

PASSIVE TRANSFER OF GROWTH-INHIBITORY ANTIBODIES RAISED AGAINST YEAST-EXPRESSED RECOMBINANT *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-1₁₉

ALFONSO GOZALO, CARMEN LUCAS, MARLENE CACHAY, BRUCE T. WELLDE, TED HALL, BRIAN BELL, JAY WOOD, DOUGLAS WATTS, MARK WOOSTER, JEFFREY A. LYON, J. KATHLEEN MOCH, J. DAVID HAYNES, JOSEPH S. WILLIAMS, CAROLYN HOLLAND, EUGENE WATSON, KENT E. KESTER, DAVID C. KASLOW, AND W. RIPLEY BALLOU

U.S. Naval Medical Research Institute Detachment, Lima, Peru; Department of Immunology, and Department of Biologics Research, Walter Reed Army Institute of Research, Washington, District of Columbia; Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland

Abstract. Purified rabbit immunoglobulin raised against yeast-expressed recombinant FVO or 3D7 *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) 19k-D C terminal fragment (MSP-1₁₉) was transfused into malaria-naive *Aotus nancymai* monkeys that were immediately challenged with FVO asexual stage malaria parasites. Control monkeys received rabbit immunoglobulin raised against the sexual stage antigen Pfs25 or *Aotus* hyperimmune serum obtained from monkeys immunized by *P. falciparum* infection and drug cure. Passive transfer of rabbit anti-MSP-1₁₉ failed to protect against homologous or heterologous challenge and, when compared with negative controls, there were no differences in prepatent periods or time to treatment. Interestingly, rabbit anti-MSP-1₁₉, but not anti-Pfs25, immunoglobulin, and immune monkey serum prevented the development of antibodies directed against MSP-1₁₉ fragment by infected monkeys, indicating that the antibodies were reactive with native MSP-1₁₉ antigen *in vivo*. The prepatent period and time to treatment was greatly delayed in the two monkeys that received *Aotus* immune serum, both of which developed a chronic intermittent low level infection. *In vitro* parasite growth inhibition assays (GIAs) confirmed the presence of inhibitory activity (40% maximum inhibition) in concentrated anti-MSP-1₁₉ immunoglobulin (4.8 mg/ml), but the peak concentrations we achieved *in vivo* (1 mg/ml) were not inhibitory *in vitro*. Subinhibitory levels of anti-MSP-1₁₉ antibodies achieved by passive transfer were not protective against *P. falciparum* challenge.

The merozoite surface protein-1 (MSP-1), a 200-kD surface-associated antigen, is expressed during schizogony of all species of *Plasmodium* as a precursor protein that undergoes processing and cleavage during merozoite invasion. A C-terminal 19-kD fragment (MSP-1₁₉) remains bound to the merozoite as it invades red blood cells (RBCs).¹ Several lines of evidence suggest that antibodies directed against MSP-1₁₉ can block merozoite invasion of RBCs. The antigen has been extensively modeled in the murine *P. yoelli* system, where active immunization confers partial to complete protection against asexual stage challenge.^{2,3} Murine monoclonal antibodies against the antigen passively protect against challenge in mice and inhibit invasion of RBCs *in vitro*.⁴ The amino acid sequence of MSP-1₁₉ is highly conserved along most of its length, with four major allelic types defined on the basis of non-synonymous substitutions at a few key base pairs, three of which appear to occur in nature (Table 1).⁵ Because a limited number of *in vitro* culture-adapted strains of *P. falciparum* are infectious to *Aotus* monkeys, preclinical studies have been designed around the MSP-1₁₉ sequences for the FVO and 3D7 alleles, representing the extremes of sequence diversity for this fragment (Q-KNG versus E-TSR). Recombinant MSP-1₁₉ expressed in yeast as a fusion protein with P30 and P2, tetanus toxoid universal T cell epitopes, have been shown to protect *Aotus nancymai* against homologous challenge when administered with Freund's complete adjuvant.⁶ Because protection seemed to be independent of antibody responses, and Freund's adjuvant is known to stimulate both strong cellular immune responses and antibodies, we sought to further characterize the relative contribution of antibody alone to protection in this model.

MATERIALS AND METHODS

Parasites. *In vitro*-adapted cultures of the *P. falciparum* strains CAMP, 3D7, and FVO were used in these studies. Mefloquine-sensitive FVO strain asexual stage parasites were used for challenge studies because a standard inoculum induces highly reproducible patent infections in *Aotus* monkeys.⁷ The FVO, 3D7, and CAMP parasites were used in growth inhibition assays as described below.

Primates. Captive-born *A. nancymai* monkeys for challenge studies were obtained from the Peruvian Primate Center in Iquitos, Peru and quarantined and acclimated at the U.S. Naval Medical Research Institute Detachment for 60 days prior to initiating the study. Ketamine (10 mg/kg) given intramuscularly was used to chemically immobilize the monkeys each time they were removed from their cages. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

Production of hyperimmune serum and immunoglobulin preparations. The three immunogens (FVO MSP-1₁₉, 3D7 MSP-1₁₉, and Pfs-25) were expressed in yeast as previously described and purified by affinity nickel chromatography.⁶ The antigens were individually emulsified in Freund's complete adjuvant and used to immunize adult NZW rabbits (two per antigen). Antigen-specific sera were pooled and immunoglobulin preparations were obtained by treatment with caprylic acid and subsequently by precipitation with ammonium sulfate.⁸ The immunoglobulin solution was dialyzed against isotonic saline, filtered through a 0.2- μ m filter, and stored at -70°C . Products were sterile, did not

TABLE 1

Amino acid substitutions among three merozoite surface protein-1 antigens*

Pf. strain	Amino acid position			
	1644	1691	1700	1701
Camp	E	K	N	G
FVO	Q	K	N	G
3D7	E	T	S	R

* Pf. = *Plasmodium falciparum*; E = glutamic acid; K = lysine; N = asparagine; G = glycine; Q = glutamine; T = threonine; S = serine; R = arginine.

agglutinate or lyse *Aotus* RBCs *in vitro* and were nontoxic when injected intravenously into mice.

The FVO hyperimmune *Aotus* sera obtained from two monkeys that had been infected with the FVO strain of *P. falciparum*, drug cured, rechallenged, and allowed to resolve their infections without further treatment. Sera from five bleedings were pooled, filtered, and stored at -70°C . The monkeys had not received mefloquine for at least six months prior to the time that sera were obtained. These reagents were tested for reactivity against *P. falciparum*-infected human erythrocytes by immunofluorescence and ELISA.

Preparation of *P. falciparum* inoculum for challenge. A donor monkey was inoculated intravenously via a saphenous vein with 2×10^8 *P. falciparum* (FVO) parasitized RBCs (pRBCs) erythrocytes recovered from a stabilate preserved in liquid nitrogen. Parasitemia and hematocrit were monitored daily until 1% of the monkey's erythrocytes were infected. Two milliliters of peripheral blood were obtained to prepare a parasite inoculum. The hematocrit was measured and the percent parasitemia was determined using Giemsa-stained thin blood smears. The donor blood was diluted in RPMI 1640 medium containing 5% normal *Aotus* monkey serum to yield an inoculum containing 1×10^4 *P. falciparum*-infected RBCs per 0.5 ml. The inoculum was kept on ice until injected into the recipient monkeys. The donor monkey was then cured with mefloquine (20 mg orally).

Baseline and postchallenge sampling. Two preimmunization blood samples (2 ml) were drawn from the monkey's femoral vein during the two-month quarantine and acclimation period. The blood was used for hematology and serum collected was stored at -20°C . Beginning on day 4 after challenge with *P. falciparum*, blood samples (0.05 ml) were obtained daily from a saphenous vein for preparation of Giemsa-stained thick and thin blood smears and for microhematocrit determinations. Blood (0.5 ml) was drawn from the femoral vein one and four days postchallenge, and weekly thereafter, for serum collection and hematology. Monkeys were monitored daily for 30 days followed by three times per week for days 31-277.

Passive transfer and challenge. Monkeys were randomly assigned to four groups of two animals each and bled for 2.0 ml of blood just prior to infusion of rabbit immunoglobulin or monkey serum. Group I received rabbit anti-FVO MSP-1₁₉ immunoglobulin, Group II received rabbit anti-3D7 MSP-1₁₉ immunoglobulin, Group III received rabbit anti-Pfs25 immunoglobulin, and Group IV received *Aotus* anti-FVO serum. Monkeys were injected over a 1-min period via the saphenous vein with 2.5 ml of immune *Aotus* serum or 30 mg of experimental rabbit immunoglobulin contained in

a volume of 2.5 ml. Thirty milligrams of immunoglobulin infused into a 1-kg monkey would result in approximately 1 mg/ml of immunoglobulin/ml of plasma, assuming 6% of the body weight to be blood and 50% of that being plasma. Two minutes after the infusion was completed, monkeys were challenged via the contralateral vein with 1×10^4 *P. falciparum* (FVO)-infected erythrocytes obtained from the donor monkey. Beginning four days after challenge, the monkeys were monitored daily for parasitemia and hematocrit.

Detection of parasitemia. Giemsa-stained thick smears were examined microscopically under oil immersion (200 fields) to detect parasites. Low parasitemias were estimated by assuming that 200 fields of a thick blood smear contained approximately 0.2 μl of blood. Subsequently, when parasitemias increased, the percentage of parasitized pRBCs on thin blood smears was calculated directly from the smear.

Serology. A standard ELISA was modified for use with recombinant MSP-1₁₉ (FVO) or Pfs25 as capture antigens. Affinity-purified, peroxidase-labeled goat anti-rabbit (heavy plus light chain) gamma globulin (Bio-Rad, Hercules, CA) and affinity-purified rabbit anti-*Aotus* monkey (heavy plus light chain) gamma globulin (Kirkegaard & Perry, Gaithersburg, MD) were used to detect and distinguish rabbit and monkey antibodies. Triplicate assays were averaged for each serum sample and a single positive and negative control serum on each plate were used to control variability.

***In vitro* growth inhibition.** Growth inhibition assays (GIAs) were performed as described previously, except that the sera and antibodies were dialyzed three times using SpectraPor #7 (50,000 molecular weight cut-off value; Spectrum Medical Industries, Los Angeles, CA) against 250 volumes of RPMI 1640 medium without bicarbonate (bicarbonate was added immediately before use in culture), and the cultures were prepared in static 96-well plates.⁹ Calculated final concentrations of test sera of immunoglobulin took into account the dilution that occurred due to adsorbing twice with erythrocytes.

Briefly, all microwell cultures contained 100 μl of normal culture media containing 10% heat-inactivated normal pooled human sera, 1.5% human A+ erythrocytes approximately 0.5% of which contained mature schizonts, and added immunoglobulin or sera. After invasion overnight, the media was replaced with 10% normal human serum-RPMI 1640 medium in the morning. Slides were made 24 hr later so that the resulting mature trophozoites and early schizonts could be more easily read microscopically than could the rings immediately after invasion. There were rarely more than 0.06% ring forms in the slide made at the initiation of culture, but the percentage of initial rings were subtracted from the final parasitemias, so that the formula for % inhibition was reduced to:

$$\% \text{ inhibition} = \frac{100 \times (\text{final control \%} - \text{final test \%})}{(\text{final control \%} - \text{initial \% rings})}$$

Growth inhibitory activities less than 15% were considered negative in this assay.

Antimalarial treatment. Infected monkeys were treated with mefloquine (20 mg orally) when the percentage of parasitized erythrocytes reached levels of 3-5% or if the hematocrit decreased to 50% of preinfection values.

TABLE 2

Development of *Plasmodium falciparum* (FVO) in *Aotus nancymai* passively immunized with rabbit anti-merozoite surface protein-1 (MSP-1) immunoglobulin

Monkey	Group*	1st day of parasitemia	Days to parasitemia >100,000/ μ l	Day of treatment	Parasitemia	
					%	Total/ μ l
471	I	5	14	14	4.2	242,228
547	I	5	11	12	5.9	389,536
550	II	4	11	11	4.2	292,600
579	II	5	10	10	4.0	255,024
481	III	4	11	12	14.2	991,200
489	III	5	11	11	4.6	360,640
477	IV	14	>277	>277	0.54	36,288†
496	IV	18	43	43	2.10	108,780

* Group I = rabbit anti-FVO MSP-1₁₉ (homologous); Group II = rabbit anti-3D7 MSP-1₁₉ (heterologous); Group III = rabbit anti-PfS25 (negative control); Group IV = *Aotus* anti-FVO strain (positive control).
 † Peak parasitemia (never treated).

RESULTS

All eight monkeys tolerated the infusion of rabbit immunoglobulin or monkey antiserum, developed patent parasitemia (Table 2) and recovered from infection with *P. falciparum* (FVO) without ill effects. Seven monkeys required mefloquine treatment while one positive control monkey self-cured the infection.

Recipients of MSP-1₁₉ hyperimmune immunoglobulin (Groups I and II). Two monkeys received rabbit anti-MSP-1₁₉ (FVO) immunoglobulin and two received rabbit anti-MSP-1₁₉ (3D7) immunoglobulin (Group II). Rabbit antibodies reactive with MSP-1₁₉ were detected by ELISA and were highest one and four days after transfer, gradually decreasing to undetectable levels over the next 48–104 days.

There appeared to be no difference in clearance times between FVO and 3D7 antibodies. Following challenge, infections in these monkeys were similar to those of control monkeys. The prepatent periods ranged from four and five days in all four monkeys, and all developed rapidly increasing parasitemias that required treatment between days 10 and 14. Data for monkey #547 are illustrative of the experimental groups and are depicted in Figure 1.

Recipients of negative control Pfs25 hyperimmune immunoglobulin (Group III). The infusion of rabbit anti-Pfs25 immunoglobulin resulted in high titers of rabbit antibody reactive with Pfs-25 that were detectable in the monkey's serum at days one and four postinfusion, and that gradually decreased until they were undetectable after 85–97 days. Following challenge, the prepatent periods were four or five days and parasitemia subsequently increased rapidly. The two monkeys were treated on days 11 and 12 postinfection when parasitemias had increased to high levels, which is typical for infections with the FVO strain. The course of parasitemia and antibody titers for this group are represented by the data for monkey #489 in Figure 2.

Recipients of positive control FVO hyperimmune serum (Group IV). *Aotus* antibodies against MSP-1₁₉ (FVO) were detectable at low levels on days 1 and 4 and persisted at decreasing levels for 27–34 days. Following challenge, prepatent periods were markedly prolonged and both monkeys initially developed persistent but low level parasitemias. Monkey #496 had a prepatent period of 18 days followed by a low level parasitemia (≤ 585 pRBCs/ μ l) until day 40, when parasitemia levels began to increase to 108,780 pRBCs/ μ l by day 43 when the monkey was treated. The monkey's hematocrit had decreased from a preinfection level

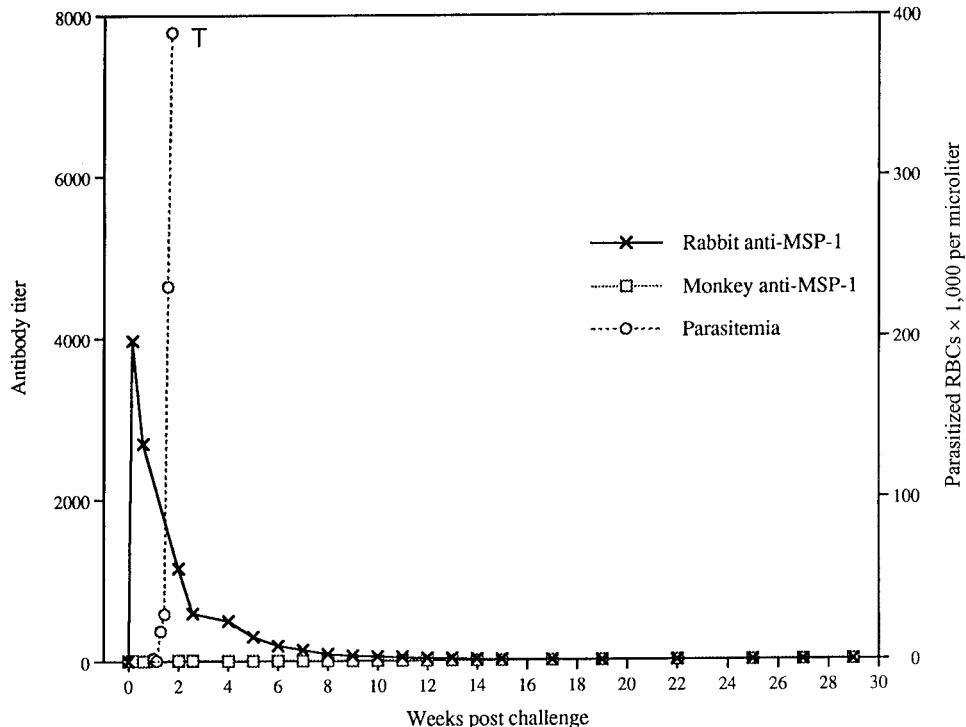


FIGURE 1. Parasitemia and antibody levels in experimental monkey #547 (group 1). T = day of treatment; MSP-1 = merozoite surface protein-1; RBCs = red blood cells.

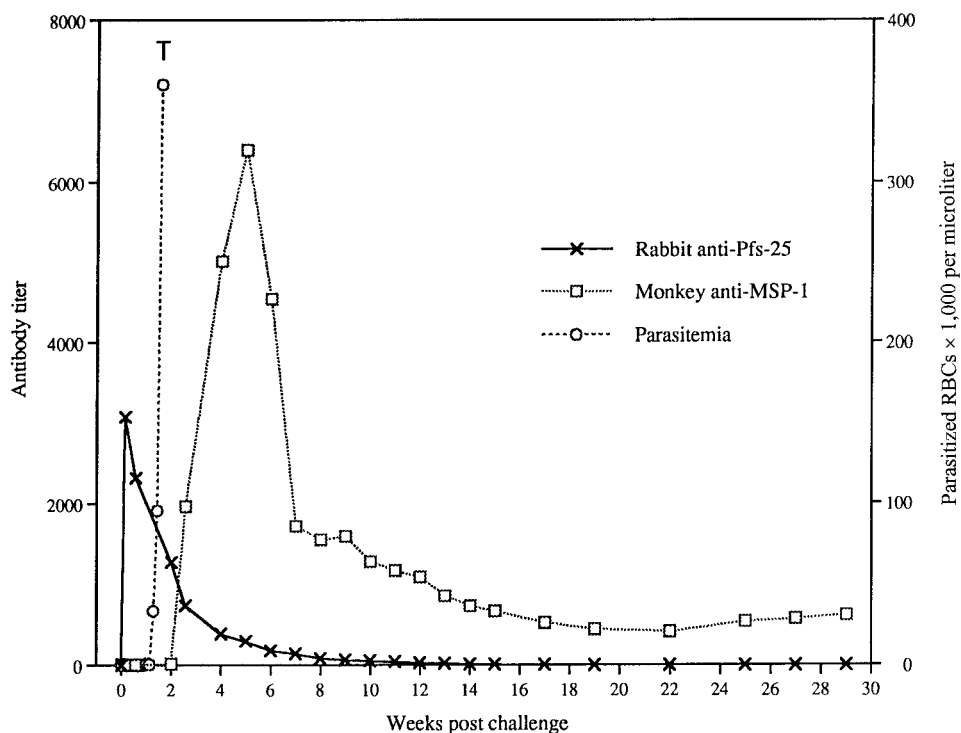


FIGURE 2. Parasitemia and antibody levels in negative control monkey #489 (group 3). T = day of treatment; MSP-1 = merozoite surface protein-1; RBCs = red blood cells.

of 52.5% to 37% at the time of treatment. Although this monkey did not reach the requirements for therapy contained in the Materials and Methods, the monkey had become anorexic and was treated out of compassion. Monkey #477 also had a long prepatent period (14 days) and a low level parasitemia that peaked on day 21 (36,288 pRBCs/ μ l). The peak parasitemia subsided and until day 133 the daily parasitemia fluctuated between five and 13,000 pRBCs/ μ l. Blood smears were negative from day 134 until day 155 when parasites were again found at low levels. Parasitemia was present intermittently until day 277, after which time parasites were no longer detectable.

Development of anti-MSP-1₁₉ responses following infection. The development of autologous antibody responses to MSP-1₁₉ following challenge differed significantly among the four groups. Although the prepatent periods and the duration and level of infection before treatment was similar in Groups I–III, three of the four monkeys receiving rabbit anti-MSP-1₁₉ immunoglobulin (Groups I and II) did not develop an antibody response to MSP-1₁₉, despite experiencing significant parasitemias. The fourth monkey (#471) developed a low titer to MSP-1₁₉ on day 55 that peaked at a titer of 153 (day 154) and had decreased to 118 on day 205.

In contrast, the monkeys in Group III that received anti-Pfs25 and developed a brisk anti-MSP-1₁₉ response which was first detected on days 14–18 and was characterized by a rapidly increasing titer that peaked on day 127 and returned to a relatively low titer for the duration of the experiment (Figure 2). Both monkeys in Group IV developed detectable antibodies to MSP-1₁₉. Monkey #477 (Figure 3), which eventually self-cured the infection, displayed a rapidly

increasing antibody titer that peaked at 7,800 and remained positive at a high titer throughout the experiment. Monkey #496 was treated on day 40 when the antibody titer was 187. One week later, the titer peaked at 1,244 and then decreased gradually to a titer of 60 at the end of the experimental period.

***In vitro* growth inhibition.** Rabbit anti-MSP-1₁₉ immunoglobulin was tested for *in vitro* growth inhibition over a wide range of immunoglobulin concentrations (Table 3). The growth of Camp and 3D7 strains was inhibited from 44% to 46% by maximal concentrations (6.4 mg/ml) of anti-3D7 MSP-1₁₉ immunoglobulin. Inhibitory activity was lost when the concentration of anti-3D7 MSP-1₁₉ immunoglobulin was decreased to 2 mg/ml. Inhibition of FVO parasites was difficult to interpret because significant nonspecific inhibition occurred with the negative control Pfs25 immunoglobulin. As additional positive and negative controls for the GIAs, and in agreement with previous strain-specific GIAs, we have performed, *Aotus* serum from FVO-infected and drug-cured monkeys inhibited the homologous, but not the two heterologous strains.

To demonstrate the specificity of the 3D7 inhibition, increasing amounts of recombinant 3D7 MSP-1₁₉ antigen were added to the *in vitro* cultures. Growth was inhibited between 34% and 38% by 4.8 mg/ml of anti-3D7 MSP-1₁₉ immunoglobulin in the absence of exogenous antigen, but the inhibition was completely reversed by the smallest dose of antigen tested (0.01 mg/ml, Table 4). Thus, while invasion inhibition by the immunoglobulin was antigen-specific, very little of the immunoglobulin may have been of the desired specificity.

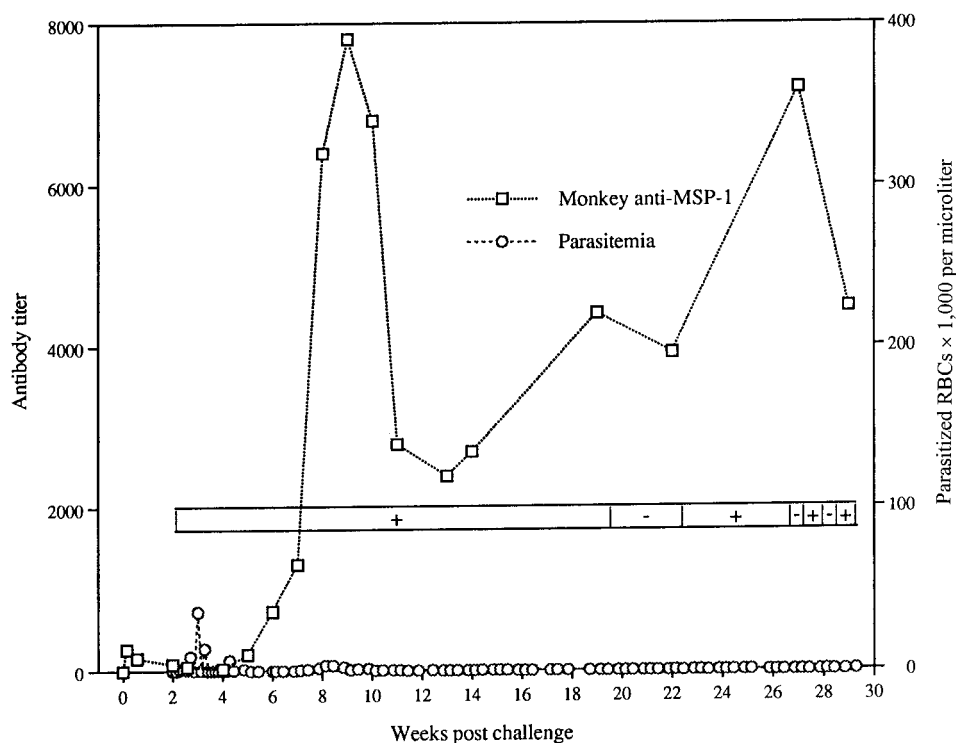


FIGURE 3. Parasitemia and antibody levels in positive control monkey #477 (group 4). Positive and negative symbols indicate periods of microscopically detectable and undetectable parasitemia. MSP-1 = merozoite surface protein-1; RBCs = red blood cells.

DISCUSSION

While infection followed by drug cure or immunization with irradiated parasitized erythrocytes induce resistance to reinfection without the use of adjuvants, most other asexual stage malaria vaccines (including MSP-1) have required Freund's adjuvant to induce protection in *Aotus* monkeys.¹⁰⁻¹⁵ It was therefore important to determine the relative contribution of the antibodies induced by MSP-1₁₉ in limiting infection. This is a key question because adjuvants that induce strong cellular responses are potentially more toxic than those, such as aluminum hydroxide, that principally induce antibody responses.

This study was designed to assess the role of antibodies raised against recombinant MSP-1₁₉ in *A. nancymai*, which can be reliably infected with *P. falciparum* asexual stage parasites. Two rabbit hyperimmune immunoglobulins (het-

erologous or homologous) administered as a single dose were ineffective in controlling parasitemia. *In vitro* characterization of these immunoglobulins showed that they had inhibitory activity, albeit at concentrations that were unachievable *in vivo*. Although the activity of these antibodies might have been enhanced through cellular mechanisms such as ADCC, the rabbit immunoglobulin may have had limited ability to bind to *Aotus* Fc receptors, thereby minimizing this potentially important functional activity. We used *Aotus* immune serum from monkeys that had been infected and drug cured as a positive control. Although hyperimmune serum from monkeys apparently completely resistant to challenge had a marked effect, it did not eliminate parasitemia in recipient monkeys.¹⁶ It appeared that parasitemia that could not be controlled eventually developed in one monkey. However, the second monkey was able to suppress parasitemia to low levels and eventually self-cure.

TABLE 3
Results of *in vitro* growth inhibition assays

Reagent*	mg/ml or v/v% added	% Inhibition		
		Camp (1.2%)†	FVO (3.2%, 3.7%)	3D7 (5.5%)
Rabbit α-MSP-1 (3D7) Ig	6.4	44%	36%, 50%	46%
Rabbit α-MSP-1 (FVO) Ig	4.8	4%	21%, 53%	19%
Controls				
Rabbit anti-Pfs25 Ig	6.0	-5%	29%	-10%
Human serum (normal)	10%	-6%	11%	7%
<i>Aotus</i> serum (normal)	8%	8%	4%	-5%
<i>Aotus</i> serum (anti-FVO)	8%	9%	38%	-9%

* MSP-1 = merozoite surface protein-1; Ig = immunoglobulin.

† *Plasmodium falciparum* strain (final parasitemia % in the control media cultures with 10% normal human sera, which was included in all cultures). Initial parasitemias in all cultures were approximately 0.5% schizonts. All cultures were performed in triplicate. Strain FVO was tested twice with rabbit anti-MSP-1₁₉Ig.

TABLE 4

Reversal of inhibition of IgG with recombinant MSP-1₁₉ antigen*

Rabbit α-MSP-1 ₁₉ (3D7) Ig (mg/ml)	MSP-1 ₁₉ (3D7) antigen (mg/ml)	% inhibition of control	
		FVO (3.7%)	3D7 (4.4%)
4.8	0	50%	34%
4.8	1.00	44%	11%
4.8	0.10	—	14%
4.8	0.01	—	2%

* Ig = immunoglobulin; MSP-1 = merozoite surface protein-1; — = not done.

This study provided an opportunity to prospectively evaluate the GIA in an antigen-specific fashion tied to protection. In this assay, one exposes invading schizonts/merozoites to antibodies to look at inhibition of invasion, as we have done here, or it can be designed with longer exposures and growth periods, with or without mononuclear cells, to look at inhibition of intracellular growth. Our data indicate that inhibitory activity was indeed present in the rabbit hyperimmune immunoglobulin, but inhibition was conferred by a very small fraction of the infused immunoglobulin. Given that GIA-active monoclonal antibodies react with conformation-dependent epitopes, one possible explanation for the discrepancy between very high ELISA titers and low MSP-1₁₉-specific GIA activities is optimal conformational folding of only a small fraction of the recombinant MSP-1₁₉ antigen used to immunize the rabbits. This possibility is being actively investigated.

Although the passively transferred rabbit antibodies to the MSP-1₁₉ construct persisted at high levels throughout the period of infection, they did not protect *Aotus* monkeys against challenge with malaria. They did, however, prevent the recipient monkeys from mounting their own antibody response to native MSP-1₁₉, an indication that they had combined with native MSP-1₁₉ *in vivo*. Passively administered antibody is known to markedly hasten the decrease in the number of antibody-forming cells in the recipient host, suggesting that these extraneous antibodies exert control on antibody synthesis to a particular antigen.¹⁷ This may also be due to the formation of immune complexes between the rabbit antibodies to MSP-1₁₉ and antigen produced by the parasite during infection, although it seems unlikely that this control is achieved wholly by neutralization of the antigen since complete immunoglobulin is much more effective than F(ab)₂ fragments in reducing the number of antibody-forming cells.¹⁷

This study was designed to extend the results of active immunization studies in *Aotus* and to determine the relative contribution of antibody to protection against challenge induced by Freund's adjuvant and MSP-1₁₉ in the *Aotus* model. One interpretation of our data suggests that if antibodies directed against MSP-1₁₉ are a principal mechanism by which such vaccines will confer protection, formulations containing conformationally optimal antigen and potent adjuvants that are capable of inducing strong immune responses (humoral and cellular) may be required.

Acknowledgment: We thank Dr. William Collins for providing the *P. falciparum* FVO parasites and for advice on the management and treatment of malaria-infected monkeys.

Financial support: This study was supported by the U.S. Naval Medical Research and Development Command, National Naval Medical Center (Bethesda, MD) and the Walter Reed Army Institute of Research (Washington, DC).

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Authors' addresses: Alfonso Gozalo, Carmen Lucas, Marlene Cachay, Douglas Watts, and Mark Wooster, U.S. Naval Medical Research Institute Detachment, APO AA 34031-3800. Bruce T. Wellde, Ted Hall, Jeffrey A. Lyon, J. Kathleen Moch, J. David Haynes, Joseph S. Williams, Carolyn Holland, Eugene Watson, Kent Kester, and W. Ripley Ballou, Department of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307-5100. Brian Bell and Jay Wood, Department of Biologics Research, Walter Reed Army Institute of Research, Washington, DC 20307-5100. David C. Kaslow, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD 20892.

Reprint requests: W. Ripley Ballou, Department of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

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