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Immunogenicity and Antigenicity in Rabbits of a Repeated Sequence of *Plasmodium falciparum* Antigen Pf155/RESA Fused to Two Immunoglobulin G-Binding Domains of Staphylococcal Protein A

ANDERS SJÖLANDER,[1]* STEFAN STÅHL,[2] PER-ÅKE NYGREN,[2] LENA ÅSLUND,[3] NIKLAS AHLBORG,[1] BIRGITTA WÅHLIN,[1] ARTUR SCHERF,[4] KLAVS BERZINS,[1] MATHIAS UHLÉN,[2] AND PETER PERLMANN[1]

Department of Immunology, University of Stockholm, S-106 91 Stockholm,[1] Department of Biochemistry, Royal Institute of Technology, S-100 44 Stockholm,[2] and Department of Medical Genetics, University of Uppsala, S-751 23 Uppsala,[3] Sweden, and Unité de Parasitologie Expérimentale, Institut Pasteur, Paris, France[4]

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A synthetic gene encoding a tetramer of the repeated subunit EENVEHDA of the *Plasmodium falciparum* antigen Pf155/RESA was expressed in a dual-expression system. The resulting fusion proteins, designated ZZ-M1 and BB-M1, comprised the EENVEHDA repeats and either two immunoglobulin G-binding domains from staphylococcal protein A or the human serum albumin-binding domains from streptococcal protein G, respectively. The soluble fusion proteins were affinity purified to homogeneity in one-step procedures. ZZ-M1 was used for immunization of rabbits. The rabbit antisera reacted with BB-M1 in an enzyme-linked immunosorbent assay and with Pf155/RESA in immunofluorescence of infected erythrocytes and immunoblotting. Inhibition studies revealed that the antibodies mainly recognized epitopes formed by two or more EENVEHDA subunits and were remarkably specific for Pf155/RESA. Importantly, the antibodies also inhibited *P. falciparum* merozoite reinvasion in vitro efficiently, indicating that they reacted with biologically important epitopes exposed on the native antigen. Immunization with Freund complete adjuvant resulted in high levels of specific immunoglobulin G antibodies over a 1-year period, whereas the antibody response obtained after immunization without adjuvant was generally weaker, immunoglobulin G and M mediated, and not sustained for longer periods. However, these titers were restored after booster injection. Taken together, the results support the usefulness of recombinant gene constructs of this type as immunogens for malaria vaccines.

The global increase during the past 15 years of *Plasmodium falciparum* malaria, the most lethal of the human malarias, has highlighted the need of an effective vaccine against this disease. Since the asexual blood stages of the parasite cause the clinical disease, identification and definition of vaccine candidates from these stages are of great importance (13). The *P. falciparum* antigen Pf155/RESA, which is deposited in the erythrocyte membrane during merozoite invasion (7, 17), is considered to be a candidate for a blood-stage vaccine (13, 16). The C-terminal part of Pf155/RESA comprises a region of repeated subunits of eight, four, or three amino acids (8). The octapeptide subunit EENVEHDA is immunogenic in rabbits, and a large fraction of human antibodies to Pf155/RESA also react with this linear sequence, suggesting that the octapeptide is part of a major antigenic region of Pf155/RESA (2, 3). Furthermore, β-galactosidase fusion proteins of DNA clones encoding Pf155/RESA sequences partially protected *Aotus* monkeys from *P. falciparum* challenge. Protection correlated with the presence of antibodies reactive with Pf155/RESA repeat sequences, including the octapeptide (6).

To obtain immunogens based on the EENVEHDA sequence, a synthetic gene encoding a tetramer of the octapeptide was constructed and inserted in the expression vector pATH 11 (1). Immunization of rabbits with a partially purified fusion protein resulted in high titers of antibodies to Pf155/RESA (1).

* Corresponding author.

For further studies of the antigenicity and immunogenicity of the EENVEHDA repeat subunit and to establish the specificity of the immune response to this sequence, the same synthetic gene was expressed in a dual-expression system as a fusion protein with either two immunoglobulin G (IgG)-binding domains from the staphylococcal protein A (10) (designated ZZ-M1) or with the human serum albumin (HSA)-binding part of the streptococcal protein G (designated BB-M1) (15, 21). This strategy allows affinity purification to homogeneity of the soluble fusion proteins in one-step procedures. Furthermore, the immune response induced by immunization with ZZ-M1 can be analyzed with BB-M1, thus eliminating any background originating from the protein A carrier part. In the present study, the humoral response in rabbits immunized with affinity-purified ZZ-M1 was studied in detail over a 1-year period, with special attention given to the specificities, isotypes, and biological activities of the antibodies.

MATERIALS AND METHODS

Expression of fusion proteins. The construction, expression, and purification of the fusion proteins has been described in detail elsewhere (21). Briefly, the synthetic gene fragment encoding a tetramer of the C-terminal octapeptide repeat EENVEHDA of the *P. falciparum* antigen Pf155/RESA was cut out from a construction in the expression vector pATH 11 (1) and inserted into plasmid pEZZT308 or pB1B2mp18 (15). The resulting expression vectors, pEZZM1 and pB1B2M1, encode the octapeptide repeats and either a divalent synthetic IgG-binding domain derived from

staphylococcal protein A or the HSA-binding domains of streptococcal protein G, respectively. The two fusion proteins were directed to the periplasmic space by the secretion signal of the staphylococcal protein A operon (15), where they constituted one of the major fractions of the produced proteins. After expression in *Escherichia coli*, the produced fusion proteins, designated ZZ-M1 (pEZZM1) and BB-M1 (pB1B2M1), were released by osmotic shock (14) and applied on columns of IgG-Sepharose (Pharmacia AB, Uppsala, Sweden) (ZZ-M1) or HSA-Sepharose (15) (BB-M1). After extensive washing with 50 mM Tris hydrochloride (pH 7.4)–0.9 M NaCl–0.05% Tween 20 and then 5 mM ammonium acetate (pH 6.0), the columns were eluted with 0.3 M acetic acid (pH 3.3; IgG-Sepharose) or 0.5 M acetic acid (pH 2.8; HSA-Sepharose). The A_{280} was measured, and relevant fractions were lyophilized. After affinity purification, the majority of the purified material was full length (data not shown).

Rabbit antisera. Two groups of four New Zealand White rabbits were immunized intramuscularly with 100 μg of the fusion protein ZZ-M1 in Freund complete adjuvant (FCA) or without adjuvant, respectively. Booster injections, in Freund incomplete adjuvant instead of FCA or without adjuvant, were given as indicated in Fig. 1 and 2. The rabbits were bled 1 week after each booster injection.

Parasite material. In vitro cultures of the Tanzanian *P. falciparum* F32 were used. For antigen analysis, merozoite-enriched fractions were prepared and processed as previously described (17, 23).

Antibody titration. Rabbit antibodies binding to the fusion protein BB-M1 were measured in an enzyme-linked immunosorbent assay (ELISA) (1). Polystyrene microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with BB-M1 (1 μg/ml). Control plates were coated with the protein G-derived part of BB-M1. After blocking with 0.5% bovine serum albumin for 3 h at 37°C, the coated plates were incubated for 1 h at 37°C with dilutions of rabbit serum. After incubation for 1 h at 37°C with sheep anti-rabbit IgG (whole molecule) conjugated with alkaline phosphatase and for 30 min at room temperature with *p*-nitrophenyl phosphate, the resulting color was registered at 405 nm. Isotypes of the reactive antibodies were determined with alkaline phosphatase-conjugated sheep IgG specific for rabbit μ or γ chains. In inhibition assays, various concentrations of inhibitor were preincubated with dilutions of antisera for 30 min at 37°C before the ELISA. Inhibitors comprised the fusion protein BB-M1 and the synthetic peptides EENVEHDA (L.-E. Larsson, Pharmacia [2]), $(EENVEHDA)_3$ (BACHEM, Bukendorf, Switzerland), $(EENV)_6$ (BACHEM), and $K(DDEHVEEPTVA)_2$, a dimer of the consensus sequence of the 5′-repeat block of Pf155/RESA. Additional inhibitors were β-galactosidase fusion proteins, purified as described by Scherf et al. (20), comprising related repeat sequences of the *P. falciparum* antigen Pf11.1 (20) or Pf332 (12) or the entire 3′-repeat region of Pf155/RESA (7, 12).

EMIF. Indirect immunofluorescence of glutardialdehyde-fixed and air-dried monolayers of *P. falciparum*-infected erythrocytes was performed as described by Perlmann et al. (17). Isotype determination of the reactive antibodies was done with biotin-labeled sheep IgG specific for rabbit μ or γ chains. For inhibition, various concentrations of inhibitor, BB-M1, EENVEHDA, $(EENVEHDA)_3$, $(EENV)_6$, $K(DDEHVEEPTVA)_2$, Pf11.1, Pf332, or 3′-Pf155/RESA were preincubated with dilutions of antisera for 30 min at 37°C before erythrocyte membrane immunofluorescence (EMIF).

Immunoblotting. Parasite extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, electrophoretically transferred to nitrocellulose, and probed with rabbit serum as previously described (1, 4).

Affinity chromatography. Antibodies were affinity purified on Sepharose beads charged with BB-M1. Purified BB-M1 was affinity bound to HSA coupled to CNBr-activated Sepharose (Pharmacia) (3.8 mg of BB-M1 per 15 mg of albumin per ml of beads) and covalently cross-linked to the HSA by glutardialdehyde (5, 21). For affinity chromatography, 2 mg of the IgG fraction isolated from rabbit serum was incubated with 1 ml of packed beads applied to a column. After extensive washing with phosphate-buffered saline, bound antibodies were eluted with 0.2 M glycine buffer (pH 2.8), followed by elution with 3 M KSCN. The buffer of the eluates was changed to phosphate-buffered saline containing 0.2% bovine serum albumin on a PD10 column (Pharmacia) according to the instructions of the manufacturer.

In vitro inhibition of *P. falciparum* merozoite reinvasion. The assay was performed in in vitro cultures with IgG fractions or affinity-purified antibodies as previously described (24).

RESULTS

Immunization of rabbits with the fusion protein ZZ-M1. Rabbits were immunized with the fusion protein ZZ-M1 in FCA or without adjuvant, and the humoral immune response was followed over a 1-year period. Immunization with ZZ-M1 in FCA resulted in high, sustaining antibody titers against the EENVEHDA repeats for all four rabbits tested, as measured in the ELISA. The typical results for one of the rabbits are presented in Fig. 1A. Serum from this rabbit reacted with the EENVEHDA repeats already after the first injection (week 3). The first booster injection increased the antibody titer (week 5), which persisted during the following 6 months (week 28). A second booster injection resulted in a further increase of the response (week 31), followed by a decrease to the week 28 level (week 52). The reactive antibodies of all four rabbits were of the IgG isotype (data not shown).

The ELISA reactivity with the EENVEHDA repeats for the rabbits immunized with ZZ-M1 without adjuvant differed in several respects from that of the rabbits immunized with ZZ-M1 in FCA (Fig. 1B). First, no antibodies could be detected until after the first booster injection. Second, the ELISA reactivity was at least 40-fold lower compared with that of the rabbits immunized with FCA. Third, the antibody levels were not sustained for longer periods, but they were restored after each booster injection. Finally, the antibodies were of both the IgG and IgM classes (data not shown).

The ELISA reactivity with protein A for antisera from all rabbits was low (data not shown). However, to avoid Fc-mediated binding of rabbit IgG to protein A, the Fc-binding domains of protein A had to be blocked with human IgG (11), which may have inhibited binding of specific antibodies. Thus, the ELISA reactivity with protein A presumably is an underestimate of the actual protein A-specific antibody response.

The reactivity of the antisera with Pf155/RESA deposited in the membrane of *P. falciparum*-infected erythrocytes was determined by using EMIF (17). Antisera from the rabbits immunized with ZZ-M1 with and without adjuvant reacted

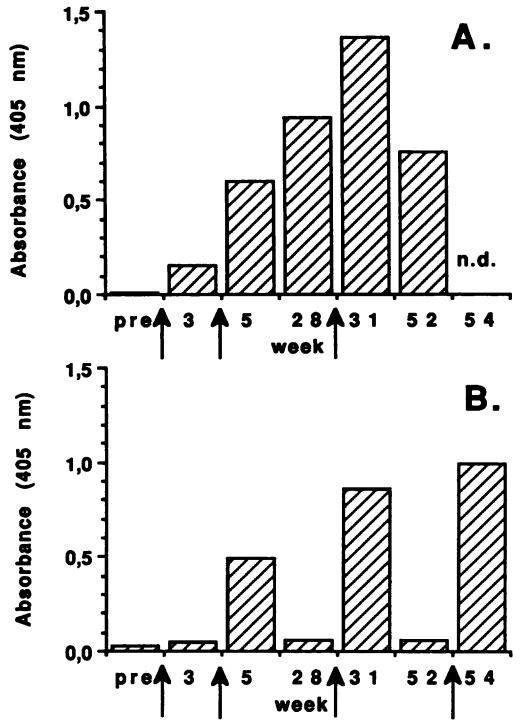

FIG. 1. Reactivity in ELISA with the fusion protein BB-M1 of antisera from rabbits immunized with the fusion protein ZZ-M1 in FCA (R27) (A) or without adjuvant (R25) (B). Sera were diluted 1:2,000 (A) or 1:50 (B). Values were reduced by subtracting the reactivity of the sera with the protein G-derived part of BB-M1 (A_{405}, 0.034 to 0.102). Arrows indicate injections with ZZ-M1.

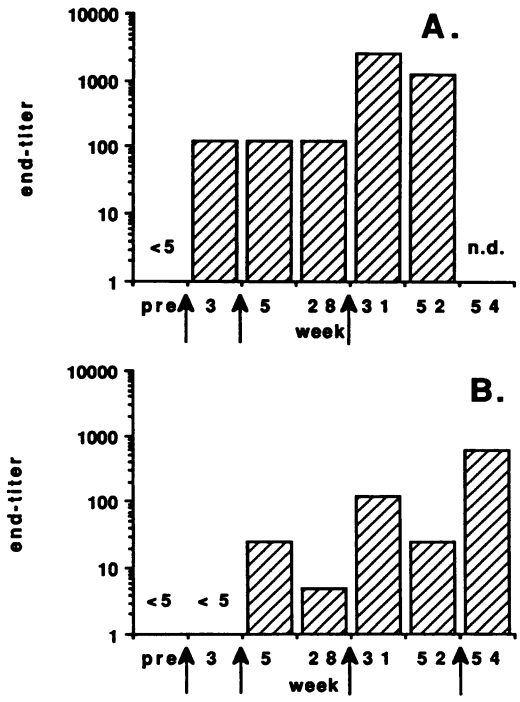

FIG. 2. Indirect immunofluorescence on air-dried monolayers of *P. falciparum*-infected erythrocytes (EMIF) of antisera from rabbits immunized with the fusion protein ZZ-M1 in FCA (R27) (A) or without adjuvant (R25) (B). Arrows indicate injections with ZZ-M1.

strongly in this assay, giving maximum endpoint titers of 1:2,500 (Fig. 2A) and 1:625 (Fig. 2B), respectively. The variation in EMIF reactivity over the 1-year period agreed well with the pattern seen for the ELISA reactivity with BB-M1. The EMIF-reactive antibodies in antisera from the rabbits immunized with FCA were of the IgG isotype, whereas the EMIF reactivity of antisera from the rabbits immunized without adjuvant was both IgG and IgM mediated.

To further elucidate the parasite specificity of the antibody response, the rabbit antisera were tested in immunoblotting for reactivity with *P. falciparum* polypeptides. Figure 3 shows the typical patterns obtained with the sera from two rabbits, one immunized with ZZ-M1 in FCA and one immunized with ZZ-M1 without adjuvant. All antisera from the rabbits immunized with ZZ-M1 in FCA strongly stained Pf155/RESA (Fig. 3A). The antisera from the rabbits immunized without adjuvant stained Pf155/RESA in a pattern compatible with their ELISA and EMIF reactivities (Fig. 3B).

Specificity of the antibody response. The specificity of the antibodies in antisera from the four rabbits immunized with ZZ-M1 in FCA was analyzed by inhibition experiments in the ELISA and EMIF (Table 1). The reactivity with BB-M1 in the ELISA of all antisera was efficiently inhibited by free BB-M1 or $(EENVEHDA)_3$, whereas the monomer of EENVEHDA only inhibited the reactivity of one antiserum (R27; Table 1, Fig. 4A). The peptides $(EENV)_6$ and $K(DDEHVEEPTVA)_2$ were essentially without effect in this assay. The inhibition pattern of the antisera in EMIF paralleled that seen in the ELISA, although EENVEHDA and $(EENV)_6$ were slightly more potent inhibitors in EMIF (Table 1).

The specificity of the antibody response for Pf155/RESA was further demonstrated by the efficient inhibition of the ELISA and EMIF reactivities of the antisera from all four rabbits immunized with ZZ-M1 in FCA by the β-galactosidase fusion protein 3′-Pf155/RESA (7, 12). In contrast, the genetically distinct but cross-reacting (12, 22) fusion proteins Pf11.1 (20) and Pf332 (12) were without effect in these assays (Fig. 4B).

Inhibition of *P. falciparum* merozoite reinvasion in vitro. EENVEHDA-reactive antibodies from human immune sera or from rabbits immunized with short synthetic or recombinant peptides have been shown to inhibit *P. falciparum* reinvasion in vitro (1, 2). The rabbits immunized with ZZ-M1

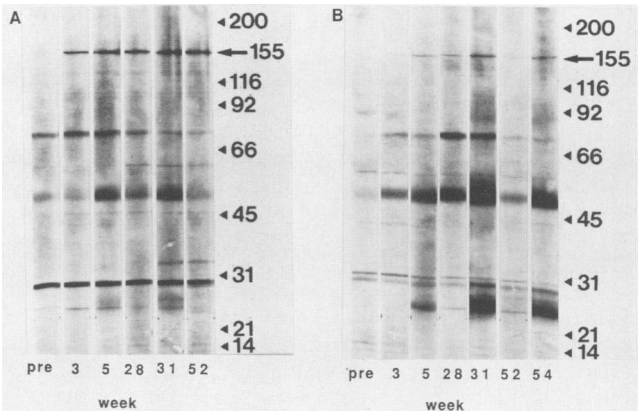

FIG. 3. Reactivity in immunoblotting with *P. falciparum* merozoite polypeptides of antisera from rabbits immunized with the fusion protein ZZ-M1 in FCA (R27) (A) or without adjuvant (R25) (B). Numbers indicate approximate molecular weights in thousands.

TABLE 1. Inhibition of the ELISA[a] and EMIF[b] reactivities of antisera from rabbits immunized with ZZ-M1 in FCA

Inhibitor	Inhibition of antiserum from rabbit:							
	R27		R47		R48		R49	
	ELISA	EMIF	ELISA	EMIF	ELISA	EMIF	ELISA	EMIF
BB-M1	+++	+++	+++	+++	+++	+++	+++	+++
EENVEHDA	+++	+++	±	++	+	++	±	−
$(EENVEHDA)_3$	+++	+++	+++	+++	++	+++	+++	+++
$(EENV)_6$	−	++	±	−	+	++	+	++
$K(DDEHVEEPTVA)_2$	−	−	−	−	−	−	−	−

[a] BB-M1 as coating antigen: −, no inhibition with 200 μM inhibitor; ±, <20% inhibition with 200 μM inhibitor; +, 20 to 50% inhibition with 200 μM inhibitor; ++, 50% inhibition with ≥25 μM inhibitor; +++, 50% inhibition with <25 μM inhibitor.

[b] −, No inhibition with 200 μM inhibitor; ±, inhibition with 200 μM inhibitor; +, inhibition with 50 to 100 μM inhibitor; ++, inhibition with 12.5 to 25 μM inhibitor; +++, inhibition with ≤6.25 μM inhibitor.

also produced antibodies that inhibited reinvasion. The inhibitory capacity of IgG fractions of antisera from one of the rabbits immunized with ZZ-M1 in FCA is shown in Fig. 5. In general, the results obtained with the different antisera were in good agreement with their reactivity patterns in the BB-M1 ELISA and in EMIF.

The inhibitory activity of the IgG can be enriched by affinity purification of the relevant antibodies (1, 2). When the IgG fraction from the week 31 bleeding in Fig. 5 was purified on immobilized BB-M1, the 50% inhibition titer for the affinity-purified antibody fraction was ~0.4 μg/ml. This represents an approximately 5×10^3-fold increase in titer compared with that of the total IgG fraction (~2 mg of IgG per ml). Of the IgG applied to the immunosorbent, ~0.6% was eluted with glycine buffer. Calculations based on the ELISA results indicated that ~40% of the reactivity with BB-M1 was recovered. However, no EMIF activity could be detected in the glycine fraction. The flowthrough fraction and the KSCN-eluted fraction lacked detectable antibody activity.

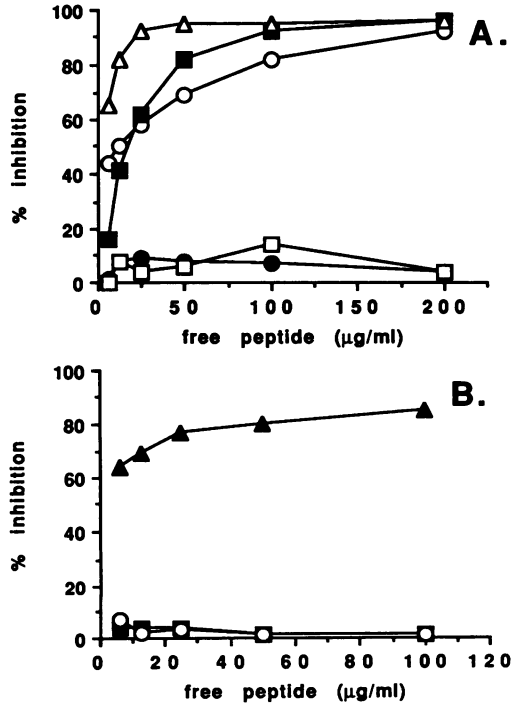

FIG. 4. Inhibition of the ELISA reactivity with BB-M1 of an antiserum from a rabbit immunized with ZZ-M1 in FCA (R27). (A) Inhibitors: ○, BB-M1; ■, EENVEHDA; △, $(EENVEHDA)_3$; ●, $(EENV)_6$; □, $K(DDEHVEEPTVA)_2$. (B) Inhibitors (β-galactosidase fusion proteins): ■, Pf11.1; ○, Pf332; ▲, 3′-Pf155/RESA.

DISCUSSION

We have used a dual-expression system to express a synthetic gene encoding a tetramer of the repeated C-terminal sequence EENVEHDA (M1) of the *P. falciparum* blood-stage antigen Pf155/RESA (21). Immunization of rabbits with ZZ-M1 (containing two IgG-binding domains from staphylococcal protein A) resulted in high antibody responses against the M1 part as measured in an ELISA with BB-M1 (containing the HSA-binding domains from streptococcal protein G). These antibodies also reacted with Pf155/RESA and were efficient inhibitors of *P. falciparum* merozoite reinvasion in vitro. In general, for each rabbit, the reactivities with BB-M1 of the sera taken at different times paralleled their reactivities with Pf155/RESA in all other test systems.

A 155-kilodalton polypeptide was the only parasite-derived antigen detected by the rabbit antisera in immunoblotting analysis of parasite extracts. Furthermore, their ELISA reactivity with BB-M1 and their EMIF reactivity were efficiently and dose dependently inhibited by a β-galactosidase fusion protein representing a major fraction of the 3′ domain of Pf155/RESA, including the complete repeat region (7, 12). In contrast, the β-galactosidase fusion proteins Pf11.1 (20) and Pf332 (12), which are genetically distinct

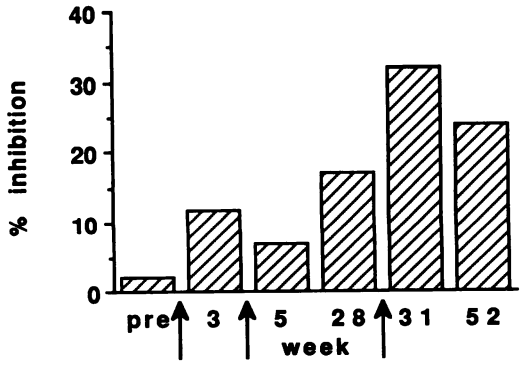

FIG. 5. Inhibition of *P. falciparum* merozoite reinvasion in vitro by IgG fractions of antisera from a rabbit immunized with ZZ-M1 in FCA (R27). The IgG concentration was 2 mg/ml. Arrows indicate injections with ZZ-M1.

antigens but which have been shown to contain epitopes cross-reacting with Pf155/RESA (12, 22), lacked inhibitory capacity in the ELISA.

Inhibition studies in the ELISA further demonstrated that the antibody response primarily was specific for epitopes formed by the octapeptide sequence. Similarly, the EMIF activity of the antisera was completely abolished by inhibition with free BB-M1 and $(EENVEHDA)_3$, demonstrating that the membrane staining of infected erythrocytes was mediated by octapeptide-specific antibodies. A weak inhibition of the ELISA and EMIF activities of some of the sera with $(EENV)_6$ presumably reflects a population of antibodies cross-reacting with both the EENVEHDA and EENV repeats.

Taken together, these results indicate that immunization with ZZ-M1, which contains a tetramer of the EENVEHDA repeat unit, gives rise to antibodies that are highly specific for Pf155/RESA. This is of considerable interest, since rabbits immunized with a synthetic monomer or dimer of EENVEHDA conjugated to a carrier protein (2) and human anti-Pf155/RESA antibodies affinity purified on such short peptides (18) usually display considerable cross-reactivity with other parasite antigens (e.g., antigen Pf11.1 or Pf332) or related peptides [e.g., EENV or $(DDEHVEPTVA)_2$]. The present findings imply that the epitope(s) expressed by Pf155/RESA in the membrane of infected erythrocytes or in immunoblotting and seen by the antibodies induced by the EENVEHDA tetramer is a linear sequence including two or more EENVEHDA units. Alternatively, the epitopes may be conformational, dependent on certain secondary or tertiary structures of the EENVEHDA tetramer. Importantly, since some of the antibodies also were efficient inhibitors of merozoite reinvasion, these epitopes are expressed on the native Pf155/RESA molecule and, therefore, are biologically significant. Antibodies reactive with the EENVEHDA sequence also seem to have a parasite-neutralizing activity in vivo, as demonstrated in the vaccination experiments by Collins et al., in which β-galactosidase fusion proteins of DNA clones encoding Pf155/RESA sequences partially protected *Aotus* monkeys challenged with *P. falciparum*-infected erythrocytes (6). Protection was correlated with the presence of serum reactivity with the Pf155/RESA repeat subunit sequence DDEHVEEPTVA of the 5′-repeat block and to some extent with serum reactivity with both the C-terminal repeat sequences EENVEHDA and EENV but not with serum reactivity with only the EENV sequence (6). In contrast, results from merozoite invasion experiments in vitro indicate that antibodies specific for the EENV sequence are more potent inhibitors of invasion than antibodies specific for the EENVEHDA sequence (3).

The apparently conflicting results regarding the anti-parasitic activities in vivo and in vitro of anti-repeat antibodies of different specificities urge a closer analysis of parasite-neutralizing immune responses induced by immunogens based on Pf155/RESA sequences. The possibility of using the present expression system for production of large amounts of well-defined and purified fusion proteins comprising the different repeat sequences of Pf155/RESA or combinations thereof will greatly facilitate the further analysis of the antibody response to these sequences. Several such constructs are presently being tested.

Affinity purification on immobilized BB-M1 of IgG fractions from the antisera resulted in a complete depletion of the peptide- and parasite-reactive antibodies. Approximately 40% of the ELISA reactivity with BB-M1 could be eluted with glycine buffer. These eluates were also strongly enriched in reinvasion-inhibitory activity but contained no EMIF reactivity. This suggests that the EMIF-reactive antibodies bind with high affinity to the EENVEHDA tetramer and, thus, cannot be eluted in an active form from the affinity column.

Immunization with ZZ-M1 in FCA resulted in high titers of IgG antibodies, which were strongly increased by booster injections and sustained over 1 year or longer. The antibody response without adjuvant was also significant but weaker. It required at least two immunizations, was mediated by both IgG and IgM, and was not stable over time. However, the antibody titers were restored by boosting during a 1-year period, indicating the existence of B- and/or T-cell memory. In any event, the striking enhancement of the antibody response after each booster injection in the FCA-treated rabbits could be due to T-cell activation by the staphylococcal protein A-derived ZZ domains or due to T-cell epitopes in the EENVEHDA repeats. Epitopes in this sequence seen by T cells from malaria-primed humans have recently been described (9, 19). Moreover, in preliminary experiments, the rabbits used in this study could be boosted efficiently with BB-M1, indicating that the EENVEHDA repeats have the potential to maintain and enhance the antibody response in the absence of ZZ. This further supports the notion that gene constructs of the type studied herein, together with a proper adjuvant, may be suitable components in a subunit malaria vaccine. Extensive studies with ZZ fusion proteins in combination with adjuvants that have been proposed for use in humans are presently under way to find an alternative adjuvant to FCA.

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