also demonstrated in an earlier paper, A/J mice immune to sporozoites were completely susceptible to challenge with erythrocytic parasites (Nussenzweig et al., 1969). Erythrocytic parasitemia developed normally in these animals, thus confirming the previous study.

Effects of sporozoite induced immunity on sexual stages

(a). *Morphological Alteration of Parasites by Immune Serum*

There was no evidence of morphological alteration of gametocytes either with those from non-immune animals incubated in vitro with serum from animals immunized with 18-day sporozoites or in the case of gametocytes developing in vivo in these hyperimmune animals. In the latter case, the number and morphological appearance of gametocytes developing in the hyperimmune animals were comparable with what occurred in non-immune animals.

Ookinetes incubated in vitro with immune serum showed no evidence of morphological alteration and no loss of the normal mobility which was previously described (Vanderberg, 1965).

(b). *Development and Infectivity of Parasites*

Infectivity and viability of all sexual stages were normal in the case of gametocytes that had developed in hyperimmune mice. Exflagellation occurred in vitro in blood samples, as well as in vivo in midguts of mosquitoes that had fed on these mice. Normal ookinetes were seen at about 24 hours, oocyst development proceeded normally as previously described (Vanderberg, Rhodin and Yoeli, 1967), and the salivary gland sporozoites that ultimately developed were found to be infective to A/J mice.

Discussion

Demonstration of different antigens by the CSP reaction

The simplest interpretation of the CSP reaction data as indicated by Figures 1 and 2

is that two distinct antigens or groups of antigens are involved in these reactions. The first, as indicated by reactions of 10-day oocyst sporozoites, makes its appearance at about the time of differentiation of the oocyst sporozoites. This antigen(s) remains in the oocyst sporozoites, then sharply decreases after maturation of the sporozoites and their movement to the salivary glands. We propose to call this the immature sporozoite antigen(s).

The second antigen(s) is also detectable early in sporogonic development, but persists and increases somewhat in sporozoites that have moved to the salivary glands, as indicated both by higher titers of antibodies in immunized animals, and higher percentages of reacting sporozoites. We propose to call this the mature sporozoite antigen(s).

Both of these antigens appear to be released from one end of the sporozoite into the external medium. It seems reasonable to speculate that the immature CSP antigen may be associated with a mechanism for invasion of the salivary gland by the immature sporozoites that have developed in the oocyst. This could account for the sharp decrease in this antigen detectable after the sporozoites have invaded the salivary gland. The mature CSP antigen could conceivably be associated with a mechanism for invasion of the liver by the mature salivary gland sporozoite. The presence of this "mature" antigen in the oocyst sporozoite could indicate a function for it at this point in development, or could simply be a case of early "packaging" for later functioning. The environment of the mosquito salivary gland, and the environment of the mammalian liver are so totally different that one might expect the sporozoite to arm itself with two different antigens for these two different tasks of invasion.

The mature sporozoite CSP antigen is not found at exactly the same times as the functional antigen responsible for protective immunity. Both are at their maximum level in the mature salivary gland sporozoite. However, the mature sporozoite CSP antigen appears in reasonably high titers early during sporogonic development in the oocyst (Figs. 1 and 2), a time when the protective immunogenicity of the sporozoites is relatively low (Table 6). When we first described the CSP reaction in 1969, we reported that there

appeared to be a correlation between CSP reactivity and protective immunity. We entertained the hope at that time that, "Should further data confirm this relationship between protective immunity and CSP reactivity, then this reaction would provide us with a valuable and convenient tool for assaying the immune status of vaccinated animals." However, the data presented in this paper, as well as other studies (Spitalny and Nussenzweig, 1972a and b, and Rivera-Ortiz, unpublished observations) now indicate that the CSP antigen and the functional sporozoite antigen are not identical.

Erythrocytic parasites, gametocytes, and ookinets failed to become morphologically altered when incubated in immune serum with high titers of CSP antibodies. This indicates that these stages do not possess the responsible antigens in common with the sporozoites, or if present, the antigens are not released as readily from these stages as from the sporozoites. Whether these antigens are present in the exoerythrocytic form has yet to be determined.

These findings are somewhat similar to those of Entner and Gonzalez (1966) and Entner (1968) who found "young" antigens characteristic of "young" trypanosomes, and then later in development "adult" antigens characteristic of "adult" trypanosomes in immunodiffusion precipitin studies of *Trypanosoma lewisi*. This technique, as well as an electrophoretic analysis of the antigen in the different stages of *P. berghei*, might be useful in further elucidating the patterns of antigen formation during the differentiation and maturation of the *P. berghei* sporozoite.

The predictable appearance of new antigens in the sporozoite at specific points in development opens up a series of still other possible future studies. It may, for instance, be possible to demonstrate differences between the oocyst and salivary gland sporozoites and their CSP reactions by means of histochemical procedures. Our initial results suggesting that a lipid may be a component of the CSP reaction is of interest in this regard. Preliminary investigations of these and other problems have already been initiated in our laboratory.

Protective immunity

These studies clearly demonstrate the high degree of stage specificity that exists in sporo-

zoite-induced protective immunity. Total protective immunity is attained only with mature salivary gland sporozoites as the immunogen, though oocyst sporozoites have the capacity to induce a slight degree of protective immunity (Table 6). The dramatic difference in immunogenicity between 14-day oocyst vs. salivary gland sporozoites from the very same mosquitoes is striking.

We have previously shown that sporozoite-immunized mice are totally susceptible to challenge with erythrocytic parasites (Nussenzweig et al., 1969). These observations are now extended by our present findings that gametocytes which develop in such hyperimmune mice are infective to mosquitoes feeding on them, and eventually develop into infective sporozoites.

The functional antigen(s) that induces protective immunity to sporozoites appears not to be present in significant amounts in the undifferentiated 7-day oocyst. This antigen seems to first appear in small functional amounts with the differentiation of sporozoites in the oocyst, and then sharply rises to its peak after the final maturation of the sporozoites and their movement to the salivary glands. Other explanations are that the functional antigen may actually be present from a relatively early stage of sporogonic development onwards, but is either a) more effectively released by the more differentiated salivary gland sporozoite, and/or b) must be associated with the invasiveness of these more mature sporozoites to be carried to the primary site for the induction of protective immunity by the mammalian host. The finding by Spitalny and Nussenzweig (1972a) that disrupted salivary gland sporozoites (with presumably available functional antigen) are not capable of inducing protective immunity mitigates against alternative a.

It is interesting to note that there is a good correlation between the increasing development of protective immunogenicity during sporozoite development (Table 6), and the pattern of increasing infectivity of sporozoites as they develop (Vanderberg, unpublished observations). It is quite conceivable that sporozoite invasiveness is a prerequisite of sporozoite immunogenicity, or indeed even that the functional antigen(s) may actually be a component of the invasive mechanisms

of the sporozoite. Other evidence tending to support this hypothesis is the demonstration by Spitalny and Nussenzweig (1972a) that attenuated but intact sporozoites (either X-irradiated or heat-inactivated) are capable of inducing protective immunity, whereas disrupted sporozoites are not. The work of Cohen, Butcher, and Crandall (1969) with *in vitro* cultures of *P. knowlesi* is also relevant. Their studies suggest that protective antibody to erythrocytic stage infection may act by preventing the reinvasion of erythrocytes by merozoites. The morphological similarity between the presumptive invasive apparatus (toxonomes leading to paired organelles) of both sporozoites and erythrocytic merozoites is well known (review by Aikawa, 1971). Perhaps substances released from these parasite organelles are antigenic, and play some role in the induction of protective immunity. The CSP reaction may represent a component of this released antigen binding to homologous antibodies.

Our findings may have practical significance if this system of immunization with X-irradiated sporozoites is ever able to be applied to humans. If the patterns of development of the human plasmodia are similar to what we have observed in *P. berghei*, then it would not be sufficient to develop a sporozoite harvesting technique utilizing whole mosquitoes infected with both oocyst and salivary gland sporozoites. A technique designed to harvest salivary gland sporozoites at the point when they are most immunogenic might be necessary.

If the growth of the sporogonic cycle in tissue culture ever becomes feasible, it might not be sufficient to merely allow the sporozoites to develop to the point where they become differentiated and free. Their maturation process might have to be supported to a point of peak immunogenicity and infectivity. It should be pointed out, however, that the patterns of differentiation of human plasmodia need not necessarily follow the same time course as in rodent malaria. There is no reason to suppose that the entire pattern of development of human plasmodial species may not be shifted forward to an earlier development of immunogenicity in oocyst sporozoites. The question will have to be answered as we cautiously move from rodent, to non-human

primate, and perhaps eventually to human malaria.

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Attempted Immunization of Rhesus Monkeys Against Cynomolgi Malaria with Irradiated Sporozoites

R. A. WARD AND D. E. HAYES

Department of Entomology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: *Anopheles stephensi* mosquitoes containing *Plasmodium cynomolgi bastianellii* sporozoites were irradiated with 10 kilorads. *Macaca mulatta* monkeys received $7.5-9 \times 10^4$ irradiated sporozoites through the bite of infected anophelines to determine if irradiated sporozoites would protect monkeys from sporozoite challenge. At this level protective effects of the immunization treatment were not evident. It is estimated that 2.5×10^8 irradiated sporozoites will be required to immunize a single rhesus. Methods of culturing and harvesting this quantity of sporozoites are discussed.

Irradiation of erythrocytic stages of human malarial parasites with gamma irradiation have produced avirulent strains that maintain their immunogenic properties. Protection against erythrocytic parasite challenge following various immunization schedules has been demonstrated against *Plasmodium falciparum* in *Aotus trivirgatus* (Sadun et al. 1969). Sporozoite challenge has not been achieved with the *Aotus*-*falciparum* malaria system due to an inability of the *Aotus* liver to sustain complete development of the exoerythrocytic schizont of *P. falciparum* (Sodeman et al. 1969).

The successful immunization of rodents by irradiated *P. berghei* sporozoites against experimentally induced sporozoite infection poses

the question as to whether or not comparable results may be observed with primates and their malarial parasites. At the present time, the *P. cynomolgi*-rhesus-anopheline model is the one most comparable to a human infection. The relatively large size of the host, its predictable gametocytemia and the close relation between *P. vivax* and *P. cynomolgi* act in favor of this model. Although *P. vivax* can now be routinely cyclically passaged through *Aotus*, extreme variations in the length of the prepatent period create problems in the interpretation of experiments (Ward et al. 1969).

This preliminary experiment was designed to determine whether or not immunization of rhesus monkeys by irradiated *P. cynomolgi* sporozoites would protect monkeys from sporozoite induced challenge.

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Table 1. The course of infection in rhesus monkeys immunized with *Plasmodium cynomolgi* irradiated sporozoites.

Group (Treatment)	Animal number	Prepatent period (days)	Day of peak parasitemia	Maximum no. parasites/mm ³	Days parasitemia 1000/mm ³
Irradiated mosquitoes (Normal)	LO-88	9	16	339,000	24
	LO-89	9	17	334,800	22
Irradiated mosquitoes (Sporozoites)	LO-46	12	17	361,200	19
	LO-47	8	11	200,000	21
	LO-489	9	16	158,400	16

Materials and Methods

Macaca mulatta monkeys* weighing 2.3–2.8 kg were used. The isolate of *P. cynomolgi* *bastianellii* was received from Peter G. Contacos in 1968 and has been maintained by mosquito transmission since that time. *Anopheles stephensi* (India strain) was utilized as the vector. The rearing procedures are detailed in Gerberg (1970) and methods of holding infected mosquitoes, assessing levels of oocyst and sporozoite infection are described by Rutledge et al. (1970).

Fourteen days after an infective blood meal, mosquitoes with heavy salivary gland infections (mean level of infection = $3 +$ or 10^3 – 10^4 sporozoites) were irradiated at a dosage of 10 kilorads in a "Gammacell 220" cobalt irradiation source which produced essentially pure gamma radiation. The mosquitoes were exposed to the source in pint-sized cardboard containers covered with nylon mesh at a dose rate of approximately 8,700 rads per minute. Mosquitoes resting on the sides of the container received a dosage 5% less than that of the source. As a control, mosquitoes of the same age which had received a non-infective blood meal were exposed to a similar radiation schedule.

To circumvent any possible immunogenic effect of mosquito body tissues or salivary gland cells, sporozoites were transferred to monkeys directly by mosquito bite rather than by dissection and trituration of infected salivary glands. Three monkeys (LO-46, LO-47 and LO-489) received irradiated sporozoites and two (LO-88 and LO-89) were bitten by

non-infected mosquitoes. One hour after exposure the irradiated mosquitoes and their controls were allowed to feed upon the respective monkeys on days 0, 14 and 42 of the experiment. 25–30 *A. stephensi* were permitted to feed to repletion on each animal on a given date. If an insufficient number fed, additional mosquitoes were added to the feeding cages. On day 56, all monkeys were challenged with the bite of 15–20 infected anophelines. Blood films were examined from all animals biweekly from the commencement of the experiment until day 60; after which blood smears were made daily. Parasite counts were recorded in terms of number of parasites per 100 white blood cells on giemsa stained thick films. At selected intervals, when gametocytemia was evident, cages of *Anopheles stephensi* were fed on the monkeys to evaluate gametocyte infectivity.

Results

The results of the experiment are summarized in Table 1 and Figures 1 and 2. Prepatent periods were similar in both groups of rhesus monkeys. The slight delay in LO-46 is within the normal range of variation. The date of peak parasitemia was similar in monkeys receiving irradiated sporozoites. Rhesus LO-489 attained a peak parasitemia only 50% as high as the other animals. This is well within normal variation as levels as low as 50,000/mm³ are observed in approximately 10% of normal rhesus exposed to *P. cynomolgi* challenge. Similarly, no appreciable differences were present in days of parasitemia in excess of 1000 parasites/mm³. This latter attribute is a good indicator of an immune response. An inspection of Figures 1 and 2 reveals no significant differences in the pattern of parasitemia in sporozoite immunized animals

* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

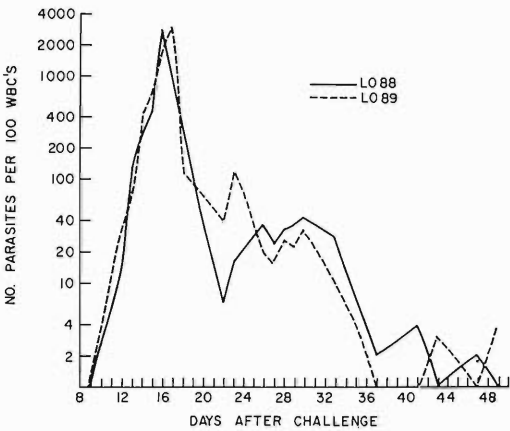


Figure 1. Parasitemias in control rhesus monkeys receiving *Plasmodium cynomolgi bastianellii* sporozoite challenge.

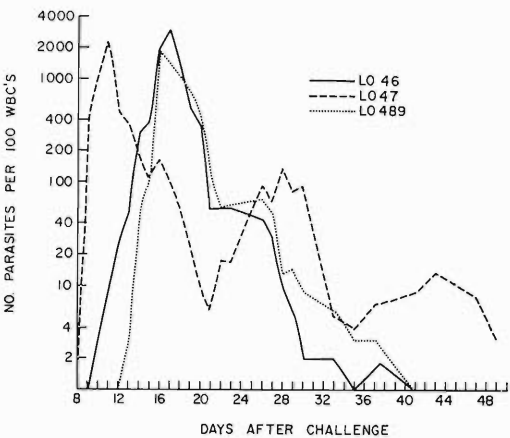


Figure 2. Parasitemias in rhesus monkeys exposed to 3 doses of irradiated *Plasmodium cynomolgi bastianellii* sporozoites prior to sporozoite challenge.

as compared to normal animals. On the basis of these observations it is evident that this immunization schedule did not elicit a protective immunity against sporozoite challenge. Feeding trials of *Anopheles stephensi* upon treated and control monkeys indicated that both groups were equally efficient as gametocyte carriers (Table 2). Rhesus LO-46 was a particularly poor gametocyte donor for an unknown reason. This refractoriness has been observed at occasional intervals in our experience.

Discussion

The selection of 10 kilorads as the minimal dose to inactivate *P. cynomolgi bastianellii* sporozoites was based upon the experience of Warren and Garnham (1970). They observed that X-irradiation of sporozoites of *P. c. cynomolgi* (M-strain) in *Anopheles atroparvus* at doses of 5 and 6.5 kilorads resulted in smaller exoerythrocytic schizonts than observed in controls but normal patent infections developed. At a dosage of 10 kilorads, there was no development of either exoerythrocytic schizonts nor a patent parasitemia. Studies with the langur strain of *P. cynomolgi* indicated a slightly different dose response to irradiation which was suggestive of a different susceptibility to radiation. It is difficult to extrapolate from the studies of Nussenzweig et al. (1969), who worked

with *P. b. berghei* (NK65 strain) and A/J mice, to the present primate model. Firstly, the rodent malarial parasites which are members of the subgenus *Vinckeia* have a primary exoerythrocytic cycle of 43–60 hours while primate malarial parasites of the subgenera *Plasmodium* and *Laverania* have exoerythrocytic cycles which range from 132–300 hours depending upon host and parasite species. This five-fold difference in time span may appreciably influence action of immunogenic materials upon exoerythrocytic parasite development. If the effect of protective immunity is directed towards the invading sporozoite, then this is immaterial. Another factor in determining effective dosage is the relative difference between the size of a mouse (25 g) and a rhesus monkey (2500 g)—a 100 fold difference. Based upon

Table 2. Susceptibility of *Anopheles stephensi* to *Plasmodium cynomolgi bastianellii* gametocytes from immunized rhesus monkeys.

Group (Treatment)	Animal no.	Peak mean no. oocysts/ mosquito midgut	Days after challenge
Irradiated mosquitoes (Normal)	LO-88	106.1	16
	LO-89	115.8	17
Irradiated mosquitoes (Sporozoites)	LO-46	1.6	19
	LO-47	266.5	6
	LO-489	102.8	26

immunological investigations with plague and arboviruses it would be expected that a monkey would require an immunization dose approximately 10 times (one log) greater than required in a mouse (Cavanaugh, D. C. and Schneider, I., personal communication). On the basis of the berghei model, which requires the use of 2.5×10^5 irradiated sporozoites to protect one mouse against a *P. berghei* sporozoite challenge, 2.5×10^6 irradiated sporozoites would be required for a single monkey. Assuming that sporozoites of *P. cynomolgi* from dissected salivary glands of *Anopheles stephensi* are as antigenically active as those of *P. berghei*, 100 heavily infected mosquitoes would have to be dissected for every booster for each rhesus monkey. If our system of feeding mosquitoes directly upon a monkey were to be used, 2,500¹ infected mosquitoes would be required for each feed.

Inspection of the immunization schedule of the present experiment indicates that each monkey received $7.5\text{--}9 \times 10^4$ irradiated sporozoites or approximately only 1% of those required by the above assumptions.

Over the past few years, the development of mass rearing procedures for *Anopheles stephensi* by Gerberg et al. (1968) and our laboratory have facilitated the production of large numbers of mosquitoes with minimal effort and manpower. One individual can rear 100,000 adults weekly. However, the production of large quantities of infected mosquitoes and the extraction of sporozoites still offer problems. A single rhesus monkey can only serve as an efficient gametocyte carrier for a 4–6 day period (i.e., produce oocyst counts of 100 or more which produce 3 + salivary gland infections). Approximately 4,000 anophelines may be fed upon a monkey daily without deleterious effects upon the donor. Of the 4,000 mosquitoes, only 80–90% will have fed, and after a 14 day incubation period, 1,500 surviving mosquitoes may be available for sporozoite isolation. It is evident that a large rhesus colony with a rapid turnover of animals is necessary to produce the infected donors.

¹The salivary glands of a heavily infected *Anopheles stephensi* are estimated to contain 10,000 sporozoites. It may be assumed that each mosquito inoculates 10% of its sporozoites (10^3) while feeding. Thus $\frac{2.5 \times 10^6}{10^3} = 2,500$ infected female anophelines would be used.

The application of density gradient centrifugation has provided a new technique for the mass isolation of sporozoites from infected mosquitoes (Chen and Schneider, 1969; Schneider and Chen, 1969). With this procedure it is possible to provide large quantities of extremely clean sporozoite suspensions for immunological and biochemical studies. It has been estimated that a technician can daily harvest 2×10^7 sporozoites with existing facilities (Schneider, I., personal communication). With some modification in the basic transferring procedures of infected *Anopheles*, the yield can be appreciably increased. It is not known whether sporozoites processed in such a manner differ immunogenically from material collected by standard procedures.

The fact that immunization with irradiated sporozoites against sporozoite infection was not achieved in this simian model was not unexpected. It is now technically feasible to produce the quantities of infected anophelines required for such studies, and optimistically, within the near future, it should be possible to produce large quantities of immunologically active sporozoites.

Acknowledgments

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Requirements for Induction of Immunity to *Plasmodium berghei* Malaria by Irradiated Parasitized Erythrocytes

B. T. WELLDE, C. L. DIGGS, E. RODRIGUEZ, JR., N. T. BRIGGS, R. M. WEBER,
AND A. E. VON DOENHOFF, JR.

Department of Medical Zoology,
Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT: Gamma irradiated *Plasmodium berghei* parasitized erythrocytes lost the capacity to immunize mice against challenge under the following conditions: (a) when disrupted by passage through a pressure cell, (b) when lysed by freezing and thawing, (c) after gamma irradiation at very high dose levels, or (d) after fixation in glutaraldehyde. Increasing levels of gamma irradiation (40-200 Krad) also resulted in both progressive hemolysis and loss of protein synthetic capacity as measured *in vitro*. Administration of sulfadiazine to mice during immunization with intact irradiated parasitized cells did not inhibit immunogenesis, nor did the drug inhibit parasite protein synthesis *in vitro*. No protective effect could be demonstrated in mice splenectomized either before or after the immunizing regimen. These findings are discussed with respect to characteristics of irradiated parasitized erythrocytes responsible for immunogenicity.

Artificial immunization of mice against *Plasmodium berghei* infections has proved to be a useful model in the study of immunity to malaria. Protection from this infection has been demonstrated in mice allowed to recover with the aid of chemotherapy (Box and Gingrich, 1958; Cox, 1958; Briggs et al. 1960), in animals administered irradiated parasitized erythrocytes (Wellde and Sadun, 1967; Wellde et al. 1969), and with parasites attenuated in a tissue culture system (Weiss and DeGiusti, 1965). More recently, reports of protection induced by parasite fractions have appeared in the literature (D'Antonio et al.

1969, 1970). Similar procedures have been used to immunize rats against the parasite (Zuckerman et al. 1967; Corradetti et al. 1966; Corradetti et al. 1969). No direct comparisons of the immunogenic efficacy of intact versus disrupted parasitized erythrocytes have been reported. These experiments were designed to make such comparisons and to explore other characteristics of the host-parasite system which are required for an optimal immune response.

Materials and Methods

Parasite

The NYU-2 strain of *Plasmodium berghei* was used in all the studies reported herein.

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Procedures for maintenance of the parasites have been previously described (Wellde et al. 1966).

All experiments were conducted using random bred albino mice* reared at the Walter Reed Army Institute of Research (Wrm:- (ICR)BR).

Immunization

Immunization of animals was achieved by the injection of parasitized erythrocytes which had been irradiated using a CO-60 source as previously reported (Wellde and Sadun, 1967; Wellde et al. 1969). The immunizing schedule involved five injections over a two and one-half week period. Unless otherwise noted, the total dose was 1×10^9 parasitized erythrocytes. In some of the experimental groups, the inocula were disrupted either in a pressure cell (French and Milner, 1951) or by freeze-thawing by immersion in a CO₂-ethanol bath with subsequent thawing; a total of three freeze-thaw cycles was employed. Morphological observations on these preparations were made after concentration of the residue by centrifugation at $27,000 \times g$ and staining with Giemsa's stain.

In experiments involving the use of sulfadiazine, the animals were injected with 0.2 ml of a 10 mg/ml solution intraperitoneally on the day prior to exposure to parasites and then daily until one week after the last exposure; challenge was deferred until one week after the last dose of drug.

Assay of immunity

Groups of animals were tested for immunity to *Plasmodium berghei* by intraperitoneal challenge with 2×10^4 parasitized erythrocytes. Significant prolongation of survival time beyond that of control animals was taken as evidence of immunity. The percentage of animals surviving for 30 days was another useful index of protection; animals alive at that time usually survived.

* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

Assays of protein synthesis *in vitro*

The protein synthetic capacity of parasitized erythrocytes was assayed by the incorporation of C-14 isoleucine (301 mc/mM) into trichloroacetic acid (TCA) insoluble material. Erythrocytes collected in heparin from heavily parasitized mice were washed and suspended at a concentration of 2.5×10^7 /ml in Hank's buffered salt solution with added glucose (1.75 mg/ml), potassium penicillin-G (500 μ /ml) and streptomycin sulfate (500 μ gm/ml). C-14 isoleucine was added to a concentration of 0.2–0.3 μ c/ml at 37°C in a metabolic water bath (Dubnoff) shaken at 60–100 cycles/min. One half ml samples were removed to 5 ml cold 0.01 M isoleucine in 0.15 M NaCl at intervals thereafter. The cells were sedimented at $800 \times g$, washed in an additional 5 ml isoleucine and lysed with 2.5 ml H₂O. The solution was brought to 10% TCA, the resulting precipitate sedimented at $800 \times g$, washed twice in 5 ml 10% TCA and dissolved in 1.0 ml of a quaternary ammonium base solvent, NCS (Amersham/Searle Corporation, Arlington Heights, Ill.). The preparations were washed into a scintillation fluid consisting of 0.3 gm/l of p-Bis (2-(5-phenyl-oxazolyl)/benzene (PO-POP) and 5 gm/l of 2,5-diphenyloxazole (PPO) in toluene and counted in a Packard liquid scintillation counting system.

Other procedures

Hemolysis was estimated by absorbance measurements of supernatant fluids at 412 nM.

Chemical fixation of irradiated parasitized cells was performed in a 2.5% solution of glutaraldehyde in 4% sucrose in 0.05 M sodium phosphate, pH 7.1–7.2 for 10 minutes at 4°C. The glutaraldehyde was removed by centrifugation and washing with 0.075M sodium phosphate, pH 7.3 in 0.075M NaCl.

Splenectomies were performed under sterile conditions on mice anesthetized with Relaxans (Pitman Moore, Indianapolis, Indiana). The spleens were exteriorized through a small incision and were removed by cautery. Sham-operated control mice received the same treatment with the exception that the spleen was not removed. Surgical skin clips were used to close the incisions. Splenectomies were

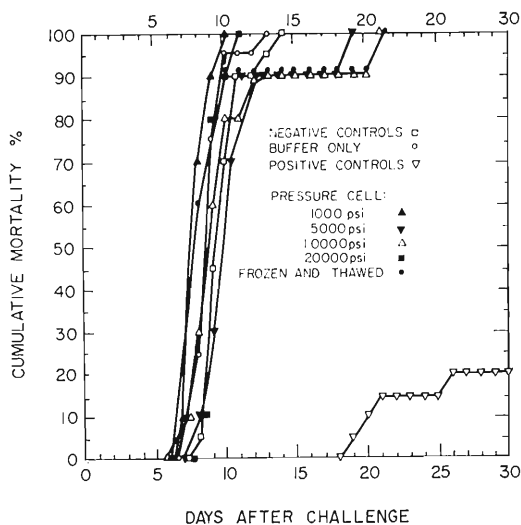


Figure 1. Mortality in groups of mice immunized as follows: (1) with intact irradiated *P. berghei* parasitized erythrocytes (positive controls) (20 mice), (2) injected with portions of the same cell suspensions disrupted at various pressures in a pressure cell, or (3) by freezing and thawing (10 mice/group), (4) injected with buffer only (20 mice) or (5) untreated (20 mice). Animals were challenged one week after the last immunizing injection.

done either one week before or 3 to 5 days after immunization.

Results

Effect of hemolysis on immunogenicity of irradiated parasitized erythrocytes

The degree of immunity induced by irradiated parasitized erythrocytes was compared with that obtained with lysates of the same preparations. Two experiments were performed in which disruption of the parasite-host cell complex was accomplished either by passage through the French pressure cell or by freezing and thawing. Mortality curves for animals in comparable groups were similar and the data from these two experiments were pooled (Fig. 1). In spite of the survival of 80% of the animals which received intact irradiated parasitized cells (positive controls), all other animals died within 21 days. The median survival times of negative controls (animals given no pretreatment) and experi-

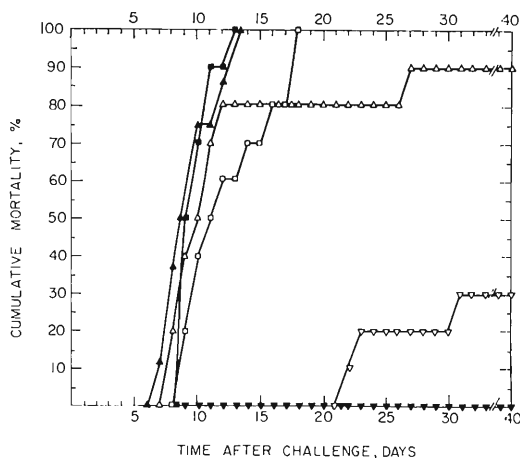


Figure 2. Mortality in mice immunized with intact parasitized erythrocytes (Positive controls, ∇ and \blacktriangledown), portions of the same cell suspensions disrupted at 1000 psi in a pressure cell (\triangle and \blacktriangle) or untreated (negative controls, \square and \blacksquare). Animals challenged 4 (open symbols) or 8 (solid symbols) weeks after the last immunizing injection. Each group consisted of 10 mice except for \blacktriangledown and \blacktriangle with 9 and 8 animals respectively.

mental groups were similar (8 to 10 days) but three mice from three different experimental groups survived longer than any negative control animals. Both methods of disruption resulted in complete hemolysis as judged from the gross appearance of the preparations. Microscopic observations revealed many intact parasites after either freeze-thaw or treatment at 1000 pounds per square inch (psi) in the French pressure cell; higher pressures resulted in essentially complete disruption of parasites.

In order to determine whether or not a greater time interval between immunization and challenge enhances immunity after treatment with antigens derived from disrupted cells, we performed a similar experiment in which the prechallenge period was extended. Irradiated parasitized erythrocytes were lysed at 1000 psi and used to immunize mice; positive and negative controls were included as before. Four weeks after immunization one half of the animals in each group were challenged; the remaining animals were challenged eight weeks after the last immunizing injection. The results are presented in Fig. 2. It

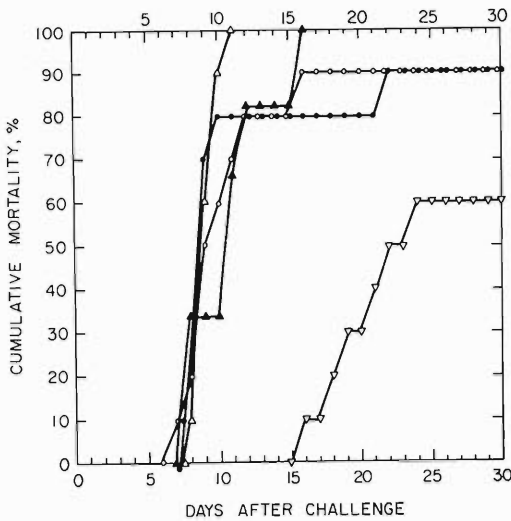


Figure 3. Mortality in mice immunized with intact irradiated parasitized erythrocytes (positive controls) (∇), portions of the same cell suspensions disrupted in a pressure cell at 1000 psi (\blacktriangle), or the supernatant fluid (\circ) or sediment (\bullet) from disrupted cells. Negative controls (buffer treatment), (\triangle) were also included. Ten mice per group except for \blacktriangle which consisted of 6 animals.

is evident that little immunity could be detected even when challenge was delayed. The survival of the single mouse in the experimental group challenged at four weeks is significant, however, since an untreated animal has never survived in any of our experiments. The survival of 100% of the positive control animals after challenge at 8 weeks is also noteworthy.

In order to further investigate the immunogenicity of disrupted parasites, an experiment was performed in which irradiated parasitized cells were disrupted at 1000 psi, the resulting preparation centrifuged at $27,000 \times g$, and the supernatant fluid and sediment each used as experimental immunogens. Control groups were also included, and the animals were challenged one week after the last injection. As illustrated in Fig. 3, median survival times were similar in negative control and experimental groups, but one mouse each in the supernatant fluid and sediment groups survived for 30 days.

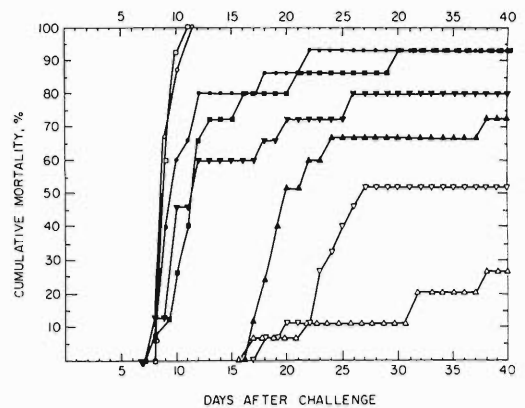


Figure 4. Mortality in mice immunized with *P. berghei* parasitized erythrocytes irradiated with 20 (\triangle), 60 (∇), 100 (\blacktriangle), 200 (\blacktriangledown), 400 (\blacksquare), or 800 (\bullet) Krad of γ -irradiation or treated with buffer only (\circ) or untreated (\square). Animals challenged one week after the last immunizing infection. Fifteen mice per group.

Effects of irradiation dose on immunogenicity and protein synthesis

Two experiments were performed to investigate the effect of increased levels of irradiation on the immunogenicity of parasitized erythrocytes. The first experiment assessed levels up to 200 Krad; in the second experiment, 400 and 800 Krad were also used. Because of the time required for the latter large irradiation doses, measures were taken to maintain all preparations at near 0°C during irradiation; heating effects can therefore be ruled out as a cause of the observed impairment of immunogenicity. The median survival times of negative controls were similar in the two experiments (9 and 11 days respectively). In both experiments, a progressive diminution in immunogenicity was noted as the irradiation doses increased (Fig. 4).

In the third experiment, *in vitro* protein synthesis by irradiated parasitized cells was studied. Heavily parasitized and uninfected control heparinized mouse erythrocytes were washed in 0.15M NaCl and adjusted to $5 \times 10^8/\text{ml}$. Aliquots of the parasitized cells were irradiated at seven different dosages. After removal of samples for estimates of hemolysis, the suspensions were assayed for protein syn-

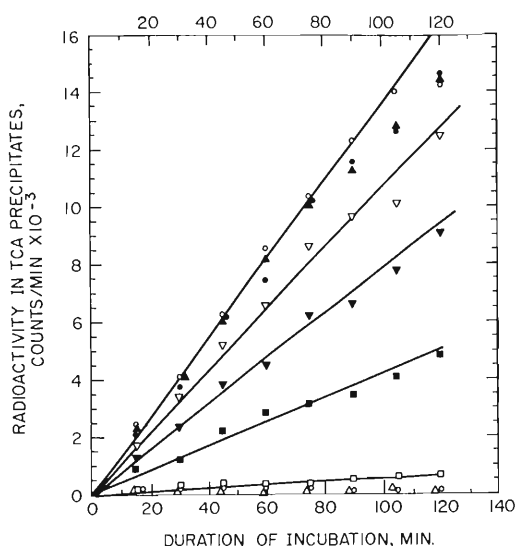


Figure 5. Incorporation of C^{14} -isoleucine into TCA precipitable material by *P. berghei* irradiated with zero (\circ), 20 (\blacktriangle), 40 (\bullet), 60 (∇), 80 (\blacktriangledown), 100 (\blacksquare), 150 (\square), or 200 (\odot) Krad of γ -irradiation. Data on a control suspension of uninfected non-irradiated erythrocyte is also included (\triangle).

thetic capacity. The results (Fig. 5) indicate little or no difference in the initial rate of isoleucine incorporation by nonirradiated cells or cells irradiated at 20 or 40 Krad, although the data suggest a diminution in rate in the irradiated cells toward the end of the experiment. Higher levels of irradiation resulted in a progressive loss in protein synthetic capacity until at 200 Krad incorporation was indistinguishable from the uninfected erythrocyte control values. It was noticed during the course of this experiment that hemolysis was taking place in the reaction mixtures. Estimates were made of hemolysis in cell suspension samples taken after irradiation and held at room temperature in saline until the last sample was taken for estimates of isoleucine incorporation (Fig. 6). Also plotted is the percentage inhibition in the rate of isoleucine incorporation as determined from the initial slopes of the plots in Fig. 5. It is apparent that both protein synthesis and hemolysis are functions of irradiation dosage but that the latter is more resistant to changes in dosage than the former.

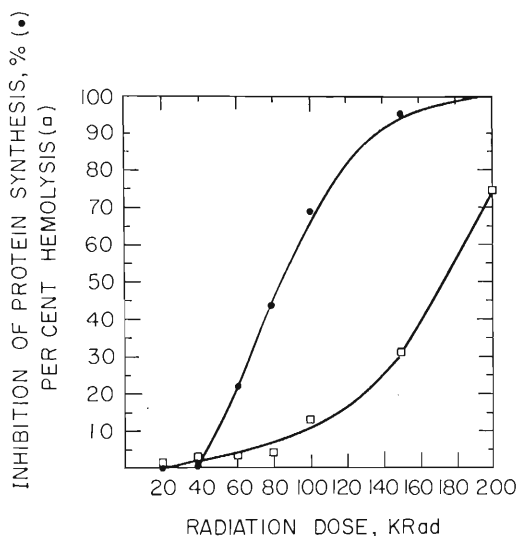


Figure 6. Degree of hemolysis (\bullet) and inhibition of the rate of protein synthesis (\square) induced by γ -irradiation expressed as percentages of control values.

Effect of sulfadiazine on immunogenicity of irradiated parasitized erythrocytes

These experiments were designed to test the possibility that normally metabolizing parasites are required for immunogenicity. Since sulfadiazine is highly effective against *P. berghei* infections, it was reasoned that the drug would also inhibit metabolism in irradiated parasites. An experiment was therefore performed in which large doses of sulfadiazine were injected into mice prior to, during, and after immunization with irradiated parasitized erythrocytes. In addition to (a) positive and (b) negative controls as described above and (c) experimental mice immunized with irradiated parasites and given drug, two additional control groups were employed; these consisted of (d) animals which were treated with drug alone and (e) animals which were given nonirradiated parasitized erythrocytes and sulfadiazine. These last two groups were included to (1) serve as "drug only" controls to detect possible persistence of drug and to (2) test the efficacy of the dose of drug used in preventing parasitemia due to nonirradiated parasites. One day prior to challenge, blood films were collected from

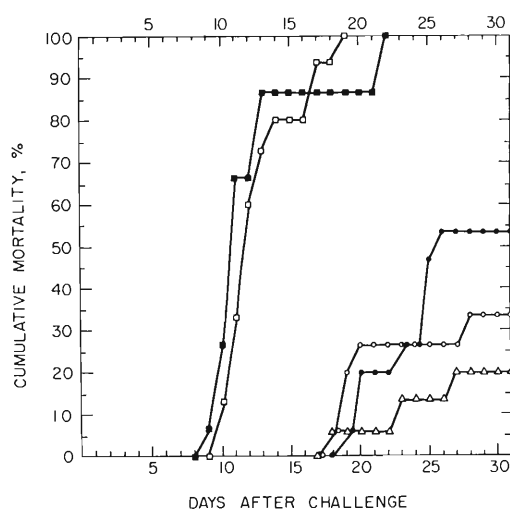


Figure 7. Mortality in mice immunized with irradiated intact parasitized erythrocytes in the presence (○) or absence (●) of sulfadiazine administration and with nonirradiated intact parasitized erythrocytes in the presence of sulfadiazine (△). Controls were treated with sulfadiazine only (□) or were untreated (■). Fifteen animals per group.

the group (e) of animals which received non-irradiated parasites and sulfadiazine; all animals were negative.

The results (Fig. 7) demonstrate that immunization was achieved even though the animals were being treated with the drug. It can also be seen from these data that non-irradiated parasitized erythrocytes were immunogenic in drug treated animals. A total of three experiments of this type all indicated that sulfadiazine does not inhibit immunogenicity.

In an *in vitro* experiment, protein synthesis by nonirradiated parasites in the presence of sulfadiazine was investigated. As shown in Fig. 8, no inhibition was observed during the 2½ hour exposure to drug.

Effect of glutaraldehyde fixation on immunogenicity of irradiated parasitized erythrocytes

Since the integrity of the parasite-host cell complex may be crucial to immunogenicity, parasitized cells fixed in glutaraldehyde were tested for ability to induce a measurable im-

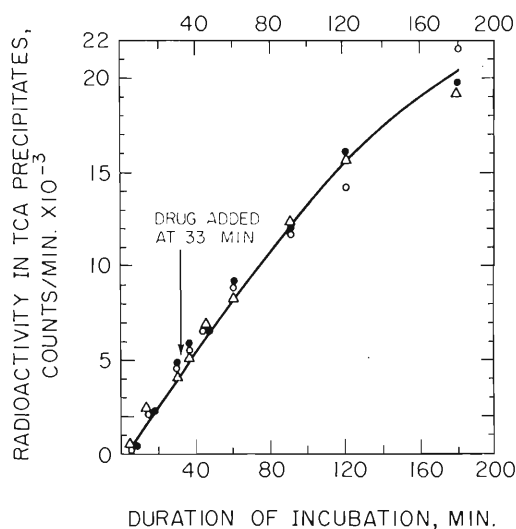


Figure 8. Incorporation of C^{14} -isoleucine into TCA precipitable material by *P. berghei* and after the addition of sulfadiazine to a concentration of 10 (●) or 100 (○) μ gm/ml. Saline was added to a control flask (△).

munity to challenge. In order to take advantage of any increase in sensitivity to be obtained from an extended time interval prior to challenge, groups of animals were held for either one week or four weeks before they were challenged. All positive control animals challenged at four weeks survived. Although median survival times were similar in the experimental and negative control groups, a single experimental mouse survived (Fig. 9).

Effect of splenectomy on the resistance produced by irradiated parasitized erythrocytes

Four separate experiments were undertaken to determine the effects of splenectomy before and after immunization on the development and maintenance of immunity produced by irradiated parasitized cells. Immunized mice were given a total of approximately 5×10^8 irradiated parasitized cells. Negative control mice received injections of irradiated normal mouse erythrocytes or were untreated. The results of these experiments are presented in Fig. 10. Mice splenectomized before immunization were not resistant to the challenging in-

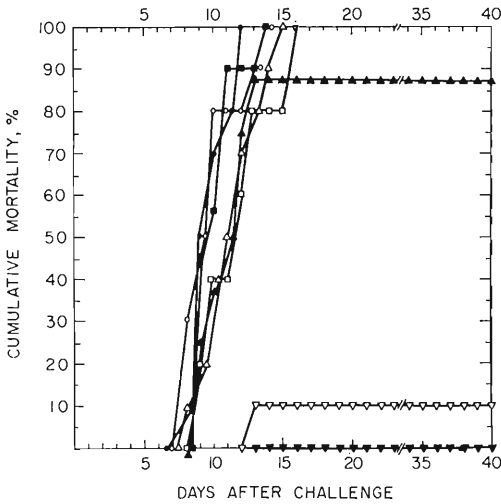


Figure 9. Mortality in mice injected with glutaraldehyde fixed (Δ and \blacktriangle) or unfixed (∇ and \blacktriangledown) intact parasitized erythrocytes, buffer (\circ and \bullet) or given no treatment (\square and \blacksquare) and challenged one (open symbols) or four (solid symbols) weeks later. Ten mice per group except for \blacktriangledown and \blacktriangle which consisted of 7 and 8 animals respectively.

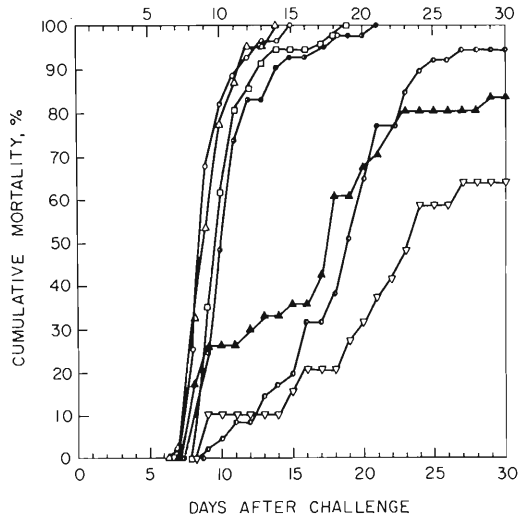


Figure 10. Mortality in mice splenectomized (Δ) (45 mice) or sham operated (\blacktriangle) (31 mice) before immunizations, and in mice splenectomized (\circ) (41 mice) or sham operated (\bullet) after immunization. Negative control groups include untreated animals (\square) (48 mice) and irradiated normal blood recipients (\circ) (28 mice). Positive controls (∇) (19 mice) were immunized and given no further treatment.

fection and their median survival time (9 days) was similar to that in untreated mice (10 days) and mice receiving normal irradiated blood (9 days). On the other hand the median survival time of sham operated immunized mice was 18 days. Splenectomy after immunization also severely affected the expression of immunity. Mice in this group died at a rate similar to that of negative control mice. The median survival time for mice splenectomized after immunization was 11 days, as compared to 19 days for sham operated mice.

Sham operation procedures conducted on mice either before or after immunization appeared to have an effect on resistance since there was a greater percentage survival and a greater median survival time in the positive control mice (untreated immunized) than in either of the two operated groups.

Discussion

These studies demonstrate that optimal immunogenicity of irradiated parasitized erythrocytes is dependent on an intact parasite-host

cell complex (Fig. 1-6). The immunogenic effect persists in the presence of treatment with a chemotherapeutic agent (Fig. 7). Disruption of parasitized erythrocytes either by passage through a pressure cell or by repeated freezing and thawing resulted in extensive, but not complete, loss of immunogenicity.

It is of interest to compare these findings with those of D'Antonio et al. (1969, 1970) who have detected immunogenic activity in fractions derived from parasitized mouse erythrocytes. Whereas these workers performed their studies with subfractions of the erythrocyte parasite complex, we worked either with the whole disruptates or with the sediment and supernatant fluids thereof. It is possible that in the present study the crucial antigenic substances were somehow destroyed or that their activity was masked by extraneous protein or by other material present in the suspension. However, activity was not significantly enhanced when the supernatant fluid containing the bulk of the hemoglobin was removed (Fig. 3). A more likely ex-

planation for the apparent discrepancy between these and the above mentioned findings is the fact that the other workers employed a host-parasite system in which virulence was less marked than in that reported here.

A possible explanation for the higher efficacy of intact parasitized erythrocytes as opposed to lysates is that antigen synthesis continues *in vivo* during the early hours (or days) after injection of irradiated parasites into the recipient. The fact that relatively low levels of irradiation only slightly inhibit protein synthesis has been demonstrated in *P. knowlesi* by Trigg et al. (1972) who suggest that this might account, in part, for immunogenicity. If this were the case, then "exoantigens" could be present in small quantities in the parasite extracts which show low level immunogenicity. The progressive reduction of immunogenicity with increasing radiation dose might indicate a progressive loss of synthetic capacity, and this is indicated in this investigation (Fig. 4 and 5). However, since lysis also increased with increasing levels of irradiation (Fig. 5), a postulated direct inhibitory effect cannot be distinguished from inhibition associated with cell disruption. Interpretation of the interrelationship between these two phenomena is complicated since lysis proceeds during the protein synthesis experiments, and, in fact, probably continues to occur for many hours (Kollmann et al. 1969). Studies of protein synthesis in irradiated parasitized erythrocytes under conditions of reduced lysis through the use of SH protective agents (Kollmann et al. 1969) might be informative.

Pertinent to these considerations are the experiments which indicate no effect of sulfadiazine on immunogenicity (Fig. 7). Since sulfadiazine would be expected to interfere with biosynthetic functions, it seems likely that the parasites of animals which received sulfadiazine on a daily basis would have an impaired ability for antigen synthesis. This speculation must be tempered by the fact that a prompt inhibition of protein synthesis by *P. berghei* is not induced by sulfadiazine *in vitro* (Fig. 8). It has also been reported that protein synthesis in malaria parasites is not inhibited by other drugs with similar sites of action (Canfield et al. 1970; Gutteridge and Trigg, 1972). In any case, animals so treated were effectively immunized.

A recent contribution indicates that heat inactivated *P. berghei* parasitized erythrocytes are immunogenic (D'Antonio, 1972). This observation may also help determine if there is a requirement for metabolizing parasites in the induction of immunity. Quantitative comparisons between the immunogenicity of heated and nonheated parasitized cells will be necessary to allow relevant interpretations.

Another hypothesis which can be offered to account for the requirement for intact cells concerns the fate of the damaged erythrocyte *in vivo*. Whereas minimally damaged erythrocytes are sequestered primarily in the spleen, more extensive erythrocyte alterations result in hepatic uptake (Ullmann and Gordon, 1965). The spleen is clearly required for the induction of resistance by irradiated parasitized cells (Fig. 10). Treatments which damage the parasitized erythrocyte membrane (i.e. increased radiation, fixation, pressure cell) may alter the patterns of sequestration and destruction of the irradiated parasitized cells in the recipient animal. This in turn may greatly effect the ability of the host to respond adequately to the challenging infection.

The loss of immunity observed when splenectomy was performed after immunization coincides with the work of others (reviewed by Zuckerman, 1970) and emphasizes the importance of the spleen in the maintenance of immunity to malarial parasites.

Although glutaraldehyde fixation is routinely used to preserve ultrastructure, it did not preserve immunogenicity of the irradiated parasitized erythrocytes (Fig. 9). This finding can be interpreted equally well in terms of inactivation of (a) biosynthetic capacity or (b) preformed antigen. It suggests that particle size alone is not the crucial factor responsible for the immunogenicity of intact parasitized erythrocytes. The significant protective effect observed in one mouse is interesting. Whether it is the result of specific immunization or nonspecific stimulation of indifferent defense mechanisms remains to be determined.

Enhancement of immunity by extension of the prechallenge interval is suggested in several of the experiments reported in this communication (Fig. 2 and 9). This is in concert with reports of D'Antonio et al. (1970) who

deferred challenge until 15 weeks after administration of antigen.

The finding that nonirradiated parasites in the presence of sulfadiazine are at least as effective as immunogens as irradiated parasites (Fig. 7) is interesting. Although this treatment resulted in 80% survival as compared with approximately 50% in the positive control group, no conclusion regarding comparative efficacy can be made.

The loss in immunogenicity of parasitized cells after lysis remains unexplained. If exoantigen production proves not to be involved, the problem can be thought of in terms of lability of antigen rather than in terms of the metabolic capability of the parasite. It is too early to speculate as to whether such lability, if applicable, might prove to be defined in terms of a relationship between organ or cell distribution and immunogenicity, protein denaturation, autolysis through hydrolytic enzymes, a combination of these considerations or some other as yet unsuspected factor(s).

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Comments on Vaccination Attempts

A. CORRADETTI

Department of Parasitology, Istituto Superiore di Sanita, Roma, Italy

Rivera-Ortiz and Nussenzweig have studied the stimulating effect of X-irradiated sporozoites of *P. berghei* on the RES of mice by employing the carbon clearance method. They have found that X-irradiated salivary glands of mosquitoes, both infected or uninfected, produced increased clearance rates and increased spleen weights. These effects, which are more pronounced when the salivary glands are infected, reach a maximum two days after the injection and return to slightly above normal values after approximately one week. Multiple sporozoite salivary gland injections do not enhance the effect.

By employing the carbon clearance method in a different model (rats and *P. berghei* X-irradiated erythrocytic parasites), we (Biozzi et al., 1970) had previously obtained an increased phagocytic activity up to the 20th day following vaccination; the stimulation of the RES falling thereafter rapidly to normal values. That the difference in the response should be attributed to the differences in the model (animal host and stage of the parasite), as suggested by Rivera-Ortiz and Nussenzweig, seems to be reasonable, although more investigations are needed.

The fact that uninfected X-irradiated salivary glands, when injected into mice, produce a non-specific stimulation of the RES makes it advisable to employ sporozoites which are not contaminated with salivary gland material. Some years ago we described a method by which uncontaminated free sporozoites can be obtained in physiological solution (Corradetti et al., 1964).

Spitalny and Nussenzweig have attempted various routes of immunization and methods

of parasite attenuation for developing protection against *P. berghei* sporozoites in mice.

X-irradiated or heat inactivated sporozoites, when injected by the intravenous route, protected mice completely against the intravenous challenge of viable sporozoites, while freeze-thawed sporozoites injected by the same way did not protect 60 per cent of the animals. Practically no protection was evident when the injection of the X-irradiated, or heat inactivated, or freeze-thawed sporozoites was given intra-peritoneally. In another series of experiments the immunogenicity of sporozoites subjected to sonication or homogenization was tested, with negative results for protection against the challenge.

From this last series of experiments Spitalny and Nussenzweig conclude that sporozoites must be intact in order to stimulate protection. They show the tendency to admit the hypothesis that intact attenuated sporozoites may retain the capacity to undergo some degree of development in the liver or elsewhere, development which might be essential for the induction of an immune response. Personally I am reluctant to accept this explanation without an experimental demonstration, because it would imply the active penetration of the sporozoite in a liver cell and the starting of a schizogonic cycle with one or more nuclear divisions; all events which are unlikely to occur after irradiation.

The failure of sporozoites subjected to sonication or homogenization to produce protective immunization may depend on biochemical changes or denaturation of the sporozoite proteins occurred during these operations.

The paper of Vanderberg, Nussenzweig,

Sanabria, Nawrot and Most deals with the stage specificity of antisporeozoite antibodies in *P. berghei* malaria and its relationship to protective immunity. It was found that sporozoites develop precipitation at one end of their body after in vitro incubation in immune serum (circumsporozoite precipitation reaction: CSP). Immature sporozoites in oocysts and mature sporozoites in the salivary glands constitute two distinct CSP antigens.

Protective immunity is produced almost exclusively by sporozoites moved to salivary glands. The antibodies formed after immunization of mice with mature salivary gland sporozoites have no effect on the morphology or development of erythrocytic parasites, gametocytes or ookynets.

These results, correlated with others reported in the paper of Spitalny and Nussenzweig, show that the CSP antigen and the sporozoite protective immunogenic antigen are not identical. Consequently the hope is lost of having detected a protective antibody against *P. berghei* in the serum of mice immunized with sporozoites.

Ward and Hayes have attempted to immunize rhesus monkeys with irradiated sporozoites of *P. cynomolgi bastianellii*. The immunized animals did not resist challenge and became infected. The authors believe that the failure depended on the dose of the sporozoites, which ranged from 75,000 to 90,000. They calculate that, owing to the weight of a rhesus monkey (2,500 g), the number of sporozoites needed for immunization should be around 2,500,000, and consequently 2,500 infected mosquitoes would be required for each feed. We look forward to new experiments in this specific field. They could confirm that the sporozoite dose was responsible for the failure, but they could alternatively reveal the inability of the rhesus monkey to build up a degree of immunity sufficient to prevent infection when challenged with viable sporozoites. In this connection I wish to state that Verolini and myself, in unpublished experiments, obtained no protection in rhesus monkeys vaccinated with irradiated *P. knowlesi* erythrocytic parasites against the challenge with viable erythrocytic schizonts of the homologous strain.

The paper of Welde and collaborators is

dealing with the protective immunogenicity of *P. berghei* erythrocytic parasites. They have found that gamma irradiated parasitized mouse erythrocytes lose protective immunogenicity when lysed by passage through a pressure cell or by freezing and thawing, when gamma irradiated at greatly increased dose levels, or when fixed in glutaraldehyde.

In relation to the passage through a pressure cell, the authors quote our experiments (Corradetti et al., 1969, 1969a) in which we obtained protection by employing the non-hydro-soluble fraction of *P. berghei* erythrocytic parasites. Many conditions were different in our experiments: 1) we used the Istisan strain of *P. berghei*, 2) the parasites were not irradiated, 3) the host was the rat, 4) the pressure cell was the "Refrigerated Ribi Cell Fractionator" operating at the temperature of 6 C and at the pressure of 20,000 psi.

Welde and collaborators give an interesting contribution which provides some light on the mechanism of the immunogenesis induced by the irradiated erythrocytic parasites. Their experiments on the continuation of the antigen synthesis *in vivo* during the early time after injection of irradiated parasites demonstrate that irradiated blood schizonts remain active for a period of time in the body of the new individual host in which they have been introduced.

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Comments on Vaccination Attempts

H. C. GOODMAN

Chief Immunology, WHO, Geneva, Switzerland

This symposium, like previous recent symposia on malaria sponsored by the Walter Reed Army Institute of Research (1, 2) presents and reviews not only contributions on the biology of human and experimental malaria which may have relevance for developing improved methods of chemotherapy or chemoprophylaxis, but has, like the preceding symposia, reserved an important part of the program for the subject of the immunology of malaria. No one underestimates the contribution that immunology research can make in developing improved immunodiagnostic methods for epidemiological studies and for the surveillance necessary for follow-up and maintenance of malaria control measures. Immunology research has also already contributed to an improvement of our understanding of immunopathological mechanisms in malaria, especially in malaria-associated nephritis (3). When we turn to the subject of immunization against malaria, however, I find a change from previous symposia, where doubts were openly expressed about the feasibility of developing a malaria vaccine. Now I sense that there is an assumption that a successful vaccine is only a matter of time. So today, I should emphasize that there is a great deal of work still to be done, and should stress the need to increase the number of immunologists working to help solve the problems ahead. But, in the past, most immunologists have "smelled an impossible task," to quote the words used by James Watson to account for why, also in the past, most basic scientists failed to undertake cancer research. I think that the fact that there is now good evidence that immunity to erythrocytic stages can be induced in rodents and primates (2) and that sporozoite immunization can be effectively induced in rodents (2 and this symposium) should reassure the basic immunologist about the potential for solution to this important problem. And in my remarks, I will try to focus, as did Dr. Stechschulte, on the need to study basic mechanisms of immunity by applying some of the recently developed *in*

vitro methods to models of human and experimental malaria.

I can do this because Dr. Corradetti has already covered the detailed discussion of the preceding five papers. But first I would like to bring up one interesting question from these papers for which I have no answer. The striking difference between the immunity induced by intravenous inoculation of irradiated sporozoites as compared to intraperitoneal, intramuscular or intracutaneous inoculation is puzzling. Is there some reason why a large number of sporozoites must be introduced into the blood stream at a given moment in order to overcome some mechanism that can prevent their contact with the cells essential for the induction of a protective immune response? Perhaps this is one factor which might explain the failure to immunize with mosquito-introduced irradiated sporozoites of cynomolgus malaria in rhesus monkeys. Have any experiments been done with labelled sporozoites to study their fate when introduced by intravenous in comparison to other routes?

To return to the model systems which might provide insight into mechanisms by which the immune system disposes of the malaria parasite, Vanderberg, et al. (4) raised the possibility of the production of "sporozoite-neutralizing" antibodies. To solve the problem of detecting "merozoite-neutralizing" antibodies for the erythrocytic forms of *P. knowlesi* in monkeys, Cohen and Butcher (5) developed the first fully *in vitro* technique for assessing the biological function of malaria antibodies. Would it be possible to develop an *in vitro* technique to measure "sporozoite-neutralizing" antibodies? This would require maintenance in tissue culture of the mammalian or rodent liver cells which the sporozoites enter.

If one of the mechanisms of effective immunity involves antibody which can prevent the entrance of merozoites or sporozoites into host cells, other mechanisms may be required to destroy and dispose of the parasites. Cris-

well, et al. (6) put *P. berghei* infected red blood cells plus macrophages in millipore membrane chambers into the peritoneal cavities of mice. They found that macrophages obtained from mice with chronic malaria destroyed parasitized erythrocytes even when the chambers were placed in the peritoneal cavities of normal mice. Macrophages from normal mice destroyed parasitized erythrocytes if the chambers were placed in the peritoneal cavities of mice with chronic malaria. Antibodies, which in this case would be antibodies cytophilic for macrophages, could account for these findings, but there are two other mechanisms which could be responsible and for which *in vitro* methods of study exist.

Antibody-dependent cell-mediated cytotoxicity is a term used to describe the destruction of an antibody-coated target cell by normal lymphoid cells (7). Perhaps Dr. Cohen can tell us whether in his system, he has observed release of radioactive label (destruction of merozoites) in the presence of antibody plus normal lymphoid cells. This system does not require complement.

Macrophages may be "activated" to kill phagocytized organisms in the presence of sensitized lymphocytes plus antigen as shown by Mackenness (8). The antigen which has stimulated delayed type hypersensitivity (specifically sensitized lymphocytes) does *not* need to be related to the organism ingested by the macrophage. Perhaps the *in vitro* method recently developed by Simon and Sheagren (9) for measurement of the killing of macrophage-ingested *Listeria monocytogenes* in the presence of sensitized lymphocytes and their specific antigen could be modified to study the possible role of activated macrophages in destroying malaria parasites. Parasitized erythrocytes could be fed to peritoneal macrophages to determine whether the addition of specifically sensitized lymphocytes plus antigen will increase the rate of destruction of the parasite. The method is already under study to learn how macrophages handle toxoplasma in further experiments like those reported by Remington (10) and also in leishmania (11).

Finally, the possibility that specifically sensitized lymphocytes might destroy malaria parasites directly, without the mediation of antibody or added complement could be tested

by modifying the *in vitro* method which Brunner and his colleagues developed to show direct cytotoxicity of sensitized lymphocytes for tumor target cells (12).

Before closing, I would like to focus on another puzzling feature of the immune response to malarial infection; the greatly increased synthesis of immunoglobulins (13). In malaria increased synthesis of IgG is well documented, and IgM levels are also increased although not to the extraordinary levels found in another protozoal disease, trypanosomiasis. It has not been possible to date to explain the increase in these two immunoglobulins by their content of specific antibody to parasite antigens, and one wonders whether the parasites could produce stimulation of the lymphoid cells in some way analogous to the way phytohemagglutinin and other lectins stimulate lymphoid cells to blast transformation. It is even possible that this kind of stimulation of lymphoid reactivity accounts for some of the reported immunosuppressive effects of malaria infection (14) by providing a generalized non-specific stimulus to the lymphoid system, rather than permitting the expansion of the clone of specifically sensitized cells which could lead to specific immunity. Again it has been suggested (15) that an *in vitro* approach might answer this question. Sensitized lymphocytes of malaria-infected animals could be exposed to malaria antigens (or whole parasites) to stimulate them to blast transformation. To a portion of the lymphocytes anti-Ig antiserum could be added to determine whether the lymphocyte stimulation is prevented. If the anti-Ig serum prevented the lymphoid stimulation, it would indicate that the stimulation is due to antigen combining with immunoglobulin receptors on the lymphocyte cell membrane, and is presumably part of a specific immune response. Failure to block the stimulation by parasites (plus stimulation of lymphocytes by malaria parasites or parasite antigens when the lymphocytes are obtained from normal individuals who were never exposed to malaria infection), would favor the interpretation that the stimulus is an immunologically non-specific (lectin-like) effect of the parasite on cell membrane receptors other than immunoglobulins.

In the past, vaccination procedures were developed empirically. It may be that by further

research on the use of irradiated parasites, successful vaccination procedures will be developed for human malaria. But I am sure that the investigators who are doing this pioneer work are trying themselves and would agree that others should also try to ensure reaching the desired goal by devoting additional resources and attention to research on the basic mechanisms of immunity in malaria. Such research will not only contribute to the development of protective vaccines, but also to better diagnostic techniques in sero-epidemiology, further understanding of immunopathological changes, and, hopefully, to an understanding of whether a disturbance in the immune response is involved in the relationship of malaria to other disorders, such as Burkitt lymphoma.

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IX

SEROLOGY

Evaluation of the Results of an Indirect Hemagglutination Test for Malaria in an Ethiopian Population

J. C. ARMSTRONG

LCDR, MSC, USN, U.S. Naval Medical Research Unit, No. 3,
Field Facility, Addis Ababa, Ethiopia

ABSTRACT: Gambela, Ethiopia was described as the setting for a serosurvey performed on samplings of the town's mobile, multi-ethnic population. The results of an indirect hemagglutination (IHA) test for malaria were provided by Dr. H. M. Mathews, Center for Disease Control, Atlanta, Georgia, for comparison with the parasitologic findings on 514 blood specimens collected toward the end of a peak malaria season. Data on 117 immigrants to Gambela from the non-malarious highlands of Ethiopia will be included in a separate report. Presented here are data on 397 lowlanders native to the Gambela region.

Blood film examinations gave a crude parasite rate of 59% for the native lowlanders whereas 77% of the matching filter paper blood specimens were positive by the IHA test using *Plasmodium knowlesi* as antigen. Mean positive titers of IHA antibody rose with age as parasite densities declined. Epidemiological observations were offered in explanation for certain deviations in the gradual accumulation of IHA antibody with age.

Among the lowlanders, the IHA test appeared to be less sensitive in detecting infections with *P. falciparum* than with the other three species endemic in Gambela or mixed infections including *P. falciparum*. This apparent deficiency was especially prominent in the 5–14 year age group where 19 of 58 subjects with *P. falciparum* were IHA-negative. Whether the deficiency resides in the test or in the immunological competence of the test subjects could not be determined. In general, there was an excellent correlation of IHA test results with other malarimetric data collected during and previous to the serosurvey.

Increasingly over the past dozen years, serology, in particular the fluorescent antibody test, has gained acceptance among research malariologists as a valuable malarimetric adjunct. Yet serologic tests remain little used on the local, operational level in malarious areas because of the special training and expensive materials required to perform them. Given stable reagents, the indirect hemagglutination (IHA) test, which is technically simple, rapid and inexpensive, might be adapted for use in small field laboratories. The advantages of the malaria IHA test are well recognized (Bruce-Chwatt, 1970), but difficulties have attended the development of a stable antigen-red cell carrier system (Bray and El Nahal, 1966). Under carefully controlled laboratory conditions, the IHA tests reported by Rogers *et al.* (1968) and by Welde *et al.* (1969) have shown a high de-

gree of sensitivity and genus specificity in assays of serum from subjects with induced (Sadun *et al.*, 1969) and naturally acquired (Kagan *et al.*, 1969; Mathews *et al.*, 1970) malaria infections.

This report, and a subsequent one, will compare IHA test results with parasitologic findings on blood specimens collected in Gambela, Ethiopia in 1970. The town of Gambela was selected as the test locality because a considerable amount of malarimetric data have been accumulated there since the establishment in 1966 of a field station by the U. S. Naval Medical Research Unit No. 3. The main purposes of the present report are (1) to describe the study locality and (2) to evaluate a portion of the IHA test results in light of what was already known of the epidemiology of malaria in the test locality.

Study Locality

Gambela is located at the base of the western escarpment of the central Ethiopian massif on the Baro River, 500 km west and a bit south of Addis Ababa, near latitude 8°

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N and longitude 35° E. The elevation of Gambela is 500 meters. Annual rainfall generally exceeds 100 cm and is distributed mainly between late April and mid-November. Diurnal temperature fluctuations range from 12°–42°C with highest temperatures registered in March–April, at the end of the dry season, and lowest temperatures in July–August at the peak of the rainy season. The mean relative humidity remains at about 75% during the wet months and decreases only to about 45% during the dry months owing to the proximity of the Baro River, large areas of seasonal and permanent swamp and a stream which bisects Gambela as it empties into the Baro. Moisture is also conserved in the shade provided by an abundance of *Acacia*, *Combretum* and *Gardenia* and by *Mangifera*, *Ficus* and *Ceiba*, the predominant native and introduced trees, respectively.

The population of Gambela is estimated at 1800. Members of the Anuak tribe, a primitive Nilotic people with an agricultural economy, comprise nearly three-quarters of the town's population and almost all of the sparse population in a large area to the south. Other Nilotic tribes represented by small numbers in Gambela include the Nuers and Shiluks from the western borderlands with the Sudan, whose economies are based on cattle, and the agricultural Komos and forest-dwelling Mesingos from areas north, east and southeast of Gambela. Members of Ethiopian highland tribes, Hamitic-Semitic peoples, comprise nearly a quarter of the town's population and occupy almost all official positions. The interests of the central government are served by the district governor and his staff, a large contingent of district police, the Baro River Authority, telegraph and post offices, a bank, an elementary school, and a health clinic. The Sudan also maintains a small consular staff in Gambela.

The town is imperceptibly subdivided into 14 named villages, each inhabited by people of similar ethnic and geographic origins. In keeping with the cross-roads nature of Gambela, its population is highly mobile. A majority of the residents consider their permanent homes to be elsewhere, and it is common practice for members of the lowland tribes, especially, to pack off for extended visits with their families. In addition to the river and

well-worn footpaths, Gambela is accessible by airplane on regularly scheduled commercial flights and by way of a treacherous dry-season road which winds down the escarpment from the cool highlands. Due to its location and relative accessibility, Gambela has long served as the major marketing center in an area of roughly 10,000 square kilometers.

Maize, the dietary staple, is grown year-round in small hand-cultivated patches, mainly in the alluvial soil along the river banks. Millet is grown in smaller quantities, stored and eaten when maize is in short supply. Mangos and the less abundant bananas and papayas are eaten in season. Fish caught in the Baro River during most of the year are eaten fresh or sun-dried. Cattle are driven in from tsetse-free regions west of Gambela, sold and slaughtered for meat which few of the native lowlanders can afford. For them, the cane rat (*Thryonomys* sp.) supplements fish as the main sources of protein in an otherwise starchy diet.

In addition to malaria, bacterial pneumonias, a wealth of arboviruses and the usual enteric protista and helminths are present in Gambela. Other important endemic infections include tuberculosis, leprosy, syphilis, trachoma, schistosomiasis mansoni, onchocerciasis, and Bancroftian filariasis; loiasis and dracunculiasis have been diagnosed in the area. Smallpox and yaws are rarely seen. Baker *et al.* (1970) and Hutchinson (1971) reported the recent introduction of Rhodesian trypanosomiasis into Illubabor Province, Ethiopia and described the ecological highlights of this area which includes Gambela.

Health services in Gambela are available at a Government Health Center staffed by one or two well-trained Health Officers, a Community Nurse, a mid-wife, a Sanitarian and usually two dressers and two clerks. A number of infections, including malaria, are treated free of charge, but the 50 cent (U.S. \$0.20) registration fee per month inhibits attendance by many of the native lowlanders for whom money is a scarce commodity and, indeed, a relatively new concept. Chloroquine is dispensed liberally for fevers or other symptoms suggestive of malaria and is taken regularly as a prophylactic by many of the more sophisticated highlanders who have not encountered malaria prior to their arrival in Gambela.

Krafsur (1970a, 1970b) carried out an intensive entomological study of malaria transmission in Gambela in 1967–68. Based on vector densities in dwellings, man-biting frequency and sporozoite rates, Krafsur estimated that in the course of one study year, *Anopheles gambiae* contributed five infective bites per person and *A. funestus* two infective bites per person, and that three quarters of these bites were inflicted during the three months (September to December) following the peak rains. Of less importance as vectors were *A. nili* and *A. pharoensis*, both avid outdoor biters, and *A. wellcomei* and *A. coustani*. On the basis of the seasonal transmission data of Krafsur (1970a) and concurrent parasitologic data (unpublished), the malaria IHA test survey was scheduled for early January as the period in time most likely to reveal high antibody titers before parasitemias declined.

Materials and Methods

Blood specimens were collected from 514 residents of Gambela by finger-prick or venipuncture and stored on filter paper rectangles as described as Mathews *et al.* (1970). The coded filter papers were air-dried overnight, then held at 5–10°C in the field and at minus 50°C in Addis Ababa prior to air shipment to the Center for Disease Control, Atlanta, Georgia, U.S.A. where the bloods were eluted with buffered saline and titrated against *Plasmodium knowlesi* antigens using the indirect hemagglutination (IHA) test of Rogers *et al.* (1968). Antibody levels were summarized as the geometric mean of the reciprocals of the titers (GMRT). Sera that were non-reactive at the first dilution (1:16) were considered negative and arbitrarily assigned a titer of 1:8 for computation purposes. Thick and thin blood films, prepared simultaneously with the filter paper specimens, were Giesma-stained within 18 hours and examined later in Addis Ababa. Asexual parasites were counted against leucocytes for 3–5 minutes in a number of thick film fields dependent upon the parasite density. These counts were converted to numbers of parasites per cmm, assuming 8000 leucocytes per cmm of blood, and summarized as the parasite density index (PDI) by the method of Bruce-Chwatt (1958). In order to compare the

serologic GMRT with a similar parasitologic index, the PDI of Bruce-Chwatt was modified by the addition of a parasite density class, designated 0.5, to include all presumptive negative blood smears.

Study subjects were accepted on a voluntary basis from numerous sites in Gambela, omitting the local Health Center. At frequent intervals, the blood collecting team moved to a different section of the town in an effort to reduce any sampling bias caused by our attraction to the sick. Each volunteer was interviewed and visitors to Gambela were excluded. A record was entered for each subject containing name, code number, age, sex, tribe, duration of residence in Gambela, travel history, oral temperature, and spleen size (Hackett's classification) in the sitting position.¹ The age recorded for each subject was the product of refined guesswork from an assemblage of dated events, e.g. number of rainy seasons since birth or puberty. Special pains were taken in the age estimation of subjects placed in the 10–14 year and 15–19 year age groups because it was apparent from data collected previously (see Tables 1 and 2) that adult levels of resistance to malaria were generally achieved by Anuaks in Gambela at an age approximately midway between 10 and 20 years.

The present report concerns itself with 397 members of the Anuak tribe living in Gambela and native to the region. Another paper will compare the serologic and parasitologic findings from these same lowlanders with similar data from 117 subjects also residing in Gambela, but native to the non-malarious highlands of Ethiopia.

Results

An indirect hemagglutination (IHA) test for malaria was performed by Dr. H. M. Mathews, CDC, Atlanta on 514 coded filter paper bloods and completed in a period of two days.

Reported here are the results on blood specimens collected from 202 females and 195 males of the Anuak tribe in Gambela, Ethiopia. The sexes were represented by equal or nearly equal numbers in each group, and

¹ Spleen palpations were performed by Senior Chief Hospitalman T. D. Carpenter under the tutelage of Captain D. C. Kent, Medical Corps, U.S. Navy.

Table 1. Malaria prevalence rates among Anuaks by age group during the peak malaria seasons¹ of 1967-68 and 1968-69 in Gambela, Ethiopia.

Age group in years	n =	Blood smear			Modified PDI ³
		No. pos.	% pos.	PDI ²	
<1	9	2	(22)	2.00	0.83
1	15	6	40.0	4.67	2.17
2	8	5	(63)	4.75	2.63
3-4	53	34	64.2	5.50	3.33
5-9	90	61	67.8	4.66	3.23
10-14	70	44	62.9	3.71	2.44
15-19	76	38	36.8	2.76	1.24
20-29	82	21	25.6	2.63	0.99
30-39	48	4	8.3	1.67	0.57
40-49	32	13	40.6	2.45	1.17
50+	12	2	16.7	4.50	1.17
Totals	495	220	44.4	3.98	1.93

¹ Dec 67; Jan, Feb, Dec 68; Jan, Feb 69.

² Parasite density index, after Bruce-Chwatt (1958).

³ PDI modified by the addition of a class, designated 0.5, to include all presumptive negative blood smears.

nothing of interest was derived from an analysis of results by sex.

Table 1 shows the crude parasite rates for eleven age groups of Anuaks in Gambela during the two peak malaria seasons previous to the present survey. Also shown are the conventional parasite density index (PDI), computed from counts of asexual parasites on positive smears, and the modified PDI which includes all blood smears, the negative smears

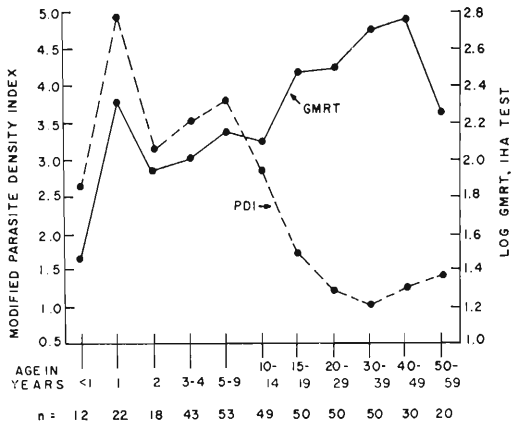


Figure 1. Modified parasite density index (density class 0.5 created for negative blood smears) and log GMRT (log titer 0.9 assigned to negative sera) of 397 Anuak residents of Gambela, Ethiopia, calculated from blood smear examinations and IHA test results in the peak malaria season of 1970, by age group (n = number of subjects in each age group).

Table 2. Malaria species prevalence among Anuaks by age group in the peak malaria seasons of 1967-68, 1968-69, and early 1970, Gambela.

Date	Age group	n =	Plasmodium species prevalence (%)				
			falciparum	malariae	ovale	vivax	Mixed
Dec 67,	<5	52	51.9	19.2	-	-	17.3
Jan 68,	5-14	73	60.3	16.4	-	4.1	13.7
Feb 68	15-29	84	17.9	-	-	-	-
	30+	59	13.6	5.1	-	-	1.7
Dec 68,	<5	33	54.5	15.2	3.0	-	15.2
Jan 69,	5-14	87	64.4	12.6	5.7	-	16.1
Feb 69	15-29	74	44.6	1.4	-	-	-
	30+	33	24.2	3.0	-	-	-
Jan 70	<5	95	60.0	23.2	3.2	-	22.1
	5-14	102	70.6	19.6	1.0	1.0	13.7
	15-29	100	42.0	3.0	-	-	-
	30+ 100		43.0	7.0	-	1.0	3.0

being assigned a class value of 0.5. An abrupt fall-off in parasite rate and density occurs at about age 15 years (Table 1) which can be seen even more clearly in Table 2 where the prevalences of the four plasmodia endemic in Gambela are arranged by age group for three successive peak malaria seasons.

Figures 1, 2 and 3 were plotted from the data presented in Table 3. Comparable indices were used in each figure to express the parasitologic and serologic data by age group. In Figures 1 and 2, curves describing rates and levels of parasitemia and antibody show similar trends until about age 14 years from which point the curves become mirror images. Figure 3 includes only those subjects who were serologically or parasitologically positive. In this figure, the decreasing trend

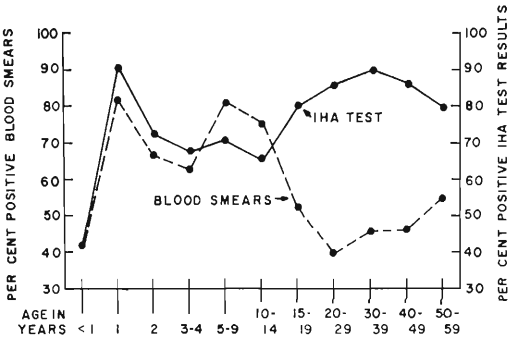


Figure 2. Malaria parasite and IHA antibody rates among 397 Anuak subjects by age group, Gambela (IHA test considered positive at titer of 1:16 or greater).

Table 3. Malaria parasite rates and densities, and IHA test rates and mean titers among Anuak study subjects by age group, Gambela.

Age group	n =	Blood smear				IHA test			
		No. pos.	% pos.	PDI ¹	Modified PDI ²	No. pos.	% pos.	GMRT ³ pos. sera	GMRT all sera
<1	12	5	41.7	5.60	2.63	5	41.7	191	30
1	22	18	81.8	6.56	4.91	20	90.9	288	208
2	18	12	66.7	4.42	3.11	13	72.2	214	71
3-4	43	27	62.8	5.46	3.50	29	67.4	377	107
5-9	53	43	81.1	4.56	3.79	38	71.7	466	147
10-14	49	37	75.5	3.97	2.85	32	65.3	523	122
15-19	50	26	52.0	2.81	1.70	40	80.0	733	297
20-29	50	20	40.0	2.44	1.20	43	86.0	570	313
30-39	50	23	46.0	1.68	1.02	45	90.0	794	501
40-49	30	14	46.7	2.23	1.25	26	86.7	1142	589
50-59	20	11	55.0	2.18	1.43	16	80.0	404	184
Totals	397	236	59.4	3.83	2.37	307	77.3	540	207

¹ Parasite density index, after Bruce-Chwatt (1958).
² PDI modified by the addition of a class, designated 0.5, to include all presumptive negative blood smears.
³ Geometric mean of the reciprocal of the titer.

in parasite density is matched by an increasing trend in mean titer with age up to about age 50 years.

Table 4 compares rates and levels of IHA antibody with spleen size in the 2-9 year age group. The increase in mean titer with spleen size suggests a direct relationship. With the older age groups no correlation was found.

Table 5 presents the frequency distribution of parasite densities and IHA titers of the 397 Anuak subjects in a condensed form. This form was used in order to reduce the number of intervals at which errors occurred in age estimations, parasite counts and IHA endpoint determinations, each of these measurements being of a continuous and imprecise nature.

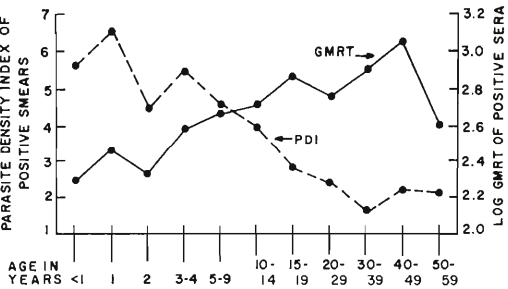


Figure 3. Parasite density indices for a total of 225 subjects whose blood smears revealed asexual stages, and mean log titers for a total of 307 subjects with IHA test-positive sera, by age group, Gambela.

Blood smears presumed, but not known to be negative were placed in parasite density class 0.5. Similarly, sera presumed, but not known to be negative were assigned a titer of 1:8. In each case, the “negatives” were given one-half the weight of the lowest positive value. By this arrangement, all test subjects were accounted for and the modified PDI became comparable to the GMRT. Differences between the adjacent age groups in Table 5 were analyzed statistically using Student’s t distribution. Significant differences in parasite density and mean IHA titer were found only between the 5-14 year and 15-29 year age groups.

Returning to Table 3 and Figure 2, it may be noted that in the 5-14 age group a higher proportion of blood films than serum specimens was positive. In Table 6, parasite rates and densities for 307 subjects with positive sera and for 90 subjects with negative sera are presented separately. Of the 90 persons

Table 4. IHA antibody rates and mean positive titers among 114 Anuaks, 2-9 years of age, by spleen size using Hackett’s classification, Gambela.

Spleen size	n =	IHA test		GMRT of pos. sera
		No. pos.	% pos.	
0	38	24	63.2	234
1	49	38	77.6	483
2	24	16	66.7	501
3	3	2	(67)	(250)

Table 5. Frequency distribution of parasite densities and IHA titers among 397 Anuaks by age group, Gambela.

Age group	n =	Parasite density class						Modified PDI	S.D.	P value (t test)
		0.5*	1 & 2	3 & 4	5 & 6	7 & 8	9 & 10			
<5	95	36	9	8	19	14	9	3.642	3.214	>.4
5-14	102	27	13	29	23	6	4	3.338	2.443	
15-29	100	56	23	16	5			1.450	1.527	<.001
30-59	100	54	33	10	2	1		1.170	1.208	>.1

* No parasites found.

Age group	n =	Reciprocal of titer by IHA test							Log GMRT	S.D.	P value (t test)
		8*	16-32	64-128	256-512	1024-2048	4096-8192	16384-32768			
<5	95	28	12	18	17	12	4	4	2.008	0.983	>.4
5-14	102	32	7	17	14	20	7	5	2.129	1.080	
15-29	100	17	9	9	19	33	10	3	2.484	1.000	<.02
30-59	100	13	4	11	19	41	8	4	2.634	0.892	>.2

* No antibody detected.

with non-reactive sera, 46 had positive blood films and 23 of these were in the 5-14 year age group. Children under 3 years of age with negative sera had significantly fewer and lighter parasitemias than children with positive sera in the same age group. A similar trend continued with the 3 and 4 year olds, but the differences were not significant.

Table 7 was made in order to examine the possibility that Anuak subjects with positive blood films and negative sera might have been suffering the acute stages of their first malaria infections. Since the gametocytes of *Plasmodium falciparum* are not evident until about

ten days after an infection has become patent (Boyd, 1949), one might expect to find antibody with the gametocytemia even in a primary falciparum infection. In Table 7, the gametocyte rate of children under age 5 with negative sera was one-half the rate of those with positive sera. This admits the possibility that some primary infections in this age group were detected in blood films before antibody appeared at measurable levels. In the 5-14 age group, however, the gametocyte rates were the same for IHA-positive and IHA-negative children.

Antigens of the simian parasite, *P. knowlesi*,

Table 6. Malaria parasite rates and densities among Anuak subjects with positive and negative IHA test results, by age group, Gambela.

Age group	IHA positive (titer ≥1:16)					IHA negative (titer <1:16)				
	n =	Blood smear				n =	Blood smear			
		No. pos.	% pos.	PDI	Modified PDI		No. pos.	% pos.	PDI	Modified PDI
<3	38	30	78.9	6.00	4.84	14	5	35.7	2.00	0.82
3-4	29	21	72.4	5.65	4.05	14	6	42.9	4.83	2.36
5-9	38	31	81.6	4.16	3.49	15	12	80.0	5.58	4.57
10-14	32	26	81.3	5.78	3.45	17	11	64.7	2.78	1.71
15-19	40	21	52.5	2.67	1.64	10	5	50.0	3.40	1.95
20-59	130	61	46.9	2.19	1.24	20	7	35.0	1.17	0.70
Total	307	190	61.9	4.03	2.52	90	46	51.1	3.68	1.95

Table 7. *P. falciparum* gametocyte rates among Anuak subjects with positive and negative IHA test results, by age group, Gambela.

Age group	IHA positive			IHA negative		
	n =	<i>P. falcip.</i> gametocytes		n =	<i>P. falcip.</i> gametocytes	
		No. pos.	% pos.		No. pos.	% pos.
<5	67	25	37.3	28	5	17.9
5-14	70	18	25.7	32	7	21.9
15-29	83	6	7.2	17	0	-
30-59	87	9	10.3	13	3	23.1
Totals	307	58	18.9	90	15	16.7

were employed in the IHA test under evaluation. It was of interest to determine, if possible, whether or not this heterologous antigen system was equally reactive in detecting antibody to each of the malaria species endemic in Gambela. Table 8 presents the rates and levels of IHA antibody separately for three groups of Anuaks: (A) those with negative blood films, (B) those with *P. falciparum* only, and (C) those with *P. malariae*, *P. ovale*, *P. vivax* or mixed infections including *P. falciparum*. Adult age groups were included for the sake of completeness, but it was among the children that the lower sensitivity of the IHA test for *P. falciparum* was demonstrated. Among children under the age of 5 years, those in group A had a significantly lower GMRT ($P < .001$) and rate of serum positivity ($P < .001$) than those in group C; those in group B had an intermediate rate and GMRT, neither differing significantly from the values in the other groups. With the 5-14 year olds, the GMRT of children in group B was slightly higher than that of children in group A, but significantly lower ($P < .05$) than that in group C.

Discussion

The rates and levels of malaria antibody in a population can provide information similar to that inferred from parasite rates and densities, from sporozoite rates and vector densities, and from spleen rates and sizes. In the survey reported here, an indirect hemagglutination (IHA) test (performed at CDC, Atlanta, Georgia) gave results which indicated many of the conclusions arduously arrived at by entomologic (Krafsur, 1970a) and parasitologic (Tables 1 and 2; unpublished data)

Table 8. Comparison of IHA test rates and mean titers among Anuak subjects with (A) negative blood smears, (B) *P. falciparum* only, and (C) smears positive for other species including mixed infections with *P. falciparum*, by age group, Gambela.

(A) Blood smear presumed negative				
Age group	n =	IHA test		GMRT
		No. pos.	% pos.	
<5	33	16	48.5	40
5-14	22	13	59.1	67
15-29	54	42	77.8	182
30-59	52	46	88.5	501
Totals	161	117	72.7	162
(B) Smear positive for <i>P. falciparum</i> only				
Age group	n =	IHA test		GMRT
		No. pos.	% pos.	
<5	38	27	71.1	113
5-14	58	39	67.2	116
15-29	42	38	90.5	663
30-59	40	35	87.5	415
Totals	178	139	78.1	232
(C)* Smear positive for other species and mixed				
Age group	n =	IHA test		GMRT
		No. pos.	% pos.	
<5	24	24	100.0	316
5-14	22	18	81.8	402
15-29	4	3	(75)	(90)
30-59	8	6	(75)	(200)
Totals	58	51	87.9	297

* Includes 38 mixed infections, mainly *P. falciparum* with *P. malariae*.

means. However, no single approach can entirely supplant another. Nor can malaria serology be interpreted accurately without additional knowledge of the local epidemiology (Voller and Bruce-Chwatt, 1968).

Tables 3, 4 and 5 and Figures 1, 2 and 3 show a good correlation between parasitologic and serologic data when the comparable indices are examined and when some factors of the local epidemiology are reviewed. At least three explanations can be offered for the peculiar (and statistically significant) elevations in the rates and levels of parasitemia and antibody for one-year-old children in the Anuak population sample. Perhaps one-third of these children, whose estimated ages were 12-23 months, were experiencing their first

peak malaria season without benefit of maternal antibody. All of them lacked the protection from mosquito bites afforded during the previous season by infant swaddling clothes. Added to these factors was a high probability of sampling bias: mothers of febrile babies quite likely volunteered their children for examination more readily than did mothers of healthy children. Since toddlers and older children are generally left unattended or under the casual supervision of siblings, this bias is believed to have affected only the sampling of babes-in-arms.

A second abrupt increase in the rate and level of IHA antibody was found among the 15–19 year old subjects, but in this instance it was accompanied by an equally abrupt decrease in the rate and density of parasitemias. This can be explained, partially, by two interdependent observations. First, young adults are attracted to Gambela from neighboring villages by the possibility of employment and to attend the school. Secondly, Krafsur (1970b) estimated an annual sporozoite inoculation rate of about 70 per person in some nearby villages as opposed to about 7 infective bites per person within Gambela. The influx of these hyperimmunized young adults was probably reflected in the test sample. A third factor, quite apart from the above, may involve an age-linked resistance to malaria as suggested by Voller *et al.* (1971).

Taliaferro (1949) noted the inverse relationship between the severity of avian malaria infections and age of the host, and added that in man it is difficult to separate age-related immunity from acquired immunity. However, as Voller *et al.* (1971) point out, the clinical and serological responses of adults to repeated experimental infections indicate a rapid attainment of resistance to malaria (cf. Jeffery, 1966) which stands in sharp contrast to the years of exposure to malaria required by children in the tropics before similar levels of resistance become apparent.

The concept of an age-related immunity can be applied very conveniently in the present study to explain the apparent sudden acquisition of protective antibody (resistance) to malaria at about the age of 15 years. It cannot be reconciled easily with the IHA antibody pattern in Anuaks with regard to the one year age group and the 10–14 year

age group. In the former, experience with malaria appears to be overestimated by the IHA test; in the latter group, this experience is clearly underestimated (Fig. 2). The underestimation, furthermore, appears to be related to infections with the predominant species, *P. falciparum* (Table 8), which reaches its highest prevalence in the 5–14 year old children (Table 2). As was noted earlier, of 90 subjects with IHA-negative sera, 46 had positive blood films and 23 of these were in the 5–14 age group (Table 6). Whether the failure of the IHA test to detect antibody in these individuals was a reflection of the test's lack of sensitivity or evidence of the immunological immaturity of these subjects could not be proven with the data in hand.

It is possible that the sensitivity of the IHA test might have been improved by the provision of serum as opposed to whole blood dried on filter paper, and further, by the use of a local antigen as opposed to the *P. knowlesi* antigen. However, these changes would have sacrificed the main advantages of the test without necessarily improving the results. Negative serologic results on subjects with patent or recent parasitemias have been reported by McGregor *et al.* (1965) and by Otieno *et al.* (1971) who used whole serum or plasma with local *P. falciparum* as antigen in the indirect fluorescent antibody test.

In conclusion, the simplicity of the filter-paper blood collection method (Mathews *et al.*, 1970) permitted its use in the field without prior training or special equipment. The rapidity of the IHA test procedure made possible the titration of over 500 sera in two days' time. And, most important, the results of the IHA test were compatible with, and complementary to, the parasitologic data and the epidemiologic observations.

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Fractionation of the Serologically Reactive Antigens of *Plasmodium falciparum*

R. O. McALISTER

Walter Reed Army Institute of Research,
Walter Reed Army Medical Center, Washington, D.C. 20012

ABSTRACT: The antigenic components detectable using the Indirect Hemagglutination (IHA) test and the Soluble Antigen Fluorescent Antibody (SAFA) test were isolated from erythrocytes of owl monkeys (*Aotus trivirgatus*) infected with *Plasmodium falciparum*. The components reactive in the two tests were found to be solubilized using either repetitive freeze-thaw or disruption in a French pressure cell for lysis of washed erythrocytes. The components were insoluble in $(\text{NH}_4)_2\text{SO}_4$ concentrations of greater than 50% at 0 C. Gel filtration studies demonstrated all reactive material to be of a molecular weight greater than the upper exclusion limit of Sephadex G-200.

Preliminary results obtained using Sepharose 6B chromatography indicate the reactive components to lie in or near the void volume eluted from this gel. The finding that these materials are of such uniformly high molecular weight is discussed.

Following the initial report (Geiman and Meagher, 1967) of susceptibility of a simian host, *Aotus trivirgatus*, to infection with *Plasmodium falciparum*, considerable advances along several avenues have been accomplished relating to the biology of this host-parasite complex. Pathological (Jervis et al., 1972), hematological (Geiman et al., 1969) and biochemical alterations (Geiman, 1969) resulting from this infection have recently been reported. The nature of the immune responses of this host to the parasite has also been studied, and a protective response by the use of γ -irradiated inocula has been obtained (Sadun et al., 1969). A degree of cross-protection was also found to exist between different strains in passive transfer experiments (Diggs et al., 1972).

Previous studies of the antigens of *P. falciparum* (Mahoney et al., 1966; Wellde et al., 1969) have been conducted using lysates obtained from infected chimpanzee blood demonstrating relatively lower parasitemias (Hickman, 1969). The high parasitemias which occur during the course of this infection in owl monkeys coupled with the greater availability of these animals, present a unique opportunity to obtain large quantities of parasite material. The properties of antigens obtainable in this host-parasite system were therefore studied. The following report details the results of initial investigations into the purification and

properties of the serologically reactive components obtained from *P. falciparum*-infected *Aotus* erythrocytes.

Materials and Methods

A. Erythrocyte isolation and lysis

The Camp. strain of *P. falciparum* was utilized for these studies. *Aotus* monkeys were housed and cared for as previously reported (Hickman, 1969).^{*} An appropriate dose of parasites obtained from either fresh blood from a donor animal or from a frozen stabulate was administered intravenously. Infections were monitored daily using Giemsa-stained films of peripheral blood. When more than 20% of the erythrocytes were parasitized, the animals were euthanized by femoral vein and cardiac puncture; blood was collected in heparinized syringes.

The heparinized plasma was collected following centrifugation at 600–800 \times g for 15 min at 2–5 C. The erythrocytes were resuspended with 50 ml cold 0.15 M NaCl and centrifuged as above for 10 min. After washing 4 consecutive times under identical conditions, the cells were centrifuged for 15 min and the packed cell volume measured. The cells were then reconstituted in 4 volumes of

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^{*} In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animals Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

PBS (0.01M Na_2HPO_4 - NaH_2PO_4 , pH 7.5-0.15 M NaCl). Erythrocytes were lysed either by repetitive freeze-thaw (Wellde et al., 1969) or by use of a French pressure cell at 1000 lbs/in². After lysis, erythrocyte counts were determined using a hemacytometer. The disrupted material following lysis was centrifuged at $15,000\text{--}23,000 \times g$ at 1-2 C. The supernatant fluid was carefully removed from the pellet containing erythrocyte membrane fragments and free parasites, and stored at -70 C for subsequent studies. The pellet was suspended in an equal volume of PBS. Thin films were subsequently prepared and stained with Giemsa to ascertain obvious morphological effects of the lytic process on the parasites.

B. Fractionation and purification

All ammonium sulfate fractionations were carried out at 0 C. Gel filtration studies were performed at room temperature (22-26 C) using dextran and agarose gels (Sephadex^{T.M.} and Sepharose^{T.M.} respectively).

The gels were equilibrated in 0.1 M Tris (hydroxymethyl) amino methane-HCl, pH 8.0-0.022% (w/v) NaN_3 ("Tris") or in this buffer containing 0.15 M NaCl ("Tris-saline"). Fractions were collected with a portable fraction collector (Buchler Instruments) set for drop counting. Protein analyses were conducted using the method of Lowry et al. on washed TCA precipitates, employing crystalline bovine serum albumin as standard, or alternatively by absorbance at 280 nm, using as $E_{1\text{cm}}^{1\%}$ the value of 14.6, a published value for human γ -globulin (McDuffie and Kabat, 1956). Absorbances were measured with a Zeiss PMQII Spectrophotometer equipped with quartz cells of 1 cm light path. Volume reduction of antigenic fractions, when necessary, were carried out in cellulose dialysis tubing of pore size 48 Å avg, under reduced pressure.

C. Assay of antigenic activity

All antigenic preparations were tested in duplicate against pools of normal and hyper-immune human sera. The normal sera were obtained from either healthy individuals who had undergone physical examinations, or from donated plasma obtained from the blood bank at Walter Reed General Hospital. The hyper-

immune serum pool was obtained from a blood bank located in a highly endemic area in Nigeria.

The soluble antigen fluorescent antibody (SAFA) test (Sadun and Gore, 1968) was performed with the following modifications: All sera (normal and immune) were tested at a single dilution of 1:5 unless otherwise specified. The fluorometer dial was set to zero by interrupting the light path with an opaque barrier. Results of dial readings for both normal and immune sera were then recorded, and the difference in fluorescence dial reading (Δ FDR) between the two was used as an expression of serologic activity. Normal serum pool FDR values varied from 9.0-14.5 units, depending on antigen concentrations used.

For testing of antigenic fractions, the standard 47 mm Millipore^{T.M.} filters (pore size 0.45μ) were cut into 6.5-7 mm discs. To each of these discs was applied 0.010 ml of the antigen solution to be tested. After a five minute absorption period, the discs were dried at 37 C for 30 min.

The indirect hemagglutination test (IHA) was performed according to the published method (Wellde et al., 1969) with the following exceptions: The sheep cells used for the sensitization step were preserved by glutaraldehyde fixation (Bing et al., 1967) and stored at 4 C as a 30% suspension (v/v) in distilled H_2O . All sera were absorbed with an equal volume of this 30% suspension for 10 min. The normal rabbit serum used in the diluent was obtained from a large pool obtained from a commercial source. Hemagglutination patterns were developed at room temperature, and serum titers were expressed as the reciprocal of the highest serum dilution giving complete agglutination. Optimal antigen concentrations for use in sensitizing the preserved erythrocytes was determined for each antigen preparation. The use of concentrations above that found to be optimal resulted in nonspecific agglutination in the presence of both immune and normal sera. All antigen fractions were tested in duplicate versus the paired hyperimmune and normal serum pools; the initial serum dilution utilized was in all cases 1:20. The test was carried out using the Microtiter^{T.M.} (Cooke Engi-

Table 1. Antigen reactivity of lysates and (NH₄)₂ SO₄ Fractions.

Animal #	Terminal parasitemia	SAFA (ΔFDR)*		Protein (mg/ml)	
		Ly-sate	(NH ₄) ₂ SO ₄ precipitate	Ly-sate	(NH ₄) ₂ SO ₄ precipitate
129	21%	15	16	26.2	2.2
130	38%	16	14	34.1	2.4
131	70%	37	39	32.4	1.8
006	53%	22	41	18.1	2.1
007	68%	21	33	37.7	2.0
008	58%	18	22	24.3	2.4
009	66%	53	70	19.7	1.8
631	44%	46	66	20.4	2.2
374	35%	33	38	29.6	1.6

* Mean values of duplicate samples.

neering Co., Alexandria, Virginia) apparatus and "U" type plates (Linbro Chemical Co., New Haven, Connecticut).

Results

A. Isolation of antigenic material

The freeze-thaw method of lysis as a means for initial isolation of antigen was investigated due to the success reported in previous studies (Welde et al., 1969). Erythrocyte counts made on the lysate prior to centrifugation revealed the degree of erythrocyte lysis to be in most cases 99% or more; however, Giemsa-stained preparations revealed the pellet to contain many intact parasites which appeared unaffected by the lytic process. By contrast, it was found that 1000 lbs/in² using the French pressure cell gave not only 100% lysis of the erythrocyte, but resulted in disruption of a number of parasites as well, when comparisons were made between the morphological appearance of pellets obtained after aliquots of suspended erythrocytes were lysed by each method. A small amount of additional protein and antigenic activity measurable with the IHA test was recovered in the French pressure cell-lysed preparation. These differences were deemed insignificant, since both preparations showed identical fractionation properties following (NH₄)₂ SO₄ treatment.

Protein measurements on lysates were performed on TCA precipitates using the Lowry method (1951). It was considered appropriate, however, to use a spectrophotometric assay following (NH₄)₂ SO₄ precipitation and subsequent dialysis.

B. Antigen reactivity and (NH₄)₂ SO₄ fractionation

Initial studies using 10 monkeys were conducted to determine the relative reactivity of various antigen preparations from different animals. The potential use of (NH₄)₂ SO₄ fractionation to remove contaminating host hemoglobin (Welde et al., 1969) was also investigated.

It was empirically determined that most of the host hemoglobin was soluble in 62% (3.34 M) (NH₄)₂ SO₄ at 0 C. An aliquot of each lysate preparation was subjected to (NH₄)₂ SO₄ precipitation at this concentration at 0 C for 30 min, followed by centrifugation at 11,000 × g for 15 min. The red supernatants were decanted and the pellets washed 3 times with 70% (3.75 M) (NH₄)₂ SO₄ with intermittent centrifugation and finally re-dissolved in a minimal quantity of PBS. Following dialysis against a large volume of PBS, the dissolved precipitates were adjusted back to the original volume and were tested for SAFA reactivity, along with the original lysate and the dialyzed (NH₄)₂ SO₄ supernatants. The precipitate fractions were assayed for their protein content. It was found (Table 1) that the antigens detectable in all preparations were insoluble at this salt concentration. The pellets recovered following salt precipitation varied in color from pale brown to deep red; the amount of contaminating hemoglobin visually seen appeared to be less of a problem when using small initial volumes of lysate for the precipitation. In all cases, negligible activity could be detected in the supernatant following precipitation, even when pressure dialysis was utilized to return the supernatant fluid volume back to that of the original lysate.

For further investigation of the properties the antigen in the presence of (NH₄)₂ SO₄, experiments were designed to elucidate antigen solubility over a wide range of salt concentrations.

A preparation of lysate obtained from an animal which had a terminal parasitemia of 84% was divided into 1 ml aliquots. Each aliquot initially contained 16.5 mg protein/ml. Saturated (NH₄)₂ SO₄ was then added to a final concentration of from 20%–75% M at 0 C at 5% intervals. Following centrifugation

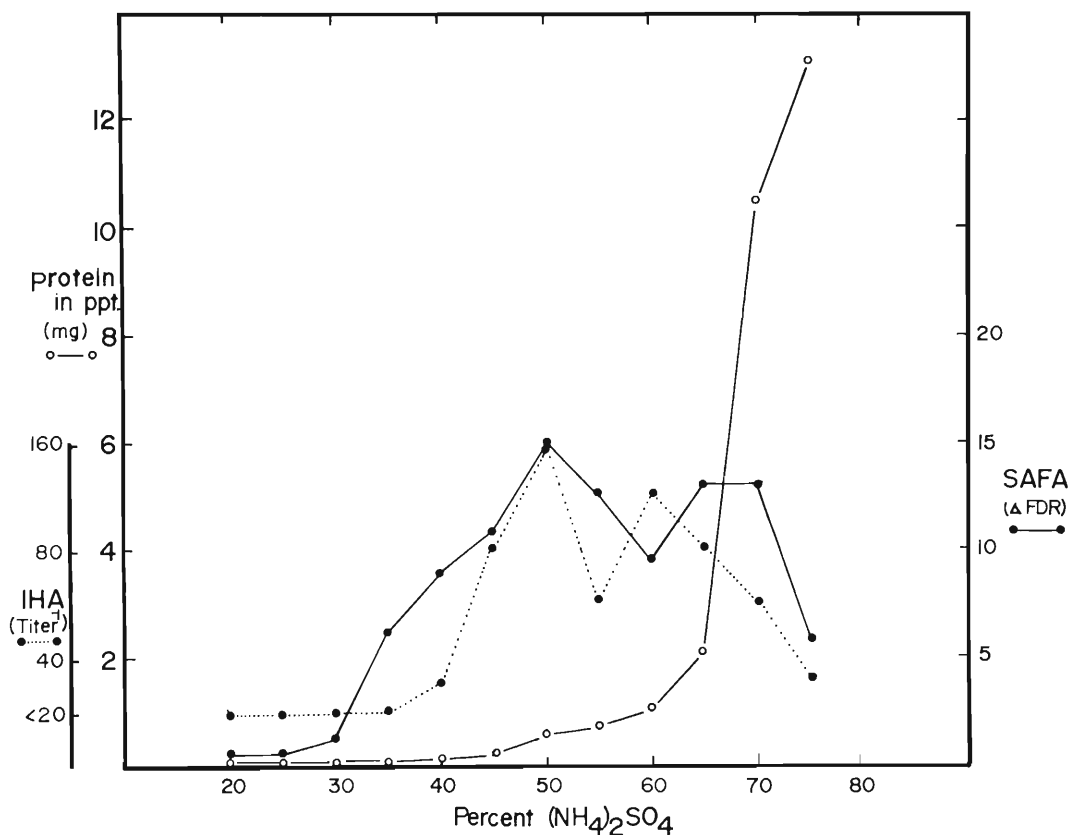


Figure 1. Solubility of antigenic material in $(\text{NH}_4)_2\text{SO}_4$. Serological values are means of duplicate samples. Each lysate sample contained 16.5 mg protein in total volume of 1 ml. Following 30 min at 0 C, pellets were removed by centrifugation and redissolved in 1 ml of PBS, except those samples at 65%, 70%, and 75%, which were redissolved in 2 ml. Protein determinations and serological tests were performed following dialysis. Each sample was tested undiluted in the SAFA test versus a 1:5 dilution of normal and immune sera. IHA values were obtained using a 50-fold dilution of each sample.

and dialysis, the paired antigen precipitates at each point were redissolved in 1 ml of PBS unless the pellet size required the use of a larger volume. All antigen precipitates including the paired unfractionated control samples were tested for reactivity in the SAFA test and in the IHA test against the pooled immune and normal sera. Following testing, protein concentrations were determined and expressed as total protein (mg) in each pellet. Each fraction was used undiluted for soaking discs to be used in the SAFA test; each preparation was diluted 50 fold for sensitization in the IHA test. The results

expressed as mean values in Fig. 1 indicate all antigenic material to be insoluble at salt concentrations above 50% saturation. When this experiment was repeated utilizing a preparation from another animal which had a terminal parasitemia of 83% and a lysate protein concentration of 7.6 mg/ml, similar curves were obtained. Preliminary studies suggest that the antigens are stable frozen at -70°C . Preparations stored in this fashion have shown no detectable loss in serological reactivity after periods of up to five months. The $(\text{NH}_4)_2\text{SO}_4$ precipitated material after dialysis, appears stable for at least several

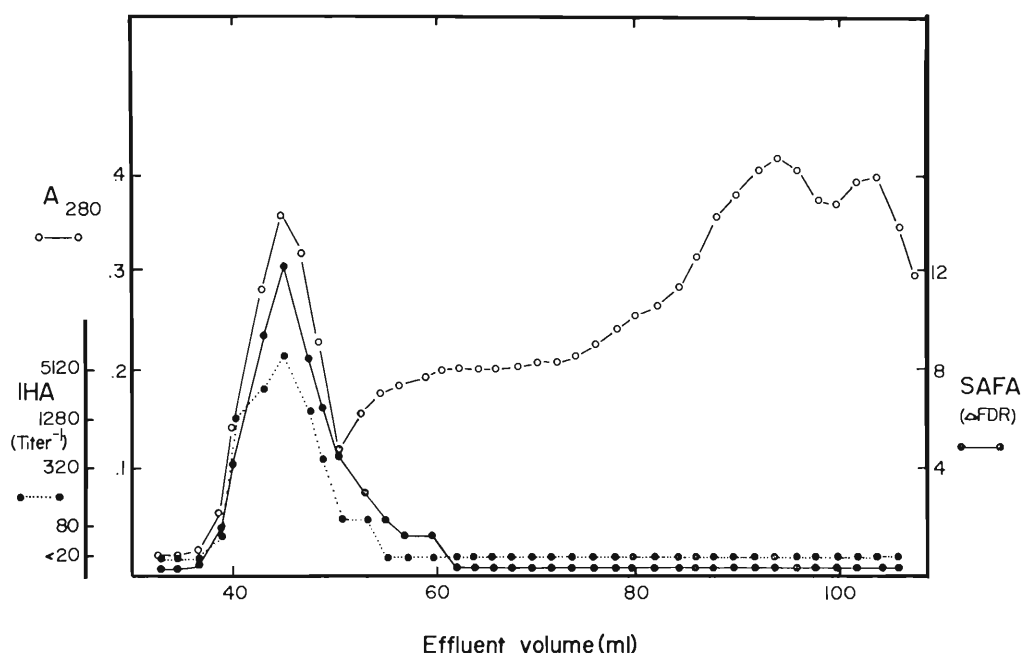


Figure 2. Elution profile of protein and antigenic activity after chromatography on Sephadex G-200. Serological values are means of duplicate samples. Starting material was $(\text{NH}_4)_2\text{SO}_4$ purified and contained 16.1 mg protein in total volume of 3 ml. Absorbance at 280 nm was performed on each 2 ml fraction. Fractions were tested undiluted versus a 1:5 dilution of normal and immune sera. IHA values were obtained using a 5-fold dilution of each fraction.

hours at room temperature, and several of these preparations have been repeatedly frozen at -70°C and thawed with no detectable ill effects on measurable activity. No change in reactivity was detected when one aliquot of the antigen was heated at 56°C for 30 min and tested in parallel with an unheated aliquot.

C. Gel filtration studies

To establish the molecular sieve fractionation properties of ammonium sulfate-purified antigen, a pool of lysate taken from three different animals was batch-treated with 62% $(\text{NH}_4)_2\text{SO}_4$ as described above and dissolved in PBS to a final concentration of 5.34 mg/ml. Three ml (16.1 mg protein) of this preparation were applied to a Sephadex G-200 column of dimensions 2.5×38 cm. The gel was equilibrated in Tris buffer and the column was pre-calibrated using 3 ml of a 0.2% solution of Blue Dextran 2000 using a flow

rate of 2 ml/min. Each 2 ml fraction was read undiluted at 280 nm. Fractions were tested undiluted in the SAFA test, and diluted 1:5 for testing IHA reactivity. As seen in Fig. 2, a major part of the activity detectable was present in the first protein peak. Curves are drawn through mean values obtained in the serological tests. This experiment was subsequently repeated two times using different antigen pools, each purified with ammonium sulfate. In every case, all detectable serological activity showed similar fractionation properties. Following testing, fractions containing activity were pooled and protein concentrations were determined. It was noticed that 2–3 fractions within the initial peak eluting from G-200 contained sufficient turbid material to interfere with absorbance measurements. This material could be removed by centrifugation at $20,000 \times g$ with no detectable loss in serological activity.

In all cases, detectable serological activity

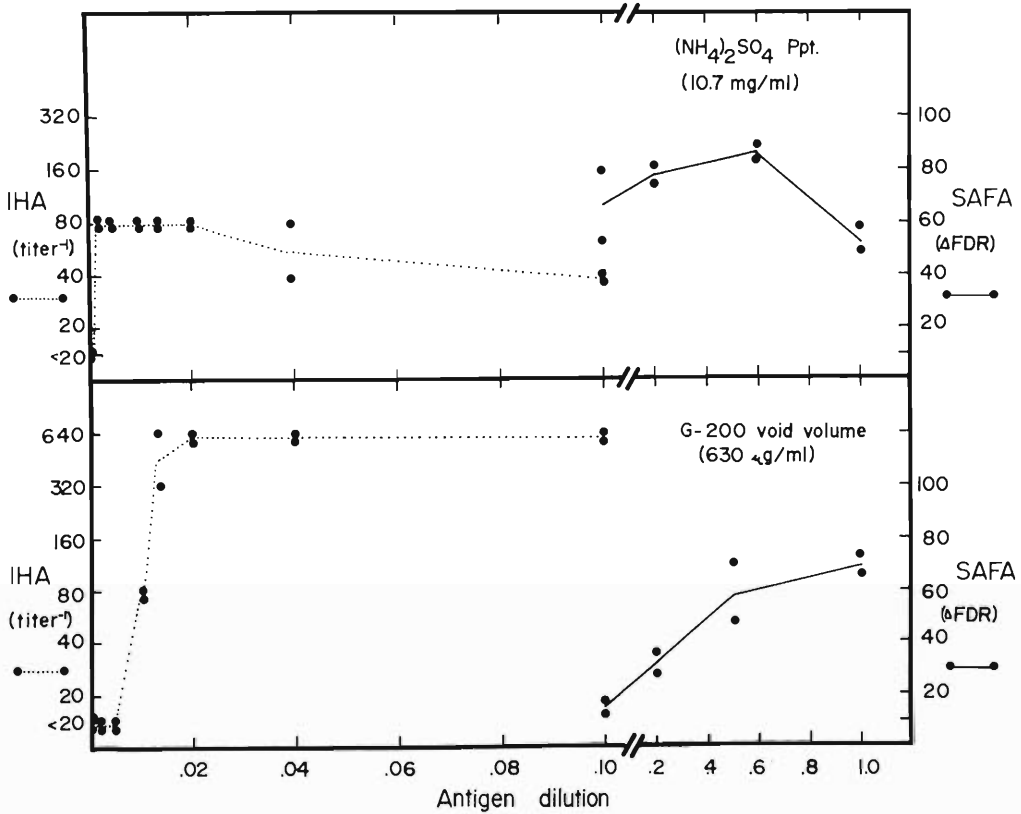


Figure 3. Comparison of effect of dilution on serological activity on $(\text{NH}_4)_2\text{SO}_4$ purified antigen and $(\text{NH}_4)_2\text{SO}_4$ purified G-200 void volume material. Dilution of antigen was effected over the range of 1:10–1:1000 for IHA test, and undiluted–1:10 for the SAFA test. Each point represents one determination. Values in parentheses represent initial undiluted protein concentrations of the respective antigenic preparations. SAFA values were obtained using 1:5 dilution of immune and normal sera. Normal serum FDR values ranged from 9.5–12.5.

was localized in a peak containing 17–27% of the protein present in the $(\text{NH}_4)_2\text{SO}_4$ purified material added to the column, regardless of whether the gel was equilibrated in Tris buffer alone or in Tris-buffered saline. The lower half of Fig. 3 illustrates the reactivity of a representative preparation obtained from the void volume following G-200 gel filtration. The upper part of the figure shows values obtained with several dilutions of the $(\text{NH}_4)_2\text{SO}_4$ purified starting material. The SAFA curves were obtained using normal and immune serum diluted 1:5.

Antigenic fractions obtained from G-200 were studied for fractionation properties on

agarose as follows: An infected monkey with a terminal parasitemia of 71.5% was exsanguinated and the lysate prepared as described previously. Following precipitation with $(\text{NH}_4)_2\text{SO}_4$, 10.9 ml of antigen were obtained with a protein concentration of 10.7 mg/ml. Five ml of this solution (53 mg) were fractionated on a 2.5×87 cm Sephadex G-200 column equilibrated in Tris-saline. After collection and pooling of the active fractions and centrifugation at $20,000 \times g$ — 1 hr, the preparation had a concentration of $370 \mu\text{g/ml}$ in a total volume of 53 ml. Five ml (1.85 mg) of this preparation were then loaded on Sepharose 6B column of dimensions

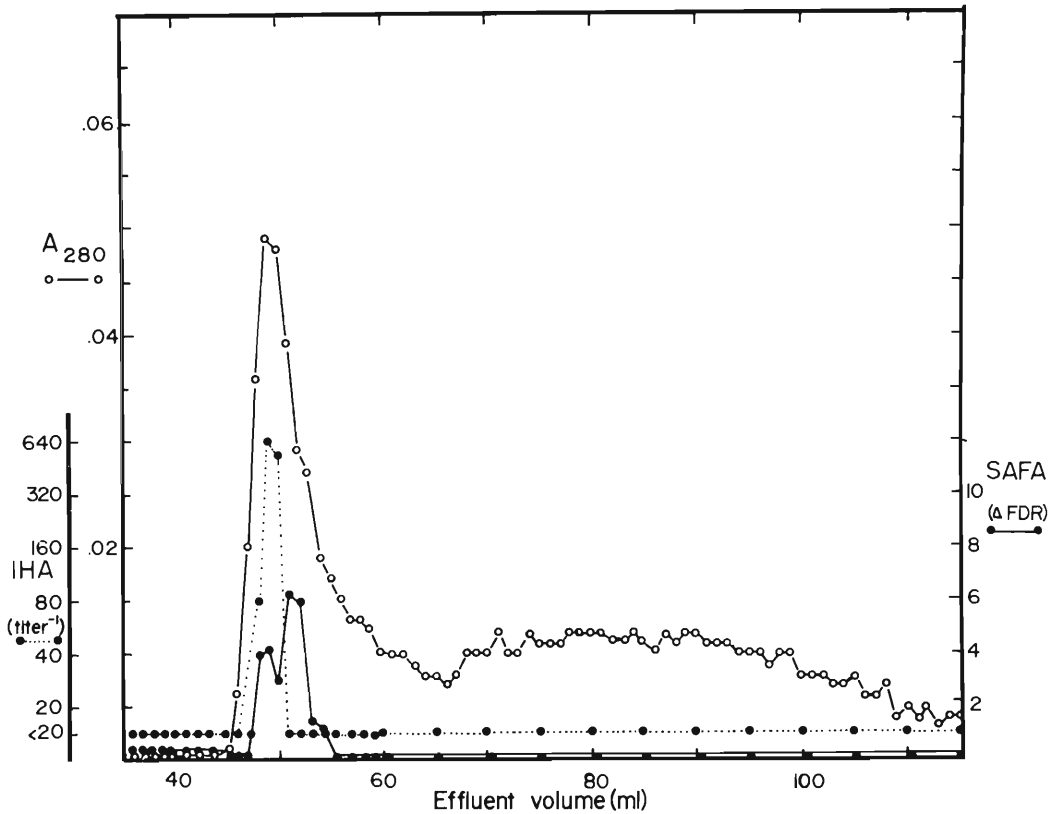


Figure 4. Elution profile of protein and antigenic activity after Sepharose 6B chromatography. Serological values are means of duplicate samples. Starting material was $(\text{NH}_4)_2\text{SO}_4$ and Sephadex G-200 purified and contained 1.85 mg protein in a total volume of 5 ml. Absorbance at 280 nm was measured on each 1 ml fraction. Fractions were tested undiluted in the SAFA test versus a 1:5 dilution of normal and immune sera. IHA values were obtained using a 5-fold dilution of each fraction.

2.5 × 38 cm. The gel had been previously equilibrated with Tris-saline and had been calibrated with a solution of Blue Dextran 2000 purified with Sepharose 6B to contain only material of a molecular weight above the upper fractionation limit of this gel. A flow rate of 10 ml/hr was maintained and 1 ml fractions were collected. Measurements of absorbance at 280 nm were performed, and each fraction was tested undiluted for SAFA reactivity and at a dilution of 1:5 for IHA response. The results are reported in Fig. 4; all antigenic material was present in the initial peak of protein, corresponding to material voided by the gel.

Discussion

Repetitive freeze-thaw was found to be an effective means for the initial isolation of serological antigens. These observations are in agreement with earlier results using lysates obtained from chimpanzee blood (Welde et al., 1969). It was additionally determined that disruption effected by gentle lysis with the French pressure cell gave similar antigenic preparations. Lysates prepared using the French cell were of slightly larger volume, due to the more compact nature of the pellet. This effect was judged due to the much smaller erythrocyte membrane fragments present following French cell treatment. The

shearing forces present at the orifice of the French cell apparently yield simultaneous fragmentation of the erythrocyte membranes at several points. By contrast, the pellet following centrifugation of the freeze-thaw isolated lysate contained a dense area of free parasites and a few unlysed erythrocytes overlaid with an amorphous stroma layer. This amorphous layer was composed of large erythrocyte "ghosts." Based on serological activities, it was concluded that either method of antigen isolation was suitable for initial solubilization of reactive components.

The degree of variation observed (Table 1) in SAFA reactivity of preparations obtained from different animals show no obvious relationship to parasitemias observed in the animal at the time of sacrifice. It is possible that the serological activity measured in the SAFA test is produced at some discrete stage in the life cycle of the plasmodium. The somewhat asynchronous nature of this infection is compatible with this hypothesis.

Following the initial studies, the IHA test was incorporated as an additional means of assaying antigenic activity. The additional sensitivity available using this simple test, as well as the relatively small amounts of antigen necessary and the desirability of assaying the reactive material using two independent and widely used tests for malaria antibody, influenced this decision.

The finding that both SAFA and IHA activities were insoluble in concentrations of $(\text{NH}_4)_2\text{SO}_4$ above 50% (2.67 M) at 0°C (Fig. 1) confirmed the use of 62% $(\text{NH}_4)_2\text{SO}_4$ as a valid salt concentration for initial removal of host hemoglobin. SAFA and IHA values of Fig. 1 are not corrected for volume differences in the re-dissolved pellets; these differences became significant only when concentrations of salt (> 67%) of sufficient size were used to bring down the host hemoglobin. If the assumption that serologic activity is diluted proportionately with volume changes and the values obtained above 65% $(\text{NH}_4)_2\text{SO}_4$ are corrected for volume differences, the serological values still do not approach those measured at the lower salt concentrations.

The findings reported following Sephadex G-200 molecular sieve chromatography indicate that the reactive elements fractionate in or near the upper exclusion limit of this gel.

No obvious differences in molecular size could be detected using pools composed of lysates obtained from different donor animals.

Comparison of the reactivity of the different antigen preparations following $(\text{NH}_4)_2\text{SO}_4$ purification and Sephadex G-200 chromatography indicate similar behavior over a variety of dilutions in both tests. The SAFA reactivity of both preparations was found to be comparable at several dilutions. Similarly, both preparations gave a maximum titer in the IHA test over a wide range of concentrations of antigen. The G-200 purified antigen gave a higher maximum titer with the hyperimmune pool than did the antigen which had been purified only with $(\text{NH}_4)_2\text{SO}_4$. This finding indicates that one or more of the lower molecular weight components removed by G-200 gel filtration may partially inhibit the sensitization step with the reactive elements of the antigen, probably due to competition for available sites in the surface of the glutaraldehyde-preserved erythrocytes.

The coupling of salt fractionation with molecular sieve chromatography gives rise to material which has been markedly purified with respect to protein concentration. The $(\text{NH}_4)_2\text{SO}_4$ fractionation normally precipitates around 5–10% of the protein, and of that material, 15–20% of the protein normally appears in the void volume region of dextran chromatography. More precise estimates concerning the actual degree of purification await studies designed to elucidate protein content of the antigenic material.

The results of the Sepharose 6B study, if substantiated, indicate that all antigenic reactive in the two tests employed in these preparations is composed of very high molecular weight components. The upper exclusion limit of Sephadex G-200 is approximately 800,000 for globular proteins. The consistent findings of all serological activity in or near the void volume following G-200 gel filtration imply that all antigens detected in the tests described were of a minimum molecular weight of 800,000. Furthermore, the preliminary result observed using material from one animal, if determined to be representative, imply the reactive components to be at or near the upper exclusion limit of Sepharose 6B as well. The upper exclusion limit for Sepharose 6B is approxi-

mately 4×10^6 for globular proteins. Regardless of the final precise estimate of molecular weight, it is obvious that the reactive serological antigens obtained in this study are uniformly of at least 800,000 molecular weight.

Investigations designed to elucidate some of the chemical and biological properties of this partially purified material are currently under way. Considering the methods used for lysis and the number of intact trophozoites seen in these preparations following centrifugation, it is presumed that a major part of the reactive material present in these lysates originates either in the erythrocyte cytoplasm outside of the parasite itself, or else comes from stages of the parasite which are sufficiently fragile to be disrupted by the lytic process, with concomitant release of serologically detectable material.

The finding that the reactive material or materials are of such high molecular weight ($> 800,000$) may indicate a spontaneous aggregation of smaller reactive molecules has occurred during the preparative procedure. Studies are currently under way to investigate if disaggregation into smaller molecules without concomitant losses in the chemical and physical properties of this material is possible.

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Isolation of *Plasmodium berghei* by Use of a Continuous-flow Ultrasonic System: A Morphological and Immunological Evaluation

R. B. PRIOR AND J. P. KREIER

The Ohio State University, College of Biological Sciences,
Department of Microbiology, 484 W. 12th Avenue, Columbus, Ohio 43210

ABSTRACT: The continuous-flow sonication system developed in this study was suitable for production of large quantities of intact plasmodia free from their host cell membranes. The free parasites were harvested by simple differential centrifugation. High-power electron micrographs revealed that the internal structure of the parasites was well preserved, while low power ones revealed the freedom of the parasite preparation from membrane contamination. The free-parasite antigen was anti-complementary at low dilutions, but not when properly diluted. Block type titration showed the optimal antigen dilution to be that dilution which eliminated anticomplementary activity. The serum of none of the normal rats tested fixed complement with the free-parasite antigen while all sera from rats recovering from acute *Plasmodium berghei* infection fixed complement with the antigen. Titers varied among the animals tested and with the stage of recovery of the donor rat. Lysates of free *Plasmodium berghei* were unstable when stored frozen or when heated. Lyophilization of the antigen in the absence of protective agents destroyed the CF activity, but the addition of 2% PVP to the antigen before lyophilization partially protected it. The antigen reactivity was best preserved by freezing (-20°C) the intact free parasites. Most of the parasites freed by the continuous-flow system of sonication were infectious.

Investigators have tried various physical, chemical, and immunolytic means to effect separation of malaria parasites from their host erythrocytes; but, they reported loss or change of the antigenic structure, low reproducibility of test results, damage to the parasites, or incomplete separation (Cook *et al.*, 1969). In an effort to overcome the problems encountered with other means of separation, some investigators have tried to use high-frequency sonic waves to effect separation of plasmodia from host erythrocytes (Verain and Verain, 1956; Kreier *et al.*, 1965; and Rutledge and Ward, 1967). Since ultrasonic energy is mechanical in nature, disruption of malaria-infected erythrocytes by ultrasound should not adversely affect the chemical composition of the parasites. However, although each investigator reported release of malaria parasites from erythrocytes by ultrasound, in the batch treatment used by these investigators many free parasites were degraded following release thereby precluding the use of

such systems to obtain large quantities of free parasites.

In the study to be reported, ultrasonic energy was used to free malaria parasites from their host erythrocytes, but a continuous-flow system of sonication was designed and built to replace the batch system of sonication used in previous studies. In the continuous-flow system of sonication, each erythrocyte receives approximately equal exposure to ultrasonic energy, and the parasites freed from their host erythrocytes are then rapidly removed from the cavitating ultrasonic field and its destructive forces.

Materials and Methods

Description of continuous-flow sonication system

The continuous-flow sonication system consisted of a pump, ultrasonic generator, and continuous-flow sonication chamber.

The pump used was a Harvard Rotary Peristaltic Pump Model 1225 (Harvard Apparatus Co., Inc., Millis, Massachusetts), and the ultrasonic generator used was a commercially available Bronwill Biosonik Model

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BP 1 generator (Bronwill Scientific, Rochester, New York). The power dial on the generator was divided into 100 increments which represented relative acoustic intensity. The transducer was equipped with a standard $\frac{3}{8}$ " probe tip, and the frequency of the ultrasonic waves was 20,000 Hertz (cycles per second).

The continuous-flow unit was constructed of 316 stainless steel. The passage through which the specimen passed was a simple tubular bore 0.11 inches in diameter which passed just under the tip of the probe. This design was selected to reduce the eddying motions of the specimen within the chamber and to permit direct contact of the specimen with the transducer probe tip. The unit was constructed so that when the probe tip was inserted into it the only place of direct contact between the unit and the transducer was the point where curvature begins on the probe. At this point, an "O" ring sealed the chamber and secured the unit to the probe tip. This design feature allowed the transducer to vibrate freely. The entrance to the chamber accommodated the standard $\frac{1}{4}$ " \times $\frac{1}{8}$ " Tygon tubing from the peristaltic pump. Void volume of the actual sonication chamber was 0.0138 cubic inches or 0.2 cubic centimeters. A diagram with the dimensions of the chamber is available (Prior and Kreier, 1972).

Preparation of specimens for sonication

Charles River inbred rats 5 to 10 weeks of age were the experimental animals used in this study. The rats were maintained on a diet of Purina Lab Chow, and water was given *ad libitum*. The *Plasmodium berghei* strain used was originally obtained through the courtesy of Dr. Herbert Cox, then of the New York University, and was maintained in susceptible rats by injecting approximately 5×10^7 to 1×10^8 parasitized erythrocytes intraperitoneally.

Infected blood was collected by cardiac puncture using disodium ethylenediamine tetraacetate as the anticoagulant (1 mg/ml of whole blood). The whole blood from 2 or 3 rats was pooled and centrifuged at 4°C at $500 \times G$ for 15 min. Following centrifugation, the supernatant plasma and buffy coat were removed. The erythrocytes were subsequently washed twice ($500 \times G$ for 15 min.) in Alsever's solution pH 7.2.

Leukocytes not removed by aspiration were removed by filtration of the washed erythrocytes through columns of packed powdered filter paper (Fulton and Grant, 1956; Cook *et al.*, 1969). Approximately 2 cubic centimeters of Whatman Column Chromedia CF-11 Fibrous Cellulose Powder were tightly packed in a 10 ml syringe over a plug of glass wool. The washed blood was poured into the inverted syringe and forced with the plunger through the filter paper column at a rate of about 10 ml of washed blood per minute. The washed, filtered erythrocytes were then made up to a concentration of 10.0 ± 0.5 percent by volume. The washed erythrocytes could be stored in the cold (4°C) until used but not for longer than 24 hours.

Determination of flow rate and sonic intensity

The flow rate was set at 29.6 ml/min., the maximum possible with our pump to minimize the duration of direct exposure to the cavitating ultrasonic field. With flow rate held constant, the power dial was advanced stepwise, samples of the sonicated specimen were collected, and the degree of hemolysis was determined. The percent hemolysis was then plotted against the power dial setting (relative acoustic intensity). Sonication at power levels which caused over 80% hemolysis caused significant debris formation. As the blood passed through the chamber, a characteristic "buzzing" or "hissing" sound was heard, and the bright red of the specimen changed to a darker colored red. The rapid flow of the precooled specimen was sufficient to dissipate heat generated in the chamber without raising the temperature of the specimen significantly.

Harvesting the free parasites by differential centrifugation

The sedimentation rate of washed rat erythrocytes was determined in standard microhematocrit capillary tubes to be 1.5 mm/hr. at $1 \times G$ force. The spin time necessary to sediment all the erythrocytes at $250 \times G$ force was estimated from these data.

Following sonication, the specimens were placed in centrifuge tubes, the height of the specimen was measured, and the specimens

were centrifuged at $250 \times G$ (avg.) for the time determined from the sedimentation rate data. Immediately following this initial spin, the red supernatant fluids were decanted carefully and placed in other centrifuge tubes. The pellets were discarded.

The next centrifugation was carried out at $600 \times G$ for 3 minutes for each centimeter of height. The red supernatant was discarded, and the free-parasite pellet was then washed twice in 5 ml Alsever's pH 7.2 ($600 \times G$ for 15 min.).

The bottom of the parasite pellet was dark brown; above this was a layer which was light brown; and above this was a gray-colored layer. Occasionally, a thin, white, fluffy layer was observed atop the free parasite pellet. This layer which contained erythrocyte ghost cells was routinely aspirated. The free-parasite pellet was smooth in consistency and dispersed quite readily in the washing fluids.

Preparation of specimen for microscopic examination

Thin films of the free-parasite pellet were prepared on pre-cleaned microslides, Giemsa stained, and examined with a Zeiss standard WL microscope. Wet mount preparations of the free parasites were also made and examined by interference-contrast microscopy. For study of thin-sections by electron microscopy, the free-parasite pellets were processed by the method described by Cook *et al.* (1969). The carbon replica technique used was the one described by Bradley and Williams (1957) except that germanium was used for shadowing. The carbon replicas and the thin sections of the free plasmodia were examined with the Zeiss EM 9S electron microscope.

Preparation of specimens for complement fixation tests

One volume of packed free parasites was suspended in 19 volumes of Triethanolamine-buffered salt (TBS) solution pH 7.3 to yield a 5 percent by volume suspension. Following adjustment of parasite concentration, the parasites were lysed by placing them in a CO_2 -acetone bath until frozen, then into a 56°C water bath until thawed for three complete

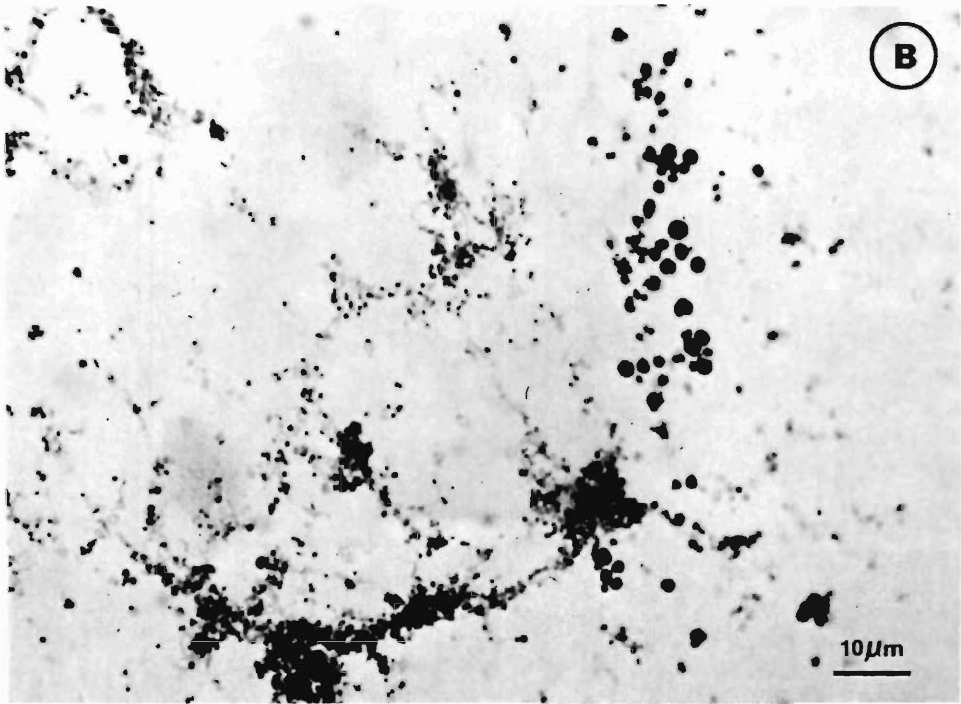
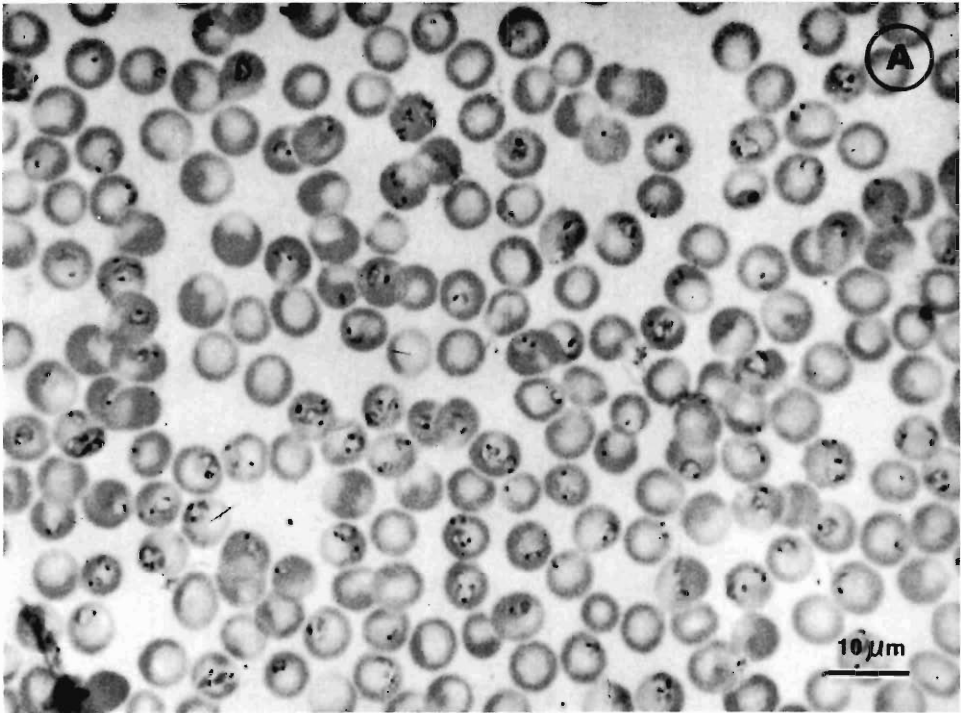
cycles. The light-brown liquid yielded was the free parasite antigen used in the complement fixation tests.

For control purposes, a lysed parasitized erythrocyte antigen was prepared employing hypotonic lysis (Dulaney and Morrison, 1944). Infected rat blood was washed three times in Alsever's solution pH 7.2. The packed erythrocytes were lysed in 10 volumes of double distilled water and immediately centrifuged in the cold (4°C) at $1500 \times G$ for 15 minutes. The supernatant hemoglobin solution was removed by aspiration, and the sediment was then washed three times in TBS pH 7.3. The parasite pellet was then resuspended in 19 volumes of TBS pH 7.3. This 5 percent packed parasitized lysed erythrocyte suspension then was solubilized as were the free parasites.

Membranes of erythrocytes of normal rats were also employed as control antigens. Erythrocytes of normal rats were washed three times in Alsever's pH 7.2. Ten volumes of Alsever's solution were added to each volume of washed cells. The cells were lysed by one cycle of freeze-thawing (-20°C to 56°C). The membranes were washed three times to remove the hemoglobin. One volume of membranes was suspended in 19 volumes of TBS. Anti-rat erythrocyte membrane serum was produced by immunizing a rabbit with rat erythrocyte membrane antigen.

Complement fixation test procedure

The complement-fixation (CF) test procedure used was the one described by Kent and Fife (1963). Anti-sera (optimum dilution) were serially diluted in TBS and antigen (optimum dilution) was then pipetted into each tube. This was followed by the addition of 5 units of complement to each tube. All tubes were then incubated for 18 hours at 4°C . Sensitized sheep erythrocytes (5×10^8 erythrocytes per ml) were then added to each tube. The total reaction volume in each tube was 1.5 ml. The mixtures were incubated for 30 minutes at 37°C , centrifuged at $1000 \times G$ for 5 minutes, and read by visual comparison with standards. Titers given are the reciprocal of the highest dilution of anti-sera giving less than 65 percent hemolysis.



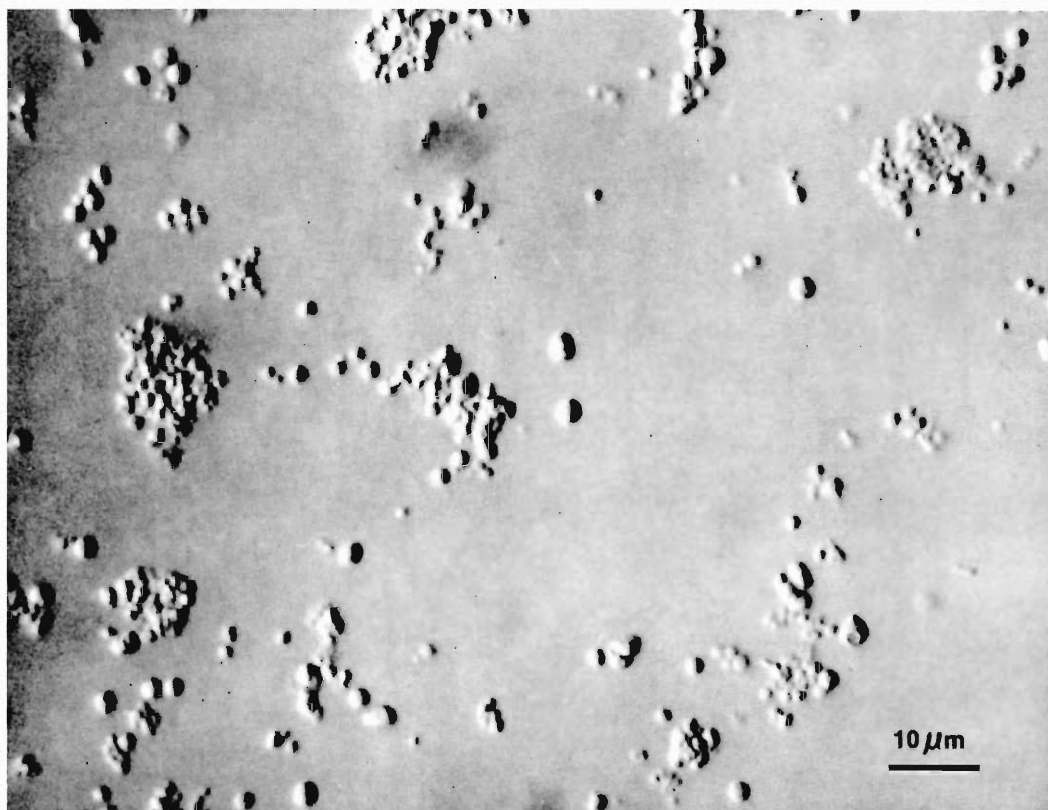


Figure 2. Interference-contrast micrograph of *Plasmodium berghei* freed from host erythrocytes by ultrasound showing clumping of some freed parasites.

Infective test of free parasites

The infectivity of the ultrasonically freed parasites was estimated by comparing the pre-patent periods and the mean survival times of mice infected with the free parasites with those given comparable numbers of parasites in infected rat blood.

Infected blood was drawn from a donor rat, washed three times in Alsever's solution pH 7.2, and the percent parasitemia was determined. The blood was then counted using a hemocytometer, and a dilution was made to yield approximately 1×10^6 parasites per

0.5 ml. The diluted specimen was then kept in the cold (4°C) until 3 hours after the original blood collection. (This was approximately the time required to process the free parasites.)

The free parasites were prepared by the methods previously described. A dilution was then made in Alsever's solution pH 7.2 to yield approximately 1×10^6 free parasites per 0.5 ml. Quantities of 0.5 ml of the diluted free parasite suspension or the same volumes of the parasitized erythrocyte suspension were then injected IP into 20 to 30 gram mice.

←

Figure 1. Photomicrographs of Giemsa-stained rat erythrocytes infected with *Plasmodium berghei* (1-A) and *Plasmodium berghei* freed from host erythrocytes by ultrasound (1-B). The staining characteristics of the freed parasites are similar to those of intraerythrocytic plasmodia.

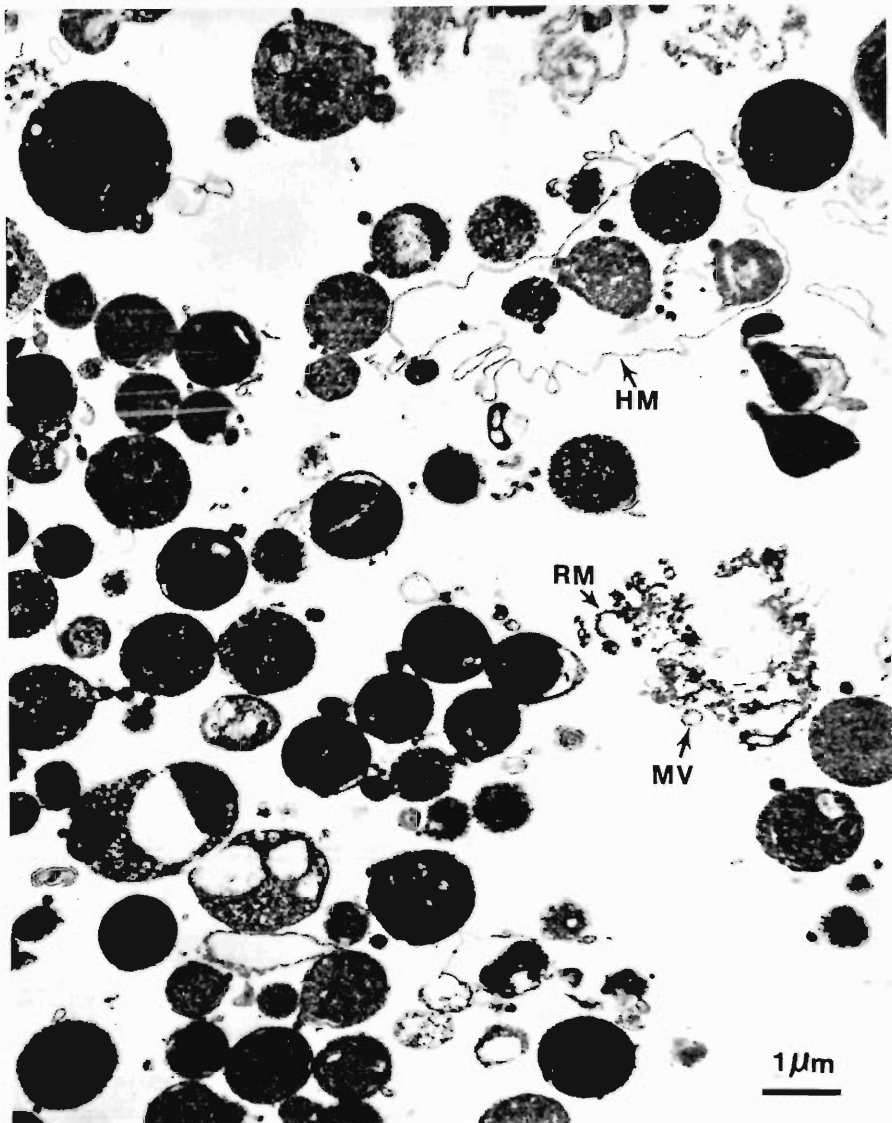


Figure 3. Low-power electron micrograph of a thin section of *Plasmodium berghei* freed from host erythrocytes by ultrasound. Some parasites, however, are not free and are surrounded by host membrane (HM). Small microvesicles (MV) and reaggregated membranes (RM) can be seen. A schizont (S) is also shown.

Results and Discussion

Microscopic examination of free parasites

Photomicrographs of Giemsa-stained unsonicated *Plasmodium berghei*-infected erythrocytes and free parasites are shown in Figures

1-A and 1-B respectively. The free parasites can be seen to retain the staining characteristics of the intraerythrocytic parasites. The free parasites are round and range in diameter from about 1 to 3 μm . The smaller forms are most plentiful. Examination of Giemsa-

stained preparations did not reveal any evidence that the act of freeing the parasites caused physical damage to them. While the evaluation of Giemsa-stained preparations alone is not sufficient to conclude that the freed parasites were not damaged, the demonstration of normal staining characteristics does strongly suggest that the free parasites are physically unaltered.

By interference-contrast microscopy (Fig. 2), the parasites appear free of host cell membranes, globular in shape, and undamaged. The free parasites disperse when mixed, but they clump together subsequently. Clumps or aggregates of free parasites can be seen both in Figure 2 and Figure 1-B. A possible cause of clumping is low surface charge on the free parasites.

Figure 3 is a low-power electron micrograph of a thin section of the freed malaria parasite preparation. Small merozoites predominate in the field, although a larger schizont (S) can also be seen. The merozoites measure approximately 1 μm in diameter and appear to be bounded by a discrete outer membrane complex. A few of the parasites are completely surrounded by host membrane (HM). The area within the host membrane has the same electron density as the surrounding medium indicating complete loss of hemoglobin from the erythrocytes. No internal erythrocyte structure is seen, nor do the parasites appear to be attached to the host erythrocyte membrane. A few microvesicles (MV) and some reaggregated membrane components (RM) are also visible in the micrograph. Kirk (1968) showed microvesicles and reaggregated erythrocyte membrane complexes in sonicated erythrocyte ghost preparations. This researcher pointed out that cations such as Ca^{++} are required for the reaggregation of membrane components. Rosenberg and McIntosh (1968) reported that sonication of red blood cell membranes breaks them into small vesicles and linear fragments which have intact unit membrane structure. It is because such pieces of erythrocyte membrane do not sediment readily in a high gravitational field that the free parasites can be obtained easily by differential centrifugation. Preparations obtained by differential centrifugation such as those shown in Figure 3 contain very few microvesicles and reaggregated membrane

components and are satisfactory as antigens for serological tests. However, for studies of the enzymes of the free parasites, further purification may be necessary to remove even these trace amounts of contaminants.

Figure 4 is a higher-power electron micrograph of a thin section of ultrasonically-freed parasites which shows their fine structure. All of the parasites shown are free of entrapping host erythrocyte membranes. The outer surfaces of most of the free parasites appear to be smooth. Most of these free parasites possess a pellicular complex (P). Aikawa (1971) defines the pellicular complex as a wall which delineates the parasite cytoplasm from its surroundings. Aikawa (1967 and 1971) showed by thin-sectioning techniques that the pellicular complex of the erythrocytic merozoites is composed of three layers: a thin outer membrane, a thick interrupted inner membrane, and a row of microtubules. The merozoites in Figure 4 do not show microtubules but nevertheless appear to be surrounded by three discrete membranes, some of which have discontinuities. The internal structure of the free parasites is well preserved. The nuclei (n) are less electron dense than the cytoplasm and stain evenly. The nuclei are bounded by a double-layered nuclear membrane and are located eccentrically in the parasites.

In order to determine the sizes of the free parasites and to observe their outer surfaces, carbon replicas were made of the preparations. The free parasites seen in the electronmicrograph of the carbon replica shown (Fig. 5) range in size from about 1 μm to 2 μm in diameter with the smaller sizes most plentiful. They are in clusters or aggregates and most are spherical. Buds protrude from the surfaces of some parasites (arrows), and these range in size from about 0.1 to 0.3 μm in diameter. The surfaces of the free parasites have an "orange-peel" appearance. This pebbled surface could possibly result from adhesion of the outer membrane to the thick interrupted inner membrane of the pellicule complex which Aikawa (1971) describes as a labyrinthine structure.

Evaluation of free parasites as CF antigens

The lysed free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane an-

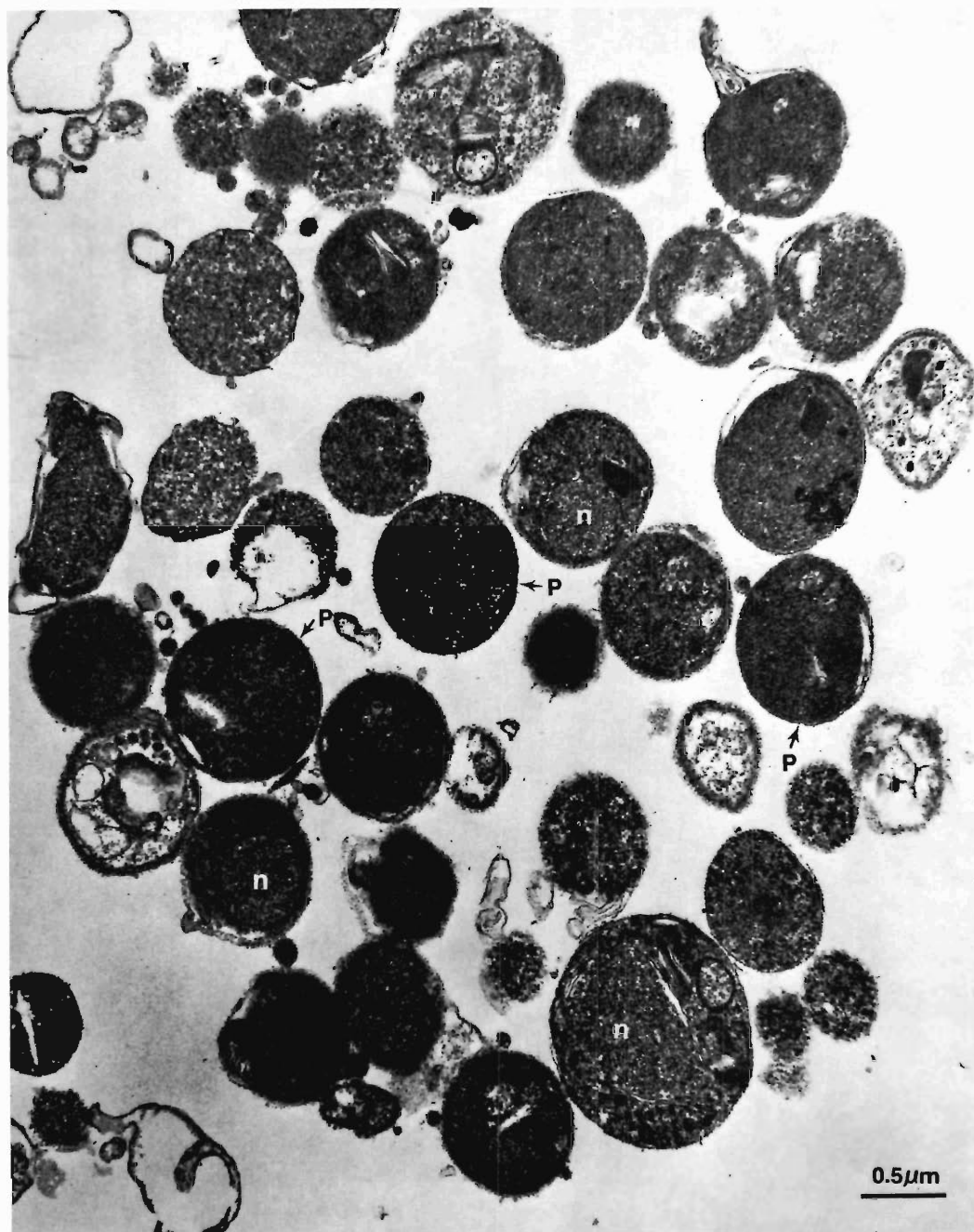


Figure 4. High-power electron micrograph of a thin section of free malaria parasites showing the pellicular complex (P) of the merozoites; nuclei (n); and preservation of fine internal structure.

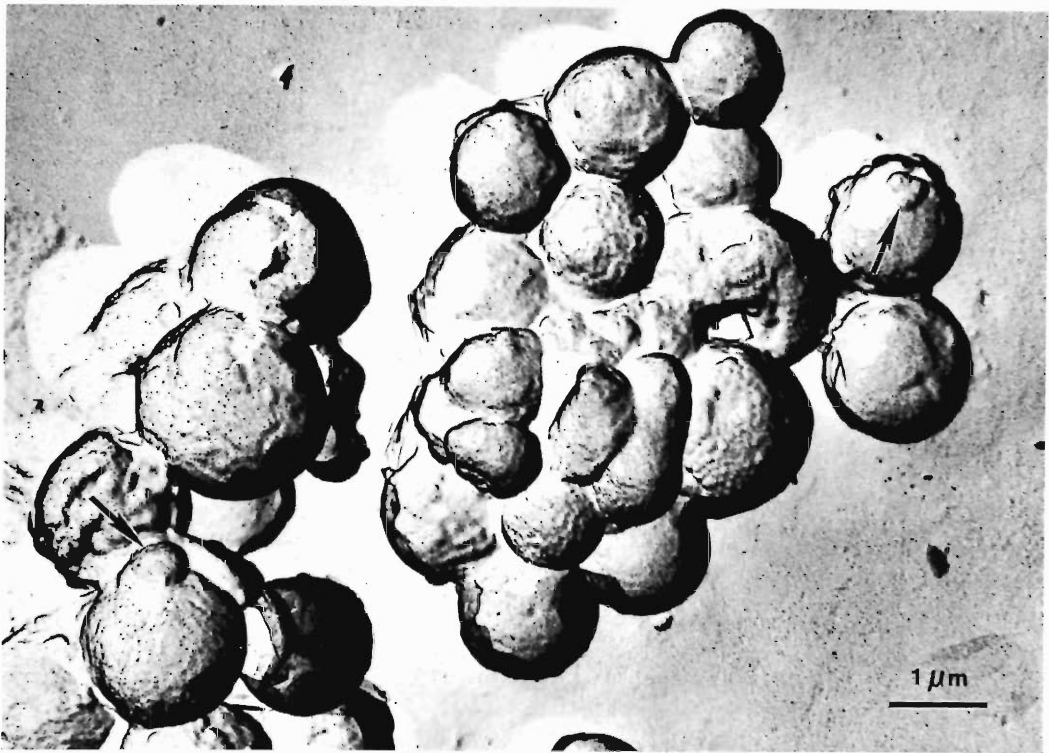


Figure 5. Carbon replica of *Plasmodium berghei* freed from host erythrocytes by ultrasound. The surfaces of most freed parasites resemble an "orange peel." Buds can be seen protruding from the surface of some free parasites (arrows).

tigens were titrated to determine their anti-complementary activity. The anticomplementary titer was defined as that dilution of antigen which demonstrated no fixation of complement in the presence of 2.5 units of complement. Results of a typical test (Table 1) show the anticomplementary titers of the free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane antigens to be 160, 80, and 80 respectively. However, some batches of free parasite antigens had anticomplementary titers as low as 80.

To determine the optimal concentration of antigen for use in the test, block titrations using the free parasite antigen and antiserum were carried out (Table 2). Both antigen and antiserum were diluted and tested for fixation of complement. The initial dilution of antigen in the test shown in Table 2 was 1:80 since the particular batch of antigen

tested had an anticomplementary titer of 80. The antigen diluted 1:80 reacted with the rat anti-*Plasmodium berghei* serum to a titer of 64. Further dilution of the antigen resulted in reduction in serum titer. One two-fold serial

Table 1. Anticomplementary titers of free-parasite antigen, lysed parasitized erythrocyte antigen, and rat erythrocyte membrane antigen using 2.5 units of complement. Results are typical of those obtained with each batch of antigen.

Antigen	Antigen diluted 1:							
	5	10	20	40	80	160	320	640
Free-parasites	4	4	3	2	1	—	—	—
Lysed parasitized erythrocytes	4	3	2	1	—	—	—	—
Rat erythrocyte membranes	4	3	2	1	—	—	—	—

4 = Complement fixation (no hemolysis).
— = No fixation (100% hemolysis).

Table 2. Block titration of free-parasite antigen and rat *Plasmodium berghei* antiserum.

Antigen diluted 1:	Antiserum diluted 1:							Antigen control (2.5 C'H50)
	2	4	8	16	32	64	128	
80	4	4	3+	3	2+	2	1+	—
100	4	3+	3	2+	2	1+	1	—
120	3	2+	2	1+	1	1	±	—
140	2+	2	1+	1	1	±	±	—
160	2	1+	1	1	±	—	—	—
Serum control (5C'H50)	—	—	—	—	—	—	—	—

4 = Complete fixation (no hemolysis).
— = No fixation (100% hemolysis).

dilution of antigen beyond its anticomplementary titer caused a reduction of the titer to two. In the CF procedure used (Kent and Fife, 1963), the optimal concentration of antigen is defined as that concentration of antigen giving maximal reaction with a threshold amount of antibody. Therefore, the optimal concentration of this batch of free parasite antigen was a 1:80 dilution of the antigen. This dilution was also the anticomplementary titer of the antigen.

Comparative CF titers of free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane antigens against rat anti-*Plasmodium berghei* and rabbit anti-rat erythrocyte membrane sera were determined. Results of a typical test are shown in Table 3. In this titration, free-parasite antigen reacted strongly with the rat anti-*Plasmodium berghei* serum (titer 256) while the lysed parasitized erythrocyte antigen reacted poorly (titer 4), and rat erythrocyte membrane antigen showed only a

Table 3. Comparative CF titers of free-parasite antigen, lysed parasitized erythrocyte antigen, and rat erythrocyte membrane antigen against rat anti-*Plasmodium berghei* serum and rabbit anti-rat erythrocyte membrane serum.

Antigen	Antiserum	
	Anti- <i>Plasmodium berghei</i>	Anti-rat erythrocyte membrane
Free-parasites	256	64
Lysed parasitized erythrocyte	4	256
Rat erythrocyte membranes	WR	256

WR = Weakly reactive.

Table 4. CF titers of normal and *Plasmodium berghei*-infected rat sera using the free-parasite antigen.

Group	No. in group	Days post infection	Titer
Normal rats	20	—	NT
Infected rats	4	19	128 128 128 256
Infected rats	3	26	128 256 256
Infected rats	4	30	64 128 128 128
Infected rats	4	37	64 64 128 256

NT = No titer at a 1:2 dilution of serum.

slight reaction. The weak reaction of erythrocyte membrane antigen with anti-*Plasmodium berghei* antiserum may have resulted from blood group antibody in the antiplasmodia serum which was obtained from a rat infected with infected red cells, or could have resulted from antierythrocyte autoantibodies (Kreier *et al.*, 1966; Seed and Kreier, 1969). The lysed parasitized erythrocyte and rat erythrocyte membrane antigens reacted strongly with rabbit anti-rat erythrocyte membrane serum (titer 256) while free-parasite antigen reacted to a lesser degree with this antiserum (titer 64). The titer of the lysed parasitized erythrocyte antigen against anti-rat erythrocyte membrane serum was identical with the titer of the rat erythrocyte membrane antigen. The free-parasite antigen also reacted with anti-rat erythrocyte membrane serum. Such a reaction was expected even from a preparation of plasmodia free from their host erythrocytes since the outer membrane of these parasites is probably host cell in origin (Bahr, 1966; Aikawa, 1971), and host cell materials are in food vacuoles in the parasite (Rudzinska and Trager, 1957; Ristic and Kreier, 1964; Rudzinska *et al.*, 1965; and Aikawa *et al.*, 1966). The presence of some microvesicles and ghost cells in the free-parasite preparations may have also contributed to the reaction with antierythrocyte antiserum.

Sera from 15 rats which had been infected with *Plasmodium berghei* were tested using the free-parasite antigen. These sera were

collected from the rats at various times during the recovery period and titrated for complement-fixing antibodies. Sera were also collected from twenty normal rats and tested for complement-fixing antibodies using the free-parasite antigen. The results obtained are shown in Table 4. None of the normal rat sera tested fixed complement while all of the sera from rats infected with *Plasmodium berghei* fixed complement in the test. While titers vary somewhat among animals, the titers are generally higher than those reported by others using other antigens (D'Antonio *et al.*, 1966; Mahoney *et al.*, 1966).

The lysed free-parasite antigen is heat labile. The activity of the free-parasite antigen was greatly reduced by heating at 56° C for 1 hour (titer 2), and all reactivity was lost within 2 hours when the antigen was heated at 56° C. The activity was also lost when the antigen was heated to 100° C for 5 min. D'Antonio *et al.*, (1966a) reported the *Plasmodium knowlesi* isolated by a French Pressure Cell technique did not lose complement fixing activity after heating at 56° C for 1 hour. These investigators reported that only slight loss of serological activity was caused by heating the antigen at 100° C for 5 min. Their antigen was fractionated by passage through a Sephadex G-200 column, and this may have contributed to the stability. Stability of *Plasmodium knowlesi* and *Plasmodium berghei* may, of course, be inherently different.

The CF titer of lysed free-parasite antigen was reduced from 64 to 8 after 1½ months of storage at both -20° C and -70° C. Lyophilized lysed free-parasite antigen did not react with the antiserum at all after storage, thus the lysed free-parasite antigen was not stable when stored at either -20° C or -70° C or when lyophilized. The addition of 2% polyvinylpyrrolidone (PVP) did preserve some CF activity in lyophilized free-parasite antigen (titer 8) but appeared to be detrimental to the stability of lysed free-parasite antigen stored at -20° C. Although the PVP helped preserve the activity of the lyophilized antigen, the antigen was more stable if it were simply frozen without PVP. Intact free-parasites frozen at -20° C without PVP were more stable than lysed free parasites stored under the same conditions. The intact frozen

parasites stored for 3 weeks showed a reduction of CF activity of less than one dilution.

Infectivity of free parasites

The mean prepatent period of the mice inoculated with 1×10^6 *Plasmodium berghei* in rat erythrocytes was 3.0 days, and the mean death time was 8.2 days. The mean prepatent period of the mice receiving 1×10^6 sonically-freed *Plasmodium berghei* was 3.9 days, and the mean death time was 10.3 days. All mice inoculated in this study died.

The longer prepatent period and time to death observed in the mice infected with free parasites may, in part, be a result of damage to parasites by ultrasound. However, Moulder (1962) suggests that intracellular parasites may have permeable membranes and lose vital constituents when removed from cells even if the membranes are not damaged. Some parasites were probably phagocytized and destroyed *in vivo* after injection also. Trubowitz and Masek (1968) have reported that parasites free of erythrocytes are at greater risk of ingestion than parasites contained in erythrocytes. Martin *et al.* (1971) reported longer survival time in mice injected with parasites which were freed from erythrocytes by ammonium chloride lysis than in mice injected with *Plasmodium berghei*-infected erythrocytes. These investigators suggested that phagocytosis and destruction of some of the free parasites probably accounted for the prolongation of survival time. The survival time reported by Martin *et al.* (1971) was 11 days for an inoculum of 5×10^6 free parasites. It appears that the sonically-freed parasites are less damaged than those freed by ammonium chloride lysis.

The free parasites obtained by the continuous-flow system of sonication are infectious. Thus not only are the parasites not grossly damaged morphologically, but they are sufficiently undamaged to be functional. Thus these free parasites may be useful in studies of drug therapy, physiology, and cultivation of malaria parasites on artificial media.

Acknowledgments

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Comments on Serology

T. LEPES

Director, Division of Malaria Eradication,
World Health Organization, Geneva, Switzerland

Malaria serology has a long tradition, although since Pewny developed the precipitin test in 1918 this technique, as well as others such as the complement fixation test or melanoflocculation test, were not much utilized in practical application for diagnostic or epidemiological purposes in relation to malarial infection. It was only with the development of the immunofluorescence technique that a revival of malaria serology came into being in the early sixties. Intensive research carried out in the past decade significantly increased our knowledge of measuring and interpreting the immune response of the host following natural infection. In addition to the indirect fluorescent antibody technique, other serological (immunological) techniques were developed, such as the indirect haemagglutination, the SAFA, precipitin (Ouchterlony) test, and the capillary agglutination test. To this list should be added the development of laboratory techniques for the estimation of the level of immunoglobulin classes that are not malaria specific but the synthesis of some of these is, no doubt, particularly stimulated by circulating plasmodia in an infected individual.

Before going on to the three papers presented this afternoon, may I first of all discuss some points considered to be of primary importance in the development of malaria serological techniques, their possible application and limitations and interpretation of the results obtained.

Amongst the serological techniques, the indirect fluorescent antibody test had been more widely used than the other techniques during the past ten years. Applying this test, the development and persistence of antibodies had been studied in the course of a natural infection, as well as in a blood-induced infection, without specific antimalarial treatment or in an infection interrupted by the administration of antimalarial drugs shortly after the infection had manifested itself. Studies were also extended to the cross-reactivity of different species of plasmodia or

different developmental stages of plasmodia. The general conclusion of most of the authors was that the indirect fluorescent antibody (IFA) technique is a sensitive and malaria specific test, that this test does not measure the protective antibodies but that there is a correlation between the antigenic stimulus (circulating plasmodia) and the height of positive titre, although there is no direct proportion between the height of the positive titre and recognisable parasitaemia. Furthermore, most research workers are in agreement that the IFA should be used for the purpose of malaria research or epidemiological evaluation. In connexion with this latter aspect a number of authors compare IFA with splenometry.

It may appear, on account of the experience gained and the number of papers published on the IFA, that this test could be widely used, at least for the purposes of epidemiological evaluation. However, in spite of the significant development achieved, there are a few problems which have to be solved before the results obtained through the IFA can be compared when obtained in different laboratories. This applies particularly to the standardization of the antigen and the fluorescein-labelled conjugate. There is no doubt that the use of homologous species of plasmodia as antigen will provide higher positive titre but there are unknown strain differences that may contribute to the difficulty of comparing the results obtained. In this connexion efforts are being made for the mutual exchange of positive sera with determined end point of fluorescence to specified antigen, as well as for the exchange of antigens. This, however, is only one aspect of standardization. Difficulties are also being encountered in attempts to standardize fluorescein-labelled conjugates. The commercially available conjugates, for example, are far from being standardized and, in some instances, are not even satisfactory. The question of utilizing specific antihuman immunoglobulin class G and M is intriguing

and requires further study. The impression, gathered previously, that anti IgM conjugate would better reflect the recentness of the infection could not subsequently be confirmed. However, in spite of all those limitations, the IFA has a definite place in the epidemiological evaluation of malaria programmes, partly for the purposes of delimitation of malarious areas (survey) sampling different age groups and partly for longitudinal studies in malaria eradication programmes approaching the late consolidation phase, to estimate the residual potential parasite reservoir within the population studied. In all fairness it should be pointed out that most of the sero-epidemiological studies undertaken so far with IFA refer to single surveys, which no doubt proved to be useful for the evaluation of the applicability of serological techniques in epidemiological evaluation. The technique proved itself very valuable in the screening of blood donors or in instances of cases of dubious origin. I think it is worth mentioning here a case of *P. vivax* infection in the suburbs of Tunis (Professor Ambroise Thomas, personal communication) in which 26 individuals in the neighbourhood of a confirmed case were epidemiologically investigated. Microscopic examination of the stained blood films did not provide any lead in respect of the possible source of infection, although there were indications that the case might be an indigenous one. IFA was carried out on the sera of these 26 individuals and led to the detection of one positive serum with a titre of 1:2560 and four others with a titre of 1:320. There are other examples of cases of accidentally-induced infections through blood transfusion in which the IFA has detected the source of infection amongst healthy donors without apparent history of malaria infection. Amongst other examples could be mentioned the case of congenital quartan malaria in a two month old baby born in California, in which the IFA led to the conclusion of the source of infection, as both the mother and the baby had equally positive sera.

Desowitz and Stein in 1962 were the first to introduce the indirect haemagglutination test (IHA) into malaria serology. However, because of difficulties experienced with the low stability of the antigen and the consequently poor reproducibility, this procedure

was almost abandoned until Rogers and collaborators came up with a modified procedure in 1968, thus paving the way for a broader application of this test for epidemiological purposes. Since then, a number of sero-epidemiological studies were carried out on sera from Argentina, Brazil, Colombia, Trinidad and Tobago, and Philippines. All these studies were carried out using antigen prepared from *P. knowlesi*. The results obtained so far indicate that, as with IFA, the experience of an individual with malarial infection is being measured but that the antibodies thus bound do not exclusively represent protective antibodies. The test has proved to be malaria specific and sensitive enough to be used for epidemiological purposes (Sadun *et al.*, 1969, Wilson 1971). Yet, due to difficulties encountered in certain laboratories in the preparation of stable antigen, the IHA in malaria serology has not had as wide an application as would have been expected. In this connexion it is worth mentioning the study carried out by Meuwissen *et al.* (1972), exploring various possibilities for the improvement of the test, including source of blood cells, source of antigen (species of *Plasmodium*), concentration of sensitized cells, absorption procedure and the importance of pH during the process of sensitization of tanned cells. This study, while underlining important procedural aspects of the technique, confirms the usefulness of the IHA for the purposes of sero-epidemiology of malaria. It was further confirmed by this study that the IHA test in malaria is hampered more by the influence of heterophile antibodies than by non-specificity. For this reason, the authors have suggested that, for the application of the IHA test, both specifically sensitized tanned sheep cells and tanned cells sensitized with control antigen should be used. It may be of interest to the participants of this meeting to know that in a further study Meuwissen and Leeuwenberg succeeded in preparing a freeze-dried antigen sensitized tanned sheep erythrocytes and have described their procedure in a recent note. A limited quantity of ampoules of lyophilized sensitized cells can be obtained upon request from Dr. J. H. E. Tr. Meuwissen, Laboratory of Medical Parasitology, Medical Faculty of the University of Nijmegen, Geert Grooteplein -Z 24, Nijmegen, Netherlands.

Coming now to the three papers for discussion this afternoon, they refer partly to the results of a malaria sero-epidemiological study and partly to studies on laboratory procedures aimed at obtaining specific purified antigens for serological tests.

A detailed epidemiological study of malaria in Gambela, Ethiopia, was carried out by J. C. Armstrong. The paper entitled "Evaluation of the results of an indirect hemagglutination test for malaria in an Ethiopian population" describes in some detail the population inhabiting the area, their characteristics, as well as general aspects of the malaria endemicity of the area. The author has, applying IHA, simultaneously examined the blood slides and sera of nearly 400 individuals, covering all age groups. This is a straightforward study which confirmed the observations made previously in respect of the correlation between microscopic parasitaemia and seropositivity, between spleen enlargement classified according to Hackett and GMRT of seropositivity, as well as the relation between the general parasite rate and seropositivity of different age groups. The author brings up an interesting and, until now, unrecorded observation that in a certain number of *P. falciparum* infections, particularly in the 5-14 age group, microscopic parasitaemia was not accompanied in all cases by a positive IHA titre. The author has rightly attempted to explain this finding as caused by a very recently acquired infection and for that purpose he used the gametocyte index, as indication of the age of infection. While this approach was, no doubt, correct other possibilities should also be explored. The argument that the failure of the IHA to detect antibodies might have been caused by the low sensitivity of the antigen prepared from *P. knowlesi* could be dismissed as its sensitivity was proved in all the rest of the sera studied. Nevertheless, it is desirable to use homologous antigens in malaria serology and it is hoped that a *P. falciparum* antigen for IHA will be developed in due time. The fractionation and purification of antigens suitable for reproducible serological techniques in malarial infection has long preoccupied the attention of research workers. In this connexion McAlister will be presenting to us the

procedure he has utilized for the evaluation of antigenic activity, using IHA and SAFA. These are, in fact, preliminary results referring to the question of solubility of antigenic material and to technical procedures in obtaining the most reactive fractions, and to the high molecular weight (800 000) estimated for the reactive material. This study revealed a number of points that deserve to be further studied and classified, as for example, the chemical and biological properties of partially purified material, and the long-standing question of whether the antigenic reactivity originates in the parasitized erythrocyte cytoplasm outside of the parasite itself or whether it originates from the cytoplasm of the parasite itself and, if so, which developmental stage of blood form of plasmodia is most antigenic?

An interesting laboratory procedure for the isolation of free parasites was presented by R. B. Prior and J. P. Kreier. Their paper is entitled "Isolation of *P. berghei* by use of a continuous flow ultrasonic system: a morphological and immunological evaluation." The procedure described by the authors is no doubt an elegant one, which was designed to eliminate drawbacks of the previously applied procedures by which not only the host cells but also the parasites were disrupted. The electron micrograph (fig 4) attached to the paper illustrates the efficiency of the procedure.

The immunological evaluation of the antigenic activity of lysed free-parasites was carried out using the complement fixation technique and studying, at the same time, its anti-complementary activity. To compare the antigenic reactivity of the lysed free-parasites the authors have used antigens prepared from rat erythrocyte membrane and from lysed parasitized erythrocytes. These antigens were tested against rat anti-*P. berghei* and rabbit anti-rat erythrocyte membrane serum. The results indicated that lysate of free-parasites does not constitute potent antigen for malaria serology. Nevertheless, the authors suggest that the procedure may prove to be very useful in preparing inocula for *in vitro* cultivation of plasmodia and subsequently the *in vitro* testing of anti-plasmodial activity of tested compounds.

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RECAPITULATION AND CONCLUDING REMARKS

Recapitulation and Concluding Remarks

E. H. SADUN

Walter Reed Army Institute of Research,
Walter Reed Army Medical Center, Washington, D.C. 20012

Six years after the publication of the first volume entitled "Research in Malaria" and 3 years after the second volume entitled "Experimental Malaria" we have prepared this third volume which comprises the working papers and prepared discussions of the Panel Workshop held at the Walter Reed Army Institute of Research on June 7, 8 and 9, 1972. As in the past, this publication reflects the scope, size and diversity of current investigations on basic research aspects of malaria carried out under the sponsorship of the U.S. Army Medical Research and Development Command. It would be unrealistic to expect a balanced presentation in a field in which the state of our knowledge is so uneven. Whereas in the previous publications epidemiologic and chemotherapeutic studies were purposely omitted since detailed discussion of these subjects had occurred elsewhere, in the present volume we have included a large section dealing with basic research in chemotherapy.

Great progress has occurred since the inception of this program. As an example, in the first volume (Sadun, 1966) we felt the need to explain why we were devoting space to immunology and serology in spite of the fact that means for practical immunizing procedures could not be foreseen. Yet, we stated that this possibility obviously deserved continued and extensive investigations. I am delighted that the studies reported in this volume permit us to assume a more optimistic attitude toward the role of the applications of immunology and serology to malaria diagnosis, management and control. In fact, we need now to remind ourselves not to be unduly elated by preliminary successes obtained in some experimental animals and that progress in this promising field will require patient, thorough stepwise investigations.

The question must be asked again whether the luxury of long term basic studies is appropriate and justifiable at a time when government research funding policy has been re-

duced. Six years ago, in attempting to define the scope of our malaria research we stated (Sadun, 1966) that we were not looking for an open end documentation of data but for the solution of research problems, conceptual or technical, that are relevant to man's struggle against malaria. Such studies can be appropriately classified as applied research in that they do not merely satisfy a desire to increase man's knowledge of nature but are oriented around the disease with a goal of developing methods or products for the prevention, management and control of malaria. The dilemma of whether funds and human resources are better employed in fostering basic or disease oriented research was examined in a recent editorial by the publisher of Science (Bevan, 1972) in which he stated that ". . . to dichotomize scientific research into pure and applied is to defy a specious distinction, for science is, in its most fundamental sense, an approach to solving problems. Its goals may be both specific and general, concrete and abstract, practical and theoretical, and immediate and long-range, and several centuries of experience have demonstrated a significant reciprocity between conceptual and practical advance." In a specific message to Congress on March 16, 1972, the President disavowed the policy of dichotomization in research funding and reported that all agencies and departments would support basic research. He indicated the necessity to "give an important place to basic research and to exploratory experiments which provide the new ideas on which our edifice of technological accomplishment rests" and emphasized "the importance of maintaining that spirit of curiosity and adventure which has always driven us to explore the unknown."

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The present publication will be distributed free of charge to interested persons and may be obtained by writing to Dr. E. H. Sadun, Special Assistant to the Director, WRAIR, for Basic Research in Malaria, Walter Reed Army Institute of Research, Washington, D.C., 20012.

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