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Anti-Idiotypic Antibodies Counteract the Invasion Inhibition Capacity of Antibodies to Major Epitopes of the *Plasmodium falciparum* Antigen Pf155/RESA

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Rabbits were immunized with the synthetic peptide EENVEHDA or $(EENV)_2$, corresponding to a tandemly repeated sequence in the C-terminal part of the *Plasmodium falciparum* antigen Pf155/RESA, or with *Escherichia coli*-derived fusion proteins containing the corresponding repeats. For all sera, the capacity of the total immunoglobulin G fractions to inhibit *P. falciparum* merozoite invasion in vitro was similar and relatively low. Affinity purification of Pf155/RESA-specific antibodies on parasite-infected erythrocyte monolayers or on peptide columns increased the inhibitory capacity 50 to 5,000 times, whereas the immunofluorescence titers were increased only 10 times. The addition of small amounts of total immunoglobulin G to the affinity-purified antibodies gave a marked and dose-dependent reduction of the inhibitory capacity of the purified antibodies. However, this reduction was only seen in combinations where the immunoglobulin G fraction was from the same serum as the affinity-purified antibodies, suggesting that it was mediated by anti-idiotypic antibodies reacting with non-cross-reacting idiotopes of the invasion-inhibiting antibodies.

Pf155/RESA is one of the prime candidate antigens of *Plasmodium falciparum* asexual blood stages being investigated as potential components in a malaria vaccine (18, 24). The antigen contains two regions of tandemly repeated amino acid sequences (Fig. 1), one in the carboxy-terminal region (3′ repeat region) predominantly consisting of the octamer Glu-Glu-Asn-Val-Glu-His-Asp-Ala (EENVEHDA) and the related tetramer Glu-Glu-Asn-Val (EENV), and one in the middle of the molecule (5′ repeat region) consisting of the undecamer Asp-Asp-Glu-His-Val-Glu-Glu-Pro-Thr-Val-Ala (DDEHVEEPTVA) and related sequences (12). A majority of the antibodies to Pf155/RESA are directed against epitopes within the repeat regions (2, 6, 7). Seroepidemiological studies have suggested an association of antibodies to Pf155/RESA with protective immunity (8, 28). This is also indicated by the high capacity of both monoclonal and polyclonal antibodies to this antigen to inhibit merozoite invasion of erythrocytes in *P. falciparum* in vitro cultures (2, 25, 29). Thus, interest has been focused on synthetic peptides as well as various recombinant gene products containing Pf155/RESA repeat sequences as immunogens for eliciting potentially protective antibody responses (1). Partial protection against *P. falciparum* challenge was obtained in monkeys in a vaccination trial with recombinant fusion proteins, and protection was correlated with production of antibodies against EENVEHDA and DDEHVEEPTVA repeats (5).

Evaluation of the presence of potentially protective anti-Pf155/RESA antibodies in human immune sera as well as in antisera from experimental animals by means of the in vitro invasion inhibition assay have shown that antibodies are often not efficient inhibitors as components in total immunoglobulin preparations but become very efficient when affinity purified (2, 23, 29). This difference could be due to the presence in the sera of both reinvasion-inhibiting antibodies and of antibodies promoting reinvasion and parasite growth (3, 30). The existence of this latter type of antibodies was recently demonstrated by using monoclonal antibodies to an asparagine-rich protein (Ag10b) of *P. falciparum*; antibodies to certain epitopes efficiently inhibited merozoite reinvasion, whereas antibodies to another epitope in the same antigen were very efficient promotors of reinvasion (13). Furthermore, the malaria parasite infectivity on the level of gametes and sporozoites may be either enhanced or suppressed by monoclonal antibodies depending on the antibody concentration (19, 20).

In this study we used total immunoglobulin G (IgG) fractions and affinity-purified antibodies from rabbit antisera to synthetic peptides corresponding to the repeat sequences of Pf155/RESA or to fusion proteins containing the corresponding repeats to further analyze the reinvasion inhibition capacity in vitro of the immunoglobulins. By affinity purification of Pf155/RESA-specific antibodies, the reinvasion inhibition titers were strongly enhanced. The presence in the total IgG fractions of a factor counteracting the antibody-mediated invasion inhibition was demonstrated. The specificity of this counteraction for antibodies only from the homologous serum suggested the involvement of anti-idiotypic antibodies.

MATERIALS AND METHODS

Parasites. *P. falciparum* of the Tanzanian strain F32 (15) was cultured in vitro in erythrocytes of blood group O+ as described by Trager and Jensen (27).

Synthetic peptides. Peptides were synthesized by the standard method of Merrifield (17) and were purified and analyzed for homogeneity by high-pressure liquid chromatography (2). The peptides were obtained from different sources as follows: EENVEHDA and $(EENV)_2$ from L.-E. Larsson, Pharmacia, Uppsala, Sweden; $K(EENVEHDA)_2$ and $K(EENV)_4$ from T. Bartfai and A. Undén, Department

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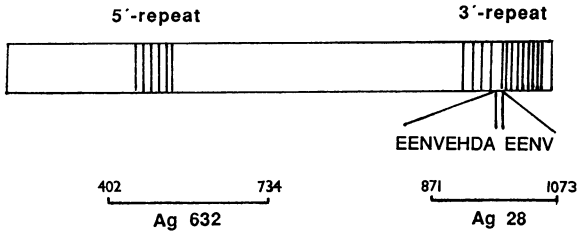

FIG. 1. Schematic presentation of Pf155/RESA. The amino acid positions of FP Ag632 and FP Ag28 are shown. The 5' repeat block consists of the consensus sequence DDEHVEEPTVA repeated twice and five similar sequences with one or two deletions each. The 3' repeat block at the C terminus consists of the sequence EENVE HDA repeated 5 times and the sequence EENV repeated 29 times plus eight variants of the latter (12).

of Biochemistry, University of Stockholm; K(DDEHVEE PTVA)$_2$ from G. Westin Sjödahl, Kabi, Stockholm, Sweden; L-N$^{\alpha}$,N$^{\varepsilon}$-dipalmitoyl-KGGEENVEHDA from A. C. Allison, Syntex Research, Palo Alto, Calif. The purity of the peptides ranged between 60 and 90%. some of the peptides were conjugated with keyhole limpet hemocyanin or tetanus toxoid as described previously (2).

Recombinant fusion proteins. The deduced primary structure of the fusion proteins (FP) Ag28 and Ag632 produced by recombinant clones and their extension in the Pf155/RESA molecule (Fig. 1) have been described previously (5, 7).

Antibodies. Rabbits were immunized with the conjugated synthetic peptides EENVEHDA-keyhole limpet hemocyanin (R988), EENVEHDA-tetanus toxoid (R989), (EENV)$_2$-keyhole limpet hemocyanin (R996), and dipalmitoyl-KG GEENVEHDA (R003) as described previously (2) or with the *Escherichia coli*-derived fusion proteins FP Ag632 (R3 and R730), FP Ag28 (R749 and R999), and both FP Ag632 and FP Ag28 (R773 and R727). Each rabbit was given three to five injections, the first in Freund complete adjuvant and the boosters in Freund incomplete adjuvant. The IgG fractions of the sera were prepared by the method of Harboe and Ingild (14), including ammonium sulfate precipitation and ion-exchange chromatography on DEAE-Sephadex (Pharmacia). IgG fractions from the preimmune sera were used as controls. For affinity purification of antibodies the IgG fractions were used (see below).

Affinity chromatography. The synthetic peptide EENVE-HDA or (EENV)$_2$ was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by Berzins et al. (2). The bound antibodies were eluted with 3 M KSCN–phosphate-buffered saline (0.15 M NaCl in 0.015 M phosphate buffer [pH 7.4]) containing 0.1 M glycine. The eluted fractions were then extensively dialyzed against phosphate-buffered saline containing 0.1 M glycine. IgG concentrations were determined by a sandwich enzyme-linked immunosorbent assay (23).

Elution of antibodies from erythrocyte monolayers. Antibodies binding to glutaraldehyde-fixed erythrocyte monolayers were eluted with 0.2 M glycine buffer at pH 2.8, neutralized with 2 M Tris base, and dialyzed extensively overnight against tissue culture medium (21).

EMIF. The indirect erythrocyte membrane immunofluorescence (EMIF) assay was performed on glutaraldehyde-fixed and air-dried monolayers of erythrocytes infected with *P. falciparum* of primarily early stages as previously described (21).

Merozoite invasion inhibition assay. The merozoite invasion inhibition in vitro assay was performed as described previously (29). In brief, *P. falciparum* cultures were set up in microculture plates (100 μl per well) at a 2% hematocrit and a starting parasitemia of approximately 0.5%, the cultures consisting primarily of late trophozoites and early schizonts. After incubation at 37°C for 20 h in 100 μl of complete tissue culture medium supplemented with different dilutions of the various purified antibody preparations or normal IgG, the microcultures were washed twice by centrifugation. Glutaraldehyde-fixed and air-dried erythrocyte monolayers were prepared on multitest slides. The parasites were stained with acridine orange, and the percentage of newly infected erythrocytes was analyzed in a fluorescence microscope. Reinvasion inhibition was calculated as 100 × (percent parasitemia in control − percent parasitemia in test)/(percent parasitemia in control). Quadruplicates were set up of all cultures, and the percentages of parasitemia given are the means from the 40,000 erythrocytes that were screened. The effect of adding the total IgG fraction to affinity-purified antibodies was analyzed by mixing the affinity-purified antibodies at fixed and slightly suboptimal concentrations with subinhibitory amounts of the total IgG fraction before addition to the *P. falciparum* cultures. For reversion of the inhibition with synthetic peptides, 100 μl of a fixed concentration of antibodies was mixed with 10 μl of synthetic peptide, K(EENVEHDA)$_2$, K(EENV)$_4$, or K(DD EHVEEPTVA)$_2$, at various concentrations. The mixtures were incubated for 30 min before addition to the *P. falciparum* cultures.

RESULTS

IgG fractions were isolated from sera of rabbits immunized with different *E. coli*-derived fusion proteins (FP Ag632, containing the 5' repeat block of Pf155/RESA (R3 and R730); or FP Ag28, containing the 3' repeat block (R749 and R999) [Fig. 1]) as well as from sera of rabbits (R773 and R727) immunized with a mixture of these proteins. IgG fractions were also isolated from sera of rabbits immunized with the synthetic peptide EENVEHDA (R988, R989, R003) or (EENV)$_2$ (R996), corresponding to sequences of the 3' repeat block of Pf155/RESA (12). The IgG fractions were analyzed for reactivity in the in vitro reinvasion inhibition assay (Fig. 2). All of the IgG fractions gave inhibition, with a maximum of 25 to 45% inhibition at about 1 to 2 mg of IgG per ml. The inhibitory effect could not be enhanced by increasing the dose of the inhibitors. However, the inhibitory activity was highly enriched by prior elution of antibodies from monolayers of infected erythrocytes (Fig. 2a) or by affinity chromatography on the immobilized peptide EEN VEHDA or (EENV)$_2$ (Fig. 2b). Moreover, all of the eluates inhibited parasite reinvasion almost completely at antibody concentrations that varied for the different antibody preparations from 0.5 to 100 μg of IgG per ml.

The EMIF titers (concentrations of IgG at the endpoint titer) and the 25% reinvasion inhibition titers from 10 different rabbits are shown in Table 1. The most efficient antibodies in both assays were those raised against fusion proteins containing the 3' repeat region or both the 3' and 5' repeats. Antibodies from these rabbits were approximately 100 times more efficient in both assays as compared with antibodies from rabbits immunized with the octapeptide EENVEHDA or (EENV)$_2$. Different rabbits immunized with the same antigen gave no big differences in their reinvasion inhibition or EMIF titers. Although the 25% reinvasion inhibition titers were increased 50 to 50,000 times by the affinity purification procedures, the EMIF titers were

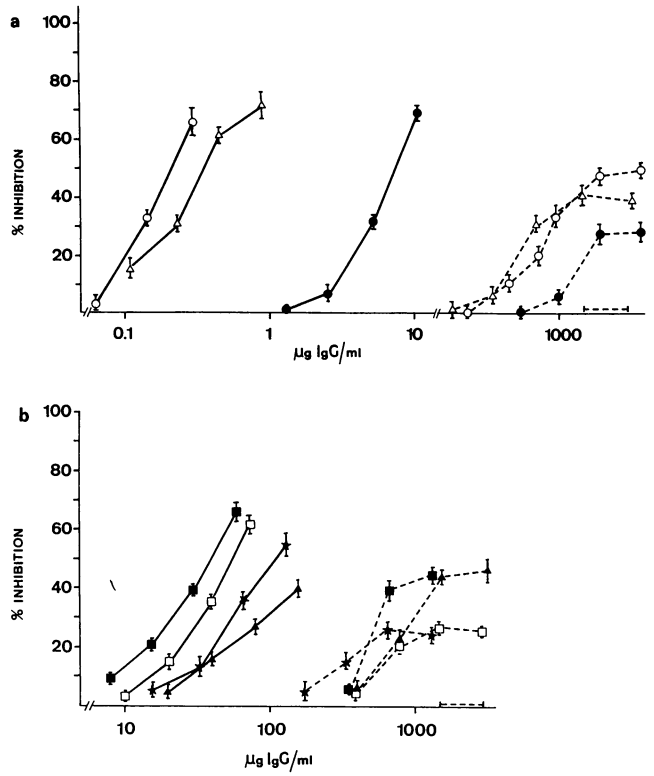

FIG. 2. Merozoite reinvasion inhibition in vitro of *P. falciparum* cultures by total IgG fractions (– – –) or by affinity-purified antibodies on parasite-infected erythrocyte monolayers or peptide columns (——). (a) Percent invasion inhibition by rabbit R749 (anti-3′ repeat) (○), R730 (anti-5′ repeat) (●), R773 (anti-5′ and anti-3′ repeats) (△), or normal rabbit serum IgG (|). (b) Percent invasion inhibition by rabbit R988 (anti-EENVEHDA) (□), R989 (anti-EENVEHDA) (▲), R003 (anti-EENVEHDA) (★), R996 [anti-$(EENV)_2$] (■), or normal rabbit serum IgG (|). The percent inhibition (ordinate) was analyzed after 20 h of incubation. Vertical bars indicate standard errors of the means from three experiments.

only increased approximately 10 times. This lack of correlation between EMIF and reinvasion inhibition titers was found for all sera tested (Table 1). The results indicate that the total IgG fractions contain a factor that counteracts the reinvasion inhibition and that is removed by the affinity purification.

To test for the presence of such a factor in the IgG preparations, subinhibitory amounts of the total IgG fractions were added to suboptimal concentrations of affinity-purified antibodies in the reinvasion inhibition assay (Fig. 3). A marked reduction (ranging between 55 and 80%) of the inhibitory effect of the eluted antibodies was found; this reduction was only obtained when the IgG fraction was from the same serum as the affinity-purified antibodies. Furthermore, IgGs from rabbits immunized with the same antigen had only a reducing effect on the homologous purified antibodies; for example, R749 eluate was affected by R749 IgG but not by R999 IgG.

Eluates from all rabbits (Table 1) showed the same kind of serum specificity for reverting the inhibition of invasion as those in Fig. 3 (data not shown). The concentration of the IgG used in these experiments was the highest that could be used without giving more than 5% reinvasion inhibition by itself (ranging from 200 to 600 μg of IgG per ml for different

TABLE 1. Concentration of total IgG and affinity-purified antibodies determined by EMIF and merozoite reinvasion inhibition

Rabbit	Antibody	EMIF results (μg/ml)		25% Reinvasion inhibition (μg/ml)	
		Total IgG	Eluate	Total IgG	Eluate
R749	Anti-3′[a]	0.14	0.03	700	0.12
R999	Anti-3′	4.6	0.6	725	0.9
R3	Anti-5′[b]	120	22	1,500	25
R730	Anti-5′	24	3.5	1,600	5
R773	Anti-5 + anti-3′[c]	4	0.4	600	0.2
R727	Anti-5 + anti-3′	12	0.3	1,000	0.65
R988	Anti-8p[d]	550	120	1,200	30
R989	Anti-8p	630	160	900	60
R003	Anti-8p	600	190	780	82
R996	Anti-2x4p[e]	510	60	520	18

[a] Rabbit immunized with Ag28 (Fig. 1).
[b] Rabbit immunized with Ag632.
[c] Rabbit immunized with Ag28 plus Ag632.
[d] Rabbit immunized with EENVEHDA.
[e] Rabbit immunized with $(EENV)_2$.

sera) (Fig. 2). The effect of the IgG fractions on the reinvasion inhibition titer was dose dependent (Fig. 4). By decreasing the amount of IgG added, the effect on the inhibitory activity was reduced. No effect with the heterologous IgG fractions was found at any concentration tested. The addition of higher concentration of any of the IgG fractions to the purified antibodies had an additive effect on the reinvasion inhibition, reflecting the activity of the eluted antibody plus the activity of the total IgG (data not shown). In the EMIF, the eluted antibodies at their endpoint titers (range, 0.03 to 190 μg of IgG per ml; Table 1) were mixed with different serum IgGs at various concentrations below their endpoint titers (range, 0.1 to 600 μg of IgG per ml). However, no influence on the reactivity of the affinity-purified antibodies in the EMIF was found in any antibody-IgG combinations (data not shown).

To ascertain the specificity of the eluted antibodies, they were mixed with graded amounts of different peptides before being used in the reinvasion assay. The peptides used were either $K(EENV)_4$ or $K(EENVEHDA)_2$, the major amino acid repeat areas of the 3′ repeat block, or $K(DDEHVEE\ PTVA)_2$, representing a dimer of the consensus sequence of the 5′ repeat block of Pf155/RESA. Figure 5 shows the typical results from one experiment with affinity-purified antibodies from R749, R773, R3, and R988. The inhibition with eluted antibodies from R749, immunized with the fusion protein corresponding to the 3′ repeat, was reversed by peptides $K(EENVEHDA)_2$ and $K(EENV)_4$ but not by $K(DD\ EHVEEPTVA)_2$, whereas inhibition with antibodies from R3, immunized with the 5′ repeat block fusion protein, was only reversed by the latter peptide. Inhibition with antibodies from R773, immunized with both types of antigens, was reversed by all three peptides. Inhibition with antibodies from R988, immunized with the peptide EENVEHDA and eluted from the octapeptide column, was totally reversed by the corresponding peptide $K(EENVEHDA)_2$ but only slightly influenced by the $K(EENV)_4$ peptide. These results were paralleled by the capacity of these peptides to inhibit the EMIF activity of the different eluates (Fig. 5).

DISCUSSION

We demonstrate in this study that the IgG fraction of antisera raised in rabbits against repeated sequences in

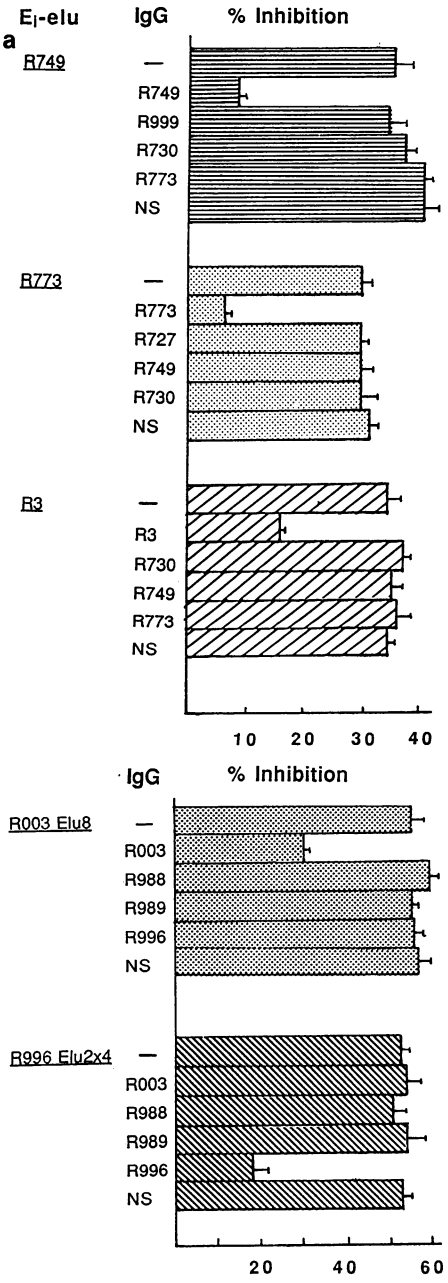

FIG. 3. Reversal of reinvasion inhibition. Suboptimal concentrations of affinity-purified antibodies were mixed with subinhibitory amounts of the total IgG fractions (200 μg of R749 per ml, 200 μg of R773 per ml, 600 μg of R3 per ml, 200 μg of R003 per ml, and 300 μg of R996 per ml). For specificity of the different rabbit IgG fractions, see Table 1. (a) Antibodies affinity purified on monolayers of parasite-infected erythrocytes: R749 (0.2 μg/ml) (▤), R773 (0.3 μg/ml) (▩), and R3 (30 μg/ml) (▨). (b) Antibodies affinity purified on EENVEHDA-Sepharose R003 (85 μg/ml) (▩) or on $(EENV)_2$-Sepharose R996 (20 μg/ml) (▩). The abscissa shows the percent reinvasion inhibition after 20 h of incubation. Smaller bars indicate standard deviations of the means from quadruplicate tests.

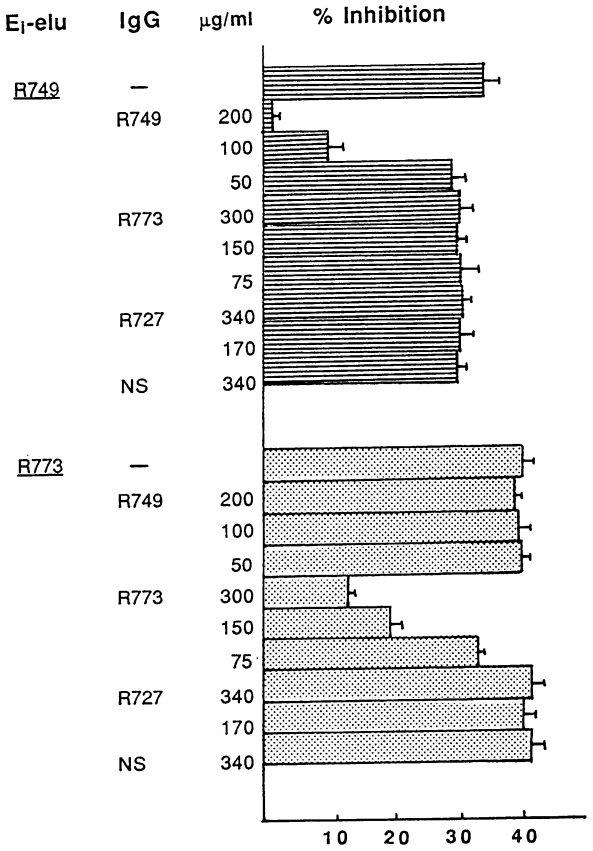

FIG. 4. Reversal of reinvasion inhibition. Fixed suboptimal concentrations of IgG from R749 (0.2 μg/ml) (▤) or R773 (0.3 μg/ml) (▩) eluted from infected erythrocyte monolayers were mixed with different concentrations of IgG from R749, R773, R727, or normal rabbit serum IgG (NS). For specificities of the different rabbit IgGs, see Table 1. The abscissa shows the percent reinvasion inhibition after 20 h of incubation. Smaller bars indicate standard deviations of the means from quadruplicate tests.

Pf155/RESA contains a factor that counteracts the merozoite reinvasion inhibition capacity of affinity-purified antibodies. This factor was removed when affinity purifying the parasite-specific antibodies. The inhibition-reverting effect was dose dependent and specific for homologous IgG-antibody combinations, whereas IgGs from other antisera were without effect, suggesting that reversion was mediated by anti-idiotypic antibodies. The specificity of the reversal of inhibition shows that it was not due to parasite-specific antibodies enhancing merozoite invasion or parasite growth-enhancing effects of high IgG concentrations (3, 13, 16, 30).

The reverting effect of the anti-idiotypic antibodies on the reinvasion inhibition indicates that they react with or close to the paratope of the anti-repeat antibodies. Immunization of rabbits with isolated anti-idiotypic antibodies will be needed to determine whether they are antiparatopic and carry an internal image of the antigen (10). Although the immunogens used in different rabbits were the same or very similar, the antibody responses were distinct (Fig. 5), indicating the absence of dominating idiotypes in these responses.

Whereas the reinvasion inhibition activity of the antibodies increased dramatically after affinity purification, their EMIF activity did not show a corresponding high increase. Furthermore, the total IgG fractions did not inhibit the EMIF activity of affinity-purified antibodies. This lack of correlation between reinvasion inhibition activities and EMIF activities in the different fractions may be explained by the difference in the sensitivity of the two assays. However, although both types of activities were completely removed from the nonbinding fractions in the affinity purifi-

FIG. 5. Reversal of reinvasion inhibition by peptides. Fixed suboptimal concentrations of IgG from R749 (0.2 µg/ml) (▤), R773 (0.3 µ/ml) (▩), or R3 (30 µg/ml) (▨) eluted from infected erythrocyte monolayers or IgG from R988 (35 µg/ml) (▨) eluted from EENVEHDA-Sepharose were mixed with different concentrations of peptides. The abscissa shows the percent invasion inhibition by antibody without peptide in the presence of 2 × 8 K(EENVE HDA)$_2$, 4 × 4 K(EENV)$_4$, or 2 × 11 K(DDEHVEEPTVA)$_2$ peptides. The figure also shows the EMIF activity of the antibody-peptide mixtures: +, strong staining; +/−, weak staining; −, no staining. Smaller bars indicate standard deviations of the means from quadruplicate tests.

cations, only about 10 to 20% of the EMIF activity was recovered in the eluted fraction, whereas the reinvasion inhibition activity was often higher than that of the original IgG fraction (data not shown). Thus, the two activities seem, at least to some extent, to be mediated by different antibody populations differing in fine specificity and/or affinity (22).

Similar affinity fractionations of human antibodies from sera of *P. falciparum*-immune Liberian donors have shown the same kind of difference in the reinvasion inhibition capacity between total IgG and purified antibodies as with the rabbit antisera (22). However, antibodies affinity purified from human sera contained only a part of the reinvasion inhibition activity, and the IgG fractions passing the peptide columns showed an inhibition capacity similar to that of the original IgG fraction, indicating the presence of antibodies that were inhibitory but specific for other parasite antigens. Nevertheless, the inhibition activity of the affinity-purified human antibodies was enriched 200- to 700-fold. At present we do not know whether the total IgG fractions of human immune sera also contain anti-idiotypic antibodies that counteract reinvasion inhibition. However, antibodies enhancing merozoite reinvasion have been demonstrated to be present in some human immune sera (3, 30).

The merozoite reinvasion inhibition assay in *P. falciparum* in vitro cultures is thought to represent a measure of the potential parasite-neutralizing capacity of antibodies in vivo. Fandeur et al. (11) found no correlation in the *Saimiri* monkey between protection in vivo and the capacity of the serum immunoglobulin to inhibit reinvasion in vitro. The effect of the in vitro reinvasion-enhancing antibodies and of anti-idiotypic antibodies against these on the parasite-neutralizing capacity of antibodies in vivo is not known. Passive immunizations with total IgG fractions from *P. falciparum*-immune individuals have, however, demonstrated their in vivo parasite-neutralizing capacity (4, 9), although they may have a moderate reinvasion inhibitory capacity in vitro (Berzins et al., unpublished data).

Observations made both in vitro and in vivo (2, 5, 8, 28, 29) have suggested that antibodies reactive with Pf155/RESA may be associated with protective immunity. Our recent results with passive immunization of *Aotus* monkeys with affinity-purified human antibodies to Pf155/RESA repeats also support these findings (Berzins et al., unpublished data). Nevertheless, our present results underline the importance of considering the effects of anti-idiotypic antibodies on the anti-parasitic response when using subunit vaccines. Most of the rabbit antisera used in this study were taken after several (three to five) immunizations. Recent immunization experiments in rabbits with recombinant fusion proteins containing multiple copies of Pf155/RESA repeats have shown that long-lasting high-titered antibody responses may be obtained with these immunogens (26). The presence of anti-idiotypic antibodies in these sera has not yet been studied, but it is possible that such antibodies may have been responsible for the prolonged maintenance of the anti-parasitic responses in these systems.

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