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Protection of *Aotus* Monkeys from Malaria Infection by Immunization with Recombinant Hybrid Proteins

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On the basis of investigations of the malarial blood-stage antigens SERP, HRPII, and MSAI from *Plasmodium falciparum*, we chose two *Escherichia coli*-expressed hybrid proteins containing selected partial sequences of these antigens. Antibodies raised against both hybrid proteins in rabbits and *Aotus* monkeys recognize the corresponding *P. falciparum* polypeptides. In two independent trials with 13 animals, immunization of *Aotus* monkeys with either of the two hybrid proteins administered in a well-tolerated oil-based formulation protected the animals from an experimental *P. falciparum* infection.

Malaria tropica, the most widespread and dangerous tropical parasitic disease, is caused by the protozoan *Plasmodium falciparum*. Resistance of the parasites to a wide range of drugs has rapidly increased in recent years and keeps spreading. To protect the health of those at risk, vaccines are urgently needed for malarial control. A number of antigens from the asexual blood stages of the malarial parasite are being considered as candidates for the development of a vaccine.

SERP (13) (also known as SERA [3]), a protein characterized by a serine stretch, is the major component of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified protein band of 140 kDa that was shown to induce protective immunity in *Saimiri* monkeys (19). The antigen is localized within the parasitophorous vacuole, where it may function as a cysteine proteinase during merozoite release (7, 14). In a previous study, we obtained partial protection of *Aotus* monkeys immunized with a recombinant protein containing a region of SERP (unpublished results). This sequence carries two T-cell epitopes (22) and seems to be conserved among different *P. falciparum* strains (3). The protective potential of SERP has further been supported by recent data demonstrating protective immunity in *Aotus* monkeys after immunization with two N-terminal fragments of this protein (12).

The histidine- and alanine-rich protein HRPII, another malaria vaccine candidate, is characterized by an extended repeat region consisting of the tripeptide units Ala-His-His and Ala-Ala-Asp (15, 25), which show only slight variability in number and arrangement among different *P. falciparum* strains (15). HRPII was shown to be released from infected erythrocytes (11); however, some of the antigen stays associated with the outer surface of the erythrocyte membrane (21). A recombinant protein containing the C-terminal half of HRPII partially protected *Aotus* monkeys against a parasite challenge (15).

The merozoite surface antigen MSAI is a polymorphic glycoprotein of 195 kDa (9, 20); the processing products of MSAI are the main constituents of the merozoite surface (16). The antigen purified by a monoclonal antibody protected *Aotus* monkeys from malaria (23).

In this report we describe the construction of two hybrid proteins combining partial sequences of the malaria vaccine candidate antigens SERP, HRPII, and MSAI, all of which are known to induce partial protection in *Aotus* or *Saimiri* monkeys against an experimental infection and their evaluation as recombinant vaccines in the *Aotus* monkey model. In comparison to the control animals, all monkeys immunized with either of the two recombinant hybrid antigens showed significantly reduced parasitemia after challenge infection with *P. falciparum*.

MATERIALS AND METHODS

Sources of DNA. Three recombinant plasmids were used as sources of DNA: pUC-SERP, a pUC18 plasmid that contains a 5.6-kb genomic *Xba*I fragment that carries the complete SERP gene (13); pUC-HRPII, a pUC8 plasmid that contains a 600-bp fragment encoding the C-terminal part of HRPII (15); and pEX-31-1-lrd, which encodes an N-terminal partial sequence of MSAI (15). The expression vector pEX31b (24) contains the lambda p_L promoter followed by the coding sequence for the N-terminal 99 amino acids of the bacteriophage MS2 polymerase and a synthetic polylinker.

Construction of the MS2-SERP-HRPII hybrid gene. The plasmid pUC-SERP was digested with restriction enzymes *Eco*RI and *Pst*I to produce a 787-bp fragment, which was ligated between the *Eco*RI and *Pst*I sites of the vector pEX31b to obtain plasmid pEX-SERP. The plasmid pUC-HRPII was completely digested with the restriction enzyme *Hin*dIII and partially digested with *Pst*I (0.05 U for 10 min at 37°C) to produce 600-bp fragments that carry *Pst*I sites at their 5′ ends and either a *Hin*dIII site (partial digestion) or a *Pst*I site (complete digestion) at their 3′ ends. The *Pst*I-*Hin*dIII fragment was introduced between the corresponding sites of the plasmid pEX-SERP to obtain plasmid pEX-SERP/HRPII. After transformation of POP2136 cells (Stratagene), individual clones were grown overnight at 28°C in L broth containing 100 μg of ampicillin per ml and analyzed for expression upon heat induction as described previously (15).

Construction of the SERP-MSAI-HRPII hybrid gene. The oligonucleotides p1 (5′-CGTCCCATGGAATTCTTACAAA TTATTGAAGAT-3′; *Nco*I restriction site at the 5′ end; corresponding to bases 2641 through 2667 of the SERP gene [13]) and p2 (5′-TCCTTCGCTATTCACATAATTACCAT AACCAACAATATTAACTGCATG-3′; complementary to bases 2998 through 3045 of the SERP gene) and 10 ng of the plasmid DNA pUC-SERP were employed for a polymerase

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chain reaction, using the Perkin-Elmer Cetus Gen-Amp kit, which yielded a 400-bp fragment. A second polymerase chain reaction was performed with 10 ng of plasmid pEX-31-1-lrd (15) in combination with the oligonucleotides p3 (5′-GGTAATTATGTGAATAGCGAAGGAGAACTCTTT GATTTAACCAATCATATG-3′; nucleotides 1 through 24 correspond to bases 3022 through 3045 of the SERP gene and nucleotides 25 through 51 correspond to bases 226 through 252 of the MSAI partial sequence 31-1-lrd) and p4 (5′-GGGGTCGACGG ATCC G G TACCAA G CTTACTTCCT TCAATTAATTCATTTATATTTGC-3′; complementary to bases 538 through 567 of the 31-1-lrd sequence; carries *Sal*I, *Bam*HI, *Kpn*I and *Hin*dIII restriction sites at the 5′ end), yielding a 360-bp fragment. Samples (150 ng) of both polymerase chain reaction products and oligonucleotides p1 and p4 were used for a third polymerase chain reaction by the method of Horton et al. (10). The amplified 760-bp fragment was digested with *Nco*I and *Sal*I and introduced into the vector pTRC99 (1) to obtain plasmid pTC. The 830-bp *Hin*dIII-*Sca*I fragment of vector pTC was replaced by one of three *Nco*I-*Sca*I fragments isolated from the pTRC99A, B, and C plasmids (1) to yield pTC1, 2, and 3, respectively. The *Hin*dIII and *Nco*I sites of the combined fragments were filled in with the Klenow fragment of DNA polymerase I before ligation. A 610-bp *Bam*HI-*Hin*dIII fragment was isolated from plasmid pUC-HRPII and ligated into the pTC2 plasmid to obtain vector pTC2-HRPII. *E. coli* RB791 cells (2) were transformed with this plasmid, and bacteria were induced by isopropylthiogalactoside (1).

Purification of the hybrid proteins. For large-scale antigen production, 5-liter bacterial cultures containing the pEX-SERP/HRPII and pTC2-HRPII plasmids were induced; after disruption of the cells, the fusion proteins were partially purified by using stepwise increasing concentrations of urea as described previously (15). The solubilized antigens were dialyzed against 3 M urea and used for the immunization of rabbits and monkeys. Western immunoblot analysis was performed as described previously (15).

Immunization of monkeys. Laboratory-raised monkeys (*Aotus azarae boliviensis*) were immunized three times (days 1, 22, and 43) subcutaneously with 0.1 mg of the partially purified hybrid proteins dissolved in 1 ml of phosphate-buffered saline–3 M urea per dose or with the diluent alone. Before immunization, all vaccine preparations were mixed with 0.1 ml of polyalphaolefine (6) as an adjuvant. After splenectomy on day 50, monkeys were challenged on day 56 with 5×10^6 (trial 1; age of animals, >3 years) or 2×10^6 (trial 2; age of animals, 6 to 30 months) parasitized erythrocytes of the *P. falciparum* strain Palo Alto, obtained from an infected donor monkey. Parasitemia was determined on Giemsa-stained blood smears. Monkeys with serious symptoms caused by high parasitemias were treated with mefloquine.

Serum samples were collected weekly for serological evaluation by an enzyme-linked immunosorbent assay with the hybrid antigens or the SERP, MSAI, or HRPII moiety expressed as an MS2-polymerase fusion protein; for determination of malaria-specific responses, sera were preabsorbed with bacterial proteins (including the MS2-polymerase fusion part) purified the same way as the recombinant malarial proteins. Indirect immunofluorescence assays were performed on acetone-fixed blood smears as described previously (15).

RESULTS

The construction of a SERP-HRPII hybrid gene and its expression in the pEX31b vector as an MS2-polymerase fusion protein in *Escherichia coli* are outlined in Fig. 1A. This fusion protein comprises amino acids 631 through 892 of the SERP antigen (13), a region which includes two T-cell epitopes between amino acid positions 640 and 700 (22) and a sequence homologous to cysteine proteinases from amino acid positions 745 through 786 (7, 14). Additionally, the hybrid gene encodes the 189 C-terminal amino acid residues of HRPII (15).

The construction of a SERP-MSAI-HRPII hybrid gene and its expression using the pTRC vector are outlined in Fig. 1B. This hybrid antigen carries amino acids 630 through 764 of SERP (13), initiating translation at Met-630, followed by amino acid residues 146 through 260 from MSAI (9), a conserved region that includes two additional T-cell epitopes (5). The resulting hybrid antigen carries at least four T-cell epitopes and was combined with the 189 C-terminal amino acid residues of HRPII (15).

The hybrid antigens MS2-SERP-HRPII and SERP-MSAI-HRPII migrate as 75- and 62-kDa proteins in sodium dodecyl sulfate-polyacrylamide gels, respectively, and were detected by Western blot analysis with antisera raised against the corresponding partial sequences of SERP, HRPII, and MSAI. Both hybrid proteins induced antibodies in rabbits that recognized the corresponding malarial proteins SERP, HRPII, and MSAI from *P. falciparum* to a similar extent, as determined by Western blot analysis (Fig. 2). This result shows that none of the fusion partners of the hybrid protein dominates the immune response.

To assess the ability of each of the hybrid proteins to induce a protective immune response, we performed vaccination experiments with *Aotus* monkeys, which are susceptible to the human parasite *P. falciparum* (Fig. 3); we chose an oil-based formulation with polyalphaolefine, which was previously shown to be well tolerated in different species and to induce a long-lasting immune response (6). In trial 1, two animals were immunized with the fusion protein MS2-SERP-HRPII and two other animals served as controls. After challenge with infected erythrocytes, the two control animals showed a rapid increase in parasitemia up to 28 and 14%, respectively. The animal with the higher parasitemia was treated with mefloquine to prevent death, whereas the animal with the lower parasitemia was able to control the infection without treatment. In contrast, parasitemias of only up to 2% were detected in the two monkeys immunized with the hybrid protein MS2-SERP-HRPII.

In trial 2, three groups of three animals each were immunized with MS2-SERP-HRPII or SERP-MSAI-HRPII or served as controls. Upon challenge, the three control animals developed parasitemias of 12, 18, and 23% and had to be treated with mefloquine. One animal in each of the immunized groups showed parasitemias of 2 and 2.8%, respectively, whereas the other two monkeys of each group developed parasitemias of less than 1%.

Serum samples from the monkeys immunized with MS2-SERP-HRPII and SERP-MSAI-HRPII showed high antibody levels in the enzyme-linked immunosorbent assay against the corresponding recombinant malarial antigens, arriving at a plateau after the second immunization. Immunofluorescence titers were low but significant during the immunization period (data not shown). Prechallenge sera

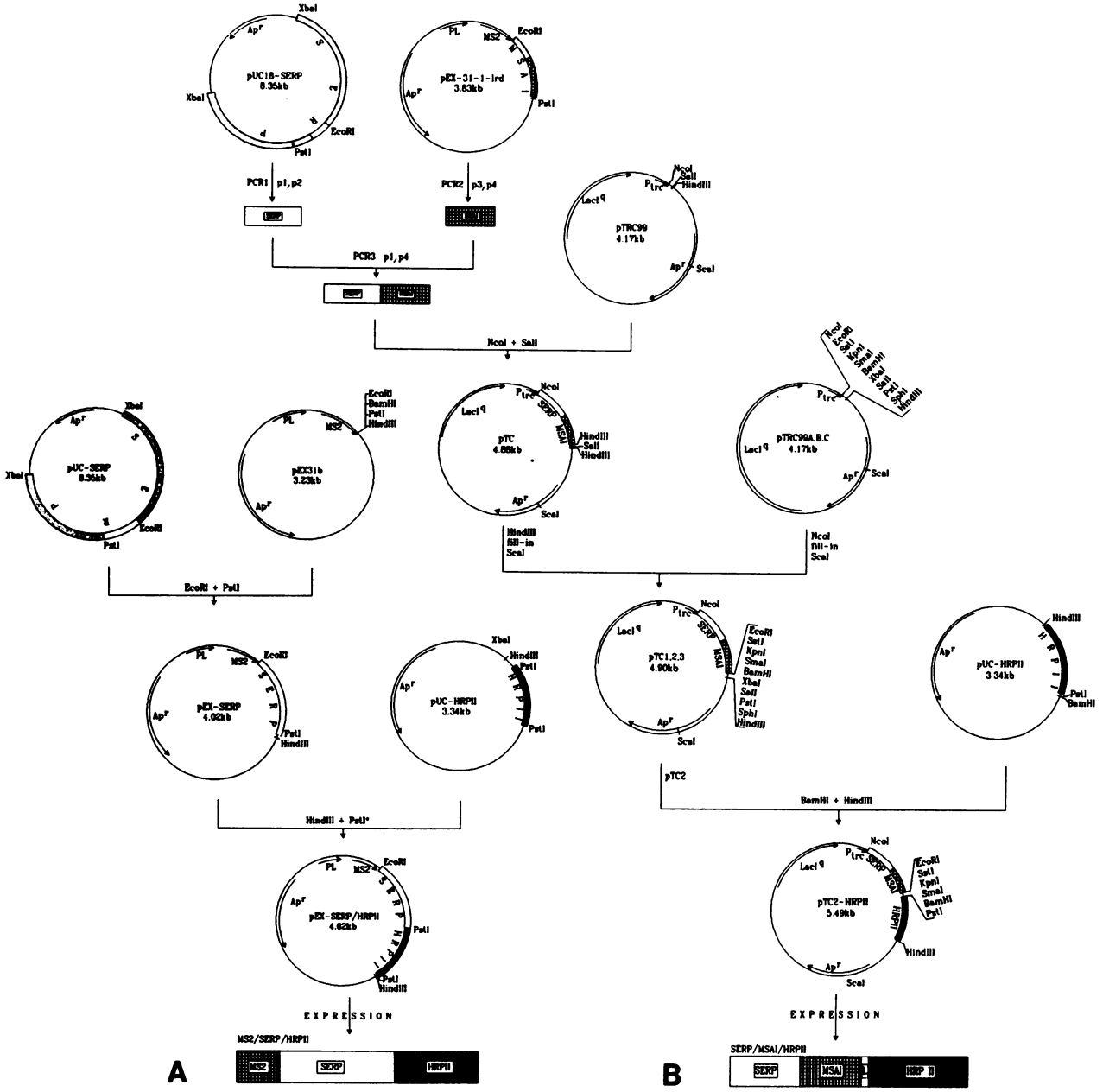

FIG. 1. Construction of the MS2-SERP-HRPII (A) and SERP-MSAI-HRPII (B) hybrid genes. Plasmids were constructed as described in Materials and Methods. Bacteria harboring pEX-SERP/HRPII express a 75-kDa fusion protein that contains parts of three different proteins: 99 amino acids of the MS2-polymerase (amino acids 1 through 99), 262 amino acids of SERP (amino acids 100 through 361), and 189 amino acids of HRPII (amino acids 364 through 552). The amino acid positions 362 and 363 are encoded by a linker region that originates from the cloning procedure. Bacteria containing plasmid pTC2-HRPII express a 62-kDa protein consisting of parts of the three malarial proteins SERP (amino acids 1 through 135), MSAI (amino acids 136 through 250), and HRPII (amino acids 267 through 455) preceded by a linker sequence L (amino acids 251 through 266).

analyzed by Western blots on *P. falciparum* schizonts detected the corresponding malarial antigens SERP, HRPII, and MSAI (Fig. 2).

DISCUSSION

We have demonstrated that recombinant genes coding for sequences from three different malarial vaccine candidate antigens, MSAI, SERP, and HRPII, can be expressed in *E. coli* to produce hybrid polypeptides. Antibodies raised by immunization of rabbits and *Aotus* monkeys with these proteins react with the corresponding *P. falciparum* antigens. The results of the vaccination experiment in *Aotus* monkeys demonstrate that both hybrid proteins induce protective antibodies that effectively prevent a lethal infection after challenge with *P. falciparum*. These experiments were performed with 10% polyalphaolefin as a well-tolerated adjuvant that efficiently stimulates the immune system.

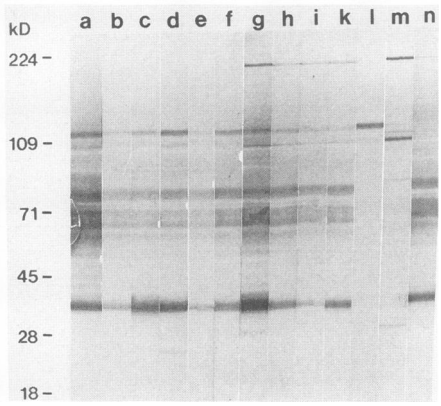

FIG. 2. Western blot analysis of polypeptides from *P. falciparum* schizonts with rabbit antisera raised against the MS2-SERP-HRPII hybrid protein (a) and against the SERP-MSAI-HRPII hybrid protein (g) as well as with prechallenge sera from *Aotus* monkeys from trials 1 (b, c) and 2 (d through f) vaccinated with the hybrid protein MS2-SERP-HRPII and with prechallenge sera from *Aotus* monkeys from trial 2 (h through k) vaccinated with the hybrid protein SERP-MSAI-HRPII. Antisera raised against the individual recombinant SERP (l), MSAI (m), and HRPII (n) fragments react only with their corresponding antigens.

It is generally accepted that a future malarial vaccine needs to contain a combination of different antigens to combine the protective potential of different antigens and to minimize the risk of parasite escape by mutation under immunological pressure. Hybrid antigens have the considerable advantage of production of just one protein compared with the production of several polypeptides for a cocktail vaccine. With this approach, partial sequences of the circumsporozoite protein and the blood-stage antigens MSAI (8) and 5.1 (4) have been combined with recombinant hybrid proteins. Patarroyo et al. used synthetic peptides composed of antigenic motifs from different malarial proteins for the immunization of *Aotus* monkeys and human volunteers (17, 18).

Antigenic structures to be selected as vaccine components should be accessible to the immune system and should contain strong B- and T-cell epitopes; furthermore, they should be conserved among different isolates. The SERP fragments used for the construction of the hybrid antigens described in this study contain a region that carries two T-cell epitopes (22), and in addition they react strongly with antibodies from immune sera of patients and infected monkeys (unpublished results). In addition to having this high epitope density, this region seems to be extremely conserved, as shown by sequence comparison of six *P. falciparum* isolates from different endemic areas; only one conservative amino acid exchange was found within the 262-amino-acid fragment (unpublished results). With the same field isolates, the protective C-terminal sequence of HRPII was shown to be well conserved; only a slight variation in number and arrangement of the repeat units was observed. The MSAI fragment selected as a component of the second hybrid protein is part of the conserved N-terminal region of this antigen contributing two more T-cell epitopes (5).

No difference in protection was observed between the hybrid antigen composed of partial sequences of SERP and HRPII and the construct additionally containing the MSAI moiety. Whether this component provides additional T-cell help could be shown by long-term immunization studies in spleen-intact animals with subsequent challenge infection. Such experiments will be needed to select antigens that induce long-term immunity. Other hybrid constructs with various combinations of the described antigens and addi-

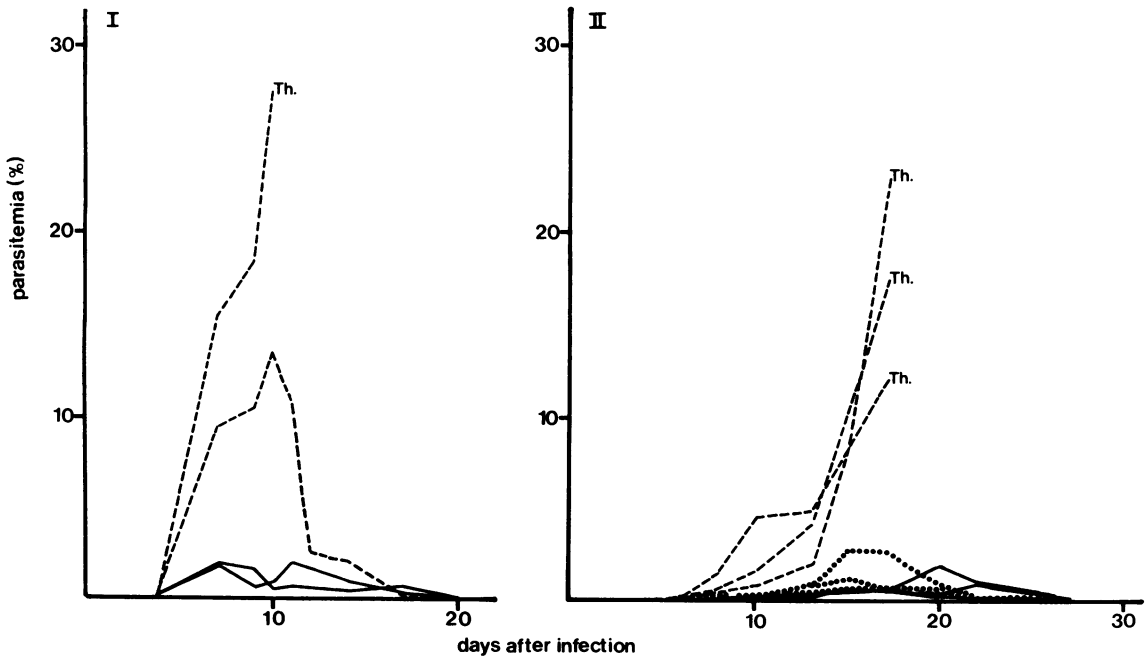

FIG. 3. Parasitemia after *P. falciparum* challenge infection of *Aotus* monkeys immunized with the MS2-SERP-HRPII hybrid protein (——) and with the diluent alone as control (– – –) in trial 1 (I) and of *Aotus* monkeys immunized with the hybrid proteins MS2-SERP-HRPII (——) and SERP-MSAI-HRPII (. . .) and with the diluent alone as control (– – –) in trial 2 (II). Th., mefloquine therapy.

tional malarial epitopes are under preclinical investigation to select a vaccine candidate for clinical trials.

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