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ANTIBODIES TO REPEATED AMINO ACID SEQUENCES IN Pf155, A MEROZOITE ASSOCIATED ANTIGEN OF *PLASMODIUM FALCIPARUM*

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Rabbits were immunized with the synthetic peptides EENVEHDA and $(EENV)_2$, *corresponding to tandemly repeated amino acid sequences in the C-terminus of Pf155. The resulting antibodies reacted in peptide-ELISAs mainly with the peptide used for immunization, but showed also some cross-reaction with the other peptide. Both sera reacted with Pf155 as detected by immunoblotting and immunofluorescence. Affinity purified antibodies from the two sera inhibited merozoite reinvasion in* P. falciparum in vitro *cultures, the anti-*$(EENV)_2$ *antibodies being more efficient. Two mouse monoclonal antibodies to EENVEHDA showed little cross-reactivity with* $(EENV)_2$. *They both reacted with Pf155 and inhibited parasite reinvasion* in vitro. *Analysis of human sera in peptide-ELISAs with the two peptides showed that reactivity to* $(EENV)_2$ *was predominating, although many sera also showed reactivity to EENVEHDA. Affinity purification of these peptide reactive antibodies revealed at least three populations of antibodies, one specific for EENVEHDA, one specific for* $(EENV)_2$ *and one reactive with both peptides. Both anti-EENVEHDA and anti-*$(EENV)_2$ *antibodies inhibited merozoite reinvasion, but the latter antibodies with higher efficiency. Our results indicate that the C-terminal region of amino acid repeats in Pf155 comprises antigenic epitopes which could form the basis for a synthetic vaccine against* P. falciparum *malaria.*

Intensive efforts have been made during recent years to identify antigens of *Plasmodium falciparum* asexual blood stages with possible involvement in protective immunity. On one hand, studies have been performed to find correlations between the degree of exposure to *P. falciparum* malaria and the presence in the serum of antibodies to specific *P. falciparum* antigens (Gysin, Dubois & Pereira da Silva, 1982; Perrin & Dayal, 1982; Wahlgren et al., 1986). On the other hand, inhibition of parasite reinvasion in *P. falciparum in vitro* cultures has been used as a measure of putative *in vivo* protective antibodies. Correlations have been sought between inhibitory sera or immunoglobulin fractions and the presence of specific antibodies (Brown et al., 1982; Myler et al., 1982; Reese, Motyl & Hofer-Warbinek, 1981; Wåhlin et al., 1984). In several cases the involvement of antibodies to specific antigens in the reinvasion inhibition has been confirmed using monospecific antibodies, either monoclonal or polyclonal (Berzins et al., 1986; Perrin & Dayal, 1982; Saul et al., 1984; Schmidt-Ullrich et al., 1986; Udomsangpetch et al., 1986).

We have identified a merozoite associated antigen of Mr 155.000 D, designated Pf155 (Perlmann et al., 1984), which fulfils the above mentioned criteria for an antigen being involved in protective immunity. A study of Liberian children showed that the appearance of antibodies to Pf155 correlated with their acquisition of clinical immunity to *P. falciparum* (Wahlgren et al., 1986). Furthermore, antibodies to Pf155 are efficient inhibitors of *P. falciparum* reinvasion in *in vitro* cultures (Berzins et al., 1986; Udomsangpetch et al., 1986; Wåhlin et al., 1984).

Pf155 is the same antigen as RESA, the "ring stage erythrocyte surface antigen", described by a research group at the Walter and Eliza Hall Institute, Melbourne, Australia (Coppel et al., 1984). This group has cloned the gene for RESA and determined its nucleotide sequence (Cowman et al., 1985). From their results we know that Pf155 contains two regions of tandemly repeated sequences, one in the C-terminus of the molecule consisting mainly of the octapeptide subunit EENVEHDA repeated 4-5 times, followed by 30-40 repeats of the tetrapeptide subunit

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EENV (Coppel et al., 1984). Towards the N-terminus is a second repeat region consisting of six imperfect 11 amino acid subunits (Cowman et al., 1985).

The present report reviews some of the results we have obtained with antibodies reactive with the C-terminal repeat region of Pf155. These antibodies include, on one hand, both rabbit and mouse antibodies to synthetic peptides corresponding to repeat subunits of the C-terminus and, on the other hand, human antibodies against Pf155 elicited by exposure to *P. falciparum* parasites. Peptide reactivities were analyzed by dot immunoblotting (Berzins et al., 1986) and peptide-ELISAs (H. Perlmann et al., in preparation) while the reactivity with Pf155 was assessed by immunoblotting (Berzins, Wahlgren & Perlmann, 1983) or indirect immunofluorescence on glutaraldehyde fixed and air dried monolayers of *P. falciparum* infected erythrocytes (Perlmann et al., 1984). The capacity of the different antibodies to inhibit parasite reinvasion in *P. falciparum in vitro* cultures was determined (Wåhlin et al., 1984).

RESULTS

Rabbit antibodies – Rabbits were immunized with the synthetic octapeptides EENVEHDA and $(EENV)_2$ coupled to keyhole limpet hemocyanine (KLH). The resulting antisera were tested for their anti-peptide reactivity in peptide-ELISAs (H. Perlmann et al., in preparation) and dot-immunoblotting (Berzins et al., 1986) using octapeptide-BSA conjugates as antigen. The antisera gave the highest reactivity with the peptide used for immunization, but a certain degree of cross-reactivity with the other peptide was also seen (Table I).

Both antisera reacted with Pf155 as detected either by immunoblotting (Fig. 1) or indirect immunofluorescence on glutaraldehyde fixed and air dried monolayers of *P. falciparum* infected erythrocytes (Table I). In the immunofluorescence assay the anti-$(EENV)_2$ serum gave higher titers. Furthermore, when antibodies were affinity purified on the corresponding peptides, the anti-$(EENV)_2$ antibodies were of higher titers, giving an immunofluorescence down to 30μg/ml as compared to 70μg/ml for the anti-EENVEHDA antibodies (Table I).

A similar difference was seen in the capacity of antibodies from the two different rabbit antisera to inhibit *P. falciparum* reinvasion *in vitro*, the anti-$(EENV)_2$ antibodies being ten-fold more efficient on the basis of the concentration of IgG needed for 50% inhibition (Table I). This difference in efficiency was not seen unless affinity purified antibodies were used. Although the affinity purification of antibodies gave a 100-1000-fold enrichment of the 50% reinvasion inhibition titer the immunofluorescence titer increased only about 10-fold. These results indicate that the total IgG-fraction contains a factor which counteracts the reinvasion inhibition and which is removed by the affinity purification.

Wåhlin et al. (unpublished data) demonstrated the presence of such a factor by adding subinhibitory amounts of the total IgG-fraction to affinity purified anti-peptide antibodies and obtained a marked reduction in the inhibitory capacity of the latter. Furthermore, this reduction was only obtained when the IgG-fraction was from the same serum as the affinity purified antibodies. The nature of the factor is not known as yet. However, the serum specificity in the reversal of the reinvasion inhibition suggests that it might be mediated by anti-idiotypic antibodies.

Upon affinity purification of IgG from human sera, we have occasionally seen a similar discrepancy in the enrichment of reinvasion inhibitory activity as compared with the immunofluorescence activity (Wåhlin et al., 1984). Whether this is due to a similar serum specific factor as in the rabbit antisera is, however, not known.

TABLE I

Analysis of antibodies reactive with the synthetic peptides EENVEHDA or $(EENV)_2$

Antibodies	Specificity	ELISA[a] aEENVEHDA:a$(EENV)_2$	IF Titer[b]		50% Reinvasion Inhibition[b]	
			Total IgG	Aff.IgG[c]	Total IgG	Aff.IgG[c]
Rabbit 988	EENVEHDA	1.9	530	70	> 8000	90
Rabbit 996	$(EENV)_2$	0.12	510	30	> 8000	9
Mouse mab 1F1	EENVEHDA	6.3	NT[d]	1	NT	80
Human IN	EENVEHDA	0.65	NT	0.9	NT	5.5
Human IN	$(EENV)_2$	0.10	NT	0.2	NT	0.5

a) Ratio OD_{405}; b) μg/ml; c) IgG affinity purified on immobilized synthetic peptides; d) Not tested.

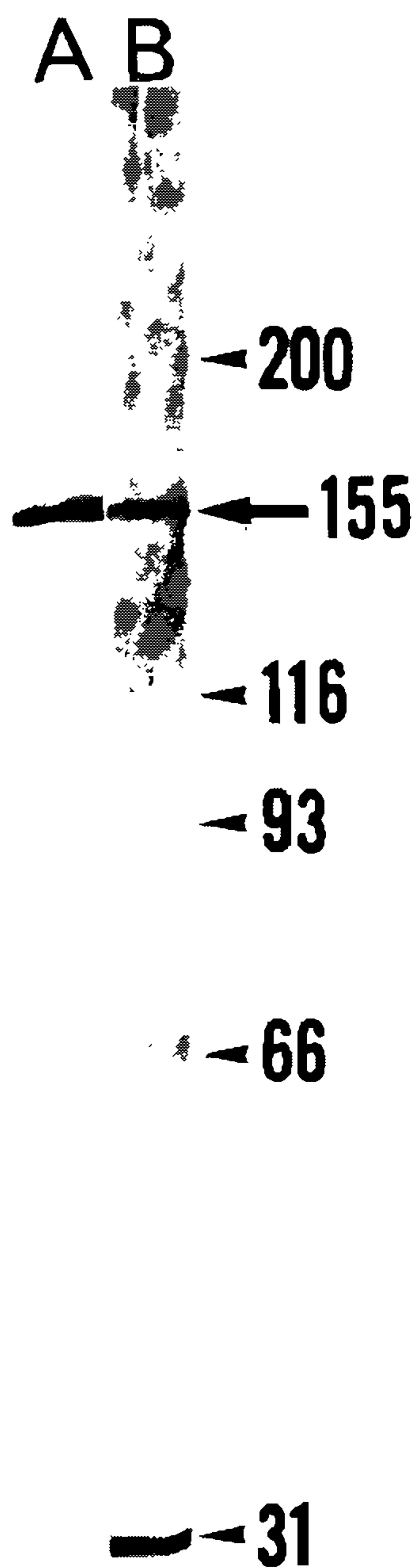

Fig. 1: Immunoblotting with a *P. falciparum* merozoite extract separated in SDS-PAGE and electroblotted onto nitrocellulose. Probing was done with (A) rabbit antiserum to EENVEHDA. Numbers indicate approximate molecular weights x 10^{-3}.

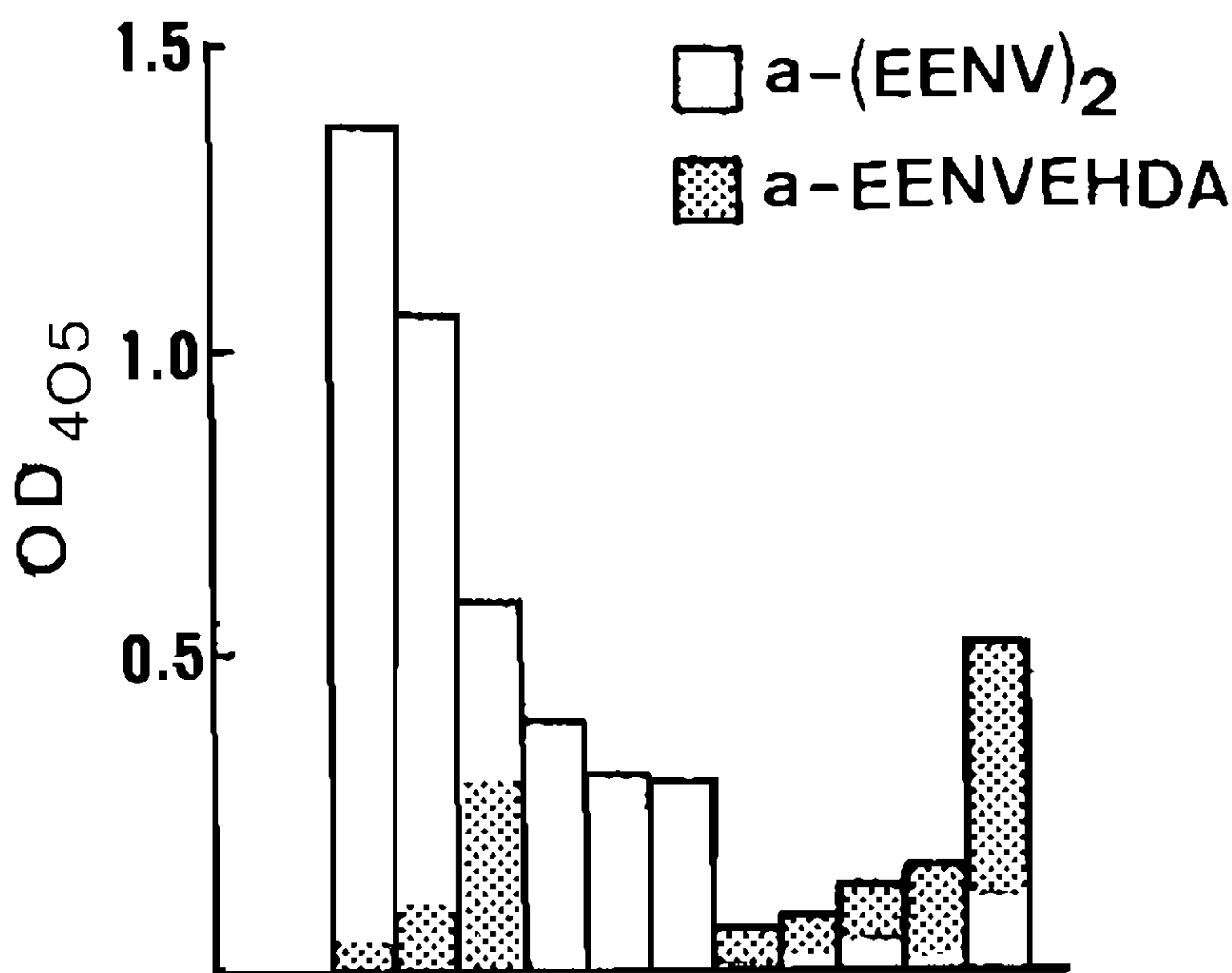

Fig. 2: Peptide-ELISAs with BSA-conjugates of the synthetic peptide EENVEHDA and $(EENV)_2$ as antigen. Analysis was done with 11 mothers from the Liberian village Kinon. Open bars represent the OD_{405} -values obtained with $(EENV)_2$ as antigen and shaded bars represent OD_{405} -values with EENVEHDA as antigen.

Mouse monoclonal antibodies – Two monoclonal mouse antibodies to the synthetic octapeptide EENVEHDA were produced (W. Ruangjirachuporn et al., in preparation). The spleen cell donor was a mouse immunized with a octapeptide-KLH conjugate. The two monoclonal antibodies reacted with the octapeptide as detected by dot-immunoblotting and peptide-ELISA, but showed poor or no reactivity with the $(EENV)_2$ peptide (Table I). They were indistinguishable in inhibition ELISA experiments with various related synthetic peptides. Both monoclonal antibodies

reacted with Pf155 as detected either by immunoblotting or surface immunofluorescence of ring-infected erythrocytes. The reactivity with Pf155 was also indicated by the capacity of the antibodies to inhibit merozoite reinvasion in *P. falciparum in vitro* cultures (Table I). The antibody concentration needed for 50% reinvasion inhibition, 80μg/ml, is high as compared to the immunofluorescence titer, 1 μg/ml. This indicates that these antibodies react better with Pf155 when this has been modified by glutaraldehyde treatment or denaturation, than when it occurs in its native form.

Human antibodies – We have previously shown that individuals exposed to *P. falciparum* elicit antibodies reactive with the octapeptide EENVEHDA as detected by dot immunoblotting with octapeptide-BSA as antigen (Berzins et al., 1986). The anti-octapeptide antibody titers correlated well with the titers of Pf155 reactive antibodies as detected by immunofluorescence. Peptide-ELISAs were used to analyse sera from Liberian donors for antibody reactivities against the synthetic peptides EENVEHDA and $(EENV)_2$. Reactivity with the tetrapeptide dimer was usually predominating, although most sera also showed reactivity with the octapeptide (Fig. 2). In some sera, however, there was a low reactivity with $(EENV)_2$ and the octapeptide reactivity dominated. This is in contrast to the results reported by Anders et al. (1986) which showed that naturally occuring antibodies in the serum of adult Papua New Guineans bound to a tetramer of EENV but not to the octapeptide EENVEHDA.

Affinity purification of the peptide reactive antibodies on immunoadsorbents containing peptide-BSA conjugates as ligands revealed at least three populations of anti-peptide antibodies. Analysis by either dot-immunoblotting or peptide-ELISAs showed one antibody population specific for EENVEHDA, one specific for $(EENV)_2$ and one recognizing both peptides. These data suggest that the C-terminal region of repeats in Pf155 comprises at least three different epitopes.

When such affinity purified human peptide reactive antibodies were tested in immunofluorescence and in *P. falciparum* reinvasion inhibition, they showed the same pattern of reactivities as the rabbit antibodies. Thus, antibodies purified on $(EENV)_2$ immunoadsorbent were more efficient in immunofluorescence and had a ten-fold higher capacity in the reinvasion inhibition as with antibodies isolated on the EENVEHDA immunoadsorbent (Table I).

CONCLUSIONS

Our results show that antibodies reactive with the C-terminal repeat region of Pf155 are potent inhibitors of *P. falciparum* reinvasion *in vitro*. Such antibodies have been obtained either by immunization of rabbits and mice with synthetic peptides corresponding to repeating subunits in the antigen or by affinity purification from the serum of individuals with previous exposure to *P. falciparum* malaria (Berzins et al., 1986; Wåhlin et al., in preparation). The *in vitro* reinvasion inhibition assay with antibodies is believed to reflect a potentially protective anti-parasite capacity of the antibodies *in vivo*, but the *in vivo* relevance of the anti-repeat antibodies in parasite elimination has yet to be demonstrated. With the possibility to affinity purify large amounts of anti-repeat antibodies from human sera, tests of the *in vivo* activity of these antibodies by passive transfers are feasable within a near future.

With the two synthetic peptides used in this study we have demonstrated that the C-terminal region of repeated sequences in Pf155 contains at least three different antigenic epitopes. As detected by immunofluorescence and reinvasion inhibition, antibodies reactive with epitopes related to the $(EENV)_2$ sequence were more efficient in their reaction with Pf155 than antibodies reactive with epitopes related to the EENVEHDA sequence. This might be expected as the $(EENV)_2$ sequence is present in about 18 copies while the EENVEHDA sequence is only repeated four times in Pf155 (Cowman et al., 1985). Delineation of the amino acid sequence of the optimal epitopes for anti-Pf155 antibodies remains, however, to be performed.

Using short synthetic peptides for immunization of rabbits gave high titers of anti-peptide antibodies, while only a minor fraction of these antibodies reacted with the Pf155 antigen. Attempts are being made to obtain a higher proportion of biologically active antibodies using for immunizations synthetic peptides comprising several repeat subunits. Such longer peptides are expected to present a secondary structure more similar to that of the repeat region in Pf155. Furthermore, as has been shown with synthetic peptides corresponding to repeat subunits in the *P. knowlesi* circumsporozoite antigen (Clough et al., 1985), with the choice of adjuvant for the immunizations the immune response can be directed towards the production of preferred types of antibodies.

In conclusion, our results indicate that the C-terminal region of amino acid repeats in Pf155 comprises antigenic epitopes which could form the basis for a synthetic vaccine against the erythrocytic stages of *P. falciparum*.

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