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Protective Immunity Induced in *Aotus* Monkeys by a Recombinant SERA Protein of *Plasmodium falciparum*: Adjuvant Effects on Induction of Protective Immunity

JOSEPH INSELBURG,[1]* IAN C. BATHURST,[2]† JOE KANSOPON,[2] GAIL L. BARCHFELD,[2] PHILIP J. BARR,[2]† AND RICHARD N. ROSSAN[3]

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755-3842[1]; Chiron Corporation, Emeryville, California 94608-2916[2]; and Gorgas Memorial Laboratory, Panama City, Republic of Panama[3]

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We report the results of vaccination trial 2 of Panamanian *Aotus* monkeys with a recombinant blood-stage antigen, SERA 1, of the malaria parasite *Plasmodium falciparum*. Monkeys were immunized with SERA 1, a 262-amino-acid fragment (amino acids 24 to 285) of the 989-amino-acid SERA protein produced by the Honduras 1 strain of the parasite. Immunization mixtures contained 100 μg of recombinant SERA 1 protein per dose mixed with one of five different adjuvants. The protein mixed with either Freund's adjuvant or MF75.2 adjuvant stimulated protective immunity. When other *P. falciparum* antigens were included in the SERA 1-Freund's adjuvant mixture, no protective immunity was observed, although high anti-SERA 1 antibody titers were produced. Three other adjuvants mixed with SERA 1 failed to induce a protective immune response. These results, their relationship to those reported previously in the first vaccination trial (trial 1), and their relationships to the quantitative measurement of anti-SERA 1 antibodies in enzyme-linked immunosorbent assays provided insights into the induction of a protective immune response in vaccinated monkeys.

The increasing incidence of malaria drug resistance expressed by *Plasmodium falciparum*, the most virulent malaria species that infects human, has stimulated a search for a malaria vaccine.

We originally identified a parasite blood-stage protein that reacted with a parasite inhibitory monoclonal antibody in vitro (2). The complete gene encoding this protein was subsequently cloned and sequenced (9, 22, 31). The gene, in the Honduras 1 strain, encoded SERA (serine repeat antigen), a protein produced in large amounts in the trophozoite-schizont blood stages (9). The protein contained 989 amino acids which included a signal peptide sequence. The gene and an allele have also been completely sequenced by Knapp et al. (28, 29). Through comparisons with other *P. falciparum* gene sequences, it was found that SERA is essentially identical to three other antigens identified separately as SERP or p140, p126, or p113 (11, 12, 28, 40). SERA is secreted into the parasitophorous vacuole of the parasite and eventually found in a processed form in the extraerythrocytic environment (16, 17). The observations that parts of the amino acid sequence of SERA are very similar to those of proteinases found in different organisms (18, 21) and that proteinase inhibitors inhibit parasitic growth or development in vitro suggest why antibodies directed against SERA may be parasite inhibitory (3, 14, 15). We had synthesized parts of the SERA protein in the yeast *Saccharomyces cerevisiae* and shown that two proteins, SERA 1 (containing SERA amino acids 24 to 285) and γSERA N (containing amino acids 24 to 506), induce high titers of parasite-inhibitory antibodies in mice when vaccination is performed with either Freund's adjuvant or the muramyl tripeptide-based adjuvant MF59 (5). We subsequently reported in our first monkey vaccination trial, trial 1, that nonsplenectomized Panamanian *Aotus* monkeys immunized with a mixture of either SERA 1 or γSERA N and Freund's adjuvant were protected from challenge with blood-stage parasites (24). Protection was manifested by an absence of a countable parasitemia, by a 10- to 1,000-fold reduction in countable parasitemia in the experimental animals when compared with controls, and by delays in the appearance of countable parasites in the experimental animals. We now report a second vaccination trial, trial 2, in which we have examined the effects of various adjuvants and an antigen mixture on the induction of the SERA 1-induced protective immune response. These results are presented here. In another vaccination trial, trial 3, described in an accompanying paper (24a), we present further evidence that extends our results from trials 1 and 2 that SERA 1 can provide a protective immune response in Panamanian *Aotus* monkeys.

MATERIALS AND METHODS

Monkeys. Panamanian *Aotus lemurinus lemurinus* monkeys were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Panama (37). A total of 23 monkeys (adult males and females, weighing 600 to 1,033 g) were divided into seven groups of three animals each that were injected intramuscularly with antigen(s) or adjuvant or both. One group of two monkeys was used as the completely naive control. The monkeys had had no previous exposure to *P. falciparum*.

Antigens. The SERA-derived antigen, SERA 1, was expressed from a cloned portion of the Honduras I SERA gene in *S. cerevisiae* and purified as described previously (5). The purified SERA 1 protein contains 262 amino acids (molecular weight, 26,976) of SERA (989 amino acids) starting from amino acid 24 (Gly) and ending with amino acid 285 (Asp) (5, 24). SERA 1 was dissolved in phosphate-buffered saline

* Corresponding author.
† Present address: LXR Biotechnology Inc., Richmond, CA 94806.

(PBS)–0.05% sodium dodecyl sulfate. Three other malaria antigens were also used to immunize monkeys in the antigen mixture group (see Table 1). All were also recombinant proteins produced in *S. cerevisiae*. Falc 2.3 (6) is derived from the major circumsporozoite protein. It is a 348-amino-acid molecule representing the portion of the natural *P. falciparum* circumsporozoite protein from amino acid 43 to amino acid 391 (13). gp195A (10) is a 50-kDa protein that contains the first 415 amino acids of the N-terminal region of the major merozoite surface antigen gp195 (38). Pfs 25B (4) is a transmission-blocking antigen derived from a 25-kDa sexual-stage surface protein (27) that is secreted from *S. cerevisiae* as a genetically engineered 19-kDa protein. All proteins were purified by standard chromatography procedures, lyophilized, and stored at 4°C.

Adjuvants. Freund's complete and incomplete adjuvants were described previously (5). Two muramyl tripeptide-based oil-in-water emulsions, MF59.2 and MF75.2, were used as adjuvants to immunize monkeys. MF59.2 is a modification of a previously described adjuvant, MF59, used in a rodent immunogenicity trial (5) and contains 5% squalene, 0.5% Tween 80, 0.5% Span (all purchased from Sigma Chemical Co., St. Louis, Mo.), and 200 μg of muramyl tripeptide-phosphatidyl ethanolamine CG19835A; CIBA-GEIGY, Basel, Switzerland) per ml in PBS. MF75.2 is based on formulations described by Byars and Allison (8) and contains 10% squalene, 5% Pluronic blocked polymer L121 (BASF Corp, Parsippany, N.J.), and 0.4% Tween 80 in PBS. Muramyl tripeptide-phosphatidylethanolamine at a final concentration of 200 μg/ml was added after the emulsification. The emulsion was created by five passages through a M110Y microfluidizer (Microfluidics, Newton, Mass.) at 12,000 lb/in^2. The resulting emulsion was passed through a 0.22-μm-pore-size polycarbonate syringe filter (Nuclepore; Costar, Cambridge, Mass.). Two liposome-based adjuvant preparations were prepared. In one liposome preparation (referred to as fusogenic liposomes in Table 1), egg phosphatidylethanolamine, dioleoyl glycerol succinate (Avanti, Polar Lipids Birmingham, Ala.), and cholesterol (Calbiochem, La Jolla, Calif.) at 8:2:1 weight ratios were combined and the organic solvent was removed by evaporation under N_2. Small unilamellar vesicles were formed by resuspending the lipids in PBS (pH 8.2), vortexing, sonicating for 4 min, and adding sufficient 1 N NaOH to thoroughly hydrate the lipids. SERA 1 at 400 μg/ml was added to lipids at 11 mg/ml, and the liposomes were frozen in a dry ice-ethanol slurry and thawed at room temperature seven times. The multilamellar vesicles were then passed through a 0.45-μm-pore-size polycarbonate syringe filter (Nuclepore; Costar) seven times, with the final passage performed aseptically. In the other liposome preparation (referred to as nonfusogenic liposomes in Table 1), the multilamellar liposomes containing monophosphoryl lipid A were prepared by the method of Alving et al. (1). Briefly, egg phosphatidylcholine, egg phosphatidylglycerol (Avanti), and cholesterol at 9:1:3.75 weight ratios were combined and the organic solvent was removed by evaporation under N_2. Monophosphoryl lipid A from *Salmonella typhimurium* (80 μg/ml; RIBI, Hamilton, Mont.), SERA 1, aluminum hydroxide (1 mg/ml; Sergeant, Clinton, N.J.), and PBS (pH 7.4) were added to 13.75 mg per ml of lipid. The liposomes were stored at 4°C until injected into monkeys.

Immunization. Antigen was injected intramuscularly on days 0, 28, and 56 of the experiment. Each dose of antigen, 100 μg, was in a final volume of approximately 0.5 ml. It was divided into two 0.25-ml portions that were injected intramuscularly into two sites in one thigh of the monkey. The booster injections were in the alternate thigh. All animals were bled prior to the start of immunization on day 0. The animals were subsequently bled 26 or 27 days after the primary vaccination injection and the first booster vaccination and 12 or 13 days after the second booster injection. Monkeys were also bled at 3-week intervals after parasite challenge. The responses to immunizations of the monkeys are shown in Table 1. Antigen and Freund's adjuvant were mixed immediately before injection. Monkeys that received Freund's adjuvant received Freund's complete adjuvant in the primary injection and Freund's incomplete adjuvant in the booster injections. Monkeys in one control group received the antigen carrier solution mixed with Freund's

TABLE 1. Antibody titers in monkeys prior to parasite challenge measured by ELISA

Monkey group	Antigen/adjuvant[a]	Titer[b]	
		Prebleed	Final bleed prior to challenge
1	No Ag/no Adj	188	167
		132	53
2	O/FA	<10	92
		<10	88
		<10	81
3	S1/FA	<10	465,021
		<10	909,318
		19	783,661
4	S1/MF75.2	102	27,502
		<10	4,689
		161	61,629
5	S1+Ag mixture/FA, including:		
	Falc 2.3	317	4,983
		18	1,295
		317	2,975
	gp195A	21	456,757
		37	247,661
		129	498,688
	S1	32	169,212
		34	481,731
		230	166,400
	Pfs 25B	191	1,193,234
		28	293,279
		208	1,157,289
6	S1/MF59.2	191	20,258
		336	13,718
		<10	59,625
7	S1/Fu Lip 59.2	13	141,295
		24	254,074
		<10	112,430
8	S1/Non-Fu Lip	<10	69,221
		<10	101,426
		17	30,641

[a] Ag, antigen; Adj, adjuvant; FA, Freund's adjuvant, complete and incomplete; S1, SERA 1; Fu Lip, fusogenic liposomes; Non-Fu Lip, nonfusogenic liposomes.

[b] Anti-S1 antibody titers are shown for all groups. Also, titers of the three group 5 monkey serum antibodies were determined separately, using either Falc 2.3, gp 195A, or Pfs 25B antigen.

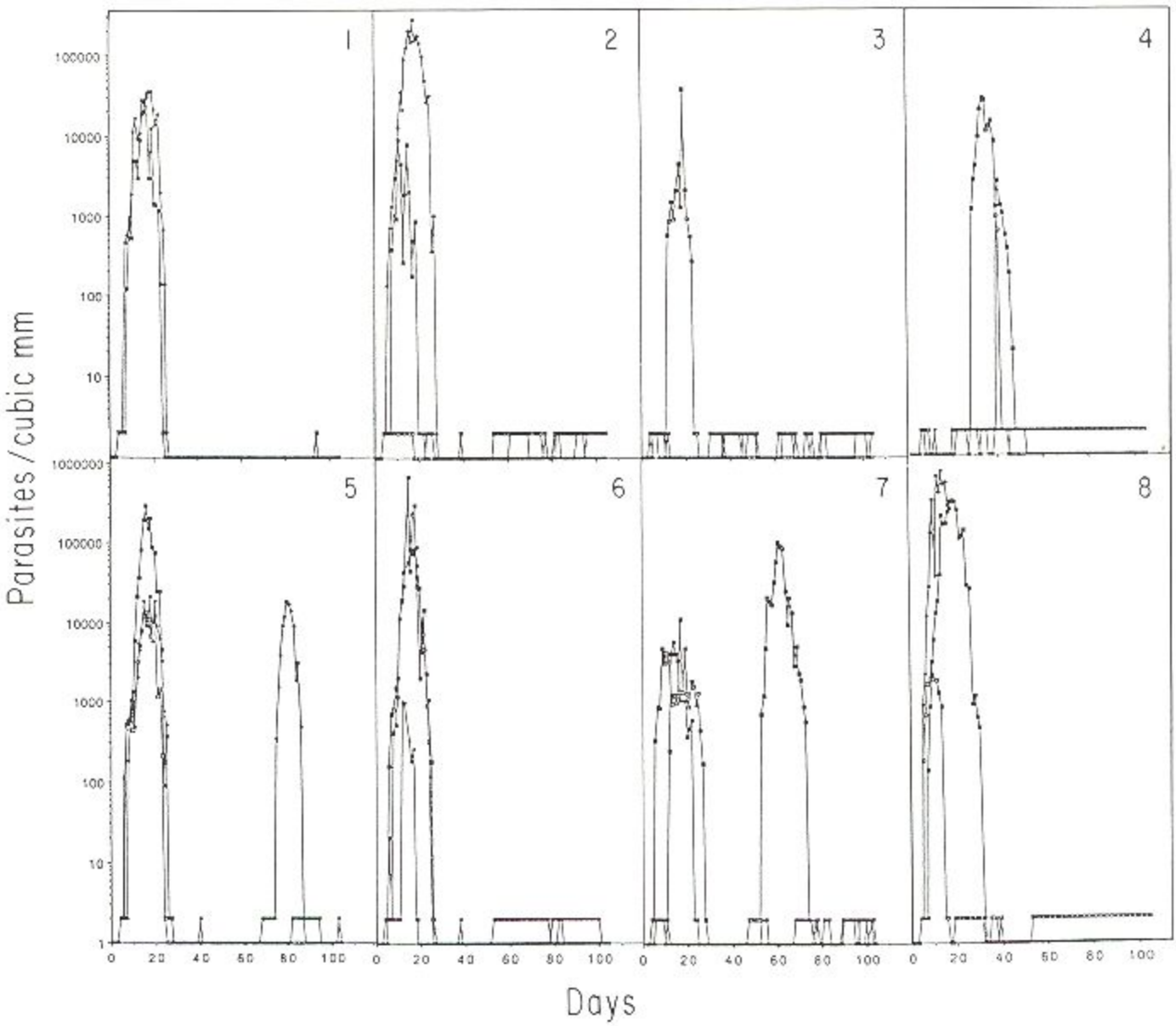

FIG. 1. Chronology of the course of parasitemia in eight groups of monkeys. Each monkey was infected with 5×10^4 Honduras I-infected erythrocytes obtained from the same infected monkey. In the semilog plot of parasites per cubic millimeter versus days, the symbols at a value of 1 indicate that no parasites were seen, while the symbols at a value of 2 indicate that fewer than 10 parasites per mm^3 were seen (see Materials and Methods). The number in the right top corner of each plot indicates the monkey group number (Table 1).

adjuvant in each injection. The monkeys in the naive control group received neither antigen nor adjuvant.

Parasites. *P. falciparum* Honduras I (23) was used for challenge. This strain was chosen after passage through splenectomized and then nonsplenectomized *Aotus* monkeys (25) until it was capable of developing a high level of parasitemia in the latter. Furthermore, since the two SERA-derived antigens were encoded by the cloned Honduras I SERA gene, this experiment represented a homologous challenge.

Parasite challenge. Fourteen days after the second booster injection, each monkey was injected intravenously with 5×10^4 parasites. Parasitized erythrocytes from an infected monkey with a moderate and increasing parasitemia were used as the challenge to ensure parasite viability. After challenge, the parasitemia was monitored daily by both thick and Earle-Perez films (19) that were stained with Giemsa. We evaluated parasitemias as follows. 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 the figures. A parasitemia of about 10 parasites per mm^3 is recorded as 2 in the log scale in the figures if parasites could be demonstrated only in a thick blood film. Parasite numbers of >10 per mm^3 determined by the Earle-Perez method are recorded in the figures.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (20). The antigens used to coat the wells of the ELISA plates (1 μg/well) were SERA 1 or, when necessary, Falc 2.3, gp195A, or Pfs 25B. Titers were calculated at 0.2 absorbancy unit with a V_{max} microtiter plate reader (Molecular Devices, Palo Alto, Calif.) programmed to read at 650 nm with

TABLE 2. Antibody titers in monkeys after parasite challenge measured by ELISA[a]

Monkey group	Antibody titer at given days after parasite challenge			
	20	41	63	83
3	76,156	91,481	24,736	15,436
	117,213	85,971	39,941	37,560
	106,831	89,711	37,283	35,012
4	1,245	7,158	933	492
	377	299	<100	<100
	3,161	1,179	574	248
6	37,191	31,355	2,441	800
	739	507	252	215
	14,675	14,871	1,539	1,231
7	495,678	101,470	15,990	—[b]
	31,700	22,575	5,078	4,951
	23,655	26,090	7,202	4,460
8	94,207	113,638	20,540	9,271
	—	—	—	—
	7,529	47,074	2,635	991

[a] Antibody titers prior to challenge are shown in Table 1.
[b] —, Sample not done.

subtraction of the 490-nm reading. The preimmunization serum of each monkey was used as the control for each postimmunization serum.

RESULTS

SERA 1 or an antigen mixture including SERA 1 was mixed with different adjuvants to test the induction in monkeys of a protective immune response to the *P. falciparum* Honduras I strain. The treatments of the groups of monkeys used in the study and a summary of the humoral immune status of each monkey prior to parasite challenge as measured by ELISA are shown in Table 1. The ELISA titers of the control serum collected 2 days prior to the initiation of vaccination (prebleed) and of the postimmunization sera collected just prior to the parasite challenge are shown.

The development and course of infection monitored in all monkeys for 100 days after challenge are shown (Fig. 1). Parasitemias developed in all monkeys. In Fig. 1, the data are plotted on a daily basis as described in Materials and Methods. Several general observations can be made regarding the response of the monkeys to parasite challenge. Patent parasitemia occurred in all monkeys by day 6 after parasite challenge as determined by either a positive thick smear or a countable parasitemia. In all groups except group 4 (immunized with SERA 1 plus MF75.2), a countable parasitemia began to develop after no more than 10 days. The countable parasitemias that developed in two monkeys in group 4 were delayed, beginning 29 and 48 days after challenge. Many of the vaccinated monkeys, after the initial parasitemia, had a recurrent parasitemia that was observed only in a thick smear.

Previously, we observed a similar result in trial 1 (24). We therefore measured the anti-SERA 1 titers in serum taken at various times after the initial parasite challenge and the appearance of the primary countable parasitemia to determine whether a correlation between them could help explain the observation. In Table 2 are shown the anti-SERA 1 titers of serum taken 20, 41, 63, and 83 days after parasite challenge. All sera, whether from animals that had or from those that had not exhibited a low-level parasitemia, showed a general decline in titer and an absence of a boosting effect. The result is consistent with the failure to achieve a sterile immunity in the animals.

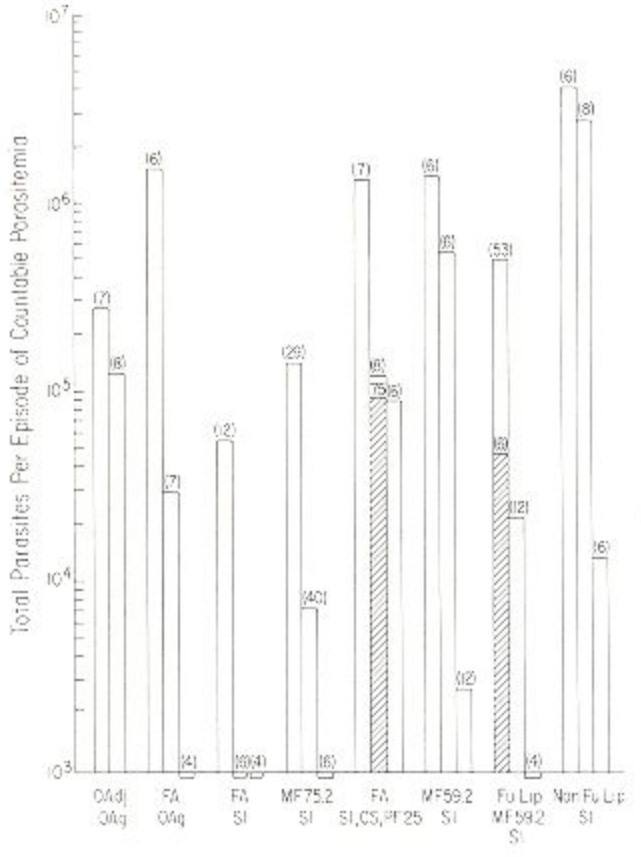

FIG. 2. Sum of parasites per episode of countable parasitemia in trial 2. The data were compiled from data in Fig. 1. A bar below the baseline, set at 10^3, indicates that no countable parasitemia was observed in that monkey. The number in parentheses represents the first day of the episode of countable parasitemia. A single open bar represents a single parasitic episode. A hatched bar superimposed on an open bar represents a second episode of parasitemia. The adjuvant and antigen used in treatment of the respective monkey groups (1 to 8) are noted below the baseline. FA, Freund's adjuvant; Fu-Lip, fusogenic liposomes; Non-Fu Lip, nonfusogenic liposomes; SI, SERA 1 antigen; CS, Falc 2.3 antigen; PF25, Pfs 25B antigen.

The major criterion we used to determine the efficacy of vaccination protection has been the level of countable parasitemia in the challenged animals. In Fig. 1 the chronological record of the countable parasitemia shows the peak and duration of the infection. We found, however, that a better way to display the data for comparison of parasitemias in the different groups was to sum the parasites per cubic millimeter in each countable peak of parasitemia observed. The sum of all parasites in the peak provides a comparison of the animal's ability to control the developing "parasite load." The data prepared in that way, and derived from data in Fig. 1, are plotted in Fig. 2. The day after challenge on which the peak parasitemia began to develop is shown at the top of the bar. The data in Fig. 2 show that the parasitemias exhibited by monkeys immunized with SERA 1 and either Freund's (group 3) or MF75.2 (group 4) adjuvant were significantly lower than parasitemias exhibited by either of the controls

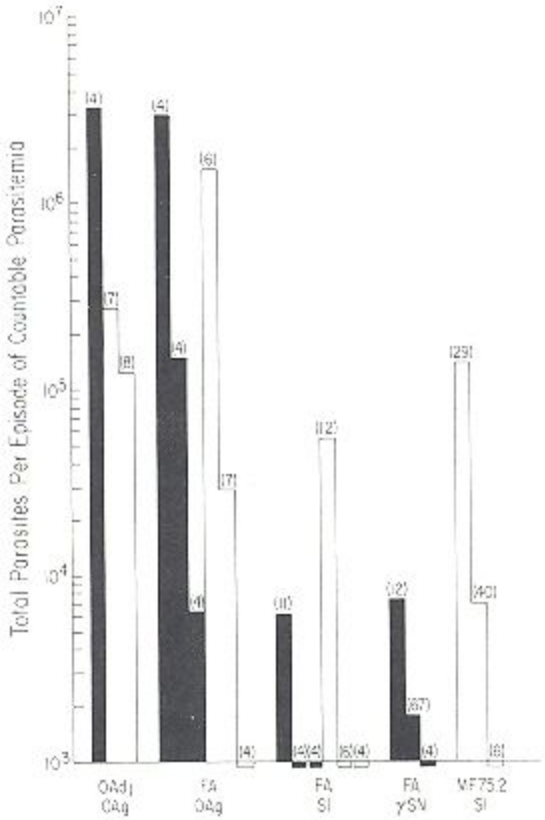

FIG. 3. Comparison of the sum of parasites per episode of countable parasitemia in monkeys vaccinated with either Freund's adjuvant or MF75.2 in trial 1 (24) and trial 2. The data were compiled from data in Fig. 1 for trial 1 and some of the data from Fig. 2 of trial 2. Black bars represent data from trial 1; open bars represent data from trial 2. γSN, γSERA N antigen (24); OAdj, O adjuvant; OAg, O antigen; FA, Freund's adjuvant; S1, SERA 1.

(groups 1 and 2) or the monkeys immunized with SERA 1 and the other adjuvants (groups 6 through 8).

Since the number of monkeys in each comparably treated group in trials 1 (24) and 2 is small, we have regraphed them all in Fig. 3 in the format of Fig. 2 to facilitate the comparison. In Fig. 3 are data from trial 1 for monkeys that were either in a naive control group, a Freund's adjuvant control group, or the experimental groups that received either SERA 1 or γSERA N plus Freund's adjuvant. The combined results clearly reinforce the interpretation that immunization with SERA 1 and with either Freund's or MF75.2 adjuvant provides significant protection compared with controls.

Monkeys immunized with Freund's adjuvant and SERA 1 mixed with three other antigens (group 5) were not protected (Fig. 1 and 2). The amount of SERA 1 injected into monkeys receiving either SERA 1 alone or SERA 1 in the antigen mixture was the same. One possible explanation for the difference in protection could have been that anti-SERA 1 antibody titers in animals receiving the antigen mixture could have been reduced because of the presence of one or more of the other antigens. The anti-SERA 1 titers in monkeys immunized with the antigen mixture (Table 1, group 5) were generally lower than in the monkeys receiving only SERA 1. It is clear, however, that those reduced anti-SERA 1 titers were still higher than the anti-SERA 1 titers achieved in the protected monkeys immunized with SERA 1 plus Freund's adjuvant in trial 1 (258,710, 97,740, and 151,530) (24). Also, the anti-SERA 1 titers that are induced in the monkeys protected after vaccination with the mixture of SERA 1 and MF75.2 (Table 1, group 4) are much lower than the titers induced by SERA 1 plus Freund's adjuvant in either trial 1 or trial 2.

DISCUSSION

Previous studies have shown that malaria parasite growth can be influenced by antiparasite humoral antibodies in vivo and in vitro (2, 32, 34). While cellular immune responses may also play a role in regulating malaria infection (7, 26), we have interpreted our present results in terms of the effects the vaccinations have had on the induction of the humoral immune response to the SERA 1 antigen.

The results, presented here, of the induction of protective immunity by a combination of SERA 1 and Freund's adjuvant were consistent with the protection observed in trial 1 with either SERA 1 or γSERA N (5, 24). In trial 1, we noted a correlation between the levels of induced anti-S1 antibody titer and the ability of monkeys to suppress parasitemia. Since Freund's adjuvant is not acceptable for human use, we examined the possible use of four other adjuvants. While the numbers of monkeys used in each group in these preliminary tests were small, the considerable differences in protection seen in monkeys vaccinated with SERA 1 and different adjuvants suggested that MF75.2 provided protection comparable to that provided by Freund's adjuvant while neither the liposome adjuvants nor the MF59.2 adjuvant provided significant protection.

In this trial the anti-SERA 1 titers in the monkeys of group 3 (SERA 1 and Freund's) were, in general, much higher than those in the monkeys of group 5 (SERA 1 plus mixed antigens and Freund's) (Table 1), providing a potential explanation for the absence of protection observed in group 5 monkeys. However, the anti-SERA 1 antibody titers associated with good protection in trial 1 were in the range of the titers produced in monkeys in group 5, in which essentially no protection was observed. This suggested that predicting protection by the comparison of absolute titers of anti-SERA 1 antibody in monkeys in which the same adjuvant was used but other vaccination conditions were changed may not be reliable. We therefore considered that the presence of the additional antigens in the group 5 vaccine mixtures may have led to a change in the serotypes of anti-SERA 1 antibodies. This did not seem to be the case since examination of the serotypes of the immunoglobulin G (IgG) antibodies in the monkeys of groups 5 and 3 showed that the amounts of IgG1, IgG2, IgG3, and IgG4 antibodies in most monkeys were the same (data not shown). We also considered that mixing SERA 1 with other antigens could, through protein-protein interactions in the vaccine antigen mixture, lead to the recognition of different SERA 1 epitopes that the anti-SERA 1 antibodies effectively recognize. Unfortunately, we presently have no knowledge of which SERA 1 epitopes may be recognized by the animal's immune system. That the quality rather than the quantity of anti-SERA 1 antibody may be important was further considered in view of the much lower anti-SERA 1 titers present in vaccine-protected monkeys in which MF75.2 rather than Freund's was the adjuvant (group 4). A further observation that we made (5; unpublished data) was that, when mice were immunized with SERA 1 antigen and either MF59.2 or Freund's adjuvant, the anti-SERA 1 titers and the in vitro parasite-inhibitory properties of those murine sera were

essentially identical while MF59.2 stimulated little or no protection in monkeys vaccinated with it plus SERA 1.

All of these observations suggest that the many variables that may affect how a particular antigen is recognized and responded to by the vaccinated animal makes prediction of the efficacy of a defined antigen questionable and makes the extrapolations of immunologic responses between animal systems difficult or impossible.

In the results obtained in a subsequent vaccination trial, trial 3, presented in the following article (24a), we report more extensive data about protective immunity induced by SERA 1 mixed with either MF75.2, MF59.2, or Freund's adjuvant that further show that SERA is a promising candidate vaccine antigen.

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