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Passive Immunization of *Aotus* Monkeys with Human Antibodies to the *Plasmodium falciparum* Antigen Pf155/RESA

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In order to assess the protective effects of anti-Pf155/RESA antibodies of different specificities in vivo, passive immunizations of *Aotus* monkeys were performed. Antibodies reactive with the Pf155/RESA repeat sequences $(EENV)_2$ and EENVEHDA were isolated from the immunoglobulin G (IgG) fraction of a pool of plasmas from Liberia by affinity chromatography on synthetic peptides. The two fractions of antibodies differed in specificity but displayed similar capacities to inhibit merozoite invasion in *Plasmodium falciparum* in vitro cultures. Four groups of monkeys (named groups I to IV) were injected with (i) 160 mg of total control IgG, (ii) 2 mg of IgG affinity purified on $(EENV)_2$, (iii) 2 mg of IgG affinity purified on EENVEHDA, and (iv) 160 mg of total immune IgG, respectively. The monkeys were then challenged with *P. falciparum*-infected erythrocytes, and the levels of parasitemia and hematocrits as well as other serological parameters were determined daily. Although all groups developed parasitemia, groups II and IV tended to show lower mean daily levels. Three monkeys of group II and two monkeys (each) of groups III and IV self cured the infections, but so did one monkey from the group treated with control IgG (group I). The serum levels of transfused antibodies were low at the peak of parasitemia, suggesting that clearance of parasites was mediated by immune responses mounted by the monkeys. The results indicate that antibodies to epitopes formed by repeats of Pf155/RESA may depress *P. falciparum* parasitemias and thus that immunogens based on such repeats should be suitable components in a subunit vaccine against asexual stages of *P. falciparum*.

Malaria caused by *Plasmodium falciparum* is a major health problem in many parts of the developing world, with an enormous toll of debiliating morbidity and mortality, particularly among children in Africa. However, immunity to this disease may be acquired after a long time of repeated exposure to the parasite. Part of this immunity is mediated by antibodies, as demonstrated in the classical passive transfer experiments of Cohen, Carrington, and McGregor (7, 8, 16). These authors showed that immunoglobulin G (IgG) fractions from malaria-immune adults could, on passive transfer to children suffering from severe *P. falciparum* infections, drastically lower the parasitemia and alleviate the disease (7, 8, 16). Similarly, protection against *P. falciparum* challenge was obtained in monkeys by passive transfer of human IgG from immune individuals (11, 25). However, the mechanism for these effects as well as the specificities of the antibodies which provided protection remains unknown.

Several different *P. falciparum* blood stage antigens have been suggested as targets for protective antibodies (18, 24), and limited vaccination trials in monkeys or humans have confirmed the potential of some of the antigens to induce partial or, in some instances, complete protection to *P. falciparum* challenge (for a review, see references 18 and 24). One of the candidate antigens for a malaria vaccine is Pf155/RESA (18, 23, 24), which is deposited in the erythrocyte membrane during or shortly after merozoite invasion (1, 10, 19). Pf155/RESA contains two regions of tandemly repeated amino acid sequences; one is in the carboxy terminus (3′ repeat region), and one is in the middle of the antigen (5′ repeat region) (14). These regions are antigenically dominant (20) and highly conserved in different parasite strains and isolates (21). Antibodies to Pf155/RESA repeats have a high capacity to inhibit merozoite invasion of erythrocytes in vitro (3, 20). By using β-galactosidase fusion proteins containing the repeat regions of Pf155/RESA in a vaccination trial in *Aotus* monkeys, partial protection against *P. falciparum* challenge was obtained with some of the immunogens (9).

In this study we have performed passive immunization of *Aotus* monkeys with affinity-purified human antibodies reactive with Pf155/RESA repeats, which was followed by challenge with *P. falciparum*-infected erythrocytes. The study was performed in order to assess the possible protective capacity in vivo of antibodies of different specificities which are reactive with repeated sequences of Pf155/RESA.

MATERIALS AND METHODS

Plasma. Plasma units were collected from healthy adult blood donors at the LAMCO Hospital, Yekepa, Liberia. Plasma units showing high reactivity to Pf155/RESA as assayed by erythrocyte membrane immunofluorescence (EMIF) were sent to Stockholm by air and then stored at −20°C. Units proved negative for hepatitis B surface antigen (by radioimmunoassay; Abbott) and antibodies to human immunodeficiency virus type 1 (by enzyme-linked immunosorbent assay [ELISA]; Organon) were processed further.

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IgG preparation. IgG was prepared at Kabi, Stockholm, Sweden, by applying the procedures used for production of Gammaglobulin Kabi 16.5%, including cold ethanol fractionation by a modified Cohn method and passage over a DEAE-Sephadex (Pharmacia, Uppsala, Sweden) column.

Synthetic peptides. Peptides were synthesized by the method of Merrifield (17) and were purified and analyzed by high-pressure liquid chromatography (HPLC) (3). The peptides were obtained from the following sources: EENVE HDA and $(EENV)_2$ were from Pharmacia, K(EENVE HDA)$_2$ and K$(EENV)_2$ were from T. Bartfai and A. Undén, Department of Biochemistry, University of Stockholm, Stockholm, Sweden, and K$(DDEHVEEPTVA)_2$ was from Kabi.

Affinity chromatography. Affinity chromatography was performed with sterile buffers under aseptic conditions. The synthetic peptide EENVEHDA or $(EENV)_2$ was coupled to CNBr-activated Sepharose 4B (Pharmacia) (0.2 mg of peptide per ml of packed beads) according to the instructions of the manufacturer. Packed beads (15 ml) were incubated with 4 g of IgG in 125 ml of buffer (20 mM Tris-acetate buffer containing 80 mM glycine [pH 7.4]) for 1 h in the cold. The beads were then centrifuged at 100 × *g* for 5 min, and the supernatant with unbound IgG was removed. The beads were suspended in the Tris-acetate-glycine buffer, packed, and washed in a column (K26/25; Pharmacia) until the optical density at 280 nm of the effluent reached close to zero.

IgG ELISA. IgG concentrations were determined by a sandwich ELISA as described previously (12, 27).

Peptide ELISA. The peptide ELISA assay was performed essentially as described by Wahlgren et al. (27). Peptides conjugated to bovine serum albumin (BSA; fraction V, Boehringer Mannheim) at an approximate molar ratio of 40:1 by means of glutaraldehyde were used for coating micro-ELISA plates (Dynatech, Alexandria, Va.) at a concentration of 10 μg of conjugate per ml. The coated plates were incubated with serial dilutions of the different IgG fractions. Binding antibodies were detected with alkaline phosphatase-conjugated rabbit antibodies to human IgG and *p*-nitrophenyl phosphate as a substrate. All determinations were done in duplicates. The level of background binding was assessed on a plate coated with glutaraldehyde-treated BSA run in parallel.

EMIF. Indirect immunofluorescence was performed on glutaraldehyde-fixed and air-dried monolayers of erythrocytes infected with *P. falciparum* of primarily early stages, as previously described (19). Different antibody preparations were assayed in fivefold dilution steps for determination of endpoint titers.

Immunoblotting. The reactivities of antibodies with *P. falciparum* polypeptides were analyzed by immunoblotting, as described earlier (5).

Merozoite invasion inhibition. This assay was performed with the Tanzanian *P. falciparum* strain F32, as described by Wåhlin et al. (29). The specificities of the inhibitory antibodies were analyzed by mixing the antibodies at fixed and slightly suboptimal concentrations with synthetic peptides, and the antibodies were then added to the *P. falciparum* cultures.

Passive immunization. *Aotus* monkeys (*Aotus nancymai*, karyotype I) were imported from Peru and housed at the Walter Reed Army Institute of Research primate facility for 4 months prior to use. During the quarantine period, the monkeys were examined and health statuses were assigned. Hematology, clinical chemistries, and fecal examinations for ova and parasites were performed, and blood smears were examined for the presence of trypanosomes, microfilaria, and malaria parasites. The sera of the monkeys were tested for anti-malarial antibodies by indirect immunofluorescence. Sixteen healthy monkeys were randomly assigned to four groups of four monkeys each. Monkeys in the first group (group I) each received 160 mg of IgG prepared from plasma of European donors who had not experienced malaria, whereas monkeys in group IV each received 160 mg of IgG prepared from Liberian donors living in an area holoendemic for *P. falciparum* malaria. Monkeys in groups II and III each received 2 mg of Liberian IgG affinity purified on $(EENV)_2$ or EENVEHDA, respectively. The immunoglobulins were administered in 5-ml volumes via a saphenous vein. Monkeys were challenged 1 h later via the contralateral vein with 4×10^6 *P. falciparum* (Camp strain)-infected erythrocytes from a donor monkey. Blood smears were made daily, and blood for measurement of hematological parameters and serum samples was collected at intervals during the course of infection. The presence of human IgG in the monkeys' sera was detected and measured by radial immunodiffusion with commercial reagents (Kallestad Laboratories Inc., Austin, Tex.). Although some cross-reactions with a substance in *Aotus* serum (presumably *Aotus* IgG) were evident, the cross-reactions did not interfere with quantitative estimates when measurements of human IgG were made in the presence of monkey serum. Antibodies to Pf155/RESA were detected either by using synthetic peptides conjugated to BSA as an antigen in an ELISA procedure as described above (27) or by EMIF (19). Monkeys were treated with chloroquine hydrochloride intramuscularly at 5 mg per monkey daily for 3 days if parasitemia reached 10% of erythrocytes parasitized or if packed-cell volumes decreased to 20% or less.

TABLE 1. Antibody activities and yields determined by EMIF and ELISA

Fraction	IgG yield (%)	Relative sp act (% yield)		
		EMIF	ELISA	
			Anti-$(EENV)_2$	Anti-EENVEHDA
Total IgG	100	1 (100)	1 (100)	1 (100)
$(EENV)_2$ eluate	0.6	160 (80)	163 (74)	53 (31)
EENVEHDA eluate	0.2	80 (14)	65 (11)	213 (32)

RESULTS

Peptide-reactive antibodies were purified from a pool of IgG from 16 Liberian donors by affinity chromatography on either of the synthetic peptides, EENVEHDA or $(EENV)_2$, coupled to Sepharose. Out of a total of 8,800 and 4,000 mg of IgG applied to the EENVEHDA and $(EENV)_2$ columns, respectively, 14 and 24 mg were retrieved in the fractions eluted from the columns. The eluted fractions were analyzed for reactivity with the two peptides by ELISA and for reactivity with Pf155/RESA by EMIF (Table 1). Although less than 1% of the total IgG was eluted from the peptide-charged columns, this material was highly enriched in both peptide reactivity and EMIF reactivity. About 80% of the EMIF reactivity of the total IgG fraction was recovered in the antibodies eluted from the $(EENV)_2$ column, whereas

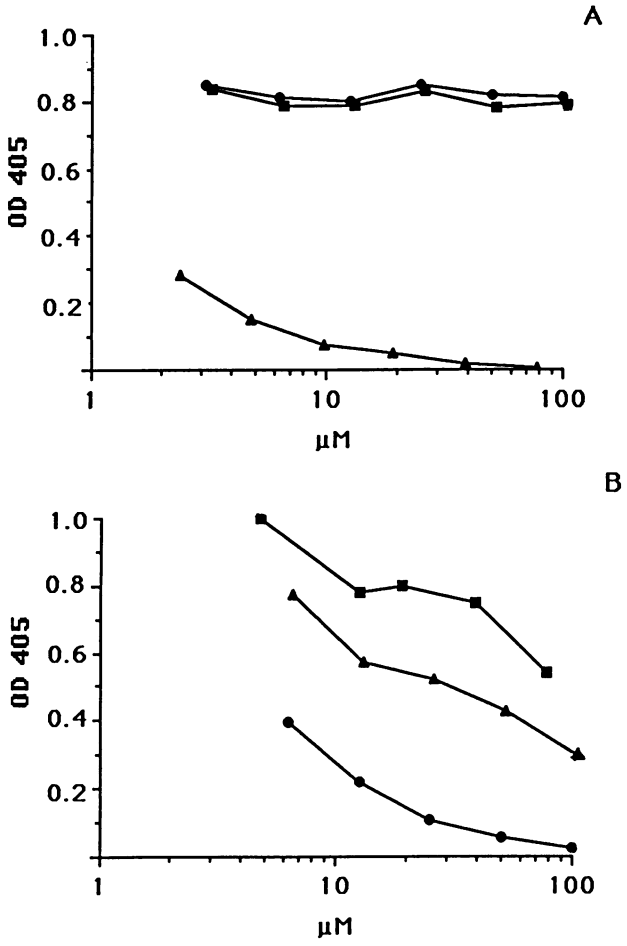

FIG. 1. Analysis of the specificities of affinity-purified antibodies by inhibition of their binding in ELISAs. (A) Antibodies purified on $(EENV)_2$ reacted with $(EENV)_2$-BSA. (B) Antibodies purified on EENVEHDA reacted with EENVEHDA-BSA. Synthetic peptides used for inhibition were $(EENVEHDA)_2$ (●), $(EENV)_2$ (▲), and $K(DDEHVEEPTVA)_2$ (■). OD_{405}, Optical density at 405 nm.

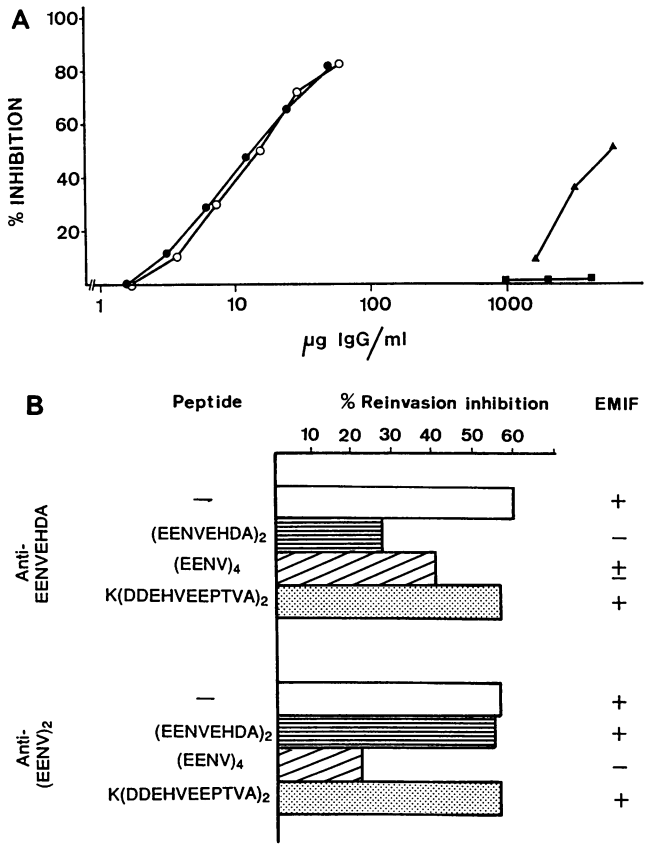

FIG. 2. (A) Invasion inhibition in vitro by total unfractionated IgG from Liberian donors (▲), total unfractionated IgG from European donors (■), antibodies affinity purified on $(EENV)_2$ (●), and antibodies affinity purified on EENVEHDA (○). The mean parasitemia in the control was 0.58% ± 0.009% at time zero and 2.14% ± 0.21% after 20 h. (B) Reversion of invasion inhibition or EMIF by antibodies purified on $(EENV)_2$ or EENVEHDA with the synthetic peptides $(EENVEHDA)_2$, $(EENV)_4$, and $K(DDEHVEEPTVA)_2$. The mean parasitemia in the control was 0.71% ± 0.07% at time zero and 1.92% ± 0.16% after 20 h.

the antibodies eluted from the EENVEHDA column contained 14% of that activity. The antibodies eluted from the $(EENV)_2$ column contained 74% of the anti-$(EENV)_2$ activity of the total IgG fraction but also 31% of the anti-EENVEHDA activity, as measured by ELISA (Table 1). Although only 32% of the total IgG anti-EENVEHDA activity was recovered in the eluate from the EENVEHDA column, these antibodies displayed a different pattern of specificities since they only contained 11% of the anti-$(EENV)_2$ activity.

The specificities of the eluted antibodies were analyzed by inhibition of their binding to $(EENV)_2$ or EENVEHDA in ELISAs using the peptides $(EENV)_4$, $(EENVEHDA)_2$, and $K(DDEHVEEPTVA)_2$ as inhibitors; the latter peptide corresponds to a dimer of a subunit of the 5′ repeat region of Pf155/RESA. While the antibodies binding to $(EENV)_2$ were specific for the homologous sequence (Fig. 1A), the antibodies binding to EENVEHDA were cross-reactive with the heterologous sequences (Fig. 1B).

Analysis of the capacity of the affinity-purified antibodies to interfere with *P. falciparum* merozoite invasion in vitro showed that this activity was similar in the two fractions (50% inhibition at about 15 μg/ml) and was enriched by about 400 times compared with that of the unfractionated Liberian IgG (50% inhibition at about 6 mg/ml [Fig. 2A]). Total IgG from European donors showed no effects in this assay. The synthetic peptides $(EENV)_4$, $(EENVEHDA)_2$, and $K(DDEHVEEPTVA)_2$ reverted the invasion inhibition of the affinity-purified antibodies and inhibited their EMIF reactivities (Fig. 2B) in a pattern similar to that obtained in the ELISA (Fig. 1). Thus, the antibodies purified on $(EENV)_2$ appeared specific for the homologous sequence, whereas the antibodies purified on EENVEHDA were cross-reactive with the heterologous sequences.

Four groups of *Aotus* monkeys were challenged with *P. falciparum*-infected erythrocytes 1 h after the monkeys each received 160 mg of total IgG from European donors (group I) or from Liberian donors (group IV) or 2 mg per monkey of IgG affinity purified on $(EENV)_2$ (group II) or on EENVEHDA (group III). Figure 3 shows the development of parasitemia in the individual monkeys. All monkeys but one in group IV developed parasitemia, and whereas the prepatency period was similar in groups I, II, and III, it was one day longer in group IV. Although there were no statistically

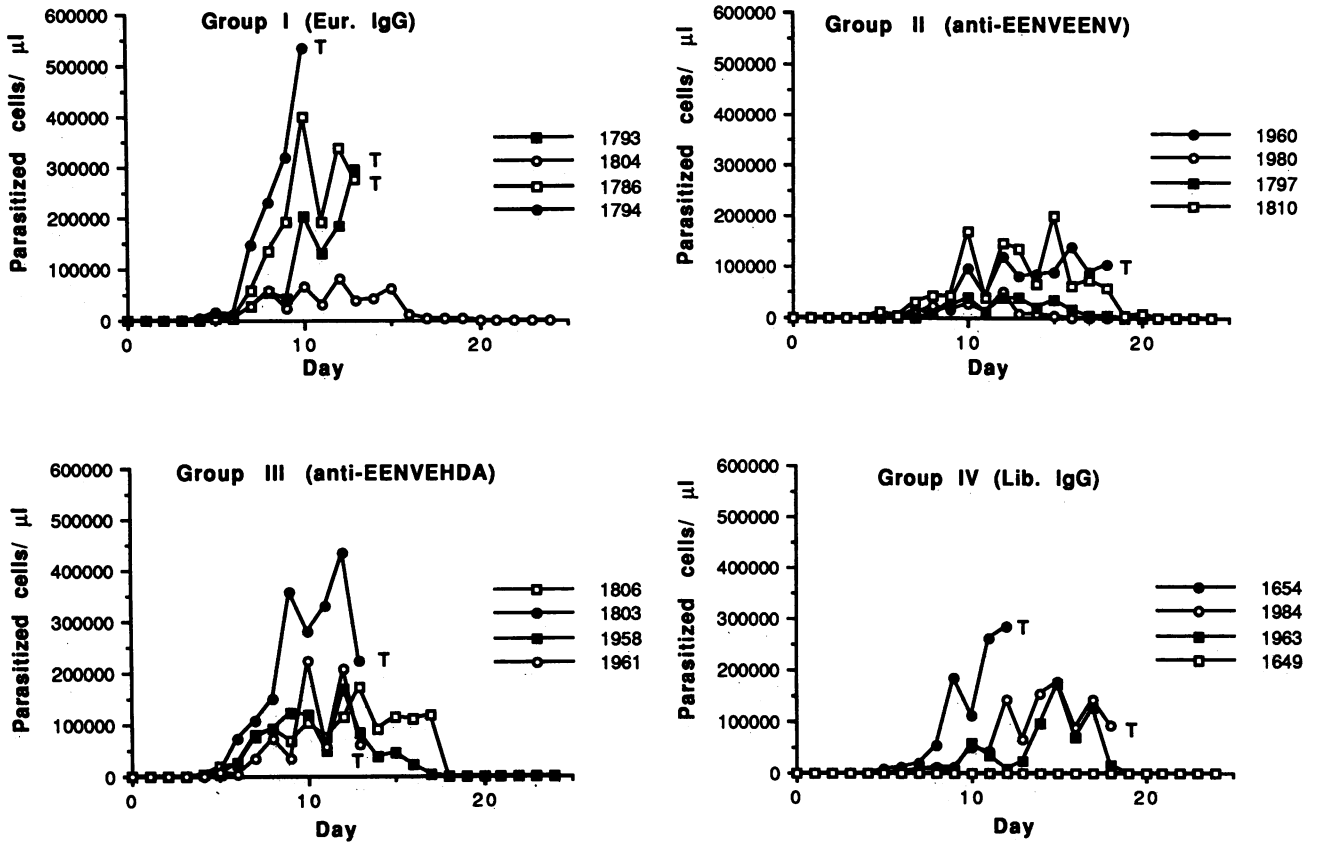

FIG. 3. Daily parasitemias in individual passively immunized *Aotus* monkeys after challenge with *P. falciparum*-infected erythrocytes. T, Initiation of treatment with anti-malarial drugs.

significant differences between the four groups of monkeys with respect to average daily parasitemia (12 days) or loss of packed-cell volume (14 days) by analysis of variance ($F = 2.35$ and 1.63, respectively), there was a trend for lower mean parasitemias in the groups receiving antibodies, especially groups II and IV. Thus, the mean parasitemia counts between days 3 and 11 of groups II and IV were consistently lower than those of groups I and III (Fig. 4). Importantly, three of four monkeys in group II self cured without any need of drug treatment, whereas two monkeys each in groups III and IV self cured. One monkey in the control group self cured as well, whereas the infection in the three other monkeys in this group had a regular course and passed 10% parasitemia between days 11 and 14. While one of the self-cured monkeys in group IV never showed parasitemia, all other self-cured animals displayed parasitemias up to days 19 to 20. Of the monkeys needing drug treatment, the one in group II and one each in groups III and IV were treated because of low hematocrits.

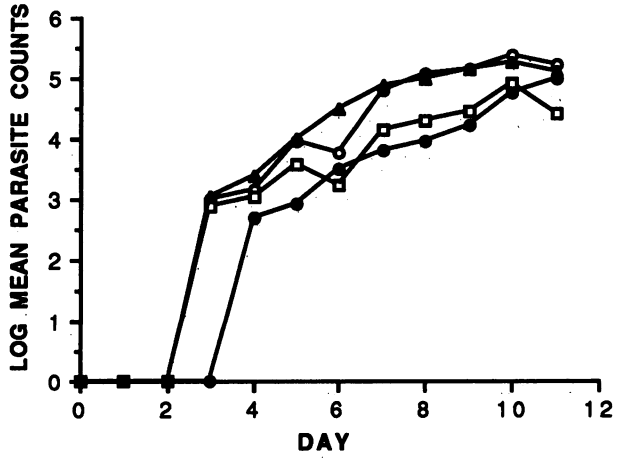

FIG. 4. Log of mean daily parasite counts of the four groups of passively immunized *Aotus* monkeys up to day 11 after challenge with *P. falciparum*-infected erythrocytes. Symbols: ○, group I; □, group II; ▲, group III; ●, group IV.

Determination of the concentrations of human IgG in the sera of the monkeys indicated that the passively transferred IgG persisted for a long time in the circulatory system (Fig. 5). The antibody reactivities against the synthetic peptides $(EENVEHDA)_3$, $(EENV)_6$, $(EENVEHDA)_2(EENV)_2$, and $(DDEHVEEPTVA)_2$ were measured by ELISA using for antibody detection either anti-squirrel monkey IgG (detecting *Aotus* IgG but not human IgG) or anti-human IgG (detecting both human and *Aotus* IgG) antibodies. The reactivity of the monkeys' own IgG was generally of low titer and usually appeared late (days 27 to 33). Some animals did not, however, react with any of the peptides. The reactivity of the transfused human IgG with the peptides was detected at low titers in the groups receiving Liberian

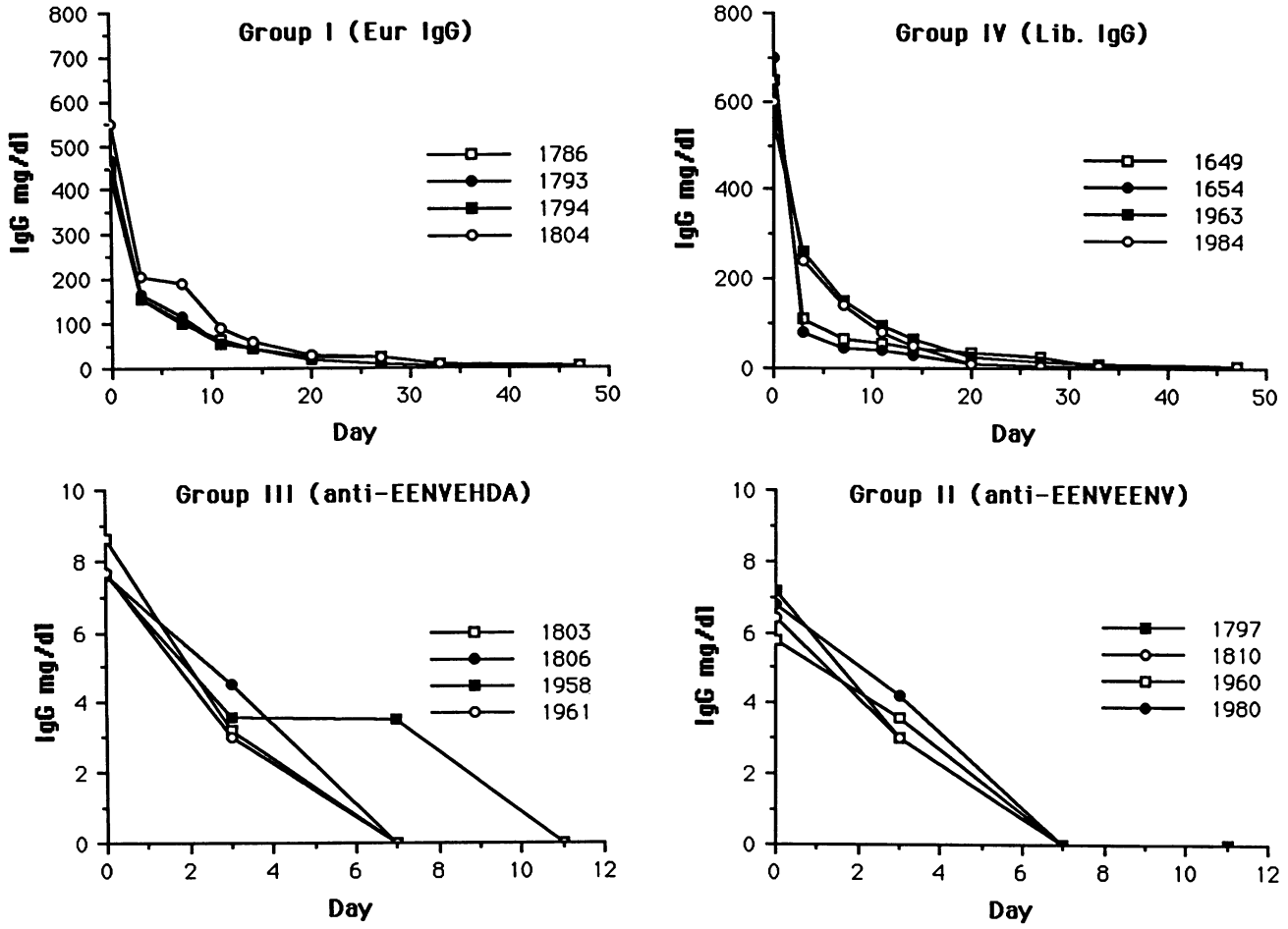

FIG. 5. Concentrations in serum of human IgG in individual passively immunized *Aotus* monkeys. Groups I and IV received 160 mg of IgG per monkey, and groups II and III received 2 mg of IgG per monkey.

immunoglobulin, whereas the control group was negative. As was found before passive transfer, no reactivity was seen with the 5′ repeat peptide. No differences between animals that were treated or self-cured animals were found in these assays.

DISCUSSION

We have in this study provided evidence for a parasite-neutralizing capacity in vivo of antibodies to repeated sequences in Pf155/RESA. Although all monkeys but one developed parasitemias with similar prepatency periods, most animals given antibodies showed depressed levels of infected erythrocytes compared with animals given nonimmune IgG. However, results from experiments involving relatively small numbers of *A. nancymai* (karyotype I) must be interpreted with some caution. The course of infection with *P. falciparum* (Camp strain) in these monkeys is more variable than in other more-susceptible *Aotus* monkeys (karyotypes II, III, and IV). Recent work with *A. nancymai* in our laboratory (Washington, D.C.) indicates that approximately 30% of control animals survive malaria infection without reaching requirements for treatment (parasitemia of ≥10% or packed-cell volume of ≤20%). The mechanisms by which some monkeys are able to control parasitemia and anemia to a greater extent than others of the same karyotype are not understood, and this compounds the difficulty in interpreting data in which differences between experimental and control groups are relatively small.

Most importantly, on passive transfer, 2 mg of affinity-purified anti-$(EENV)_2$ antibodies had effects similar to those obtained with 160 mg of total immune IgG. The levels of parasitemia peaked between days 10 and 15, and clearance of parasites occurred when the levels of transfused antibodies were low, indicating that clearance was not mediated by these antibodies. Thus, our data suggest that the transfused antibodies did not neutralize the challenged parasites efficiently but appeared to depress the parasitemia to a level low enough for the monkeys' immune systems to cope with the infection. In previous experiments with passive immunization of *Aotus* monkeys with human immune IgG, a different pattern of development of parasitemia with a marked delay in the onset of parasitemia was seen (11, 25). This difference may probably be ascribed to different virulences of the parasites used for challenge.

Only low concentrations in serum (60 to 80 μg/ml) of anti-$(EENV)_2$ antibodies were needed to obtain a depression of parasitemia after challenge with a large load of infected erythrocytes. Similar concentrations of antibodies with this specificity are often found in adult Liberians who are considered clinically immune to *P. falciparum* (20). However, some individuals are low responders with regard to antibod-

ies to Pf155/RESA but are still clinically immune, indicating that other antigens and antiparasitic mechanisms participate in mediating protection.

Using fusion proteins containing Pf155/RESA repeats for vaccination of *Aotus* monkeys, Collins et al. obtained partial protection against *P. falciparum* challenge (9). The protected monkeys developed a depressed parasitemia in a pattern similar to that obtained in our study. Protection correlated with the presence of antibodies to a dimer of the Pf155/RESA 5′ repeat DDEHVEEPTVA and to some extent with antibodies reactive with both the 3′ repeats, EENVEHDA and $(EENV)_4$, but not with antibodies reactive with $(EENV)_4$ only (9). These data appear to be in contradiction with our results suggesting that antibodies affinity purified on $(EENV)_2$ seemed to confer protection with higher efficiency than those affinity purified on EENVEHDA. Whereas the latter antibodies cross-reacted with $(EENV)_2$ and to some extent with $(DDEHVEEPTVA)_2$, the antibodies isolated on $(EENV)_2$ were specific for this peptide. Thus, although the differences in results between the studies may have several reasons, one explanation may be differences in the fine specificities of the antibodies.

Although the antibodies purified on $(EENV)_2$ and EENVEHDA had a similar capacity to inhibit merozoite invasion in vitro, the $(EENV)_2$ antibodies appeared to have a higher parasite-neutralizing capacity in vivo. In previous analyses of in vitro invasion inhibition with affinity-purified antibodies from individual serum samples, antibodies eluted from $(EENV)_2$ columns usually were more efficient than antibodies eluted from EENVEHDA columns (4, 20, 22). In passive transfer experiments in *Saimiri* monkeys with total IgG fractions from immune and nonimmune animals, others have also reported that there is no direct correlation between in vivo protective and in vitro invasion inhibitory effects of antibodies (13). In experiments with total IgG fractions from *P. falciparum* immune sera, antibodies to Pf155/RESA exhibit only a low efficiency in in vitro inhibition assays but become very effective when affinity purified (3, 29). Such differences are probably due to the presence in such sera of both inhibitory antibodies and antibodies which promote merozoite invasion and parasite growth (6). Furthermore, anti-idiotypic antibodies may be present in the sera and may counteract inhibition in the in vitro assay (28). How these different factors affect parasite neutralization in vivo is unknown.

Because of extensive antigenic cross-reactions between Pf155/RESA repeats and repeat sequences of other *P. falciparum* blood stage antigens (2, 15, 26), the actual target of the parasite-neutralizing antibodies is not easily determined. Recent in vitro studies indicate that the merozoite invasion process involves both Pf155/RESA and cross-reacting antigens (27a). Regardless of whether or not Pf155/RESA is a primary antibody target for in vivo protection and although the mechanism of this protection remains unknown, it is evident that some Pf155/RESA repeat sequences may elicit *P. falciparum*-neutralizing immune responses.

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