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Involvement of Pf155/RESA and Cross-Reactive Antigens in *Plasmodium falciparum* Merozoite Invasion In Vitro

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Lines of *Plasmodium falciparum* FCR3 either expressing or not expressing the blood-stage antigen Pf155/RESA were used to analyze the possible involvement of this antigen in the merozoite invasion process in vitro. Antibodies from human sera, affinity purified on synthetic peptides corresponding to C-terminal repeated sequences in Pf155/RESA, were shown to inhibit merozoite invasion of both types of parasites with similar efficiency. Reversal of the invasion inhibition by fusion proteins containing repeated sequences of Pf155/RESA but not of the cross-reactive antigens Ag332 and Pf11.1 indicated that the inhibitory antibodies had similar target antigens in both Pf155/RESA$^+$ and Pf155/RESA$^-$ parasites that involved cross-reacting epitopes present in Pf155/RESA. Rabbit antibodies specific for Pf155/RESA repeats inhibited merozoite invasion of Pf155/RESA expressing parasites efficiently but had no or very small effect on the invasion of Pf155/RESA-deficient parasites. In contrast, rabbit antibodies specific for Ag332 repeats as well as human antibodies affinity purified on synthetic Ag332 peptides inhibited merozoite invasion of both types of parasites with high efficiency. A similar inhibition pattern was seen with the human monoclonal antibody 33G2, which has specificity for Ag332 but also cross-reacts with Pf155/RESA and Pf11.1. Taken together, our data suggest that Pf155/RESA and related cross-reactive antigens as well as Ag332 are involved in the merozoite invasion process and may constitute targets for invasion inhibitory antibodies.

Analysis of antibody-mediated inhibition of merozoite invasion in vitro in *Plasmodium falciparum* cultures is a method commonly used to select antigens with the potential to induce protective immune responses. Polyclonal as well as monoclonal antibody (MAb) preparations to several *P. falciparum* antigens have been demonstrated by this assay to possess the capacity to interfere with the erythrocytic life cycle (14). However, extensive antigenic cross-reactions occurring between different malarial antigens (3, 5, 18) can make it difficult to determine which antigen is the actual target of the inhibitory antibodies.

Antibodies reactive with the *P. falciparum* blood-stage antigen Pf155/RESA have been shown to be very efficient inhibitors of merozoite invasion in vitro (6, 9, 24, 33, 35), indicating that Pf155/RESA is involved in the merozoite invasion process. However, this involvement is difficult to envision, as the antigen appears to be translocated from dense granules in the apical part of the merozoites to the intracellular surface of the erythrocyte membrane during or shortly after invasion (2, 12) and thus should not be exposed to the outside medium during the invasion process. Recently, a line of *P. falciparum* FCR3 which does not express Pf155/RESA was identified (7). As this parasite line grows well in in vitro cultures, Pf155/RESA seems not to be obligatory for merozoite invasion under these conditions.

In this study, we have made use of the Pf155/RESA-deficient parasite line in order to analyze the possible involvement of Pf155/RESA and related antigens in the merozoite invasion process. Human and rabbit antibodies reactive with Pf155/RESA and to different degrees with other cross-reactive blood stage antigens were analyzed for their capacities to inhibit merozoite invasion of Pf155/RESA$^+$ and Pf155/RESA$^-$ parasites. The results indicate that the invasion process involves both Pf155/RESA and antigens cross-reacting with this antigen.

MATERIALS AND METHODS

Parasites. *P. falciparum* strains were cultivated in vitro in erythrocytes of blood group O$^+$ according to the method of Trager and Jensen (31). The strains used were FCR3-RESA$^+$ (Gambia) and a variant line of this strain that does not express Pf155/RESA antigen (7), FCR3-RESA$^-$. The latter parasite line was kindly provided by R. F. Anders, Walter and Eliza Hall Institute, Melbourne, Australia.

Synthetic peptides. The peptides EENVEHDA and $(EENV)_2$ were synthesized by the standard method of Merrifield (19) and were obtained from L.-E. Larsson, Pharmacia, Uppsala, Sweden. The peptide SVTEEIAEEDKSV IEEAV (designated LJ14) was synthesized by the "tea bag" method (16) and was obtained from R. Houghten, Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, Calif.

Recombinant fusion proteins. For immunization of rabbits, three different *Escherichia coli*-produced fusion proteins comprising various Pf155/RESA repeat sequences (M) C-terminally linked to two immunoglobulin G (IgG)-binding domains (ZZ) from *Staphylococcus aureus* protein A (27–29) were used: ZZ-M1 (M1 = $[EENVEHDA]_4$), ZZ-M2 (M2 = $[EENV]_8$), and ZZ-M3 (M3 = $[EENVEHDA]_5[EENV]_{10}$). β-Galactosidase fusion proteins used for analyses of antibody specificities were purified as described by Scherf et al. (26). They comprised the related repeat sequences of the *P. falciparum* antigen Pf11.1 (26) or Ag332 (18) or the entire 3′ repeat region of Pf155/RESA (10, 18).

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TABLE 1. Immunofluorescence reactivity and invasion inhibition activity on FCR3-RESA$^+$ and FCR3-RESA$^-$ parasite strains of total IgG fractions or affinity-purified antibodies from human sera[a]

Antibody	Minimal concn (μg/ml) required for positive reaction in:				% Invasion inhibition		
	EMIF		PARIF				
	FCR3-RESA$^+$	FCR3-RESA$^-$	FCR3-RESA$^+$	FCR3-RESA$^-$	Highest concn (μg/ml) used	FCR3-RESA$^+$	FCR3-RESA$^-$
Kinon IgG	12	290	7.2	7.2	100	88	78
W183 IgG	64	340	8.4	8.4	4,200	46	40
W183 Elu8[b]	20	Neg	1.0	1.0	50	93	88
W86 IgG	61	304	7.6	7.6	3,800	36	48
W86 Elu2×4[c]	14	Neg	0.7	0.7	18	25	37
Lib29 IgG	56	1,400	ND	ND	3,500	36	41
Lib29 Elu2×4	0.2	Neg	ND	ND	21	49	42
Lib29 Elu8	1.4	Neg	ND	ND	ND	ND	ND
W186 IgG	74	Neg	15.0	15.0	1,000	85	62
W186 Elu-LJ14[d]	Neg	Neg	0.8	0.8	40	93	75
33G2 IgM	0.25	Neg	0.03	0.03	15	66	56

[a] ND, not done.
[b] Affinity purified on EENVEHDA.
[c] Affinity purified on $(EENV)_2$.
[d] Affinity purified on SVTEEIAEEDKSVIEEAV.

Antibodies. IgG was prepared from plasmas from Liberian donors by ammonium sulfate precipitation and ion-exchange chromatography on DEAE-Sephadex (Pharmacia, Uppsala, Sweden) (15). Antibodies were affinity purified on the synthetic peptide EENVEHDA, $(EENV)_2$, or SVTEEIAEEDK SVIEEAV (LJ14) coupled to CNBr-activated Sepharose 4B (Pharmacia) (6). Bound antibodies were eluted with 3 M KSCN in phosphate-buffered saline (PBS) containing 0.1 M glycine. The eluted fractions were extensively dialyzed against PBS containing 0.1 M glycine. Rabbits were immunized with the fusion protein ZZ-M1, ZZ-M2, or ZZ-M3 or with the synthetic peptide SVTEEIAEEDKSVIEEAV (LJ14). IgG from each rabbit antiserum was isolated on protein A-Sepharose (Pharmacia). IgG from the LJ14-immunized rabbit was further affinity purified on LJ14-coupled Sepharose 4B (see above). A human MAb (IgM; 33G2) was obtained from a cloned B-cell culture derived from Epstein-Barr virus-transformed B-lymphocytes of a *P. falciparum*-immune donor and purified by ammonium sulfate precipitation as described previously (33).

Immunofluorescence. Erythrocyte membrane immunofluorescence (EMIF) on glutaraldehyde-fixed and air-dried monolayers of *P. falciparum*-infected erythrocytes and conventional parasite immunofluorescence (PARIF) on unfixed and air-dried monolayers of *P. falciparum*-infected erythrocytes were performed as previously described (20).

Merozoite invasion inhibition assay. Merozoite invasion inhibition in vitro was performed as described earlier (35). In brief, *P. falciparum* cultures of late trophozoites and early schizonts were set up in microculture plates (100 μl per well) at a 2% hematocrit and a starting parasitemia of 0.5%. The cultures were incubated for 20 h at 37°C in 100 μl of complete tissue culture medium supplemented with different dilutions of various purified antibody preparations. The microcultures were washed twice, and monolayers were prepared on multitest slides by glutaraldehyde fixation and air drying. Parasites were stained with acridine orange, and the percent parasitemia was calculated with a fluorescence microscope. Each culture was set up in quadruplicate, and the percentage of parasitemia represents the mean from 40,000 erythrocytes screened. Invasion inhibition was calculated as 100 × [(percent parasitemia in control − percent parasitemia in test)/(percent parasitemia in control)]. For analyses of antibody specificities in the merozoite invasion inhibition, purified recombinant fusion proteins (Pf332, RESA-3′, and Pf11.1) at various concentrations were mixed with 100 μl of a fixed concentration of antibodies. The mixtures were incubated for 30 min before being added to the *P. falciparum* cultures. Each experiment setup was repeated at least three times. The standard deviation of the invasion inhibition values within the quadruplicates, calculated from all the experiments included in this study, varied from 0.2 to 3.7%, while the standard deviation between experiments varied from 3.2 to 7.1%.

RESULTS

Total IgG fractions of sera from Liberan donors as well as antibodies from these sera, affinity purified on synthetic peptides corresponding to repeat units in the *P. falciparum* antigen Pf155/RESA or Ag332, were analyzed by indirect immunofluorescence (EMIF and PARIF) on FCR3-RESA$^+$ and FCR3-RESA$^-$ parasite-infected erythrocytes. All but one of the total IgG fractions gave EMIF reactions with both parasites, but the reactivity with FCR3-RESA$^-$ was very weak and gave considerably lower titers in all cases (Table 1). In contrast, antibodies affinity purified on Pf155/RESA repeat peptides gave distinct EMIF staining with FCR3-RESA$^+$ parasites but were completely negative in this assay with FCR3-RESA$^-$. Also, the human MAb 33G2 was totally negative in EMIF with the FCR3-RESA$^-$ parasites but had a high EMIF titer against the FCR3-RESA$^+$ parasites (Table 1). Furthermore, the various human antibodies were tested for their reactivities with the intracellular parasites by using unfixed and air-dried monolayers of infected erythrocytes. As can be seen from Table 1, the titers in PARIF were similar with both parasites for all the antibodies tested.

The total IgG fractions and affinity-purified antibodies were also tested for their capacities to inhibit FCR3-RESA$^+$ and FCR3-RESA$^-$ merozoite invasion in vitro (Table 1). All antibodies, regardless of whether they reacted with the FCR3-RESA$^-$ parasites in EMIF, inhibited invasion of these parasites efficiently and to a degree similar to that obtained with the Pf155/RESA-expressing parasites. Also, the human

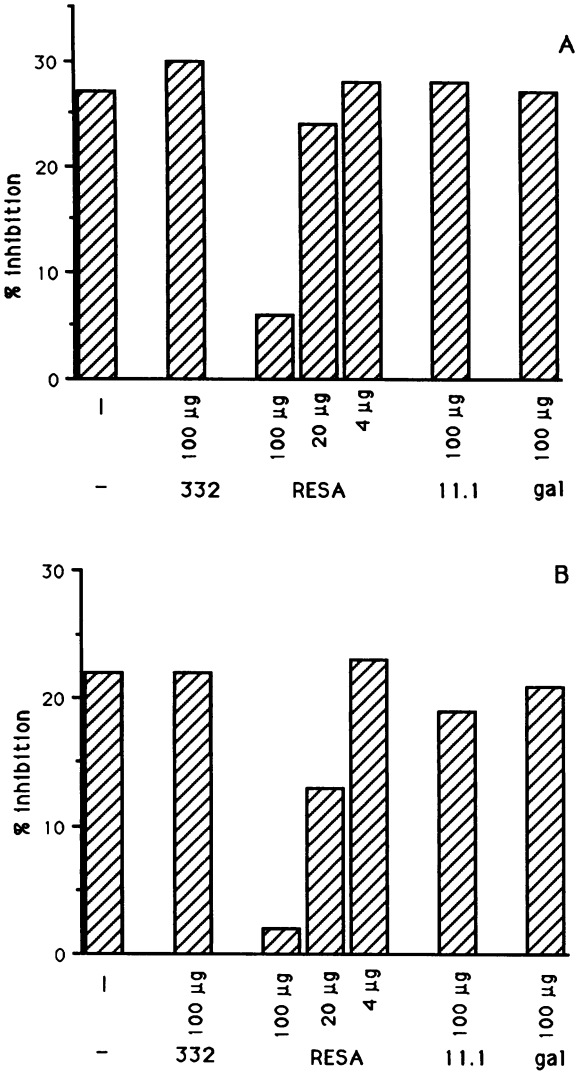

FIG. 1. Reversal by recombinant fusion proteins of the invasion inhibitory activity of human antibodies (donor W86) affinity purified on the peptide $(EENV)_2$. Two lines of *P. falciparum* FCR3 were used: FCR3-RESA$^-$ (A) and FCR3-RESA$^+$ (B). A suboptimal concentration (18 μg/ml) of the antibody was mixed with different amounts of various fusion proteins. The ordinate shows percent invasion inhibition by antibodies without fusion protein added or in the presence of Pf332, RESA-3′, Pf11.1, or β-galactosidase (gal). Parasitemia in the control at time zero and after 20 h was, respectively, 0.31 and 1.14% in panel A and 0.49 and 1.46% in panel B.

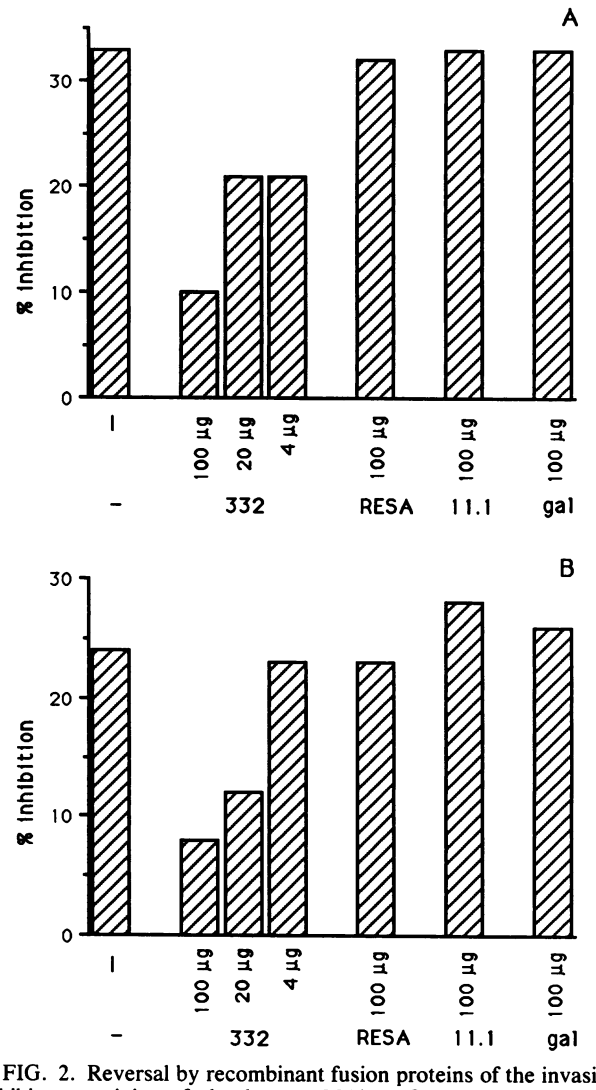

FIG. 2. Reversal by recombinant fusion proteins of the invasion inhibitory activity of the human MAb 33G2. Two lines of *P. falciparum* FCR3 were used: FCR3-RESA$^-$ (A) and FCR3-RESA$^+$ (B). A suboptimal concentration (8 μg/ml) of the antibody was mixed with different amounts of various fusion proteins. The ordinate shows percent invasion inhibition by MAb 33G2 without fusion protein added or in the presence of Pf332, RESA-3′, Pf11.1, or β-galactosidase (gal). Parasitemia in the control at time zero and after 20 h was, respectively, 0.80 and 2.19% in panel A and 0.88 and 3.38% in panel B.

MAb 33G2 inhibited the merozoite invasion of both types of parasites to a similar degree.

To further analyze specificity in the inhibition of the merozoite invasion, human antibodies affinity purified on Sepharose columns charged with $(EENV)_2$, the major C-terminal repeat motif of Pf155/RESA, were mixed with three different recombinant fusion proteins, Pf332, RESA-3′, and Pf11.1 (Fig. 1). These antibodies were EMIF positive on the FCR3-RESA$^+$ parasites and EMIF negative on the FCR3-RESA$^-$ parasites but were equally good in the invasion inhibition assay against both parasites (Table 1). The invasion inhibitory activities of these $(EENV)_2$ reactive antibodies was reversed only by the RESA-3′ fusion protein, and the reversal was similar for both the FCR3-RESA$^+$ and the FCR3-RESA$^-$ parasites. The invasion inhibition was reduced by 85 to 90% of that of the control in the presence of 100 μg of RESA-3′. Neither of the other two fusion proteins had any effect on invasion inhibition (Fig. 1). These results were parallelled by the capacity of these peptides to inhibit the EMIF activity of the affinity-purified antibodies on FCR3-RESA$^+$ parasites (data not shown).

Similarly, antibodies with high reactivity for Ag332 (1, 17, 32), the human MAb 33G2, and human antibodies affinity purified on columns charged with SVTEEIAEEDKSVI EEAV (LJ14), a repeated motif of Ag332 (17), were analyzed for their merozoite invasion inhibitory capacities after being mixed with the fusion proteins (Fig. 2 and 3, respectively). While the MAb 33G2 was EMIF positive on the FCR3-RESA$^+$ parasites and EMIF negative on the FCR3-RESA$^-$

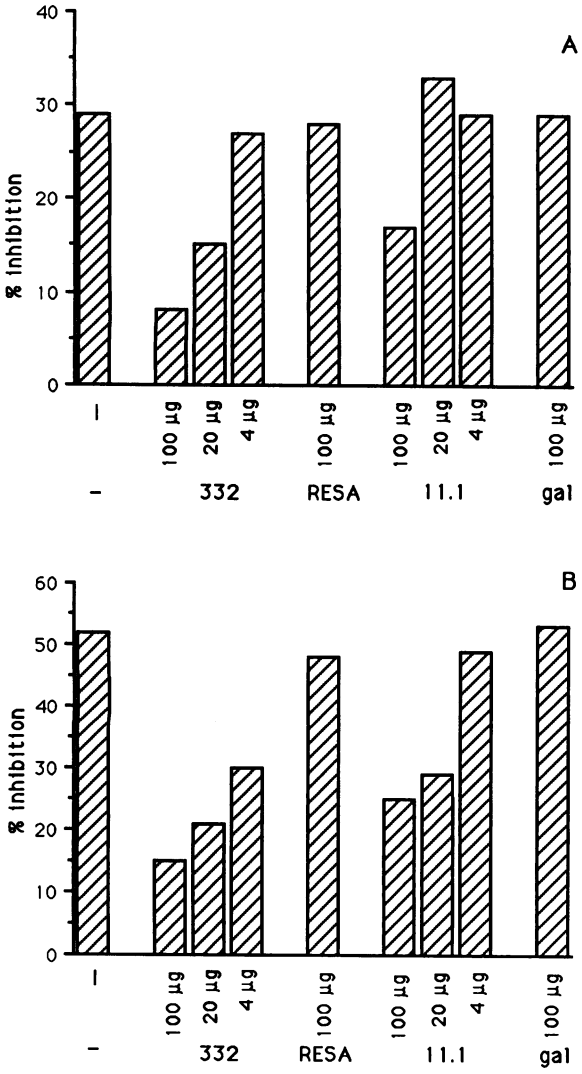

FIG. 3. Reversal by recombinant fusion proteins of the invasion inhibitory activity of human antibodies (donor W186) affinity purified on the peptide LJ14 (SVTEEIAEEDKSVIEEAV). Two lines of *P. falciparum* FCR3 were used: FCR3-RESA$^-$ (A) and FCR3-RESA$^+$ (B). A suboptimal concentration (20 μg/ml) of the antibody was mixed with different amounts of various fusion proteins. The ordinate shows percent invasion inhibition by antibodies without fusion protein added or in the presence of Pf332, RESA-3′, Pf11.1, or β-galactosidase (gal). Parasitemia in the control at time zero and after 20 h was, respectively, 0.34 and 1.03% in panel A and 0.56 and 1.76% in panel B.

parasites, antibodies affinity purified on the LJ14 peptide were negative in EMIF on both lines of the parasite (Table 1). However, in the merozoite invasion inhibition assay, both antibodies efficiently inhibited both the FCR3-RESA$^+$ and the FCR3-RESA$^-$ parasites. The invasion inhibitory activity of MAb 33G2 was reversed only by the fusion protein Pf332, while the other fusion proteins were totally ineffective. This pattern of reversal was similar for both parasite strains (Fig. 2). In the case of the LJ14-binding antibodies, the Pf332 fusion protein also efficiently reversed merozoite invasion inhibition (Fig. 3). The Pf11.1 protein had less effect, while RESA-3′ was totally ineffective.

A direct involvement of Pf155/RESA in the invasion process was indicated by using IgG from rabbit antisera containing antibodies with high specificities for this antigen (Table 2). IgGs from rabbit antisera against different recombinant fusion proteins expressing repeat sequences of Pf155/RESA were analyzed by EMIF and PARIF with the two lines of *P. falciparum*. While all IgGs showed EMIF reactivity with FCR3-RESA$^+$ parasites, none showed any such reactivity with the FCR3-RESA$^-$ parasites. In contrast, all antibodies stained the intracellular parasites of either FCR3 line in PARIF, but considerably higher IgG concentrations were needed to get positive immunofluorescence with the FCR3-RESA$^-$ parasites. When the IgG fractions were analyzed in the invasion inhibition assay, efficient inhibition was obtained with FCR3-RESA$^+$, while the invasion of FCR3-RESA$^-$ merozoites was intact or very little affected. The IgGs that had some inhibitory reactivity against the Pf155/RESA$^-$ parasites had less than 30% of the activity with these parasites that they had with the Pf155/RESA$^+$ parasites.

Affinity-purified antibodies from a rabbit immunized with LJ14 peptide (Ag332) gave no reaction in EMIF against any of the parasites. However, the invasion inhibitory capacities of these antibodies against both FCR3-RESA$^+$ and FCR3-RESA$^-$ parasites were very high (Table 2). This is in good agreement with the reactivity patterns of human antibodies purified on LJ14 (Table 1). Thus, when Ag332 repeat sequences were used for selection of antibodies, most of the inhibitory activities of these antibodies were presumably due to reaction with Ag332 and not with the Pf155/RESA antigen.

Analysis of the reactivity of the different antibodies by immunoblotting confirmed the absence of Pf155/RESA in FCR3-RESA$^-$ parasites. Thus, apart from reactivity with the 155-kDa polypeptide and its breakdown products, the antibodies showed identical reactivity patterns with the RESA$^+$ and RESA$^-$ parasites (data not shown; 18, 27, 29, 33).

DISCUSSION

We have in the present study made use of a line of *P. falciparum* FCR3 that does not express Pf155/RESA to confirm and extend our previous findings that Pf155/RESA is the major antigen recognized at the surface of newly infected erythrocytes in EMIF (10, 20, 23) and that it is a target for antibodies inhibiting merozoite invasion in *P. falciparum* in vitro cultures (6, 9, 24, 35). Not unexpectedly, however, Pf155/RESA is not the only parasite antigen giving the erythrocyte surface staining characteristic for EMIF. Thus, total IgG fractions from malaria-immune individuals also gave EMIF staining of erythrocytes infected with the FCR3-RESA$^-$ parasites, indicating the presence of other parasite-associated antigens at a location similar to that of Pf155/RESA. One such antigen may be the rhoptry protein of 110 kDa, which has been shown to be translocated to the intracellular surface of the erythrocyte membrane during invasion (25). Still other *P. falciparum* antigens, e.g., MESA and HRP-1, are translocated from the mature stages of the parasite to become associated with the erythrocyte skeleton (11, 17). However, there is presently no evidence that antibodies specific for the latter antigen are EMIF positive.

Direct evidence for the involvement of Pf155/RESA in antibody-mediated invasion inhibition was obtained by using rabbit antibodies against its C-terminal repeat sequences. These antibodies gave EMIF-positive reactions with Pf155/RESA$^+$ parasites but not with those lacking this antigen. Similarly, they efficiently inhibited invasion of antigen-pos-

TABLE 2. Immunofluorescence reactivity and invasion inhibition on FCR3-RESA$^+$ and FCR3-RESA$^-$ parasite strains of IgG from rabbit antisera

Rabbit antiserum	Antigen	Minimal concn (μg/ml) required for positive reaction in[a]: EMIF		PARIF		% Invasion inhibition		
		FCR3-RESA$^+$	FCR3-RESA$^-$	FCR3-RESA$^+$	FCR3-RESA$^-$	Highest concn (mg/ml) of IgG used	FCR3-RESA$^+$	FCR3-RESA$^-$
R27	ZZ-M1	30	Neg	1.2	6.0	1.0	61	0
R49	ZZ-M1	42	Neg	4.2	21.0	1.0	27	6
R60	ZZ-M2	18	Neg	0.7	18.0	0.9	41	16
R69	ZZ-M2	14	Neg	7.0	35.0	0.4	34	0
R73	ZZ-M3	45	Neg	3.2	10.6	1.2	51	13
R74	ZZ-M3	35	Neg	3.5	17.5	1.3	44	8
R45 Elu[b]	LJ14	Neg	Neg	1.3	ND	0.07	93	94

[a] ND, not done.
[b] Affinity purified on SVTEEIAEEDKSVIEEAV (Lj14).

itive parasites while that of antigen-negative parasites was significantly lower or nil. The weak inhibition of Pf155/RESA$^-$ parasites may be due to antibody binding to cross-reacting antigens, as reflected by the low-titered intracellular immunofluorescence staining (PARIF) of these parasites. Although our experiments strongly support the importance of Pf155/RESA in invasion, they do not exclude involvement of other antigens, which may be recognized by these rabbit antibodies but may also be missing in the FCR3-RESA$^-$ parasites (2a).

For the extensively cross-reacting human antibodies, the results were more complicated. Although human antibodies affinity purified on the C-terminal repeats of Pf155/RESA did not react in EMIF with erythrocytes infected with the Pf155/RESA-deficient parasites, they inhibited merozoite invasion of these parasites as efficiently as that of parasites expressing the antigen. These antibodies stained intracellular parasite antigens at similar titers in both antigen-positive and antigen-negative parasites. The results indicate that invasion inhibition of the Pf155/RESA$^-$ parasites is due to the binding of inhibitory antibodies to cross-reactive antigens possessing epitopes very similar to those made up by the C-terminal repeat sequences of Pf155/RESA (Fig. 1). Whether invasion inhibition by these antibodies of Pf155/RESA$^+$ parasites was due to their binding to this antigen, to cross-reacting antigens, or to both cannot be decided from these experiments. In any event, as shown in Fig. 1, the epitopes seen by the inhibitory antibodies were not present in the two cross-reacting fusion proteins (Ag332 and Pf11.1) used in attempts to abolish inhibition.

Several *P. falciparum* blood-stage antigens have been shown to contain epitopes cross-reacting with epitopes within the repeat regions of Pf155/RESA. In addition to Ag332 and Pf11.1, these include FIRA, CARP, and a 210-kDa protein (1, 3, 4, 18, 34). Because of these antigenic cross-reactivities, it has been difficult to assess the locations of the individual antigens in the infected erythrocytes and the possible involvement of the antigens in the invasion process. However, we show here that antigen Ag332 also appears to be an important target for invasion inhibitory antibodies in both Pf155/RESA-positive and -negative parasites. Thus, the human MAb 33G2 (1, 32) efficiently inhibited invasion of both parasite lines. Very similar results were obtained with human antibodies affinity purified on an 18-amino-acid-long synthetic peptide representing a repeated sequence in Ag332 and with rabbit antibodies raised by immunization with the same peptide. As neither these human antibodies nor the rabbit antibodies cross-reacted with Pf155/RESA (in EMIF), these results provide good evidence for the involvement of Ag332 in the invasion process.

Pf155/RESA has recently been localized to dense granules in the apical complex of the merozoites (2, 12). Some time during or shortly after merozoite invasion, the antigen is translocated by an unknown mechanism to the cytoplasmatic face of the erythrocyte membrane, where it associates with the cytoskeleton by a binding involving spectrin (13, 23). In ultrastructural studies of *Plasmodium knowlesi*, the contents of the dense granules appear to be released into the parasitophorous vacuolar space after merozoite entry into the erythrocyte (30). Thus, antigens present in the dense granules appear not to be directly involved in the invasion process, which is inconsistent with Pf155/RESA being a target antigen for merozoite invasion inhibitory antibodies. However, Pf155/RESA is found in large amounts in spent medium from *P. falciparum* cultures (8, 20), and the antigen can be detected associated with the membrane of erythrocytes which a merozoite has attached to but not yet entered (20), indicating that the pathway of antigen release from dense granules in *P. falciparum* might be different from that described for *P. knowlesi* (30) or, alternatively, that Pf155/RESA may also be present at other locations not yet defined by electron microscopy.

Parasites deficient in Pf155/RESA grow, multiply, and invade new erythrocytes well in in vitro cultures, indicating that the antigen is not obligatory for parasite survival under these conditions. In contrast, it appears that these parasites do not grow in vivo in monkeys (9a). Moreover, this lack of Pf155/RESA expression has been observed only in the FCR3 strain (7, 22), a parasite kept in in vitro culture for many years, while so far no parasites deficient of this antigen have been identified among >200 field isolates (21, 29a). The possibility that low numbers of Pf155/RESA$^-$ parasites exist in vivo cannot be excluded, but their rare occurrence also suggests that they do not possess any advantage for survival under immunological pressure.

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