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Protective Immunity Induced in *Aotus* Monkeys by Recombinant SERA Proteins of *Plasmodium falciparum*

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We describe the vaccination of Panamanian monkeys (*Aotus* sp.) with two recombinant blood stage antigens that each contain a portion of the N-terminal region of the SERA (serine repeat antigen) protein of the malaria parasite *Plasmodium falciparum*. We immunized with either a 262-amino-acid SERA fragment (SERA 1) that contains amino acids 24 to 285 of the 989-amino-acid protein or a 483-amino-acid SERA fragment (SERA N) that contains amino acids 24 to 506 as part of a fusion protein with human gamma interferon. The recombinant proteins were shown to stimulate protective immunity when administered with complete and incomplete Freund adjuvant. Four of six immunized monkeys challenged by intravenous inoculation with blood stage *P. falciparum* developed parasitemias that were reduced by at least 1,000-fold. Two of six immunized monkeys developed parasitemias which were comparable to the lowest parasitemia in one of four controls and were 50- to 1,000-fold lower than in the other three controls.

The threat of the increasing incidence of malaria drug resistance has stimulated the search for a malaria vaccine. Most of that work has been directed towards a vaccine against the most virulent malaria parasite species, *Plasmodium falciparum*. Antigens of the parasite blood stage forms that can react with parasite-inhibitory antibody and can be produced by cloned genetic information represent a source of candidate antigens to be tested for use in a vaccine.

Work began by isolating a mouse hybridoma that produced a parasite-inhibitory monoclonal antibody to identify a potential vaccine antigen (1). The cDNA nucleotide sequence that encoded the complete protein antigen recognized by the monoclonal antibody was cloned, and the genomic DNA nucleotide sequence of the gene was cloned and sequenced as well (3, 12, 17). That protein, which contains 989 amino acids including a putative signal peptide sequence, has a molecular weight (MW) of 111,000, and has an 11% serine content with a long serine repeat of 35 serine residues, was named SERA (serine repeat antigen). It is a major protein in that at least 1.5% of mRNA in trophozoites and schizonts is SERA mRNA (3, 17). Immunologic data and nucleotide sequence data have shown that the SERA protein and three other proteins. P126 (6, 7, 12, 23), P113 (4), and P140 (5, 15, 19), that were originally identified by their relative size in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, are the same protein. Comparisons of the published SERA amino acid sequences from different *P. falciparum* strains suggest that most of the amino acid sequences are highly conserved, with the exception of the region in and around the serine repeat sequence, which contains multiple point and deletion differences. The protein accumulates in the parasitophorous vacuole of trophozoites and schizonts and is released into culture medium at or near the time of merozoite release when it undergoes processing into several smaller polypeptides (6, 7). The amino acid sequence of a portion of the molecule is similar to that of certain proteinases (8, 11, 18), suggesting a function

and the possible reason why antibodies directed against it may interfere with parasite growth. Perrin et al. (19) immunized *Saimiri* monkeys with the purified whole P140 protein isolated from cultured parasites and showed it could provide partial immunological protection against malaria infection. We have synthesized parts of the SERA protein in the yeast *Saccharomyces cerevisiae*, purified them, and shown that they stimulate parasite-inhibitory antibodies in mice that are effective in vitro (2). We report here the immunization of Panamanian Owl monkeys (*Aotus* sp.) with two of those purified proteins and the protection of those immunized monkeys from infection by the Honduras I (13, 14) strain of *P. falciparum*.

MATERIALS AND METHODS

Monkeys. Panamanian *Aotus lemurinus lemurinus* monkeys were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Panama. A total of 10 animals (adult males and females, weighing from 759 to 970 g) were divided into three groups of three each that were injected intramuscularly with antigen(s) and/or adjuvant and one group of one monkey which was used as a completely naive control. The monkeys had had no previous exposure to *P. falciparum*.

Antigens. Two SERA-derived antigens, SERA 1 and gamma interferon (IFN-γ)–SERA N, were expressed from cloned portions of the Honduras I SERA gene in *S. cerevisiae* and purified (2). The purified SERA 1 protein contains 262 amino acids (MW, 26,976) of SERA (989 amino acids) starting from amino acid 24 (Gly) and ending with amino acid 285 (Asp) (3, 17). The purified IFN-γ–SERA N protein contains 483 amino acids (MW, 52,520) of SERA starting from amino acid 24 (Gly) and ending with amino acid 506 (Pro) (3, 17). The sequence is fused at its N-terminal end to IFN-γ (MW, 16,704), thus giving a fusion protein with a MW of 69,224. SERA 1 was dissolved in phosphate-buffered saline (PBS) and 0.05% SDS, while IFN-γ–SERA N was dissolved in PBS and 0.1% SDS. Both antigens were dissolved at 800 μg/ml. The purified antigens separated by
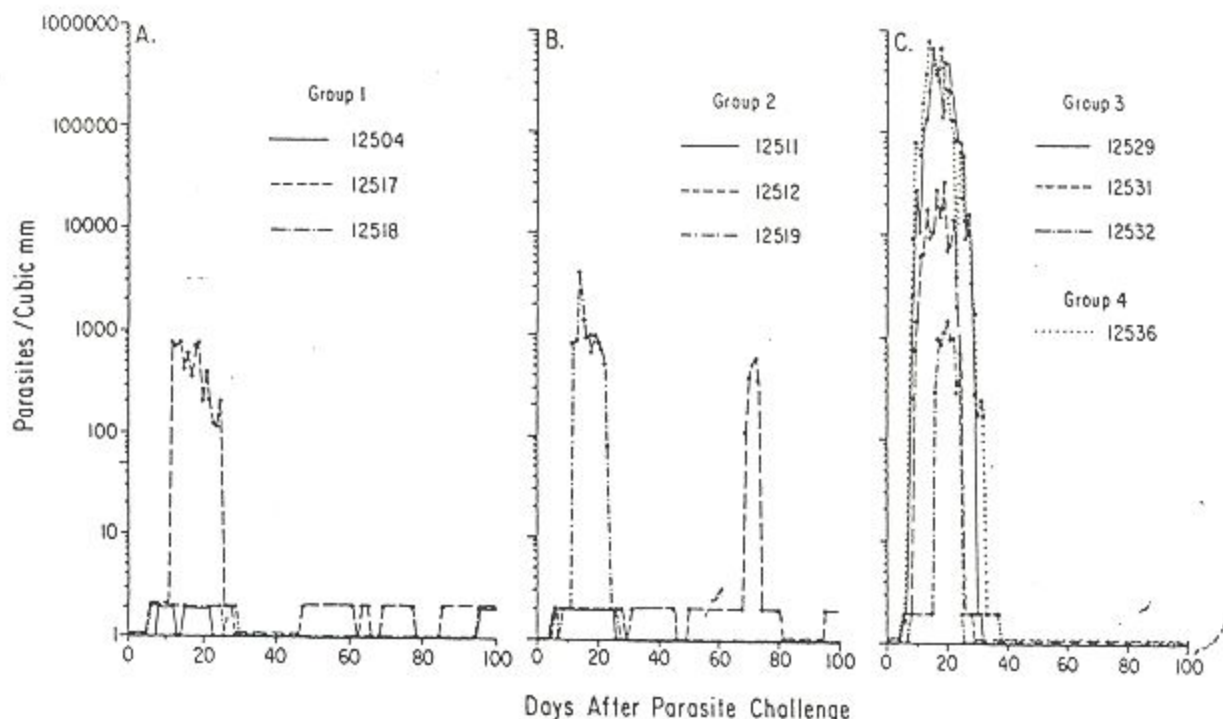
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FIG. 1. Course of parasitemia in four groups of monkeys. Each monkey was infected with 5×10^4 Honduras I-infected erythrocytes obtained from the same infected monkey. In the $\log_{10}$ plot of parasites per mm³, the symbols at a value of 1 indicate that no parasites were seen, while the symbols at a value of 2 indicate that fewer than 10 parasites per mm³ were seen (see Materials and Methods).

SDS-PAGE (16) gave a single band, as determined by Coomassie blue staining and Western blot (immunoblot) analysis (2, 22).

Immunization. Antigen was injected intramuscularly on days 0 and 21 of the experiment. Each dose of antigen was in a final volume of 0.5 ml that contained 200 µg of the appropriate antigen(s) in 0.25 ml that was mixed with 0.25 ml of adjuvant immediately before a vaccination injection. Monkeys in groups 1, 2, and 3 received complete Freund adjuvant (CFA) in the primary injection and incomplete Freund adjuvant (IFA) in the booster injection. Monkeys in group 3 received the antigen carrier solution mixed with the Freund adjuvant in each injection. The monkey in group 4 received neither antigen nor adjuvant. Each dose for injection was divided into two 0.25-ml portions and injected intramuscularly into two sites in one thigh of the monkey. The booster injection was in the other thigh. All animals were bled 5 days prior to the start of immunization on day zero, 18 days after the primary vaccination injection, 21 days after the single booster injection, and at appropriate times after the parasite challenge.

Parasites. *P. falciparum* Honduras I (13) was used for challenge. It was chosen because it had been passaged through splenectomized and then nonsplenectomized *Aotus* monkeys (14) until it produced a good parasitemia in the latter and because the two SERA-derived antigens were encoded by the cloned Honduras I SERA gene.

Parasite challenge. Twenty-two days after the booster injection, each monkey was injected intravenously with 5×10^2 parasites. Parasitized erythrocytes from an infected monkey with a moderate and increasing parasitemia were used in the challenge to ensure parasite viability. After challenge, the parasitemia was monitored daily by both thick and Earle-Perez films (9) stained with Giemsa. We evaluated

parasitemias by several criteria. A negative parasitemia is reported if no parasites were seen after examining a thick blood film for at least 5 min. It is recorded as 1 in the log scale in Fig. 1. A parasitemia of <10 parasites per mm³ is reported if parasites could be demonstrated only in a thick blood film. It is recorded as 2 in the log scale in Fig. 1. Parasite numbers of >10 per mm³ determined by the Earle-Perez method (9) were recorded in Fig. 1.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as previously described (10). A_{650} was measured. The preimmunization serum of each monkey was used as the control for each postimmunization serum.

RESULTS

Four groups of monkeys were used to examine the ability of either purified SERA 1 or IFN-γ-SERA N proteins mixed with Freund adjuvant to induce a protective immune response in monkeys to *P. falciparum* Honduras I. A presentation of the different groups of monkeys that were used, their respective treatments prior to the challenge infection with the Honduras I strain, and a summary of the monkeys' humoral immune status as measured by ELISA are shown in Table 1. Control serum was taken 5 days prior to the initiation of vaccination (Table 1, prebleed). Postimmunization sera were taken 18 days after the primary vaccination (Table 1, bleed 1) and 21 days after the single boost injection (Table 1, bleed 2), which was just prior to the parasite challenge.

The development and course of infection was monitored in all monkeys for 100 days after challenge (Fig. 1). All monkeys developed parasitemias. The prepatent period was between 6 and 8 days for monkeys in all groups.

Two of three monkeys immunized with SERA 1 antigen

TABLE 1. Antibody titers in monkeys measured by ELISA[a]

Monkey	Antigen/adjuvant	Titer		
		Prebleed(s)	Bleed 1	Bleed 2
Group 1	S1/CFA+IFA			
12504		<100[b]	117,512	258,710
12517		438	70,301	97,740
12518		<100	42,168	151,530
Group 2	γSN/CFA+IFA			
12511		104	214,772	254,320
12512		<100	43,772	54,350
12519		153	47,882	57,740
Group 3	None/CFA+IFA			
12529		<100/<100[c]	<100/<100	<100/100
12531		<100/<100	<100/<100	<100/<100
12532		<100/<100	<100/<100	<100/<100
Group 4	None/None			
12536		ND	<100/<100	<100/<100

[a] S1, SERA 1; γSN, IFN-γ–SERA N; ND, not determined.
[b] ELISA values are the dilutions of serum that gave an optical density at 650 of 0.200.
[c] ELISA titers were measured in assays against both SERA 1 and IFN-γ–SERA N antigen.

(group 1, 12504 and 12518) developed peak parasitemias of fewer than 10 parasites per mm^3, and the third one (12517) developed a peak parasitemia of about 1,000 parasites per mm^3 within the first 20 days after challenge (Fig. 1A). Two of three monkeys immunized with IFN-γ–SERA N (group 2) developed peak parasitemias of fewer than 10 parasites per mm^3 during the first 20 days, and subsequently one of those (12512) then developed a brief parasitemia with a peak of 590 parasites per mm^3 between days 69 and 73. The third monkey (12519) developed a parasitemia of about 800 parasites per mm^3, with a brief peak of 4,000 parasites per mm^3 during the first 20 days (Fig. 1B). Monkeys 12517 and 12512 continued to show parasitemias that fluctuated between less than 10 parasites per mm^3 and undetectable levels during much of this 100-day period, while the other four immunized monkeys from both groups had, with one brief exception for 5 days (12518), no detectable parasitemias during the period following the original peak parasitemias.

Two of three monkeys in the control group that had received only Freund adjuvant (group 3) developed much higher peak parasitemias than did those in groups 1 and 2. The third monkey in group 3 developed a relatively low parasitemia that was comparable to but still higher than the highest parasitemias exhibited by monkeys in groups 1 and 2 (Fig. 1C). The monkey in group 4 that had received neither an antigen nor adjuvant injection developed a peak parasitemia that was also much higher than those in the monkeys in groups 1 and 2 (Fig. 1C). All the peak parasitemias in the control groups also occurred within the first 20 days after challenge. After the subsidence of those initial parasitemias in all the controls, no parasites were subsequently seen. Not only were the parasite levels in the two monkeys from groups 1 and 2 much lower than those in the controls, but the times during which parasitemias in monkeys were greater than 500 parasites per mm^3 were short compared with the times of higher parasitemias in group 3 and 4 monkeys (Fig. 1). These results suggest that the early parasite loads experienced by challenged monkeys may influence the subsequent course of parasite growth in the animal.

The three immunized monkeys in groups 1 and 2 whose prechallenge antibody levels were above 150,000 (12504, 12518, and 12511) did not develop countable parasitemias, while the other three monkeys (12517, 12512, and 12519) in those groups with prechallenge antibody levels between 50,000 and 100,000 did (Table 1; Fig. 1). These results suggest a relationship between the level of the humoral immune response to the SERA proteins and the manifestation of immune protection.

DISCUSSION

We have shown that the SERA 1 and IFN-γ–SERA N proteins induce a protective immune response in a Panamanian Aotus sp. when immunization is done with CFA and IFA. These results are consistent with our original observation that the parasite-inhibitory mouse monoclonal antibody, 43E5, reacted with a SERA polypeptide (1) encoded by cDNA clone 366 (3) (SERA amino acids 23 to 357), which includes some of the same amino acid sequences as those encoded by the overlapping SERA 1 (amino acids 24 to 285) and IFN-γ–SERA N (amino acids 24 to 506) sequences (2). The observed protection is also consistent with the report of Perrin et al. (19), who showed that immunization with the whole P140 protein, purified from cultured schizonts of P. falciparum SGE2 from Zaire, could partially protect Saimiri monkeys from a blood stage infection. They reported that three monkeys developed peak parasitemias of less than 3% and one monkey developed a peak parasitemia of 4.5%. Assuming an average erythrocyte concentration of 5.0×10^6 parasites per mm^3 in the Aotus sp. (21), the monkeys in groups 1 and 2 had peak parasitemias of less than approximately 0.05%. While the results of Perrin et al. (19) cannot be readily compared to ours, as the genera of the monkeys, the antigens, the immunization protocols, and the challenge infections were all different, each result suggests that SERA antigens from different parasite strains, with documented differences in amino acid composition (3, 5, 15, 17, 23), can confer protective immunity to parasite challenge.

The fusion of IFN-γ to SERA was necessary to obtain high levels of the SERA N fragment in yeast cells (2). The IFN-γ portion of IFN-γ–SERA N has interferon activity in culture (unpublished data). IFN-γ has been reported to be capable of enhancing the protective immune response of mice to Plasmodium yoelii (20). The IFN-γ–SERA N protein provided a level of protection against infection (e.g., levels of parasitemia) and an immune response (e.g., antibody titers) that were similar to those of SERA 1 (Fig. 1; Table 1). Because we could not prepare sufficient amounts of the nonfused SERA N protein and because IFN-γ might effect the immune response, we do not know what effect the additional amino acids in SERA N, compared with those in SERA 1, had on the immune response.

We observed that the immunized monkeys with ELISA titers that were less than 100,000 sustained low-grade parasitemias for much of the period during which they were observed. The immunization in those animals was apparently sufficient to provide significant but incomplete suppression of parasite growth during the period of observation. The low-level parasitemias that subsequently occurred in these immunized animals were apparently unable to provide a further immunological stimulus that could completely inhibit the parasite such as was apparently produced by the much greater parasite loads that occurred in the control monkeys. The interesting question of the induction of a sterile versus a premunition immunity by a vaccine must be considered and

will be addressed as we test other adjuvants that are acceptable for use in humans.

The delayed appearance of a brief, low-level countable parasitemia in one immunized monkey (12512) was probably a consequence of changes in competing interactions of immunologic and physiologic factors that control parasite growth in a partially immunized, infected monkey. The brevity and low level of the episode, which occurred during a long, fluctuating low-level parasitemia, does not suggest the appearance of a parasite mutant that was selected for because of its lack of response to the anti-SERA immunity. The cloning of parasites isolated during such an episode and the sequencing of the SERA gene may be of interest.

The reason for the unexpectedly low-level parasitemia seen in one control animal (12532) is not known. It may reflect a natural resistance of some animals or a problem in injecting the parasite challenge. Whatever the explanation, it does not alter the interpretation of the preponderance of the evidence that the SERA protein in this system provided immunologic protection against the Honduras I parasite.

It is not known why antibody that interacts with part of the SERA protein, a major processed, exported *P. falciparum* protein, inhibits parasite growth in cultures and can provide protective immunity in monkeys. While the protein is found principally in the parasitophorus vacuole, it has also been found associated with intraerythrocytic but not free merozoites in culture (1, 7). The fragility of free merozoites in culture may explain the inability to detect the association with them.

Higgins et al. (11) reported that the amino acid sequence in the region of the carboxyl end of the SERA protein suggests that the molecule may be a cysteine proteinase. Others (8, 18) have pointed out that the structure of the active site of the presumed proteinase suggests that it is really a serine proteinase that may have evolved from a cysteine proteinase. The immunological protection produced by anti-SERA antibodies may be related to the inhibition of an essential proteinase activity provided by SERA even though the antibodies are directed against the region of the amino end of the molecule. If that is the cause of immunological protection, then perhaps a vaccine based on the SERA protein will not be readily compromised by changes in the antigen structure, because the important parts of the antigen and enzyme structure of SERA may both be conserved. Comparison of known SERA sequences from different parasite strains have indicated that most of its amino acid sequence is highly conserved (3, 5, 15, 17, 23). Further tests of these antigens with appropriate adjuvants acceptable for human use are now being conducted.

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