INFECTION AND IMMUNITY, Jan. 1996, p. 253–261 Vol. 64, No. 1
0019-9567/96/$04.00+0

A Recombinant Baculovirus 42-Kilodalton C-Terminal Fragment of *Plasmodium falciparum* Merozoite Surface Protein 1 Protects *Aotus* Monkeys against Malaria

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Received 7 August 1995/Returned for modification 29 September 1995/Accepted 31 October 1995

The immunogenicity and protective efficacy of a baculovirus recombinant polypeptide based on the *Plasmodium falciparum* merozoite surface protein 1 (MSP-1) has been evaluated in *Aotus lemurinus griseimembra* monkeys. The MSP-1-based polypeptide, BVp42, corresponds to the 42-kDa C-terminal processing fragment of the precursor molecule. Immunization of *Aotus* monkeys with BVp42 in complete Freund's adjuvant resulted in high antibody titers against the immunogen as well as parasite MSP-1. Fine specificity studies indicated that major epitopes recognized by these antibodies localize to conserved determinants of the 19-kDa C-terminal fragment derived from cleavage of the 42-kDa processing fragment. Effective priming of MSP-1-specific T cells was also demonstrated in lymphocyte proliferation assays. All three *Aotus* monkeys immunized with BVp42 in complete Freund's adjuvant showed evidence of protection against blood-stage challenge with *P. falciparum*. Two animals were completely protected, with only one parasite being detected in thick blood films on a single day after infection. The third animal had a modified course of infection, controlling its parasite infection to levels below detection by thick blood smears for an extended period in comparison with adjuvant control animals. All vaccinated, protected *Aotus* monkeys produced antibodies which inhibited in vitro parasite growth, indicating that this assay may be a useful correlate of protective immunity and that immunity induced by BVp42 immunization is mediated, at least in part, by a direct effect of antibodies against the MSP-1 C-terminal region. The high level of protection obtained in these studies supports further development of BVp42 as a candidate malaria vaccine.

The *Plasmodium falciparum* merozoite surface protein (MSP-1) is a major candidate for a blood-stage malaria vaccine. Previous studies have shown that nonhuman primates can be protected against *P. falciparum* infection by vaccination with MSP-1 isolated from cultured parasites (10, 12, 31). However, similar vaccine experiments using recombinant MSP-1 polypeptides produced in bacterial expression systems have not generated a level of immunity to *P. falciparum* infection comparable to that obtained with parasite-derived MSP-1 (10, 13–15, 21–23). Differences in protective efficacy between parasite MSP-1 and recombinant polypeptide MSP-1 fragments may have been due to the absence of sequences encoding protective MSP-1 epitopes in the recombinant polypeptides tested in vaccination experiments. Most constructs tested thus far have been based on amino-terminal segments of the MSP-1 precursor molecule. Additionally, the *Escherichia coli* expression system is generally considered suboptimal for expression of disulfide-dependent conformational epitopes because of the oxidizing environment within the bacterial cytoplasm (33).

Studies performed with the analogous MSP-1 protein of rodent malaria parasites have indicated that the highly conserved C-terminal region of this molecule contains epitopes critical for the generation of a protective immune response (8, 25). Preservation of disulfide-dependent conformational determinants of the C-terminal region of MSP-1 is critical for optimal antigenicity and immunogenicity of the recombinant polypeptide. For *P. falciparum*, we have shown in previous studies that a baculovirus recombinant polypeptide corresponding to the 42-kDa MSP-1 C-terminal processing fragment (BVp42) closely approximates the antigenicity and immunogenicity of the native MSP-1 C-terminal processing fragment (5). Furthermore, these studies showed that antibodies produced by rabbits immunized with this baculovirus recombinant polypeptide have a direct inhibitory effect on in vitro *P. falciparum* growth. Recently, vaccination of *Aotus nancymai* monkeys with a mixture of *Saccharomyces cerevisiae* recombinant polypeptides consisting of the P30 and P2 universal T-helper-cell epitopes fused to the 19-kDa MSP-1 C-terminal fragment as well as cleaved molecules consisting of three P30P2 amino acids (E-V-E) followed by the 19-kDa MSP-1 sequence enabled these animals to self-resolve an otherwise lethal *P. falciparum* infection (23).

The present vaccination experiment evaluates the immunogenicity and protective efficacy of the BVp42 recombinant polypeptide in complete Freund's adjuvant (CFA). The experimental model used in this study is the New World primate *Aotus lemurinus griseimembra*, which is highly susceptible to

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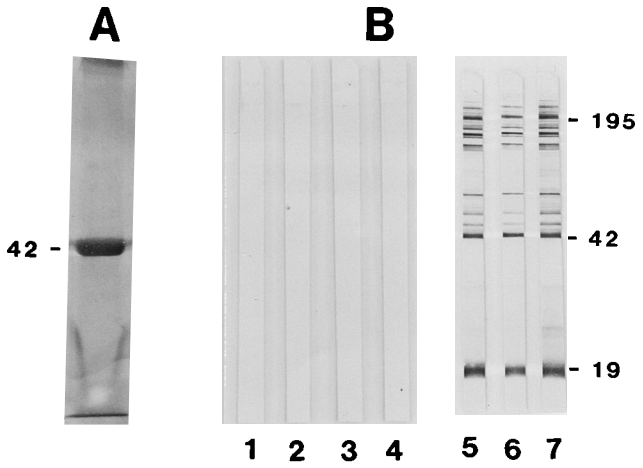

FIG. 1. Immunoblots of *Aotus* quaternary sera with purified parasite MSP-1 obtained from the *P. falciparum* FUP isolate. (A) Purified BVp42 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained. (B) Purified MSP-1 was similarly electrophoresed, transferred to nitrocellulose, and reacted with quaternary day 14 sera of *Aotus* monkeys immunized with saline plus CFA (lane 1, no. 20; lane 2, no. 80475; lane 3, no. 81560; lane 4, no. 81261) or BVp42 plus CFA (lane 5, no. 81269; lane 6, no. 81512; lane 7, no. 417). Numbers at each side of the panel are molecular mass in kilodaltons.

infection with the human malaria parasite *P. falciparum* and has been shown to be a suitable animal model for evaluation of human malaria vaccines (11, 29). In addition, the relationship between the in vitro growth inhibition of sera from vaccinated animals and in vivo protection against parasite infection was investigated.

MATERIALS AND METHODS

Antigen preparation. The recombinant p42 antigen BVp42 is based on the C-terminal MSP-1 sequence of the Uganda-Palo Alto *P. falciparum* isolate (FUP) (7). BVp42 corresponds to the p42 coding region from Ala-1333 to Ser-1705 cloned into the *Autographa californica* nuclear polyhedrosis virus polyhedrin promoter-regulated expression system (26). The recombinant baculovirus was grown in Sf9 cells, and the recombinant polypeptide was isolated from culture supernatants by affinity chromatography as described previously (31). Antigen purity was assessed by silver staining of antigen preparations separated by polyacrylamide gel electrophoresis (Fig. 1A).

The MSP-1 protein and its processing fragments were purified from in vitro cultured parasites (*P. falciparum* isolates FUP, Vietnam-Oak Knoll [FVO], and 3D7) by monoclonal antibody affinity chromatography procedures (31).

The rMSP1$_{19}$ construct corresponds to the FUP MSP-1 coding region from Asn-1613 to Ser-1705 (numbered as per reference 7) and is expressed as a fusion protein with the preprosequence of yeast alpha factor and a C-terminal, six-histidine-residue tag (20). Yeast culture supernatants were incubated with nickel-nitrilotriacetic acid agarose, the agarose was centrifuged at low speed and washed four times with 2× phosphate-buffered saline, and rMSP1$_{19}$ was eluted with 0.2 M sodium acetate-saline, pH 4.0.

Immunization and challenge. *Aotus lemurinus griseimembra* monkeys of karyotypes II and III with a history of no previous malaria exposure were screened for lack of antibody reactivity with *P. falciparum* antigens by indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA), ability of serum to support in vitro parasite growth, normal blood chemistry and hematology values, absence of blood and intestinal parasites, absence of cardiovascular abnormalities, and overall good health. *Aotus* monkeys passing this screening process were stratified by sex and colony origin before being randomly assigned to the control group injected with saline and CFA (four animals) or the experimental group injected with BVp42 in CFA (four animals). All animals were maintained in accordance with National Institutes of Health and institutional guidelines. Immunizations consisted of 0.5 ml of borate-buffered saline (BBS) (controls) or 100 μg of BVp42 in 0.5 ml of BBS (experimental) emulsified in 0.5 ml of CFA. The mycobacterium content in the Freund's adjuvant was halved with each successive immunization to minimize granuloma formation. Each dose was given at 21-day intervals intramuscularly into a thigh (first and second immunizations) or in multiple subcutaneous sites on the back (third and fourth immunizations) for a total of four immunizations. Approximately 3-ml blood samples were collected from the femoral vein 7 and 14 days after each immunization. To generate the parasite challenge inoculum, a frozen stabilite of in vivo-passaged FUP isolate parasites was thawed and inoculated into a naive *Aotus* monkey. After 5 days, peripheral blood containing parasitized erythrocytes was collected from this animal and transferred to a second *Aotus* monkey. Seven days later, an inoculum was collected from this animal and adjusted to 7×10^5 parasites per ml, and 0.5 ml was inoculated into the saphenous vein of control and experimental animals 29 days after the final immunization. Parasitemias were monitored by blood smears which were randomly coded before being submitted to microscopists. The experimental endpoint for this vaccination study was defined as either parasitemia of ≥10%, an erythrocyte count of ≤2.5×10^6/μl, or serious illness as determined by the attending veterinarian. Antimalarial therapy was chloroquine (10 mg/kg of body weight per day for 4 days). The experiment was terminated by antimalarial drug treatment of remaining, untreated animals on day 97 after parasite challenge.

ELISA. Serum antibodies produced against MSP-1, BVp42, or rMSP-1$_{19}$ were assayed by an ELISA described previously (6). Briefly, vinyl plates were coated with 0.08 μg of purified, parasite MSP-1, recombinant BVp42, or recombinant MSP-1$_{19}$ per ml; washed with BBS; and blocked with 1% bovine serum albumin in BBS (167 mM borate, 134 mM NaCl, pH 8.0). *Aotus* sera were serially diluted in 1% bovine serum albumin–BBS. Fifty microliters of diluted sera was added to antigen-coated wells and incubated for 1 h at room temperature. Plates were washed with BBS containing 0.5 M NaCl, and an appropriate dilution of peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) (heavy and light chain specific) (Cappel, Durham, N.C.) was added and incubated for 1 h at room temperature. Plates were washed in BBS containing 0.5 M NaCl and finally in BBS. One hundred microliters of peroxidase substrate solution [H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] was added to each well, and the A_{410} was determined with a Dynatech 605 ELISA reader. The endpoint of ELISA titers for *Aotus* sera was designated to be the serum dilution producing an absorbance value of 0.1. This cutoff corresponded to >3 standard deviations above background absorbance readings for control, antigen-coated wells incubated with the anti-monkey IgG-peroxidase conjugate, and the peroxidase substrate.

The ELISA inhibition assay was performed by diluting *Aotus* antisera to a point on the descending portion of the ELISA titration curve. The diluted sera were mixed with various concentrations of inhibitor, incubated for 1 h, and added to BVp42-coated plates. The rest of the assay was carried out as described above.

IFA. Assays were performed on acetone-fixed thin blood smears of schizonts and merozoites of the FUP isolate as described previously (30). Endpoint IFA titers correspond to the final serum dilution producing parasite immunofluorescence above background levels obtained with preimmune sera.

In vitro inhibition assay. In vitro parasite growth inhibition assays were performed by culturing parasites in the presence of preimmune and immune (quaternary day 14) *Aotus* serum by a modification of established methods (18). *Aotus* sera were heat inactivated and absorbed with a 1/10 volume of washed human erythrocytes before use. For in vitro assays using purified *Aotus* immunoglobulin, gamma globulins from 0.4 ml of serum or plasma were precipitated with an equal volume of saturated ammonium sulfate. The precipitate was brought up to 0.4 ml in 0.85% NaCl, dialyzed against 16 liters of 0.85% NaCl, and then dialyzed overnight against RPMI 1640 culture medium. Parasite cultures were synchronized by sorbitol lysis. Infected human erythrocytes were adjusted to an initial parasitemia of 0.5% and a hematocrit of 1.6% in culture medium containing 0.5% AlbuMAX (Gibco BRL, Grand Island, N.Y.). One hundred microliters of the cell suspension was added in duplicate wells containing either 100 μl of culture medium–0.5% AlbuMAX (positive controls) or 60 μl of culture medium–0.5% AlbuMAX plus 40 μl of test sera or purified Ig in a 96-well microtiter plate. Cultures were incubated for 72 h, and the parasitemia of Giemsa-stained thin smears of cultured erythrocytes was determined by microscopy.

Immunoblotting of MSP-1 antigens. Purified MSP-1 and BVp42 polypeptides were dissolved in Laemmli's buffer and separated on Na-dodecyl-SO_4 polyacrylamide gels (24). The separated proteins were electrophoretically blotted onto nitrocellulose (32) and reacted with goat anti-monkey Ig and rabbit anti-goat Ig-alkaline phosphatase conjugate as previously described (6).

T-cell proliferation assay. *Aotus* lymphocytes were purified from 3 ml of heparinized blood by centrifuging the buffy coat over a Histopaque gradient (Sigma Diagnostics, St. Louis, Mo.). All antigen concentrations were initially made up to 10 μg/ml in culture medium. Lymphocytes were cultured in triplicate in a total volume of 200 μl of RPMI 1640 containing 5% fetal bovine serum and 5 μg of antigen per ml and at a cell density of 1.8×10^6/ml in 96-well microculture plates. Cultures were incubated in a 8% CO_2 humidified incubator at 37°C for 4 days and pulsed with [^{3}H]thymidine (1 μCi/50 μl; 5 Ci/mmol) for 18 h. Cells were harvested, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

RESULTS

Physical status, hematology, and blood chemistries of vaccinated animals. Blood chemistry values measured after the primary and secondary immunization were similar for both experimental and control animals and were generally within

TABLE 1. Antibody responses of *Aotus* monkeys vaccinated with BVp42 in CFA (quaternary day 14 response)

Animal no.	Immunogen	IFA titer[a]	ELISA titer[b]			Immunoblot[c]
			MSP-1	BVp42	rMSP-1_{19}	
20[d]	CFA	1:320	1:250	1:250	1:200	−
80475		—	1:<50	1:50	1:110	−
81261		1:50	1:<50	1:50	1:<50	−
81560		—	1:<50	1:200	1:450	−
417	BVp42-CFA	1:6,400	1:800,000	1:3,000,000	1:800,000	+
81269		1:6,400	1:500,000	1:2,500,000	1:450,000	+
81512		1:6,400	1:410,000	1:3,000,000	1:400,000	+

[a] Titers for IFA correspond to the highest serum dilution producing merozoite fluorescence. Absence of reactivity by IFA is indicated by a dash.
[b] ELISA titers correspond to the highest serum dilution producing an absorbance reading of 0.1 absorbance units for plates coated with equal amounts of parasite MSP-1 (MSP-1), recombinant baculovirus p42 (BVp42), or recombinant yeast p19 (rMSP-1_{19}).
[c] Results expressed as presence (+) or absence (−) of antibody reactivity with parasite MSP-1 by immunoblot.
[d] Results for *Aotus* monkey 20 are for the secondary response since quaternary serum was not available for reasons cited in the text.

the normal range. However, decreased concentrations of phosphorus and potassium were noted in most experimental and control animals, along with elevated leukocyte counts accompanied by an inverted lymphocyte/neutrophil ratio indicative of an immune response. Also noted during immunization were elevated eosinophils, reduced hematocrits, and the detection of circulating reticulocytes consistent with slight erythrocyte regeneration. At the time of parasite challenge, the erythrocyte counts of all animals were within the normal range. One control animal (no. 20) experienced lethargy and muscular weakness after the third injection. To avoid additional stress in this animal, the fourth injection of saline plus adjuvant was not administered and no subsequent blood samples were drawn from this animal before challenge. *Aotus* monkey 20 subsequently recovered and was in good health at the time of parasite challenge. One experimental animal (no. 119) expired following handling for venipuncture 2 weeks after the third immunization; the cause of death was determined to be respiratory distress. All other animals remained in good overall health during the immunization regimen.

Immunogenicity studies. The immunogenicity of the BVp42 antigen in CFA in *Aotus* monkeys was examined by evaluating serum antibodies produced by vaccinated animals and the antigen-specific proliferative response of their peripheral blood lymphocytes. The antibody responses generated after the series of four immunizations as measured by ELISA titers, indirect immunofluorescence antibody titers, and immunoblot results are summarized in Table 1. Preimmune sera were negative in all assays (data not shown). Uniformly high antibody responses were obtained for all *Aotus* monkeys immunized with BVp42 in CFA. ELISA antibody titers of greater than $1:10^6$ were produced against the BVp42 immunogen. These antibodies displayed extensive cross-reactivity by ELISA with MSP-1 purified from the homologous FUP parasite isolate, and with a yeast recombinant 19-kDa, C-terminal MSP-1 polypeptide (rMSP1_{19}) based on the homologous FUP sequence. Controls immunized with saline and CFA were either negative or developed low ELISA titers against BVp42, rMSP-1_{19}, and parasite MSP-1 antigens.

Antibody titers were also determined by an IFA with FUP-infected erythrocytes. IFA titers of 1:6,400 were obtained for all three animals immunized with BVp42. The staining pattern corresponded to the characteristic merozoite surface fluorescence reported for the MSP-1 antigen. Two control animals (no. 20 and 81261) had a low IFA titer (1:50 to 1:320) while the other controls were negative.

Antibody reactivity of *Aotus* sera against purified, parasite MSP-1 was evaluated by immunoblot analysis. In addition to reactivity with molecular species corresponding to the C-terminal 42-kDa MSP-1 processing fragment, reactivity was also noted with the high-molecular-weight precursor molecule, a series of intermediate processing fragments, and smaller species migrating as a broad band of approximately 19 kDa (Fig. 1). Similar patterns have been reported for rabbits and mice immunized with native and recombinant MSP-1 antigens (5, 6). Controls displayed no significant reactivity with MSP-1 by immunoblot. Sera which reacted with parasite-derived MSP-1 also reacted by immunoblot with BVp42 (data not shown).

The kinetics of the antibody responses of control and experimental animals are shown in Fig. 2. MSP-1-specific antibody titers of *Aotus* monkeys were detected after the first immunization and were boosted with each subsequent dose of BVp42. ELISA titers reached 1:20,000 to 1:500,000 after the second immunization and, in most cases, gradually increased with subsequent immunizations, achieving maximal titers after the fourth immunization. Among the controls, low titers were observed for no. 20 (approximately 1:200) while the other controls remained at background levels during the immunization period.

Aotus peripheral blood lymphocytes obtained before immunization and 14 days after the secondary immunization were cultured with various MSP-1 antigens to determine the level of antigen-specific cellular activation. No significant antigen-specific proliferation was detected in preimmune baseline cultures (data not shown). Table 2 presents the proliferation assay results for *Aotus* monkeys after the second immunization. Animals in the experimental group displayed variable levels of cellular activation; however, highly significant stimulation was observed for three of the four BVp42-CFA-vaccinated animals, particularly for parasite MSP-1 and recombinant BVp42. A consistent pattern could be seen for each animal with several MSP-1-based antigens. For example, *Aotus* monkey 81269 had a very high proliferative response to three of the four antigens, with similar levels of proliferation noted for both parasite MSP-1 and recombinant BVp42. No significant proliferation was detected in cultures containing rMSP-1_{19}. Stimulation indexes obtained with animal 81512 were similar to those obtained for most of the normal controls. In contrast to the experimental group, lymphocytes obtained from the majority of control animals did not proliferate when cultured with parasite MSP-1 or recombinant BVp42, although slight proliferation was noted with the whole parasite antigen. The exception was animal 81560, for which there was significant proliferation with the whole parasite as well as both MSP-1-related antigens.

Parasite challenge. All *Aotus* monkeys were challenged with an intravenous inoculum of 3.5×10^5 infected erythrocytes 29

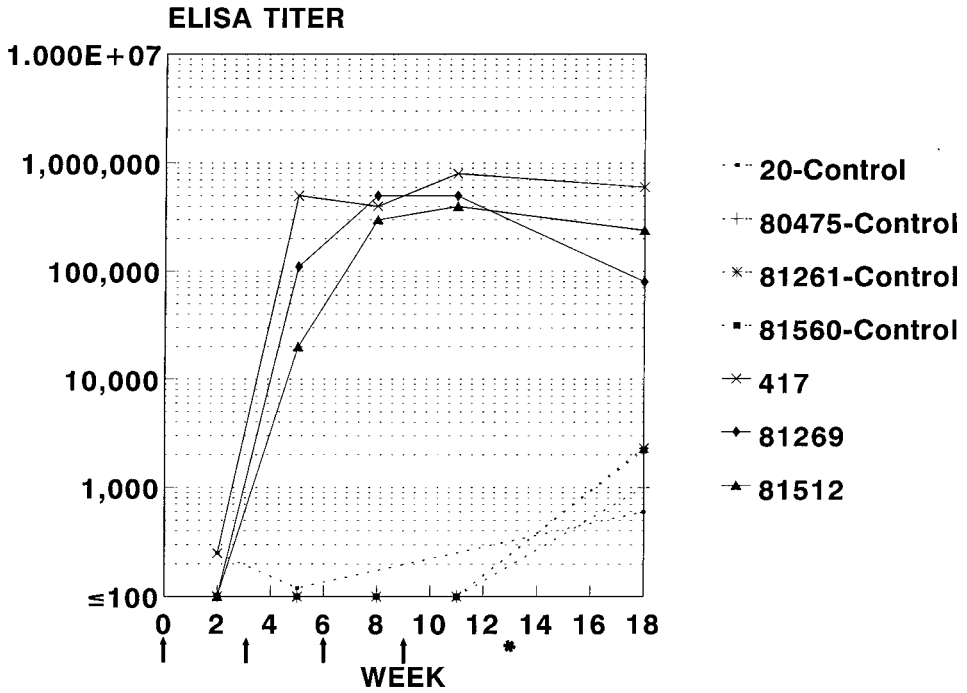

FIG. 2. Kinetics of the antibody responses of control *Aotus* monkeys immunized with saline plus CFA (dashed lines) or experimental *Aotus* monkeys immunized with BVp42 plus CFA (solid lines). ELISAs for MSP-1 antibody reactivity were carried out as described in Materials and Methods. Immunizations were given at weeks 0, 3, 6, and 9 as indicated by the arrows, and parasite challenge was administered during week 13 (asterisk). 1.000E + 0.7 equals 10^7.

days after the fourth immunization. Adjuvant control animals developed patent parasitemias between days 4 and 5 after parasite challenge and reached a level of 1% parasitemia within 7 to 10 days (Fig. 3; Table 3). Thereafter, controls followed two distinct courses of infection. Some control animals rapidly reached the treatment level of 10% parasitemia within 10 to 15 days (no. 80475 and 81560) and were drug treated at that point. However, a second subset of controls (no. 20 and 81261) displayed a slower course of infection, maintaining lower parasitemias over an extended period (Fig. 3) before drug treatment was required for anemia. In contrast, all three *Aotus* monkeys in the BVp42-CFA group displayed immunity to parasite infection although the degree of protection was variable. After a single day of very low parasitemia by thick smear (one parasite per thick film for *Aotus* monkey 417 on day 8 and for *Aotus* monkey 81512 on day 6), *Aotus* monkeys 417 and 81512 became aparasitemic, remaining free of detectable parasites throughout the remaining observation period (3 months following parasite challenge). Blood samples taken from these animals on postchallenge day 34 were negative when cultured for parasites. *Aotus* monkey 81269 had an extended prepatent period (15 days) before parasites were seen in thick smears, and over the following 3 days, thick smears from this animal continued to have very few parasites. However, higher parasitemias eventually developed, with 1% parasitemia being reached by day 22 and 10% by day 24, requiring drug treatment of this animal. BVp42-immunized animals as a group experienced a longer prepatent period than adjuvant control animals although the most dramatic effects of vaccination were the control of parasitemias at subpatent levels for an

TABLE 2. Antigen-specific lymphocyte proliferation assay of *Aotus* monkeys immunized with BVp42 in CFA[a]

Aotus monkey no.	Immunogen	[^{3}H]thymidine incorporation (mean dpm ± SD [stimulation index])				
		No antigen	Whole parasite	Parasite MSP-1	BVp42	rMSP-1_{19}
20	CFA	7,510 ± 2,977	11,411 ± 2,826 (1.5)	7,460 ± 2,243 (1.0)	5,903 ± 664 (0.8)	5,591 ± 1,300 (0.7)
80475		12,295 ± 2,292	17,930 ± 1,261 (1.5)[b]	13,815 ± 2,814 (1.1)	13,762 ± 2,031 (1.1)	12,025 ± 2,494 (1.0)
81261		8,770 ± 533	13,467 ± 1,397 (1.5)[b]	11,208 ± 1,658 (1.3)	8,568 ± 1,023 (1.0)	7,399 ± 1,482 (0.8)
81560		27,848 ± 4,558	95,422 ± 9,372 (3.4)[c]	121,994 ± 8,709 (4.4)[c]	256,062 ± 32,758 (9.2)[c]	22,608 ± 2,916 (0.8)
119	BVp42-CFA	17,785 ± 3,135	34,230 ± 4,504 (1.9)[d]	47,263 ± 3,324 (2.7)[c]	69,270 ± 10,474 (3.9)[c]	21,907 ± 1,864 (1.2)
417		5,907 ± 1,219	25,583 ± 580 (4.3)[c]	28,516 ± 4,843 (4.8)[c]	55,185 ± 6,286 (9.3)[c]	4,757 ± 784 (0.8)
81269		12,022 ± 2,009	99,281 ± 10,880 (8.3)[c]	205,345 ± 15,024 (17.1)[c]	254,861 ± 30,375 (21.2)[c]	15,578 ± 1,217 (1.3)
81512		8,128 ± 1,655	15,195 ± 1,779 (1.9)[c]	9,897 ± 2,056 (1.2)	11,362 ± 895 (1.4)[d]	13,940 ± 6,716 (1.7)

[a] Lymphocytes were cultured in medium alone (no antigen controls) or with 5 μg of whole parasite antigen, parasite MSP-1, or recombinant BVp42 or rMSP-1_{19} per ml. Results are expressed as the mean disintegrations per minute of triplicate cultures ± 1 standard deviation and as the stimulation index (disintegrations per minute of cultures containing antigen/disintegrations per minute of cultures containing no antigen).

[b] $P < 0.05$ versus control by Student's t test.

[c] $P \leq 0.001$.

[d] $P < 0.010$.

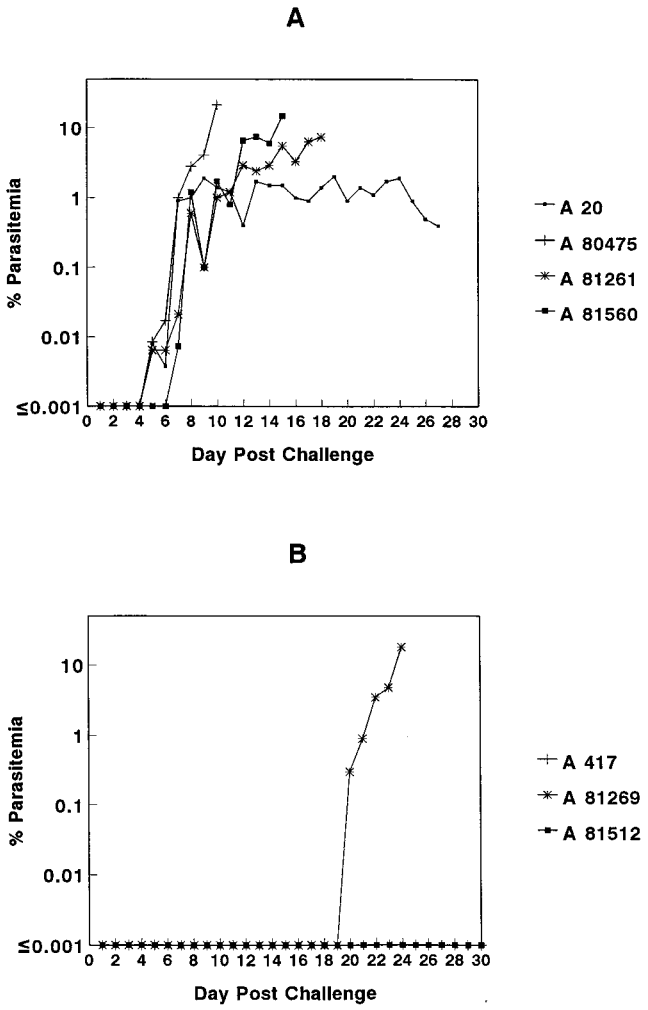

FIG. 3. Course of infection of monkey-passaged *P. falciparum* (FUP isolate) in *Aotus* monkeys immunized with saline plus CFA (A) or BVp42 plus CFA (B). Animals were challenged with an intravenous inoculum of 3.5×10^5 infected erythrocytes on day 29 after the fourth immunization, and parasitemias were monitored for 3 months following challenge.

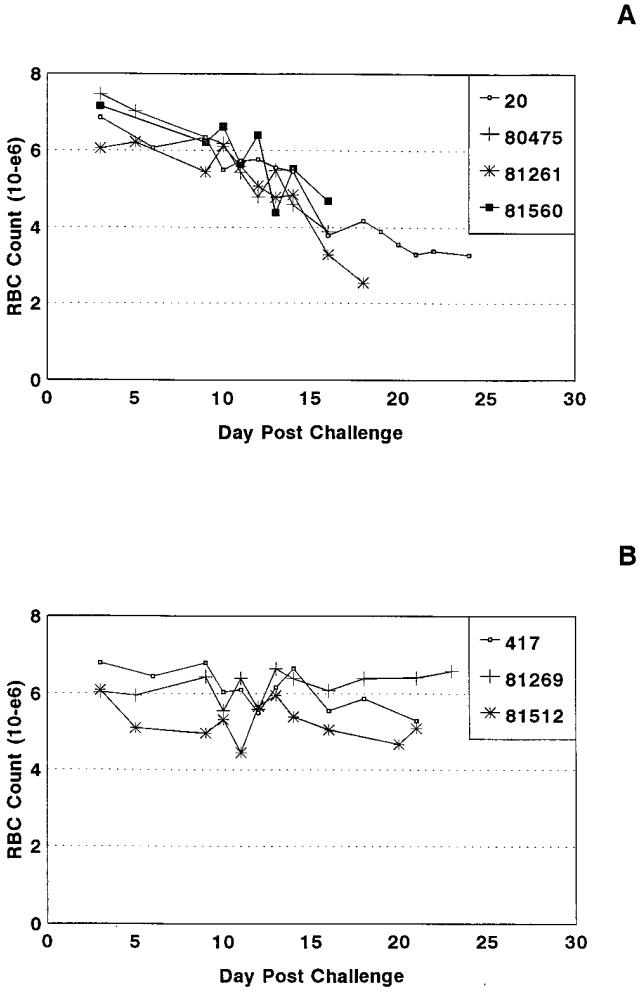

FIG. 4. Erythrocyte counts of *Aotus* monkeys immunized with saline plus CFA (A) or BVp42 plus CFA (B) following challenge with an intravenous inoculation of *P. falciparum* (FUP isolate). Erythrocyte counts are shown as number of erythrocytes multiplied by 10^6 per cubic millimeter.

extended time period and, in some cases, complete parasite clearance following a minimal, transient parasitemia.

In addition to monitoring parasitemias, we also monitored erythrocyte counts as an indicator of malaria infection and disease (Fig. 4). In adjuvant controls, erythrocyte counts began to decline during the second week of infection. In general, this decline did not appear to correlate with the level of parasitemia, since animals with relatively low parasitemias such as no. 20 experienced a decline in erythrocyte count which paralleled that of animals with higher parasitemias (no. 81261 and 81560). In contrast, stable erythrocyte counts were maintained in all animals within the protected BVp42-CFA group, including *Aotus* monkey 81269, which eventually required drug treatment on day 24.

In vitro parasite growth inhibition. Immune sera of vaccinated *Aotus* monkeys obtained after the fourth immunization and prior to parasite challenge were evaluated for possible effects on in vitro parasite growth. In duplicate inhibition experiments, parasite growth inhibition was obtained for sera of all three animals vaccinated with BVp42-CFA (Table 4). However, a higher level of inhibition was obtained for sera of *Aotus* monkeys 417 and 81269 than for monkey 81512. A lower parasitemia was noted for the preimmune *Aotus* monkey 417 sample than for other animals because it was necessary to use plasma rather than serum for the preimmune cultures of no.

TABLE 3. Course of parasitemia for *Aotus* monkeys immunized with saline plus CFA (controls) or BVp42 plus CFA (experimental)

Animal no.	Immunogen	Prepatent period (days)	Period to 1% parasitemia (days)	Period to 10% parasitemia or maximum parasitemia (days)
20	CFA	4	8	19 (2%)[a]
80475		4	7	10
81261		5	10	18 (7%)[a]
81560		5	8	15
417	BVp42-CFA	8[b]		
81269		15	22	24
81512		6[b]		

[a] Maximum parasitemia: no. 20, 2%; no. 81261, 7%.
[b] Parasites were detected by thick smear on a single day for *Aotus* monkeys 417 and 81512.

TABLE 4. In vitro parasite growth inhibition assay of sera of *Aotus* monkeys vaccinated with various BVp42-adjuvant formulations[a]

Aotus monkey no.	Immunogen	Expt 1			Expt 2			Expt 3		
		% Parasitemia		% Growth inhibition	% Parasitemia		% Growth inhibition	% Parasitemia		% Growth inhibition
		Preimmune serum	Immune serum		Preimmune serum	Immune serum		Preimmune Ig	Immune Ig	
80475	CFA	9.4	11.2	0	8.4	9.5	0	ND[b]	ND	
81261		9.3	9.8	0	8.1	8.3	0	ND	ND	
81560		6.8	8.1	0	8.4	8.2	0	ND	ND	
417	BVp42-CFA	1.4	0.2	100	2.4	0.2	100	14.0	1.6	93
81269		9.7	0.2	100	8.1	0.2	100	13.4	1.2	96
81512		6.7	3.2	57	7.0	2.4	70	13.4	1.8	91

[a] Parasites were cultured in the presence of 0.5% AlbuMAX supplemented with 20% preimmune or immune *Aotus* serum (experiments 1 and 2) or the equivalent volume of preimmune or immune *Aotus* Ig (experiment 3). Parasitemia was determined at 72 h of culture by microscopy. In the equation below, S is the starting parasitemia of cultures at 0 h, P is the parasitemia of cultures supplemented with preimmune serum or Ig, and I is the parasitemia of cultures supplemented with immune serum or Ig. Starting parasitemias in these experiments were 0.3 to 0.5%.

$$\% \text{ inhibition} = \left(1 - \frac{I - S}{P - S}\right) \times 100$$

[b] ND, not done.

417; however, the inhibition noted for *Aotus* monkey 417 immune serum was still highly significant. In contrast, the quaternary sera of CFA control animals had no effect on parasite growth. To confirm that the observed parasite growth inhibition was antibody mediated, inhibition experiments for the three positive samples were also performed with purified *Aotus* Ig (Table 4, experiment 3). High levels of growth inhibition (>90%) were noted for all three immune Ig preparations. Preimmune Ig, including that obtained from *Aotus* monkey 417, had no effect on parasite growth and supported a higher parasitemia in the presence of 0.5% Albumax than *Aotus* whole serum. The experiments using purified Ig were performed three times with identical results (data not shown).

Specificity analysis of anti-BVp42 antibodies. We were also interested in determining the specificity of antibodies produced by protected animals. To address this issue, antibodies of vaccinated *Aotus* monkeys were evaluated in a competitive inhibition ELISA in which binding to BVp42 was carried out in the presence of increasing concentrations of various MSP-1 antigens. Inhibition curves were similar for all three animals immunized with BVp42-CFA as shown in Fig. 5. Antibody binding was nearly completely inhibited by high concentrations (≥1 μg/ml) of MSP-1 purified from the homologous FUP parasite isolate as well as by heterologous MSP-1 obtained from parasite strains differing in the allelic blocks 15 and 16 (FVO) and in variant sequences located within the C-terminal 19K region block 17 (FVO and 3D7) (20). The degree of inhibition was comparable for BVp42 and parasite MSP-1. The similar inhibition curves obtained for the homologous FUP MSP-1 and the two heterologous MSP-1 molecules indicated that the majority of *Aotus* antibodies induced by BVp42 were directed against conserved MSP-1 epitopes of the C-terminal, 19K region. This is consistent with the high titers measured against the yeast rMSP-1_{19} in the ELISA (Table 1).

DISCUSSION

Previous studies have established parasite-derived MSP-1 as a protective malarial antigen in nonhuman primates (10, 12, 31). We now show that a baculovirus recombinant polypeptide based on the C-terminal processing fragment of *P. falciparum* MSP-1 is highly immunogenic in *Aotus* monkeys and confers on them a high degree of protective immunity against malarial infection. Complete protection was achieved in two of three animals immunized with BVp42 in CFA as reflected by the effective control and elimination of the parasite infection as shown previously for purified parasite MSP-1 (31). A third animal displayed a markedly modified course of infection in comparison with controls, controlling its parasitemia for an extended period before circulating parasites could be detected. Although previous studies have reported modifications in the course of infection by vaccination with recombinant MSP-1 antigens (10, 13–15, 21) or a self-resolving course of infection (23), none have achieved the level of protection obtained in the present experiment.

Protected animals maintained normal erythrocyte counts, signifying the absence of disease in these animals. Therefore, vaccination with BVp42 in CFA also provided protection against development of the anemia characteristic of malarial infection. This confirms that the assessment of protection based on peripheral blood smears accurately reflected the absence of a significant parasite load, thereby making subpatent infection or sequestration unlikely in these animals. Further evidence for an aborted infection in protected animals was lack of parasite growth in blood cultures obtained from these animals and the failure of postchallenge sera of the two completely protected animals to recognize an N-terminal MSP-1 recombinant polypeptide while postchallenge sera of all other animals showed strong reactivity with this antigen (data not shown).

The immune response of *Aotus* monkeys vaccinated with BVp42 in CFA was investigated by a variety of approaches measuring both quantitative and qualitative aspects of immunity. Very high serum ELISA titers (>$1:10^6$) against the BVp42 immunogen were attained in this study, and immunization was accompanied by a strong booster effect. High titers were measured against purified parasite MSP-1 by ELISA and by indirect immunofluorescence of merozoites, indicating extensive cross-reactivity of antibodies produced against the recombinant polypeptide with native MSP-1. It is noteworthy that this potent MSP-1 immune response did not require the incorporation of extraneous carrier sequences into the antigen but was elicited entirely by epitopes encoded by *P. falciparum* MSP-1. These primate data are consistent with previous studies with rabbits (5) and increase the likelihood of a similar level of immunogenicity and cross-reactivity in humans vaccinated with a BVp42 malaria vaccine provided that a clinically acceptable alternative adjuvant formulation to CFA can be identified.

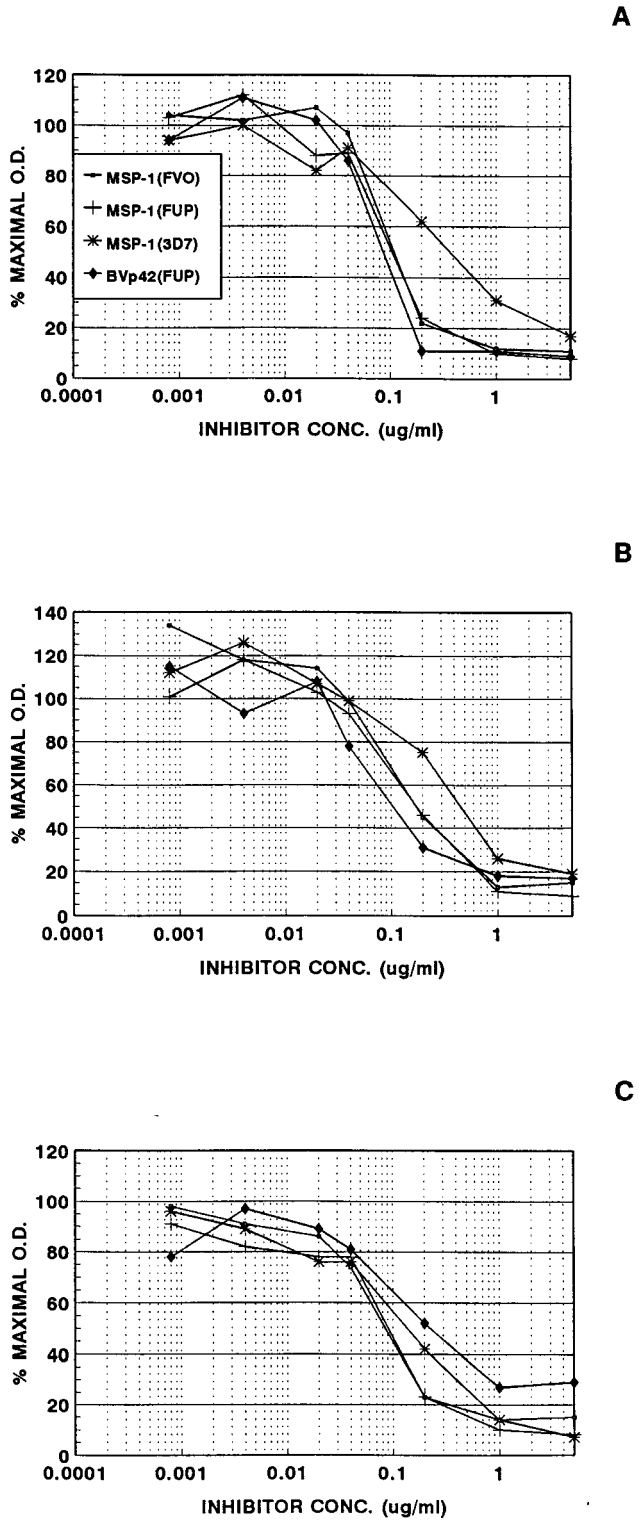

FIG. 5. ELISA inhibition of *Aotus* anti-MSP-1 antibodies by purified FUP parasite MSP-1, purified FVO parasite MSP-1, purified 3D7 parasite MSP-1, and recombinant BVp42. (A) *Aotus* monkey 417; (B) *Aotus* monkey 81269; (C) *Aotus* monkey 81512. For the ELISA inhibition assay, diluted *Aotus* quaternary day 14 sera were mixed with various concentrations of the indicated inhibitor proteins, incubated for 1 h, and added to BVp42-coated plates. O.D., optical density.

Aotus anti-BVp42 antibodies recognized a variety of MSP-1 processing fragments as well as the high-molecular-weight precursor molecule by immunoblot. However, ELISA and competitive inhibition results showed that the major epitopes recognized by these antibodies localize to the 19-kDa C-terminal processing fragment represented by yeast rMSP-1_{19} and correspond to conserved determinants shared with heterologous MSP-1 molecules. The importance of conserved conformational epitopes within the MSP-1 19-kDa C-terminal region has been suggested by in vitro growth inhibition studies carried out with different isolates of *P. falciparum* (16, 17). Furthermore, a yeast recombinant polypeptide based on this 19-kDa region induced an immune response which protected monkeys against parasite challenge (23). The parasite MSP-1 proteins evaluated in the ELISA competitive inhibition studies represent the three major variant sequences of the C-terminal region (19, 20), and one of these (3D7) is derived from a parasite clone used in human clinical trials of potential malaria vaccines. These findings have important implications in the vaccine design since they suggest that anti-MSP-1 antibodies directed against a single sequence within the 19-kDa C-terminal region may be effective against most *P. falciparum* isolates in nature.

The stimulation of antigen-specific T cells was also assessed in vaccinated animals by the lymphocyte proliferation assay. T-cell priming was evident in most animals immunized with BVp42 in CFA. Peripheral blood lymphocytes of these animals responded to both recombinant BVp42 and purified parasite MSP-1, indicating that primates may process the recombinant and native antigens similarly, resulting in the generation of common antigenic peptides on the surface of antigen-presenting cells that are readily recognized by BVp42-primed T cells. High levels of antigen-specific IgG antibodies were observed in all vaccinated animals, suggesting effective induction of T helper cells although the lymphocyte proliferation assay performed after the second immunization detected variable levels of antigen-specific T cells in peripheral blood. One of the four control animals, *Aotus* monkey 81560, appeared to have expanded an MSP-1-reactive T-cell population as a result of priming with CFA alone since this animal had not responded to MSP-1 antigens in preimmunization, baseline proliferation studies. BVp42-specific T cells were detected in all vaccinated *Aotus* monkeys in follow-up T-cell proliferation studies carried out after completion of the vaccination experiment (data not shown). Effective priming of T helper cells by BVp42 fulfills a critical requirement for any malaria vaccine formulation for which boosting by natural infection is desired. It was also noted that antigen-primed lymphocytes of these animals did not undergo proliferation when restimulated in vitro with rMSP-1_{19}, suggesting that the 19-kDa polypeptide either does not contain a sequence corresponding to a major BVp42 T-cell epitope or is not processed in a manner which generates antigenic peptides containing epitopes common with BVp42. We have previously reported that a variety of mouse major histocompatibility complex haplotypes are capable of generating an antibody response to parasite MSP-1 (6). Similar studies carried out with BVp42 have shown that B10 congenic mice representing seven different major histocompatibility complex haplotypes were all able to produce a vigorous antibody response (unpublished data), indicating that responsiveness to BVp42 T-cell epitopes also is not restricted to a few major histocompatibility complex haplotypes. A major question which remains to be addressed is whether or not BVp42 T-cell epitopes are shared among various MSP-1 isolates, and this issue is currently under investigation.

The in vitro parasite inhibition assay has been useful in the

evaluation of antibodies for potential direct effects on parasite invasion and/or growth (2, 4, 18, 27). However, the relevance of this assay to the mechanism(s) of immunity in vivo is controversial (3). In the present study, whole sera of two of three of the protected *Aotus* monkeys consistently produced complete inhibition of parasite growth. The serum of the third protected *Aotus* monkey (no. 81512) appeared to be less inhibitory, generating 57 and 70% parasite growth inhibition in duplicate experiments. However, Ig purified from immune sera of all three protected animals displayed a similar high level of inhibitory activity. The difference in the degree of inhibition measured with purified Ig compared with whole serum primarily reflected the higher control parasitemias achieved in cultures containing purified Ig. It was apparent that serum components removed by ammonium sulfate precipitation had a slight inhibitory effect on in vitro parasite growth. Removal of these components facilitates the assessment of specific antibody effects in the absence of non-specific inhibitors. Thus, protected animals as a group tended to produce antibodies which were capable of affecting in vitro parasite growth while control sera had no inhibitory effect. The level of inhibition did not completely correlate with the degree of protection against infection, since the sera of *Aotus* monkey 81269 were strongly inhibitory of in vitro parasite growth and yet this animal was only partially protected against infection. These results suggest that complete resolution of infection may require other mechanisms, such as cell-mediated immunity, which may have been suboptimal in *Aotus* monkey 81269. However, it should be noted that this animal had a very high antigen-specific T-cell proliferative response, indicating effective T-cell priming. Of the three *Aotus* monkeys immunized with BVp42-CFA, only *Aotus* monkey 81269 showed markedly decreased antibody titers 5 weeks after parasite challenge. Although this decline may have been due to antibody absorption by circulating parasites, it may also have reflected a lower antibody titer at the time of challenge. It may be difficult to accurately predict the level of antimalarial immunity by evaluating the growth inhibitory activity of sera collected several weeks before in vivo parasite challenge. Despite the incomplete correlation of the level of in vitro parasite inhibition with the degree of protection, our results clearly showed that all protected *Aotus* monkeys produced antibodies which had a direct effect on parasite growth and that this direct mechanism of antibody-mediated growth inhibition appears to be relevant to in vivo immunity. These results differ from those reported for the yeast 19-kDa polypeptide by Kumar et al. (23), which induced protective immunity in the absence of detectable parasite-inhibitory serum antibodies, and suggest that immunity in these two vaccination experiments may have been due to distinct mechanisms. It will be important to completely define the fine specificity, isotype, and functional properties of the inhibitory antibodies and to determine whether protection induced by BVp42 vaccination is achieved primarily via a direct effect of antibodies on parasite growth or invasion or may also involve other indirect antibody-mediated mechanisms (3) or cell-mediated mechanisms as suggested by recent results in the rodent malaria model (9).

Although the production of antibodies recognizing conserved C-terminal MSP-1 epitopes represents the majority of antibodies induced by BVp42-CFA immunization, the recognition of the C-terminal 19K region by these antibodies is not sufficient for protective immunity to malarial infection on the basis of the results of parallel studies of *Aotus* monkeys vaccinated with BVp42 formulated with certain synthetic adjuvants (15a). In these studies, not all *Aotus* monkeys producing high-titered antibodies of the above specificity were protected against infection. Thus, antibodies critical for protective immunity to malaria may represent a specific subset of the antibody population recognizing conserved C-terminal MSP-1 epitopes which are distinct in fine specificity or functional properties.

Recent longitudinal studies of serological responses to MSP-1 indicate a correlation between MSP-1 C-terminal region-specific antibody titers and resistance to subsequent malarial infection (1, 28), suggesting that development of immunity to malaria induced by natural parasite exposure may parallel the immunity developed by vaccination with recombinant BVp42. It will be of interest to compare MSP-1-specific mechanisms of immunity developed in naturally immune humans and vaccinated nonhuman primates and their relative efficiencies in protection against *P. falciparum* infection.

ACKNOWLEDGMENTS

We thank Sylvia Kondo and the University of Hawaii Laboratory Animal Services staff for their assistance, the blood banks of Tripler Army Medical Center and Queen's Medical Center for their cooperation, and David Kaslow for the generous gift of $rMSP1_{19}$ used in this study. We also thank Wasim Siddiqui for his critical reading and helpful discussions of the manuscript. We acknowledge Daiichi Pharmaceutical Co. for purchase and partial support of some of the monkeys used in this study.

This work was supported by the Malaria Vaccine Development Program of the U.S. Agency for International Development through Cooperative Agreement no. HRN-6001-A00-202-00 and Chiron Corporation.

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Editor: J. M. Mansfield