GREEN, Theodore James, 1935-
STUDIES ON THE NATURE OF THE IMMUNE RESPONSE TO PLASMODIUM BERGHEI IN THE RAT.
The Ohio State University, Ph.D., 1977
Microbiology

University Microfilms International, Ann Arbor, Michigan 48106

© 1978

THEODORE JAMES GREEN

ALL RIGHTS RESERVED
STUDIES ON THE NATURE OF THE IMMUNE RESPONSE TO PLASMODIUM BERGHEI IN THE RAT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Theodore J. Green, B.S., M.S.

* * * * *

The Ohio State University

1977

Reading Committee:
Dr. Julius P. Kreier
Dr. Robert M. Pfister
Dr. Matthew C. Dodd
Dr. Bruce S. Zwilling
Dr. Melvin S. Rheins

Approved By
Adviser

Department of Microbiology
DEDICATION

This work is dedicated to my lovely wife, Windy, and to my two fine sons, Ted and Bill, and especially to my beautiful little daughter, Ginna, who was born just in time to help us begin the new life ahead.
ACKNOWLEDGMENTS

I am privileged to thank Dr. Julius P. Kreier, my adviser, for his patience and generosity in sharing his considerable knowledge and for extending his friendship. I cannot repay his many kindnesses. Neither can I ever repay the members of the Faculty of Microbiology for their timely and unhesitating support and encouragement. And I am also pleased to thank Kathryn Brown for her unfailing help and friendship.
VITA

October 29, 1935 .......... Born - North Adams, Massachusetts

1957 .................... B. S., Cornell University, Ithaca, New York

1957-1968 ............... Research Associate, Sterling Winthrop Institute, Rensselaer, New York

1968-1974 ............... Head, Animal Care and Research, Warren-Teed Pharmaceuticals, Inc. Columbus, Ohio

1974-1975 ............... Vivarium Supervisor, College of Biological Sciences, The Ohio State University, Columbus, Ohio

1975 ..................... M. S., The Ohio State University, Columbus, Ohio

1975-1977 ............... Graduate Research Associate, Department of Microbiology, The Ohio State University Columbus, Ohio

PUBLICATIONS


J. P. Kreier, J. Hamburger, T. M. Seed, K. Saul and T. J. Green.


PRESENTATIONS AND PUBLISHED ABSTRACTS


# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER 1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2. &quot;The infectious properties of \textit{Plasmodium berghei} merozoites free by continuous flow sonication&quot;</td>
<td>9</td>
</tr>
<tr>
<td>CHAPTER 3. &quot;The demonstration of the role of cytophilic antibody in resistance to malaria parasites (\textit{Plasmodium berghei}) in rats&quot;</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 4. &quot;A characterization of anti-malarial (\textit{Plasmodium berghei}) opsonic and macrophage-cytophilic antibodies by fluorescent antibody techniques&quot;</td>
<td>44</td>
</tr>
<tr>
<td>CHAPTER 5. &quot;The effects of adoptive transfer of macrophages on the immune response to \textit{Plasmodium berghei} malaria in the weanling rat&quot;</td>
<td>69</td>
</tr>
<tr>
<td>CHAPTER 6. Discussion</td>
<td>81</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>89</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>The effects of incubating <em>Plasmodium berghei</em> infected erythrocytes suspended in Hanks' Balanced Salt Solution at various temperatures upon their infectivity to mice</td>
</tr>
<tr>
<td>2</td>
<td>The effects of incubating sonically released free <em>Plasmodium berghei</em> parasites suspended in Hanks' Balanced Salt Solution at various temperatures upon their infectivity to mice</td>
</tr>
<tr>
<td>3</td>
<td>Titer determination of hyperimmune serum conjugate by direct fluorescent antibody technique</td>
</tr>
<tr>
<td>4</td>
<td>Titer determinations of hyperimmune serum by indirect fluorescent antibody technique</td>
</tr>
<tr>
<td>5</td>
<td>Experimental groups used in tests for complement fixing ability of opsonic antibody on fixed parasitized blood films</td>
</tr>
<tr>
<td>6</td>
<td>Experimental groups used in tests for complement fixing ability of opsonic antibody on free parasites</td>
</tr>
<tr>
<td>7</td>
<td>Experimental groups used to test for complement fixing ability of macrophage-bound cytophilic antibody</td>
</tr>
<tr>
<td>8</td>
<td>Experimental groups used to test the specificity of cytophilic antibody for <em>P. berghei</em> antigens</td>
</tr>
<tr>
<td>9</td>
<td>The binding affinities of fluorescein-labeled macrophage-cytophilic and opsonic antibodies from <em>P. berghei</em> hyperimmune serum as determined by spectrofluorometry</td>
</tr>
<tr>
<td>10</td>
<td>Experimental groups used in various tests for protective effects of adoptive transfer into weanling rats of unsensitized or sensitized weanling or adult macrophages</td>
</tr>
<tr>
<td>11</td>
<td>Variation in the ability of macrophages for immature rats to bind fluorescein-conjugated macrophage-cytophilic antibodies</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure                                                                                           Page
1  The mean Plasmodium berghei parasitemia curves of mice inoculated with either infected erythrocytes, infected erythrocyte filtrate, or filtered ultrasonically released free parasites 16
2  The effects of cytophilic antibodies upon the ability of macrophages to retain free Plasmodium berghei parasites 30
3  The pH elution pattern of hyperimmune rat serum from QAE-Sephadex A-50 31
4  The precipitin pattern formed in gel by eluted macrophage-cytophilic antibodies and QAE-Sephadex hyperimmune serum fractions when reacted against anti-IgG antiserum 33
5  The effect of adsorption with macrophages upon the protective capacity of Plasmodium berghei immune serum in rats. 34
6  The protective effects against Plasmodium berghei of macrophage-adsorbed and macrophage-eluted hyperimmune serum fractions in rats 35
7  The protective effects against Plasmodium berghei in rats of QAE-Sephadex hyperimmune serum fractions 37
8  The binding affinities of fluorescein-labeled macrophage-cytophilic and opsonic antibodies from P. berghei hyperimmune serum as determined by spectrofluorometry 64
9  The effect of adoptive transfer of weanling or mature macrophages into weanling rats on the subsequent course of a challenge infection with Plasmodium berghei 75
10 The effect of adoptive transfer of weanling or mature rat macrophages with hyperimmune serum into weanling rats on the subsequent course of a challenge infection with P. berghei 76
11 The effect of adoptive transfer into weanling rats of antibody sensitized or unsensitized adult macrophages treated with antigen on the subsequent course of a challenge infection with Plasmodium berghei 77
CHAPTER I.

INTRODUCTION

Malarial parasites, the descendents of coccidian ancestors, are found in a wide variety of vertebrates including reptiles, birds and mammals. These parasites are notably host-specific and many species are found in the primates, with four species being adapted specifically to man, i.e. *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *Plasmodium falciparum* and *P. vivax* are considered to be of the greatest importance from the public health and economic viewpoints. Although malaria is not a major cause of disease in the United States today, it was not always thus. Andrews (1963) has estimated that in 1915 there were 4 million cases of malaria in the United States, with a fatality rate of 10 to 20%. He also estimates the economic loss in the United States in 1938 due to malaria at $500 million dollars. Historically, malaria has also been a major disease in other parts of the world, affecting many millions of persons and causing untold economic losses.

The earliest efforts at malaria control were directed towards vaccine development, but without success. In the 1940's due to the exigencies of wartime the major research emphasis was shifted to chemotherapy and vector control. The combined effects of chemotherapy, application of the results of epidemiologic studies and an incredibly large scale vector control program accomplished enormous strides toward malarial eradication.
between the mid 1950's and the early 1970's. There developed a complacency about malaria control but not without some cause for by 1975, 727 million persons in previously malarious areas were now malaria free and another 814 million persons were considerably benefited by the programs.

It should be noted, however, that at least 272 million persons were still living under malarious conditions with no special efforts being made on their behalf. These people represent a vast reservoir of infection. Another significant reservoir of infection in areas where eradication is attempted may be found to consist of infected non-human primates.

By 1970, many species of anopheline malaria vectors were showing resistance to the more effective insecticides, and resistance was developing to the best chemotherapeutic agents by all of the species of plasmodia causing human malaria (Peters, 1972). The concurrent withdrawal of chlorinated hydrocarbon insecticides from vector control also was having a tremendous impact, and malaria began a worldwide resurgence. The number of cases recorded in the United States rose from 62 in 1960 to 4247 in 1970, due in part to the nation's military involvement in southeast Asia and with the great majority of cases occurring among military personnel. By 1973 the military cases had fallen below the number of civilian cases in the U. S. with the total being 216. By 1976 the civilian cases in the U. S. had risen to 401 of the 406 cases reported (C D C Malaria Surveillance). While this represents a notable increase in civilian malaria, it is not alarming.
However, the situation existing in other areas does raise cause for alarm. For example, India went from a pre-eradication level of 75 million cases per year to a low of 150,000 cases in 1964, and is now returned to an estimated incidence of 5 million cases. Pakistan fell from 15 million pre-eradication cases annually to 150,000 cases and is now back to an estimated 8 million cases annually (WHO, 1975). This circumstance has been repeated in many parts of the world. The WHO Weekly Epidemiological Record (1976, 1977) lists 106 countries where the risk to travelers of contracting malaria is substantial. The need for an effective malaria vaccine therefore has become a worldwide priority.

There are two possible approaches to vaccination against malaria. The first is immunization against the vector-borne sporozoite stage (Nussenzweig, 1972), and the second is immunization against the asexual erythrocytic forms which actually account for the pathology seen in malarial infection. We have arbitrarily elected to study the erythrocytic forms which actually account for the pathology seen in malarial infection. Before a vaccine against malaria can become a worldwide reality there are several problems which must be solved. These are 1) the production of a merozoite or merozoite-rich antigen of uniformly high quality which is free of host contaminants, 2) the production of this antigen in batch quantities in the amounts needed to immunize 1 billion or more people, and 3) a clear understanding of the immune response of the host to malaria to enable the most effective and efficient use to be made of the antigen available, integral to which
is the identification of the specific protective antigen. It is to these latter considerations that I have addressed myself.

Antigen production from infected host erythrocytes has been attempted in a variety of forms, including saponin lysis (Speck et al, 1946); ammonium chloride lysis (Martin et al, 1971); explosive decompression (D'Antonio et al, 1966); continuous flow sonication (Prior and Kreier, 1972) and natural release \textit{in vitro} (Dennis et al, 1975). Several of these methods are basically unacceptable for vaccine production due to the presence of contaminating reagents or chemicals. The continuous flow sonication method produces a high quality preparation, but contains only 10 to 30% of the merozoite form which is considered by many to be desirable for effective immunization (Mitchell et al, 1974; Mitchell et al, 1975; Cohen, 1974, Miller et al, 1975; Siddiqui, 1977). The method of natural release first described by Dennis et al offers the best hope for the production of immunizing forms using the available technology. Even though the chimpanzee and the Aotus monkey are susceptible to \textit{P. falciparum}, the most severe of the human malarias and the species toward which the most effort is directed for a vaccine, there is no hope of producing enough antigen through the use of these hosts' blood for even a national scale immunization effort, let alone a worldwide program. The most recent major achievement in this area was reported by Trager and Jensen (1976). These workers developed a technique for the continuous \textit{in vitro} culture of \textit{P. falciparum} malaria in human erythrocytes, thus providing the potential for antigen production on a mass scale although at present the technique is relatively inefficient.
While the immune response to malaria is known to depend upon the production of humoral antibody (Cohen et al, 1974; Diggs and Osler, 1975) there has been a poor correlation between antibody titer and resistance to infection (Cohen and Butcher, 1971) and passive transfer has not proven to provide reliable protection. The work of Criswell et al (1971), of Brown and Phillips (1974) and of Hamburger and Kreier (1976) indicated that factors in addition to the anti-parasite antibody were necessary for resistance to malarial infection. Trubowitz and Masek (1968) have shown that blood phagocytes will ingest some parasites (probably trophozoites) readily, and Chow and Kreier (1972) have demonstrated that phagocytosis of free parasites is greatly enhanced by immune serum and complement. Thus we have undertaken in this project to examine not only the humoral immune response of the rat to Plasmodium berghei (the model system for the present study), but also have attempted to relate the humoral response to the function of the macrophage in phagocytosis as well as to examine the interaction of the macrophage and the antigen during the induction of the immune response. Using both in vitro techniques and in vivo models we have attempted to determine the relationship between macrophage-cytophilic and opsonic antibody in the elimination of parasites, and to determine how these antibodies interact with macrophages in both the induction phase and the effector phase of the immune response.


CHAPTER 2

"THE INFECTIOUS PROPERTIES OF PLASMODIUM BERGHEI MEROZOITES FREED BY CONTINUOUS FLOW SONICATION"

There currently is a great deal of interest in the production of malarial parasites which are free of contaminating host materials and which are as undamaged as possible. Although electron microscopy can provide evidence of the presence of contaminating host membranes, the most exacting method of assessing the quality of the free merozoites produced by any method is by determining the extent of their invasive capacity; that is, their ability to initiate an infection in the appropriate host erythrocyte. The quality of the free parasites of the noninvasive stages must of course be evaluated by other criteria. A variety of methods for achieving the production of free parasites from infected erythrocytes have been developed. These include saponin lysis (Christophers and Fulton, 1939), immune lysis (Speck et al, 1946), ammonium chloride lysis (Martin et al, 1971), and natural release in in vitro culture (Dennis et al, 1975). An additional method, continuous flow ultrasonic lysis (Prior and Kreier, 1972) has been developed by and is currently in use in this laboratory. This method has the advantages of providing parasites which are nearly devoid of contaminating host materials and of being free of added chemical or other lytic reagents. This also remains the only method in which parasites of all stages of growth can be obtained from erythrocytes without the use of membrane dissolving reagents. The condition of these parasites, when produced in a properly controlled ultrasound field, is of very high quality. A
properly controlled field is produced in a continuous flow chamber with a very small void volume when the flow rate and sonic energy are properly adjusted. In our hands sonically released merozoites have consistently been capable of producing infections in susceptible hosts. Recent reports in the literature (McAlister and Gordon, 1977; McAlister, 1977) have suggested that such infectivity is in fact due to the presence of contaminating infected erythrocytes rather than to viable infective merozoites in the free parasite population. In this paper we demonstrate that the merozoites freed by ultrasound from their host cells are capable of producing infections in susceptible hosts, and that the viability characteristics of such merozoites are distinct from those of infected erythrocytes.

MATERIALS AND METHODS:

The Walter Reed strain of *Plasmodium berghei*, uniformly lethal for mice, was used throughout the study. Infections were initiated from an aliquot of reference stabilite held in liquid nitrogen, and retrieved by injection of rapidly thawed material into a mouse. Mice were infected with either $5 \times 10^2$ infected red blood cells (IRBC) or $5 \times 10^5$ free parasites freed by the continuous flow ultrasonic lysis method of Prior and Kreier (1972) as modified by Hamburger et al (1976). Filtration of the FP or the IRBC was accomplished by passage of the suspension through a 2um Millipore filter. Examination for the presence of contaminating IRBC in the free parasite preparation was done on thick smears made on glass slides which were searched exhaustively after Giemsa staining.
Percent parasitemia was determined by counting the number of infected erythrocytes per 1000 erythrocytes counted on a Giemsa-stained thin blood film, and dividing the number of infected cells by 10. Films were taken between 10 a.m. and 12 noon for 14 days following infection of the test suspensions. Mice which did not show a parasitemia by day 14 were deemed uninfected.

Experiment #1. Thirty-eight young adult Swiss white mice were used to assay the infectivity of the parasitized erythrocyte preparation after incubation for various times at 1°C, 22°C or 37°C. The percent parasitemia of the infectious IRBC inoculum was determined by examination of a Giemsa-stained smear of the material. The concentration of the inoculum was then adjusted by dilution with Hanks' Balanced Salt Solution (HBSS) at 1°C, 22°C or 37°C so that 5 x 10^2 IRBC were contained in 0.2 ml of the various suspensions. This volume was immediately injected into one mouse for each incubation temperature, and at 30 minute intervals over a period of 2.5 hours, and then into 2 mice at hourly intervals up to 5 hours and again at the 12, 24, 48 and 72 hours. The inocula were held at 1°C, 22°C or 37°C for the duration of the procedure. Percent parasitemias were determined daily as described above.

Experiment #2. Thirty-one young adult Swiss mice of either sex were used to assay the viability of the free parasite preparation after various incubation times at 1°C, 22°C or 37°C. The free parasites were harvested from heavily infected blood by the continuous flow sonication method previously described. The parasites were held at 1-2°C throughout this procedure, and when the harvest was complete
(45 minutes after sonication) the parasites were counted by standard hemocytometer technique and diluted to the appropriate volume with HBSS, pH 7.2, at 1°C, 22°C or 37°C and held at the temperature for the duration of the experiment. Percent parasitemias were determined daily as described above.

Experiment #3. Group 1. Free parasites prepared by the continuous flow sonication procedure were adjusted to a concentration $5 \times 10^5$ parasites per 0.2 ml. The unfiltered free parasite suspension was injected i.v. into each of 4 mice.

Group 2. An inoculum containing $5 \times 10^2$ IRBC per 0.2 ml was prepared as in Experiment 1 above. Each of 4 mice in the group received $5 \times 10^2$ IRBC i.v.

Group 3. An inoculum containing $5 \times 10^2$ IRBC per 0.2 ml was prepared as in Experiment 1 above. This suspension was then filtered with a 2 um Millipore filter. Two-tenths of a ml of the filtrate were used to inject each of the 5 Swiss white mice in the group.

Giemsa-stained blood films from all of the animals in the above groups were examined daily to determine the percent parasitemias.

RESULTS:

Table 1 is a tabular representation of the effects on parasite viability of incubating IRBC at either 1°C, 22°C or 37°C for intervals of up to 12 hours. The results are presented as the time required to achieve 1% parasitemia in the infected mice. As the time required for the parasitemia to achieve 1% is inversely proportional to the number of infectious units which survive the incubation, it is apparent that
Table 1. The effects of incubating *Plasmodium berghei* infected erythrocytes suspended in Hanks' Balanced Salt Solution at various temperatures upon their infectivity to mice.

<table>
<thead>
<tr>
<th>Duration of Incubation in Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0.5 1.0 1.5 2.0 2.5 3.0 4.0 5.0 12.0</td>
</tr>
<tr>
<td>37°C 4.7* 6.2 NI **</td>
</tr>
<tr>
<td>22°C 4.2 5.3 6.3 NI</td>
</tr>
<tr>
<td>1°C 3.6 4.0 4.3 4.9 5.3 5.2 6.2 6.4 7.1 NI</td>
</tr>
</tbody>
</table>

* Results are in days to 1% parasitemia of test mice given inoculum consisting of $5 \times 10^2$ IRBC given i.v.

** NI = not infective
in all cases the infectivity of the inoculum decreased with increasing incubation time. The time required for the inoculum to become non-infectious was greatest at 1°C and least at 37°C.

Table 2 is a tabular representation of the effects on parasite viability of incubating FP in HBSS at either 1°C, 22°C or 37°C for intervals of up to 5 hours. The results are presented as the time required to achieve 1% parasitemia in the infected mice. At no temperature of incubation was there a progressive loss of infective units with time, rather there was an abrupt loss of all infectivity after some period of incubation. The time to the abrupt loss of infectivity of the inoculum was inversely proportional to the temperature of incubation. Those free parasites which were incubated at 1°C survived the longest and those which were incubated at 37°C survived for the shortest time.

Figure 1 is a graphic representation of the mean parasitemia curves of groups of mice inoculated with either IRBC, filtered FP or IRCF filtrate. The infective free parasites passed the 2 μm filter, whereas the IRBC did not.

DISCUSSION:

The data derived from the in vitro incubation experiments indicate that free merozoites survive longer at low temperatures than at higher temperatures. The more rapid exhaustion of the merozoites energy stores by increased metabolic rates at the higher incubation temperatures may be the reason for the shorter survival times at the higher temperatures. If all merozoites had a similar energy store and a minimum amount of energy was required for penetration, this would account for
### Table 2. The effects of incubating sonically released free *Plasmodium berghei* parasites suspended in Hanks' Balanced Salt Solution at various temperatures upon their infectivity to mice. Results are in days to 1% parasitemia of test mice.

<table>
<thead>
<tr>
<th>Duration of Incubation in Minutes</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
<th>210</th>
<th>240</th>
<th>270</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>6.1</td>
<td>6.2</td>
<td>6.1</td>
<td>6.3</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22°C</td>
<td>6.9</td>
<td>6.4</td>
<td>7.1</td>
<td>7.1</td>
<td>6.3</td>
<td>7.0</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1°C</td>
<td>6.2</td>
<td>6.9</td>
<td>6.2</td>
<td>6.1</td>
<td>7.1</td>
<td>7.1</td>
<td>6.4</td>
<td>5.9</td>
<td>5.2</td>
<td>5.1</td>
<td>5.1</td>
<td>5.6</td>
<td>4.9</td>
<td>5.3</td>
<td>5.3</td>
<td>5.4</td>
<td>5.9</td>
<td>5.5</td>
<td>NI</td>
</tr>
</tbody>
</table>

* NI = not infective
Figure 1. The mean Plasmodium berghei parasitemia curves of mice inoculated with either infected erythrocytes, infected erythrocyte filtrate, or filtered ultrasonically released free parasites.
a sudden complete loss of infectivity. Direct incubation of the parasites in HBSS may also be harmful. The survival times of the free parasite inocula were markedly less than the survival times of infected erythrocytes at all incubation temperatures tested.

The heterogeneity of the parasites in the IRBC inoculation may reflect different sensitivities of the metabolic systems of the different developmental forms of the parasites to heat. Maturation and death of merozoites in vitro could also occur. Merozoites were not sensitive to cold, in fact they survived better at low temperatures. The most probable reason for a gradual loss of infectivity in the IRBC inoculum would be differential ability of the various intracellular forms of the parasites to survive in HBSS.

The two populations studied exhibit distinct patterns of loss of infectivity. Exhaustion of a stored energy reserve could explain the synchronous loss of infectivity in the homogeneous free merozoite population, and the deleterious effects of incubation in HBSS on the metabolisms of a heterogeneous parasite population could explain the progressive loss of viability of the intraerythrocytic population.

The infectivity of the free parasite preparation was clearly demonstrated by the fact that parasites passed through a 2 um filter which was demonstrated to retain erythrocytes. The free parasite preparation also demonstrated survival characteristics which differed markedly from those of infected erythrocytes. It is possible that only fully mature merozoites in the free parasite preparation are capable of initiating infections as the immature forms do not possess the necessary apparatus for penetration. However, all of the stages
of the parasite in the infected red cell possess the potential to initiate infections since they may complete their development in the new host.

This work demonstrates that the infective capacity of the free parasites prepared by continuous flow sonication is not due solely to the presence of contaminating infected erythrocytes for if the infectivity of the free parasite preparation were due to contaminating IRBC, then the characteristics of the infective capacities of the two preparations would be expected to be similar. The failure of others (McAlister et al, 1977) to demonstrate infectivity of parasites released by continuous flow sonication could be due to the large void volume of the chamber they used, the power output of the sonic oscillator they used, and the flow rate at which they pumped the infected blood through the chamber as well as their failure to achieve a proper separation of parasites from erythrocytes (Abdalla et al, 1977 in press, Journal of Parasitology).
LITERATURE CITED


It is established that antimalarial antibodies are capable of protecting rodents against malarial infections (Diggs and Osler, 1969; Zuckerman, 1970), and serum antibodies have been shown to coat free parasites. Therefore, the suggestion has been made that protection is achieved at the merozoite stage (Cohen and Butcher, 1970; Miller et al, 1975). While previous work (Miller et al, 1975; Jerusalem et al, 1971; Hamburger and Kreier, 1975) also has shown that free parasites which have been coated with immune serum in vitro remained capable of initiating infection, Hamburger and Kreier, 1975, have additionally demonstrated that such antibody coated parasites do not retain full infectivity if they were accompanied into the test animal by immune serum. Chow and Kreier, 1972, also concluded that immune serum enhanced the phagocytic capability of both normal and immune macrophages.

Immunoglobulins associated with macrophages first were reported by Girard and Murray in 1954; then Boyden (1963) introduced a rosette-forming procedure utilizing macrophage monolayers to conveniently demonstrate the presence of such antibodies. The definition of cytophilic antibody proposed by Boyden, 1963, and Sorkin, 1963 stated explicitly that the binding of antigen to cytophilic antibody takes place subsequent to the cellular fixation of the antibody, with the
implication that these antibodies are capable of cellular fixation prior to their combination with antigen (Tizard, 1972). This usage of the term "cytophilic antibody" has been accepted by the present authors.

Both Parrish (1965) and Tizard (1969) have reported that opsonizing antibodies are not cytophilic prior to combination with specific antigen, and therefore are not identical with the antibodies detected by Boyden's macrophage rosette system.

According to Tizard (1972) the amount of cytophilic antibody found in the serum will depend upon both the avidity with which the antibodies bind to the macrophage receptors and the availability of those receptors. High binding avidity and ample receptor availability may result in undetectable levels of cytophilic antibody in the serum, with those antibodies which are found being of perhaps the lowest binding avidity for the macrophage receptors. For these reasons we have chosen to use hyperimmunized rats for our source of cytophilic antibody in an effort to effect a surplus of a relatively high-avidity cytophilic antibodies in the serum.

In the present studies we investigated the possibility that immune serum might directly arm macrophages against *P. berghei* parasites by the agency of a macrophage-cytophilic antibody, and we undertook to demonstrate by various procedures the presence of a macrophage-cytophilic antibody on non-immune macrophages which had been pre-sensitized in immune serum. In *vitro* experiments which demonstrated the capacity of a specific macrophage-cytophilic antibody to promote adherence of free parasites to macrophages were performed first, after which an attempt was made to adsorb the cytophilic antibody component out of hyperimmune serum (HIRS) with normal macrophages. The effectiveness of the adsorption was judged
by the blockade of subsequent fluorescent staining of the macrophages by fluorescein-conjugated hyperimmune serum globulins which contained macrophage-cytophilic antibodies. Physical separation of the cytophilic antibodies from the opsonic antibodies in hyperimmune serum was attempted by both column chromatography and by adsorption-elution with macrophages. Boyden (1963) and Sorkin (1963) have reported that heating a macrophage suspension to 56°C for 30 minutes will cause elution of cytophilic antibodies. Hyperimmune serum, serum fractions and eluates were tested in vivo for protective effects.

MATERIALS AND METHODS:

Malaria parasites. The Plasmodium berghei strain used in this study was obtained from M. Aikawa (Case Western Reserve University, Cleveland, Ohio) and originated from the Walter Reed Army Institute of Research. A pool of infected mouse blood in 10% glycerol which was aliquoted and frozen in liquid nitrogen served as the reference stabilate source for all infectious inocula used in this study. Thawed material was inoculated first in a mouse, then passed into a weanling rat, whose infected blood served as the inocula for test animals and for animals which subsequently served as either a source of immune serum or of parasites. Free parasites were injected into some animals as the infectious inoculum and were obtained by harvesting the blood of infected adult rats after parasitemia exceeded 50%. These high levels of parasitemia were obtained by pretreatment of rats with 30 mg/kg of phenylhydrazine-HCl intraperitoneally five days and three days before intravenous infection with 0.5 ml of heavily infected blood. Three days after infection, the parasitemia exceeded 50% and the animals were exsanguinated into Alsever's solution for the parasite
harvest. Parasites were isolated from the blood by a modified continuous flow procedure (Prior and Kreier, 1972a; 1972b). The only modification involved filtration of the blood sonicate through a glass wool pad prior to centrifugation for 10 minutes at 1020 x g to remove unbroken erythrocytes after which the supernatant was centrifuged for 10 minutes at 3300 x g to pellet the parasites. The parasites then were washed twice and resuspended in Hanks' Balanced Salt Solution (HBSS), and were used immediately or were held at 1° no longer than 2 hours. Centrifugations were accomplished in a refrigerated Sorval RC-2 centrifuge.

In vitro macrophage-parasite adherence tests. CDF rats were anesthetized with ether and sacrificed by exsanguination. Using aseptic technique, the abdomen was opened and normal, unstimulated macrophages were removed by lavage with 8-10 ml of Tissue Culture Medium 199 (M-199) (Grand Island Biological Co., Grand Island, New York 14072) containing 10% heat inactivated fetal calf serum and antibiotics (penicillin was used at 100 units/ml). The cells were pelleted by centrifugation for 3-5 minutes at 1000 x g and resuspended in Tris-ammonium chloride buffer (1 part 0.1M Tris, pH 7.2 : 9 parts 0.83% ammonium chloride). After 10 minutes in the Tris-ammonium chloride buffer at 37°C, the cells were pelleted by centrifugation as above, and resuspended in M-199. Cell numbers and viability determinations were made in a standard homocytometer by counting cells suspended in buffer containing 1% trypan blue stain.

Two x 10^6 unstimulated peritoneal "washout" cells containing 80-85% macrophages as determined by May-Greenwald staining characteristics were suspended in 2 ml of M-199 and incubated in plastic 35 x 10 mm tissue culture dishes for 30 minutes at 37°C in an atmosphere of 5% CO₂.
Non-adherent cells were removed by 3 washes in HBSS, leaving primarily macrophages adherent to the culture dishes.

The macrophage monolayers were sensitized with 1 ml of either normal or hyperimmune serum diluted with 2 ml of M-199 and the sensitized cell layers were incubated for 30 minutes at 37°C in 5% CO₂. Following this, the cells were washed 3 times in cold HBSS and 2 x 10⁸ free parasites in 1 ml of cold HBSS were layered over the macrophages and incubated at 4°C for 30 minutes. The monolayers then were fixed with methanol and stained by the Giemsa technique. Two hundred cells showing typical macrophage morphology were counted and the presence and number of adherent parasites on each plate were recorded. Each experiment was performed in duplicate and the data were averaged.

Animals. Male inbred CDF rats (Charles River Breeding Laboratories) were used throughout the study. Protection assays were performed with rats 4-5 weeks of age, while parasites and various sera were obtained from adult rats (see below).

Sera. Normal serum was harvested from adult CDF rats, aliquoted and stored at -20°C. Hyperimmune serum was obtained by challenging rats (50-75 per group) newly recovered from P. berghei infection with 20 x 10⁶ infected CDF rat erythrocytes weekly for three weeks. The animals were bled approximately 2 weeks later, the sera separated, pooled and stored in 2 ml aliquots at -20°C until needed. Fluorescent serum globulins were prepared from hyperimmune anti-P. berghei serum by the method of Cherry et al, 1966. Rabbit anti-rat gamma globulin fluorescein-conjugated IgG was obtained from Microbiological Associates, Walkersville, Md. and rabbit anti-rat IgG serum for immunoelectrophoresis was obtained from
Miles Laboratories, Inc., Elkhart, Ind.

Chromatographic separation of HIRS. Four ml of CDF rat anti-
P. berghei hyperimmune serum (pooled) was dialyzed for 3 days against ethylenediamine acetate buffer (EDA) and then separated on a QAE-Sephadex A-50 column which had been swollen in EDA buffer. The buffer was prepared as follows: 2.88 g of EDA was dissolved in 73 ml of glacial acetic acid and the volume adjusted to 1 liter with distilled water to achieve an ionic strength of 0.1, pH 7.2. Elutions of serum fractions from the column were accomplished with EDA buffer adjusted to pH 5.0 by adding acetate buffer, and with acetate buffer at pH 4.0. Acetate buffer was prepared by adding 435 ml of 0.6 M acetic acid to 130 ml of 0.6 M Na acetate and adjusting the volume to 1 liter with distilled water to give an ionic strength of 0.1, pH 4.0. Serum fractions were examined and identified by immunoelectrophoresis and by indirect fluorescent antibody technique.

Immunoelectrophoresis. Immunoelectrophoresis was accomplished using 0.65% Difco agar (Difco Laboratories, Detroit, Mich.) in Gelman high resolution buffer, pH 8.8 Gelman Instrument Co., Ann Arbor, Mich.) on an LKB electrophoresis apparatus, Type 3290B LKB Produkter, Stockholm, Sweden. The test samples were electrophoresed at 200V, 20 mA for 60 minutes and then reacted with rabbit anti-rat IgG serum at 25°C in a humidity chamber for 24 hours.

Fluorescent antibody techniques. Indirect fluorescent antibody assay for the opsonic antibody was conducted by incubating fixed films of parasitized blood for 30 minutes at 37°C in hyperimmune serum followed by 2 washes of 10 minutes duration in phosphate buffered saline, pH 7.2. The film was then incubated with rabbit anti-rat gamma globulin fluorescein-
conjugated IgG for 30 minutes at 37°C. The slides were again washed as above and examined for fluorescein on a Carl Zeiss Model WL standard microscope fitted with a mercury vapor illuminator, and using excitor filters BG-38 and BG 12 in conjunction with a darkfield condenser and barrier filters 50 and 44. Indirect fluorescent antibody assay for the macrophage-cytophilic antibody was conducted by incubating macrophage monolayers for 30 minutes at 4°C in hyperimmune serum followed by 2 rapid washes in phosphate buffered saline, pH 7.2. The cells were then incubated with rabbit anti-rat gamma globulin fluorescein-conjugated IgG for 30 minutes at 4°C. The cells were again washed as above and examined as previously for fluorescence.

Adsorption of hyperimmune rat serum with macrophages. Peritoneal cells consisting of at least 80% macrophages were collected from normal CDF rats in M-199, centrifuged at 1000 x g for 2 minutes, resuspended in Tris-ammonium chloride buffer for 10 minutes at 37°C to lyse erythrocytes, centrifuged at 100 x g for 2 minutes and resuspended in M-199. The cells then were plated in 35 x 10 mm plastic tissue culture dishes and incubated for 1 hour at 37°C in an atmosphere containing 5% CO₂ to promote adherence. Non-adherent cells were washed away after this incubation and the M-199 was replaced. The plated cells were held at 4°C until used. After washing the plate in cold HBSS and draining the excess, serum was absorbed by transferring a 1 ml sample to the plate, which then was incubated at 4°C for 30 minutes. At this time another plate was rinsed in cold HBSS and the 1 ml of serum from the preceding plate was transferred to it. The serum was serially adsorbed 6 times in this manner. Each plate after treatment was washed twice in cold HBSS and incubated with
a 1:10 dilution of the fluorescein-conjugated anti-\textit{P. berghei} CDF rat serum globulin for 30 minutes at 4°C. After rinsing 3 times the plates were examined for specific macrophage fluorescent staining. The presence of cytophilic antibody bound to the macrophages during the serial adsorptions served to block the finding of fluorescein-labeled cytophilic antibodies in the conjugated globulins and thus resulted in a negative fluorescent test. Conversely, the absence of cytophilic antibody from the adsorbed serum resulted in a positive staining of the macrophages by the fluorescein-conjugated cytophilic antibodies.

**Cytophilic antibody elutions.** Macrophage-cytophilic antibody was purified by serially adsorbing a 3 ml aliquot of HIRS 6 times with 15 x $10^6$ peritoneal washout cells in suspension for 30 minutes to effect the elution of bound antibody. The supernatant containing the eluted antibodies was adjusted to 3 ml volume. Both the adsorbed serum and the eluate were tested by indirect fluorescence microscopy for specific activity. The eluate was compared to QAE-Sephadex A-50 fractions 1 and 2 for determining its composition by double diffusion in gel against rabbit anti-rat IgG antiserum.

**In vivo protection test # 1 - Adsorbed serum.** HIRS which had been adsorbed 6 times by 5 x $10^6$ peritoneal washout cells was compared with both normal serum and unadsorbed HIRS in 5 week old male CDF rats. Each test group contained 5 rats, and each rat received 5 x $10^6$ free parasites in 0.1 ml of HBSS mixed with 0.1 ml of either normal, HIRS or adsorbed HIRS. The mixtures were immediately injected intravenously. Percent parasitemia values were determined by counting 1000 erythrocytes at random on a Giemsa stained thin blood film daily. Data are plotted as daily mean % parasitemia for the group.
In vivo protection test #2 - Eluted antibody. The antibody containing eluate recovered after 3 ml of HIRS had been adsorbed 6 times with 15 x 10^6 peritoneal washout cells was compared for protective activity in vivo with the adsorbed serum, as well as with both normal and HIRS, in 5 week old male CDF rats. Each test group contained 5 rats and each animal received 5 x 10^2 infected CDF erythrocytes suspended in 0.5 ml of HBSS. The erythrocytes were given intravenously 24 hours after the rats received 0.5 ml intravenously of either NRS, HIRS, macrophage-adsorbed HIRS or macrophage eluate. Percent parasitemias were determined by counting 1000 erythrocytes at random on a Giemsa-stained thin blood film each day. Data are plotted as the mean time required for the rats in each group to reach 1% parasitemia.

In vivo protection test #3 - HIRS fractions. Fractions of HIRS which had been separated by elution chromatography on QAE-Sephadex A-50 were compared with both normal rat serum (NRS) and HIRS in 5 week old male CDF rats. Each group contained 5 rats, and each rat received 0.1 ml of either NRS, HIRS, fraction 1, 2, or 3 or 0.1 ml of fraction 1 plus 0.1 ml of fraction 2. The infectious inocula consisted of 5 x 10^6 freed parasites suspended in 0.1 ml of HBSS which was mixed with the serum or serum fraction(s) and immediately injected intravenously via the lateral tail vein. Percent parasitemias were determined by counting 1000 erythrocytes at random each day on a Giemsa-stained thin blood film. Data are plotted as the mean time required for the group to reach 1% parasitemia.
RESULTS:

**In vitro macrophage-parasite adherence tests.** The effects of arming the macrophages with macrophage cytophilic antibody specific for *Plasmodium berghei* parasites prior to their incubation with free parasites are presented in Figure 2. Twenty-five percent of the macrophages pretreated with hyperimmune serum were associated with adherent parasites as opposed to only 9% of those pretreated with normal rat serum. Of the actual parasites counted, more than three times as many parasites were found to be associated with macrophages pretreated with hyperimmune serum as with macrophages pretreated with normal rat serum.

**Chromatographic separation of HIRS.** Figure 3 is an optical density tracing at 280 nm recorded during the elution of fractions of HIRS from a QAE-Sephadex A-50 column at pH 7.2, 5.0 and 4.0 (fractions 1, 2 and 3 respectively). When examined by immunoelectrophoresis, fractions 1 and 2 were found to consist of IgG$_1$ and IgG$_2$ respectively, following the nomenclature of Bazin et al (2), whereas fraction 3 was found to contain albumin and IgM.

**Adsorption of HIRS with macrophages.** One ml of HIRS adsorbed 4 times by $5 \times 10^6$ peritoneal washout cells was found to block subsequent direct fluorescent antibody staining of macrophages, after the fifth adsorption to partially block the direct fluorescent antibody staining of the macrophages, and after the sixth adsorption to not block the direct fluorescent staining of the macrophages. Thus the macrophage cytophilic antibody was no longer present at inhibitory levels in 1 ml of HIRS after 6 adsorptions with $5 \times 10^6$ peritoneal washout cells.
Figure 2. The effects of cytophilic antibodies upon the ability of macrophages to retain free *Plasmodium berghei* parasites.
Figure 3. The pH elution pattern of hyperimmune rat serum from QAE-Sephadex A-50.
Cytophilic antibody elutions. When the eluate from peritoneal washout cells which had been used to adsorb HIRS was compared by the indirect fluorescent antibody technique to the adsorbed HIRS, it was found that the eluate contained primarily macrophage-cytophilic antibodies, whereas the adsorbed HIRS contained primarily opsonic antibodies. When the same eluate was compared by double diffusion in gel with fractions 1 and 2 obtained by QAE-Sephadex A-50 chromatography of HIRS, fraction 1 was shown to contain antibodies identical with the antibodies eluted from the macrophages, whereas fraction 2 was shown to contain antibodies distinct from both the eluate and fraction 1. However, all antibodies were identified as belonging to the IgG class (Figure 4).

In vivo protection test #1 - Adsorbed serum. The protective effects of HIRS which had been depleted of macrophage-cytophilic antibodies was reduced from the level of protection exhibited by unadsorbed HIRS (Figure 5) as was demonstrated by the earlier onset and rise of parasitemia after the challenge infection was initiated.

In vivo protection test #2 - Eluted antibody. When eluted macrophage-cytophilic antibodies were compared with HIRS depleted of macrophage-cytophilic antibodies it was found that the protective capacity of HIRS depleted of macrophage-cytophilic antibodies was diminished, and that the eluate which contained only the eluted macrophage-cytophilic antibodies had no protective capacity (Figure 6).

In vivo protection test #3 - HIRS fractions. Fraction 1, containing the macrophage cytophilic antibodies, had no protective capacity against P. berghei infection in vivo, and fraction 2 contained only moderate protective capacity. However, when fractions 1 and 2 were recombined
Figure 4  The precipitin pattern formed in gel by eluted macrophage-cytophilic antibodies and QAE-Sephadex hyperimmune serum fractions when reacted against anti-IgG antiserum.
Figure 5. The effect of adsorption with macrophages upon the protective capacity of *Plasmodium berghei* immune serum in rats.

Values plotted are means (± S.E.M.) obtained from counts of 1000 cells on each film. (n=5).
Figure 6. The protective effects against Plasmodium berghei of macrophage-adsorbed and macrophage-eluted hyperimmune serum fractions in rats.

Values plotted are means (± S.E.M.) obtained from counts of 1000 cells on each film (n=5).
in vivo, a synergistic effect was seen, resulting in a greater protective capacity than would be indicated by the expected additive effects of the individual fractions. Fraction 3 contained minimal protective capacity which can be ascribed to the presence of IgM antibodies (Figure 7).

DISCUSSION:

Our findings indicate that there are two major and distinct types of IgG antibody which provide protection against P. berghei in the rat. The first of these is an opsonic antibody which has as its specific ligand some site on the parasite surface. The second is a macrophage-cytophilic antibody which is unable to bind to parasites until after it has become attached to the surface of a macrophage. Sodeman and Haferkamp (1969) have reported finding in rats macrophage-cytophilic antibodies to tuberculoprotein after BCG immunization and after appropriate immunization to Group A Streptococcus carbohydrate. Binding of the cytophilic antibody to the macrophage surface is probably mediated by an Fc receptor (Davey and Asherson, 1967; Dissanayake and Hay, 1975). Davey and Asherson concluded that the macrophage receptor for macrophage-cytophilic antibodies against sheep red blood cells in the guinea pig might be a phospholipid or phospholipoprotein, and Arend and Mannik (1973) found that cytophilic antibody receptors on macrophages were destroyed by phospholipase. We have not yet attempted to determine the nature of the receptors in our system.

Macrophage-cytophilic antibodies cannot be adsorbed out of serum by antigen; however, upon attachment to the macrophage surface membrane the antibodies presumably undergo steric rearrangement whereupon they
Figure 7. The protective effects against Plasmodium berghei in rats of QAE-Sephadex hyperimmune serum fractions. Values plotted are means (± S.E.M.) obtained from counts of 1000 cells on each film. (n=5).
become capable of specifically binding parasite receptors. However the protective nature of this interaction is not manifested in the absence of specific opsonic antibody. Indeed it is established that some cytophilic antibodies fix antigens to macrophages without inevitably stimulating phagocytosis (Tizard, 1972). Levenson and Braude (1967) observed that anti-\textit{S. typhi} Vi polysaccharide obtained early in immunization could induce rosette formation of Vi-coated erythrocytes around macrophages and that prolonged incubation of these rosettes at 37°C did not lead to phagocytosis. Internalization may be dependent upon the presence of complement-fixing anti-parasite antibodies at the macrophage-parasite interface. Ehlenberger and Nussenzweig (1977) have recently demonstrated synergy between Fc and C3 receptors in the phagocytic process. Bianco et al (1975) have concluded that the process of phagocytosis is accompanied by a change in the macrophage C3b receptor that allows a shift in function from binding to both binding and internalization.

Miller et al (1977) recently have reported a lack of correlation between functional immunity \textit{in vivo} in monkeys, and the presence of antibodies capable of inhibiting merozoite invasion \textit{in vitro}. In the immune response of the rat to \textit{P. berghei} a synergistic relationship exists between the macrophage-cytophilic and the opsonic antibodies in which the combined protective effect is greater than the sum of the individual protective capacities. The exact nature of this interaction remains unclear. It has been proposed (Cohen and Butcher, 1970; Miller et al, 1975) that the antibody coating the parasites may physically interfere with penetration of the erythrocyte by the merozoite form of
the parasite. We have observed in this study that the opsonic antibody is necessary for the manifestation of the protective effect of the macrophage-cytophilic antibodies. Perhaps the macrophage-cytophilic antibodies promote only the adherence of parasites to the phagocytic cells, and not their subsequent ingestion. Stossel (1976) has observed that under differing conditions "some particles stick tightly to phagocytes but are not ingested, some stick and are partly ingested and still others stick and are completely ingested". The opsonic antibodies may trigger the actual ingestion of the parasites by the macrophages, but may be less efficient alone in promoting adherence. It has been stated that the internalization of particles attached to macrophages is triggered by IgG, and that different roles are assigned for the macrophage receptor sites for complement (C3) and for immunoglobulin (Mantovani et al, 1972; Scribner and Farney, 1976).

Destruction of the infective merozoites is necessary to control an infection by malarial parasites. Electronmicroscopic studies in this laboratory (Brooks, 1977) have indicated that although trophozoites in a free parasite preparation will adhere to macrophages in the presence of either normal or immune serum, merozoites are found to be adherent to macrophages only in the presence of immune serum. A capsule has been demonstrated around the infective merozoite stage of Plasmodium (Miller et al, 1975). This capsule may very well confer antiphagocytic properties upon the merozoite. Immune serum has been shown to contain protective antibodies which bind to this capsule (Miller et al, 1975), yet these antibodies are not capable of direct merozoite neutralization. However, these antibodies may serve to reduce or eliminate the
anti-phagocytic nature of the capsular material either by reduction of
the hydrophilic characteristics of the capsular material thereby favoring
the physical processes of phagocytic engulfment (Mudd and Mudd, 1933), or
by stabilization of the amorphous capsular material thereby facilitating
adhesion and subsequent engulfment, or by both mechanisms.

Although the macrophage-cytophilic antibodies may facilitate the
attachment of the merozoites to the macrophage, alteration of the capsular
material (perhaps by complement fixation) may not occur in the absence of
the opsonic antibody and ingestion of the parasite would therefore be
less likely. Furthermore, should the merozoite be swept loose, leaving
some capsular material attached at the macrophage surface, the recogni-
tion capability of the cytophilic antibody-armed macrophage would be
reduced. Thus it becomes apparent that cytophilic antibody-mediated
attachment of infective merozoites would result in enhancement of the
protective effects of the opsonic antibody, but would probably not be
protective alone.

From this laboratory Hamburger and Kreier (1976a) have demonstrated
that passive immunity to malaria cannot be conferred to mice by immune
serum of rat origin, with the implication that the site of protective
action of antimalarial antibodies is species-specific. This action is
probably at the level of phagocytosis, i.e. the macrophage. Further
work (Hamburger and Kreier, 1975) suggested that either the parasite
opsonizing antibodies were of low avidity and quickly became dissociated
from the parasites in vivo, or that a cytophilic antibody was involved
in phagocytosis, or both. Low opsonizing antibody avidity indeed was
demonstrated by these investigators in a subsequent publication
(Hamburger and Kreier, 1976b), and now we have additionally demonstrated
the presence and role of a macrophage-cytophilic antibody.


CHAPTER 4

"A CHARACTERIZATION OF ANTI-MALARIAL (PLASMODIUM BERGHEI) OPSONIC AND MACROPHAGE-CYTOPHILIC ANTIBODIES BY FLUORESCENT ANTIBODY TECHNIQUES"

There are a variety of tests in use for the measurement of anti-malarial antibodies but serological evaluations of people in areas where malaria is endemic have not been shown to correlate strictly with protection, and passive transfer of serum has not been very successful in protecting non-immune persons. Additionally, serum from immune rats has been shown to provide only limited protection to non-immune rats, and was shown to be non-protective in mice (Hamburger and Kreier, 1976). These investigators also determined that binding of antibody to parasites in vitro did not affect their infectivity unless the recipient animal also received some immune serum, suggesting an additional in vivo requirement for antibody, perhaps in association with macrophages. A host-selective effect seemed to be involved. The work of Chow and Kreier (1971) also suggested that the role of the macrophage might be more complex than merely that of phagocytizing opsonized parasites. Subsequent work in this laboratory (Green and Kreier, 1977) established the presence of a macrophage-cytophilic component in immune serum and also established that the cytophilic antibody functions in a protective capacity in the host in concert with the opsonic antibody component. The definition of cytophilic antibody
proposed originally by Boyden (1963) and by Sorkin (1963) stated explicitly that the binding of antigen to cytophilic antibody takes place subsequent to cellular fixation of the antibody. Thus all macrophage-cytophilic antibodies are recognized by and bound to a common type of macrophage receptor and undergo a subsequent steric rearrangement to expose their antigen-specific antigen binding sites. This definition of the term "cytophilic antibody" has been accepted by the present author.

MATERIALS AND METHODS:

Conjugation of serum immunoglobulins with fluorescein isothiocyanate. The procedure used was a modification of that published by Cherry et al. 1966. Ten ml of specific immune serum was diluted with 10 ml of distilled water and chilled in an ice bath in a foil-wrapped 50 ml Erlenmeyer flask. The diluted serum was stirred with a magnetic stirrer while an equal volume of saturated ammonium sulfate at room temperature was added dropwise. Stirring was continued for 20 minutes, then the precipitated globulins were pelleted by centrifugation at 20,000 x g for 15 minutes and the supernatant fluid was discarded. The pellet was redissolved in 21.2 ml of ice cold distilled water. Saturated ammonium sulfate solution (18.8 ml) was added dropwise to achieve a 47% saturation while stirring slowly. The stirring was continued for 20 minutes, after which the suspension again was centrifuged (as above) and the supernatant fluid was discarded. No hemoglobin was visible in the sample. The globulin pellet was redissolved in phosphate buffered saline, pH 7.5, (PBS) to the original volume and dialyzed at 4°C against
200 volumes of PBS, pH 7.5 for six hours to eliminate ammonium sulfate ions. A Lowry's protein assay of the globulin solution was performed and the protein concentration was adjusted to 10 mg/ml by dilution with PBS and a volume of 0.5M carbonate-bicarbonate buffer, pH 9.0, equal to 10% of the globulin solution. The buffered globulin solution was kept chilled in an ice bath and stirred gently while adding 25ug of dry fluorescein isothiocyanate per mg of total globulin protein. Slow stirring was continued overnight at 4°C. The fluorescein/globulin conjugate then was layered onto a Sephadex G-25 column which had been equilibrated with PBS, pH 7.5. The column was run at 30 dpm and the labeled conjugate was collected with the void volume, whereas the unconjugated fluorescein was retained in the column. The volume of the collected conjugate was adjusted to twice the original volume with PBS, filtered through a 0.22mu Millipore filter and aliquoted in small volumes for storage at -20°C.

Titer determination of hyperimmune serum conjugate by direct fluorescent antibody technique.

Opsonic antibody. Since hemoglobin has the effect of quenching the fluorescence produced by fluorescein isothiocyanate, the infected blood films used as antigen for the test first were fixed in methanol for 2 minutes, air dried, and then dehemoglobinized by immersion in 0.1% HCl for 30 seconds followed by 2 rinses in 0.15M NaCl and 1 rinse in distilled water. The slides were air dried prior to use. An area of approximately 1 cm² was marked on the warmed slide with a wax pencil and a solution of the hyperimmune serum globulin/fluorescein isothiocyanate conjugate (HIRS/FITC) was placed on the enclosed area and the treated
blood film was incubated at 37°C in a humid chamber for 30 minutes. The slides then were rinsed twice in PBS by immersing them in a staining dish on a shaker table and shaking gently for 2 minutes. The slides were blotted dry and coverslips were mounted in a drop of 90% glycerol in PBS, pH 7.5. The films were examined for fluorescence immediately on a Carl Zeiss Model WL Standard microscope fitted with a mercury vapor illuminator.

**Cytophilic antibody.** Macrophages for use as targets for cytophilic antibody binding were obtained by peritoneal lavage of unstimulated CDF rats (Charles River Breeding Laboratories, Inc.) with Tissue Culture Medium 199 (M-199). The peritoneal washout cells, containing approximately 80-85% macrophages as determined by examination of May-Greenwald stained films were washed once in M-199 and then plated out onto 30 mm plastic tissue culture dishes (Falcon: 1950 Williams Dr., Oxnard, CA) at the rate of approximately 1 x 10^6 cells per dish in 2 ml of M-199. These then were cultured for 30 minutes and washed to remove non-adherent cells, leaving primarily macrophages. These cells were cultured in fresh M-199 without added serum for 2 hours at 37°C, in 5% CO₂. Following culture, the monolayers were washed by immersion in Hanks' Balanced Salt Solution (HBSS), and the appropriate dilution of HIRS/FITC was added. The cells were incubated at 37°C, 5% CO₂, for 30 minutes, again washed 3 times by immersion in HBSS, drained and mounted with coverslips in a drop of 90% glycerol in PBS, pH 7.5. The preparations were examined for fluorescence and were graded from 0 to 4+, with 1+ being considered positive but minimal fluorescence while 4+ was considered maximal fluorescence present on the surface of the macrophages.
Titer determinations of hyperimmune serum by indirect fluorescent antibody technique.

Opsonic antibody. Free *P. berghei* parasites were prepared by the continuous flow sonication method of Prior and Kreier (1972). The parasites were aliquoted as $5 \times 10^7$ parasites per tube and incubated with the appropriate dilution of HIRS for 30 minutes at 37°C. The parasites were washed twice by centrifugation in PBS and reincubated at 37°C for 30 minutes with rabbit anti-rat gamma globulin fluorescein conjugate diluted 1:4 in PBS and which had been previously adsorbed with rat liver powder. The parasites again were washed twice in PBS, resuspended in 90% glycerol in PBS, pH 7.5, and examined for fluorescence.

Cytophilic antibody. Macrophage monolayers were prepared as described above and incubated with appropriate dilutions of hyperimmune serum in M-199 at 4°C for 30 minutes. The monolayers were washed 3 times by immersion in HBSS and then reincubated at 37°C, 5% CO₂ for 30 minutes with a 1:6 dilution of fluorescein-conjugated rabbit anti-rat gamma globulin in HBSS, drained of excess buffer and mounted with coverslips in a drop of 90% glycerol in PBS pH 7.5. The plates were then examined for fluorescence and graded as before.

Fluorescent staining of fixed complement: the ability of opsonic vs cytophilic antibody to fix complement.

Infected blood films were fixed for 2 minutes in methanol and dehemoglobinized for 30 seconds in 0.1% HCl. Normal and hyperimmune rat serum were inactivated by incubation at 56°C for 30 minutes.
just prior to use. Complement was obtained from guinea pig serum which was diluted 1:10 in PBS, pH 7.5, and aliquoted into 2 ml volumes and stored frozen at -20°C. Normal and hyperimmune sera were diluted in PBS, pH 7.5 and fluorescent conjugated rabbit anti-G.p.IgG was diluted in PBS containing 2% Tween 80. Fixed guinea pig complement was stained with fluorescein-conjugated rabbit anti-guinea pig gamma globulin (Cappel Laboratories, Inc. Downingtown, Pa.) at a 1:4 dilution in PBS with 2% Tween 80.

Complement fixing ability of opsonic antibody on fixed parasitized blood films.

A variety of tests for complement fixing ability of the opsonic antibody were done (Table 3). The tests were performed as follows: fixed dehemoglobinized P. berghei-infected blood films were flooded with either NRS, HIRS or PBS plus an equal volume of a 1:10 dilution of guinea pig complement and were incubated at 37°C for 30 minutes in a humidity chamber, washed again as above and blotted. Coverslips were mounted in 90% glycerol in PBS, pH 7.5 and the preparations were examined for fluorescence with a Zeiss fluorescence microscope.

Complement fixing ability of opsonic antibody on free P. berghei parasites.

Free parasites prepared by the continuous flow sonication method at a concentration of \(5 \times 10^8\) parasites/ml were washed 3 times in PBS. Each tube received \(5 \times 10^7\) free parasites. The parasites were incubated for 1 hour at 37°C with a 1:2 dilution of HIRS plus either a 1:10 dilution of guinea pig complement, or PBS. After incubation the parasites were washed twice in PBS by centrifugation at 1000 x g for 3 minutes each
centrifugation and resuspended in a 1:4 dilution of rabbit anti-guinea pig gamma globulin/FITC and incubated at 37°C for 30 minutes. The parasites again were washed 2 times as above and were resuspended in 0.1 ml of PBS. A drop of this suspension was mixed with a drop of 90% glycerol in PBS, pH 7.5 on a glass slide, coverslipped and examined for fluorescence. The test was set up as outlined in Table 4 (Page 56).

Complement fixing ability of macrophage-bound cytophilic antibody when reacted with free parasites (P. berghei).

This test was designed to determine the ability of both the cytophilic and the opsonic antibodies to fix complement in the presence of antigen. A sandwich technique was used in which macrophages were sensitized with cytophilic antibody by incubation with a 1:4 dilution of heat inactivated HIRS for 45 minutes at 4°C. The plates were then washed 3 times by immersion in cold HBSS and the exposed to antigen in the form of $5 \times 10^7$ freeze-thawed free P. berghei parasites for 30 minutes at 4°C. The plates were then washed 3 times by immersion in cold HBSS and then exposed to antigen in the form of $5 \times 10^7$ freeze-thawed free P. berghei parasites for 30 minutes at 4°C. The plates were again washed 3 times and then flooded with a 1:60 dilution of guinea pig complement and incubated at 4°C for 45 minutes. Additionally, cytophilic antibody sensitized macrophages which had been allowed to bind antigen were incubated with opsonic antibody in a 1:2 dilution of HIRS which contained guinea pig complement at a 1:60 dilution. This was to provide a test for the ability of opsonic antibody to fix complement under the same conditions, as well as to prove that cytophilic antibody-antigen complexes
were indeed present on the macrophage surface. After incubation with
guinea pig complement or guinea pig complement plus opsonizing antibody,
the plates were again washed twice in cold HBSS and then flooded with a
1:8 dilution of rabbit anti-guinea pig gamma globulin/FITC and incubated
for 45 minutes at 4°C. Following incubation the preparations were washed
twice in HBSS, drained and coverslips were mounted in a drop of 90%
glycerol in PBS, pH 7.5. The preparations were then examined for fluores­
cence. The experimental groups tested are outlined in Table 5 (Page 58).

Test of specificity of cytophilic antibody for *P. berghei* antigen.

This test employed the technique of sandwiching antigen between the
malaria-specific cytophilic antibody on the sensitized macrophages and
opsonic antibody which was labeled with FITC. Four x 10^6 peritoneal
washout cells in M-199 containing approximately 80% macrophages were
plated onto 30 mm plastic culture dishes and incubated for 30 minutes.
The plates were then washed 3 times by immersion in cold HBSS, more M-199
was added and the cells were incubated an additional 2 hours. After this
point, 2% sodium azide was added to all the plates to stabilize the
macrophage membranes (Taylor et al, 1971). The monolayers were then
sensitized with a 1:2 dilution of HIRS or NRS in M-199 by incubation
at 4°C for 30 minutes. The plates were then washed 3 times in cold
HBSS and approximately 5 x 10^7 freeze-thawed free *P. berghei* parasites
in 2 ml of M-199 were added to the appropriate plates, and the plates
were incubated at 4°C for 30 minutes. The plates were again washed
3 times in cold HBSS and either HIRS/FITC or NRS/FITC at a 1:4 dilution
in HBSS was added to each plate and incubated at 4°C for 30 minutes.
The plates were washed twice in cold HBSS, drained and coverslips were mounted in a drop of 90% glycerol in PBS, pH 7.5. The preparations were immediately examined for fluorescence on a Zeiss fluorescence microscope.

If the cytophilic antibody on the macrophage was specific for *P. berghei*, then the parasite material would be bound, and could be stained by fluorescein-tagged opsonic antibody. False positives due to direct macrophage staining by fluorescein-tagged cytophilic antibody were minimized by blocking the receptors on the azide-stabilized macrophage membrane with non-labeled antibody at the time of macrophage sensitization. The staining of parasite material on the macrophage surface would indicate that the cytophilic antibody specifically bound the *P. berghei* antigens.

The experimental groups tested are outlined in Table 6 (Page 59).

**Specificity test of cytophilic antibody for *P. berghei* antigen by C\(^{1}\) staining.**

A second test for malarial specificity of the cytophilic antibody is contained in the complement staining test for the ability of cytophilic and opsonic antibodies to fix complement given above. Specificity can be demonstrated by the presence of parasite antigens bound to the surface of the macrophages as demonstrated by the presence of fixed complement on the macrophage surface after reacting opsonic antibody and guinea pig complement with the bound antigen.

**Determination of the binding affinity characteristics of opsonic and cytophilic antimalarial antibody.**

The proportions of fluorescein-labeled antibody bound to the macrophages or parasites when equilibrated at various temperatures were determined
spectrofluorometrically in a Mark I Spectrofluorometer, Farrand Optical Co., Inc., New York. The wavelength of the exciting light was 490 μm and that of the emitted light was measured at 535 μm.

Cytophilic antibody affinity. Unstimulated peritoneal washout cells containing approximately 85% macrophages were collected in M-199 and incubated for 1 hour at 37°C, 5% CO₂ in 12 x 75 mm polypropylene tubes (Falcon: 1950 Williams Dr., Oxnard, Co.). The tubes, each containing 2.32 x 10⁷ macrophages in 2 ml of solution were then equilibrated with HIRS/FITC for 90 minutes at incubation temperatures of 1°C, 12°C, 24°C or 37°C. The 56°C sample was sensitized at 1°C and then eluted for 30 minutes at 56°C. This was done to provide an estimate of antibody recovery at elution temperatures of 56°C. After incubation the cells were washed twice in ice cold HBSS by centrifugation at 1000 x g for 3 minutes. A control tube, containing macrophages but no HIRS/FITC was included and incubated at 1°C. This tube served as a zero blank to determine amount of scattered light which was present when the test was read in the spectrofluorometer. The sensitized macrophages in the 1°C incubation tube were used to determine the maximal fluorescence reading at 1.000 microamperes and the remaining tubes were read immediately in the range between 0.000 and 1.000 microamperes. All tubes were held in an ice bath after washing, until read.

Opsonic antibody affinity. Free P. berghei parasites, freshly prepared by the continuous flow sonication procedure of Prior and Kreier (1972) and at a concentration of 1.65 x 10⁸ parasites per tube were incubated in 2 ml of undiluted HIRS/FITC for 60 minutes at temperatures of 1°C, 12°C, 24°C and 37°C. The parasites were then washed twice in ice cold HBSS by centrifugation for 5 minutes at 1000 x g and then resuspended
in cold HBSS. A control tube containing parasites but no HIRS/FITC was included. This tube served as a zero blank to determine the small amount of light scatter present when the test was read in the spectrofluorometer. The 37°C tube was used to set the maximal fluorescence reading at 1.000 microamperes, and the remaining tubes were read immediately in the range between 0.000 and 1.000 microamperes. The tubes were held at room temperature until read.

RESULTS:

Titer determination of hyperimmune serum conjugate by direct fluorescent antibody technique.

Table 3 presents the results of titering the anti-*P. berghei* hyperimmune conjugate for the activity of both opsonic and cytophilic antibodies.

The titers were expressed as the reciprocal of those dilutions which gave 1+ fluorescence and by ** in Table 3. Thus the direct fluorescent antibody titer of the opsonic antibodies was 160, and that of the macrophage-cytophilic antibodies was 320.

Titer determination of hyperimmune serum by the indirect fluorescent antibody technique.

Table 4 presents the results of titering the anti-*P. berghei* hyperimmune serum for the activity of both opsonic and cytophilic antibodies. The titer was expressed as the reciprocal of those dilutions which gave a 1+ fluorescence and are indicated by ** in Table 4. Thus the indirect fluorescent antibody titer of the opsonic antibodies is 640 and that of the macrophage-cytophilic antibodies is 80.
Table 3. Titer determinations of hyperimmune serum conjugate by direct fluorescent antibody technique.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Opsonic antibody assay</th>
<th>FAT reaction</th>
<th>Dilution</th>
<th>Cytophilic antibody assay</th>
<th>FAT reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:8</td>
<td></td>
<td>3+</td>
<td>1:8</td>
<td></td>
<td>4+</td>
</tr>
<tr>
<td>1:20</td>
<td></td>
<td>3+</td>
<td>1:20</td>
<td></td>
<td>4+</td>
</tr>
<tr>
<td>1:40</td>
<td></td>
<td>3+</td>
<td>1:40</td>
<td></td>
<td>4+</td>
</tr>
<tr>
<td>1:80</td>
<td></td>
<td>2+</td>
<td>1:80</td>
<td></td>
<td>3+</td>
</tr>
<tr>
<td>1:160**</td>
<td></td>
<td>1+</td>
<td>1:160</td>
<td></td>
<td>2+</td>
</tr>
<tr>
<td>1:320</td>
<td>+/−</td>
<td></td>
<td>1:320**</td>
<td></td>
<td>1+</td>
</tr>
<tr>
<td>1:640</td>
<td>−</td>
<td></td>
<td>1:640</td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>1:1280</td>
<td>−</td>
<td></td>
<td>1:1280</td>
<td></td>
<td>−</td>
</tr>
</tbody>
</table>

**Titer was expressed as the reciprocal of these dilutions.
Table 4. Titer determinations of hyperimmune serum by indirect fluorescent antibody technique.

<table>
<thead>
<tr>
<th>Opsonic antibody assay</th>
<th>Dilution</th>
<th>IFAT reaction</th>
<th>Cytophilic antibody assay</th>
<th>Dilution</th>
<th>IFAT reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>4+</td>
<td>1:10</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:20</td>
<td>4+</td>
<td>1:20</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:40</td>
<td>4+</td>
<td>1:40</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td>3+</td>
<td>1:80**</td>
<td>1+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:160</td>
<td>3+</td>
<td>1:160</td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:320</td>
<td>2+</td>
<td>1:320</td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:640**</td>
<td>1+</td>
<td>1:640</td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1280</td>
<td>+/-</td>
<td>1:1280</td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Titer was expressed as the reciprocal of these dilutions.
Complement fixing ability of opsonic antibody when reacted with fixed parasitized blood films.

There was fluorescent staining for complement on films of fixed parasites treated with antibody and complement. Thus guinea pig complement was fixed by the reaction of opsonic antibody with parasite antigens in fixed films of parasitized erythrocytes. The various control groups were negative for fixed complement (Table 5).

Complement fixing ability of opsonic antibody when reacted with free P. berghei parasites.

No specific fluorescent staining for complement was observed in the control preparations which consisted of free parasites treated with antibody but no complement or complement but no antibody; however, very strong fluorescent staining was seen in the test (Table 6) which employed antibody coated free parasites and active complement.

Complement fixing ability of macrophage-cound cytophilic antibody when reacted with free P. berghei parasites.

No specific fluorescent staining for complement was observed in any of the macrophage, cytophilic antibody, parasite preparations unless opsonic antibody was bound on the parasites (Table 7). Thus the cytophilic antibody on macrophages did not fix complement even after reaction with P. berghei antigens. However, the positive control, i.e. that test in which opsonic antibody coated the parasites bound to the macrophages, showed fixation of complement. The antigen-antibody complexes which coated the macrophages stained brightly with anti-guinea pig complement fluorescent stain. These test results are presented in Table 7.
Table 5. Experimental groups used and results obtained in tests for complement fixing ability of opsonic antibody on fixed parasitized blood films.

<table>
<thead>
<tr>
<th>Number</th>
<th>Status</th>
<th>Step 1. (37°C; 30 min.)</th>
<th>Step 2 (37°C; 30 min.)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control NRS (inact.) 1:2 + C' 1:10</td>
<td>Rab. anti-C'/FITC 1:4</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Control NRS (inact) 1:2 + C' (inact) 1:10</td>
<td>Rab. anti-c'/FITC 1:4</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Control PBS + C'' 1:10</td>
<td>Rab. anti-C'/FITC 1:4</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Control HIRS (inact) 1:2 + PBS</td>
<td>Rab. anti-C'/FITC 1:4</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Control HIRS (inact) 1:2 + C' (inact)</td>
<td>Rab. anti-C'/FITC 1:4</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Test HIRS (inact) 1:2 + C' 1:10</td>
<td>Rab. anti-C'/FITC 1:4</td>
<td>positive</td>
<td></td>
</tr>
</tbody>
</table>
Table: 6. Experimental groups used and results obtained in tests for complement fixing ability of opsonic antibody on free parasites.

<table>
<thead>
<tr>
<th>Number</th>
<th>Status</th>
<th>Step 1 37°C; (1 hr.)</th>
<th>Step 2 37°C; (30 min.)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>parasites + HIRS (inact) 1:2 + PBS</td>
<td>rab. anti-guinea pig C' 1:8</td>
<td>negative</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>parasites + HIRS (inact) 1:2 + C' (inact.)</td>
<td>rab. anti guinea pig C' 1:8</td>
<td>negative</td>
</tr>
<tr>
<td>3</td>
<td>Test</td>
<td>parasites + HIRS (inact) 1:2 + C'</td>
<td>rab. anti-guinea pig C' 1:8</td>
<td>positive</td>
</tr>
</tbody>
</table>
Table 7. Experimental groups used and results obtained in tests for complement fixing ability of macrophage-bound cytophilic antibody.

<table>
<thead>
<tr>
<th>Number</th>
<th>Status</th>
<th>Step 1 (45 min, 4°C)</th>
<th>Step 2 (4°C, 45 min)</th>
<th>Step 3 (45 min, 4°C)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control HIRS (inact)/wash/ag M-199 rab. anti-g.pig C' 1:8</td>
<td>negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control HIRS (inact)/wash/ag C'(inact) 1:60 rab. anti-g.pig C' 1:8</td>
<td>negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Test HIRS (inact)/wash/ag C' 1:60 rab. anti-g. pig C' 1:8</td>
<td>negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Control HIRS (inact)/wash/ag M-199+HIRS (inact) 1:4 rab. anti-g. pig C' 1:8</td>
<td>negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Control HIRS (inact)/wash/ag C'(inact)l:60+HIRS(inact)l:4 rab. anti-g. pig.C'1:8</td>
<td>positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Test/Con. HIRS (inact)/wash/ag C' 1:60 +HIRS (inact)1:4 rab. anti-g. pig C 1:8</td>
<td>positive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Test for specificity of cytophilic antibody for *P. berghei* antigen by antigen staining.

Cytophilic antibody armed macrophages fluoresced after they were *P. berghei* antigens and subsequently treated with fluorescent labeled anti *P. berghei* antibody. This indicates that *P. berghei* antigens were bound to the surface of the cytophilic antibody-sensitized macrophages. The various control preparations which were reacted with NRS/FITC were completely negative, whereas preparation exposed to fluorescent-labeled antibody were weakly positive (Table 8).

**Complement staining test for *P. berghei* specificity of macrophage-cytophilic antibody.**

Macrophages to which cytophilic antibody had bound free *P. berghei* parasites were able to fix opsonic antibody and complement (Table 9). This reaction is in effect a test for the specificity of cytophilic antibody to react with *P. berghei*. The fact that parasite material was demonstrated on the surface of macrophages which were sensitized with immune cytophilic antibody proves anti-*P. berghei* specificity to be present in the cytophilic antibody population.

**Determination of the binding affinity characteristics of opsonic and cytophilic anti-*P. berghei* antibodies.**

The binding affinity characteristics of macrophage-cytophilic and opsonic antibodies as determined by spectrofluorometer measurements of the relative amounts of fluorescein-labeled antibodies bound at various temperatures are presented in Table 9 and Figure 8.
Table 8. Experimental groups used and results of tests of the specificity of cytophilic antibody for *P. berghei* antigens.

<table>
<thead>
<tr>
<th>Number/status</th>
<th>Step 1. (4°C/1 hr)</th>
<th>Step 2. (4°C/1 hr)</th>
<th>Step 3. (4°C/1 hr)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Test</td>
<td>MOs + HIRS (1:2)</td>
<td>freeze/thawed <em>P. berghei</em></td>
<td>HIRS/FITC (1:4)</td>
<td>4+</td>
</tr>
<tr>
<td>2 Control</td>
<td>MOs + NRS (1:2)</td>
<td>freeze/thawed <em>P. berghei</em></td>
<td>HIRS/FITC (1:4)</td>
<td>-</td>
</tr>
<tr>
<td>3 Control</td>
<td>MOs + HIRS (1:2)</td>
<td>freeze/thawed <em>P. berghei</em></td>
<td>NRS/FITC (1:4)</td>
<td>+/-</td>
</tr>
<tr>
<td>4 Control</td>
<td>MOs + NRS (1:2)</td>
<td>freeze/thawed <em>P. berghei</em></td>
<td>HIRS/FITC (1:4)</td>
<td>+/-</td>
</tr>
<tr>
<td>5 Control</td>
<td>MOs + HIRS (1:2)</td>
<td>M-199; no <em>P. berghei</em></td>
<td>HIRS/FITC (1:4)</td>
<td>-</td>
</tr>
<tr>
<td>6 Control</td>
<td>MOs + NRS (1:2)</td>
<td>M-199; no <em>P. berghei</em></td>
<td>HIRS/FITC (1:4)</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 9. The binding affinities of fluorescein-labeled macrophage-cytophilic and opsonic antibodies in *P. berghei* hyperimmune serum as determined by spectrofluorometry.

<table>
<thead>
<tr>
<th>Equilibrium Temperature</th>
<th>Fluorescent emission intensity—microamperes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytophilic antibody</td>
</tr>
<tr>
<td>1°C</td>
<td>1.000</td>
</tr>
<tr>
<td>12°C</td>
<td>0.970</td>
</tr>
<tr>
<td>24°C</td>
<td>0.690</td>
</tr>
<tr>
<td>37°C</td>
<td>0.570</td>
</tr>
<tr>
<td>56°C</td>
<td>0.410</td>
</tr>
</tbody>
</table>
Figure 8. The binding affinities of fluorescein-labeled macrophage-cytophilic and opsonic antibodies from P. berghei hyperimmune serum as determined by spectrofluorometry.
Macrophage-cytophilic antibody bound most efficiently at low temperatures, and bound much less at 37°C. The parasite opsonizing antibody bound most efficiently at 37°C, and much more weakly at 0°C.

**DISCUSSION:**

A series of replicate titrations of cytophilic and opsonic antibodies in a sample of hyperimmune serum were consistent when determined by the direct fluorescent antibody technique. When the indirect method was used the titer of opsonic antibody was equal to or greater than the titer determined by the direct technique whereas the cytophilic antibody titer was consistently lower by approximately two dilutions when determined by the indirect technique. The higher opsonic titer obtained by the indirect technique is probably due to amplification of the response by attachment of more than one fluorescent tagged anti-IgG molecule per molecule of bound opsonic antibody, and the fact that the temperature optima for both the opsonic and the anti-IgG antibodies are the same, i.e. 37°C minimizes loss of labeled antibody during incubation. On the other hand, the optimum binding temperature for the cytophilic antibody is much lower than that of the anti-IgG used to visualize the attached cytophilic antibody. Consequently, there is the risk that cytophilic antibody/anti-IgG complexes will be shed during incubation at the higher temperatures which are most desirable for the binding of the labeled anti-IgG. At lower temperatures the cytophilic antibody is bound more firmly, but the binding of the labeled anti-IgG is much less efficient. Additionally, capping and loss of the lattice like cytophilic antibody/anti-IgG complexes may also be a factor in reducing the efficiency of this technique. Capping has been observed
in our system at both ends of the temperature range studies, although the use of 2% sodium azide seemed to reduce the losses due to capping.

The ability of the opsonizing antibody to fix complement was observed using both unfixed free parasite antigens and fixed infected blood film parasite antigens. This result was not unexpected due to the recognized usefulness of the standard serological complement fixation test for the detection of anti-malarial antibodies. The failure of cytophilic antibody to fix complement in the presence of antigen seems logical. It would certainly not be in the best interest of the host mounting an immune response if complement were to be fixed on the surface of its own macrophages. This fixation would cause membrane damage. On several occasions when opsonized parasites were bound to macrophages in the presence of complement we observed damaged macrophages. Various phenomena probably operate in vivo to avoid such damage. First of all, cytophilic antibodies do not trigger the fixation of complement, secondly the more avid opsonic antibody will probably have triggered the complement cascade before the parasite becomes associated with a macrophage, and lastly rapid internalization of bound parasites may remove the antigen-antibody complexes from the macrophage surface before damage occurs.

The proof of malarial specificity of the macrophage-cytophilic antibody, or at least some portion of the population of cytophilic antibodies bound to the macrophages, rests on the considerable difference in fluorescence observed between the various negative control tests and the positive test for bound malarial antigen. That some small amount of fluorescein-tagged cytophilic antibody would become associated with
macrophages in the control groups where this reagent was used is unavoidable, but these problems were minimized by blocking macrophages with the labeled reagent at low temperatures and adding sodium azide to the incubation medium. We did not observe significant fluorescence of macrophages which were reacted with NRS/FITC. We have consistently observed a very low level of cytophilic antibody in normal serum. Apparently cytophilic antibody becomes abundant in the serum only in response to certain types of antigenic stimulation. The demonstration of macrophage-cytophilic antibody specificity for plasmodium using the complement staining technique gave an independent positive test which was clearly unambiguous. This test, although somewhat cumbersome is highly specific.

The binding affinities of opsonic and cytophilic antibodies were clearly different. However, as the opsonizing antibody was measured bound to specific antigen, and the cytophilic antibody was measured bound to macrophages the results are not strictly comparable. A strict comparison of the binding characteristics of the two types of antibody for the parasite is essentially impossible as the macrophage-cytophilic antibody must be bound to the macrophage before it will bind to the malarial parasite. Thus, the affinity of the antibody for the parasite antigen cannot be measured independently of its macrophage binding affinity. Nevertheless, the two antibody types are clearly distinguishable in their affinity characteristics.
LITERATURE CITED


CHAPTER 5

"THE EFFECT OF ADOPTIVE TRANSFER OF MACROPHAGES ON THE IMMUNE RESPONSE TO PLASMODIUM BERGHEI IN THE WEANLING RAT"

Recent work done in this laboratory (Green and Kreier, 1976; Green and Kreier, 1978; Wange, 1976; Brooks, 1977) has demonstrated that macrophage-cytophilic antibodies as well as opsonizing antibodies are involved in the immune response of the rodent to Plasmodium berghei. Cytophilic antibodies act in concert with the opsonizing antibodies. Gravely and Kreier (1976) have demonstrated by adoptive transfer of lymphocytes that T-lymphocytes are necessary for the production of an immune antibody response to Plasmodium berghei in rats. The present study employed the fluorescent staining of macrophage-cytophilic antibody on macrophages and adoptive transfer techniques to study the role of the macrophage in the induction of the immune response to malaria in the rat, and to detect if there was any correlation between the inability of weanling rats to mount an efficient immune response to P. berghei infection and the status of their macrophage populations.

MATERIALS AND METHODS:

All rats used in this study were of the inbred CDF strain obtained from Charles River Breeders, Inc. The Walter Reed strain of Plasmodium berghei, obtained from M. Aikawa, Case Western Reserve University, Cleveland, Ohio, was used throughout the study. A reference stabilitate of infected mouse blood in 10% glycerol was held in liquid nitrogen and
parasites were retrieved as needed by intraperitoneal injection of the rapidly thawed material into a Swiss white mouse. A CDF rat then was infected from the mouse, and $5 \times 10^2$ infected CDF red blood cells were given i.v. as the infectious inoculum to test animals. Free parasites were prepared by the continuous flow method of Prior and Kreier (1972). Unstimulated macrophages for cell transfer were harvested from weanling (40-50 gm) or adult (270-280 gm) CDF rats of either sex by peritoneal lavage with Tissue Culture Medium 199. Parasites and macrophages were counted, and macrophage viability determinations using 1% trypan blue in Hanks' Balanced Salt Solution (HBSS) were made in a standard hemocytometer. Hyperimmune sera were collected and pooled from young adult CDF rats which had recovered from *P. berghei* infection and which had been given three weekly challenges with $20 \times 10^6$ infected red blood cells (IRBC) following recovery from the initial infection. Serum was collected 2 weeks after the initial challenge. Cytophilic antibody staining of the macrophages was done by incubation of macrophages at $4^\circ C$ in fluorescein-isothiocyanate conjugated hyperimmune serum gamma globulin (HIRS/FITC).

**Macrophage staining experiment.** Macrophages from immature CDF rats weighing from 15 to 60 gms were examined for the presence of cytophilic antibody receptors as determined by fluorescent cytophilic antibody staining technique. Unstimulated peritoneal macrophages were collected by peritoneal lavage with M-199, washed once by centrifugation at 1000 x g for 3 minutes, resuspended in M-199 and plated out at the rate of $1-2 \times 10^6$ cells/dish in 33 mm plastic tissue culture dishes (Falcon: Downingtown, Pa.). The cells were cultured for 30 minutes at $37^\circ C$ in
5% CO\textsubscript{2}, washed 3 times by immersion of the plates in HBSS, and recultured as above for 2 hours in M-199. The cells were then washed 3 times by immersion of the plates in HBSS and flooded with HIRS/FITC at a dilution of 1:4 in HBSS. After incubation at 4\textdegree C for 30 minutes the plates were washed 3 times by immersion in HBSS, drained, and coverslips were mounted in the dishes in a drop of 90% glycerol in PBS, pH 7.5.

Peritoneal macrophages from rats of both sexes weighing 25, 35, 46 and 60 gms were examined. Total cell numbers per field were determined by phase contrast microscopy, and the fluorescent cell ratios of the same fields were determined by dark field fluorescence microscopy, using a Zeiss Standard WL microscope fitted for both phase contrast and fluorescence microscopy. Intensity of staining by fluorescent antibody (F.A.T. reaction) was ranked from 4 to 4+.

**Macrophage transfer experiment.** All injections of macrophages, serum or buffer were given slowly in 1 ml volumes via the lateral caudal vein of the weanling recipient rats 24 hours prior to administration of the infectious inoculum. Hyperimmune serum alone was given at a concentration of 10% in HBSS; that is, 0.1 ml of HIRS was administered in 1 ml volume in HBSS. Macrophages were introduced at the rate of 10 x 10\textsuperscript{6} viable cells suspended in 1 ml of HBSS or serum dilution in HBSS.

Macrophages and parasites were sensitized with HIRS by first culturing the macrophages for 2 hours in M-199 at 37\textdegree C, 5% CO\textsubscript{2}, and then washing the macrophages by centrifugation at 1000 x g for 3 minutes and resuspending in 10% HIRS in M-199 with 5 x 10\textsuperscript{8} free parasites. The mixture was incubated for 1 hour at 37\textdegree C, 5% CO\textsubscript{2} and then washed 4 times in cold HBSS by centrifugation at 1000 x g for 3 minutes, resuspended in HBSS to
Table 10. Experimental groups used in various tests for protective effects of adoptive transfer into weanling rats of unsensitized or sensitized weanling or adult macrophages.

<table>
<thead>
<tr>
<th>Group # (n)</th>
<th>Treatment day -1</th>
<th>Treatment day 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (4)</td>
<td>HBSS i.v.</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>2 (4)</td>
<td>$10 \times 10^6$ weanling macrophages in HBSS i.v.</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>3 (4)</td>
<td>$10 \times 10^6$ adult macrophages in HBSS i.v.</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>4 (4)</td>
<td>$10%$ HIRS i.v.</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>5 (4)</td>
<td>$10 \times 10^6$ weanling macrophages in $10%$ HIRS</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>6 (4)</td>
<td>$10 \times 10^6$ weanling macrophages</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>7 (4)</td>
<td>$10 \times 10^6$ HIRS sensitized adult macrophages and parasites</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
<tr>
<td>8 (4)</td>
<td>$10 \times 10^6$ unsensitized adult macrophages and parasites</td>
<td>$5 \times 10^2$ IRBC i.v.</td>
</tr>
</tbody>
</table>
Table 11. Variation in the ability of macrophages for immature rats to bind fluorescein-conjugated macrophage-cytophilic antibodies.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Body Weight</th>
<th>F.A.T. Reaction</th>
<th>% Cells Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 gm</td>
<td>negative</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>35 gm</td>
<td>+/-</td>
<td>2-5%</td>
</tr>
<tr>
<td>3</td>
<td>46 gm</td>
<td>2+</td>
<td>12%</td>
</tr>
<tr>
<td>4</td>
<td>60 gm</td>
<td>2+</td>
<td>25%</td>
</tr>
</tbody>
</table>
a concentration of $10 \times 10^6$ macrophages / ml and injected in 1 ml aliquots. The infectious inoculum consisted of $5 \times 10^2$ infected red blood cells and was given 1 day after the administration of the various treatments. Infections were monitored daily from this time. Percent parasitemia was determined by counting the number of infected erythrocytes per 1000 erythrocytes counted on Giemsa-stained thin blood films and converting the result to percent. All treatments were made and all blood films were taken between 11 am and 1 pm daily. Treatments were as shown in Table 10.

RESULTS:

**Macrophage staining experiment.** Peritoneal macrophages from immature rats weighing 25, 35, 46 and 60 gms differed in their ability to bind fluorescein-conjugated macrophage cytophilic antibody (Table 11). Peritoneal macrophages from the rat weighing 25 gms did not bind fluorescein-conjugated cytophilic antibody, indicating that there were few or no receptors for cytophilic antibody present on the surface of these macrophages. Weak binding of fluorescein-labeled antibody was observed on a small percentage of the macrophages obtained from the 35 gm rat. Progressively more cells were observed to bind fluorescein-labeled antibody in the 46 and 60 bm rats, and at a more intense level, indicating a denser population of receptors on the macrophage surfaces of the older rats than on the macrophages of the less mature rats.

**Macrophage transfer experiments.** Young rats which received adult macrophages were able to terminate their infections slightly earlier than young rats which did not receive adult macrophages and suffered
Figure 9. The effect of adoptive transfer of weanling or mature macrophages into weanling rats on the subsequent course of a challenge infection with *Plasmodium berghei*. 
Figure 10. The effect of adoptive transfer of weanling or mature rat
macrophages with hyperimmune serum into weanling rats on
the subsequent course of a challenge infection with *P. Berghei*. 
Figure 11. The effect of adoptive transfer into weanling rats of antibody sensitized or unsensitized adult macrophages treated with antigen on the subsequent course of a challenge infection with Plasmodium berghei.
no mortality. Whereas young rats which did not receive macrophages had mortality rates of 50% (Figure 9). There was no mortality in rats which received HIRS with either adult or weanling macrophages, but macrophages with HIRS did not give as good protection then as HIRS alone (Figure 10). The administration of HIRS did lessen the severity of the infection in all rats and also delayed the day of appearance of the infection. Pre-treatment of young rats with a mixture of unsensitized adult macrophages and free parasites reduced the severity of the infection more than pre-treatment with only unsensitized adult macrophages. However, administration of adult macrophages which were pre-sensitized by incubation with parasites together in 10% HIRS dramatically ameliorated the course of the infection (Figure 11). By day 11 the parasitemia was noticeably milder and by day 14 the mean parasitemia was declining in rats which had received macrophages, parasites and antibody. In contrast, severe infections occurred in the rats which received macrophages and parasites without antibody. Additionally, the rats which received HIRS-sensitized macrophages and parasites terminated their infections earlier than any of the other rats.

DISCUSSION:

Very young rats are incapable of mounting an efficient immune response to *P. berghei* infection (Gravely and Kreier, 1976) whereas mature rats do mount an efficient response. The scarcity of cytophilic antibody receptors on the macrophages in very young rats suggested that there might be some correlation between the ability to bind cytophilic antibody and the poor immunologic response of such rats. Seeding of immature rats with mature macrophages did serve to eliminate the
mortality observed in rats which received either no macrophages or immature macrophages; however, the course of the infection was only slightly affected by adult macrophage transfer. The addition of HIRS to the transferred cell suspension delayed the onset of the parasitemia by several days and lessened the severity of the infection throughout its course and eliminated mortality. However, injection of either weanling or adult macrophages with HIRS was not as beneficial as injection of HIRS alone. The slightly increased parasitemia seen in rats given massive macrophage pre-treatment is similar to that seen with large adoptive transfers of lymphocytes by Gravely and Kreier (1976). Perhaps the efficiency of the macrophage-lymphocyte interaction in the induction of the immune response is reduced by the imbalance in the normal macrophage-lymphocyte ratio created by the adoptive cell transfer.

When unsensitized macrophages incubated with free parasites were transferred, there was only a slight beneficial effect observed. However when macrophages and parasites which were presensitized with HIRS were transferred, there was a dramatic effect on the subsequent course of the P. berghei infection.

Recent reports in the literature have described a capsule on plasmodial merozoites (Ladda et al, 1969). The merozoite is released when a fully mature schizont bursts. It reinvades a new erythrocyte to initiate another asexual reproductive cycle. The extracellular merozoite is the stage in the asexual malarial cycle which may be affected by the host's immune response. Our data, and that of C. Brooks (1977), indicate that this stage is capable of resisting
phagocytosis by normal macrophages; therefore, the capsule present on
the surface of this stage must certainly have anti-phagocytic
properties analogous those of bacterial antiphagocytic capsules.
The macrophage-cytophilic antibodies as well as opsonizing antibodies
and fixed complement allow the macrophages to bind and phagocytize the
parasites. These antibodies act to increase the phagocytosis of
merozoites (Chow and Kreier, 1972; Brooks, 1977). The adoptive transfer
work of Gravely and Kreier (1976) has clearly shown that immunity to
malaria in rats is mediated by antibody, and that the production of
protective antibody is T-lymphocyte dependent. Macrophages are an
obligatory component of T-lymphocyte dependent immune response
(Rosenstreich and Oppenheim, 1976; Yoshinaga et al, 1972). The work in
the present study indicates that the macrophages function poorly in
presenting the protective antigen to lymphocytes in the absence of
antibody, but that antibody sensitization of the macrophages and
parasites results in the rapid production of a good protective immune
response. A role for cytophilic antibody in antigen presentation by
macrophages has been suggested before (Tizard, 1971). The apparent
scarcity of antibody receptors on the macrophages of very young rats
could seriously impair their ability to process antigen even when small
amounts of antibody were present. Thus the feed back effect of antibody
during the initial stages of the immune response would be poor. Thus we
propose that the antigen which would stimulate the protective response is
the merozoite capsular material, and that this capsule endows the merozoite
with anti-phagocytic properties. We further propose that specific anti-plasmodial antibodies function at least in part by promoting a prompt and effective immune response in the presence of malarial parasites. We believe they do this by making it possible for macrophages to process and present the protective antigen to lymphocytes efficiently.
LITERATURE CITED


Malaria is a protozoan disease of vertebrates which is found in reptiles, birds and mammals and which is remarkably host-species specific. Four plasmodial species are specifically adapted to man. These are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The most clinically severe form is *P. falciparum*, with *P. vivax* also being quite important clinically. Plasmodial species which have been of the greatest use in animal model systems for the study of malarial infection and the host immune response are the rodent malarias *Plasmodium berghei* and *P. vinckei*, and the primate malaria *P. knowlesi* which produces an extremely pathogenic infection in rhesus monkeys, thereby presenting the worker in malaria with a very demanding model for immunization studies. More recently the human plasmodium *P. falciparum* has been found to infect *Aotus* and other South American monkeys (Geiman and Meagher, 1967). The *Aotus trivirgatus-Plasmodium falciparum* system is rapidly assuming an important role in the research efforts for the development of a *P. falciparum* vaccine.

Various workers have indicated that the merozoite form of the asexual cycle is the stage at which the mammalian host directs the protective immune response (Mitchell et al, 1974; Mitchell et al, 1975; Cohen, 1974; Miller et al, 1975; Siddiqui, 1977). The only way to procure pure undamaged merozoites at present is by natural release in culture (Dennis et al, 1975). The culture method of Trager and Jensen
(1976) offers the possibility of continuous production of *P. falciparum*
infected human erythrocytes, although the method is still inefficient
and being performed on a very small scale.

The protective immune response to malaria is known to depend upon
the production of humoral antibody (Diggs and Osler, 1975; Cohen et al,
1974; Gravely and Kreier, 1976) and yet, paradoxically, passive transfer
of immune serum has not proven to be very efficacious (Cohen and Butcher,
1971). Factors other than serum antibodies have been shown to be
necessary for resistance to malaria infection (Criswell et al, 1971;
Brown and Phillips, 1974). Phagocytosis of free parasites is greatly
enhanced by immune serum *in vitro* (Chow and Kreier, 1972). Anti-
malarial antibody production is T-lymphocyte dependent (Gravely and
Kreier, 1976) and macrophage dependent (Rosenstreich and Oppenheim,
1976; Yoshinaga et al, 1972). Immune serum contains both opsonizing and
macrophage-cytophilic antibodies which are capable of promoting the
adherence of free parasites to the phagocytic macrophage (Green and
Kreier, 1978). Macrophage-cytophilic antibodies are found in the IgG\textsubscript{1}
subclass in the rat (Green and Kreier, 1978) and those serum fractions
which contained the greatest amount of IgG\textsubscript{1} were the most protective
(Phillips and Jones, 1972). The attachment of the invasive merozoite
form to macrophages only occurs in the presence of macrophage-cytophilic
or parasite-opsonizing antibodies. Opsonic antibody is more effective
at promoting attachment than macrophage-cytophilic antibody, and both
together give the best results (Brooks, 1977). In that study cyto-
philic antibody treatment of macrophages did not cause the phagocytosis
of the bound parasites, whereas opsonic antibodies did. Studies in
this dissertation indicate that macrophage-cytophilic antibodies are antigen specific but do not fix complement (Chapter 4). "Cell-bound cytophilic antibodies render the cell which they sensitize capable of binding to a specific antigen, and this may be the sole function of such antibodies" (Tizard, 1971). Such binding has been found to increase the protective capacity of the opsonic antibody (Green and Kreier, 1978) and may also serve to present antigen to lymphocytes during the induction of the anti-malarial immune response (Chapter 5).

An individual is infected with malaria via the bite of an infected mosquito. The infective form, located in the mosquito's salivary glands, is the sporozoite. The sporozoite does not share surface antigens with the exoerythrocytic forms, and is not affected by serum from recovered individuals. However, it is possible to immunize with sporozoite antigens and achieve protective immunity against sporozoite invasion by sporozoite neutralization (Nussenzweig et al, 1972). The sporozoite of the mammalian malarias normally localizes in the liver parenchymal tissue to establish an exoerythrocytic schizont. While the exoerythrocytic schizont is differentiating into a schizont containing many infective merozoites, virtually no response is elicited from the host beyond a mild inflammatory reaction (Yoeli and Most, 1965). No antibodies are produced against the exoerythrocytic stage. The infective merozoites which are released into the circulation invade erythrocytes and thereby initiate the asexual erythrocytic cycle. These merozoites are indistinguishable antigenically from later asexual merozoites. After penetrating an erythrocyte, the merozoite develops into a trophozoite.
The mature trophozoite differentiates into an erythrocytic schizont containing many infective merozoites; the fully mature schizont is sometimes referred to as a "segmenter". The segmenter releases its crop of merozoites to initiate yet another cycle of erythrocyte invasion and asexual reproduction. Also released at the time of segmenter rupture are a variety of materials including malarial pigment, parasite and host cell detritus and other antigens. These may include some of the anti-phagocytic capsular material which Brooks (1977) has provided some evidence by electronmicroscopy to be present in the parasitophorous vacuole. If the parasitophorous vacuole is rich in capsular material this would explain the success of Siddiqui (1977) who recently immunized monkeys with fully mature segmenters emulsified in Freund's complete adjuvant. Recovery from malaria infection depends upon the elimination of merozoites (Cohen and Butcher, 1971; Hamburger and Kreier, 1975). These merozoites possess a capsule consisting of a protein or glycoprotein (Mason et al, 1977; Miller et al, 1975).

Since the immune response to malaria is known to be T-lymphocyte dependent, and since T-dependent responses always require the cooperation of macrophages in the presentation of antigen, it becomes apparent why the immune response may not develop rapidly in young animals who have had no previous experience with malaria, and who have little or no age-dependent "natural" antibody (Trager et al, 1950; Sodeman and Jeffery, 1965). The induction of an efficient and prompt immune response will depend upon the ability of the macrophages to present an anti-phagocytic aggressin-like antigen to lymphocytes. Under natural conditions, "natural" antibody may facilitate this process, as would immune antibody
(Chapter 5). Once antibodies are present, a feedback mechanism would exist for the presentation by macrophages of more antigen to lymphocytes allowing the immune response to develop rapidly. Freund's complete adjuvant could also serve to effect this process by non-specifically activating the macrophages, thereby increasing the likelihood of capsular antigen binding. In fact, this method of immunization has been used successfully (Saul and Kreier, 1977; Siddiqui, 1977). The dearth of cytophilic receptors on the macrophages of very young rats (Chapter 5) may be associated with the defective immune response exhibited by such animals to infection with P. berghei.

Thus it would appear that the protective antigen in the malarial immune response resides in the merozoite capsular material, and that this capsule endows the merozoite with antiphagocytic properties, and that specific anti-plasmodial antibodies are capable of promoting a prompt and effective immune response in the presence of antigen by facilitating macrophage binding and processing of the antigen as well as promoting the phagocytic function of the macrophage.
LITERATURE CITED


evidence for the parasitic origin of the surface coat on malaria

114: 1237-1242.


Nussenzweig, R. S., J. Vanderberg, G. L. Spitalny, C. O. Rivera, C. Orton
and H. Most. 1972. Sporozoite induced immunity in mammalian

Phillips, R. and V. Jones. 1972. Immunity to Plasmodium berghei in rats:
Maximum levels of protective antibody activity are associated with
eradication of infection. Parasitol. 64: 117-127.

Rosenstreich, D. L. and J. J. Oppenheim. In: Immunobiology of the

rats with antigens from a population of free parasites rich in

Siddiqui, W. 1977. An effective immunization of experimental monkeys
against a human malaria parasite, Plasmodium falciparum. Science
197: 388-389.

Sodeman, W. A. and Jeffery, G. M. 1965. Immunofluorescent studies of

Tizard, J. R. 1971. Macrophage-cytophilic antibodies and the functions

Trager, W., Stauber, L. and S. Ben Harel. 1950. Innate and acquired
agglutinins in ducks to the malarial parasite Plasmodium lophurae.

Trager, W. and J. B. Jensen. 1976. Human malaria parasites in continuous


