INFORMATION TO USERS

While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. For example:

- Manuscript pages may have indistinct print. In such cases, the best available copy has been filmed.

- Manuscripts may not always be complete. In such cases, a note will indicate that it is not possible to obtain missing pages.

- Copyrighted material may have been removed from the manuscript. In such cases, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or as a 17”x 23” black and white photographic print.

Most photographs reproduce acceptably on positive microfilm or microfiche but lack the clarity on xerographic copies made from the microfilm. For an additional charge, 35mm slides of 6”x 9” black and white photographic prints are available for any photographs or illustrations that cannot be reproduced satisfactorily by xerography.
Alder, Jeffrey Dale

THE PHYSICAL CHARACTERIZATION AND IMMUNOLOGICAL EFFECTS OF PLASMODIUM BERGHEI IMMUNE COMPLEXES

The Ohio State University

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1987 by Alder, Jeffrey Dale
All Rights Reserved
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark □.

1. Glossy photographs or pages □
2. Colored illustrations, paper or print □
3. Photographs with dark background □
4. Illustrations are poor copy □
5. Pages with black marks, not original copy □
6. Print shows through as there is text on both sides of page □
7. Indistinct, broken or small print on several pages □
8. Print exceeds margin requirements □
9. Tightly bound copy with print lost in spine □
10. Computer printout pages with indistinct print □
11. Page(s) □ lacking when material received, and not available from school or author.
12. Page(s) □ seem to be missing in numbering only as text follows.
13. Two pages numbered □. Text follows.
14. Curling and wrinkled pages □
15. Dissertation contains pages with print at a slant, filmed as received □
16. Other

__________________________________________________________________________
__________________________________________________________________________
__________________________________________________________________________

University Microfilms International
THE PHYSICAL CHARACTERIZATION AND IMMUNOLOGICAL EFFECTS
OF PLASMODIUM BERGHEI IMMUNE COMPLEXES

DISSERTATION

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

by

Jeffrey Dale Alder, B.S., M.S.

* * * * *

The Ohio State University
1987

Dissertation Committee
G. Banwart
R. Pfister
M. Rheins
J. Kreier

Approved by
Julius P. Kreier, Adviser
Department of Microbiology
Copyright by
Jeffrey Dale Alder
1987
ACKNOWLEDGMENTS

I am especially grateful to Dr. Julius Kreier for his suggestions and guidance throughout my research. Gratitude is expressed to Jack Kocka for technical assistance. I wish to thank Steve Felton for being a good friend and colleague during my work. I wish to especially thank my wife Barbara for her help, both technical and moral. Finally, I offer gratitude to my parents, Ramona and the late Richard Alder, to whom I am forever indebted.
VITA

March 26, 1959 . . . . . . Born Toledo, Ohio

1981 . . . . . . . . . . . B.S., The Ohio State University
Columbus, Ohio

1983 . . . . . . . . . . . M.S., The Ohio State University
Columbus, Ohio

1983-Present . . . . . . Graduate Student, Microbiology
The Ohio State University
Columbus, Ohio

Clinical Bacteriologist
University Hospitals
Columbus, Ohio

PUBLICATIONS


# TABLE OF CONTENTS

ACKNOWLEDGMENTS ......................................... ii
VITA ..................................................... iii
LIST OF TABLES .......................................... iv
LIST OF FIGURES ......................................... v

PAGE

INTRODUCTION ........................................... 1
MATERIALS AND METHODS ................................... 9
RESULTS .................................................... 27
DISCUSSION ............................................... 170
SUMMARY ................................................... 196
LITERATURE CITED ....................................... 198
<table>
<thead>
<tr>
<th>TABLE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protein content of immune complexes in serum samples collected during the course of infection</td>
</tr>
<tr>
<td>2</td>
<td>Protein content of immune complexes precipitated at various concentrations of PEG in serum collected 5 days after challenge</td>
</tr>
<tr>
<td>3</td>
<td>Quantitation of rat IgG by radial immunodiffusion</td>
</tr>
<tr>
<td>4</td>
<td>Quantitation of IgG in immune complexes precipitated at a PEG concentration of 3% in rat serum collected during the course of infection</td>
</tr>
<tr>
<td>5</td>
<td>Assay for inhibition of <em>P. falciparum</em> growth <em>in vitro</em> by monoclonal antibody BJ</td>
</tr>
<tr>
<td>6</td>
<td>Effect of plasmodial immune complexes upon the <em>in vivo</em> immune response to SRBC as shown by the hemolysis assay</td>
</tr>
<tr>
<td>7</td>
<td>Effect of plasmodial immune complexes upon the immune response to SRBC as shown by the plaque assay</td>
</tr>
<tr>
<td>FIGURE</td>
<td>DESCRIPTION</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>Light absorbence by effluent from a HPLC column on which normal rat serum was fractionated</td>
</tr>
<tr>
<td>2</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 2 days after infection was fractionated</td>
</tr>
<tr>
<td>3</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 5 days after infection was fractionated</td>
</tr>
<tr>
<td>4</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 9 days after infection was fractionated</td>
</tr>
<tr>
<td>5</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 14 days after infection was fractionated</td>
</tr>
<tr>
<td>6</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 17 days after infection was fractionated</td>
</tr>
<tr>
<td>7</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 26 days after infection was fractionated</td>
</tr>
<tr>
<td>8</td>
<td>Light absorbence by effluent from a HPLC column on which rat serum collected 35 days after infection was fractionated</td>
</tr>
<tr>
<td>9</td>
<td>Fractions obtained by sucrose gradient centrifugation of normal rat serum</td>
</tr>
<tr>
<td>10</td>
<td>Fractions obtained by sucrose gradient centrifugation of rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>FIGURE</td>
<td>DESCRIPTION</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>11</td>
<td>Patterns produced by immunoelectrophoresis of normal rat serum and rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>12</td>
<td>Patterns produced by immunoelectrophoresis of 7s and 19s fractions of normal serum</td>
</tr>
<tr>
<td>13</td>
<td>Patterns produced by immunoelectrophoresis of 7s fractions from normal rat serum and rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>14</td>
<td>Patterns produced by immunoelectrophoresis of 19s fractions from normal rat serum and rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>15</td>
<td>Patterns produced by immunoelectrophoresis of 22s fractions from normal rat serum and rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>16</td>
<td>Patterns produced by immunoelectrophoresis using anti-rat IgG antiserum of 22s fractions of normal rat serum and rat serum collected 5 days after infection</td>
</tr>
<tr>
<td>17</td>
<td>Patterns produced by immunoelectrophoresis of the 22s fraction of rat serum collected 5 days after infection and rat IgG</td>
</tr>
<tr>
<td>18</td>
<td>Light absorbance by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 3% in rat serum collected 5 days after challenge was fractionated</td>
</tr>
<tr>
<td>19</td>
<td>Light absorbance by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 3% in normal rat serum was fractionated</td>
</tr>
<tr>
<td>20</td>
<td>Light absorbance by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 5% in rat serum collected 5 days after infection was fractionated</td>
</tr>
<tr>
<td>21</td>
<td>Light absorbance by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 5% in normal rat serum was fractionated</td>
</tr>
</tbody>
</table>
Light absorbence by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 7% in rat serum collected 5 days after infection was fractionated ........................................ 78

Light absorbence by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 7% in normal rat serum was fractionated .................. 80

Light absorbence by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 10% in rat serum collected 5 days after infection was fractionated . . . . 82

Light absorbence by effluent from a HPLC column on which the precipitate formed at a PEG concentration of 10% in normal rat serum was fractionated .................. 85

Patterns produced by immunoelectrophoresis of the precipitate formed at a PEG concentration of 3% in normal rat serum or in rat serum collected 5 days after infection ........... 88

Patterns produced by immunoelectrophoresis of the precipitate formed at a PEG concentration of 5% in normal rat serum or in rat serum collected 5 days after infection ........... 91

Patterns produced by immunoelectrophoresis of the precipitate formed at a PEG concentration of 7% in normal rat serum or in rat serum collected 5 days after infection ........... 93

Patterns produced by immunoelectrophoresis of the precipitate formed at a PEG concentration of 10% in normal rat serum or in rat serum collected 5 days after infection ........... 95

Patterns produced by PAGE of the precipitates formed at PEG concentrations of 3, 5, 7, 10 or 20% in normal rat serum or in rat serum collected 5 days after infection ............. 100

Patterns produced by PAGE of the precipitates formed at a PEG concentration of 3% in normal rat serum or in serum collected 2, 5, 9, 14 or 26 days after infection ............. 102
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>Audioradiograph of the patterns produced by PAGE of the Protein A precipitates of $^{35}$S-labeled <em>P. berghei</em> antigen reacted with rat serum collected 2, 5, 7, 9, 14, 17 and 26 days after infection . . . . . . . . . . 106</td>
</tr>
<tr>
<td>33</td>
<td>Light absorbence by the effluent from an HPLC column on which mouse ascites fluid containing monoclonal antibody B10 was fractionated . . . . 110</td>
</tr>
<tr>
<td>34</td>
<td>Light absorbence by the effluent from an HPLC column on which mouse ascites fluid containing monoclonal antibody B4 was fractionated . . . . 112</td>
</tr>
<tr>
<td>35</td>
<td>Light absorbence by the effluent from an HPLC column on which mouse ascites fluid containing monoclonal antibody B6 was fractionated . . . . 114</td>
</tr>
<tr>
<td>36</td>
<td>Light absorbence by the effluent from an HPLC column on which mouse ascites fluid containing monoclonal antibody H8 was fractionated . . . . 116</td>
</tr>
<tr>
<td>37</td>
<td>Light absorbence by the effluent from an HPLC column on which mouse ascites fluid containing monoclonal antibody BJ was fractionated . . . . 118</td>
</tr>
<tr>
<td>38</td>
<td>Reaction of immune rat serum to rat erythrocytes infected with <em>P. berghei</em> as shown by fluorescent antibody analysis . . . . 121</td>
</tr>
<tr>
<td>39</td>
<td>Reaction of normal rat serum to rat erythrocytes infected with <em>P. berghei</em> as shown by fluorescent antibody analysis . . . . 124</td>
</tr>
<tr>
<td>40</td>
<td>Reaction of monoclonal antibody H8 to rat erythrocytes infected with <em>P. berghei</em> as shown by fluorescent antibody analysis . . . . 126</td>
</tr>
<tr>
<td>41</td>
<td>Reaction of monoclonal antibody BJ to human erythrocytes infected with <em>P. berghei</em> as shown by fluorescent antibody analysis . . . . 128</td>
</tr>
<tr>
<td>42</td>
<td>Reaction of monoclonal antibody BJ to rat erythrocytes infected with <em>P. berghei</em> as shown by fluorescent antibody analysis . . . . 130</td>
</tr>
<tr>
<td>43</td>
<td>Reaction of monoclonal antibody BJ to a rat erythrocyte infected with <em>P. berghei</em> at the schizont stage as shown by fluorescent antibody analysis . . . . . . . . . . 132</td>
</tr>
<tr>
<td>FIGURE</td>
<td>DESCRIPTION</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>44</td>
<td>Reaction of monoclonal antibody H8 with protein immunoblots of <em>P. berghei</em> antigens</td>
</tr>
<tr>
<td>45</td>
<td>Reaction of monoclonal antibody BJ with protein immunoblots of <em>P. berghei</em> antigens</td>
</tr>
<tr>
<td>46</td>
<td>Effect of D5 serum given at the time of challenge on the course of subsequent parasitemia</td>
</tr>
<tr>
<td>47</td>
<td>Reaction of serum from normal rats and rats immune to malaria with <em>P. berghei</em> antigens as measured by ELISA</td>
</tr>
<tr>
<td>48</td>
<td>Effect of D5 serum given during the course of plasmodial challenge upon antibody titers as measured by ELISA</td>
</tr>
<tr>
<td>49</td>
<td>Effect of D5 serum fractions given at the time of challenge on the course of subsequent parasitemia</td>
</tr>
<tr>
<td>50</td>
<td>Effect of treatment with cyclophosphamide upon the suppressive effect of D5 serum</td>
</tr>
<tr>
<td>51</td>
<td>Effect of multiple doses of D5 serum given two weeks before challenge upon the course of subsequent parasitemia</td>
</tr>
<tr>
<td>52</td>
<td>Effect of vaccination with free malaria parasites two weeks before challenge upon the course of subsequent parasitemia</td>
</tr>
<tr>
<td>53</td>
<td>Effect of infusions of D5 serum upon subsequent parasitemia in rats vaccinated with free malaria parasites two weeks before challenge</td>
</tr>
<tr>
<td>54</td>
<td>Effect of early challenge upon subsequent parasitemia in rats vaccinated with free malaria parasites</td>
</tr>
<tr>
<td>55</td>
<td>Effect of vaccination with 1, 3 or 5 doses of formalin-fixed infected red blood cells upon the course of subsequent parasitemia following challenge</td>
</tr>
<tr>
<td>FIGURE</td>
<td>DESCRIPTION</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>56</td>
<td>Effect of infusions of D5 serum upon subsequent parasitemia in rats vaccinated with formalin-fixed infected red blood cells two weeks before challenge</td>
</tr>
<tr>
<td>57</td>
<td>Effect of early challenge upon subsequent parasitemia in rats vaccinated with infected red blood cells</td>
</tr>
</tbody>
</table>
INTRODUCTION

The protozoan disease malaria afflicts about 300 million people and kills two to four million each year. Malaria is the accepted term for the disease caused by the genus *Plasmodium*. The term "malaria" is derived from the Italian words for "bad air", which reflects an early belief that the disease was caused by wet, humid air. Malaria was once the most common killer of man, and throughout history it has killed more people than any other disease.

Malaria is undergoing a major resurgence due to degenerating health conditions in many third world nations, and to the failure of control measures. Plasmodial infection is a major risk in most tropical third world nations, including those in tropical Africa, South and Central America, Mexico, the Indian subcontinent and Southeast Asia (Wernsdorfer, 1980).

Members of the genus *Plasmodium* are obligate intracellular parasites during much of their lifecycle. These protozoans exist in both vertebrate and arthropod hosts. The *Anophelene* or *Culicine* mosquito is the arthropod host for all plasmodia. Plasmodia are transmitted by mosquito vectors to a large variety of vertebrate hosts such as
birds, reptiles and mammals, including humans. Individual species of plasmodia, however, are usually host specific.

The vertebrate host becomes infected when the plasmodial sporozoite is injected along with salivary gland secretions into the bloodstream by an infected mosquito during a bloodmeal. In mammalian hosts, the sporozoites rapidly move to the liver and enter hepatocytes and the blood is temporarily cleared of parasites. In the hepatocyte, the sporozoite nucleus divides by multiple budding, the parasite cytoplasm segregates and merozoites are formed. A single sporozoite may form up to 30,000 merozoites during this process, which is termed exoerythrocytic merogony. The infected hepatocyte ruptures and merozoites are released into the bloodstream. Exoerythrocytic merogony lasts five to ten days.

The merozoites invade the vertebrate erythrocytes and differentiate into trophozoites. Between three and five nuclear divisions occur, and the plasmodial cytoplasm segregates yielding erythrocytic merozoites. The entire erythrocytic parasite composed of differentiated merozoites is termed a meront. The infected erythrocyte bursts, and up to 32 merozoites are released to infect other erythrocytes. This entire process is termed erythrocytic merogony. The duration is 24 to 72 hours, depending upon the plasmodial species.
After leaving an erythrocyte, a merozoite may initiate additional cycles of erythrocytic merogony in the erythrocyte or may develop into sexual gametocytes. During a bloodmeal, the gametocytes are ingested by the mosquito and develop into mature macrogametes (female) or microgametes (male). Other ingested plasmodial blood forms die. The flagellated microgamete fuses with the macrogamete to form the zygote. The zygote develops into a motile ookinete which invades the mosquito's intestinal epithelium. The ookinete becomes a large tumor-like mass known as an oocyst. A single oocyst may liberate 10,000 sporozoites, some of which make their way to the salivary glands. The sporozoites in the salivary glands are capable of infecting a new vertebrate host and starting the plasmodial lifecycle over again in the new host.

The pathology of malaria in the mammalian host is associated with the rupture of erythrocytes during schizogony and the release of antigenic substances. These substances are probably not toxic in themselves, but cause reactions by complexing with antibody. These complexes cause the characteristic cycles of chills and fever of malaria. Erythrocyte destruction leads to acute anemia, the most common cause of death in man. In *falciparum* malaria, infected erythrocytes become sticky and death may be caused by the blockage of small capillaries of the brain.
by agglutinated erythrocytes. Malaria also lowers resistance to other diseases (Garnham, 1980).

Attempts to control malaria have included various means of mosquito control, chemotherapy to inhibit parasite growth in the human host, and research to produce a vaccine to induce immunity. The anti-mosquito and chemotherapy programs have typically eliminated the disease from the temperate zones, where the hold of malaria was unstable, leaving the tropical zones infested.

Attempts to control the mosquito have included modification of the environment such as the drainage of swamps to remove the normal breeding habitat of mosquitoes, the use of screening to restrict mosquito access to humans, and the use of residual insecticides. In many tropical countries, total environment modification is not practical. Initial successes with insecticides were often followed by a resurgence of the disease due to the development of insecticide resistance by the mosquitoes. The discovery of the environmental dangers of DDT and dieldrin caused a further reduction of vector control (Peters, 1972).

Chemotherapeutic treatment has been used to control malarial infection and to block transmission between man and mosquito. Chloroquine and amodiaquin are effective against the blood forms, but primaquine is needed to eliminate the tissue forms of the parasite in hepatocytes. Drug resistant strains of malaria parasites first appeared in
1960; currently, chloroquine and amodiaquin resistant strains of *Plasmodium falciparum* are common in Southeast Asia and eastern Africa. A combination of sulfadoxine and prymethamine may be used to control chloroquine resistant strains, but gametocytes are resistant to this treatment. Drug companies have little interest in developing products that will be used only in the poorest areas of the world.

Due to the failure of vector control and chemotherapy, there has been a renewed effort to produce an effective vaccine as a cost effective means to eradicate malaria. One of the earliest attempts at vaccination utilized killed sporozoites from mosquitos to immunize monkeys (Sargent and Sargent, 1910). Since that time, many attempts at vaccination have been made using whole killed or partially purified components from sporozoites (Nussenzweig, *et al.*, 1967; Clyde, *et al.*, 1975), gametocytes (Carten and Chen, 1976), and blood stage parasites (Brown, *et al.*, 1970; Grothaus and Kreier, 1980; Siddiqui, *et al.*, 1977). Most of these procedures have not protected against heterologous challenge with other strains or species of plasmodia. In addition, it is difficult to obtain the needed quantities of parasite material for large vaccination programs.

Much of the current research on malaria vaccines has centered on the use of single antigens from the surface of sporozoites. Monoclonal antibodies with protective activity have been used to identify a protein antigen on
the surface of the sporozoite with a molecular weight of 40-60 kd, depending upon the species of plasmodia (Nussenzweig and Nussenzweig, 1984). These antibodies all bound to the same site, so the sporozoite surface protein has a single immunodominant site. The sporozoite surface genes have been cloned and sequenced for *P. falciparum* (Enea, et al, 1984). The central core region of this protein, which is the main target of the immune response, is a repetitive 4-amino acid sequence. This section has been cloned and expressed in *Escherichia coli* (Young, et al, 1985) and vaccina virus (Smith, et al, 1984) as a means of mass production. In addition, synthetic peptides from various regions of the *P. falciparum* sporozoite protein have been produced and conjugated to carrier proteins (Ballou, et al, 1985; Zavala, et al, 1985). The materials produced by these techniques are immunogenic, but their value in vivo has not been proven. Past studies have shown that sporozoite vaccines produce immunity that is stage specific and of limited duration. It is also possible that the parasite could become resistant to this type of vaccine by altering the antigenic structure of the single peptide involved in the immune response. It is hoped that the polypeptide used in the vaccine will be essential to the parasite so that modification is impractical. Controlled vaccine trials with these substances are currently in progress.
Research with blood stage parasite material for use in vaccines has followed lines similar to those used for sporozoite vaccines, but has lagged behind. There are currently about 200 antigens isolated from merozoites or other malarial blood stages, and these are being evaluated for potential use in a vaccine (Kolata, 1984).

In a large malaria immunization program in an endemic area, some people infected with malaria, or at least carrying a burden of plasmodial immune complexes will unavoidably be vaccinated. In holoendemic areas, the average age at time of first infection is three to six months. People in these areas receive between 40 and 120 infective bites from mosquitoes each year. Obviously, vaccination under conditions in which the subjects are in various states of infection and recovery is quite different from the controlled laboratory conditions under which vaccines are initially tested. It is quite likely that people with a low parasitemia, and possibly carrying an immune complex burden from a recent infection would not appear as diseased in a routine pre-vaccination screen and would thus receive vaccine.

As immune complexes normally appear after the generation of an immune response, they could be expected to serve as suppression signals for the response. In addition, plasmodial immune complexes may serve to protect the parasite from clearance, thus being relevant to the generation

This study was initiated to investigate the role of Plasmodium berghei immune complexes on the development of immunity, including the response to vaccination. Both the physical and biological nature of the complexes were investigated. The development of immune complexes in relation to the course of disease was studied. To this end, serum collected at various stages of infection was assayed for immune complexes. Then, the isolation of the complexes was undertaken. The protein and immunoglobulin content of the complexes was analyzed, and the polypeptide content of the isolated complexes was analyzed. Next, the antigenic content of the complexes was studied using both polyvalent immune serum and monoclonal antibodies. Finally, the effect of immune complexes upon the immune response to infection or to vaccination was studied by various methods. These analyses revealed both the physiochemical nature of the immune complexes, and their biological effect on the immune response in rodents infected with Plasmodium berghei.
MATERIALS AND METHODS

Parasites. The *Plasmodium berghei* strain used was obtained from Dr. M. Aikawa (Case Western Reserve University, Cleveland, Ohio). The parasites were stored in liquid nitrogen (-193°C) by standard techniques (Trager and Jensen, 1980). The parasites were retrieved from storage by thawing, and then intraperitoneal injection into Swiss mice. The stock strain was passed no more than twice during these studies. This strain is highly pathogenic for Swiss mice and causes death, usually within one week of patency. Adult Sprague-Dawley rats usually survive infection while weaning rats usually die.

The *Plasmodium falciparum* strain used was obtained from Dr. T. Green (University of Columbia, Missouri). This strain was maintained exclusively *in vitro* by continuous passage in human erythrocytes in RPMI 1640 media plus 10% human serum. The parasites are cultivated in 100 mm tissue culture plates with a 5% hematocrit by the candle jar method (Jensen and Trager, 1977). Parasitemias approaching 20% are obtainable with frequent (8 hr.) changes of media. only O+ type blood and serum were used in cultivation.
Animals. Outbred Sprague-Dawley (SD) rats were used as the source of the infected red blood cells (IRBC) used in vaccine preparation and the challenge inoculum. SD rats were also the source of all serum used in the assays with *P. berghei*. Younger SD rats weighing 130 g were used in the vaccine trials.

Swiss mice were used to recover the *P. berghei* parasites from liquid nitrogen storage. Swiss mice were also used as the source of macrophages for the cultivation of hybridomas. Balb/c mice were immunized with *P. falciparum* in the production of monoclonal antibodies.

Serum collection. Rats were anesthetized with ether and blood was collected by cardiac puncture into glass tubes. The blood was incubated at 37°C for 30 minutes and then at 4°C overnight. The blood was centrifuged at 1,000 x g for 10 minutes and the serum supernatant was collected. Sera was stored at -20°C. Immune serum was collected from rats that had recovered from multiple cycles of *P. berghei* infection. These rats, which had recovered from an initial *P. berghei* infection, were inoculated intraperitoneally with $2 \times 10^9$ *P. berghei* infected erythrocytes at bi-weekly intervals for a total of four injections and five recoveries. One week after the last injection, the rats were exsanguinated and the immune sera was stored at -20°C. Immune complexes were recovered from serum from adult SD
rats inoculated intravenously with $10^8$ infected red cells. Serum was collected at various times, ranging from 2 to 35 days post infection, pooled and stored at $-20^\circ$C. Human anti-\textit{P. falciparum} serum was received from Dr. Soren Jepsen (Statens Seruminstitut, Copenhagen, Denmark). This serum was collected from clinically immune Liberians and was strongly positive at an ELISA titer of 1/5120.

\textbf{Polyethylene glycol precipitation of immune complexes.}

Immune complexes were removed from serum by precipitation with polyethylene glycol 6000 (PEG). The pooled rat serum was mixed with a sufficient amount of 40\% PEG in borate buffer (0.1 M boric acid, 25 mM disodium tetraborate, 75 mM NaCl, pH 8.4) to yield a final concentration of 3.5, 5, 7 or 10\%. The mixture was incubated at 4$^\circ$C overnight and the complexes were collected by centrifugation at 12,800 $\times$ g for 2 minutes. Both the pellet of complexes and the supernatant fluid was resuspended to the original volume of the serum with 0.85\% saline.

\textbf{Vaccine preparation.} The isolation of the \textit{P. berghei} parasites by sonic lysis of erythrocytes followed by differential centrifugation has been previously published (Prior and Kreier, 1972, 1972a). Vaccination was by a single intramuscular injection of $5 \times 10^8$ free plasmodia in 0.5 ml saline containing 0.13 mg saponin (Eastman Kodak
Co., Rochester, N.Y.). Injection was into the large hind leg muscles of the rat. Rats of the control group were injected with normal erythrocyte membranes in the same saponin adjuvant. Challenge was routinely given by an intravenous injection of $10^4$ infected erythrocytes into the tail vein 14 days after vaccination. In one trial, the challenge dose was given on the same day as the vaccine.

The technique for the preparation of the formalin-fixed IRBC vaccine was adapted from Murphy and Lefford (1979). A suspension containing $1 \times 10^9$ IRBC/ml was gently agitated at $4^\circ C$ for 24 hours in 0.1% formalin. The vaccine was aliquoted and stored at $-20^\circ C$ until used. Administration of the vaccine was by injection of $1 \times 10^8$ IRBC at weekly intervals for a total of 1, 3 or 5 injections. Rats of the control group received five weekly injections of normal red blood cells (NRBC) in 0.1% formalin. All rats were age matched so that they were of the same approximate weight at challenge. The challenge was as previously described, and was given two weeks after the last vaccine dose, except in one trial when it was given with the last injection of vaccine in a group that received three weekly injections of vaccine.

The *Plasmodium falciparum* vaccine used to immunize Balb/c mice for the production of monoclonals was similar to the *P. berghei* free parasite vaccine in saponin. When the parasitemia in culture approached 20%, the human red
cells in RPMI were recovered by centrifugation at 2,000 x g for 10 minutes. The erythrocytes were resuspended to 10% by volume in Alserver's solution. The *P. falciparum* parasites were then recovered by sonic lysis of the red cells followed by differential centrifugation as reported previously (Prior and Kreier, 1972, 1972a). Balb/c mice were vaccinated by intramuscular injection of $1 \times 10^8$ parasites in 0.02 mg saponin adjuvant.

The SRBC vaccine used to immunize the rats for the hemolysis and plaque assays consisted of 4.0 ml/Kg body weight of 0.5% SRBC in 0.85% saline injected intravenously (Garvey, *et al*, 1977). Rats were exsanguinated and splenectomized six days following vaccination.

**Administration of D5 serum.** Serum was collected from adult rats five days after an infectious dose of $1 \times 10^8$ IRBC was injected intravenously. This serum, designated D5 serum, was used in various immunosuppressive assays. The D5 serum was given in doses each consisting of three daily injections of 1.0 ml intraperitoneally. If a vaccine or challenge injection was to be given with the dose of D5 serum, then the injection was given during the second daily injection of D5 serum. Therefore, the D5 serum was given the day before, during, and the day after the vaccine or challenge injection. For simplicity, administration of the dose of D5 serum was reported to be on the day of the
second daily injection. For example, a dose of D5 serum given 14 days before challenge actually involved 1.0 ml injections administered 15, 14 and 13 days before challenge.

**Parasitemia.** Parasitemias during the vaccination trial were determined by examination of Giemsa stained thin blood films. A total of 33 fields of 300 red cells per field were examined on each slide. Determinations of parasitemia were continued until each animal had died or recovered from infection.

**High performance liquid chromatography.** Chromatography was carried out on an Altex high performance liquid chromatography (HPLC) apparatus. The buffer used was 0.1M Na₂SO₄, and 0.02M NaH₂PO₄, at pH 6.8. The Bio Rad TSK 250 column was run at 1 ml/minute and the output was continuously monitored at 200 nm. Samples consisted of 20 µl of PEG precipitate, or 20 µl of 1/3 dilutions of serum. The areas under the various eluted peaks were automatically calculated using a Hewlett-Packard integrator. Protein concentrations of the various peaks were calculated by correlating peak area of serum, IgG, IgM and albumin standards with protein concentration as calculated by the Lowry (1951) protein assay. The estimation of retention time
versus molecular weight was also based on the use of these same standards.

Sucrose gradient ultracentrifugation. The relative molecular size of immune complexes (IC) was studied by ultracentrifugation. Normal rat serum (NS) and D5 serum were diluted 1/2 in phosphate buffered saline (PBS) and 0.5 ml quantities were applied to 12.7 ml of 10-40% sucrose gradients in PBS. The gradients were formed using a two-chambered gradient forming apparatus, and the sucrose concentration of samples collected top to bottom was determined by optical density. The samples were centrifuged at 40,000 RPM for 24 hours in a SW 41 roter (Beckman Instruments, Fullerton, Calif.). The reference markers were 7s IgG and 19s IgM. Then, 0.5 ml samples were collected starting from the top of the gradients. The optical density (for calculation of sucrose concentration) and absorbance at 200 nm (for protein content) were determined. The calculations of s value were performed as in McEwen (1967). Immunoelectrophoresis was used to assay 5 μl portions of various samples from the gradient.

Immunoelectrophoresis. Immunoelectrophoresis of the sucrose gradient samples for PEG precipitates was performed using standard techniques (Garvey, 1977). Briefly, glass slides were coated with a 2% agarose layer to a depth of 1
mm. Two wells and a single trough were punched in the agarose by using a template, and the plugs from the wells were removed. The 5 µl samples were applied in the wells along with 0.05% bromphenol blue tracking dye. The samples were electrophoresed at 15 MA/slide using borate buffer in the reservoir and Whatman number 1 filter paper wicks. When the tracking dye had reached the end of the slide, after about 45 minutes, electrophoresis was discontinued. Antisera was added to the trough and the slides were incubated at 4°C in a moist chamber for five days. The slides were then washed gently in two changes each of 0.3 M and 0.15 M NaCl for 24 hours. The slides were then dried and stained with 0.5% amido carmidine.

Jerne plaque assay for immunosuppression. Splenic lymphocytes from rats immunized six days previously were used to assay D5 serum for suppressive activity in vitro. The Jerne hemolytic plaque technique (Jerne, et al, 1974) as described by Cox, et al (1983) was modified somewhat to show the ability of the splenic lymphocytes to respond to SRBC targets in the presence or absence of D5 serum. Briefly, the spleens were collected aseptically from rats immunized with SRBC and normal rats. The spleens were forced through a sterile wire mesh to yield a single cell suspension. The spleen cells were washed in minimum essential media (MEM), counted, and adjusted to 5 x 10^5 viable
cells/ml. Then, 1 ml aliquotes of the spleen cells were incubated with 1 ml of heat inactivated D5 serum or NS at 37°C for 30 minutes in a 5% CO₂ humidified incubator. The spleen cells were recovered following centrifugation at 800 x g for 10 minutes and resuspended to 1 ml in MEM. Next, 9 ml of 0.6% agarose in MEM at 40°C was added, along with SRBC to make a 1% suspension. The suspension was mixed and then layered over a 2.4% agarose base in three 100 mm plates (3.3 ml/plate). The plates were incubated at 37°C, 5% CO₂ for one hour. Then 7.0 ml of 10% rat plasma in MEM was added to each plate as a source of complement. The plates were incubated at 37°C, 5% CO₂ for 30 minutes and then counted for plaques in the SRBC layer. Spleen cells from rats vaccinated with SRBC during infusions of D5 serum or NS, or from normal rats were used in this assay. The cells were incubated in either D5 serum or normal serum as described.

Hemolysis assay. The serum from rats vaccinated with SRBC alone, or SRBC injected during infusions of D5 serum or NS was tested for ability to cause hemolysis. The technique used was similar to that of Garvey, et al (1977). Briefly, serum was collected from rats six days after vaccination with SRBC injected during infusions of D5 serum or NS as described. The complement was deactivated by heating the sera at 56°C for 30 minutes. Doubling dilutions of the
serum were made in 1 ml amounts and SRBC were added to equal 0.2% by volume. Excess guinea pig complement (0.1 ml guinea pig serum) was added to each tube which was then incubated at 37°C for 30 minutes. The tubes were serofuged for 15 seconds, and the tubes were examined for hemolysis of the SRBC as indicated by the lack of an erythrocyte pellet. Released hemoglobin was indicated by a red colored supernatant.

Enzyme Linked Immunosorbent Assay (ELISA). Indirect ELISA was used to determine the amount of anti-P. berghei antibody present in various serum samples. The technique has been published elsewhere (Alder and Kreier, 1984). Briefly, the supernatant from infected erythrocyte lysate was used as the source of antigen. The antigen was suspended at 1.0 mg/ml in coating buffer, and coated into the wells of polystyrene 96 well Dynatech U bottom Immunlon microtiter plates (Dynatech Labs) at 200 μg protein per well. The plates were incubated overnight at 4°C. The wells were washed three times with phosphate buffered saline-Tween-20 solution (PBS-Tween). Dilutions of the various serum samples were made in PBS-Tween and 200μl was added to each well. The plates were incubated at 37°C for two hours and then washed three times in PBS-Tween. Rabbit anti-rat antibody (Sigma Chemical Co.) was diluted 1:1000 and 200 μl was added to each well. The plates were incubated at 37°C
for one hour and washed three times in PBS-Tween. Goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co.) was diluted 1:500 and 200 µl was added to each well. The plates were incubated as before for one hour and washed three times with PBS-Tween. Then 200 µl of p-nitrophenyl phosphate disodium at 10 mg/ml was added to each well. The plates were incubated at room temperature for 15 minutes. The colorimetric reactions were stopped by the addition of 50 µl of 2M NaOH to each well. The plates were read on an ELISA spectrophotometer (Dynatech Labs) at 405 nm.

A similar ELISA test was used to screen monoclonal antibodies for anti-\textit{P. falciparum} activity. The antigen in this test was isolated from \textit{P. falciparum} infected erythrocytes. Cultures of infected erythrocytes at 15+% parasitemia were pooled and washed three times in 0.85% saline to remove plasma and media from the cells. The erythrocytes were resuspended in minimum volume coating buffer and sonicated four times on ice for 15 seconds each and centrifuged at 10,000 x g for 10 minutes as described (Alder and Kreier, 1984). The supernatant was resuspended at 1.0 mg protein/ml in coating buffer and coated onto ELISA plates as described previously. Undiluted ascites fluid containing monoclonal antibody, or hybridoma culture supernatant was added as the primary antibody. The detection system used rabbit anti-mouse (U.S. Biochemical Corp.) and goat
anti-rabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co.) in the same procedure described above. For detection of anti-\textit{P. falciparum} antibodies, human immune sera was used as a positive control with a rabbit anti-human and goat anti-rabbit conjugate (Sigma Chemical Co.) detection system.

**Radial immunodiffusion.** Radial immunodiffusion was used to quantitate the IgG present in PEG precipitates from rat serum collected at various points during infection. A 2\% agarose matrix containing a 1:50 dilution of rabbit anti-rat IgG (Sigma Chemical Co.) was used in 60 mm petre plates. A template was used to punch out four 1 mm wells in a square pattern in each lane. Doubling dilutions of the redissolved PEG precipitates were placed in the wells at 5 µl/well. A known concentration of rat IgG was used as a positive control. The plates were incubated at 4°C for at least five days. The areas of precipitation were examined daily until there was no longer any increase in precipitation area.

**Radioimmune precipitation (RIP) assay.** The RIP assay was used to analyze the antigens in \textit{P. berghei} immune complexes and to examine the relative antibody:antigen ratios in these complexes. \textit{Plasmodium berghei} was labeled in culture by metabolic incorporation of $^{35}$S-methionine. The details
of this procedure have been published (Alder and Kreier, 1984). Following the in vitro labeling, the infected red blood cells were lysed by sonication. The supernatant containing the labeled antigen was collected following centrifugation at 10,000 x g for 10 minutes.

The RIP assay was performed using two different procedures. The first analyzed the PEG precipitates from sera collected at various points during infection and recovery by direct reaction with labeled antigen. The PEG precipitates were resuspended to their original volume (1 ml) and a 50 µl aliquote was mixed with 50 µl of labeled antigen along with 50 µl of 2% DL methionine. The mixture was incubated at room temperature for two hours. Immune complexes were collected by the addition of 50 µl of (1) Protein A coated latex beads (Sigma Chemical Co.), (2) along with 150 µl of radioimmune precipitation assay (RIPA) buffer, (3) followed by incubation at room temperature (4) for 30 minutes. The latex beads containing bound immune complexes were pelleted by centrifugation at 12,800 x g for 2 minutes. The pellets were washed five times with 150 µl of RIPA buffer per wash. Next, the pellets were solubilized in a SDS-B mercaptoethanol buffer and then separated into individual polypeptides on a discontinuous polyacrylamide gel system (Laemmli, 1973). A 4% stacking and 10% separation gel were used, and the electrophoresis was carried out at 30 ma per gel. The gels were fixed in 10%
acetic acid overnight. The gels were processed for autoradiography by using two 30-minute changes in DMSO, followed by a three-hour immersion in DMSO + 20% PPO, followed by two 30-minute washes in distilled water as described (Alder and Kreier, 1984). The gels were dried in a Bio-Rad gel drier and exposed to Kodak X-Omat x-ray film for two weeks at -70°C. The film was developed as per manufacturer's instructions (Kodak).

The second RIP assay procedure also utilized the PEG precipitates from 1 ml sera samples collected at various points during infection. The pellets were each resuspended to 0.5 ml (1/2 original volume) in saline, to which 0.5 ml of serum from immune rats was added. This mixture was incubated for two hours at room temperature. Then 50 μl of Protein A-coated latex beads was added, followed by incubation at room temperature for 30 minutes. The latex beads were pelleted and washed as before. Then, 50 μl of labeled antigen along with 50 μl of DL-methionine and 150 μl RIPA buffer were added to each pellet. The mixtures were incubated at room temperature for two hours. The latex beads were again pelleted and washed five times. The immune complexes bound to the pellets were solubilized and analyzed by PAGE, followed by autoradiography as described above.
Fluorescent antibody assay. Fluorescent antibody was used to detect antibody bound to erythrocytes infected with *P. berghei* or *P. falciparum*. Blood was collected into Alserver's solution from rats infected with *P. berghei* at a parasitemia of about 3%. Similarly, blood was collected from cultures of *P. falciparum* at a parasitemia of about 3%. The blood was washed three times in saline to remove serum immunoglobulin, and then the washed red cells were smeared onto glass slides. The blood films were fixed in ethanol and air dried. The primary antibody, consisting of rat serum, human serum or mouse ascites fluid containing monoclonal antibody, was layered onto the slides, which were incubated at 37°C in a humidified chamber for two hours. The slides were washed in three changes of PBS for a total of 30 minutes. Then the fluorescene conjugated antibody was added to the appropriate slide. Goat anti-mouse IgG + IgM + IgA conjugate (Boehringer Mannheim) was used to detect monoclonal antibody, goat anti-rat IgG conjugate (U.S. Biochemical) was used to detect rat immunoglobulin, while goat anti-human IgG conjugate (U.S. Biochemical) was used to detect human immunoglobulin. The slides containing the fluorescene conjugates were incubated at 37°C in a humidified chamber for 30 minutes. The slides were then washed in three changes of PBS for 30 minutes, followed by three changes of distilled water for 30 minutes. The slides were then dried, and examined by
fluorescent microscopy using a 90% glycerol mounting media. The slides were then cleaned using 100% xylene, washed in distilled water and stained by the Giemsa technique. The slides were then examined by oil immersion microscopy.

**Protein Immunoblots.** The reaction of monoclonal antibodies to *P. berghei* antigens and immune complexes was examined by protein immunoblots. First, 50 µl aliquotes of rat IRBC lysate, NRBC lysate, or the PEG precipitate from 1 ml of D5 serum were solubilized in sample buffer as previously described. The samples were separated into polypeptides by PAGE on a Hoeffer minigel apparatus. The peptides were electrophoretically transferred from the acrylamide gel to a sheet of nitrocellulose using a Hoeffer Transblot apparatus. The open protein binding sites on the nitrocellulose were blocked with a 3% skimmed milk solution for one hour. The nitrocellulose was overlaid with 50 ml of a 10% ascites solution in a Tris-Tween-20 buffer (TTBS). This solution was incubated overnight at room temperature. The nitrocellulose was washed three times in TTBS and then 50 ml of a 1:3000 dilution in TTBS of goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad Labs) was added. This solution was incubated at room temperature for 30 minutes, followed by three washings in TTBS. The development reagent was made up according to manufacturer's instructions (Bio-Rad). The development solution was added
to the nitrocellulose in the dark for 15 minutes at which
time the reaction was stopped by washing with distilled
water.

**Production of monoclonal antibodies.** Monoclonal antibody BJ was produced by the fusion of mouse myeloma cells of the A-1 line with spleen cells from a Balb/c mouse immunized against *P. falciparum*. The fusion was performed using polyethylene glycol-4000 as described by Goding (1982). The media was adjusted to select for the growth of hybridomas by the addition of hypoxanthine-aminopterin-thymidine (HAT) to the stock RPMI 1640 + 10% foetal calf serum as described (Goding, 1982). The HAT media was changed in the 96 well plates every three days.

Two to four weeks after the last fusion, and three days after the last media change the culture supernatants from the 96 well plates that appeared positive for growth were assayed for anti-*P. falciparum* activity. An indirect ELISA assay was used as described elsewhere. The cells that produced supernatants that yielded anti-*P. falciparum* activity were cloned by the technique of limit dilution cloning (Goding, 1982). Macrophages were used as the feeder cells since hybridomas do not grow well at low cell densities. Dilutions of the hybridomas were prepared to contain 50, 10 or 5 hybridoma cells/ml along with $10^7$ macrophages/ml. The solutions were plated into 96 well
plates at 1/20 ml (1 drop) per well. After 7-14 days in culture, the supernatants were again tested by ELISA. Those cells that produced supernatants still positive by ELISA were expanded into 24 well plates and eventually T-25 tissue culture flasks (Corning). Aliquots were frozen in liquid nitrogen by standard methods (Goding, 1982).

The hybridomas were expanded in vivo by the production of ascites in Balb/c mice. The mice were primed by an intraperitoneal (IP) injection of 0.5 ml pristane (Sigma Chemical Co.) 10 days before injection of hybridoma cells. The hybridoma cells were recovered from culture and adjusted to 5 x 10^5 viable cells/ml. Each mouse was injected IP with 0.5 ml of solution. Swelling of the abdomen was apparent in one to three weeks. Ascites fluid was collected using an 18 G needle, aliquoted and stored at -20°C.

Ascites fluid containing monoclonal antibodies B4, B10, E6 and H8 was received from Dr. D. Taylor (Georgetown University).
RESULTS

Analysis for the presence of immune complexes in serum collected during malaria infection. We carried out a high performance liquid chromatography (HPLC) analysis of rat sera collected during the course of malaria infection (Figures 1-8). Normal rat serum fractionated into four major peaks (Figure 1). These fractions correspond in molecular weight to the major serum proteins IgM (greater than 350 kd), IgG (150-160 kd), albumin (50-70 kd), and small glycoproteins (less than 50 kd). This pattern was used as the reference to identify relative changes in the levels of IgG and high molecular weight components in subsequent serum samples.

Serum collected two days after challenge, just as parasitemia is beginning to develop (0.5%) already differed from normal serum. The percentage of protein in the mass range of free IgG (150-160 kd) has decreased slightly, while the total amount of protein in the serum nearly doubled. The amount of protein in a high molecular weight (MW) configuration (greater than 350 kd) also increased more than two-fold (Figure 2).

In serum collected five days after challenge as the
Figure 1.

Light absorbence by effluent from a HPLC column on which normal rat serum was fractionated. The peak at 6.49 min. represents all serum protein of molecular weight greater than 350 kd (primarily IgM). The peak at 8.44 represents serum protein in the 140-160 kd range (primarily IgG). The large peak at 11.09 represents serum protein in the 50-70 kd range (serum albumin), while the peak at 14.60 shows serum protein of less than 50 kd (various glycoproteins). This figure shows the relative amount of IgG, the major component at 150 kd, and of higher molecular weight components greater than 350 kd.
Figure 1

NORMAL RAT SERUM

*retention time (min)
molecular weight
Figure 2.

Light absorbance by effluent from a HPLC column on which rat serum collected two days after maria challenge was fractionated. Parasitemia was 0.5% when the serum was collected. The amount of protein in the molecular weight range of IgG (140-160 kd, peak at 8.43) was slightly lower than in normal serum, while the amount of protein of molecular weight greater than 350 kd (peak at 6.53) is 2.5 times the normal level.
Figure 2

RAT SERUM D2

molecular weight

retention time (min)

350kd 150kd 65kd 40kd

A280

0.5

0.25

0.20

0.15

0.10

0.05

0.00

6.23*

8.43

11.28

14.38

*Retention time (min)
parasitemia continued to develop (3.2%), the percentage of protein in the weight range of free IgG was clearly depressed. The amount of protein greater than 350 kd reached a maximal level both in concentration (8.0 mg/ml) and percentage of total serum protein (9.5%). The concentration and percentage of normal serum protein with molecular weights greater than 350 kd were 2.16 mg/ml and 3.6%, respectively.

In serum collected nine days after challenge as the parasitemia reached 11%, the percentage of protein of the same mass as free IgG reached a state of maximal depression which was maintained through day 17 (Figure 4). The concentration of protein mass greater than 350 kd dropped to 4.4 mg/ml. Protein with mass greater than 350 kd was 6.8% of the total serum protein.

In the serum collected 14 days after challenge as the parasitemia approached 19%, the concentration of protein of mass greater than 350 kd was only 3.8 mg/ml (Figure 5).

By 17 days after challenge, the parasitemia had reached a maximal value of 21.5%. The amount of protein of the same mass as free IgG (150-160 kd) remained depressed while the amount of protein greater than 350 kd increased to 4.6 mg/ml (Figure 6).

At 26 days after challenge, the rats have recovered from the infection and the parasitemia was 0%. The level of protein of the same mass as free IgG began to return to normal. However, the amount of protein of mass greater
Figure 3.

Light absorbence by effluent from a HPLC column on which rat serum collected five days after malaria challenge was fractionated. Parasitemia was 3.2% when the serum was collected. The amount of protein in the MW range of IgG (140-160 kd, peak at 8.08) was much less than normal, while the material of molecular weight greater than 350 kd (peak at 6.43) was at the maximal level it obtained during the infection which was about four times the normal amount.
Figure 3

RAT SERUM D5

*retention time (min)
molecular weight

A280

0.5

0.25

1 - 0.5

6.43*
Figure 4.

Light absorbence by effluent from a HPLC column on which rat serum collected nine days after malaria challenge was fractionated. Parasitemia was 11.7% when the serum was collected. The amount of IgG (peak at 8.20) was less than in normal serum, while the amount of protein with molecular weight greater than 350 kd was two times the normal amount.
Figure 4

RAT SERUM D9

*retention time (min)  molecular weight

350kd  150kd  65kd  40kd

6.32*  8.28  10.59  14.62
Figure 5.
Light absorbance by effluent from a HPLC column on which rat serum collected 14 days after malaria challenge was fractionated. Parasitemia was 18.9% when the serum was collected. The amount of IgG (peak at 8.25) was below normal, while the amount of protein of molecular weight greater than 350 kd was slightly less than two times the normal level.
**Figure 5**

- **RAT SERUM D14**

- Molecular weight
  - 350kd
  - 150kd
  - 65kd
  - 40kd

- Retention time (min)
  - 6.46
  - 8.65
  - 18.97
  - 14.63

---

*retention time (min)  molecular weight*
Figure 6.

Light absorbence by effluent from a HPLC column on which rat serum collected 17 days after malaria challenge was fractionated. The parasitemia was 21.5% when the serum was collected. The amount of IgG (peak at 8.24) was below normal, while the amount of protein of molecular weight greater than 350 kd was slightly more than two times the normal amount.
Figure 6

RAT SERUM D17

*Retention time (min)
than 350 kd has remained essentially stable at 4.5 mg/ml (Figure 7).

In the final serum sample collected 35 days after challenge, both the amount of protein of mass equal to free IgG had increased to the normal level, and the amount of protein of mass greater than 350 kd had decreased to the normal level (1.9 mg/ml). The parasitemia had been 0% for about two weeks at the time this sample was collected (Figure 8). The protein contents of the fractions are listed in Table 1.

**Analysis for presence of immune complexes in serum by sucrose gradient centrifugation.** Based on the results of the HPLC analysis of serum collected during the course of infection, serum collected during the fifth day of infection (D5) was chosen for further study. Sucrose gradient centrifugation across a 10-40% sucrose pad was used to compare normal serum to D5 serum. Normal serum separated into three major fractions and one minor fraction based upon sedimentation coefficients. The major fractions were 4s, 7s, and 19s (Figure 9). The minor fraction was at 22s. The 7s and 19s fractions were tested for the presence of immunoglobulin by immunoelectrophoresis (Figures 11-17).

Serum collected five days after malaria challenge (D5) resolved into three major fractions and one minor fraction based upon sedimentation coefficient as did the normal
Figure 7.

Light absorbence by effluent from a HPLC column on which rat serum collected 26 days after malaria challenge was fractionated. Parasitemia was 0% when the serum was collected. The level of IgG (peak at 8.31) was almost normal, while the amount of protein of molecular weight greater than 350 kd was about two times the normal level.
RAT SERUM D26

*retention time (min)  molecular weight

Figure 7
Figure 8.

Light absorbance by effluent from a HPLC column on which serum collected 35 days after malaria challenge was fractionated. Parasitemia was 0% when the serum was collected. The level of IgG (peak at 8.39) was normal, and the amount of protein of molecular weight greater than 350 kd has returned to normal.
Figure 8

RAT SERUM D35

-0.5
-0.25
0
A280

*retention time (min)
molecular weight

350kd 150kd 65kd 40kd

11.85
14.68
Table 1
Protein content of immune complexes in serum samples collected during the course of infection.

<table>
<thead>
<tr>
<th>day of serum collection</th>
<th>total protein (mg/ml)</th>
<th>protein $&gt;$ 350kd (mg/ml)</th>
<th>% of total protein $&gt;$ 350kd</th>
<th>parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal serum</td>
<td>60.0</td>
<td>2.16</td>
<td>3.6%</td>
<td>0.5%</td>
</tr>
<tr>
<td>2</td>
<td>116.8</td>
<td>5.12</td>
<td>4.4%</td>
<td>3.2%</td>
</tr>
<tr>
<td>5</td>
<td>84.0</td>
<td>8.00</td>
<td>9.5%</td>
<td>11.7%</td>
</tr>
<tr>
<td>9</td>
<td>64.8</td>
<td>4.36</td>
<td>6.8%</td>
<td>18.9%</td>
</tr>
<tr>
<td>14</td>
<td>65.6</td>
<td>3.80</td>
<td>5.8%</td>
<td>21.5%</td>
</tr>
<tr>
<td>17</td>
<td>74.8</td>
<td>4.56</td>
<td>6.1%</td>
<td>0.0%</td>
</tr>
<tr>
<td>26</td>
<td>79.8</td>
<td>4.48</td>
<td>5.7%</td>
<td>0.0%</td>
</tr>
<tr>
<td>35</td>
<td>57.6</td>
<td>1.88</td>
<td>4.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
Figure 9.

Fractions obtained by sucrose gradient centrifugation of normal rat serum. The serum resolved into three major components. These components had sedimentation constants of 4s, 7s and 19s.
serum. The major fractions were somewhat different being 4s, 19s, and 22s. The 7s fraction, which was a major component in the normal serum sample, was a minor component in the D5 sample, which contained an additional major fraction at 22s (Figure 10).

Analysis by immunoelectrophoresis of the fractions obtained by sucrose gradient centrifugation. Serum samples and sucrose fractions of the samples were analyzed for the presence of immunoglobulin by immunoelectrophoresis (Figures 11-17). Whole normal serum (b) and D5 serum (a) when reacted with anti-rat whole serum (c) following electrophoresis yielded electrophoretic mobility patterns with major precipitation arcs that corresponded to albumin and IgG. Albumin typically migrated rapidly towards the positive electrode while IgG migrated slowly towards the negative electrode (Figure 11).

The 7s fraction (a) and the 19s fraction (b) of normal serum both reacted with anti-rat whole serum (c) following electrophoresis. The 7s fraction yielded three precipitation arcs that corresponded to IgG subclasses (α1, α2, α3) based upon electrophoretic mobility. The 19s fraction yielded a single arc that corresponded approximately to IgM (β1) based upon electrophoretic mobility (Figure 12).

An immunoelectrophoresis pattern was obtained with the 7s fractions from normal serum (a) and D5 serum (b),
Figure 10.

Fractions obtained by sucrose gradient centrifugation of rat serum collected five days after malaria challenge (D5). The serum resolved into three major components. These components had sedimentation coefficients of 4s, 19s and 22s. The 7s fraction which was a major component of normal serum was largely absent from this sample.
Figure 10

- A280
- % w/w Sucrose
- 4s
- 7s
- 19s
- 22s

5 Sample Volume (ml)

5 10 13
Figure 11 (Slide #1).

The pattern produced by immunoelectrophoresis of serum obtained five days after plasmodial infection. Well (a) contained rat serum collected five days after malaria challenge (D5); well (b) contained normal rat serum; the trough (c) contained rabbit anti-rat whole serum. Both samples yielded multiple and nearly identical precipitation arcs.
Figure 12 (Slide #2).

The patterns produced by immunoelectrophoresis of fractions of normal serum yielded by sucrose gradient centrifugation. Well (a) contained the 7s fraction from normal serum; well (b) contained the 19s fraction from the same serum sample; the trough (c) contained rabbit anti-rat whole serum. Well (a) yielded several precipitation arcs, those of IgG subclasses (arrows a1, a2, a3). Well (b) yielded a single faint precipitation arc, presumably that of IgM (arrow b1). The positive electrode was positioned to the left edge of the slide.
following electrophoresis and reaction with anti-rat whole serum (c) (Figure 13). The 7s fraction from normal serum again showed three precipitation arcs (a1, a2, and a3, IgG subclasses) as in Figure 12. The 7s fraction of D5 serum yielded only two precipitation arcs, which corresponded to IgG subclasses based upon electrophoretic mobility. This D5 7s fraction was missing a precipitation arc that was present in the corresponding 7s normal serum fraction.

The 19s fractions of normal (a) and D5 (b) serum reacted weakly with anti-rat IgM (c) (Figure 14). This reaction was carried out as a control on the fractionation process.

We compared an IgG standard (a) against the 19s fraction from normal serum (b) as a control on the sucrose fractionation process. Both samples were electrophoresed and then reacted against anti-rat IgG antiserum (c). The IgG standard showed a strong precipitation arc (a1), while the 19s normal serum fraction showed no reaction (Figure 15).

The 22s fractions of D5 serum (a) and normal serum (b) were reacted with anti-rat whole serum (c). The 22s fraction from the D5 serum yielded a single precipitation arc (a1) that corresponded to the IgG based upon electrophoretic mobility. The 22s fraction from normal serum yielded no reaction (Figure 16).
Figure 13 (Slide #5).

The patterns produced by immunoelectrophoresis of serum fractions yielded by sucrose gradient centrifugation. Well (a) contained the 7s fraction from normal rat serum; well (b) contained the 7s fraction from D5 serum; the trough (c) contained rabbit anti-rat whole serum. Well (a) yielded three arcs, presumably those of IgG subclasses (a1, a2, a3). Well (b) yielded only two arcs, presumably IgG subclasses (b1, b2).
Figure 14 (Slide #3).

The patterns produced by immunoelectrophoresis of serum fractions yielded by sucrose gradient centrifugation. Well (a) contained the 19s fraction from normal serum; well (b) contained the 19s fraction from D5 serum; the trough contained rabbit anti-rat IgM antiserum. Very faint precipitation arcs (a1, b1) developed with the 19s fractions of both sera.
Figure 14
Figure 15 (Slide #9).

The patterns produced by immunoelectrophoresis of serum fractions yielded by sucrose gradient centrifugation. Well (a) contained purified rat IgG; well (b) contained the 19s fraction from normal serum; the trough (c) contained rabbit anti-rat IgG. Well (a) yielded a single precipitation arc (a1); well (b) showed no reaction.
Figure 16 (Slide #4).

The patterns obtained by immunoelectrophoresis of serum fractions yielded by sucrose gradient centrifugation. Well (a) contained the 22s fraction from D5 serum; well (b) contained the 22s fraction from normal serum; the trough (c) contained rabbit anti-rat whole serum. Well (a) yielded a single precipitation arc (a1); well (b) yielded no reaction.
An IgG standard (a) and the 22s fraction of D5 serum (b) both yielded arcs following reaction with anti-rat IgG (c). These single precipitation arcs (a1, b1) were identical in mobility and corresponded to IgG (Figure 17).

HPLC fractionation of polyethylene glycol precipitates of rat serum. The precipitates obtained from serum collected five days after malaria challenge (D5) and from normal serum at various concentrations of polyethylene glycol (PEG) (3-10%) were analyzed by HPLC. The HPLC analysis was used to identify the PEG concentration that precipitated protein of molecular weight greater than 350 kd (immune complexes), without precipitation of large amounts of other material.

Two peaks resulted from HPLC fractionation of the 3% PEG precipitate from D5 serum. One of these peaks corresponded to MW greater than 350 kd (peak at 6.29). This peak contained 0.22 mg/ml of protein, which presented 19.4% of the total protein (1.2 mg/ml) of the precipitate. This was the highest concentration of high MW components obtained using PEG with D5 serum (Figure 18).

Only small amounts of protein (0.3 mg) were precipitated by 3% PEG from normal serum. Only trace amounts of the precipitated proteins were greater than 350 kd in mass (Figure 19).
Figure 17 (Slide #6).

The patterns produced by immunoelectrophoresis of serum fractions obtained by sucrose gradient centrifugation and IgG standards. Well (a) contained the 22s fraction from D5 serum; well (b) contained purified rat IgG; the trough contained rabbit anti-rat IgG. Wells (a) and (b) yielded single and identical precipitation arcs (a1, b1).
Figure 18.

Light absorbence by effluent from a HPLC column on which precipitates from rat serum collected five days after malaria challenge (acute phase serum-APS) were fractionated. The precipitate was formed in the presence of 3% PEG. A total of 1.2 mg of protein was precipitated. Over 19% of the 3% PEG precipitate had a mass greater than 350 kd (peak at 6.29).
Figure 18

**retention time**

molecular weight

**APS 3% PEG**

<table>
<thead>
<tr>
<th>A280</th>
</tr>
</thead>
</table>

- 350kd
- 150kd
- 65kd
- 40kd

*retention time*
Figure 19.

Light absorbence by effluent from a HPLC column on which precipitates from normal serum (NS) were fractionated. The precipitates were formed in the presence of 3% PEG. A total of 0.3 mg was precipitated. Only trace amounts of protein of mass greater than 350 kd were precipitated.
Figure 19

- Retention time
- Molecular weight

- A280
- NS 3% PEG
The quantity of protein precipitated from D5 serum by 5% PEG was more than three times as large as the quantity precipitated by 3% PEG, but the concentration of protein of mass greater than 350 kd decreased to 16.5% (Figure 20). Therefore, 5% PEG precipitated a higher concentration of protein less than 350 kd than did 3% PEG.

The HPLC fractionation of the 5% PEG precipitate from normal serum revealed three major peaks, including one that corresponded to protein of molecular weight greater than 350 kd (Figure 21). This peak comprised 34% of the protein precipitated. However, the total amount precipitated from normal serum was only 25% that precipitated from D5 serum.

The amount of material precipitated (8.1 mg) from D5 serum by 7% PEG was twice the amount precipitated by 5% PEG. However, the amount of protein of mass greater than 350 kd dropped to 14.7% of the total (Figure 22).

A total of 3.0 mg of protein was precipitated from normal serum by 7% PEG. This amount is less than half of that from the 7% PEG D5 sample. Only about 9% of the total protein was of mass greater than 350 kd (Figure 23).

The 10% PEG precipitate from D5 serum had a total protein concentration of 9.8 mg/ml, but the amount of protein greater than 350 kd in mass dropped to 13.1% of the total (Figure 24).

The concentration of the protein precipitated from normal serum by 10% PEG was 7.7 mg/ml, and the proportion
Figure 20.

Light absorbence by effluent from a HPLC column on which precipitates from serum collected five days after malaria challenge (APS) were fractionated. The precipitates were formed in the presence of 5% PEG. Over three times more protein (3.9 mg) was precipitated by 5% PEG than by 3% PEG, but the fraction of mass greater than 350 kd (peak at 6.33) was only 16.5%.
Figure 20

A280

- retention time
- molecular weight
Figure 21.

Light absorbence by effluent from a HPLC column on which precipitates from normal serum (NS) were fractionated. The precipitates were formed in the presence of 5% PEG. A total of 1.0 mg. of protein was precipitated, of which about one-third was of mass greater than 350 kd (peak at 6.13).
Figure 21: Graph showing the retention time vs. molecular weight for NS 5% PEG with peaks at 6.13, 11.21, and 14.35. The Y-axis represents absorbance at 280 nm (A280) and the X-axis represents molecular weight (350kd, 150kd, 65kd, 40kd).
Figure 22.

Light absorbence by effluent from a HPLC column on which precipitates from serum collected five days after malaria challenge (APS) were fractionated. The precipitate was formed after addition of polyethylene glycol to 7% of the total concentration (7% PEG). A total of 8.1 mg of protein was precipitated. The fraction of protein in the precipitate of MW greater than 350 kd (peak at 6.34) was 14.7%.
Figure 22

- A280
- Retention time
- Molecular weight

APS 7% PEG

350kd 150kd 65kd 40kd
Figure 23.

Light absorbence by effluent from a HPLC column on which the precipitate from normal serum (NS) was fractionated. The precipitate was formed by the addition of PEG to 7% of the total concentration. A total of 3.0 mg of protein was precipitated. The fraction greater than 350 kd (peak at 6.24) was 9.3%.
Figure 23
Figure 24.

Light absorbence by effluent from a HPLC column on which the precipitate from serum collected five days after malaria challenge (APS) was fractionated. The precipitate was formed by the addition of PEG to the serum to a concentration of 10%. The amount of protein precipitated was 9.8 mg. The fraction greater than 350 kd (peak at 6.30) was 13.1%.
Figure 24

A280

-0.5

-0.25

350kd 150kd 65kd 40kd

APS 10% PEG

retention time

molecular weight

Figure 24
with mass greater than 350 kd was 7.1% of the total (Figure 25).

Table 2 summarizes the data on concentrations of total protein and of proteins of mass greater than 350 kd in the various PEG precipitates. All protein concentration estimates were based upon absorbance at 280 nm. With the D5 serum, as the concentration of PEG increased, so did the amount of total protein precipitated. However, as the amount of total protein precipitated increased, the percentage of this protein which had a mass greater than 350 kd decreased. This same pattern was evident in the PEG precipitates of normal serum. The amounts of protein precipitated from NS were less than the amounts precipitated from D5 serum.

Analysis of PEG precipitates by immunoelectrophoresis. The 3% PEG D5 precipitate (b) developed a strong precipitation arc (bl) after electrophoresis and reaction with anti-rat IgG. The precipitate from normal serum (a) developed only a very weak precipitation arc under the same conditions (Figure 26). The 5% PEG D5 serum precipitate also yielded a strong IgG precipitation arc (bl) while the NS precipitate yielded a weak arc (a1). The 7% and 10% PEG precipitates from both NS and D5 serum yielded strong precipitation arcs with anti-rat IgG. Thus, the greatest difference
Figure 25.

Light absorbence by effluent from a HPLC column on which precipitates from normal serum (NS) were fractionated. The precipitates were formed by the addition of PEG to the serum to the concentration of 10%. The amount of protein precipitated was 7.7 mg. The fraction of mass greater than 350 kd was 7.1%.
Figure 25

Retention time vs. molecular weight for NS 10% PEG.
Table 2
Protein content of immune complexes precipitated at different concentrations of PEG from serum collected 5 days after challenge.

<table>
<thead>
<tr>
<th>day of serum collection</th>
<th>%PEG</th>
<th>total protein (mg/ml)</th>
<th>protein &gt; 350kd (mg/ml)</th>
<th>% of total protein &gt; 350kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>84.0</td>
<td>8.00</td>
<td>9.5%</td>
</tr>
<tr>
<td>5</td>
<td>3%</td>
<td>1.2</td>
<td>0.22</td>
<td>19.4%</td>
</tr>
<tr>
<td>5</td>
<td>5%</td>
<td>3.9</td>
<td>0.64</td>
<td>16.5%</td>
</tr>
<tr>
<td>5</td>
<td>7%</td>
<td>8.1</td>
<td>1.19</td>
<td>14.7%</td>
</tr>
<tr>
<td>5</td>
<td>10%</td>
<td>9.8</td>
<td>1.28</td>
<td>13.1%</td>
</tr>
<tr>
<td>normal serum</td>
<td>0%</td>
<td>60.0</td>
<td>2.16</td>
<td>3.6%</td>
</tr>
<tr>
<td>normal serum</td>
<td>3%</td>
<td>0.3</td>
<td>0.00</td>
<td>0.0%</td>
</tr>
<tr>
<td>normal serum</td>
<td>5%</td>
<td>1.0</td>
<td>0.34</td>
<td>34.0%</td>
</tr>
<tr>
<td>normal serum</td>
<td>7%</td>
<td>3.0</td>
<td>0.28</td>
<td>9.3%</td>
</tr>
<tr>
<td>normal serum</td>
<td>10%</td>
<td>7.7</td>
<td>0.55</td>
<td>7.1%</td>
</tr>
</tbody>
</table>
Figure 26 (Slide #10).

The patterns produced by immunoelectrophoresis of precipitates from serum. Well (a) contained the 3% PEG precipitate from normal serum; well (b) contained the 3% PEG precipitate from D5 serum; the trough (c) contained rabbit anti-rat IgG. Well (a) yielded a weak precipitation arc (a1); well (b) yielded a much stronger precipitation arc (b1). This result indicates that a 3% PEG concentration precipitates little IgG from normal serum but much more from D5 serum.
Quantitation of IgG in PEG precipitates by radial immunodiffusion. Radial immunodiffusion (RID) was used as a means of quantitating the amount of IgG in the 3% PEG precipitates. A standard curve was constructed showing the relationship between amount of IgG and area of precipitation. There was an approximate linear relationship between IgG concentration and area of precipitation (Table 3). This data was used to calculate IgG concentration in the 3% PEG precipitates assayed by RID (Table 4). The maximum amount of IgG (111.3 mg/ml) was precipitated from the D5 serum sample. This amount was more than 10x higher than the total amount of IgG in NS. No IgG was detected in the 3% PEG supernatant fractions from D2-D26 serum. Whole normal serum contained 10.0 mg/ml of IgG. A concentration of 7.9 mg/ml IgG was detected in the 3% PEG supernatant from NS, while no IgG was found in the precipitate.

As the disease progressed and the animals began to recover, the quantity of IgG precipitated from their serum by 3% PEG decreased. However, IgG did not reappear in the supernatant fraction until 35 days after infection (D35) when 7.9 mg/ml was again detected.
Figure 27 (Slide #11).

The patterns produced by immunoelectrophoresis of precipitates from serum. Well (a) contained the 5% PEG precipitate from normal serum; well (b) contained the 5% PEG precipitate from D5 serum; the trough (c) contained rabbit anti-rat IgG. Both wells yielded precipitation arcs (a1, b1), with well (b) showing a slightly stronger pattern. These results indicate that IgG is present in the precipitates and that at 5% PEG, less IgG is precipitated from normal serum than from D5 serum.
Figure 28 (Slide #12).

The patterns produced by immunoelectrophoresis of precipitates from serum. Wells (a) and (b) contained the 7% PEG precipitates from normal and D5 serum, respectively. The trough (c) contained anti-rat IgG. Both wells yielded strong precipitation arcs (a1, b1). These results indicate the presence of IgG in both precipitates.
Figure 29 (Slide #13).

The patterns produced by immunoelectrophoresis of precipitates from rat serum. Wells (a) and (b) contained the 10% PEG precipitates from normal and D5 serum, respectively. The trough contained anti-rat IgG. Both wells yielded strong precipitation arcs (a1, b1), indicative of IgG in the precipitates.
Table 3
Quantation of rat IgG by radial immunodiffusion.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area of precipitation (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG, 1000 μg/ml</td>
<td>1520.0</td>
</tr>
<tr>
<td>IgG, 100 μg/ml</td>
<td>153.0</td>
</tr>
<tr>
<td>IgG, 10 μg/ml</td>
<td>15.9</td>
</tr>
<tr>
<td>IgG, 1 μg/ml</td>
<td>0.0</td>
</tr>
<tr>
<td>BSA, 1000 μg/ml</td>
<td>0.0</td>
</tr>
</tbody>
</table>

IgG = rat IgG samples standardized to indicated concentrations.
BSA = bovine serum albumin.
<table>
<thead>
<tr>
<th>day of serum collection</th>
<th>sample</th>
<th>area of precipitation (mm²)</th>
<th>amount of IgG precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>PEG ppt.</td>
<td>12.6</td>
<td>7.9 ug/ml</td>
</tr>
<tr>
<td>5</td>
<td>PEG ppt.</td>
<td>177.0</td>
<td>111.3 ug/ml</td>
</tr>
<tr>
<td>9</td>
<td>PEG ppt.</td>
<td>154.0</td>
<td>96.7 ug/ml</td>
</tr>
<tr>
<td>14</td>
<td>PEG ppt.</td>
<td>57.0</td>
<td>35.8 ug/ml</td>
</tr>
<tr>
<td>26</td>
<td>PEG ppt.</td>
<td>50.0</td>
<td>31.4 ug/ml</td>
</tr>
<tr>
<td>35</td>
<td>PEG ppt.</td>
<td>0.0</td>
<td>0.0 ug/ml</td>
</tr>
<tr>
<td>NS</td>
<td>whole, 1/1000</td>
<td>15.9</td>
<td>10.0 mg/ml</td>
</tr>
</tbody>
</table>

PEG ppt. = precipitates from rat serum at PEG concentration of 3%.  
NS = normal rat serum.  
whole, 1/1000 = whole normal rat serum diluted 1/1000.
Analysis of PEG precipitates by PAGE. The material precipitated by PEG at concentrations of 3, 5, 7, 10 or 20% was analyzed by PAGE (Figure 30). As the concentration of PEG increased from 3% to 20%, the amount of material precipitated also increased. This relationship held in general for NS (tracks 5 to 1, respectively) and for D5 serum (tracks 6 to 10, respectively). Little change was noted in the amount of material precipitated from NS at PEG concentrations of 3, 5, and 7%. From D5 serum, two major polypeptides of molecular weights approximately equal to 66 kd and 55 kd were precipitated at the 3% PEG concentration. Several other peptides were present in this sample. At a PEG concentration of 5%, a third major polypeptide appeared in the 35 kd range in the precipitate from D5 serum. At PEG concentrations of 7, 10 and 20%, the quantity of material precipitated from D5 serum increased, but no new major bands appeared.

The polypeptides from D2, 5, 9, 14, and 26 serum, in addition to the polypeptides in whole D2-D26 serum precipitated at a 3% PEG concentration were further analyzed (Figure 31). The 3% PEG precipitate from NS (track 13) yielded only one major polypeptide which corresponded to albumin (66 kd) in molecular weight. The PAGE analysis of the whole NS sample (track 12) produced several major polypeptides that corresponded to albumin (66 kd), and several other serum proteins. Of the 3% PEG precipitates
Figure 30.
Polyacrylamide gel electrophoresis (PAGE) pattern of 3% to 20% PEG precipitates from normal serum (NS) or from serum collected five days after malaria challenge (D5). Starting at the left, tracks 1-5 contained precipitates from NS at 3, 5, 7, 10 and 20% PEG concentration, respectively. Tracks 6-10 contained precipitates from D5 serum at 3, 5, 7, 10 and 20% PEG concentration, respectively. As the concentration of PEG used to precipitate the serum increased, the amount of material precipitated greatly increased, especially in the case of normal serum. Most of the precipitated material was in the molecular weight range of IgG heavy (50-60 kd) and light (25 kd) chains, IgM heavy (65 kd) and light (25 kd) chains and albumin (66 kd).
Sample
% PEG
NS NS NS NS NS D5 D5 D5 D5 D5
3% 5% 7% 10% 20% 3% 5% 7% 10% 20%

Figure 30
Figure 31.

PAGE patterns of 3% precipitates from rat serum collected 2 to 26 days after malaria challenge (D2 to 26). Starting at the left, tracks 1 and 2 contained D2 serum and the 3% PEG precipitate from D2 serum; tracks 3 and 4 contained D5 serum and the 3% PEG precipitate from D5 serum; tracks 5 and 6, D9 serum, and the 3% PEG precipitate from D9 serum; track 7, molecular weight markers (M); tracks 8 and 9, D14 serum and the 3% PEG precipitate from D14 serum; tracks 10 and 11, D26 serum and the 3% PEG precipitate from D26 serum; tracks 12 and 13, normal rat serum and the 3% PEG precipitate from normal rat serum. All tracks received an equal volume of sample with precipitates reconstituted to the volume of the original sample. The quantity of protein was greatest in the PEG precipitates from D2 and D5 serum.
from D2, 5, 9, 14 and 26 serum analyzed by PAGE (tracks 2, 4, 6, 9, and 11, respectively), only the D5 serum precipitate (track 4) produced a major polypeptide in the 70 kd range. The 70 kd polypeptide was present however in smaller (D2; track 2) or trace (D9, 14, 26; tracks 6, 9, 11) amounts in the other precipitates. The D5 3% PEG precipitate also contained the largest amount of total precipitate. Qualitative differences are evident between the D2 and D5 precipitates (tracks 2 and 4). Some high molecular weight polypeptides (greater than 100 kd) are more prominent in the D2 precipitate, while other bands are more evident in the D5 precipitate. There are no such differences in the whole D2 (track 1) and D5 (track 3) serum samples.

Analysis of the antigens in immune complexes. The antigens in the immune complexes were identified with $^{35}$S-labeled P. berghei antigen and hyperimmune serum (Figure 32). The PEG precipitates from day 2, 5, 7, 9, 14, 17 and 26 serum (tracks 10-16, respectively) were reacted with $^{35}$S-labeled P. berghei antigen and then precipitated with protein A. The major polypeptide antigen detected by this reaction had a molecular weight of 16, 25 and 37.5 kd. The PEG precipitate from day 26 serum (track 16) reacted with two additional polypeptides at 30 and 42 kd.
Figure 32.

Autoradiograph of PAGE plate on which antigens in malarial immune complexes were analyzed. Immune complexes were precipitated with 3% PEG from serum collected 2, 5, 7, 9, 14, 17 and 26 days after challenge. The precipitates were resuspended and then reacted directly with \( ^{35}\text{S}-\text{labeled } P.\ berghei \) antigen (lanes 10-16, respectively), or were first reacted with hyperimmune serum, followed by precipitation with protein A and reaction with labeled antigen (lanes 2-8, respectively). Lane 1 contains the labeled \( P.\ berghei \) antigen; lane 9 contains the precipitate from normal rat serum reacted with labeled antigen. The material in lanes 2-16 were precipitated from solution using protein A. See text for details.

The control reaction (antigen reacted with normal serum) in lane 9 showed only one weak band at 25 kd. The PEG precipitates from sera collected 2, 5, 7, 9 and 14 days post challenge and not preincubated with immune serum (lanes 10-14) bound little antigen, indicating that the complexes are antigen excess. The PEG precipitates from sera collected on day 26 (lane 16) bound several antigens not bound by complexes from sera collected earlier. The binding of these antigens indicates that the immune complexes collected on day 26 were antibody excess complexes. The overall intensity of the bands is greater in lanes containing complexes pretreated with immune serum (10-16)
and more bands are observed in these lanes than in lanes containing complexes not pretreated with immune sera (2-8). The complexes collected on day 26 directly bound several new antigens as several new bands appear in the autoradiographs of these precipitates (lane 8). This result indicates that the complexes contained antibody in excess to these antigens. A prominent new band appeared at 30 kd in lanes 8 and 16 which received PEG precipitates from day 26 serum with and without treatment with hyperimmune serum before reaction with labeled antigen. This result indicates that an antibody excess to the antigen of 30 kd had developed by day 26.
Day of infection

immune serum treatment

Figure 32
When the same PEG precipitates from day 2-26 serum were first reacted with hyperimmune serum, precipitated with protein A, and then reacted with labeled P. berghei antigen (tracks 2-8), a greater quantity of labeled antigen was detected. Additional peptides at 18, 18.5, 21, and 23.5 kd were detected by day 5 (track 3). An additional peptide was detected at 58 kd in the day 26 sample (track 8). Peptides, detected in both reactions (16, 25, 37.5 kd), were present in greater quantity in the samples first incubated with hyperimmune serum. No antigen of molecular weight greater than 58 kd was detected by this method.

Analysis of antigens in immune complexes using monoclonal antibodies. Several monoclonal antibody-containing ascites fluids were fractionated by HPLC. These monoclonal antibodies were designated B10, B4, E6 and H8 and were raised against Plasmodium yoelii. A monoclonal antibody designated BJ was raised against Plasmodium falciparum. Of these five antibodies, only H8 and BJ reacted with P. berghei. All of these antibodies were determined to be of the mu (IgM) heavy chain type by ELISA (data not shown). The peak that corresponded to IgM was a minority component in B10 (6.39) and E6 (6.38), and IgM was the second most common component in B4 (6.38). Of the two monoclonal antibodies that did react with P. berghei, the peak that corresponded
to IgM was the majority component in H8 (6.33) and a minority component in BJ (Figures 33-37).

Before being used as a probe for *P. berghei* immune complex antigens, the BJ antibody was tested for ability to inhibit *P. falciparum* growth in culture (Table 5). The percent parasitized human erythrocytes in the cultures was set at 2.0%, and various substances were assayed for ability to inhibit growth over a 48-hour period. RPMI media, A-1 or SP-2 myeloma cell culture supernatants were added as negative controls. The parasitemias of these cultures increased to 5.5 to 7.3% by 48 hours. The average parasitemia of the cultures which received diluted human anti-*P. falciparum* serum was 1.3% after 48 hours. The cultures which received whole BJ ascites fluid had an average parasitemia of 7.6% at 48 hours, which was the highest parasitemia observed in this assay. This indicates that the monoclonal antibody BJ does not inhibit growth of *P. falciparum* in culture.

Normal and hyperimmune serum, and the monoclonal antibodies H8 and BJ were reacted with *P. berghei* infected erythrocytes. Each figure shows the field stained by the Giemsa method and the fluorescent antibody (FA) method.

Polyclonal hyperimmune serum reacted with both infected cells and uninfected cells (Figure 38). There was considerable reaction against the normal erythrocytes as many are clearly visible in the photograph but the infected
Figure 33.

Light absorbance by effluent from a HPLC column on which ascites fluid containing monoclonal antibody B10 was fractionated. This antibody was of mu (IgM) chain type. The peak at 6.39, which corresponds to IgM, is a minority component of the fluid.
Figure 33

A280

B10 MAb Ascites

*retention time (min)
molecular weight

350 kd 150 kd 65 kd 40 kd

6.39* 18.77 14.52
Figure 34.

Light absorbence by effluent from a HPLC column on which ascites fluid containing monoclonal antibody B4 was fractionated. This antibody was of the mu (IgM) chain type. The peak at 6.38, which corresponds to IgM, was the second most common component of the fluid.
Figure 34

B4 MAb Ascites

Retention time (min)

Molecular weight

350kD 150kD 65kD 40kD
Figure 35.

Light absorbence by effluent from a HPLC column on which ascites fluid containing monoclonal antibody E6 was fractionated. This antibody was of the mu (IgM) chain type. The peak at 6.38, which corresponds to IgM, is a minority component of the fluid.
A280

E6 MAb Ascites

*retention time (min)    molecular weight

350kd 150kd 65kd 40kd

Figure 35
Figure 36.

Light absorbence by effluent from a HPLC column on which ascites fluid containing monoclonal antibody H8 was fractionated. This antibody was of the mu (IgM) chain type. The peak at 6.33, which corresponds to IgM, is the majority component of the fluid.
A280

H8 MAb Ascites

*retention time (min)  molecular weight

Figure 36
Figure 37.

Light absorbence by effluent from a HPLC column on which ascites fluid containing monoclonal antibody BJ was fractionated. This antibody was of the mu (IgM) chain type. The peak at 6.39, which corresponds to IgM, is the second most common component of the fluid.
Figure 37
Table 5: Assay for inhibition of *P. falciparum* growth *in vitro* by BJ monoclonal antibody.

<table>
<thead>
<tr>
<th>Culture additive</th>
<th>Ave. Parasitemia $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr</td>
</tr>
<tr>
<td>RPMI 1640 media</td>
<td>4.5</td>
</tr>
<tr>
<td>A-1 myeloma cell culture supernatent</td>
<td>4.5</td>
</tr>
<tr>
<td>SP-2 myeloma cell culture supernatent</td>
<td>3.5</td>
</tr>
<tr>
<td>human anti-<em>P. falciparum</em> serum, diluted 1/4</td>
<td>2.0</td>
</tr>
<tr>
<td>BJ hybridoma ascites fluid</td>
<td>4.6</td>
</tr>
</tbody>
</table>

$^1$Average percent infected erythrocytes per triplicate cultures.
Figure 38.

Reaction of hyperimmune serum to *P. berghei*-infected erythrocytes as shown by fluorescent antibody analysis. The top photograph shows a Giemsa stain of a blood smear from a rat infected with *P. berghei*. The bottom photograph shows the same field (at a lower magnification) following reaction with rat hyperimmune serum against *P. berghei* and development with fluorescent antibody (see text for details). The hyperimmune serum reacted strongly to the two infected erythrocytes in the center of the field, but also showed a high background of reaction against uninfected red blood cells.
cells in the field appear brighter than the surrounding normal erythrocytes.

Normal rat serum did not react with either infected or normal cells. Only trace background reactions are visible (Figure 39).

The monoclonal antibody H8 reacted with the *P. berghei*-infected erythrocytes. Only the infected cells were fluorescent, and there was little background reaction. The reaction was such that sections of the infected cell were clearly illuminated, while other sections remain dark. The location of fluorescent material partly corresponded to the location of parasite material within the cell (Figure 40).

The monoclonal antibody BJ reacted with human erythrocytes in culture infected with *P. falciparum* (Figure 41). Rat erythrocytes from an animal infected with *P. berghei* also reacted with this monoclonal antibody. The infected cells were brightly fluorescent, with little background reaction in all cases. Monoclonal antibody BJ reacted with both *P. falciparum* and *P. berghei* to yield bright patches of fluorescence that only roughly outline the infected cell (Figures 42, 43). A cell, containing a late stage *P. berghei* schizont, appeared to be fluorescent on both its exterior and interior. The sections most brightly illuminated do not correspond exactly to parasite material, but many of the individual merozoites within the cell were stained.
Figure 39.

Reaction of normal rat serum to *P. berghei*-infected erythrocytes as shown by fluorescent antibody. The top photograph shows a Giemsa-stained blood smear from a *P. berghei*-infected rat. The bottom photograph shows the same field following reaction with normal rat serum and development with fluorescent antibody. The normal serum did not react to infected erythrocytes and showed only trace background reactions.
Figure 40.

Reaction of monoclonal antibody H8 to *P. berghei*-infected erythrocytes as shown by fluorescent antibody. The top photograph shows a Giemsa stain of a blood smear from a *P. berghei*-infected rat. The bottom photograph shows the same field following reaction with monoclonal antibody H8, and development with fluorescent antibody. Monoclonal antibody H8 reacted to infected erythrocytes, and very little background was apparent. The antibody reacted in a manner that clearly illuminated some sections of the infected red cell, while not reacting to other sections.
Figure 41.

Reaction of monoclonal antibody BJ to \textit{P. falciparum}-infected erythrocytes. The top photograph shows a Giemsa stain of \textit{P. falciparum}-infected human erythrocytes raised in culture. The bottom photograph shows the same field following reaction to monoclonal antibody BJ and development by fluorescent antibody. Monoclonal antibody BJ reacted with infected erythrocytes in a pattern of patches that roughly outlined the surface of the infected cell. Little background reaction is apparent.
Figure 42.

Reaction of monoclonal antibody BJ to *P. berghei*-infected erythrocytes. The top photograph shows a Giemsa-stained blood smear from a rat infected with *P. berghei*. The bottom field shows the same field following reaction with monoclonal antibody BJ and development by fluorescent antibody. Monoclonal BJ reacted to the *P. berghei*-infected red cells with the same pattern of bright patches that roughly outline the cell as seen in Figure 55.
Figure 43.

Reaction of monoclonal antibody BJ to *P. berghei*-infected erythrocytes. The top photograph shows a Giemsa stain of a blood smear from a rat infected with *P. berghei*. The bottom photograph shows the same field following reaction with monoclonal antibody BJ and development with fluorescent antibody. In this enlarged view, the reaction against the late-stage schizont appears as bright patches against a dimmer pattern that roughly outlines the surface. The reaction highlights "knobs" on the surface of the schizont.
A immunoblot assay for the reaction of monoclonal antibody H8 with *P. berghei* antigens was run (Figure 44). Track 1 contains the 3% PEG precipitate from D5 serum; track 2 contains normal erythrocyte antigens; and track 3 contains soluble *P. berghei* antigens extracted from 40% infected erythrocytes. Tracks 1B, 2B and 3B are protein immunoblots of the corresponding tracks on the gel, reacted with monoclonal antibody H8. This antibody reacted with two antigens, at 31 and 112 kd, in the tracks that corresponded to the PEG precipitate from D5 serum and the infected erythrocyte antigen (tracks 1B, 3B) (Figure 44).

The reaction of monoclonal antibody BJ to protein immunoblots of the same antigens was different. This monoclonal reacted to a single antigen at 17 kd in the tracks that corresponded to the 3% PEG precipitate from D5 serum, and to the infected erythrocyte antigen (Figure 45).

Injections of D5 serum had no effect upon the *in vivo* response to sheep red blood cells (SRBC) (Table 6). Infusions of D5 serum given during immunization with SRBC did not reduce or increase the hemolytic titer of the serum collected five days after vaccination. Serum from rats which received the SRBC vaccine, or the SRBC vaccine with infusions of D5 serum both had hemolytic titers of 1024. Serum from non-vaccinated rats had a hemolytic titer of 8.

The D5 serum did have an effect upon the plaque response of rat lymphocytes (Table 7). A total of $5 \times 10^5$
Figure 44.

Reaction of monoclonal antibody H8 with protein in immunoblots of *P. berghei* antigens. Tracks 1B, 2B and 3B are protein blots onto nitrocellulose from gel tracks 1, 2 and 3. Track 1 contains the 3% PEG precipitate from serum collected five days after challenge with *P. berghei*; track 2 contains normal rat erythrocyte protein; track 3 contains *P. berghei*-infected rat erythrocytes with a parasitemia of 40%; and track 4 contains molecular weight markers. Monoclonal antibody H8 was reacted to the protein immunoblots (see text for details). This antibody reacted to two polypeptides at 112 kd and 31 kd in lanes 1B and 3B. The 112 kd polypeptide does not appear to be a distinct component of the PEG precipitate in track 1, although the reaction is clear in track 1B.
Protein blots of tracks 1-3 reacted with MAb HB.

Figure 44

Figure legend:
- 1B: Protein band 1
- 2B: Protein band 2
- 3B: Protein band 3
- 3% PEG D5: 3% Polyethylene Glycol 5000
- NRBC: Normoblasts
- IRBC: Infected RBCs
- M: Marker

Molecular weight markers in kDa:
- 21.5
- 45
- 66.2
- 82.5

Figure 44 legend:
- 13.9
- 21.5
- 45
- 66.2
- 82.5

Figure 44 description:
- Each lane contains a protein sample reacted with MAb HB.
- Molecular weight markers are indicated at the bottom of the gel.
Figure 45.

Reaction of monoclonal antibody BJ with protein in immunoblots of *P. berghei* antigens. Tracks 1B, 2B and 3B are protein blots from gel tracks 1, 2 and 3. Track 1 contains the 3% PEG precipitate from rat serum collected five days after *P. berghei* challenge; track 2 contains normal rat erythrocyte protein; track 3 contains *P. berghei*-infected rat erythrocytes with a parasitemia of 40%; and track 4 contains molecular weight markers. Monoclonal antibody BJ reacted to a single polypeptide at 17 kd in tracks 1B and 3B. This assay was run in parallel with that illustrated in Figure 50, and the gel staining patterns (tracks 1-4) are repeated for illustrative purposes.
Protein blots of tracks 1–3 reacted with MAb BJ

Figure 45
Table 6

Effect of plasmodial immune complexes upon the in vivo immune response to SRBC as shown by the hemolysis assay.

<table>
<thead>
<tr>
<th>immunization</th>
<th>trial 1</th>
<th>trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SRBC</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>SRBC-D5 serum</td>
<td>1024</td>
<td>1024</td>
</tr>
</tbody>
</table>

1 Inverse of the highest serum dilution that yielded hemolysis of sheep red blood cells.
SRBC = sheep red blood cells.
SRBC-D5 serum = SRBC's given to rats simultaneously with infusion of serum collected from rats 5 days after infection with *P. berghei*. 
Table 7

Effect of plasmodial immune complexes upon immune response to SRBC as shown by the plaque assay.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>lymphocyte pretreatment</th>
<th>trial 1</th>
<th>trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>normal serum</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>None</td>
<td>D5 serum</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>SRBC</td>
<td>normal serum</td>
<td>69</td>
<td>78</td>
</tr>
<tr>
<td>SRBC</td>
<td>D5 serum</td>
<td>13</td>
<td>29</td>
</tr>
<tr>
<td>SRBC-D5 serum</td>
<td>normal serum</td>
<td>ND</td>
<td>31</td>
</tr>
<tr>
<td>SRBC-D5 serum</td>
<td>D5 serum</td>
<td>ND</td>
<td>33</td>
</tr>
</tbody>
</table>

1
no. plaques

1 number of hemolysis plaques/ $5 \times 10^5$ lymphocytes.

SRBC = sheep red blood cells

SRBC-D5 serum = SRBC's given during infusions of serum collected 5 days after infection with P. berghei.
normal rat lymphocytes preincubated with NS yielded 22-27 plaque forming units (PFU). Preincubation of the normal lymphocytes with D5 serum reduced the PFU response by about 10%. The number of PFU from lymphocytes from a rat vaccinated with SRBC was three times higher than observed with lymphocytes from a non-vaccinated rat. There were 69 and 78 PFU/3 plates with lymphocytes from the vaccinated rat. Preincubation of the lymphocytes with D5 serum reduced the PFU response by over 60%. A rat vaccinated with SRBC during infusions of D5 serum produced lymphocytes with a smaller proportion secreting anti-sheep antibody than did rats vaccinated but not infused. The response was about 50% that of a rat vaccinated with SRBC alone. Preincubation of the lymphocytes from the SRBC-D5 serum infusion rat with D5 serum did not further reduce the PFU response.

**Effect of immune complexes upon P. berghei infection in rats.** The D5 serum was shown to be immunosuppressive (Figure 46). Rats given infusions of D5 serum at the time of challenge had a higher parasitemia than rats given infusions of NS. Rats given immune serum (IS) at the time of challenge had the lowest overall parasitemia.

The titers of normal and hyperimmune rat serum were measured in an ELISA test with *P. berghei* antigen (Figure 47). Hyperimmune serum gave higher absorbance readings than normal serum at all dilutions tested, although the
Figure 46.

Effect of D5 serum given at the time of challenge on the course of parasitemia. Doses (see text for details) of normal serum (NS) or immune serum (IS) were infused into rats at the time of malaria challenge as negative and positive controls, respectively. Rats treated with D5 serum had significantly \((p = 0.05)\) higher parasitemia than did rats in the control group treated with NS. The IS infusion passively protected the rats from challenge \((p = 0.05)\) as the parasitemia of this group was about half that of the NS control.
Ave. percent parasitemia, days 1-15

- **NS 1x**: 2.2
- **D5**: 3.8
- **IS 1x**: 2.0
Figure 47.

ELISA reactions of *P. berghei* antigens with serum from normal rats and rats hyperimmune to malaria. Normal and hyperimmune rat sera of various dilutions were allowed to react with *P. berghei* in an ELISA test and the reactions were measured at 480 nm. The hyperimmune serum yielded stronger reactions than the normal serum at all dilutions from 1/3 to 1/7000. The 50% endpoint for hyperimmune serum occurred at a dilution of 1/700.
Figure 47

A480 vs. 1/dilution

- Hyperimmune
- Normal
difference was greatly diminished at dilutions near 1/7000. The 50% endpoint absorbance for hyperimmune serum occurred at a dilution of 1/700. At this dilution, the difference between hyperimmune serum and normal serum was significant.

Infusions of D5 serum affected the ELISA titers as well as the parasitemia levels which developed in rats following infection. Three groups of rats were challenged on day 0. Rats of one group received infusions of D5 serum on day 0; rats of a second group received infusions on day 10 (arrows), while rats of the third group received no serum. The titers equivalent in absorbance to the 50% endpoint of hyperimmune serum (Figure 47) are shown graphically, while the average parasitemias of the rats are indicated next to each point (Figure 48).

Rats of the control group which received no D5 serum had a steady increase in ELISA titer; their parasitemia peaked at day 14 and decreased to 0% by day 21. The rats of the group which received D5 serum infusions on day 0 had a lower titer and higher parasitemia on days 7 and 14 than did rats of the control group. By day 21, the titer of this group had increased to almost equal that of the control group, although the parasitemia remained at over 13%. The group which received infusions of D5 serum on day 10 had a lower titer and a higher parasitemia by day 14 than did the rats of the control group although these trends
Figure 48.

Effect of infusing D5 serum into rats during the course of plasmodial infection upon antibody titers measured by the ELISA test. Three groups of rats were challenged with *P. berghei* on day 0. Rats of one of the groups received D5 serum on day 0, while rats of the second group received D5 serum on day 10. The third (control) group received no serum infusions. The average parasitemias (indicated in parenthesis) of the three groups were recorded at seven-day intervals. Serum samples collected on the days parasitemias were recorded were tested by ELISA. The titers equivalent to the 50% endpoint titer of hyperimmune serum are shown. The control group, which received no D5 serum, showed a steady increase in titer which reached about 4000 when the parasitemia was 0 on day 21. The group which received the D5 serum infusion on day 0 had a significantly (p = 0.05) higher parasitemia and lower titer on days 7 and 14 than the control group; by day 21, the titer had increased to nearly equal that of the control group, although the parasitemia was still at 13%. The group which received the D5 serum infusion on day 10 showed a slight drop in titer between days 14 and 21 and a steady increase in parasitemia by day 14; the differences between the 10-day infusion group and the control group were significant (p = 0.05) by day 21. The titer in the 10-day infusion group remained at less than 1/500 while the parasitemia reached 63%.
No D5 serum given.

D5 serum given on day 10.

D5 serum given on day 0.
were not significant (p = 0.05) until day 21 of the infection.

The fractions of D5 serum varied in their effects on the course of infection (Figure 49). When given at the time of challenge, immune complexes precipitated by 3% PEG caused the rats to experience a higher parasitemia than that in rats that received an infusion of NS. The supernatant from the 3% PEG precipitation of D5 serum partially protected the rats from challenge, but not as much as did the infusion of IS. The infusion into the rats of whole D5 serum at the time of challenge also caused the development of higher parasitemia than did the infusion of NS.

The immunosuppressive agent cyclophosphamide (CP) when given with D5 serum at the time of challenge increased the severity of the parasitemia over that which developed in rats which received D5 serum alone. Rats given D5 serum, with or without administration of CP, also had higher parasitemias than rats given NS. The CP alone appeared to slightly suppress the ability of the rats to control parasitemia after challenge, although this trend was not significant (p = 0.05). Administration of CP at the time of administration of D5 serum did not abolish the suppressive effect of the D5 serum (Figure 50).

Singles doses of NS, D5 serum, IS, or a mixture of D5 serum and IS given two weeks before challenge did not affect the parasitemia that developed following challenge.
Effect on the course of subsequent parasitemia of giving of D5 serum fractions at the time of challenge. Doses (see text) of normal serum (NS), immune serum (IS), or serum collected five days after malaria challenge (D5) were given as controls. The D5 serum fractions were the 3% PEG precipitate from D5 serum (3% PEG ppt), and the supernatant from the 3% PEG precipitation (3% PEG supernat-D5). The average parasitemia of the group that received D5 serum was higher than that of the NS group, while the rats that received IS had lower (p = 0.05) parasitemia. The precipitated material from the D5 serum retained the suppressive effect of the whole D5 serum (3% PEG ppt-D5) as the rats of this group had the highest parasitemia of those in any group in the experiment. The group that received the supernatant from the 3% PEG precipitation was partly protected from challenge, as the parasitemia in this group was lower than that of the NS group but higher than that of the IS group.
Figure 49

Average percent parasitemia, days 1-20

- D5
- NS
- 3% PEG ppt (D5)
- 3% PEG supernt (D5)
Figure 50.

Effect of treatment with cyclophosphamide upon the suppressive effect of D5 serum. Groups of rats were given injections of normal serum, cyclophosphamide (CP), rat serum collected five days after challenge (D5-APS), or a combination of D5-APS and CP (see text for details). Challenge injections were given to all groups five days after the last injection. The parasitemias of the normal serum and CP groups did not differ significantly (p = 0.05) following challenge. Similarly, parasitemia in the rats of the group which received D5-APS with CP did not differ significantly from that in the rats which received D5-APS without CP. The two groups which received the D5-APS did have higher parasitemias (p = 0.05) than the normal serum control group.
Figure 50

Parasitemia

- Normal
- CP
- D5 APS
- D5 APS - CP

Percentage:
- 10%
- 20%
- 30%
The parasitemias of these four groups ranged from 14-17% (Figure 51). However, three weekly doses of D5 serum, the last dose given two weeks before challenge, induced an immunity which caused the parasitemia following challenge to be less than 1%.

**Vaccination studies using a single dose vaccine.** Protection was conferred by a single vaccine dose of free malaria parasites (FP 1x) (Figure 52). The parasitemia following challenge in rats receiving free parasite vaccine was about half that of rats given a mock vaccine of erythrocyte membranes (EM 1x).

The administration of D5 serum with the free parasite vaccine reduced the protection conferred by the FP vaccine (Figure 53). Simultaneous infusions of NS with the FP vaccine had no effect. Rats of the group which received the FP vaccine (FP 1x-NS) had a parasitemia about half that of the group injected with the EM vaccine along with simultaneous infusions of NS (EM 1x-NS). The infusions of D5 serum during administration of the FP vaccine abolished the protective effect of the vaccine. The parasitemia of the rats of the group given free parasite vaccine with D5 serum (FP 1x-D5) was about equal to that of the mock-vaccinated group.

The administration of FP vaccine with a simultaneous challenge (SC) injection abolished the protective effect of
Figure 51.

Effect of giving multiple doses of D5 serum two weeks before challenge upon parasitemia following challenge. Single doses (see text) of normal serum (NS), serum collected five days after malaria challenge (D5), immune serum (IS), or a mixture of D5 serum and IS (D5-IS 1x) were given to groups of rats two weeks before malaria challenge. To one group, three doses of D5 serum (D5 3x) were given weekly, the last two weeks before malaria challenge. The rats given three doses of D5 serum had a lower parasitemia after challenge (p = 0.05), than any of the other four groups, which did not differ significantly from each other.
Ave. percent parasitemia, days 1-15

Figure 51

- NS 1x: 3.6
- D5 1x: 2.3
- IS 1x: 2.6
- D5-IS 1x: 3.2
- D5-3x
Figure 52.

Effect of vaccination with free malaria parasite vaccine upon parasitemia following challenge. A single dose of $1 \times 10^8$ free merozoites in saponin (FP) was given to rats two weeks before challenge. Erythrocyte membranes (EM) were given to other rats as a negative control. Following challenge, the parasitemia of the rats of the group given FP was about 50% lower ($p = 0.05$) than that of rats of the EM group.
Figure 52

Average percent parasitemia, days 1-15

5%  10%  15%  20%

EM 1x

FP 1x

8.6

2.2
Figure 53.

Effect of infusions of D5 serum upon parasitemia following challenge in rats vaccinated with free malaria parasites. Groups of rats were vaccinated with a single injection of free parasites (FP-1x) while receiving infusions of normal serum (NS) or serum collected five days after malaria challenge (D5). A mock vaccine of erythrocyte membranes (EM) was given to one group. The infusion of NS had no effect upon the protective effect of the FP vaccine as the FP group again had about 50% lower (p = 0.05) parasitemia following challenge than the EM group. However, the infusion of D5 serum abrogated the protective effect of the FP vaccine as the parasitemia of this group (FP 1x-D5) was significantly higher than that of the group given FP and normal serum (FP 1x-NS).
Ave. percent parasitemia, days 1-15

Figure 53

- EM 1x-NS: 2.7
- FP 1x-NS: 1.5
- FP 1x-D5: 3.0
the vaccine. The parasitemia of these rats was slightly higher than in the EM mock vaccinated group. Once again, the FP vaccine group had a parasitemia about half that of the mock EM vaccine group. In these control groups, the challenge injection was given two weeks after vaccination (Figure 54).

Vaccination studies using a multiple dose vaccine. Protection was given by multiple doses of vaccine. The parasitemia after challenge in rats given formalin-fixed infected red blood cells (IRBC) was always less than that in rats given formalin-fixed normal red blood cells (Figure 55). The protection offered by the vaccine was proportional to the number of doses given. Five doses of a mock vaccine of formalin-fixed normal red blood cells (NRBC) was given to a control group. Three injections of the vaccine (IRBC 3x) gave protection comparable to that conferred by a single dose of FP vaccine (see Figure 52).

The injections of D5 serum did not reduce the protection conferred by three injections of IRBC vaccine (Figure 56). Infusions of NS also did not affect the protection given by the IRBC 3x vaccine.

When challenge was given simultaneously with the last injection of the three-dose IRBC vaccine, the vaccine conferred little benefit. Rats challenged two weeks after the last IRBC vaccine dose again had parasitemias that were
Figure 54.

Effect of early challenge upon parasitemia following vaccination with free malaria parasites. Groups of rats were vaccinated with free malaria parasites (FP) or erythrocyte membranes (EM), followed by challenge two weeks later. One group was vaccinated with FP as usual, but also received a simultaneous challenge (FP 1xSC). The FP vaccine protected the rats as the parasitemia of the (FP 1x) group was about 50% lower (p = 0.05) than that of the group which received EM. The group which received the FP vaccine with a simultaneous challenge (FP 1x-SC) had a significantly higher (p = 0.05) parasitemia than the FP group; in fact, this group had the highest overall parasitemia.
Ave. percent parasitemia, days 1-15

Figure 54

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM 1x</td>
<td>2.1</td>
</tr>
<tr>
<td>FP 1x</td>
<td>0.7</td>
</tr>
<tr>
<td>FP 1x-SC</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Figure 55.

Effect of vaccination with one or several doses of formalin-fixed infected red blood cells upon parasitemia following challenge in rats. Groups of rats were immunized with one, three or five weekly injections of $1 \times 10^8$ malaria-infected red blood cells (IRBC 1x, 3x, 5x). As a control, one group received five weekly injections of normal red blood cells (NRBC 5x). All rats were challenged two weeks after the last immunization. The parasitemia following challenge was inversely proportional to the number of IRBC vaccine doses that the rats received. The IRBC 5x group had the lowest parasitemia ($p = 0.05$), the IRBC 3x group had a higher parasitemia ($p = 0.05$), and the IRBC 1x group had the highest parasitemia ($p = 0.05$) of the three vaccinated groups. The control group NRBC 5x had a parasitemia that was higher than that of the IRBC 5x and IRBC 3x groups. The IRBC 3x group had a parasitemia that was about 50% that of the NRBC group.
Figure 56.

Effect of infusions of normal serum or D5 serum upon immunity produced by vaccination with IRBC. Groups of rats received three weekly injections of formalin-fixed infected red blood cells along with simultaneous infusions of normal serum (IRBC 3x-NS) or rat serum collected five days after challenge (IRBC 3x-D5). As a control, rats of one group received a mock immunization of three weekly injections of formalin-fixed normal red blood cells with infusions of normal serum (NRBC 3x-NS). Both groups which received the IRBC vaccine had lower (p = 0.05) parasitemias following challenge than the NRBC group; also, the two IRBC groups did not differ in average parasitemia, regardless of NS or D5 serum infusion.
Ave. percent parasitemia, days 1-15

Figure 56

NRBC 3x-NS

IRBC 3x-NS

IRBC 3x-D5
about 50% of those in rats given the NRBC mock vaccine. When the challenge injection was delivered with the last vaccine dose, the resulting parasitemia nearly equaled that of the control group (Figure 57).
Effect of early challenge upon parasitemia following vaccination with infected red blood cells. Two groups of rats received three weekly injections of formalin-fixed infected red blood cells (IRBC 3x) or normal red blood cells (NRBC 3x) followed by challenge two weeks later. One group received a challenge injection along with the last IRBC dose (IRBC 3x-SC). The rats of the group given three injections of IRBC (IRBC 3x) had a lower parasitemia ($p = 0.05$) than those of the group given NRBC three times (NRBC 3x). The group which received the simultaneous challenge with the last IRBC vaccine injection (IRBC 3x-SC) had a parasitemia that was higher ($p = 0.05$) than that of the group which received IRBC three times. The parasitemias in the rats of the group that were challenged at the time of the last vaccine injection did not differ from the parasitemia in the rats of the group given NRBC three times (NRBC 3x).
Ave. percent parasitemia, days 1-15

Figure 57

NRBC 3x

IRBC 3x

IRBC 3x-SC
DISCUSSION

Rat serum collected during the course of infection and recovery from Plasmodium berghei infection was studied for the presence of immune complexes. This analysis was conducted with three goals: 1) to determine the period during infection of greatest concentration of immune complexes in serum, 2) to determine the composition of the immune complexes, and 3) to determine when the complexes are of antigen excess and when of antibody excess. Rats with malaria have been reported to have large amounts of immune complexes in their plasma (DeGraves and Cox, 1983). The period during which the concentration of immune complexes in plasma was maximal was determined by using HPLC analysis. Then, the immune complexes were harvested from infected animals at this time for further study. The relation of concentration of immune complexes in the serum to the level of parasitemia was analyzed. This analysis was used to determine if individuals with an early infection, or who had recently recovered from infection, could be carrying a burden of plasmodial immune complexes. These apparently healthy individuals might be vaccinated during a mass immunization program.
The goal of the high performance liquid chromatography (HPLC) analysis of serum collected during the course of infection and recovery was to determine when shifts in the fractionation profile would signal that IgG decreased, while high molecular weight components increased (Figures 1-8, Table 1). The area under each peak was automatically calculated during each run. Since the upper fractionation range of the available column (Bio-Rad TSK-250) was about 350 kd, most immune complexes appeared in the same peak as IgM. Therefore further analysis was needed to confirm the presence of immune complexes.

As *P. berghei* infection developed, the first change detected by the HPLC fractionation of the serially collected serum was the depression of the peak that corresponded to free IgM in molecular weight. This depression was first detected in the serum sample collected two days after infection (Figure 2), and was prominent in the serum sample collected five days after infection (Figure 3). The IgG peak remained completely depressed until day 26, when an increase was detected (Figure 7). Reappearance of IgG in the serum was complete by day 35 after infection (Figure 8). The depression of IgG in the serum was accompanied by an increase in the amount of protein of mass greater than 350 kd. The amount of protein of mass greater than 350 kd peaked early in the infection, in the serum collected on the fifth day of infection. On this day, the animals had
an average parasitemia of about 3%. The amount of protein with mass greater than 350 kd slowly decreased to the normal amount by day 35 (Table 1). Based on molecular weight alone, it would appear that free IgG was rapidly depleted during the infection, and free IgG did not return to normal levels in the serum until almost two weeks after parasitemia was cleared. The depletion of free IgG initially occurred at the time of increase in the amount of protein in the weight range of immune complexes (greater than 350 kd). However, as infection progressed, the amount of protein in the high molecular weight peak gradually decreased to the normal level, while the free IgG level remained depressed and then rapidly increased to normal only after parasitemia was cleared.

These conditions would imply that IgG was complexed throughout the infection but that the immune complexes were cleared from the serum with greater efficiency as parasitemia increased. At the time the peak parasitemia (20+%) was cleared, the maximal amount of immune complexes should have been formed. However at days 17 and 26 when parasitemia was high and decreasing, the immune complex levels were already below maximum. The peak concentration of immune complexes was in fact detected only five days after infection when the parasitemia was only 3%. Independent analysis by PEG precipitation and radial immunodiffusion confirmed this result (Figure 3, Table 4). The fact that
the peak occurred early in the infection, before a high degree of immunity was reached would also support the contention that clearance of immune complexes, rather than rate of formation, is the prime factor in determining the concentration of immune complexes in serum. It is also noteworthy that the complexes shifted from antigen excess to antibody excess late in the infection as parasitemia fell from maximum. However, the data also indicated that not all complexes made the shift at the same time (Figure 32).

Since the HPLC analysis was an indirect method for analysis of the immune complexes, sucrose gradient centrifugation was used to confirm the presence of immune complexes. The fractionation of NS on a 10-40% sucrose pad produced an expected pattern with three major fractions based upon sedimentation coefficients: 4s, 7s, and 19s (Figures 9 and 10). This pattern is typical of that seen in normal serum with the major fractions 4s, 7s and 19s corresponding to the major proteins, albumin, IgG and IgM, respectively (Blomback and Hanson, 1980). The fractionation of the serum collected on the fifth day of infection did not produce a pattern typical of normal serum as the major fractions were 4s, 19s and 22s. This pattern would seem to suggest that free IgG was largely absent by day 5 of the infection when a high density (22s) fraction appeared in the serum. This result is in agreement with the
results of the HPLC analysis (Figure 3) which showed that the depletion of free IgG coincided with the accumulation of high molecular weight compounds. The high density components (greater than 19s) which later proved to be immune complexes have also been found in the serum of individuals infected with other diseases such as syphilis (Baughn and Musher, 1983).

As the analysis by sucrose gradient centrifugation is still an indirect analysis of immune complexes, the sucrose fractions were directly analyzed for complexes. Immuno-electrophoresis (IEP) was used to assay the various fractions for immunoglobulin content. The goal of these assays was to demonstrate the presence of IgG in high density (22s) fractions from the serum collected five days after infection, but not in the corresponding fractions from NS. The results of these assays are shown in Figures 11-17.

The overall composition of the NS and the serum collected five days after infection appears to be identical (Figure 11). Based upon known electrophoretic mobility of serum proteins, both samples contain IgG. The HPLC and sucrose gradient centrifugation analysis had suggested that very little free IgG was present in the D5 serum sample (Figures 3, 10). The immunoelectrophoresis analysis shows clearly that the IgG in the day 5 sample is in the high molecular weight fractions. It appears that the complexes dissociated during electrophoresis.
The IEP analysis of sucrose fractions demonstrate the success of the fractionation (Figures 12-15). The 7s and 19s fractions of NS were shown to contain different components (Figure 12) based upon known migration values. The 7s fraction appeared to contain IgG subclasses, while the 19s fraction contained IgM. The two 7s fractions from NS and D5 serum were compared (Figure 13). While both fractions appeared to contain IgG, the NS sample contained an additional subclass. Albumin (4s) was also found in the 7s fractions. This could have occurred due to slight lateral diffusion of the albumin in the sucrose gradient during sample collection as the 4s and 7s fractions were in close proximity in the gradient. The two 19s fractions were shown to contain IgM by using specific antiserum (Figure 14). These IgM precipitation arcs were identical to the arc observed in the 19s fraction from NS (Figure 12). Analysis using specific antiserum showed that the 19s fraction of NS did not contain IgG (Figure 15). Thus, the sucrose gradient centrifugation was shown to fractionate NS into three main characteristic fractions.

The 22s fraction which was found in the D5 serum sample was analyzed for immunoglobulin content (Figures 16 and 17). First, the 22s fraction from D5 serum was shown to yield a single precipitation arc when reacted against anti-whole serum, while the corresponding fraction from NS gave no reaction. This arc was shown to be IgG when reacted
against specific antiserum and compared with an IgG standard (Figure 17). Therefore, the 22s fraction from D5 serum contains IgG, while NS contains IgG only in the usual 7s fraction. This result suggests that the D5 serum contained IgG complexed into a high density configuration. Thus, the HPLC patterns (Figures 1-8) which showed that the free IgG disappeared at the time of the appearance of high molecular weight components is shown to be a result of the complexing of the IgG with malarial antigen to yield high molecular weight (greater than 350 kd) - high density (22s) components. The HPLC fractionation patterns, which show that the disappearance of free IgG occurs at the time of accumulation of high molecular weight components can thus be used as a guide for determining when immune complexes form. The D5 serum sample which contained the highest proportion of high molecular weight components was chosen for further study.

Precipitation with polyethylene glycol-6000 (PEG) was used to isolate immune complexes from D5 serum. PEG has been used to isolate plasmodial immune complexes before, although the final concentration of PEG used in various studies has varied (Brown and Kreier, 1982; Alder and Kreier, 1984; Cox and Hayes, 1985). Therefore, various concentrations of PEG were tested to determine the optimum concentration of PEG for precipitation of complexes with
the highest concentration of high molecular weight components while causing little or no precipitation of free IgG.

We did HPLC analysis of the precipitates from NS and D5 serum at final PEG concentrations of 3-10% (Figures 18-25 and Table 2). The two major fractions precipitated from D5 serum at all PEG concentrations corresponded to high molecular weight components (greater than 350 kd) and albumin (50-70 kd) in mass (Figures 18, 20, 22, 24). As the final concentrations of PEG increased, the proportion of the precipitate of mass greater than 350 kd decreased. Thus, precipitation with a final concentration of 3% PEG yielded the greatest concentration of immune complexes and the smallest amount of free IgG in the protein precipitate from D5 serum. The precipitates from NS showed a different pattern, with the highest concentration of high molecular weight components precipitating at a PEG concentration of 5%. However, the amount of protein precipitated from NS was always less than the amount precipitated at equal PEG concentrations from D5 serum. The greatest difference between precipitates from NS and D5 serum were observed at a PEG concentration of 3%. About four times more protein was precipitated from D5 serum at this PEG concentration than from normal serum, and nearly 20% of this precipitate was in a high molecular weight configuration. Less than 10% of the protein in whole NS is of mass greater than 350kd. Only trace amounts of high molecular weight substances
were precipitated from NS at a PEG concentration of 3%. It was desirable to avoid precipitation of uncomplexed antibody as it could interfere with subsequent immunological assays of the immune complexes. Thus, HPLC analysis suggests that precipitation of D5 serum with a final PEG concentration of 3% optimizes immune complex recovery.

The PEG precipitates were also analyzed by IEP with specific antiserum for the presence of IgG (Figures 26-29). The biggest difference in precipitates between five day and normal serum was again observed at a PEG concentration of 3%. At this PEG concentration, only a faint IgG precipitation arc is noticeable in the NS precipitate. At a PEG concentration of 5%, the IgG precipitation arcs of NS and D5 serum are nearly equal, and at PEG levels of 7% and 10%, there is no noticeable difference in the PEG precipitation arcs. Since earlier IEP analysis of NS sucrose fractions had detected no complexed IgG in NS (Figures 11-17), the conclusion is that high concentrations of PEG precipitate free IgG, in addition to immune complexes and albumin. Thus, IEP analysis of the PEG precipitate also suggests that a final concentration of 3% PEG is optimal for precipitation of complexes, since higher concentrations cause precipitation of free IgG as well.

The relative amounts of immune complexes precipitated from each serum by 3% PEG were determined using radial immunodiffusion (RID). The precipitates, or more likely
the IgG which dissociated from the complexes, were allowed to diffuse through an agarose matrix containing anti-rat IgG antiserum. The standardization of known amounts of IgG versus area of precipitation yielded a straight line with a "y" intercept that was not zero. This result is not unusual since some displacement of the antiserum around the well occurs as the antibody solution diffuses out, so that no precipitation occurs with low concentrations of rat IgG. Analysis of the 3% PEG precipitates showed that the maximal amounts of IgG were precipitated from the D5 (111 µg/ml) and D9 (96 µg/ml) samples. Apparently there was about a 20% loss of IgG during the procedure of precipitation with PEG. This was shown by the IgG content of NS (10 mg/ml) which was more than the combined IgG content of the precipitate plus the supernatant (7.9 mg/ml). However, small amounts of IgG were difficult to detect by this assay as discussed above, so the actual loss could have been smaller (Tables 3 and 4).

There is a potential inaccuracy in assuming that IgG content directly relates to immune complex content in serum samples collected over the course of infection and recovery. The IgG content of immune complexes formed early in the infection could be quite different from the IgG content of immune complexes formed late in the infection. In fact, evidence will be presented to show that immune
complexes formed early in *P. berghei* infection have a lower IgG concentration than immune complexes formed late in the infection. Taking this factor into account, the greatest quantities of serum immune complexes were clearly present early in the infection, at about the fifth day after challenge (D5 serum).

The PEG precipitates from NS and D5 serum formed at various PEG concentrations were also analyzed by polyacrylamide gel electrophoresis (PAGE) as shown in Figure 30. This analysis was useful for direct, visual comparison of the precipitated protein. However, it was not possible to distinguish between polypeptides of host or parasite origin, although certain polypeptides such as albumin (66 kd), IgG heavy (60-70 kd) and light (20-25 kd) chains appear clearly. There was an increase in material precipitated as PEG concentration increased.

The 3% PEG precipitates from serum samples collected during the course of infection were also analyzed (Figure 31). In this analysis, there are qualitative and quantitative differences in the precipitates that are not present in the whole serum samples. The analysis based upon intensity of the bands in the tracks is valid as all precipitates were resuspended to the volume of the original sample and equal volumes of sample were put on each track. The differences are especially apparent in the PEG precipitates from serum collected two and five days after infection.
(Tracks 2 and 4). As infection progressed and the animals recovered, the quantity of material in the PEG precipitates appears to decrease (Tracks 2, 4, 6, 9, 11). This correlates with the overall IgG content of the PEG precipitates as shown by RID (Table 4). The 3% PEG precipitate from D2 serum is somewhat of a paradox in that it has been shown to contain a high concentration of protein and high molecular weight components by HPLC analysis (Figure 2), but was shown to have a low complexed IgG content by RID (Table 4). In the PAGE analysis (Figure 31), the whole serum sample appears quite similar to the D5 sample (Tracks 1, 3), but the PEG precipitates are different in content (Tracks 2, 4). Early in the plasmodial infection, IgM may be the primary immunoglobulin in immune complexes. This would explain the large amount of high molecular weight components and protein content of the PEG precipitate, along with the low amount of IgG in the PEG precipitate. This analysis shows that changes occur in immune complex content as infection progresses. This demonstrates that an intricate immune response occurs, with possibly different antigens emphasized as infection progresses.

The only major polypeptide that appeared in the 3% PEG precipitate from NS (Track 13) was of the same molecular weight as albumin (66 kd). This result corresponds nicely with that of the HPLC analysis of the 3% PEG precipitate from NS (Figure 19).
The antigenic components of the immune complexes were characterized with polyvalent immune serum, and monoclonal antibody probes, and $^{35}$S labeled antigens. As antibody molecules are divalent or polyvalent, and all antigen binding sites on a single antibody molecule are identical, labeled antigen bound by antibody excess complexes will correspond to antigens in the complexes. By the same reasoning, labeled antigen bound by antibody that has bound to antigen excess complexes will also correspond to antigens in the complexes. The goal of these assays was to positively identify \textit{P. berghei} antigens in the isolated immune complexes. Since the immune complexes have been shown to be both immunogenic and suppressive, depending upon the schedule of infusion and challenge, any identified antigens could be targets for future study.

Immune serum and $^{35}$S-labeled \textit{P. berghei} antigen were used to identify the antigens in immune complexes as infection developed (Figure 32). This assay also allowed analysis of the relative antibody-antigen ratios in the PEG precipitates. An explanation of the mechanisms of the assay is helpful. First, immune complexes were precipitated out of duplicate samples of D2 to D26 serum with PEG at a final concentration of 3%. One set of complexes was then reacted with immune serum to provide excess antibody for any open plasmodial antigens in the complexes. This excess of antibody allowed the binding of labeled antigen, which
would not be possible in antigen excess complexes lacking open antibody Fab sites. Protein A-coated latex beads were used to separate these complexes from unreacted immune serum antibody. Thus, any $^{35}$S-labeled *P. berghei* antigens bound by these complexes contained identical epitopes to antigens in the complexes. Similarly, the second set of PEG precipitates were reacted directly with the labeled antigen, which shows only the antigens in the complexes that contain an excess of bound antibody with open Fab sites. Thus, these two assays showed not only the antigens present in the complexes, but also the relative amount of bound antibody with open Fab sites.

As infection progressed, the precipitated complexes bound larger quantities and varieties of labeled *P. berghei* antigen (Figure 32, Tracks 10-16). This shows that as immunity developed, the immune complexes became such that an excess of antibody Fab sites were present. This excess of Fab sites is direct evidence that the complexes contained a larger proportion of antibody as infection progressed. The new polypeptides that appeared late in the infection are indicative of immune complexes with few open Fab antibody sites during the early portion of the infection. Preincubation of the immune complexes with immune serum provided excess specific antibody, and allowed the binding of a greater quantity of labeled *P. berghei* antigen which contained identical epitopes to antigens in the
complexes. This result indicates that most of the complexes contained open antigenic epitopes. These open sites allowed the binding of antibody which in turn bound more labeled antigen. This result is demonstrated by the greater intensity of the tracks containing complexes preincubated with immune serum as compared to the tracks containing complexes not incubated with immune serum (Figure 32, Tracks 1-7, 9-15). However, complexes precipitated from serum collected after recovery did not show an increase in the quantity of antigens bound following preincubation with immune serum (Tracks 8, 16). This result indicates that as the rats recover from parasitemia, the immune complexes are saturated with antibody and contain few open epitopes. This result is consistent with what is considered the normal course of malarial infection and recovery (Zuckerman, et al, 1970). Thus, as the parasitemia ran its course, the immune complexes gradually shifted from antigen excess to antibody excess. The complexes were saturated with antibody after recovery.

An earlier study showed that P. berghei immune complexes mixed directly with immune serum inhibited the passive protection conferred on Swiss mice (Alder and Kreier, 1984). This inhibition was probably due to the binding of immune serum antibody by the complexes, which were collected from infected animals just before recovery.
As a probe for identifying specific immune complex antigens, monoclonal antibodies were used. Since all of these monoclonals were of the IgM type, HPLC fractionation was performed on the ascites fluid raised in mice to determine the relative IgM content of the fluid. The peak that corresponded to IgM ranged from a minority component (B10, E6) to the majority component (H8) of the fluid (Figures 33-37). This is not a completely accurate indicator of IgM content as other high MW components (greater than 350 kd) could also be present in the peak. No further purification of ascites components was attempted. In general, the reactivity of the monoclonals was not directly related to the size of the IgM peak. Therefore, the reactions obtained with the ascites fluid were judged to be specific antigen-antibody interactions rather than nonspecific adsorptions.

The BJ monoclonal was actually raised against *P. falciparum* blood stage parasite antigen and only later proved to react with *P. berghei*. Earlier studies have shown that *P. berghei* and *P. falciparum* share antigenic determinants (Stein and Desowitz, 1984). The BJ monoclonal did not inhibit the growth of *P. falciparum* *in vitro* (Table 5). This result is not surprising since there is no evidence that rodent and human malarias share protective antigens (Strickland and Nussenzweig, 1982).
Of the five monoclonal antibodies tested, only the H8 and BJ clones reacted against *P. berghei* infected erythrocytes as detected by fluorescent antibody (Figures 38-43). Both of these antibodies reacted in ELISA and immunoblot tests against *P. berghei* antigen from infected red blood cell lysate (Figures 44 and 45). Most importantly, these two monoclonals also reacted with *P. berghei* immune complex antigens isolated by PEG precipitation (Figures 44 and 45). It is unlikely that the reactions observed in the immunoblots are due to the anti-mouse immunoglobulin system detecting rat immunoglobulin in the complexes. The reaction occurred only in the tracks that contained parasite material. Furthermore, the precipitated polypeptides at 17, 31 and 112 kd do not correspond to immunoglobulin heavy and light chains.

Monoclonal H8 reacted against *P. berghei* polypeptides of 31 and 112 kd (Figure 44). The 112 kd polypeptide was not apparent on PAGE gels of *P. berghei*-infected red cell antigens, and is apparently a minority component in parasite material. This antibody produced an immunofluorescence pattern that roughly outlined the infected red cell (Figure 40). Thus, it appears likely that the antibody is reacting to foreign epitopes on the surface of an infected red cell. None of the internal parasite material is visible. The 31 kd polypeptide may be a cleavage product of the 112 kd polypeptide. This determinant is expressed more
or less evenly on the surface of the red cell. During acute malaria, the immune response to infected red cell antigen may be due to proteolytic modification of the erythrocyte membrane by the parasite (Kreier, et al, 1966). This condition may naturally lead to erythrophagocytosis, causing clearance of the parasitemia (Zuckerman, et al, 1969).

Monoclonal BJ reacted against a P. berghei polypeptide of 17 kd (Figure 45). This monoclonal produced an immunofluorescence pattern that was patchy, with some sections of the infected red cell clearly fluorescent, while other parts remained dark (Figures 41 and 42). Furthermore, some of the internal parasite structure is clearly visible. This very light polypeptide is apparently expressed on the surface of the parasite, and on portions of the infected red cell. This polypeptide may be a cleavage product of a larger polypeptide that is completely reduced under PAGE conditions, or it may be actively shed by the parasite, to lodge in the red cell membrane.

Rat hyperimmune serum appeared to react with normal as well as infected erythrocytes (Figure 38). Bound immunoglobulin is commonly found on red cells during acute malarial infection (Packer and Kreier, 1986; Lubicic, et al, 1977). As parasitemia develops, the erythrocytes acquire a coat of immunoglobulin from the surrounding serum (JeJe, 1983). The rabbit anti-rat IgG fluorescence conjugate did
not react with the infected or normal red cells incubated with normal serum (Figure 40) so the observed reaction is apparently due to the binding of serum antibody to the red blood cells.

Thus, two different monoclonals were produced that reacted to different immune complex antigens. These antigens appeared to be in different locations on infected red cells and probably play different roles in the development of the parasite. Their role, if any, in the observed immunosuppression caused by malarial immune complexes is undetermined. The assays do serve to show some of the diversity of antigenic material in malarial immune complexes.

The biological activity of D5 serum and immune complexes was studied next. The effect of immune complexes upon response to challenge and to vaccination was assayed. The immune response was evaluated by several criteria including response to SRBC as measured by the plaque and hemolysis assays, parasitemia following challenge, and ELISA titer.

The effect of D5 serum upon the immune response to SRBC was investigated. Whole SRBC have frequently been used as the target antigen in various immunosuppressive assays since the immune response is rapid and easy to evaluate. Infusions of D5 serum given with a SRBC vaccine did not interfere with the immune response as measured by the
hemolysis titer of serum collected five days after challenge (Table 6). However, the number of plaque forming units (PFU) produced by spleen cells from rats immunized with SRBC was reduced by over 50% by either giving the vaccine during infusions of D5 serum, or by preincubating the spleen cells in D5 serum before the plaque assays. The PFU response of normal spleen cells was also reduced by preincubation with D5 serum (Table 7).

The results of the hemolysis and plaque assays conflict. While D5 serum infusions given with the SRBC vaccine inhibited the PFU response of spleen cells from rats immunized with SRBC, there was no apparent inhibition of the hemolysis response by serum collected from rats treated in an identical manner. Since these two assays essentially measure the same phenomenon, the differing conditions of the assays must be responsible for the conflicting results. The plaque assay measures in vitro antibody production, while the hemolysis assay measures in vivo antibody production. Inhibition of the PFU response has been associated with malarial infection and plasmodial immune complexes (Cox, et al, 1983; Wedderburn, 1974). It has been postulated that immune complexes inhibit macrophage processing of antigens by blocking Fc receptors (Packer and Kreier, 1986).

The decrease in the PFU response of rats immunized with SRBC during D5 serum infusions would seem to indicate a
macrophage processing defect. This would cause a decrease in the number of B cell clones specific for SRBC, yielding a decrease in PFU. The D5 serum also caused a direct inhibition of antibody production following incubation with spleen cells from a SRBC-sensitized rat. Thus, the immune complexes apparently inhibit the response to antigen both by limiting the processing of antigen, leading to fewer specific B cell clones, and by inhibiting the production of antibody by sensitized B cells.

Doses of D5 serum given at the time of challenge were shown to inhibit the immune response to *P. berghei* challenge (Figure 46). Doses of immune serum (IS) were shown to cause the reduction of parasitemia following challenge by about 50%. A similar trial (Figure 49) showed that the suppressive fraction of D5 serum was contained in the 3% PEG precipitate, but not in the supernatant. This result indicates that immune complexes are responsible for the immunosuppressive effect of the serum.

The injection of D5 serum into rats undergoing challenge was shown to have a negative effect on the anti- *P. berghei* ELISA titer (Figure 48). Infusions of D5 serum ultimately caused a drop in titer and an increase in parasitemia, even if given ten days after challenge. This result is somewhat surprising since rats undergoing an infection ten days after challenge are already exposed to
considerable levels of plasmodial immune complexes as shown earlier.

A unique system was devised for reporting ELISA titers in this assay. The absorbance reading at the 50% endpoint for hyperimmune serum was taken as a standard (Figure 47). Then, the reciprocal of the dilution of the serum sample that gave these absorbance readings were reported as the titer. This system was used since the different sera had quite different maximal absorbance readings. For example, the 50% endpoint titer of normal serum is quite similar to the 50% endpoint titer of hyperimmune serum, although the absorbance readings of the two sera at that dilution (1/700) are quite different (Figure 47).

The infusion of additional immune complexes on day 10, which is about four to seven days before peak parasitemia is reached, was sufficient to influence the resulting parasitemia. The immune response leading to recovery in these animals is apparently in a delicate balance with the growth of the parasites. The infusion of additional immune complexes at a critical period of the infection had dramatic results in this assay.

As these conclusions were based upon observation of the parasitemia following challenge, no mechanism for the suppression was suggested. It was postulated that immune complexes from D5 serum may stimulate T-suppressor cell activity, causing an inhibition of the immune response. To
test this concept, the immunosuppressive agent cyclophosphamide (CP) was used in an attempt to inhibit T-suppressor cell activity. The idea was to stimulate T-suppressor cell activity with doses of D5 serum and to simultaneously inhibit the proliferating cells with doses of CP. The drug CP has been reported to specifically inhibit T-suppressor cell activity (North, 1982; Askenase, et al, 1975), although its activity is not entirely clear. Preliminary trials showed that the reported effective dose of 200 mg/kg body weight was lethal to rats if maintained over the three-day period required for the infusions of D5 serum (data not shown). Other trials showed that smaller amounts of CP (60 mg/kg), which were not lethal to the rats, inhibited *P. berghei* growth if given with the challenge dose (data not shown). Thus, the procedure used was three injections of D5 serum at six, five and four days before challenge given with injections of CP at 60 mg/kg body weight (Figure 50). This procedure, while not exactly the same as that used in the earlier inhibition trials utilizing infusions of D5 serum, was required so that CP could be cleared from the bloodstream by the time of challenge. The results of this assay showed that under these conditions CP did not inhibit *P. berghei* infection, and that doses of D5 serum did inhibit the response to challenge although not to the degree of earlier trials (Figures 46 and 49). The reduced effect of D5 serum was probably due to the time
delay between administration of the serum and the injection of the challenge dose. Most importantly, CP did not have any effect on the inhibition caused by D5 serum. This result argues against the involvement of T-suppressor cells in inhibition induced by D5 serum. However, this conclusion is debatable as the dosage used (60 mg/kg) was below the dosage used by others (200 mg/kg), but is still of therapeutic value (Physicians Desk Reference, 1985).

The effect of single and multiple doses of D5 serum given two weeks before challenge was studied (Figure 51). The rationale behind this trial was to study the effect of immune complexes upon the response to vaccines. Two different vaccines were used; one was given in a single dose two weeks before challenge, while the other vaccine was given in three weekly doses, the last two weeks before challenge. In order to assess the effect of D5 serum upon response to vaccination, D5 serum was first given alone to rats on the same schedule as the single or multiple dose vaccine injections. Single doses of D5 serum, NS or IS given two weeks before challenge had no effect on the response to challenge. This result is probably due to the short half-life of these injected substances in the serum. Surprisingly, the three weekly doses of D5 serum, the last given two weeks before challenge, proved protective to the rats. This protection is almost certainly due to complexed plasmodial antigens in the D5 serum which stimulated a
protective response over time. The defense of the parasite may be based upon a "smoke screen" of soluble antigens and immune complexes that blocks Fc receptors on macrophages and prevents phagocytosis (Wilson, 1974). This smoke screen defense is overcome with time as macrophages ingest immune complexes and become activated (Packer and Kreier, 1986). Residual antibodies in the D5 serum are an unlikely source of protection since whole immune serum given two weeks before challenge did not protect. Thus, D5 serum both inhibits and stimulates the immune response, depending upon the dosage schedule. The inhibition is a short-term effect as the single dose of D5 serum had to be given close to the time of challenge to suppress the immune response. The induced immunity is apparently a long-term effect stimulated over a four-week period by multiple doses of D5 serum.

A single dose of $1 \times 10^8$ free P. berghei merozoites in saponin given two weeks before challenge induced a protective immune response in the rats. The vaccinated rats suffered an average parasitemia that was 50% less than the non-vaccinated rats (Figure 52). The protective effect of this vaccine was abrogated by giving the vaccine at the same time as infusions of D5 serum were given (Figure 53). The results suggest that the response to the vaccine was inhibited by D5 serum, but the response to challenge two weeks later was unaffected. This is in agreement with
earlier data which suggested that the D5 serum has a short-term inhibitory effect. Since the vaccine functions by stimulating humoral immunity, the immune complexes could be acting to inhibit B cell function as well as macrophage function. Either of these factors would render the vaccine less effective. Plasmodial immune complexes have been shown to inhibit humoral responses to other antigens such as SRBC (Wedderburn, 1974; Lelchuk and Playfield, 1980).

Giving the single free parasite vaccine along with the challenge injection resulted in the loss of the protection induced by the vaccine. While the group that received the vaccine along with the challenge had slightly higher parasitemia than the control group, this difference was not significant. Therefore, it cannot be suggested that this vaccine caused immunosuppression in an infected animal; instead the vaccine was simply rendered useless (Figure 54).

The protection offered by multiple doses of formalin-fixed infected red blood cell vaccine (IRBC) was shown to be directly related to the number of doses of vaccine (Figure 55). While five doses of IRBC yielded the greatest protection, the three-dose vaccine was studied further since this gave protection equivalent to the single dose of free parasites. The protection yielded by the three doses of formalin-fixed IRBC was not inhibited by giving infusions of D5 serum along with the three vaccine injections.
(Figure 56). This result is not surprising in that D5 serum alone injected on this schedule induced a protective immune response (Figure 51). On this schedule, the D5 serum probably acted as a vaccine in its own right, and the immune system simply responded to the additional plasmodial antigens.

Giving the challenge injection along with the third vaccine injection of IRBC resulted in abrogation of the protection offered by the vaccine (Figure 57). Again, there was no evidence of immunosuppression. Instead, the response of the vaccinated rats was similar to that of the non-vaccinated rats. Thus, while the multiple dose IRBC vaccine was not susceptible to suppression caused by simultaneous administration of immune complexes, it does require a longer period to generate immunity than does the single dose of free merozoites.

Summary.

The highest concentration of immune complexes in serum was shown to occur early in P. berghei infection, when the parasitemia is low. This result suggests that the concentration of immune complexes is primarily determined by the rate of clearance, which is low early in the infection, rather than being determined by parasitemia. Immune complexes prevented the induction of immunity by a single dose of vaccine. Since large amounts of complexes may be
present during a low parasitemia, pre-vaccination screening should allow for detection of the subclinical infections frequently characterized by low parasitemia in infected individuals from endemic areas. *Plasmodium berghei* immune complexes also contain protective antigen, and can induce immunity with repeated exposures over time. Thus, the *P. berghei* immune complexes were shown to be immunosuppressive in the short term and immunogenic in the long term. *Plasmodial* immune complexes suppress the immune response in a variety of ways, including inhibition of production of specific B cell clones, the suppression of antibody production by differentiated B cells, and direct binding of antibody. As infection progressed, the complexes contained a greater proportion of antibodies, which saturated the antigenic determinants after recovery. Finally, the diversity of immune complex antigens was demonstrated. This result suggests that immune complexes may stimulate a variety of responses in the host, some of which are contradictory. The immune system responds in a variety of ways to malarial infection, and immune complexes have an important or even decisive role in the outcome of the disease.


