Protective Immunity Induced in Aotus Monkeys by a Recombinant SERA Protein of Plasmodium falciparum: Further Studies Using SERA 1 and MF75.2 Adjuvant

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We describe the third of three vaccination trials of Panamanian Aotus monkeys with a recombinant blood-stage antigen derived from the malaria parasite Plasmodium falciparum. Immunization was performed with an N-terminal region of the SERA antigen (serine repeat antigen protein), SERA 1, that contains a 262-amino-acid fragment including amino acids 24 to 285 of the 989-amino-acid SERA protein. Vaccinations were carried out with the recombinant protein mixed with either Freund's, MF59.2, or MF59.2 adjuvant. A control group that did not receive SERA 1 but only MF75.2 adjuvant was included. Monkeys vaccinated with the antigen MF59.2 mixture produced low anti-SERA 1 titers and were not protected. Monkeys vaccinated with antigen and Freund's adjuvant had, in general, a higher average anti-SERA 1 titer (107,278) than did monkeys immunized with SERA 1 and MF75.2 (40,143), yet monkeys in both groups were well protected. Monkeys that received only MF75.2 developed neither detectable anti-SERA 1 nor anti-P. falciparum antibodies prior to or 10 days after parasite challenge, yet were apparently protected against infection. Monkeys vaccinated with either SERA 1 and Freund's, SERA 1 and MF75.2, or MF75.2 alone and that had been challenged but did not develop a countable parasitemia were treated with a curative dose of mefloquine 100 days after parasite challenge and then rechallenged 40 days later. None of the five rechallenged monkeys that had originally received SERA 1 and Freund's developed a countable parasitemia. Only one of five rechallenged monkeys that originally received SERA 1 and MF75.2 developed a high countable parasitemia, while two animals developed a barely countable parasitemia. Four of the rechallenged monkeys that had originally received only MF75.2 developed a moderate to high countable parasitemia. The results indicate that vaccination with SERA 1 and either Freund's or MF75.2 adjuvant provides protection and vaccination with MF75.2 alone can provide a temporary protection unrelated to the induction of anti-SERA 1 or antimalarial antibodies.

We have examined the capability of parts of the Plasmodium falciparum parasite antigen SERA (3, 6, 11–13) to induce parasite-inhibitory antibodies (1, 2) and a protective immune response in Panamanian Aotus monkeys (8, 9). In a previous trial, trial 1 (9), we reported that approximately the N-terminal quarter of the molecule, SERA 1, and half of the molecule, γ-SERA N, mixed with Freund's adjuvant could induce a protective immune response. In the accompanying report, trial 2 (8), preliminary evidence showed that the MF75.2 adjuvant, when mixed with the SERA 1 portion (amino acids 24 to 294) of the complete SERA molecule (989 amino acids) (3), also induces a protective immune response to challenge with blood-stage parasites. We report here the vaccination of larger groups of monkeys than used previously, eight animals per group, with the SERA 1 antigen and either MF75.2, Freund's, or MF59.2 adjuvant. The results of vaccinating increased numbers of individuals in each group and of being able to analyze the cumulative numbers of monkeys in the three trials that had received the same antigen-adjuvant treatment have further clarified the interpretation of results. The character of the protection afforded by the antigen and the adjuvants is considered.

MATERIALS AND METHODS

Monkeys. Panamanian Aotus lemurinus lemurinus monkeys were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Panama (14). Thirty-two animals (adult males and females, weighing 637 to 1,306 g), some born in the laboratory and some caught in the wild, were without previous exposure to P. falciparum. The monkeys were not splenectomized. They were divided into four groups of eight each that were balanced for sex, size, and whether they were born in the laboratory or caught in the wild. Animals were injected intramuscularly with antigen(s) or adjuvant or both. A nonsplenectomized, malaria-infected monkey was used as the source of parasites for challenge infections.

Antigens. The recombinant SERA-derived antigen, SERA 1, was described previously (2). SERA 1 was dissolved in a carrier solution of phosphate-buffered saline and 0.05% sodium dodecyl sulfate and mixed with an equal volume of an appropriate adjuvant.

Adjuvant. Freund's (complete or incomplete), MF59.2, and MF75.2 adjuvants were described previously (2, 8, 9).

Immunization. Antigen with appropriate adjuvant was injected intramuscularly on days 0, 28, and 56 of the experiment. Each dose of antigen was in a final volume of 1.0 ml that contained 100 μg of SERA 1. Freund's complete or incomplete adjuvant and antigen was mixed immediately before a vaccination injection. The other antigen-adjuvant

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mixtures were prepared more than a week prior to injection and stored at 0°C. Monkeys received Freund's complete adjuvant in the primary injection and Freund's incomplete adjuvant in the booster injections. Monkeys in the MF75.2 control group received the antigen-carrier solution mixed with MF75.2 in each injection. Each dose for injection was divided into two 0.5-ml portions and injected intramuscularly into two sites in one thigh of the monkey. The booster injections were in the alternate thigh. All animals were bled 7 days prior to the start of immunization, which was designated as day 0, and then prior to and after parasite challenge.

Parasites. *P. falciparum* Honduras I (7) was used for challenge. It was chosen because it had been passaged through splenectomized and then nonsplenectomized *Aotus* monkeys (10) until it produced a good parasitemia in the latter and because the SERA-derived antigen was encoded by the cloned Honduras I SERA gene.

Parasite challenge and rechallenge. Twenty-nine days after the second booster injection, each monkey was injected intravenously with 5 × 10⁴ parasites as described previously (8, 9). Parasitized erythrocytes taken from the same infected monkey that had a moderate and increasing parasitemia were used in the challenge to ensure parasite viability and the same parasite dose per animal. After challenge, the parasitemia was monitored daily by both thick and Earle-Perez (4) films stained with Giemsa. We evaluated parasitemias as described previously (8, 9). A negative parasitemia is reported if no parasites were seen after examining a thick blood film for at least 5 min and is recorded as 1 in the log scale in the figures. A parasitemia of <10 parasites per mm³ is reported if parasites could be demonstrated only in a thick blood film and is recorded as 2 in the log scale in figures. Parasite numbers of >10 parasites per mm³, counted by the Earle-Perez method, are recorded in the figures. All monkeys were treated with a single curative dose of mefloquine (40-mg base/kg of body weight) 100 days after challenge. Parasite-infected monkeys that had not developed a countable parasitemia (>10 parasites per mm³) within 100 days of the initial parasite challenge were selected from three of the four groups for rechallenge and reinjected with 5 × 10⁴ parasites 40 days after the mefloquine treatment to avoid residual drug effects.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (5, 9). The A₄₅₀ was measured. The preimmunization serum of each monkey was used as the control for each postimmunization serum.

### RESULTS

Four groups of eight monkeys each were used to examine the ability of purified SERA 1 to induce a protective immune response in monkeys to *P. falciparum* Honduras I. The decision to vaccinate larger groups of monkeys than before was made to provide a basis for a more definitive test of the ability of the antigen to induce a protective immune response. The comparison of the cumulative results of the responses of monkeys receiving the same antigen-adjuvant combination in two previous vaccination trials (8, 9) with those in this trial would also provide a better sample on which to base the interpretation of the results. In Table 1 are listed the groups of monkeys (groups 1 to 4), their respective treatments with adjuvant or antigen or both, and a summary of the humoral anti-SERA 1 antibody titers as measured by ELISA 10 days prior to and 10 days after challenge infection with the Honduras I strain. The control ELISA titers were <10.

The data showing the development and course of infection monitored in all monkeys for 140 days after challenge are shown (Fig. 1). The data are plotted on a daily basis as described in Materials and Methods. On day 100 all monkeys were treated with a single curative dose of mefloquine. In group 1 monkeys, immunized with SERA 1 plus Freund's adjuvant, patent parasitemia was seen at 15 to 34 days in five monkeys. Two monkeys had no patent parasitemia. One monkey had a patent parasitemia on day 7, which was similar to previous observations of the appearance of the initial patent parasitemias (8, 9). In group 2 monkeys, immunized with S1 plus MF59.2, all monkeys had a patent parasitemia between days 5 and 7, and seven of eight monkeys went on to develop a countable parasitemia by day 12. One monkey never developed a countable parasitemia and died in an accident on day 117 (see X in Fig. 2). In group 3 monkeys, immunized with SERA 1 plus MF75.2, patent parasitemia of four monkeys was seen between days 6 and 13 and that of four others was delayed to between days 20 and 35. One monkey died accidentally on day 86 (see X in Fig. 2). Two monkeys eventually developed a countable parasitemia, one on day 53 and the other on day 84. In group 4 monkeys, immunized with only MF75.2, the initial patent parasitemia of four monkeys was seen on day 6, while in three monkeys it was delayed to days 25, 37, and 42. One monkey died of a bacterial infection on day 16, prior to any observed parasitemia (see X in Fig. 2). Two monkeys eventually developed a countable parasitemia, one on day 50 and the other on day 96. Many of the challenged monkeys

### Table 1. Anti-SERA 1 antibody titers of serum from vaccinated *Aotus* monkeys measured by ELISA

<table>
<thead>
<tr>
<th>Group 1 (FA, SERA 1)</th>
<th>Group 2 (MF59.2, SERA 1)</th>
<th>Group 3 (MF75.2, SERA 1)</th>
<th>Group 4 (MF75.2, control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>102,205</td>
<td>147,216</td>
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<td>7,022</td>
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<td>104,391</td>
<td>91,266</td>
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<td>2,100</td>
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<td>71,563</td>
<td>96,873</td>
<td>2,733</td>
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</tr>
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<td>70,921</td>
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<td>130,054</td>
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</tr>
<tr>
<td>67,521</td>
<td>77,385</td>
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<td>1,445</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>2,877</td>
<td>1,108</td>
</tr>
</tbody>
</table>

* a Titers were measured 10 days before and 10 days after parasite challenge.

* b ELISA titers prior to vaccination with SERA 1 were <10⁷.

* ND, not done.
had recurrent parasitemias that were only detected on thick smears.

Another way to display the data to compare parasitemias, and therefore the monkey's ability to control parasite growth in the different groups, is to determine the cumulative parasitemia that developed in monkeys during the periods of countable parasitemia, the "parasite load" (8). This was calculated for each monkey from the data in Fig. 1, and the data are shown in Fig. 2. The protection provided monkeys vaccinated with SERA 1 and either Freund's or MF75.2 adjuvant is consistent with results presented in trials 1 and 2 (8, 9). The failure to achieve protection in monkeys vaccinated with SERA 1 plus MF59.2 is consistent with the results presented in trial 2 (8). The protection provided monkeys vaccinated with SERA 1 and MF75.2 appears consistent with the results presented in trial 2. The apparent resistance to parasite infection of monkeys that only received MF75.2 was unexpected and raised questions as to whether SERA 1 contributed to the protection of monkeys in group 3.

We did several things to clarify the interpretation of these results. The samples used to vaccinate monkeys in groups 3 and 4 were tested for protein content to rule out either a mix-up of samples or the accidental addition of SERA 1 to the sample that should have contained only MF75.2. No detectable protein was found in the group 4 vaccine samples that were to contain only MF75.2. Furthermore, no anti-SERA 1 antibody was detectable 10 days after parasite challenge (Table 1) in monkeys that had only received MF75.2. The apparent protection of group 4 monkeys was therefore not a result of a simple mistake. We then tested the
possibility that a high level of nonspecific antimalarial antibodies were produced by monkeys in group 4 prior to or after parasite challenge. Using a preparation of total parasite antigen, which should also include some SERA 1, we measured the antiparasite antibodies by ELISA in all pre-vaccination, 10-day prechallenge, and 10-day postchallenge monkey sera. The results, in summary, showed the following: (i) none of the prevaccination sera had detectable antiparasite antibody titers (<10); (ii) monkeys that were immunized with SERA 1 and Freund’s, SERA 1 and MF59.2, SERA 1 and MF75.2, and only MF75.2 had average 10-day prechallenge antiparasite antibody titers that were, respectively, 9,746, 1,199, 3,724, and <10; (iii) monkeys that were immunized with SERA 1 and Freund’s, SERA 1 and MF59.2, SERA 1 and MF75.2, and only MF75.2 had average 10-day postchallenge antiparasite antibody titers that averaged, respectively, 11,026, 1,320, 3,479, and 90. These results do not support the possibility that the challenged monkeys receiving only MF75.2 could have been protected by a production of high levels of antimalarial antibody.

We next tested the hypothesis that monkeys that received only MF75.2 were only transiently protected against blood-stage challenge compared with monkeys that received MF75.2 plus SERA 1. Five monkeys from each of the groups (groups 1, 3, and 4) that had been vaccinated but had not developed a countable parasitemia within 100 days of the initial parasite challenge (Fig. 2, underlined bars) were treated with a single curative dose of mefloquine and then were observed for 40 days, during which time no parasites were observed. Those monkeys were then rechallenged with $5 \times 10^4$ parasites and monitored daily for 35 days. The results of that experiment are shown in Fig. 3. None of the five rechallenged monkeys from group 1 (Freund’s plus SERA 1) developed a countable parasitemia. Two rechallenged monkeys from group 3 (MF75.2 plus SERA 1) did not develop a countable parasitemia, two developed a cumulative parasitemia of 230 and 310 parasites per mm$^3$, and only one developed a cumulative parasitemia of about 10,000 parasites per mm$^3$. Three of five of the rechallenged monkeys from group 4 (MF75.2) developed a cumulative parasitemia of $>100,000$, and one developed a cumulative parasitemia of about 3,000. We interpret these results to support the interpretation that MF75.2 alone had an unknown transient effect on parasite growth in the challenged monkeys and the monkeys immunized with SERA 1 plus MF75.2 were protected through the immunization with SERA 1 and exhibited a transient adjutant effect.

**DISCUSSION**

In this report we show that the SERA 1 antigen mixed with either Freund’s or MF75.2 adjuvant provides protection to most vaccinated monkeys as measured by either the strict criterion of the vaccinated animals not developing a countable parasitemia or the less strict criterion of the challenged animals developing a delayed and significantly reduced parasitemia. The interpretation becomes more clear when the cumulative results from the two previous vaccination trials we have conducted are compared with those of trial 3 (8, 9). If one considers that vaccine-protected monkeys are only those that do not develop a countable parasitemia, then the following ratio of protected/total monkeys receiving a particular treatment in those cumulative results from trials 1, 2, and 3 has been: 0:3 naïve control animals; 1:6 animals when only Freund’s adjuvant was given; 9:14 when Freund’s adjuvant plus SERA 1 was given; 7:11 when MF75.2 plus SERA 1 was given; and 1:11 when MF59.2 plus SERA 1 was given. Overall protection afforded by SERA 1 immunization is even better than the data suggest when some animals exhibiting a delayed onset of a countable parasitemia and a low countable parasitemia are considered among the protected animals. It is clear that unknown variables, which may include individual monkey susceptibility to infection or immune system responsiveness, may occasionally influence the results and so make the interpretation of some results difficult.

While the results strongly confirm that SERA 1 and the appropriate adjuvant are very protective, we have been perplexed by our observations that immunized monkeys often do not show a sterile immune response but may show a recurring low-grade infection, as determined by positive thick smears, seen over long periods. In naturally immune humans in areas of endemicity, there is often a persistent low-level parasitemia associated with a premunition type of immunity. The anti-SERA 1 or anti-ßSERA N (9) antibodies are clearly capable of suppressing the blood-stage infection and holding it in check; however, their presence is often not sufficient to eradicate the organism. We have speculated that the balance being struck between the growth rate of the parasite and the antiparasite action of the immune system is influenced by physiological factors that would eventually be tilted in favor of the complete immune suppression of parasite infection as a result of the stimulation of either a more effective anti-SERA 1 antibody response or other antiparasite antibodies developed during the controlled low-grade infection. Measurements of the anti-SERA 1 and anti-*P. falciparum* antibodies to trophozoite and schizont antigens had been done after challenge. We examined the anti-SERA 1 antibody titers in monkeys 10 days after the challenge infection (Table 1), which was 20 days after the previous titer was measured (Table 1). The postchallenge
anti-SERA 1 titers in monkeys immunized with SERA 1 plus Freund’s adjuvant remained stable or were marginally increased. The antibody titers induced in the presence of SERA 1 and either MF75.2 or MF59.2 appeared to have undergone a general decline during the post-parasite challenge. In trial 2 the anti-SERA 1 titers significantly declined over the course of the study (8). The inability of the animals immunized with SERA 1 and different adjuvants to sustain or increase the levels of anti-SERA 1 antibody titers and to develop other antimalarial antibodies after the challenge infection may in part explain the chronic low-level infections and the delayed occurrences of countable parasitemias seen in vaccinated animals.

We suggest that these results strongly support the use of SERA antigen as a useful vaccine antigen and highlight the necessity of choosing an appropriate adjuvant to promote its use.

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