ANTIMALARIAL IMMUNITY IN SAIMIRI MONKEYS

Immunization with Surface Components of Asexual Blood Stages

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The asexual erythrocytic phase of the development of malaria parasites is responsible for the pathological manifestations of the disease in man. During the blood stage of infection, the parasite develops within the erythrocytes (RBC)[1] into three successive, morphologically distinct stages called ring forms, trophozoites, and schizonts. Mature schizonts rupture the RBC, releasing merozoites which, after a short extracellular life in the blood, invade new RBC.

Parasite components are exposed to the host immune effector mechanisms at the schizont stage, as parasite components are exposed at the surface of RBC containing schizonts (1–3), and at the merozoite stage. Immunization experiments have therefore been conducted in monkeys susceptible to *Plasmodium falciparum*, using whole schizonts and/or merozoites as immunogens (4–6). The immunized monkeys were protected from a lethal, blood-induced infection but most of them developed a moderate transient parasitemia. The usefulness of immunization with whole schizonts and/or merozoites is limited, however, by the contamination of these preparations by potentially immunogenic RBC cell components (6). This inconvenience may be avoided by using purified parasite components as immunogens. Two *P. falciparum* polypeptides were therefore selected for immunization on the basis of several observations. They are among the four polypeptides recognized by monoclonal antibodies inhibiting the in vitro growth of asexual blood stages (200,000 [200 K], 140, 82, and 41 K mol wt) (3, 7, 8); they are exposed as intact polypeptides or as processed products at the surface of merozoites and/or schizonts (3, 9–11); and they are probably involved in the binding of merozoites to RBC (10).

Material and Methods

Preparation of Schizont and Merozoite Extract. The SGE2 isolate of *P. falciparum* was adapted to in vitro culture using the blood of a patient from Zaire and was maintained in

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[1] *Abbreviations used in this paper:* NP-40, Nonidet P-40; PBS, phosphate-buffered saline; RBC, erythrocytes; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone.

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culture for 30 mo. The parasites were cultivated in 100-mm diam Falcon petri dishes using A+ human RBC (12). Cultures were initiated at a parasitemia of 0.4%, which varied between 12 and 20% after 4 d of culture. Infected RBC from 40 dishes were harvested by centrifugation at 1,500 *g* for 15 min at 4°C. A brown upper layer present on top of each pellet was collected (~1/12 of the packed RBC), resuspended at a hematocrit of 50% with culture medium, and centrifuged at 1,500 *g* for 15 min in 1-ml Wintrobe tubes. The brown layer was collected again, resuspended in 20 vol of cold phosphate-buffered saline (PBS), and centrifuged at 1,500 *g* for 10 min. The pellet was extracted with 7 vol of lysis buffer (5 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 [NP-40], and 2 mM phenylmethylsulfonyl fluoride, pH 7.2). The lysate was centrifuged at 60,000 *g* for 30 min and the supernatant was stored at −75°C. This procedure was repeated nine times and provided 90 ml of starting material. The blood smears obtained on the last pellet showed >85% schizonts, free merozoites, and pigments as well as trophozoites, rings, and normal RBC in low proportions.

Preparation of Immunoabsorbents. IgG was purified by ammonium sulfate precipitation and DEAE cellulose chromatography and then coupled to cyanogen bromide–activated Sepharose 4B at a concentration of 20 mg/ml according to the manufacturer's specifications (Pharmacia Fine Chemicals, Piscataway, NJ). Three separate immunoabsorbents were prepared in this manner using IgG from three sources: (*a*) a pool of human sera from adults living in Gambia selected for their reactivity with polypeptides of 140 and 200 K mol wt (3); (*b*) a pool of rabbit serum directed against human immunoglobulins, serum, and RBC membranes (DAKO, Copenhagen, Denmark); and (*c*) rabbit anti-*P. falciparum* antisera (see below).

[^{35}S]Methionine Labeling of P. falciparum. Unsynchronized SGE2 and FUP Palo Alto (13) isolates of *P. falciparum* cultivated in 100-mm diam petri dishes at a parasitemia of 15% were resuspended individually at a hematocrit of 6% in methionine-free minimum essential medium (Eagle's; Gibco Laboratories, Grand Island, NY) supplemented with 10% normal human serum, 40 mM Hepes, and 2 mM glutamine. L[^{35}S]methionine was added at a concentration of 100 μCi/ml (sp act 1,040 Ci/mmol; Amersham Corp., Arlington Heights, IL). After an incubation of 5 h in a candle jar at 37°C, the cells were washed twice in cold PBS and the pellet was extracted with 12 vol of lysis buffer. The lysate was centrifuged at 60,000 *g* for 30 min at 4°C and aliquots were stored at −75°C.

Preparation of Rabbit Anti-P. falciparum Antisera. 20 ml of the *P. falciparum* schizont and merozoite extract were supplemented with 1×10^7 cpm of an extract of SGE2 L[^{35}S]methionine-labeled *P. falciparum* culture. This extract was incubated overnight at 4°C with 9 ml of Gambia IgG immunoabsorbent, poured into a column, and washed with 5 vol of lysis buffer, followed by 2 vol of TNE (5 mM Tris, 50 mM NaCl, 5 mM EDTA), 0.15% NP-40 buffer. Elution was achieved at 4°C using 0.1 M glycine HCl, 0.15% NP-40, pH 3. One-milliliter fractions were collected in siliconized tubes, immediately neutralized with 0.5 M Tris, pH 9, and checked for associated radioactivity using a Beckman LS20 β counter (Beckman Instruments, Inc., Fullerton, CA) and for antigen content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The eluted fraction contained four of the main merozoite- and schizont-specific antigens of *P. falciparum* (polypeptides of 200, 160, 140, and 82 K mol wt), low concentrations of polypetides common to the various asexual development stages, and trace amounts of IgG and RBC membranes. The antigenic fraction was dialyzed against distilled water (using dialysis membranes boiled in 1% SDS and then in distilled water), lyophilized, and resuspended in 2.5 ml of PBS containing 0.15% NP-40. The IgG and RBC contaminants were removed by incubation with 4 ml of immunoabsorbent coupled with rabbit IgG directed against human IgG, human serum, and human RBC membrane. The 6 ml final preparation contained 0.11 mg/ml of proteins as measured by a protein assay (Bio-Rad Laboratories, Richmond, CA). Two rabbits were immunized four times with 0.5 ml of the malaria antigenic preparation at 2-wk intervals, the first time with Freund's complete adjuvant and the following times with Freund's incomplete adjuvant. The serum of one of the rabbits had an endpoint titer of 1/32,000 by indirect immunofluorescence (14) and was used for the preparation of an immunoabsorbent.

Purification of the 200 and 140 K Mol Wt Polypeptides. The same procedure as described above was repeated three times using for each purification 22 ml of *P. falciparum* extract supplemented with 10^8 cpm of an extract of L[^{35}S]methionine-labeled SGE2 *P. falciparum*. The supernatant was incubated and mixed on an end-over-end rotator overnight at 4°C with 25 ml of rabbit IgG anti-*P. falciparum* immunoabsorbent. The acid-eluted fraction was dialyzed against H_2O at 4°C for 24 h, lyophilized, and reconstituted in 2 ml of 20 mM Tris, pH 8, containing 2% SDS and 3% β-mercaptoethanol. 800 μl of this preparation was applied to 3-mm-thick, 200 × 160 mm, 6% SDS-PAGE (procedure repeated eight times) (15). The 140 and 200 K bands were identified on small gel strips, stained with Coomassie Blue, excised with a razor blade, and electroeluted in a dialysis bag (16). The purity of each preparation was assessed by analysis on 8% SDS-PAGE stained with Coomassie Blue and by autoradiography.

Tryptic Digestion and Reverse Phase Chromatography of Malaria Tryptic Peptides. Schizonts and merozoites were purified as described above using as starting material a 1-ml pellet of SGE2-parasitized RBC previously labeled with L[^{35}S]methionine. The 200 and 140 K mol wt enriched preparation was purified using the rabbit immunoabsorbent. This preparation was applied on 8% SDS-PAGE and the 200 and 140 K mol wt polypeptides were visualized by autoradiography of the dried gel. The regions containing the labeled polypeptides were excised, eluted, and digested for 18 h at 37°C with 50 μl of TPCK trypsin (Worthington Biochemical Corp., Freehold, NJ) (17). For the reverse phase chromatography of tryptic peptides, the peptides were dissolved for 10 min in 100 μl of 90% formic acid, and then diluted with 400 μl of H_2O. The sample was then applied to a 4.6-mm × 25-cm Spherisorb 10 ODS (C18) reverse phase column (Spectra-Physics, Inc., Mountain View, CA). The column was run at 40°C and at a flow rate of 1.4 ml/min. After injection of the sample, the column was washed for 5 min with 4.5% formic acid (PSC reagents; J. T. Baker Chemical Co., Phillipsburg, NJ) and then developed with a linear gradient from 0 to 62.5% ethanol (Photrex reagent; J. T. Baker Chemical Co.) in 4.5% formic acid over three periods of 95 min on a Spectra-Physics SP8000 high performance liquid chromatography system. Aliquots were taken from each of the fractions selected and mixed with 6 vol of Aquasol (New England Nuclear, Boston, MA) for counting in a liquid scintillation counter.

Animals and Immunization Schedule. Six male and six female Saimiri monkeys of Guyana origin, mean age 3 yr 2 mo, weighing between 600 and 800 g, and bred in the animal facilities of Hoffmann-La Roche in Basel, were divided into three groups of two males and two females. These monkeys were free of parasites commonly detected in wild monkeys (Trypanosoma, Leishmania, Filaria). The control group of four monkeys was injected subcutaneously three times in four different sites: the first time with 0.5 ml saline with Freund's complete adjuvant, the second and third times with 0.5 ml of saline mixed with 0.5 ml of Freund's incomplete adjuvant 16 and 30 d, respectively, after the first immunization. The second and third groups of animals were immunized according to the same protocol with the 200 or 140 K mol wt antigenic preparations, using 30 μg of protein in a volume of 0.5 ml mixed with an equal volume of adjuvant.

Challenge, Monitoring of the Infection, and the Antibody Response. The 12 monkeys were challenged on day 63 by an intravenous injection into the femoral vein with 2.5×10^7 parasites provided by a splenectomized monkey infected 8 d previously with the Uganda Palo Alto strain FUP of *P. falciparum* (gift of Dr. Hommel). Parasitemia was determined at various intervals on Giemsa-stained, thin blood smears. Before starting the experiment, it was decided to treat with antimalarial drugs any monkey with a parasitemia >20%. Sera collected at various intervals were checked for antimalarial antibody titers using an indirect immunofluorescence technique (14). For immunoprecipitation experiments, 4×10^5 cpm of L[^{35}S]methionine-labeled *P. falciparum* Palo Alto or SGE2 cultures solubilized with lysis buffer were incubated with 5 μl of the various monkey sera, and immune complexes were precipitated by protein A (11). The immunoprecipitates were analyzed on 8% SDS-PAGE and autoradiographed (11, 14).

Results

Isolation and Characterization of the 200 and 140 K Mol Wt Polypeptides. It has previously been shown that the 200 and 140 K mol wt polypeptides and their processed products are schizont and/or merozoite specific (3, 9–11). 400 and 520 μg, respectively, of the 200 and the 140 K polypeptides were purified from starting material corresponding to 11 ml of packed infected RBC (1.1×10^{11} RBC) containing 85% schizonts at various stages of maturation (from 4 to 32 nuclei) and numerous free merozoites. The 140 and 200 K polypeptides were detectable in the starting material analyzed on optimally loaded SDS-PAGE stained with Coomassie Blue by comparing stained gel with autoradiography. A single passage on the immunoabsorbent depleted the starting material of at least 90% of the 200 and 140 K components, since they were no longer detectable on flow-through fractions analyzed on SDS-PAGE followed by autoradiography (not shown). The material eluted by glycine HCl buffer from the rabbit anti–*P. falciparum* immunoabsorbent contained mainly the 200, 140, and 76 K polypeptides (Fig. 1). The second step of the purification (electrophoresis) gave a recovery of at least 85% as measured by determination on subsamples of the counts per minute applied and recovered. The number of molecules present per schizont is a gross approximation due to the heterogeneity of the schizont population and the presence of free merozoites in the starting material and corresponds approximately to 3×10^4 molecules for the 200 K and 6×10^4 molecules for the 140 K polypeptide. The 200 and 140 K polypeptides used for immunization were free of contaminants as shown on Fig. 1. In addition, these polypeptides still reacted after Western blotting with monoclonal antibodies directed against the 200 and 140 K polypeptides (data not shown). To investigate the structural relatedness between the 200 and 140 K polypeptides, both polypeptides were digested with TCPK trypsin and the peptides recovered were analyzed by reverse

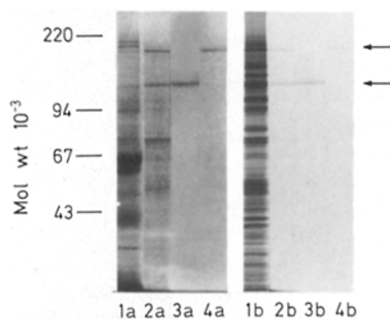

FIGURE 1. Analysis by SDS-PAGE and autoradiography of *P. falciparum* schizont and merozoite extracts and affinity-purified and electroeluted polypeptides. Samples were heated as described before application on the gel, and after electrophoresis the gel was stained with Coomassie Blue, destained, and autradiographed. The schizont and merozoite extract used as starting material for purification was supplemented with 30,000 cpm of L[^{35}S]methionine-labeled SGE2 culture extract before application. (*1a, 2a, 3a, 4a*) Commassie-stained gel, (*1b, 2b, 3b, 4b*) autoradiography. (*1*) Schizont and merozoite extract, (*2*) material eluted from the rabbit anti–*P. falciparum* immunoabsorbent, (*3*) 140 K mol wt polypeptide electroeluted, (*4*) 200 K polypeptide electroeluted. Arrows indicate polypeptides of 200 and 140 K. Reference molecular weights (Pharmacia) are indicated.

phase chromatography. As shown in Fig. 2, there is no clear homology between the peptide patterns of the 200 and 140 K polypeptides.

The course of parasitemia in the three groups of monkeys is reported on Fig. 3. Two of the control monkeys had to be treated with antimalarial drugs when their parasitemia reached >20%. Two other monkeys of this group spontaneously recovered and their maximum parasitemia reached 9.5 and 11%, respectively. Due to a technical error one of these monkeys received a paravenous challenge infection and a delay of 7 d was observed before the appearance of patent parasitemia. The eight monkeys immunized with either the 200 or 140 K polypeptides spontaneously cleared their parasites. In both groups a delayed onset of patent parasitemia (defined here as a parasitemia >0.01%) was observed. In the group of monkeys immunized with the 140 K polypeptide, peak parasitemia was <3% in three monkeys and reached 4.5% in one monkey. In the group immunized with the 200 K polypeptide, peak parasitemia was <3% in two monkeys, reached 6% in one monkey, and 11% in another. The latter monkey suffered from a large skin excoriation on its tail that appeared after the first immmunization, and the animal lost a large amount of serous fluid. Weekly monitoring of blood smears from all the monkeys for 3 mo after challenge did not reveal recrudescent patent parasitemia. The weight of the monkeys did not decrease >5% during the experiment and all gained weight in the following

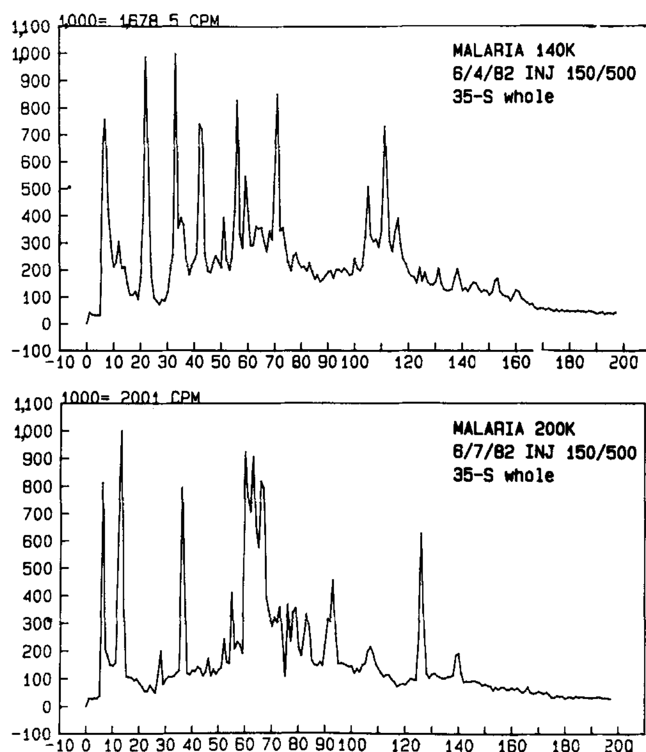

FIGURE 2. Reverse phase chromatography of L[^{35}S]methionine-labeled peptides of the 200 and 140 K polypeptides digested with TPCK trypsin. [^{35}S]methionine cpm are indicated on the ordinate axis (correction factor noted on top). Tube numbers are indicated on the abscissa.

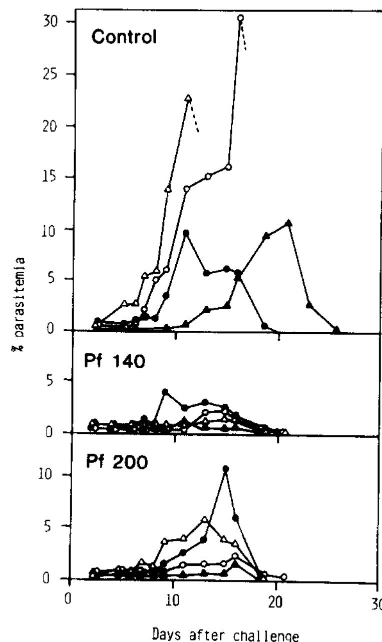

FIGURE 3. Course of parasitemia in three groups of four monkeys immunized, respectively, with saline, 140 K polypeptide, and 200 K polypeptide. Abscissa gives the days after challenge with 2.5×10^7 asexual blood forms of the isolate Palo Alto.

month. All monkeys showed a decrease of their hematocrit 13 d after challenge as compared with levels before the start of the experiment: 34 vs. 41% for the 200 K group, 30 vs. 39% for the 140 K group, and 28 vs. 39% for the control group. A return to a normal hematocrit level was observed 1 mo after challenge.

Reactivity of the Monkey Sera. The humoral response of the immunized and control monkeys was tested by indirect immunofluorescence and immunoprecipitation. None of the sera collected before immunization was positive by indirect immunofluorescence (Fig. 4). All but one of the sera of the monkeys immunized with the 200 and 140 K polypeptides were positive after the second immunization. The one negative serum was collected from the monkey immunized with the 200 K polypeptide and suffering from a large skin excoriation. A rise in antimalarial antibody titers was observed after the third immunization in all monkeys. Prechallenge sera of the monkeys immunized with the 200 and 140 K polypeptides reacted equally with schizonts and merozoites from the SGE2 isolate used for immunization, with the Palo Alto isolate used for challenge, and with three other isolates of *P. falciparum* of Asian and South American origin. The sera collected after the second and third immunization were used for immunoprecipitation experiments. Sera collected after the second immunization from monkeys immunized with the 200 or 140 K polypeptides only reacted with the polypeptides used for their immunization. A similar reactivity was observed using sera collected after the third immunization (Fig. 5) but an additional polypeptide of ~31 K was precipitated by the four sera of the monkeys immunized with the 200 K polypeptide. One of the sera of the monkeys immunized with the 140 K

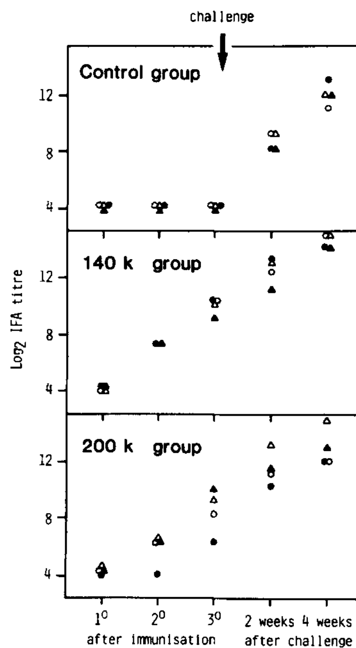

FIGURE 4. Reciprocal titers of anti-*P. falciparum* antibodies as measured by indirect immunofluorescence on sera taken at various intervals before and after challenge infection. Symbols for each monkey are the same as those used on Fig. 3.

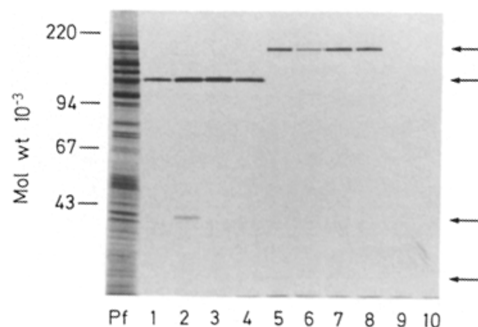

FIGURE 5. [^{35}S]methionine-labeled polypeptides immunoprecipitated by prechallenge sera of two monkeys of the control group (Nos. 9 and 10) and sera from each of the monkeys immunized with the 140 K polypeptide (Nos. 1–4) and with the 200 K polypeptide (Nos. 5–8). No. 6 corresponds to the sera (●) on Figs. 3 and 4; this monkey suffered from a large excoriation. *Pf*, starting material for immunoprecipitation: [^{35}S]methionine-labeled extract of SGE2 *P. falciparum* isolate. Arrows indicate polypeptides of 200, 140, 39, and 31 K mol wt. The 31 K polypeptide is not clearly visible on this reproduction.

polypeptide precipitated an additional polypeptide with an apparent relative molecular weight of 39,000. Similar results were obtained by using [^{35}S]methionine material from SGE2 or Palo Alto isolates.

Discussion

In this study, immunization of *Saimiri sciureus* monkeys with small amounts of merozoites and/or schizont polypeptides of *P. falciparum* conferred a significant

degree of protection against a blood-induced challenge infection. The eight immunized monkeys survived an infection that induced a life-threatening situation requiring drug therapy in two of the four control monkeys. The number of animals in the control group is relatively small but in other experiments conducted by the same investigators using the same experimental protocol, similar results were observed: five out of nine control monkeys required antimalarial therapy after reaching a parasitemia >20%. The cause of the variation in susceptibility between individual monkeys is not known but is unrelated to sex, age, weight, and origin of the monkeys.

The sera of the monkeys immunized with either the 200 or the 140 K mol wt polypeptides had a relatively good prechallenge antibody titer as measured by Freund's incomplete adjuvant and strongly precipitated the polypeptide used for their immunization. The main conclusion of this investigation is that immunization with limited amounts of defined and possibly denatured *P. falciparum* polypeptides induces in monkeys a degree of resistance at least equal to that obtained in monkeys immunized with whole schizonts and/or merozoites (4, 6). Other studies (18, 19) have previously shown, using malaria species infecting rhesus monkeys and mice, that defined antigens are indeed able to raise a protective immune response. These data present an interesting step towards the development of a malaria vaccine, since identified antigens may be produced in the future by DNA recombinant technology or direct biochemical synthesis (20).

The mechanism of protection induced by immunization with purified polypeptides from merozoites and/or schizonts is difficult to elucidate because, in vaccinated monkeys, low to substantial levels of parasitemia persisted for at least 15 d, suggesting that reinvasion continued. It may be that the level of specific antibodies induced by the low amount of probably modified antigen was not fully adequate. There are at least three other alternatives. First, antibodies are only one major element needed to achieve protection and may play a crucial role in the early control of parasite multiplication. Complete elimination of the malaria parasite, however, may depend on cellular immune response (21), macrophage activation (22), and/or cytokine production (23, 24) and these responses may be delayed or induced by antigens different from those responsible for an antibody-dependent protective response. Secondly, antigenic diversity has been shown to occur among and within various isolates of *P. falciparum* (25, 26). It is therefore possible that only some of the antibodies raised against the antigens used for immunization are effective. In the experiment reported here, the protective effect crossed different isolates of *P. falciparum*, since the purified polypeptides were obtained from an isolate (SGE2) unrelated to the isolate (Palo Alto) used for challenge. Finally, the parasite itself modifies the hosts' immune responses and this may also account for the inability to induce a sterile immunity (27).

Sera from monkeys immunized with the 200 and 140 K mol wt polypeptides reacted only with the polypeptide used for immunization, suggesting that these two polypeptides did not share major antigenic determinants. However, a recent study indicates that a 195 K mol wt *P. falciparum* polypeptide was processed into smaller molecular weight products at the end of schizogony (11). The relatedness of the 200 and 140 K polypeptides was therefore investigated; there is no clear

homology in the profile of peptides obtained after trypsin digestion of both the 200 and 140 K polypeptides. This result is in agreement with a study (28) demonstrating that a probable analogue of the 140 K *P. falciparum* polypeptide expressed at the surface of *P. knowlesi* merozoites is antigenically unrelated to other merozoite surface components. It seems, therefore, that at least two different surface components of *P. falciparum* and possibly others (the 41 K polypeptide is also a good candidate) may induce a partial protective immunity against *P. falciparum* asexual blood stages.

Summary

Plasmodium falciparum polypeptides of 200 and 140 K mol wt exposed at the surface of merozoites and/or schizonts were purified by affinity chromatography and by electroelution from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Monkeys were separated into three groups of four and immunized either with one of the two polypeptides or with saline (control). After intravenous challenge with 2.5×10^7 *P. falciparum* asexual blood stages, two monkeys of the control group had to be treated and two recovered spontaneously after peak parasitemia of 9 and 11%. The four monkeys immunized with the 140 K polypeptide recovered without treatment after peak parasitemia between 1.5 and 4.5%. Monkeys immunized with the 200 K polypeptide had similar peak parasitemia except one monkey who suffered from a large skin excoriation and who recovered spontaneously after a peak parasitemia of 11%. Prechallenge sera of the immunized monkeys reacted only with the polypeptide used for immunization except for one serum of the 140 K group, which precipitated an additional polypeptide of 39 K, and a polypeptide of 31 K weakly precipitated by the four sera of monkeys immunized with the 200 K polypeptide.

The relatedness between the 200 and 140 K polypeptides was investigated using tryptic digestion and reverse phase chromatography. No clear analogy was found between the two polypeptides, which suggests that immunization with either of two independent surface components of *P. falciparum* asexual blood stages is able to induce at least a partial protective immunity in immunized hosts.

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