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Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies

(malaria/asexual blood stage antigens/antipeptide antibodies)

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ABSTRACT **Polypeptides expressed on the surface of merozoites, the invasive stage of the asexual blood cycle, are good candidates for the development of malaria vaccines. Five synthetic peptides with predetermined specificity deduced from a genomic DNA clone coding for the NH_2-terminal portion of the main merozoite surface polypeptide of *Plasmodium falciparum* were evaluated for their capability to raise antibodies that react with the *P. falciparum* merozoites. Antibodies induced by two of the peptides (3 and 5) reacted with the membrane surfaces of seven of seven isolates of *P. falciparum* from different geographic areas. Antibodies against peptide 4, which contains a repeated amino acid sequence (Gly-Gly-Ser and Val-Ala-Ser), reacted with six of seven isolates. Structural analysis of the deduced polypeptides suggests that peptide 3 is exposed at the surface of merozoites. When it was used to immunize monkeys, three of the four animals were partially protected from a challenge infection that induced a fulminant infection in control animals.**

There is evidence to suggest that parasite components expressed at the surface of the invasive stages (sporozoite and merozoite) of *Plasmodium falciparum*, the most lethal malaria parasite species for humans, may be used for the development of malaria vaccines (1–5). Such vaccines require large amounts of antigens, which cannot be easily purified from the parasites but can be produced in large quantities by use of recombinant DNA technology (6–9).

Previously, we have isolated a plasmid (pMC31-1) that codes for a portion of the schizont (180–200 kDa) and merozoite (83 kDa)-specific polypeptide from a cDNA library constructed from mRNA purified from the asexual blood stages of *P. falciparum* (isolate SGE2 from Zaire). The 83-kDa protein is a processed product of the 180- to 200-kDa polypeptide and it is expressed at the surface of the merozoites (5, 9). The potential value for vaccine development of the malaria-specific protein encoded by pMC31-1 plasmid is substantiated by the finding that antisera raised against lysates of pMC31-1-containing bacteria react with the merozoite surface of five out of five *P. falciparum* isolates from various geographic locations and by immunization trials showing that monkeys immunized with the 180- to 200-kDa polypeptide are resistant to a blood-induced challenge infection (10). The aim of this study was to evaluate the possibility of raising antibodies against this protein by immunization with synthetic peptides of predetermined specificity containing either unique or repeated amino acid sequences deduced from the cDNA sequence. Antisera raised against the synthetic peptides were tested for their capacity to react with asexual blood stage parasites and with the 180- to 200-kDa polypeptide and its processed products. Finally, a synthetic polypeptide was selected for an immunization experiment with monkeys.

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MATERIALS AND METHODS

Parasites. Asynchronous *in vitro* cultures of seven *P. falciparum* isolates—SGE2 (from Zaire), FUP Palo Alto (Uganda), FCR3 clone A2 (West Africa), FCC2 (China), M23 (Honduras), clone Tak9.94 (Thailand), and NF54 (Africa)—were grown as described (10). When required, these cultures were radioactively labeled or were synchronized by mannitol treatment (9–11).

Schizont and merozoite preparations were used for immunoblotting experiments and for the coating of multispot slides, which were subsequently used for indirect immunofluorescence studies (12). Ring forms of SGE2 collected after mannitol treatment of asynchronous cultures of SGE2 and peripheral blood from a patient with acute *P. falciparum* infection (2% ring forms, no mature asexual blood stages) were also used for the coating of multispot slides.

Synthetic Polypeptides. Peptides were synthesized by the solid-phase method (13, 14), using a Vega model 250 synthesizer (Vega Biotechnology, Tucson, AZ). The peptides were deblocked and cleaved from the resin with liquid HF containing 10% anisole (15). The peptides were purified by gel filtration (Sephadex G-25) and medium-pressure reversed-phase chromatography. All the peptides were approximately 90% pure when analyzed by silica gel thin-layer chromatography using 1-butanol/pyridine/acetic acid/water (30:30:6:24, vol/vol) as the mobile phase. For conjugation with peptides, 10 mg of bovine serum albumin (BSA) or tetanus toxoid (Tetanus Seratoxin, Bern, Switzerland) were dissolved in 2 ml of 0.1 M sodium phosphate buffer (pH 7) and then mixed with 2 mg of peptide dissolved in 100 μl of water. During a 1-hr period, 1 ml of 2.5% glutaraldehyde was added in several portions and the mixture was stirred for another 6 hr. Finally, the conjugate was dialyzed against Dulbecco's phosphate-buffered saline (PBS; Hazelton Laboratories, Denver, PA) overnight. The 180- to 200-kDa parasite antigen and the pMC31-1-encoded fusion peptide were purified as described (9, 10).

Antisera. Rabbits were immunized with *Escherichia coli* lysate containing the fusion protein encoded by pMC31-1 (9) and with each of the five synthetic polypeptides coupled to tetanus toxoid. The rabbits were immunized three times, at 2-week intervals, with the pMC31-1 lysate (2 mg of protein) or with one of the synthetic polypeptides coupled to tetanus toxoid (1.5 mg of total protein, including the tetanus toxoid carrier). For the first immunization, 1 ml of antigen in PBS

Abbreviation: BSA, bovine serum albumin.

was mixed with an equal volume of Freund's complete adjuvant, and for subsequent immunization, the samples were mixed with Freund's incomplete adjuvant. Antisera were collected 10 days after the third immunization and absorbed at 4°C for 1 hr with one volume of human erythrocytes to remove anti-erythrocyte antibodies.

Analysis of the Antibody Response. The preimmune and immune sera were tested for antimalarial antibodies by indirect immunofluorescence, enzyme-linked immunoabsorbent assay (ELISA), immunoblotting, and immunoprecipitation. For indirect immunofluorescence, serial dilutions of the antisera in PBS supplemented with 1% BSA were applied on multispot slide preparations previously fixed with acetone (9).

For the ELISA tests, 96 wells (Linbro ELISA plates, Flow Laboratories) were coated overnight at 4°C with 100 μl of a 0.1 M bicarbonate buffer (pH 9) containing either 0.5 μg of one of the various polypeptides coupled to BSA, 20 ng of the pMC31-1 fusion protein purified by $NaDodSO_4$/PAGE, or 20 ng of the 180- to 200-kDa native protein (10). The wells were then saturated with 1% BSA in PBS for 1 hr. One hundred microliters of serial dilutions of the antisera in PBS supplemented with 0.5% BSA were incubated in duplicate wells for 2 hr at 20°C. The wells were washed five times with 0.5% BSA supplemented with 0.04% Tween 20 (Siegfried, Zofingen, Switzerland), and alkaline phosphatase-conjugated IgG fractions of goat anti-rabbit immunoglobulin at a dilution of 1:1000 (Cappel Laboratories, Cochranville, PA) was used as second antibody. Enzyme-substrate solution (*p*-nitrophenyl phosphate, Sigma) was added to each well. After 2 hr of incubation at 20°C, 50 μl of 2.5 M sulphuric acid was added to stop the reaction. The optical density of each well was read at 405 nm, using a Titertek Multiscan spectrophotometer.

For immunoprecipitation experiments, [^{35}S]methionine-labeled *P. falciparum* SGE2 extract (4×10^5 cpm) was incubated with 5 μl of rabbit antiserum. Immune complexes were precipitated with protein A (10) and analyzed by $NaDodSO_4$/8% PAGE followed by autoradiography. Immunoblot analysis was carried out as described (9), using extract from 3×10^6 schizonts or 5×10^6 merozoites per lane. After the blot was incubated with rabbit antiserum (1:50 dilution), ^{125}I-labeled goat anti-rabbit IgG was added [4 ml (400,000 cpm) per blot]. The nitrocellulose paper was then exposed to x-ray film for 3 days.

Immunization of Monkeys. Eight *Saimiri* monkeys (600–800 g each), bred in the animal facilities of Hoffmann–La Roche (Basel, Switzerland) were divided into two groups of four monkeys. The control group of four monkeys was injected subcutaneously three times at four different sites, the first time with 1.5 mg of glutaraldehyde-treated tetanus toxoid mixed with Freund's complete adjuvant, and the second and third times with 1.5 mg of tetanus toxoid mixed with Freund's incomplete adjuvant (15 and 40 days after the first immunization). The second group of animals was immunized according to the same protocol but using 1.5 mg of peptide 3 (Fig. 1) coupled to tetanus toxoid. The eight monkeys were challenged at day 60 by an injection, into the femoral vein, of 2.5×10^7 parasites provided by a splenectomized monkey injected 8 days previously with the Ugandan FUP Palo Alto strain of *P. falciparum*. Parasitemia was determined at various intervals on Giemsa-stained thin blood smears. Before starting the experiment, it was decided

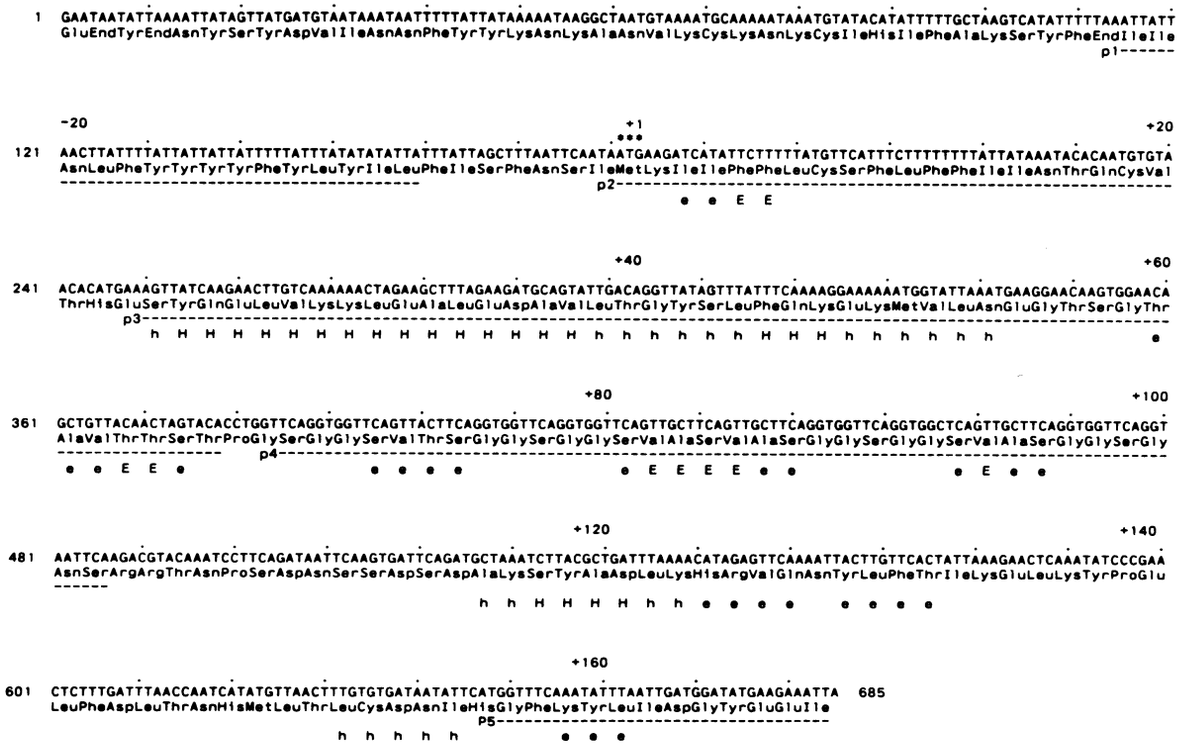

FIG. 1. Nucleotide sequence of a genomic clone and its deduced amino acid sequence. Nucleotide sequence was determined by the method of Maxam and Gilbert (19). Amino acid residue numbers are given above the nucleotide sequence, the initiation methionine (+1) codon is indicated by asterisks and the synthetic peptides (p1–p5) are underlined. Predicted α-helices (H,h; 38% of all residues) and β-strands [E,e (extended); 22%] are adapted from Garnier *et al.* (20). Helices with less than 4 residues and strands with less than 3 residues were suppressed; a very strong signal (H and E) indicates possible nucleation of secondary structure. In interpreting the prediction, note that the average three-state accuracy of this method is 56% (21). Peptide 3 (residues 24–66) is most hydrophilic from residue 24 to residue 50, with a high probability for helical secondary structure. Most of the helix (residues 26–48) would be amphiphilic, with residues like Leu, Val, Gly, and Phe on one face, and residues like Glu, Lys, Asp, Ser, and Thr on the other.

Table 1. Antimalarial antibodies measured by indirect immunofluorescence

Anti-serum	Immuno-gen*	Antibody titer, dilution^{-1}			
		P. falciparum SGE2			Patient AR rings
		Schizonts	Merozoites	Rings	
R0	pMC31-1	3200	800	100	100
R1	P1-TT	<100	<100	<50	ND
R2	P2-TT	<100	<100	<50	ND
R3	P3-TT	1600	800	100	100
R4	P4-TT	800	400	100	100
R5	P5-TT	200	100	100	ND
NRS†	None	<50	<50	<50	<50

Results are expressed as reciprocal end point dilutions. ND, analysis not done.
*Synthetic peptides (P1–P5) were conjugated to tetanus toxoid (TT). pMC31-1 represents lysate of pMC31-1-containing *E. coli*.
†Normal (preimmune) rabbit serum.

to treat any monkey with antimalarial drugs when parasitemia exceeded 20% (10).

RESULTS

Analysis of the Amino Acid Sequence of the Schizont-Specific 180- to 200-kDa Polypeptide Deduced from a Genomic Clone. A genomic library using DNA from *P. falciparum* (SGE2) was constructed in the pUC9 vector (16) by the mung bean nuclease method according to McCutchan *et al.* (17). Several genomic clones were identified by filter hybridization using ^{32}P-labeled malarial DNA present in the pMC31-1 plasmid (9). This plasmid has been shown to code for a portion of a 180- to 200-kDa schizont-specific polypeptide that is later processed into an 83-kDa polypeptide, the main parasite component expressed at the surface of merozoites. The DNA sequence of the genomic clone selected and its deduced amino acid sequence, which overlaps with the previously reported pMC31-1 plasmid sequence (9) and contains the 5′ end of the gene (18), is presented in Fig. 1. Immediately following the initiation methionine (designated amino acid +1), there is a hydrophobic region of 20 amino acid residues and a stretch of 60 residues with twenty 3-residue repeats of the type Xaa-Xaa-Thr or Xaa-Xaa-Ser (Xaa being mostly Ser, Gly, Thr, Ala, and Val), starting with Glu-Gly-Thr at residue 55 and ending with Ser-Asp-Ser at residue 114. The consensus sequence is Gly-Gly-Ser and occurs six times; the sequence Val-Ala-Ser occurs three times.

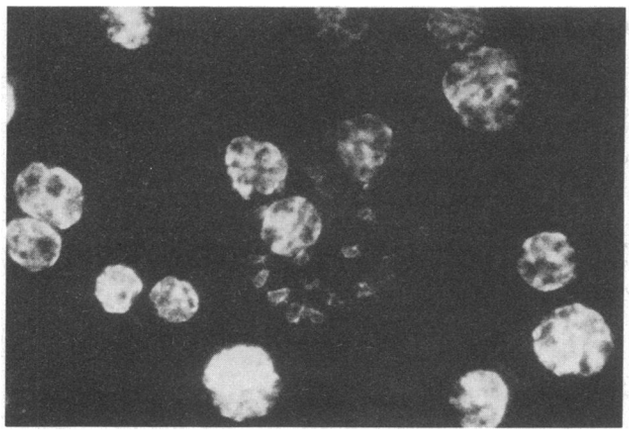

FIG. 2. Specificity of rabbit antibodies (R3) directed against peptide 3 coupled to tetanus toxoid. Acetone-fixed schizonts and merozoites were incubated with a 1:200 dilution of antiserum R3 followed by a 1:50 dilution of fluoresceinated affinity-purified goat anti-rabbit antibodies. These antibodies react with the entire surface of merozoites (center of figure) and with the membrane and internal structure of schizonts (15 of them are evenly distributed in the figure). (×1000.)

Table 2. Antipeptide antibodies measured by ELISA

Antigens for coating	Dilution^{-1}				
	R0	R1	R3	R4	R5
200 kDa*	18,000	<20	15,000	12,000	500
pMC31-1	20,000	<100	20,000	8,000	100
1	<100	6000	<100	<100	<100
3	3,000	<100	20,000	200	100
4	15,000	<50	4,000	9,000	100
5	<50	<50	200	<50	9000
BSA	<50	<50	<50	<50	<50

Results are expressed as the reciprocal of the dilution giving an absorption of 1.0 at 405 nm. R0 is antiserum to pMC31-1 fusion protein. R1, R3, R4, and R5 are antisera to peptides 1, 3, 4, and 5, respectively.
*Schizont antigen of 180–200 kDa.

To identify the potential immunogenic epitopes present on the native molecule, five peptides deduced from the genomic clone (as indicated in Fig. 1) were chemically synthesized to raise antibodies in rabbits. Peptide 1 (made from a hypothetical translation of the 5′ untranslated region of the gene) contains residues prior to the initiation methionine at positions −22 to −8 (15 amino acids); peptide 2 consists of the hydrophobic region from residue +1 to +20 (20 amino acids); peptide 3 (43 amino acids) and peptide 5 (12 amino acids) contain the nonrepetitive sequences at positions +24 to +66 and +157 to +168, respectively; and peptide 4 (34 amino acids) contains the repeat region at positions +69 to +102.

Specificity of the Antisera. *Indirect immunofluorescence.* Serial dilutions of the rabbit antisera were tested against preparations of merozoites, schizonts, and ring forms from the SGE2 isolate and against ring forms obtained from a patient (AR) with acute *P. falciparum* infection. The reciprocal end point of antibody titers is reported in Table 1. Antisera R1 and R2, which are directed against synthetic peptides derived from DNA sequences situated before (peptide 1) and after (peptide 2) the initiation methionine codon, are negative. All the other antisera (R0, R3, R4, and R5) reacted strongly with the surface of free released merozoites and with merozoites contained in mature schizonts (Fig. 2). The membrane of schizonts was positive; a diffuse and weaker cytoplasmic reactivity was observed in both the young and mature schizonts. In addition, a weak ring-shaped fluorescence was observed with two ring preparations, derived from the SGE2 isolate and from the blood of the acutely infected patient.

Antisera R0, R3, and R5 were also positive when tested against six other schizont and merozoite clones or isolates of *P. falciparum* as described in *Materials and Methods*, though the fluorescence was less intense in the case of the Tak9.94 isolate from Thailand. Further, all the isolates tested with R4 (the antiserum from the rabbit immunized with the amino acid repeats) were positive except Tak9.94.

ELISA. Serial dilutions of each of the sera were tested on ELISA plates coated with the different peptides coupled to BSA, with the 180- to 200-kDa schizont-specific polypeptide, and with the pMC31-1 fusion peptide purified from *E. coli* lysate (Table 2).

All the antisera react with the homologous synthetic peptide used for immunization. Antisera R0, R3, R4, and R5 react with the native schizont-specific polypeptide. R3 and R4 react with the pMC31-1 fusion polypeptide, which contains the amino acid sequences of the respective immunogenes; similarly, R0 reacts with peptides 3 and 4.

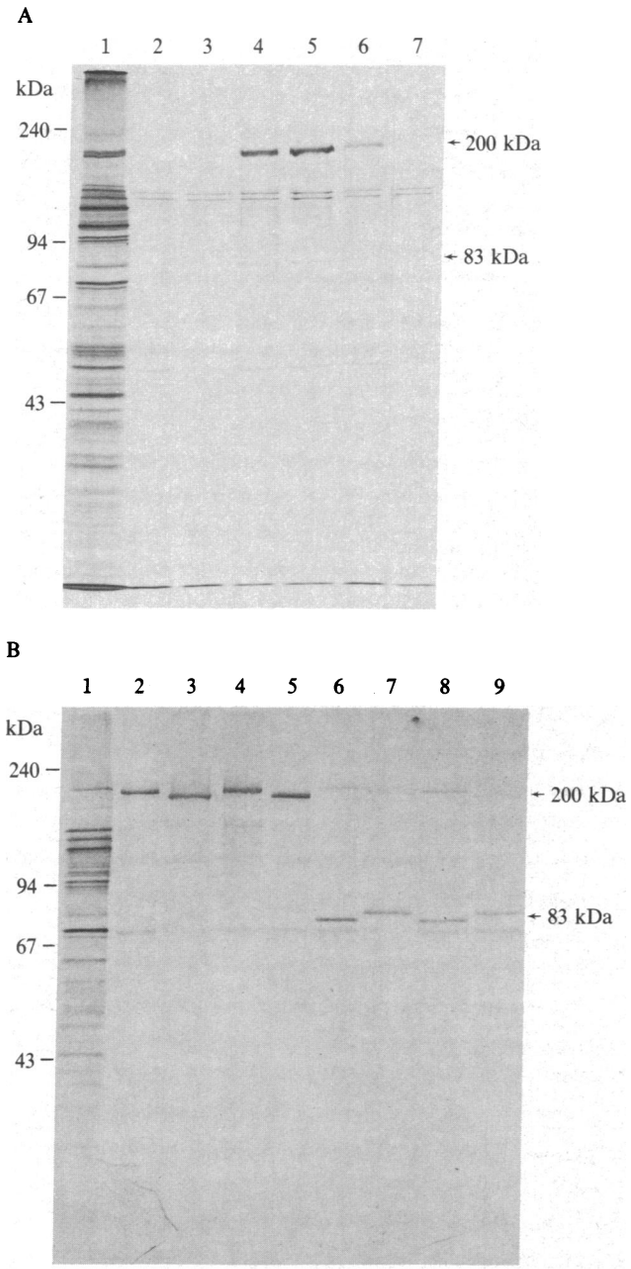

FIG. 3. (*A*) Autoradiograph of *P. falciparum* SGE2 [^{35}S]methionine-labeled polypeptides precipitated by antibodies directed against peptide 1 (lane 2), peptide 2 (lane 3), peptide 3 (lane 4), peptide 4 (lane 5) and peptide 5 (lane 6) and by nonimmune rabbit serum (lane 7). Lane 1 represents the starting material for immunoprecipitation. Positions of standards run in parallel are indicated at left. (*B*) Immunoblot analysis of enriched preparations of schizonts and merozoites from four different isolates of *P. falciparum*. Antiserum R3, directed against peptide 3, was used as first reagent, and the nitrocellulose sheet was subsequently incubated with ^{125}I-labeled affinity-purified goat anti-rabbit antibodies (New England Nuclear). Rabbit antisera directed against pMC31-1-encoded fusion polypeptide, peptide 4, and peptide 5 gave similar results, but no reactivity was observed with sera directed against peptide 1 and peptide 2 (data not shown). Lane 1: total [^{35}S]methionine-labeled lysate from SGE2. Lanes 2–5: enriched schizont preparations of NF54, SGE2, M23, and FCC2, respectively. Lanes 6–9: enriched merozoite preparations of the same isolates in the same order.

Interestingly, R3 crossreacts with peptide 4; this may be due to homology between the repeated sequences of peptide 4 and the repeated sequences at the COOH-terminal end of peptide 3.

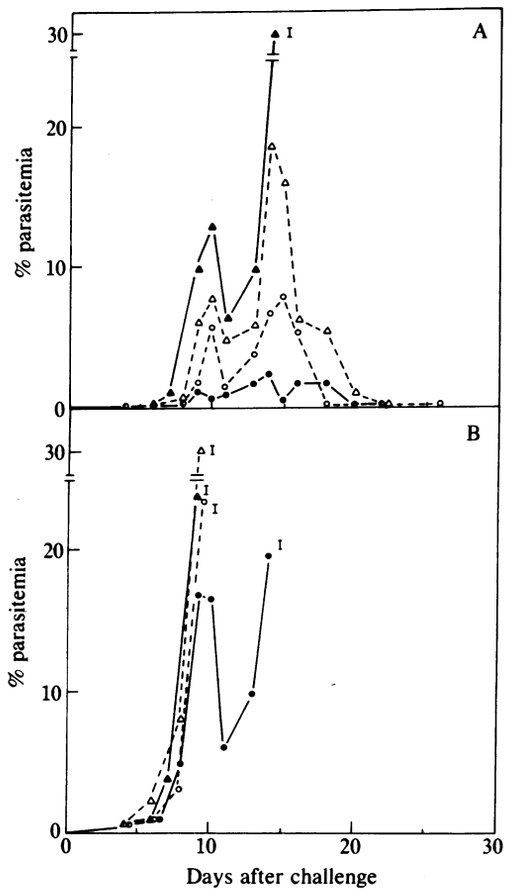

FIG. 4. Course of parasitemia in two groups of four monkeys immunized with tetanus toxoid (control, *B*) and peptide 3 coupled to tetanus toxoid (*A*). I indicates that antimalarial drugs were administered.

Immunoprecipitation and immunoblot analysis. The various antisera were tested for their capacity to precipitate [^{35}S]methionine-labeled *P. falciparum* polypeptides and to react with schizont and merozoite polypeptides transferred from $NaDodSO_4$/polyacrylamide gels onto nitrocellulose paper. Antisera R3, R4, and R5 precipitated [^{35}S]methionine-labeled polypeptides of 200 and 83 kDa (Fig. 3*A*) of SGE2 isolate. Immunoblot analysis on preparations enriched for schizonts or merozoites showed that both the 200- and the 83-kDa polypeptides were recognized by antiserum R3 from four different *P. falciparum* isolates (Fig. 3*B*). The data clearly showed that there are variations in the apparent molecular mass of the 200- and 83-kDa proteins among these isolates.

Immunization Experiment in Monkeys. The course of parasitemia in monkeys is presented in Fig. 4. All the control monkeys had parasitemia >20% by day 10 and had to be treated with antimalarial drugs. The rise of parasitemia in monkeys immunized with peptide 3 conjugates was much slower than in control animals. Also, the immunized monkeys presented different responses: one monkey required antimalarial therapy, whereas the other three had peak parasitemia of various amplitudes. By day 22, all the immunized monkeys cleared their parasitemia, and no parasites were detected in their blood samples taken over a period of 3 months. The prechallenge antibody titers as measured by indirect immunofluorescence assay were 1:100 in the sera of three monkeys and 1:200 in the serum of the monkey who later had the lower peak of parasitemia. The prechallenge antibody titers on ELISA plates coated with peptide 3

coupled to BSA were between 1:2000 and 1:10,000 in immunized monkeys and 1:<50 in control monkeys.

DISCUSSION

We have presented the sequence of a genomic clone coding for part of a *P. falciparum* schizont- and merozoite-specific polypeptide that is a good candidate for the development of a malarial vaccine based on asexual blood stage antigens (5, 9, 10). Indirect evidence based on sequence data and serology (antibody reactivity of rabbits immunized with the fusion and synthetic peptides) suggests that the genomic clone codes for the NH_2-terminal part of the 180- to 200-kDa schizont-specific polypeptide and its merozoite-specific processed product of 83 kDa. Further, peptide 1 is not part of the protein and peptide 2, which has the characteristics of a signal peptide, is either cleaved off or its conformation does not resemble that of the native protein. Recently, Holder *et al.* (18) reported that the 83-kDa polypeptide begins with Val-Thr-His-Glu, which corresponds to amino acid residues 20–23 of the deduced sequence shown in Fig. 1.

Immunization experiments in rabbits using the pMC31-1-encoded fusion protein and synthetic peptides 3, 4, and 5 coupled to tetanus toxoid led to several conclusions. First, all these peptides induce antibodies reacting with *P. falciparum* asexual blood stages, and these antibodies react with the membrane of the schizonts and merozoites from different geographic locations. Second, lower antibody titers were raised with synthetic peptides than with the fusion protein. This may be explained by the low ability of the synthetic peptides to mimic the epitopes present on the native protein. In this respect, some peptides were able to raise high antibody titers against their own structure but had few antibodies reacting with the asexual blood stages, as measured by indirect immunofluorescence (e.g., peptide 5). Third, antisera directed against the fusion polypeptide of pMC31-1 and against peptide 3 and 5 react with seven out of seven *P. falciparum* isolates or clones tested, whereas the antiserum raised against peptide 4 (composed of repeated amino acid sequences) failed to react with clone Tak9.94. Other investigators who have cloned the same portion of the gene coding for the 180- to 200-kDa protein using mRNA from other isolates found similar (18) as well as different (22) repeated sequences. This shows that there is antigenic diversity at the level of the 180- to 200-kDa polypeptide. Monoclonal antibody analysis and hybridization experi-ments using the pMC31-1 plasmid (9, 23) also suggest that the main surface component of merozoites may vary and that the repeated amino acid sequence may play a role in the observed diversity.

It has been suggested that repeated amino acid sequences are immunodominant (24) and prevent the induction of protective antibodies which have to be directed against adjacent parts of the molecule (25). We have observed that most of the sera from adult individuals living in endemic areas are directed against peptide 4 (repeated sequence) but not against peptides 3 and 5 (unpublished data). Monkeys immunized with peptides, unlike rabbits, had very low antimalarial antibody titers; however, it is encouraging to observe that three of four monkeys showed lower parasitemia and recovered without therapy when challenged.

The cloned repeated sequence is significantly homologous (Fig. 5) with the nonhelical parts of intermediate filament proteins, such as cytoskeletal keratins, desmin, and vimentin (27), and of α-fibrinogen. The common pattern is a stretch of glycine- and serine-rich repeats with a large hydrophobic residue [such as tyrosine, tryptophan, phenylalanine, valine, or arginine (which has a nonpolar "stem")] every 10–12 residues. This pattern is probably characteristic of a particular type of β-structure.

What is the functional relationship between the merozoite surface antigen and the nonhelical regions of the intermediate filament proteins? An intriguing hypothesis is that the merozoite surface has evolved to integrate into the erythrocyte cytoskeleton so as not to disrupt the host-cell structure during replication of the parasite (camouflage hypothesis). Another possibility is that the surface antigen is used to modify the erythrocyte membrane. It will be interesting to determine whether the 83-kDa merozoite surface antigen interacts with the erythrocyte cytoskeleton in a functionally specific way.

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      +          +        + +        +          +              +=V/R
..SGTA VTTSTPGSGG SVTSGGSGGS VASVASGGSG GSVASGGSGN SRRTNPS..   58-108 meroz.
  *          ***     * ***       * * * **   * **    *   *
..GGSG GGSYGGSSGG GSYGGSSGGG GSYGGSSGGG GSYGGGSSGC GGRGGGS..  464-514 keratin
         +        +          +          +          +              +=Y/R
```

FIG. 5. Sequence homology between the merozoite surface antigen (residues 58–108) and mouse type 1 cytoskeletal keratin (residues 464–514; ref. 26). Other cytoskeletal proteins have similar glycine plus serine-rich repeats with tyrosine, tryptophan, phenylalanine, valine, or arginine every 9–13 residues. Asterisks mark identical residues, plus signs mark valine or arginine in the first and tyrosine or arginine in the second sequence. The tyrosine–tyrosine spacing of 9 or 10 residues is similar to the valine–valine spacing in the first sequence. The homology continues, but less strongly, to the left of the repeat region (not shown). Standard one-letter amino acid abbreviations are used to depict the sequences.

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