Immunization of *Aotus nancymai* with Recombinant C Terminus of *Plasmodium falciparum* Merozoite Surface Protein 1 in Liposomes and Alum Adjuvant Does Not Induce Protection against a Challenge Infection

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Merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum* is an antimalarial vaccine candidate. The highly conserved 19-kDa C-terminal processing fragment of MSP-1 (MSP-1$_{19}$) is of particular interest since it contains epitopes recognized by monoclonal antibodies which inhibit the invasion of erythrocytes in vitro. The presence of naturally acquired anti-MSP-1$_{19}$ antibodies in individuals exposed to malaria has been correlated with reduced morbidity, and immunization with an equivalent recombinant *P. yoelii* antigen induces substantial protection against this parasite in mice. We have expressed *P. falciparum* MSP-1$_{19}$ in *Escherichia coli* as a correctly folded protein and immunized *Aotus nancymai* monkeys by using the protein incorporated into liposomes and adsorbed to alum. After vaccination, the sera from these animals contained anti-MSP-1$_{19}$ antibodies, some of which competed for binding to MSP-1$_{19}$ with monoclonal antibodies that inhibit parasite invasion of erythrocytes in vitro. However, after challenge with either a homologous or a heterologous strain of parasite, all animals became parasitemic and required treatment. The immunization did not induce protection in this animal model.

Malaria is a serious health problem in tropical countries, with an estimated 300 to 500 million clinical cases and 2.7 million deaths per year (9). Resistance to existing drugs is developing fast, and an effective vaccine is urgently needed. A number of antigens expressed at different stages of the parasite's life cycle have been characterized with respect to their use in a subunit vaccine against *Plasmodium falciparum* (19). Merozoite surface protein 1 (MSP-1) is one of the most promising vaccine candidates (14, 20). People naturally exposed to *P. falciparum* develop antibodies against MSP-1 (15, 27, 31–33). Furthermore, an association between a naturally acquired immune response to MSP-1 and reduced malaria morbidity has been observed (30). In a number of independent studies, immunization with purified native MSP-1 or recombinant fragments of the protein has induced at least partial protection against parasite challenge (reviewed in reference 14).

MSP-1 is one of the best-characterized *P. falciparum* proteins (reviewed in reference 10). At the time of merozoite release and erythrocyte invasion, MSP-1 is proteolytically cleaved into several fragments. Only a 19-kDa C-terminal polypeptide (MSP-1$_{19}$) is carried into newly infected erythrocytes, and the remaining fragments are shed from the parasite surface (3). The sequence of MSP-1$_{19}$ is highly conserved and composed of two motifs which have structural similarity to epidermal growth factor (4). Epitopes in this region of MSP-1 are the targets of antibodies which inhibit erythrocyte invasion in vitro (3, 7, 11, 29). Vaccination experiments with the equivalent polypeptide from *P. yoelii* expressed in *Escherichia coli* have shown that mice immunized with this recombinant protein are protected against an otherwise lethal challenge with this rodent parasite (12, 25). Protection appears to be mediated largely by antibody (16, 25). *P. falciparum* MSP-1$_{19}$ expressed in *E. coli* or *Saccharomyces cerevisiae* appears to be correctly folded (6, 23) and reacts with antibodies induced by natural infection (15, 33). In The Gambia, where malaria is endemic, the prevalence of anti-MSP-1$_{19}$ antibodies is low in children (about 20%) but is up to 60% in adults (15), and a significant correlation between anti-MSP-1$_{19}$ antibody titer and reduced malaria morbidity has been reported (16). Acquisition of high levels of antibodies to MSP-1$_{19}$ from the mother correlates with a reduced probability of an episode of clinical malaria in infants (18). In summary, in vitro studies, results obtained with a rodent malaria model, and studies on naturally acquired immune responses suggest that MSP-1$_{19}$ is an important target of antimalarial immunity and for the development of a vaccine against *P. falciparum*.

To evaluate the recombinant *P. falciparum* MSP-1$_{19}$ expressed in bacteria as a vaccine against malaria, we immunized *Aotus nancymai* monkeys and challenged them with blood stage parasites.

MATERIALS AND METHODS

Production of antigen. The antigen used in this study was the C terminus of the Wellcome type MSP-1 expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST). The protein was prepared as described previously (6). The sequence contains amino acids Asn-1631 to Asn-1726 (numbering according to reference 26). To purify the malaria polypeptide for use in enzyme-linked immunosorbent assays (ELISA), GST–MSP-1$_{19}$ was cleaved with protease factor

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IMMUNIZATION WITH RECOMBINANT MALARIAL PROTEIN PfMSP-1$_{19}$

Xa (Boehringer) and MSP-1$_{19}$ was separated from GST by gel filtration through Superdex-75 (Pharmacia).

Formulation of vaccine. GST–MSP-1$_{19}$ and GST were formulated with liposomes that were prepared as described previously (1). The liposomes were composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol in a molar ratio of 9:1:7.5 and lipid A at 13 μg/mol of phospholipid. The phospholipids were obtained from Avanti Polar-Lipids, Inc., Alabasta, Ala.; the lipid A (from *Salmonella minnesota* R595) was obtained from List Biological Laboratories, Inc., Campbell, Calif. A single dose of 250 μl of liposomes contained 215 μg of lipid A, 16.5 μmol of phospholipid, and 74 μg of the GST–MSP-1$_{19}$ fusion protein or 40 μg of GST as a control antigen, respectively. The liposomes were adsorbed to alum prior to injection.

Design of vaccine trial. *A. nancymai* monkeys were immunized in two separate trials with two different *P. falciparum* challenge strains. For homologous challenge, parasites of the FVO strain of *P. falciparum* were used; these parasites express MSP-1$_{19}$ with a sequence identical to that of the recombinant protein (23). The FVO strain is a highly virulent parasite for *A. nancymai*, producing a rapidly developing parasitemia after challenge. To preserve monkeys infected with this strain, treatment with mefloquine (20 mg/kg orally) was initiated when the percentage of infected erythrocytes approached 5%. Heterologous challenge was done with *P. falciparum* CAMP parasites, which express the MAD20 allele of the *msp-1* gene and the amino acid sequence of whose MSP-1$_{19}$ differs from that of the antigen at four positions (26). The CAMP strain is less virulent, with only about 40% of infected *A. nancymai* monkeys requiring treatment (chloroquine-HCl, 5 mg intramuscularly on each of 3 days) for parasitemias approaching 10%. Twenty-five percent require treatment for severe anemia (<20% hematocrit) with moderate parasitemia, and approximately 35% survive without treatment, never developing high parasitemia or severe anemia, but nonetheless undergo substantial infections (2, 34). Eleven animals were used: numbers 1, 2, 3, 7, and 8 were immunized with GST, and numbers 4, 5, 6, 9, 10, and 11 were immunized with GST–MSP-1$_{19}$. Monkeys 1 to 6 were challenged with the CAMP strain, and numbers 7 to 11 were challenged with the FVO strain.

Immunization and challenge protocol. The monkeys were immunized by the intramuscular route on days 1, 30, and 60 and challenged on day 67 with 10^5 parasitized erythrocytes obtained from donor monkeys. Blood samples were taken immediately before each immunization and on the day of challenge. After challenge, parasitemia was monitored with Giemsa-stained blood smears and hematocrits were determined daily. Monkeys were treated with antimalarial drugs when the parasitemia approached 10% for the CAMP strain and 5% for the more virulent FVO strain or when the hematocrit dropped to 20% or less.

ELISA. Immunolon 4 microtiter plates (Dynatech Laboratories) were coated by incubation with 3 μg of MSP-1$_{19}$ per ml or 2 μg of GST per ml in carbonate buffer (pH 9.5) at 5°C and washed three times with phosphate-buffered saline, and then uncoated sites were blocked with 1% (wt/vol) milk powder in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20. The plates were washed again, and then triplicate or duplicate wells were incubated with serially diluted sera. After washing, alkaline phosphatase-conjugated anti-monkey immunoglobulin G (Sigma) was added at a dilution of 1/1,000. Bound antibody was detected with *p*-nitrophenylphosphate (Sigma), and the A_{405} was measured.

Competition ELISA. The ability of antibody present in the monkey sera to block binding to MSP-1$_{19}$ of monoclonal antibodies (MAbs) 2.2, 7.5, 12.8, and 12.10 (a kind gift of J. S. McBride) was studied. Two of these MAbs, 12.8 and 12.10, block parasite invasion of erythrocytes in vitro (11) and inhibit the proteolytic cleavage of MSP-1 (5), whereas the others do not. Plates coated with MSP-1$_{19}$ were incubated with various dilutions of monkey sera and incubated for 5 h. After the plates were washed, ascitic fluid containing one MAb and diluted 1/4,000 was added and the plates were incubated overnight. Finally, the plates were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G diluted 1/1,000. Bound antibody was detected by using H$_2$O$_2$ and *o*-phenylenediamine (Sigma); the color reaction was stopped by addition of H$_2$SO$_4$, and then the A_{492} was measured.

RESULTS

Assessment of antibody response by ELISA. All of the animals tolerated the immunizations well, with no deleterious effects on weight or hematologic parameters. The sera collected after immunization and immediately before challenge were analyzed for the presence of specific antibodies. We determined the titers of antibodies specific for GST and MSP-1$_{19}$.

The antigen used for immunization was either the GST–MSP-1$_{19}$ fusion protein or GST alone. Therefore, to assess the immunogenicity of the vaccine formulation, we determined the response in each animal to GST by ELISA. As shown in Fig. 1a and b, all of the animals developed antibodies against GST, indicating that the vaccine formulation had stimulated an immune response. The response tended to be lower in animals

that had received the fusion protein, and monkey 11 developed a noticeably lower titer than all of the others.

We also investigated the specific antibody response to MSP-1$_{19}$ by using the cleaved and purified product as the antigen in the ELISA. All of the monkeys that had been immunized with GST–MSP-1$_{19}$ developed antibodies against this protein (Fig. 1c and d), whereas the animals immunized with GST did not. Monkey 11 also developed a lower response to GST–MSP-1$_{19}$ than did the others immunized with this antigen.

Antibodies induced by immunization compete with MAbs for antigen binding. The sera were assayed for the ability to block the binding to recombinant protein of inhibitory MAbs 12.8 and 12.10, which recognize different epitopes. The sera of monkeys immunized with GST–MSP-1$_{19}$ had at least a partial effect on the binding of both MAbs at the dilutions tested (Fig. 2), whereas the sera from the GST-vaccinated animals had no effect on MAb binding. In competition with MAb 12.8 (Fig. 2a and b), blocking was first observed with some samples at a dilution of 1/250 and was essentially complete at a dilution of 1/25, the highest concentration in serum tested. In contrast, the blocking of MAb 12.10 binding was first detected at a concentration in serum of 1/50 and at the highest concentration tested (1/25), the binding of MAb 12.10 was blocked to only 60% by four of the sera and to 45 and 35% by two others (Fig. 2c and d). The strongest blocking effect was observed for noninhibitory MAb 2.2. In this case, competition was first detected at a dilution of 1/5,000 in some samples (Fig. 2e and f).

Outcome of challenge with parasites. All six monkeys (numbers 1 to 6) in the control (GST) and immunized (GST–MSP-1$_{19}$) groups challenged with the heterologous strain (CAMP) developed detectable parasitemia in their blood on day 3 or 4 after challenge (Fig. 3a and b). While one control monkey required treatment for high parasitemia on day 15, the other two controls both recovered without treatment after undergoing substantial infections. Two of the immunized monkeys required treatment for high parasitemia on days 10 and 11 after challenge. The third immunized monkey developed moderate parasitemia but was treated because of severe anemia on day 13.

The outcome of challenge with the homologous strain (FVO) is shown in Fig. 3c and d (numbers 7 to 11). Parasites were detected in the blood of all six monkeys on day 4 or 5 after challenge, and all monkeys were treated on day 10 because of high parasitemia. While the two specific control (GST) animals had somewhat higher parasitemias than the three immunized monkeys at the time of treatment, two other control monkeys challenged at the same time with the same inoculum had parasitemias on day 10 of 195,950 and 664,620 organisms per μl of blood, respectively (data not shown).

DISCUSSION

Immunization with the C terminus of *P. yoelii* MSP-1 expressed in bacteria as a fusion protein with GST can protect mice against an otherwise lethal challenge with this parasite (12, 25). In contrast, immunization of mice with *P. chabaudi* MSP-1$_{19}$ expressed in *E. coli* as a fusion with maltose-binding protein was not effective (28). We have produced the homologous region of *P. falciparum* MSP-1 as a GST fusion protein and tested it as a vaccine in *A. nancymai* monkeys challenged with this human malaria parasite. In the protocol used here, vaccination with the C terminus of MSP-1 did not induce protection against a challenge with *P. falciparum*.

In the *P. yoelii* model, the secondary structure of the antigen is important for induction of protective immunity; for example, reduced and alkylated recombinant protein does not induce
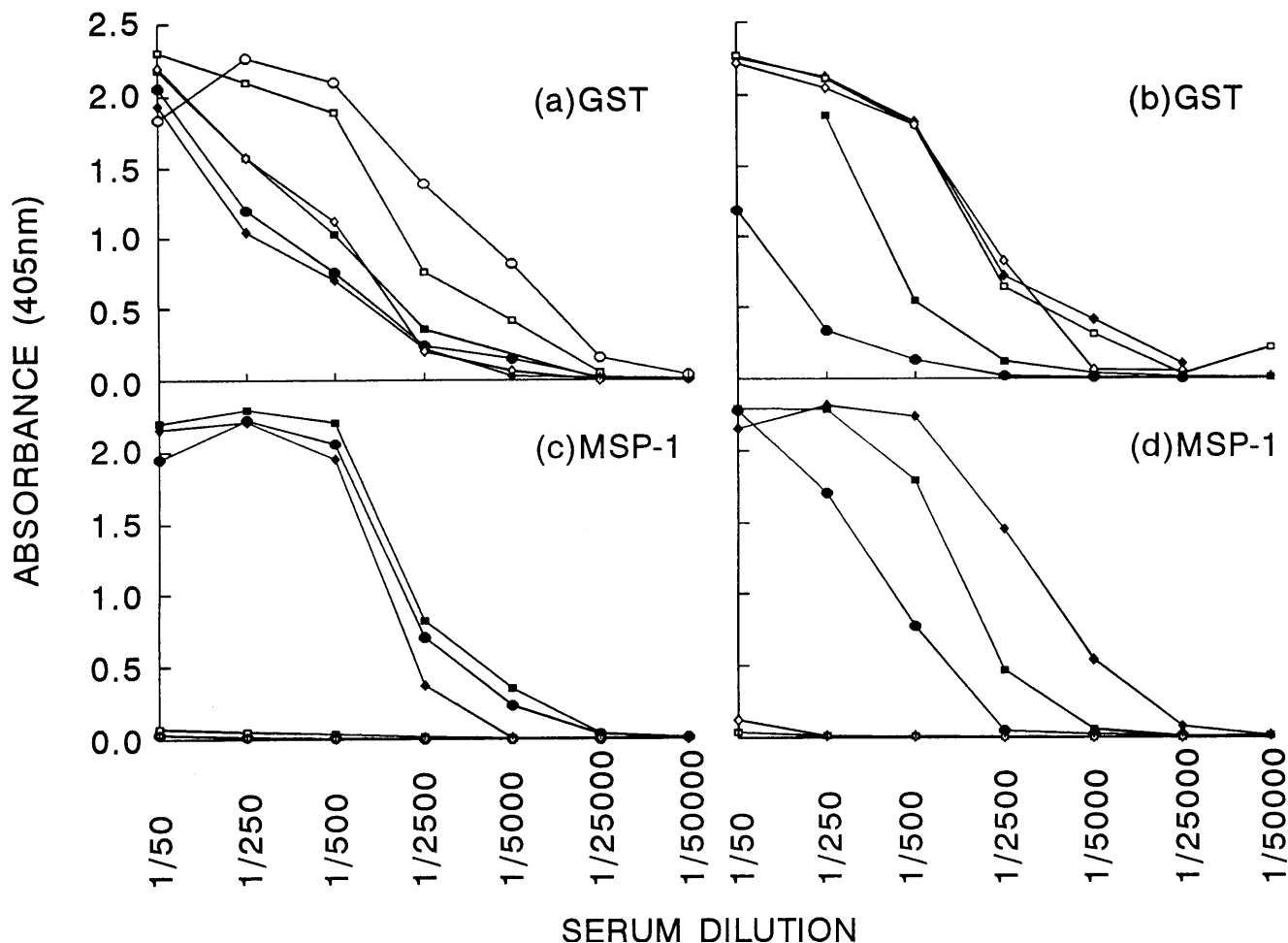
FIG. 1. Antibody titers of *Aotus* sera as determined by ELISA. The capture antigen was control protein GST (a and b) or malarial protein MSP-1$_{19}$ (c and d). Monkeys were challenged with the heterologous parasite strain (no. 1 to 6) (a and c) or given a homologous challenge (no. 7 to 11) (b and d). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST–MSP-1$_{19}$. Monkey no. (symbol): 1 (◇), 2 (□), 3 (○), 4 (◆), 5 (■), 6 (●), 7 (◇), 8 (□), 9 (◆), 10 (■), and 11 (●).

protection. *P. falciparum* MSP-1$_{19}$ contains 12 cysteine residues and it is thought that the six predicted disulfide bonds are important to determine the three-dimensional structure of the polypeptide. The recombinant protein produced in *E. coli* has been shown to contain epitopes shared with parasite-derived MSP-1 by using a panel of 12 MAbs and several immune sera (6, 15, 16, and unpublished data). Five of the MAbs recognize epitopes in the first epidermal growth factor-like motif (8), two (MAbs 91.33.5 and 94.65, a kind gift of G. S. N. Hui) are specific for the second epidermal growth factor-like motif (23), and the rest bind only when both motifs are expressed together. None of the MAbs binds when the protein has been treated with reducing and alkylating agents. These studies suggest that the recombinant protein is correctly folded and resembles the native protein.

Immunization with GST–MSP-1$_{19}$ induced the production of antibodies, some of which may be against functionally important epitopes, but the antibody titers were low compared with those in mice protected against challenge infection. In mice immunized with recombinant *P. yoelii* MSP-1$_{19}$, protection appears to correlate with the antibody response (12, 13, 25). In this study, the monkeys responded to immunization with the fusion protein. They developed antibodies to both the

GST carrier and MSP-1$_{19}$ (Fig. 1); only one monkey (number 11) did not respond well on immunization. The sera also partially blocked the binding to MSP-1$_{19}$ of MAbs that inhibit erythrocyte invasion in vitro (Fig. 2). These MAbs have different specificities; 12.8 recognizes an epitope in the first epidermal growth factor-like motif, whereas MAb 12.10 binds only to the whole MSP-1$_{19}$ polypeptide and not to either of the individual epidermal growth factor-like motifs alone (6, 8). The results of this competition ELISA indicate that the animals developed at least some antibodies recognizing epitopes that are important targets for inhibition of the invasion process, as judged by in vitro assays. However, these antibodies were present in a low concentration because high concentrations of serum were required in the blocking assay, in particular with MAb 12.10. The majority of antibodies induced by immunization may be directed against other epitopes of MSP-1$_{19}$. Consistent with this observation is the fact that the sera were able to effectively block the binding of noninhibitory MAb 2.2 and that of MAb 7.5 to a lesser extent (data not shown). Antibodies with 2.2 and 7.5 specificity compete with the inhibitory antibodies for binding and prevent the inhibition of MSP-1 processing (5) and therefore may be detrimental to protection.

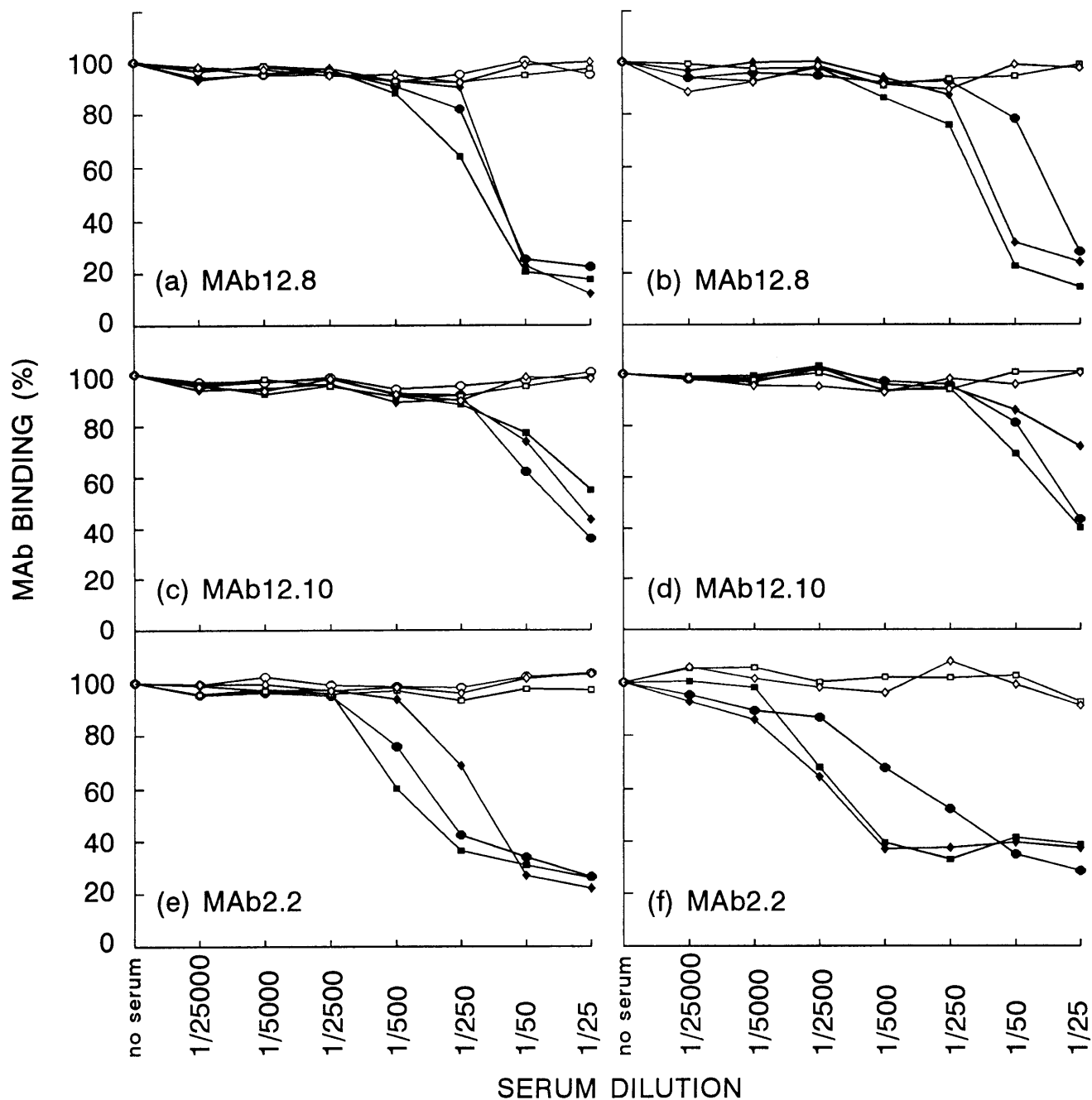After challenge, none of the monkeys that received the

FIG. 2. Competition ELISA to demonstrate that the monkey sera contained antibodies that block the binding of inhibitory MAbs 12.8 (a and b) and 12.10 (c and d) and noninhibitory MAb 2.2 (e and f). Monkeys were given a heterologous challenge (a, c, and e) or a homologous challenge (b, d, and f). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST-MSP-1$_{19}$. Monkey no. (symbol): 1 (◇), 2 (□), 3 (○), 4 (◆), 5 (■), 6 (●), 7 (◇), 8 (□), 9 (◆), 10 (■), and 11 (●).

GST–MSP-1$_{19}$ vaccine was protected and all required chemotherapy, although they had developed antibodies against the antigen. This contrasts with immunity induced by infection and cure in *A. nancymai*, which is antibody dependent and of long duration (34). Recently, Kumar and colleagues used *P. falciparum* MSP-1$_{19}$ expressed in *S. cerevisiae* (23) and formulated with Freund's adjuvant (24) to immunize *Aotus* monkeys of two different karyotypes. The two *A. nancymai* monkeys which received the MSP-1$_{19}$ vaccine had peak parasitemias of <0.1% and resolved the infection. It was suggested that the protective

immunity induced was not mediated by antibodies that block invasion. A significant difference between the two studies is the adjuvant used. Freund's adjuvant is unacceptable for human use, and therefore we used liposomes and alum, an adjuvant combination which is being developed for use in humans (17). In the *P. yoelii* model, essentially the same GST–MSP-1$_{19}$–liposome formulation did induce protection in mice (24a). For purified MSP-1 (21) and MSP-1$_{19}$ (22), the importance of an adjuvant for the level and quality of the immune response in different species has been demonstrated.
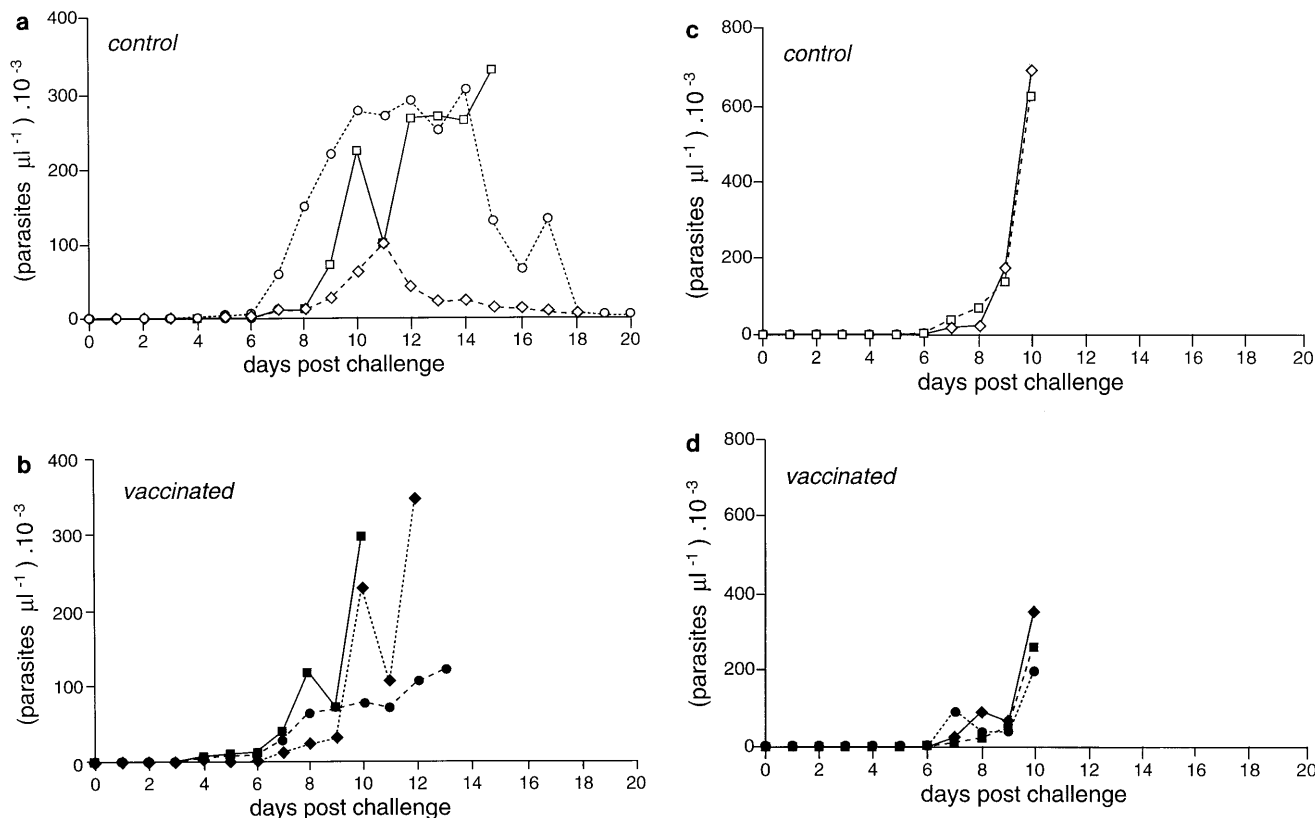
FIG. 3. Parasitemia curves of *Aotus* monkeys challenged with the heterologous CAMP strain (a and b) or the homologous FVO strain (c and d). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST–MSP-1$_{19}$. Monkey no. (symbol): 1 ($\diamond$), 2 ($\square$), 3 ($\bigcirc$), 4 ($\blacklozenge$), 5 ($\blacksquare$), 6 ($\bullet$), 7 ($\diamond$), 8 ($\square$), 9 ($\blacklozenge$), 10 ($\blacksquare$), and 11 ($\bullet$).

The vaccination of a small group of *Aotus* monkeys in this study resulted in an immune response, but there was no apparent influence on the course of the disease. When the results of in vitro assays (3, 7), observations in field studies with naturally exposed humans (16, 18, 33), results of immunization studies with two mouse models (12, 25, 28), and results obtained with *Aotus* monkeys given a strong adjuvant (24) or liposomes-alum (this work) are combined, there is still no clear picture of the importance of MSP-1$_{19}$ in immunity to blood stage *P. falciparum* malaria. The in vitro assays and results obtained with *P. yoelii* suggest that high concentrations of antibody to MSP-1$_{19}$ can prevent erythrocyte invasion. It remains to be determined whether or not the antibody concentration, the fine specificity of the humoral response (5), or the contribution of an unidentified cellular response is crucial for protection in vivo. The design of a vaccine based on the sequence of MSP-1 to elicit the production of specific inhibitory antibodies in humans remains to be implemented and requires the further development of reliable systems to test immunogenicity and efficacy in vitro or in animal models.

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