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Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria

WASIM A. SIDDIQUI*, LESLIE Q. TAM, KENTON J. KRAMER, GEORGE S. N. HUI, STEVEN E. CASE, KAREN M. YAMAGA, SANDRA P. CHANG, EUGENE B. T. CHAN, AND SIU-CHOW KAN

Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, Leahi Hospital, 3675 Kilauea Avenue, University of Hawaii, Honolulu, HI 96822

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ABSTRACT **Groups of *Aotus* (owl) monkeys were immunized with either the *Plasmodium falciparum* merozoite surface-coat precursor protein and its processing fragments or a complex of high molecular mass rhoptry proteins and challenged with a lethal infection of the homologous *P. falciparum* Uganda Palo Alto (FUP) strain. No patent parasitemia could be detected on thick blood films of monkeys immunized with the merozoite surface antigens; however, only one of three monkeys immunized with the rhoptry proteins was partially protected, while two required drug therapy. The experiment clearly demonstrates that the merozoite surface-coat precursor protein can completely protect *Aotus* monkeys against a lethal infection of the human malaria parasite.**

Because of the emergence of drug-resistant strains of the malaria parasite *Plasmodium falciparum* and insecticide-resistant mosquito vectors, malaria has become a major public health problem in developing countries. Recent advances in biotechnology have introduced the possibility that a recombinant protein or synthetic peptide malaria vaccine may supplement existing public health control measures in endemic areas. An important step in the engineering of a modern vaccine is the identification of protective antigens to which gene cloning techniques can be applied. In 1981, Holder and Freeman showed that monoclonal antibody (mAb)-purified proteins on the merozoite surface and in the merozoite rhoptries of *Plasmodium yoelii* were important classes of protective antigens in a murine malaria model (1). Subsequent studies with *P. falciparum* have shown that similar antigens on the human malaria parasite are partially protective in monkey experiments (2–5).

The major *P. falciparum* merozoite surface-coat proteins are the 83-, 42-, and 19-kDa processing fragments (6) derived from a glycosylated, polymorphic precursor molecule having a variable molecular mass of 185–200 kDa, depending on the strain studied (7–15). Immunization with the mAb-isolated precursor protein and its processing fragments (2) or with the precursor protein alone, isolated by preparative $NaDodSO_4$/PAGE (3), partially protects *Saimiri* (squirrel) monkeys against heterologous challenge and indicates that conserved, nonpolymorphic regions may have vaccine potential.

The rhoptries are a pair of electron-dense, flask-shaped organelles near the apical end of the merozoite. Ultrastructure studies indicate that the contents of the rhoptries are discharged through ducts onto the erythrocyte membrane during the invasion process and may help form the parasitophorous vacuole membrane (16, 17). mAbs have identified high molecular mass [approximately 130–155 kDa (18–20)] and low molecular mass [approximately 40–80 kDa (4, 19, 21, 22)] protein complexes in the rhoptries. The high molecular mass rhoptry proteins that are coprecipitated by mAb appear to be noncovalently associated and are not processing fragments of a single precursor protein (18). *Saimiri* monkeys were strongly protected by a "native" 41-kDa rhoptry protein against heterologous challenge; however, the $NaDodSO_4$ "denatured" protein was markedly less protective (4). A 155-kDa protein, originally described on the surface of ring-infected erythrocytes (designated RESA) (23, 24), has recently been localized in the merozoite rhoptry–microneme complex prior to erythrocyte invasion (25). Immunization with recombinant RESA polypeptides, based on the Papua New Guinea isolate, partially protects *Aotus* monkeys against a virulent Indochina strain (5).

Recently, we reported a pilot experiment in which a combination of the merozoite surface-coat precursor protein and its processing fragments together with the high molecular mass rhoptry protein complex, both isolated by mAb affinity chromatography, completely protected a karyotype VI *Aotus* monkey with no patent parasitemia when administered with a strong adjuvant (20). In this *Aotus* karyotype, the *P. falciparum* Uganda Palo Alto (FUP) isolate produces a mild infection of up to 4% parasitemia and is followed by self-cure. In the present experiment, we examined whether immunization with either antigen alone or in combination could protect *Aotus* karyotype II and III monkeys in which the same FUP infection is consistently lethal. Following homologous challenge, no patent parasitemia could be detected in thick blood films of monkeys immunized with the merozoite surface-coat precursor protein alone; however, monkeys immunized with rhoptry proteins were either partially or not protected. The results identify the merozoite surface-coat precursor protein as a strong blood-stage candidate upon which a recombinant protein or synthetic peptide malaria vaccine can be based.

MATERIALS AND METHODS

Parasites. The FUP monkey-passaged knob-positive strain was used throughout this experiment. It has been repeatedly passaged through *Aotus* monkeys since its isolation in 1967 from a patient in Palo Alto returning from Uganda. The FUP parasites were cultured by the method of Trager and Jensen (26) as modified by Siddiqui and Palmer (27). Cultures were initiated from cryopreserved blood from an infected *Aotus* monkey and maintained in continuous culture for no more than 8 weeks. During this period, all parasites were knob-positive as examined by electron microscopy. Cultures were harvested weekly or when the parasitemia rose above 10%. The infected erythrocytes were washed three times in 0.85% NaCl and lysed with a final concentration of 0.013% saponin. Whole parasites were collected by centrifugation at 2500 × *g* and subsequently washed three times in saline, quickly frozen, and stored at −70°C until extraction.

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Abbreviations: FUP, *P. falciparum* Uganda Palo Alto strain; IFA, indirect immunofluorescence assay; mAb, monoclonal antibody.

*To whom reprint requests should be addressed.

Isolation of Antigens. Approximately 2.3 × 10^{11} infected erythrocytes (containing approximately 33% each of rings, trophozoites, and schizonts) were extracted with 7 volumes of 1% Nonidet P-40 detergent containing 10 mM iodoacetamide, 5 mM EGTA, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride in borate-buffered saline (pH 8.0). Approximately 114 mg of protein, estimated by the Coomassie method (Bio-Rad), was supplemented with [^{35}S]methionine-labeled *P. falciparum* polypeptides, and the extract was passed serially through 3 ml each of high-capacity mAb 5.2 (anti-merozoite surface) and mAb 219.5 (anti-rhoptry) protein A-Sepharose columns, respectively, as described (20). To remove nonspecifically bound proteins, the columns were then washed with 50 volumes of borate-buffered saline containing 1 M NaCl and 0.1% Nonidet P-40. Bound proteins were then eluted with 50 mM diethylamine (pH 11.5) containing 10 mM iodoacetamide, 1 mM EDTA, 1 mM EGTA, and 0.1% Nonidet P-40 and were neutralized with 1 M Tris·HCl (pH 8.0). Peak radioactive tubes were pooled, dialyzed against borate-buffered saline, and concentrated by Amicon filtration. The yield was 1.9 mg of merozoite surface proteins and 3.5 mg of rhoptry proteins. The isolated antigens were characterized by silver staining and iodination methods explained in the legend to Fig. 1.

Immunization and Challenge. A total of 11 *Aotus lemurinus griseimembra* (karyotype II and III) monkeys were used in this experiment. In this *Aotus* karyotype, the FUP K^+ strain consistently produces a lethal infection without splenectomy, reaching 70–80% parasitemia. Monkeys A398, A400, and A401 were each immunized three times with 100 μg per injection of the merozoite surface-coat precursor protein and its various processing fragments. Monkeys A373, A397, and A399 were each immunized three times with 100 μg per injection of the rhoptry proteins. Monkeys A368, A372, and A375 were given three injections each containing 100 μg of the merozoite surface-coat precursor protein and 100 μg of the rhoptry proteins. Each dose was given in 1 ml of Freund's complete adjuvant (GIBCO) at 21-day intervals injected into multiple sites intramuscularly and subcutaneously. The amount of mycobacterium was approximately 250, 125, and 62.5 μg/ml of emulsion for primary, secondary, and tertiary immunizations, respectively. Two monkeys (H7 and H25) were unimmunized controls. All *Aotus* monkeys were challenged 3 weeks after the third immunization with approximately 7 × 10^5 FUP knob-positive monkey-passaged parasites.

Evaluation of Immune Responses. Approximately 1-ml serum samples were obtained 14 days after each immunization for evaluation of antibody titers by indirect immunofluorescent assay (IFA) on acetone-fixed blood smears and immunoprecipitation of [^{35}S]methionine-labeled polypeptides by methods previously described (20).

RESULTS

Characterization and Immunogenicity of mAb-Purified Merozoite Surface and Rhoptry Proteins. Primarily the merozoite surface-coat precursor protein was prominent by silver staining of the antigen preparation eluted from the mAb 5.2 (anti-merozoite surface) affinity column (Fig. 1*a*); however, additional minor 152-, 121-, and 83-kDa proteins were visualized by iodination (Fig. 1*b*). The 185-kDa precursor and the above processing fragments in the FUP strain also have been immunoprecipitated by mAb 89.1 (7), obtained from the Wellcome group (unpublished data).

Serum samples from monkeys A398, A400, and A401, immunized with the merozoite surface-coat precursor protein, precipitated the 185-kDa precursor and 152-, 121-, 83-, 73-, and 42-kDa [^{35}S]methionine-labeled proteins (Fig. 2, lanes 4–6, respectively). The 83- and 42-kDa proteins have been described as terminal-processing products of the merozoite surface-coat precursor protein (6). Intermediate processing fragments of approximately 152 kDa also have been described (7, 14), and additional 50- (13), 73-, and 45-kDa (14, 28) processing fragments also have been precip-

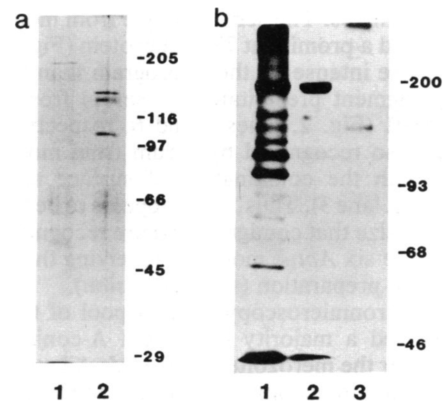

FIG. 1. Analysis of proteins affinity-purified by anti-merozoite surface mAb 5.2 (*a*) and anti-rhoptry mAb 219.5 (*b*). (*a*) Silver staining after resolution by NaDodSO$_4$/PAGE (7.5% gel). Lanes: 1, merozoite surface proteins; 2, rhoptry proteins. The molecular mass standards are myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg albumin (46 kDa), and carbonic anhydrase (29 kDa) from Sigma. (*b*) Aliquots were also iodinated by Iodobeads (Pierce) and resolved on NaDodSO$_4$/PAGE (6.25% gel) under reducing conditions. Lanes: 1, total FUP antigens affinity-purified by immune *Aotus* IgG (25); 2 and 3, iodinated merozoite surface and rhoptry proteins, respectively. The molecular mass markers are myosin (200 kDa), phosphorylase *b* (93 kDa), bovine serum albumin (68 kDa), and ovalbumin (46 kDa) from Amersham.

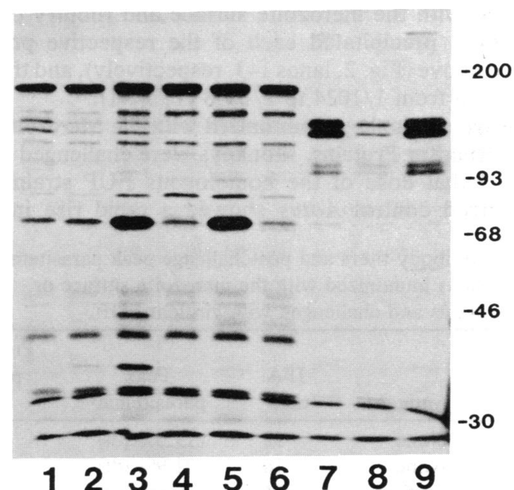

FIG. 2. NaDodSO$_4$/PAGE of the [^{35}S]methionine-labeled FUP proteins immunoprecipitated by serum samples obtained from *Aotus* monkeys 14 days after the third immunization. Lanes: 1–3, proteins immunoprecipitated by serum samples from monkeys A368, A372, and A375, respectively, immunized with the combination of merozoite surface and rhoptry proteins; 4–6, proteins immunoprecipitated by serum samples from *Aotus* monkeys A398, A400, and A401, respectively, immunized with the 185-kDa merozoite surface-coat precursor protein and its processing fragments isolated by mAb 5.2; 7–9, proteins immunoprecipitated by serum samples from monkeys A373, A397, and A399, respectively, immunized with the complex of 143-, 132-, and 102-kDa proteins isolated by anti-rhoptry mAb 219.5. The immunoprecipitation was performed by methods previously described (20). The molecular mass markers are the same as in Fig. 1*b*, except that ovalbumin (46 kDa) and carbonic anhydrase (30 kDa) were separated in the 7.5% polyacrylamide gel.

itated by other mAbs. The serum sample from monkey A400 also precipitated a prominent 73-kDa protein (Fig. 2, lane 5), which was more intense on the fluorogram than the 73-kDa processing fragment precipitated by serum from monkeys A398 and A401 (Fig. 2, lanes 4 and 6, respectively). This protein was also recognized by serum from monkey A375 immunized with the combination of surface and rhoptry proteins (Fig. 2, lane 3). Thus, there appears to be two protein species of this size that comigrate and are recognized by sera from two of the six *Aotus* monkeys receiving the merozoite surface-protein preparation (see *Discussion*).

Immunoelectronmicroscopy using a pool of these serum samples showed a majority of protein A-conjugated gold distributed over the merozoite surface (M. Aikawa, personal communication), indicating that the predominant antibody response was directed to the merozoite surface. The IFA titers on serum samples from these animals ranged from 1/4098 to 1/8192 (Table 1).

The anti-rhoptry mAb 219.5 immunoabsorbent-isolated 143-, 132-, and 102 (doublet)-kDa proteins were visualized by silver staining (Fig. 1*a*). These same proteins have been immunoprecipitated by mAb 61.3 (18), also obtained from the Wellcome group (unpublished data). The iodinated preparation showed only the 143-kDa protein in $NaDodSO_4$/PAGE; however, iodination of the rhoptry proteins caused much of the material to form large aggregates, which could not be resolved under reducing conditions.

Serum samples from monkeys A373, A397, and A399, immunized with the rhoptry proteins, precipitated the 143-, 132-, and 102-kDa proteins (Fig. 2, lanes 7–9, respectively) and had IFA titers of 1/1024 to 1/4096 (Table 1). Immunoelectronmicroscopy using a pool of these serum samples revealed protein A-conjugated gold prominently distributed in the merozoite rhoptries and rarely in the micronemes (M. Aikawa, personal communication).

Serum samples from monkeys A368, A372, and A375, immunized with the merozoite surface and rhoptry protein combination, precipitated each of the respective proteins described above (Fig. 2, lanes 1–3, respectively), and the IFA titers ranged from 1/1024 to 1/2048 (Table 1).

Challenge of Monkeys Immunized with the Merozoite Surface and Rhoptry Proteins. Monkeys were challenged with a virulent, lethal dose of the homologous FUP strain. Two unimmunized control *Aotus* showed a rapid rise in parasitemia and were treated with drugs to prevent fatal infection. No parasites could be detected in thick-blood films of monkeys immunized with the merozoite surface-coat precursor protein (Fig. 3) over a 60-day period. In contrast, monkeys A373 and A397, immunized with the rhoptry proteins, developed high parasitemias and were drug-treated, while monkey A399 had a prepatent period of 9 days (compared to the 2–6 days of prepatency in unimmunized controls) and self-cured after a 4.4% peak parasitemia (Table 1). The monkeys receiving the combination of proteins had prepatent periods of 10–17 days and peak parasitemias of about 2% (Table 1). All three monkeys cleared the infection without drug treatment by day 31.

To evaluate the possibility that a sterile immunity had been achieved, blood was withdrawn 32 days after challenge from A398, A400, and A401 monkeys, washed of autologous immune serum, and cultured *in vitro*. Parasites were detected approximately 10 days later from the blood of monkeys A400 and A401, but not A398, indicating that, although no parasites were observed on thick blood films, a sterile immunity had not been achieved.

Table 1. Antibody titers and postchallenge peak parasitemias in *Aotus* monkeys immunized with the merozoite surface or rhoptry proteins and challenged with virulent FUP

Monkey	Antigen*	IFA titer†	Peak parasitemia‡	Prepatent period, days
H7	None	32	19.6% (dt)	2
H25	None	16	21.9% (dt)	6
A398	Surface	4096	Negative	
A400	Surface	8192	Negative	
A401	Surface	8192	Negative	
A373	Rhoptry	1024	20.5% (dt)	5
A397	Rhoptry	4096	14.3% (dt)	5
A399	Rhoptry	2048	4.4%	9
A368	Both	1024	2.3%	10
A372	Both	2048	2.1%	17
A375	Both	2048	2.4%	11

*Both means immunization with a combination of surface and rhoptry proteins.

†Reciprocal of the highest dilution of the 14-day, tertiary serum giving a positive result by IFA.

‡Negative means no patent parasitemia could be detected in thick-blood films over a 60-day period; however, parasites were cultured from blood samples of monkeys A400 and A401. dt, Drug-treated.

DISCUSSION

The results of this vaccination experiment demonstrate that *Aotus* type II and III monkeys, in which infection with FUP is consistently lethal, are completely protected without patent parasitemia detectable on thick-blood films when immunized with the merozoite surface-coat precursor protein and its processing fragments. In two previous vaccination experiments with this protein, partial protection was obtained (2, 3). In one experiment, *Saimiri* monkeys were immunized with the precursor protein isolated from a Thai strain and challenged with the FUP strain. One monkey required drug therapy, and the parasitemia in the remaining two monkeys reached 10% (2). In the second experiment, *Saimiri* monkeys were immunized with the protein from a Zaire strain and challenged with FUP strain (3). Again, only partial protection was obtained, with parasitemias ranging from 2% to 10%.

In this report, the *Aotus* monkeys were immunized with the precursor protein from the FUP strain and challenged with parasites of the same strain. Although comparisons among the experiments are difficult because of differences in methodologies, one explanation for the strong protection observed in this vaccination experiment is that the critical epitopes responsible for protection may be polymorphic and that complete protection is achieved only with homologous challenge.

McBride and co-workers have classified the merozoite surface-coat precursor protein into seven serological groups exhibited by 37 strains from different geographical areas of the world, using a panel of mAbs (10). This indicates that only a limited repertoire of antigenic diversity with respect to this protein may exist in nature. Thus, antigenic polymorphism in the malaria parasite appears to differ from the continuous antigenic variation observed in the trypanosomes, and a multivalent recombinant construct or synthetic peptide malaria vaccine based on defined polymorphic determinants may be feasible.

The precise nature of polymorphism of the merozoite surface-coat precursor protein will be revealed through antigenic analysis (10, 11) and by comparison of gene structures (15) and sequences (29–32) as they become available. The complete gene sequence of the merozoite surface-coat precursor protein in the Wellcome–Lagos (29) and Thai-K1 (30) strains have been published recently. The two genes code for nearly identical amino acid sequences (99% homology), with the exception of a small repeat region bearing variations of the tripeptide Ser-Xaa-Xaa. This appears con-

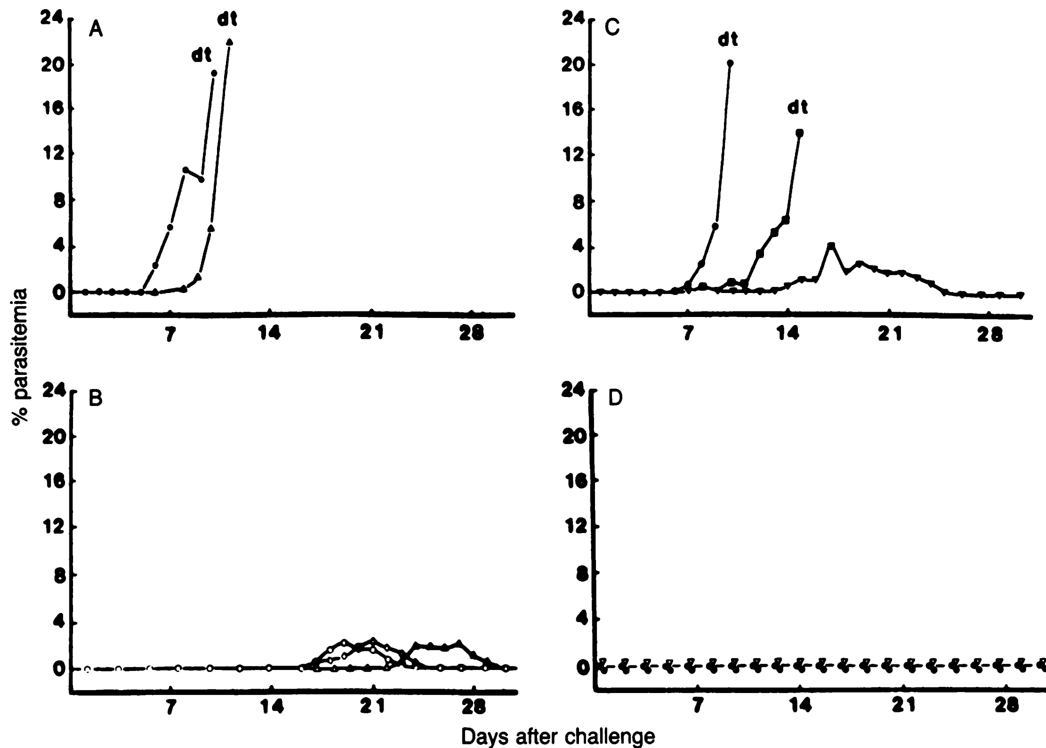

FIG. 3. Course of infection of a virulent, monkey-passaged FUP in vaccinated *Aotus lemurinus griseimembra* (karyotype II and III) monkeys. (*A*) Control. Two unimmunized monkeys, H7 (●) and H25 (▲), had high parasitemias and were drug-treated (dt) to prevent death. (*B*) Immunization with merozoite surface/rhoptry protein combination. Three monkeys, A368 (○), A372 (△), and A375 (◇), immunized with a combination of the merozoite surface and rhoptry proteins, had an increased prepatent period and peak parasitemias of about 2%, followed by spontaneous recovery. (*C*) Immunization with rhoptry proteins. Two of the three monkeys, A373 (●) and A397 (■), that were immunized with the 143-, 132-, and 102-kDa rhoptry proteins had high parasitemias and required drug treatment (dt), while the third monkey, A399 (▼), was partially protected. (*D*) Immunization with merozoite surface proteins. All three *Aotus* monkeys, A398 (▽), A400 (●), and A401 (○), immunized with the 185-kDa merozoite surface-coat precursor protein and its processing fragments were completely protected, and no patent parasitemia was detected in thick-blood films over a 60-day period.

sistent with the placement of both strains into serotype I of the McBride classification. The partial gene sequence of a 3.6-kilobase fragment encoding the N terminus of the precursor protein in the CAMP (Malaysia) strain reveals five conserved regions (>63% amino acid homology) separated by nonconserved segments (ranging from 9% to 37% amino acid homology) when compared to the Wellcome sequence (32). Preliminary studies on the gene structure of the FUP precursor protein in our laboratory indicate that differences exist between the FUP protein (serotype VI) and the Wellcome protein (serotype I). We have found that a 50-mer oligonucleotide probe, based on the Wellcome sequence and falling into the second variable region defined by the comparison of the CAMP, Wellcome–Lagos, and Thai K1 strains, failed to hybridize with the FUP genomic DNA. However, a similar probe falling within a sequence that is conserved between the CAMP, K1, and Wellcome strains identified a FUP genomic fragment of a size appropriate for the merozoite surface-coat precursor gene (unpublished data). Thus, the designing of a malaria vaccine based on this merozoite surface-coat precursor protein may require the extensive cloning and sequencing of genes in endemic areas, with attention given to diverse or strain-specific epitopes.

Although a 73-kDa processing fragment has been reported in the processing scheme of the merozoite surface-coat precursor protein (14), the prominent 73-kDa protein precipitated by the sera of monkeys A375 and A400 (Fig. 2, lanes 3 and 5) is probably not a processing fragment of the merozoite surface antigen and instead represents a second comigrating protein for the following reasons. First, this intense band was precipitated by sera from only two of the six *Aotus* monkeys immunized with the merozoite surface precursor protein and from one (K18) of two rabbits immunized with the same antigen preparation (not shown). Second, using the polyclonal antisera from rabbit K18 to screen phage λgt11 libraries, we have obtained a clone that does not hybridize with any of the clones encompassing the entire merozoite surface-coat precursor gene in FUP but encodes the partial sequence of an unrelated 73-kDa protein of *P. falciparum* (unpublished data). Furthermore, rabbit K18 antibodies, affinity-purified on this recombinant fusion protein, precipitated a 73-kDa [^{35}S]methionine-labeled FUP protein but not the merozoite surface-coat precursor protein (unpublished data). Although the mAb immunoabsorbent used to isolate the merozoite surface proteins was washed thoroughly with buffer containing 1 M NaCl, this 73-kDa protein was probably a minor contaminant in the antigen preparation. Because only two of the six *Aotus* monkeys that were protected made antibody to this protein, it is unlikely that it was involved significantly in protection.

Two of the three monkeys immunized with the rhoptry proteins required drug treatment, while one was partially protected. These results are similar to vaccination experiments with other *P. falciparum* rhoptry proteins. A NaDodSO$_4$-denatured 41-kDa rhoptry protein induced only partial protection (4), and recombinant polypeptides to RESA, a 155-kDa protein originating in the microneme–rhoptry complex prior to merozoite invasion (25), have similarly induced variable immune responses, although protection was correlated with those monkeys making immune responses to the recombinant polypeptides (5). The rhoptry proteins used in the present experiment were eluted from the mAb column with buffer at pH 11.5, and it is possible that the proteins were partially denatured. Conformation may be critical to the

antigenicity of the 143-, 132-, and 102-kDa rhoptry proteins used in this experiment as it is with the 41-kDa rhoptry protein (4).

Although merozoite surface-coat precursor protein alone induced strong immunity without patent parasitemia, the combination of surface and rhoptry proteins did not give complete protection. After an increased prepatent period, parasitemias in all three monkeys rose to about 2%. Although the degree of protection seen in these monkeys was less than that of the merozoite surface-coat precursor protein-immunized group, it was similar to the results obtained from our previous successful vaccination experiments using whole parasites as immunogen (33–35). We have found that the 143-, 132-, and 102-kDa proteins aggregate with the merozoite surface-coat precursor protein when the mixtures were iodinated (unpublished data). It is possible that the protective epitope(s) in the merozoite surface-coat precursor protein were altered upon contact with the rhoptry proteins.

This vaccination experiment demonstrates that a single, mAb-isolated protein can induce complete protective immunity to a lethal human malaria parasite. However, the strong protection was induced when using Freund's complete adjuvant. In a pilot experiment, we had reported that the same merozoite proteins induced complete protection without patency in a karyotype VI *Aotus* monkey when given with Freund's complete adjuvant, whereas the proteins given with a synthetic muramyl dipeptide derivative did not induce protection, despite similar antibody titers (20). Therefore, the protective epitope(s) on the merozoite surface-coat precursor protein may be weakly immunogenic and requires a strong adjuvant. Thus, an effective blood-stage vaccine based on this protein may depend not only on the cloning and expression of relevant epitope(s) but also on the development of a safe and effective adjuvant.

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