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Induction of Opsonizing Antibodies after Injection of Recombinant *Plasmodium falciparum* Vaccine Candidate Antigens in Preimmune *Saimiri sciureus* Monkeys

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We have previously shown that *Plasmodium falciparum* recombinant antigens PfEB200, R23, and Pfi72 inhibit opsonization of infected erythrocytes by hyperimmune *Saimiri* sera, indicating that they contain target epitopes involved in the phagocytosis of infected erythrocytes. We have investigated in this study the immune response of *Saimiri* monkeys with previous experience of malaria infections (preimmune monkeys) after injection of these recombinant antigens, administered alone or simultaneously. The humoral response to the recombinant antigens was monitored by radioimmunoassay, and the response to *P. falciparum* blood stages was assayed by immunofluorescence. The relative proportion of protective versus nonprotective immunoglobulin subtypes was investigated by using 3A2/G6 and 3E4/H8 monoclonal antibodies, and the capacity of the antisera to promote in vitro phagocytosis of infected erythrocytes was evaluated. The antigens evoked in most cases a secondary-type antibody response, resulting in important increases in antigen-specific antibody titers and concomitantly in anti-*P. falciparum* titers. The ratio of 3A2/G6 to 3E4/H8 immunoglobulin subtypes varied with the immunogen used. Opsonizing antibodies were boosted in several animals, the most promising combination being the mixture of PfEB200 and R23 that induced long-lasting production in five of five animals. The detectable opsonizing activity appearing after immunization of the animals was antigen specific, as it was lost after adsorption of the recombinant antigens. The challenge of the animals with blood stage parasites confirmed previous findings showing a correlation between the presence of detectable opsonizing antibodies in serum and protection.

In the development of an effective *Plasmodium falciparum* malaria vaccine, two distinct types of recipients have to be considered: naive individuals and preexposed, semi-immune populations living in endemic areas. The regulation of the immune response may be quite different in both cases. Concerning naive vaccinees, efforts have to be devoted to eliciting the relevant primary response stimulating protective effectors (23). In contrast, *P. falciparum* antigens (Ags) injected into semi-immune recipients face an immune system already primed by the parasite Ags and may stimulate a secondary response involved or not in protection-associated mechanisms. Recent immunization trials with individuals living in hyperendemic regions indicated that many recipients responded to the injected Ags (11, 25, 31, 33), but little is known about the functional aspects of the immune response.

There is increasing evidence that protection against blood stages of *P. falciparum* involves cooperation between antibodies (Abs) and monocytes (3, 5), resulting in phagocytosis of infected erythrocytes (9, 20, 24) or merozoites (3, 4, 6, 19). Some of the potential targets for these Abs have been identified (4, 12, 26). In a vaccination strategy aiming at inducing opsonizing Abs, the experimental host *Saimiri sciureus* is particularly relevant, as protection of these monkeys against *P. falciparum* blood stages is associated mainly with phagocytosis of the infected erythrocytes (7, 8, 10, 24). Furthermore, immunization of both naive and preimmune animals, i.e., those with previous malaria infection, can be investigated with this model.

We report here an analysis of the immune response of preimmune *Saimiri* monkeys after injection of three recombinant Ags, PfEB200, R23, and Pfi72. PfEB200 is derived from Pf332 (21), an Ag associated with the membrane of the trophozoite- and schizont-infected erythrocyte. In the late stages, it is exposed on the surface of the erythrocyte (16). R23 contains 11 repeats from Ag R45, expressed by the young trophozoites and maintained throughout schizogony (2). Pfi72 is part of the heat shock-like protein Hsp70 (12, 22). These Ags have been selected because they consistently competed with infected erythrocytes in an in vitro phagocytosis assay, suggesting that these specificities are important targets for opsonizing Abs (12). We have studied the humoral response of preimmune *Saimiri* monkeys after injection of single Ags or Ag mixtures. Immunoglobulin (Ig) subtypes were determined with specific monoclonal Ab (MAb) (14), and the capacity of the Abs to mediate opsonization was evaluated in an in vitro assay. The functionality of the immune response was examined by challenging the animals with *P. falciparum* parasites.

MATERIALS AND METHODS

Recombinant Ags used for immunization. The recombinant polypeptide PfEB200 contains a 135-amino-acid sequence from Ag Pf332 (21), Pfi72 comprises the last 153 amino acids from the Hsp70-like Pf72 Ag from *P. falciparum* (12, 22), and R23 contains 11 repeats of 6 amino acids from the R45 Ag (2). The recombinant PfEB200-i72 was constructed by PCR of the original clones with specific primers. These polypeptides were expressed as fusion proteins with glutathione *S*-transferase (GST) in the pGEX vector and were purified by affinity chromatography on glutathione agarose beads (Sigma Chemicals) as described previously (32).

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TABLE 1. Recombinant proteins used for immunizations of various groups of monkeys

Ag used for immunization	No. of monkeys	Adjuvant used for injections
R23	4	PAO[a]
PfEB200	2	PAO
GST	5	PAO
None	1	PAO
R23 + PfEB200[b]	5	PAO
Pfi72 + PfEB200	3	PAO
Pfi72 + PfEB200	3	None
PfEB200-i72[c]	3	PAO
PfEB200-i72	2	None

[a] Synthetic white oil giving a stable water-oil emulsion.
[b] Two monkeys were not splenectomized when they were immunized.
[c] One hundred micrograms of a tandem chimeric construct was injected.

Animals, immunization protocol, and serum samples. Twenty-nine squirrel monkeys, *S. sciureus* of the 14-7 karyotype and of the Guyanese phenotype, were used in this study. Their characteristics and their maintenance in facilities at the Institut Pasteur in French Guiana have been described elsewhere (13). These monkeys (splenectomized or not) had previously experienced one to five inoculations with *P. falciparum* (FUP-1 Palo Alto or IPC/RAY strains) and, for some animals, with *Plasmodium vivax*. None of the animals had experienced a malaria infection for the preceding 500 days or more, nor had they ever been injected with the recombinant Ags described herein.

On days 0 and 35 (and day 197 for animals receiving simultaneous injection of R23 plus PfEB200), the animals were injected subcutaneously with 100 μg of each recombinant Ag (Table 1) in the presence or absence of the adjuvant polyalphaolefin (PAO), a synthetic white oil, giving a stable water-oil emulsion. The use of the PAO adjuvant for immunization of *Saimiri* monkeys has already been reported (18, 27, 28). The animals were lightly anesthetized with ketamine for femoral vein puncture. Three milliliters of blood was withdrawn and was poured into dry tubes. Serum samples were kept frozen at −20°C until further use. No side effects were observed by means of clinicobiochemical investigations after immunizations with the different vaccine formulations.

Titration of Ag-specific Abs by RIA. Ab titers against selected Ags were measured by indirect radioimmunoassay (RIA) as described previously (28). Briefly, microtiter plates (MIC-2000; Dynatech, Guyancourt, France) were coated overnight at 4°C with 50 μl of Pfi72 or PfEB200 (1 μg/ml) or β-galactosidase R23 (5 μg/ml) in 0.01 M phosphate-buffered saline (PBS), pH 7.2. Before titration, anti-GST Abs were removed by affinity chromatography on a GST column. Ag-specific Abs were detected with a predetermined optimal concentration of mouse MAbs raised to squirrel monkey Ig (14) prior to incubation with ^{125}I-labelled goat anti-mouse Ig (Sigma Chemicals, l'Isle d'Abeau-Chesne, France). Total IgG was detected with MAb 3F11/G10. The relative amounts of two distinct *Saimiri* Ig types were estimated by the use of MAb 3A2/G6 (detecting opsonizing Abs) versus MAb 3E4/H8 (detecting nonopsonizing Abs) (10, 14).

Parasites and hyperimmune sera. The Uganda Palo-Alto (FUP-1 alias FUP/SP) strain of *P. falciparum* adapted to the squirrel monkey was used for challenging the animals (13). Fresh asynchronous viable parasitized erythrocytes (pRBCs) were obtained by venipuncture from an infected donor monkey and injected intravenously into the recipient monkeys. An inoculum of 10^6 viable parasites was used in splenectomized animals, and 10^8 parasites were injected in two nonsplenectomized monkeys. The evolution of the parasitemias was monitored daily by microscopic examination of Giemsa-stained blood smears. All animals had transient circulating parasites 24 h after challenge, indicating that they had been effectively inoculated. Challenged animals presenting a level of parasitemia above 15 to 20% were treated by chemotherapy. Animals with a body weight of ≤700 g were treated before the level of parasitemia reached 15%.

A pool of *Saimiri* hyperimmune sera (SHI) collected from a series of monkeys resistant to *P. falciparum* challenge was used as a reference. This pool, described elsewhere (12), was protective by passive transfer in vivo and had a marked opsonizing capacity in vitro.

Opsonizing assay. Detection of Abs involved in adhesion and/or ingestion of pRBCs (referred to as opsonins herein) was done by the method of Groux et al. (10). Phagocytic cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation and incubated in tissue culture chamber slides (Lab-Tek; Miles Laboratories, Inc., Slough, Great Britain) with sera (final dilution, 1:40) in the presence of *Saimiri* infected FUP-1 pRBC (humidified atmosphere at 37°C, 5% CO_2). The positive control consisted of the same volume and dilution of SHI, and the negative control contained pRBCs alone. The specificity of the reaction was verified by the use of uninfected *Saimiri* erythrocytes. After Giemsa staining, the numbers of bound and/or ingested pRBCs were estimated by counting 200 adherent phagocytic cells. The attachment-ingestion index (AI index), representing the percentage of phagocytic cells having bound and/or ingested pRBCs, was calculated according to the following formula: AI index = (bound and ingested pRBCs/phagocytic cells) × 100. By this technique, SHI gave an AI index of 75 to 90%.

Selective depletion of Ag-containing sera. The specificity of the detectable opsonizing activity in the sera of immunized monkeys was assessed by adsorbing these sera on the relevant recombinant Ags by standard affinity chromatography techniques. The recombinant Ags PfEB200, Pfi72, and R23 or the carrier GST was coupled to CNBr-activated Sepharose 4B columns (Pharmacia) according to the manufacturer's instructions. Sera were diluted in PBS (1:2 for recombinant Ag-containing sera and 1:100 for depletion of anti-GST Abs) and adsorbed for 60 min at room temperature with an equal volume of immunoadsorbent. The adsorbed sera were recovered by centrifugation and used in the pRBC adhesion-ingestion assay. Ag-specific Ab titers were determined by RIA, and anti-*P. falciparum* Ab titers were determined by immunofluorescence assay (IFA).

IFA. The presence of anti-*P. falciparum* Ab was detected by indirect IFA on air-dried *Saimiri* pRBCs. Briefly, serial dilutions of serum samples were incubated on pRBCs for 30 min at 37°C in a humid chamber. After a washing with PBS, anti-*Saimiri* Ig MAbs (ascitic fluids diluted 1:100) were added and incubated under the same conditions. Then, after extensive washings, fluorescein isothiocyanate-conjugated goat anti-mouse Ab (Sigma Chemicals) was applied. Total anti-*P. falciparum* IgG titers were determined with MAb 3F11/G10. The relative amounts of the two *Saimiri* Ig types were determined with MAbs 3A2/G6 and 3E4/H8.

RESULTS

Ab response to the selected Ags elicited by experimental *P. falciparum* infection. Experimental *P. falciparum* blood stage infections usually elicit Abs reacting with the Pfi72, PfEB200, or R23 recombinant Ags. The titers of Ag-specific Abs in serum samples collected in the period immediately following recovery from an infection were investigated. The Ab levels depended upon the individual animal and upon the number of previous parasite infections. After secondary challenge, anti-Pfi72 Ab titers of up to 1:6,400 were detected, while anti-R23 and anti-PfEB200 Ab titers peaked at 1:400 to 1:1,600. After additional parasite infections, the Ab levels increased to above 1:12,800 for the three Ags (data not shown). This value is comparable to the level of specific Abs detected in a pool of SHI shown to be protective against experimental infection by passive transfer (12), namely, 1:6,400 for anti-Pfi72 and anti-PfEB200 Abs and 1:3,200 for anti-R23 antibodies. However, after ≥500 days without any malaria infection, i.e., at the time when these preimmune monkeys entered this study, the levels of specific Abs had dropped to low titers (0 to 1:400), as shown in Tables 2 and 3.

Immunization of preimmune monkeys with a single Ag. R23 and PfEB200 were first injected separately into preimmune monkeys in the presence of 10% PAO. The kinetics of appearance of specific anti-R23 and anti-PfEB200 Abs, as assayed by RIA on the recombinant polypeptides, is plotted in Fig. 1. The Ags elicited a secondary-type humoral response with a rapid increase of specific IgG as early as day 7 that reached levels above those found in the pool of protective hyperimmune sera. These data indicate that injection of R23 or PfEB200 recombinants elicited a strong memory response.

The increase in Ag-specific Ab was accompanied by an increase in anti-*P. falciparum* Ab titers, assayed by IFA on pRBC as indicated in Table 2. An increase of 1 to 5 dilutions in total parasite-specific IgG levels, varying among the individuals, was observed. In order to obtain a qualitative estimate of this boosting, an IFA was performed with anti-*Saimiri* Ig MAbs 3A2/G6 and 3E4/H8, discriminating protective Ig and nonprotective Ig, respectively (14). This assay showed that the recombinant Ags induced similar titers of both types of anti-*P. falciparum* Igs. The IFA titers dropped to the preboost values after absorption on the recombinant Ags, indicating that the Abs reacting in IFA were specific for the Ag used for immunization. Surprisingly, an increase of the anti-*P. falciparum* Ab titers was also found for the five control animals that received

TABLE 2. Serological responses in preimmune squirrel monkeys after injection of a single recombinant Ag

Saimiri no.	Ag injected	IFA anti-*P. falciparum* titer[a] of Ig				RIA titer[a] of Ag-specific Abs		AI index[b] (%)	
		3F11/G10		3A2/G6, day 56	3E4/H8, day 56				
		Day 0	Day 56			Day 0	Day 56	Day 56	After immuno-adsorption
1705	R23	160	5,120	1,280	2,560	0	25,600	10	0
1624	R23	160	2,560	640	640	0	12,800	45	0
88023	R23	80	2,560	1,280	1,280	0	6,400	5	ND[c]
89043	R23	160	5,120	640	1,280	0	12,800	6	ND
1719	PfEB200	640	1,280	1,280	1,280	400	6,400	2	ND
1712	PfEB200	640	5,120	5,120	5,120	400	25,600	23	0
1518	GST	320	2,560	ND	ND	<100	<100	1	ND
88003	GST	160	2,560	ND	ND	<100	<100	0	ND
89088	GST	160	20,480	ND	ND	<100	<100	0	ND
1711	GST	640	40,960	ND	ND	<100	<100	3	ND
1715[d]	GST	640	40,960	ND	ND	<100	<100	29	27
89047	None	80	640	ND	ND	<100	<100	2	ND

[a] Titers are expressed as the limit dilution producing a signal twice that of the background signal given by a serial dilution of a pool of nonimmune sera.
[b] Mean result of at least two independent determinations done with coded sera. The standard deviations were lower than 10, the SHI had an AI index of 80 to 90%, and control pRBCs yielded an AI index of 0 to 2%.
[c] ND, determination not done.
[d] Preexisting opsonic activity (24%) was detected in the serum of this monkey before immunization with GST.

GST as the immunogen. The anti-*P. falciparum* reactivity was lost after absorption of the antisera on a GST column. A moderate increase of anti-*P. falciparum* Ab was also observed for the monkey that received the adjuvant alone and was interpreted as the result of polyclonal activation of the Ab-secreting cells by the adjuvant.

Opsonizing Abs were detected in the sera of 50% of the animals receiving R23 or PfEB200 on day 56 after immunization, in contrast to results for the serum samples collected on day 0 of the experiment, in which no such opsonizing Abs could be observed. An exception was control monkey 1715, which presented a 24% AI index before Ag injection that remained unchanged after the immunization with GST. The detectable opsonic activity of the sera was not correlated with the level of the anti-*P. falciparum* Ab measured by IFA or with the relative levels of 3A2/G6$^+$ Abs. Absorption of specific Abs on the relevant recombinant Ag resulted in a loss of the opsonizing activity of the sera, except for control monkey 1715 (Table 2), indicating that, in contrast to Abs elicited after injection of R23 or PfEB200, the anti-*P. falciparum* Abs boosted by injection of GST consisted of nonopsonizing Abs.

Immunization with Ag mixtures. (i) PfEB200 and R23 polypeptides. The PfEB200 and R23 recombinants were injected simultaneously in two groups of monkeys (three splenectomized and two nonsplenectomized animals) in the presence of 10% PAO as an adjuvant (Table 1). The results of the titration of the specific Ab response are shown in Fig. 2a and b. All five animals exhibited a secondary-type humoral re-

TABLE 3. Serological responses in preimmune squirrel monkeys after injection of Ag combinations or a chimeric construct[a]

Saimiri no.	Ag injected	IFA anti-*P. falciparum* titer of Ig:				RIA Ab titer against Ag:						AI index (%) on day of challenge
		3F11/G10		3A2/G6, day 56	3E4/H8, day 56	Pfi72		PfEB200		R23		
		Day 0	Day 56			Day 0	Day 56	Day 0	Day 56	Day 0	Day 56	
89061	R23 + PfEB200[b,c]	160	640	160	160	NA[d]	NA	400	6,400	200	6,400	15
89095	R23 + PfEB200[b,c]	40	320	160	80	NA	NA	800	3,200	800	3,200	52
88046	R23 + PfEB200[b]	1,280	5,120	640	1,280	NA	NA	800	12,800	800	6,400	31
88065	R23 + PfEB200[b]	2,560	5,120	2,560	1,280	NA	NA	3,200	12,800	1,600	>12,800	8
88072	R23 + PfEB200[b]	80	2,560	640	320	NA	NA	400	6,400	800	>12,800	21
85040	Pfi72 + PfEB200	1,280	10,240	1,280	5,120	3,200	25,600	800	800	NA	NA	6
1686	Pfi72 + PfEB200	640	10,240	1,280	5,120	800	3,200	400	3,200	NA	NA	4
1695	Pfi72 + PfEB200	320	2,560	160	1,280	800	6,400	400	6,400	NA	NA	2
1713	Pfi72 + PfEB200[b]	640	1,280	1,280	640	100	6,400	100	12,800	NA	NA	3
1703	Pfi72 + PfEB200[b]	320	1,280	1,280	1,280	100	6,400	100	3,200	NA	NA	4
1055	Pfi72 + PfEB200[b]	640	10,240	5,120	1,280	400	25,600	100	6,400	NA	NA	2
760	PfEB200-i72	2,560	5,120	640	2,560	800	800	100	6,400	NA	NA	13
1680	PfEB200-i72	1,280	1,280	640	640	400	800	800	6,400	NA	NA	4
750	PfEB200-i72[b]	2,560	5,120	160	5,120	400	1,600	800	6,400	NA	NA	19
1710	PfEB200-i72[b]	320	20,480	1,280	5,120	3,200	12,800	800	25,600	NA	NA	14
798	PfEB200-i72[b]	320	20,480	640	5,120	400	3,200	400	51,200	NA	NA	5

[a] Determinations were made as described in Materials and Methods and stated in Table 2, footnotes *a* and *b*.
[b] Ags were injected subcutaneously mixed with 10% PAO as an adjuvant.
[c] Monkeys were not splenectomized when they were immunized.
[d] NA, not applicable.

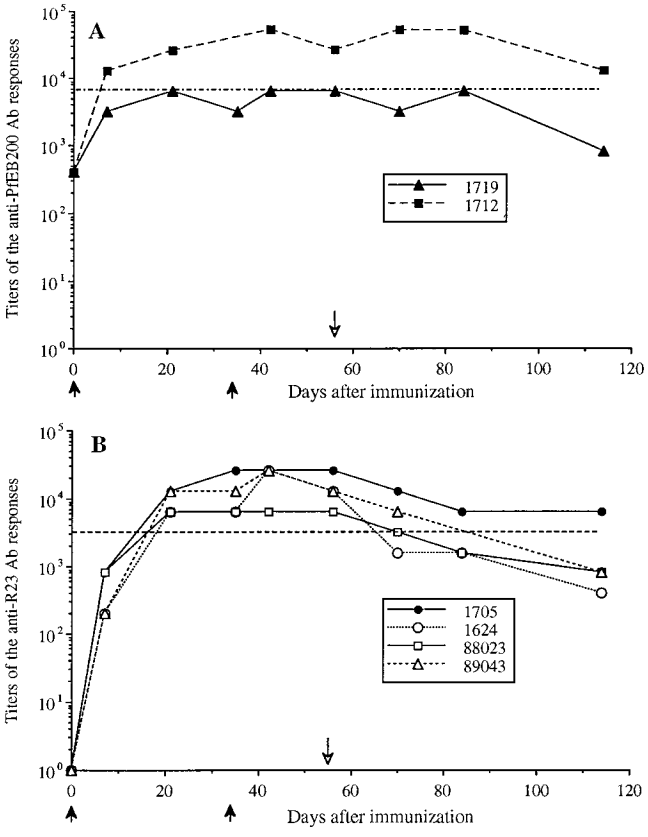

FIG. 1. Induction of anti-PfEB200 (A) and anti-R23 (B) Abs in preimmune monkeys after immunization with Ag mixed with PAO adjuvant. Preimmune *Saimiri* monkeys (numbers keyed to symbols) each received two injections of PfEB200 (A) or R23 (B) Ag on days 0 and 35 (filled arrows). Serum samples were taken at different times and tested in duplicate by the RIA. Specific Ab titers of individual serum samples were measured after serial dilutions and compared with those of a pool of sera from naive monkeys. Titers are expressed as the log 10 dilution giving a count-per-minute value twice that of the background level obtained with nonimmune sera. All animals were challenged with 10^6 viable blood stages of *P. falciparum* on day 56 (open arrows). The titers of anti-PfEB200 and anti-R23 Ab present in SHI are plotted as dashed lines (— · — [A] and – – – [B]).

sponse to PfEB200, and three responded similarly to R23. These animals had already produced anti-PfEB200 and anti-R23 Abs after their parasite infections in the past. Two animals (monkeys 89061 and 89095) exhibited a primary-type Ab response to R23, with Ab titers rising only after day 14 and boosted after the second injection. This result is consistent with the absence of detectable anti-R23 Ab in the serum samples collected following the parasite infections the animals experienced in the past.

The anti-*P. falciparum* IFA results are indicated in Table 3. A boosting of the IFA titers of all five monkeys was observed, for both 3A2/G6$^+$ and 3E4/H8$^+$ Ig types. The observed titers for nonsplenectomized animals remained relatively low in relation to the Ag-specific Ab response or the IFA titers elicited in the monkeys injected with single recombinant Ags (Table 2). Importantly however, all animals produced opsonins in response to the injection of the recombinant Ag mixture. This opsonizing activity could be partially depleted by adsorbing sera on R23 or PfEB200 immunoadsorbents and was totally eliminated by using both immunoadsorbents.

(ii) PfEB200 and Pfi72 Ags. PfEB200 and Pfi72 were injected simultaneously, in the presence or absence of 10% PAO as an adjuvant, in two groups of three splenectomized monkeys. The animals exhibited a secondary-type Ab response against both Ags, except monkey 85040, which responded against PfEB200 after the second injection only. The anti-Pfi72 Ab titers reached levels similar to or even higher than those of SHI (Fig. 2d), whereas those of Abs to PfEB200 were usually lower than those of the hyperimmune serum (Fig. 2c). As shown in Table 3, the anti-*P. falciparum* Ab reached high titers in animals receiving Ags in the absence of adjuvant. In that case, there was a trend for an increased proportion of 3E4/H8$^+$ Abs, whereas in the presence of adjuvant the trend was in favor of 3A2/G6$^+$ Abs. In contrast with the responses of the PfEB200+R23 group, simultaneous immunization with PfEB200 and Pfi72 did not lead to the production of detectable opsonins.

(iii) PfEB200-i72 fusion protein. In four of the five monkeys that received the fusion polypeptide, anti-PfEB200 Ab titers rose rapidly and reached levels similar to or significantly higher than those for SHI (Fig. 2e). In contrast, the anti-Pfi72 Ab titer tended to remain lower than that for SHI and lower than those for the groups injected separately with PfEB200 and Pfi72 (Fig. 2f). The group inoculated with the fusion protein in the presence of adjuvant produced high titers of anti-*P. falciparum* Abs. This Ab response was mostly of the 3E4/H8$^+$ type whether the fusion protein had been injected in the presence of adjuvant or not (Table 3). Opsonizing Abs were detected in some animals of both groups and, as indicated in Table 3, this was unrelated to the Ab titers.

Kinetics of opsonin production in animals injected with R23 and the R23-plus-Pf200 mixture. As indicated above, significant opsonin production was observed in all monkeys injected simultaneously with R23 and PfEB200 and in one animal (monkey 1624) that received R23 alone. In order to investigate more precisely the kinetics and duration of this response, the opsonizing activity in the various serum samples collected during the course of immunization was assayed longitudinally. The results are presented in Fig. 3. Opsonins were detected as early as day 7, and the general tendency was for an increase until the second injection. After week 8, the level of opsonizing Ab slowly decreased but was still significant at week 28 in four of five animals. A third injection of the Ag did not modify this level, apart from that of monkey 89095. The presence of opsonizing Ab for long periods in the animals that received R23 and PfEB200 simultaneously contrasted with the short-lived production of opsonins after injection of this Ag mixture into naive monkeys (data not shown).

Challenge of monkeys with FUP-1 parasites. Since high specific Ab titers had been induced in all animals, monkeys were challenged with FUP/SP pRBCs. The results are summarized in Table 4. Various outcomes were observed in all groups, including the monkeys that received the GST carrier and the adjuvant alone. Three major types could be determined. Type A infections presented an initial course of parasitemia typical of that observed in naive animals, i.e., a short prepatent period (3 to 7 days) followed by an immediate and rapid increase in the level of parasitemia. However, this course was followed in 8 of 14 animals by a self-cure, contrasting with the fulminant course of infection usually observed in naive animals. Type B infection consisted of a prolonged prepatent period of 9 to 19 days and/or a short prepatent period followed by a period of patency at low parasitemia levels lasting for at least 1 week. A late peak of low-level parasitemia on days 14 to 23 was observed and resolved without treatment. In type C, escape parasites developed rapidly after a prolonged prepatent period, requiring drug treatment.

Five of six monkeys of the control group (Table 4) showed a

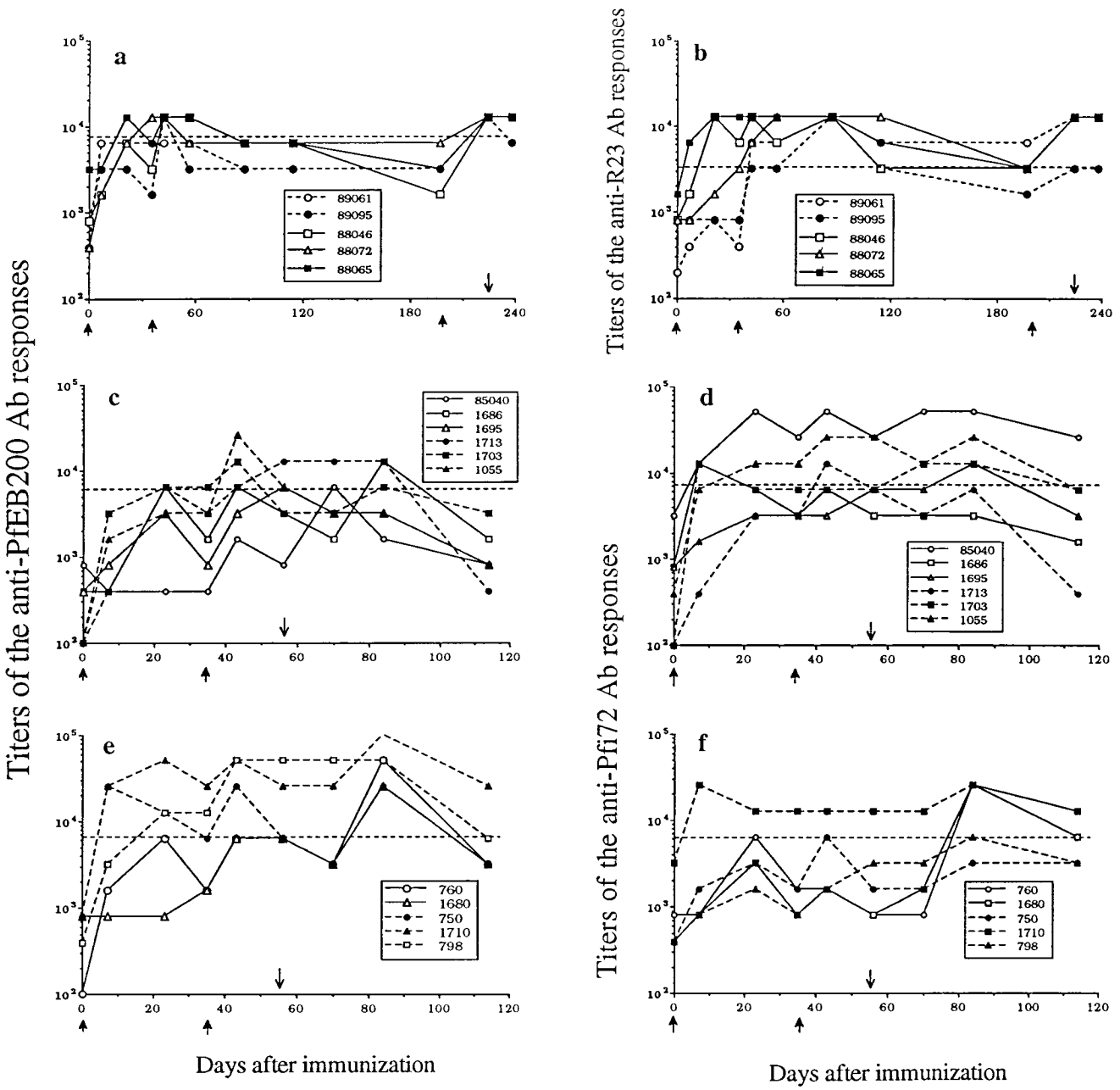

FIG. 2. Ab response against PfEB200, Pfi72, and R23 after immunization of preimmune monkeys with different Ag combinations. *Saimiri* monkeys (numbers keyed to symbols) were immunized on days 0 and 35 with PfEB200 and R23 mixed with PAO adjuvant. Recombinant PfEB200 and Pfi72 or the PfEB200-i72 tandem construct was injected in the presence or absence of the PAO adjuvant as indicated in Tables 1 and 3. Titers of the anti-PfEB200 Abs are plotted in panels a, c, and e; titers of the anti-Pfi72 Abs are plotted in panels d and f; and the titers of anti-R23 IgG are plotted in panel b. Titrations were performed for each individual monkey, and titers have been expressed as indicated in the legend to Fig. 1. The Ab responses measured of monkeys that received Pfi72, PfEB200, or PfEB200-i72 without the PAO adjuvant are plotted with solid lines (——) in panels c, d, e, and f. The levels of specific Abs found in SHI are also indicated (– – –).

type A outcome, while monkey 1715 experienced a type B outcome. As noted above, this animal had opsonizing Abs at the beginning of the experiment.

Two animals that received R23 presented opsonizing Abs on the day of the challenge. As indicated in Table 4, monkey 1705 had a type B outcome, with a very low peak of parasitemia, whereas in monkey 1624 the circulating opsonins were consumed after challenge (Fig. 3) and this monkey presented a delayed escape peak of parasitemia, requiring drug treatment on day 16. Importantly, the pRBCs recovered from this monkey did not present the same surface phenotype as those that were injected, since they failed to react with the SHI by IFA with live nonfixed pRBCs (data not shown). The other two monkeys of this group (monkeys 88023 and 89043), who did not exhibit detectable opsonizing activity, experienced delayed peaks of high-level parasitemia; the surface phenotype of their pRBCs has not been investigated.

Of the two monkeys that received PfEB200, one animal (monkey 1712) that had seric opsonins developed low-grade, delayed, self-curing parasitemia, while the other (monkey

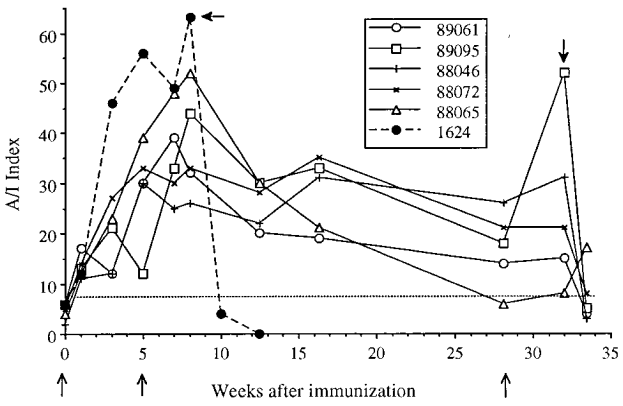

FIG. 3. Kinetics of the production of detectable seric opsonins in monkeys immunized with the mixture of R23 and PfEB200 or R23 alone. Preimmune *Saimiri* monkeys (numbers keyed to symbols) each received three injections of R23 and PfEB200 Ags on days 0, 35, and 197 (arrows under the abscissa) and were challenged with parasites on day 224 (downward arrow). Monkey 1624 was immunized with R23 alone on days 0 and 35 and challenged with parasites on day 56 (horizontal arrow). For all monkeys, Ags were mixed with PAO as an adjuvant before subcutaneous immunization. Serum samples were taken at different times, and their opsonic activity was determined and expressed as the AI index, as described in Materials and Methods. The mean AI index of the preimmune monkeys, considered a nonsignificant value, is indicated as a dashed line.

1719), in whom no opsonizing Ab could be detected, experienced a virulent type A parasitemia that had to be treated.

Among the animals that received R23 and PfEB200 simultaneously, four of five had detectable levels of opsonizing Abs on the day of challenge. No parasite development was observed after challenge of nonsplenectomized squirrel monkeys 89061 and 89095. Monkey 88046 showed a late outgrowth of escape parasites at a time when serum opsonins were no longer detectable. As observed with monkey 1624, these escape parasites were no longer recognized in live surface IFA with the SHI (data not shown). Monkey 88072 required drug treatment after a rapid parasite growth, despite having a detectable level of opsonins on the day of challenge. Monkey 88065, which did not have opsonizing Ab, experienced a type A outcome.

Various outcomes were observed in the group of six monkeys injected simultaneously with PfEB200 and Pfi72. Two monkeys (monkeys 1686 and 1703) presented a type B outcome with low-level parasitemia, while the other monkeys experienced type A outcomes, with a rapid increase of parasite densities reaching different levels in the various animals. In that group, the control of parasite growth could not be correlated with any immunological parameter assayed on the day of challenge.

The animals that were injected with the fusion protein PfEB200-i72 and that had a detectable opsonizing activity in their sera on the day of challenge (i.e., monkeys 760, 750, and 1710) also developed low-grade, delayed parasitemia and did not require any treatment (type B outcome), while those in whom no opsonins could be detected experienced a type A infection (monkeys 1680 and 798).

DISCUSSION

The data presented here indicate that the recombinant Ags R23, PfEB200, and Pfi72, injected alone or simultaneously, were able to boost an anti-*P. falciparum* Ab response previously induced by malaria infections in *Saimiri* monkeys. In all cases, injection of the immunogen was well tolerated and no adverse reactions were noted. A surprising finding was the induction of high titers of anti-*P. falciparum* Abs in control monkeys after immunization with *Schistosoma mansoni* GST. This result was unexpected, as anti-GST Abs raised in naive monkeys never showed any positive reaction to *P. falciparum* Ags by IFA or by immunoblotting. These data suggest that GST boosted the production of Abs reacting with a malaria Ag and that such Abs are not elicited by active immunization. This malaria Ag may be *P. falciparum* GST, which could share significant homologies with the *S. mansoni* GST, in view of the high degree of conservation of this enzyme in evolution (17). Importantly, unlike the recombinant Ags used for immunization, GST did not stimulate the synthesis of opsonins. It must be noted that GST and the fusion proteins do differ in their C-terminal domains. GST carries a sequence encoded by the polylinker (32) that is absent in the recombinant proteins and is highly immunogenic in mice (data not shown). We cannot rule out the possibility that this region is responsible for the reactivity observed in this study.

The three recombinant Ags did not elicit strictly similar responses. Pfi72 elicited a secondary-type response in all animals, whereas PfEB200 and R23 induced a secondary-type response in most animals, with two exceptions each. Interestingly, in some animals injected simultaneously with two Ags, a primary-type immune response to one Ag was observed whereas a secondary-type response was elicited by the other. This difference indicated that the immune responses to the Ags (both presented fused to the same carrier) were independent. *P. falciparum* IFA titers and Ig subtype responses obtained after immunization with Ag mixtures differed from those elicited by single Ags. This difference was particularly clear for the R23 and PfEB200 mixture, which induced the synthesis of opsonins in five of five animals, whereas PfEB200 alone elicited opsonins in one of two animals and R23 elicited opsonins in two of four monkeys. These opsonins were Ag specific, since they were adsorbed onto the corresponding immunoadsorbents. This was further confirmed recently, in an experiment in which immunization of preimmune monkeys with carrier-free R23 polypeptide induced the production of opsonizing Abs (26a).

The responses observed after simultaneous immunization with Pfi72 and PfEB200 also differed from those induced by PfEB200 alone or the PfEB200-i72 fusion. The tandem fusion protein elicited a limited humoral response to Pfi72 contrasting with the strong response obtained after immunization using the mixture of recombinant proteins. Conversely, the degree of anti-PfEB200 response was increased in the animals receiving the fusion. The level of production of opsonins also tended to be increased in these animals. Thus, PfEB200 immunogenicity was improved when PfEB200 was presented as a fusion protein with Pfi72. These data suggest that Pfi72, a fragment of the Hsp70-like protein from *P. falciparum*, could play the role of an enhancer of the immune response, as previously demonstrated with mice (1) and *Saimiri* monkeys (29) for Hsp70 from *Mycobacterium tuberculosis.*

The influence of the adjuvant was investigated in the group injected with the Pfi72 and PfEB200 Ags. Both the Ag mixture and the fusion induced higher anti-PfEB200 and anti-Pfi72 titers when injected in the presence of adjuvant than those induced in the absence of adjuvant. However, the levels of anti-*P. falciparum* Ab titers of all the monkeys that received the Ags with or without adjuvant were comparable. The significance of this result is still unclear. Unlike adjuvants tested recently in mice (15) and in *Saimiri* monkeys (27, 28), the PAO adjuvant did not obviously influence the quality of the Ab response to the recombinant Ags used in this study.

Information accumulated so far indicates that preimmune

TABLE 4. Outcome of challenge in monkeys immunized with single Ags, with Ag combinations, or with the fusion recombinant protein

Saimiri no.	Ag injected	Opsonizing activity[a] of serum taken on day:		Parasitemia course			Treatment for parasitemia	Type of parasite outcome
		0	Challenge	Marginal until day	Peak			
					% of maximum	Day		
1715	GST	+	+	19	1.5	23	No	B
1518	GST	−	−	5	10	11	No	A
88003	GST	−	−	5	13.5	13	No	A
89088	GST	−	−	5	5	10	No	A
1711	GST	−	−	5	20.7	14	Yes	A
89047	None	−	−	5	5	12	No	A
1705	R23	−	±	16	0.47	17	No	B
1624	R23	−	+++	9	26.6	16	Yes	C
88023	R23	−	−	5	13.5	18	No	B
89043	R23	−	−	8	12.8	19	No	B
1712	PfEB200	−	+	13	0.46	14	No	B
1719	PfEB200	−	−	5	12.3	10	Yes	A
89061[b]	R23 + PfEB200	−	+		No peak		No	NA[d]
89095[b]	R23 + PfEB200	−	+++		No peak		No	NA
88046	R23 + PfEB200	−	++	8	23.1	15	Yes	C
88072	R23 + PfEB200	−	+	4	48	10	Yes	A
88065	R23 + PfEB200	−	−	4	10.65	10	No	A
85040	Pfi72 + PfEB200[c]	−	−	3	16.8	8	Yes	A
1686	Pfi72 + PfEB200[c]	−	−	10	1.6	15	No	B
1695	Pfi72 + PfEB200[c]	−	−	5	2.4	9	No	A
1713	Pfi72 + PfEB200	−	−	6	20	10	Yes	A
1703	Pfi72 + PfEB200	−	−	9	3.8	20	No	B
1055	Pfi72 + PfEB200	−	−	5	12	13	No	A
760	PfEB200-i72[c]	−	±	11	1.75	17	No	B
1680	PfEB200-i72[c]	−	−	7	20	14	Yes	A
750	PfEB200-i72	−	+		No peak		No	
1710	PfEB200-i72	−	±	10	5	22	No	B
798	PfEB200-i72	−	−	5	22.4	15	No	A

[a] Activity levels: −, <7%; ±, 7 to 15%; +, 15 to 25%; ++, 25 to 35%; +++, >35%.
[b] Nonsplenectomized *Saimiri* monkeys challenged with 10^8 parasites each.
[c] Ags injected subcutaneously in saline without adjuvant.
[d] NA, not applicable.

monkeys such as those analyzed in this study usually cannot resist a high dose of parasite challenge ($>10^7$ parasites) (30). To date, no information is available on protection against a low-dose inoculum. In order to analyze the possible role of Ag-specific opsonins in protection, the monkeys in this study were challenged with 10^6 or 10^8 pRBCs. The outcome was variable. The spleen-intact monkeys that received a large inoculum were completely protected. We cannot draw any conclusion from these data, as our experience with challenge in intact monkeys is too limited.

In all groups of splenectomized monkeys, including the control groups, distinct types of parasitemia were observed after challenge with FUP-1 parasites, a strain inducing a lethal infection when left untreated in naive monkeys. Interestingly, control monkeys presented a type A infection with an initial course of parasitemia typical of that observed for naive animals followed in most animals by a self-cure, suggesting that a secondary response to parasite Ags was evoked after challenge and allowed the monkeys to finally eliminate the parasites. Conversely, in type B infections, the prolonged prepatent period and the late peak of low-level parasitemia suggest that the multiplication of the parasite was seriously hampered. A type B outcome was observed in a single control (monkey 1715), which was unique in exhibiting opsonizing Ab before immunization, and in many animals injected with the recombinant Ags. This observation suggests that the Ag-specific immune response was able to significantly control the initial parasite growth, in contrast with the response of the animals in the control group.

The results obtained in this study are consistent with previous findings indicating that protection against blood stages of *P. falciparum* in squirrel monkeys is usually correlated with the presence of Abs promoting phagocytosis of the pRBCs in vitro (10, 24). In all groups, apart from the group immunized by simultaneous injection of Pfi72 and PfEB200, the animals that had opsonizing Abs on the day of challenge showed a greater ability to control parasites, presenting a low-density, significantly retarded peak of parasitemia. The single exception was monkey 88072. The emergence of escape parasites presenting an altered surface (determined by IFA) in monkeys 1624 and 88046 (type C outcome) that occurred after adsorption of the circulating opsonizing Abs by parasites (and of Ag-specific Ab in monkey 1624) suggests that they have been selected under this immune pressure. It must be noted that no opsonizing Abs were detected in the group of animals injected simultaneously with Pfi72 and PfEB200, where two monkeys showed a type B outcome, indicating that control of parasite growth can occur in the absence of detectable opsonins.

Taken together, these results suggest that Pfi72, R23, and PfEB200 recombinant polypeptides induced a strong humoral

response in preimmune monkeys, resulting in a significant increase in the amount of Ab reacting with *P. falciparum* parasites. This indicates that the conformation of the recombinant Ags mimics that of their parasite counterparts so as to stimulate parasite-induced memory cells. Seroconversion to the injected Ag frequently has been observed in the human trials done so far in endemic areas, but a limited response in the IFA (i.e., a reaction to the parasite-encoded Ags) has been reported (11, 25, 31, 33, 34). The boosting of opsonins by R23, PfEB200, and Pfi72 indicates that the parasite Ags, or Ags presenting similar antigenic determinants, are accessible on the infected erythrocyte membrane. These data confirm the conclusions based on the results of the phagocytosis inhibition assay (12). Importantly, long-lasting production of opsonizing Abs was obtained for a significant proportion of animals, including those that exhibited a primary-type immune response, in contrast with the response of naive animals receiving the same immunogens (26a). This approach shows that building upon a parasite-primed immune system may help direct the immune response to the relevant immune effectors such as particular Ab subclasses (4, 9).

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