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Antibodies to a Conserved-Motif Peptide Sequence of the *Plasmodium falciparum* Thrombospondin-Related Anonymous Protein and Circumsporozoite Protein Recognize a 78-Kilodalton Protein in the Asexual Blood Stages of the Parasite and Inhibit Merozoite Invasion In Vitro

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Athrombospondin-related anonymous protein (TRAP) of the human malaria parasite *Plasmodium falciparum* shares highly conserved amino acid sequence motifs with the circumsporozoite protein of all plasmodia sequenced so far, as well as with unrelated proteins like thrombospondin and properdin. Although it was first described as an asexual blood stages protein, there has been some controversy about its expression in these stages. Pursuant to our interest in the conserved sequences within the malaria antigens, we synthesized an 18-residue peptide (18-mer) representing a conserved motif of TRAP and raised polyclonal antibodies against it. In an immunoblot assay in which we probed proteins from the asexual blood stages of the parasite, we found that this antibody recognized predominantly a 78-kDa protein in the whole parasite lysate. Furthermore, in another immunoblot, the recombinant TRAP constructs containing the conserved-motif sequence were distinctly recognized by the antipeptide antibodies, whereas a construct lacking the motif sequence was not, suggesting that the antibodies specifically cross-reacted with a protein which might be a TRAP-like protein present in the asexual blood stages of the parasite. Also, in an immunofluorescence assay, this antibody brightly stained the acetone-fixed trophozoites of the parasite. Most significantly, anti-18-mer immunoglobulin G, as well as antipeptide antibody against a smaller (nonamer) construct representing the most conserved motif within the 18-mer, inhibited the merozoite invasion of erythrocytes in a dose-dependent manner. These results provide evidence of the expression of TRAP or a TRAP-like protein in the asexual blood stages of the parasite and of a possible role of the conserved motifs in the parasite-host cell interaction during the process of invasion.

The human malaria parasite *Plasmodium falciparum* follows a complex life cycle which involves distinctly different developmental stages. A number of different antigens are expressed at particular stages; some of these are believed to play a role in specific invasion processes involving the parasite and the host cells. Thus, there has been considerable interest in proteins such as the circumsporozoite (CS) protein and the major merozoite surface proteins, MSP-1 and MSP-2, in connection with the search for suitable vaccine candidates (13, 16, 26, 32). However, extensive polymorphism in these and most other malarial antigens is a major concern in the development of a malaria vaccine based on whole antigens (14, 17, 24).

A *P. falciparum* protein described as thrombospondin-related anonymous or adhesive protein (TRAP) shares amino acid sequence motifs with region II of the CS protein of all *Plasmodium* species sequenced so far (15, 27, 29). There is a cluster of cysteine residues in and around the conserved sequence suggestive of a secondary structure. A *Plasmodium yoelii* analog of TRAP, the sporozoite surface protein-2 (SSP-2), has also been shown to contain the same conserved motifs (30, 31). Interestingly, these motifs are present in a variety of other different proteins of biological significance, such as thrombospondin (hence, the name TRAP), properdin, and terminal components of the complement pathway (15, 27, 29). An antigenic protein from *Eimeria tenella*, a parasite phylogenetically related to *Plasmodium* spp., also contains these conserved motifs (9). The nonapeptide WSPCSVTCG, which represents the most homologous sequence of the conserved motifs, is present in three copies in thrombospondin, six copies in properdin, and one copy each in the CS proteins of six species of malaria parasite (27).

The occurrence in the sporozoite and, possibly, in the asexual blood stages of *P. falciparum* of a highly conserved sequence which is also present in thrombospondin and properdin led to the speculation that TRAP may be a functionally relevant protein and that the conserved motifs may be involved in the parasite's interaction with the host cell membrane. In fact, two laboratories have provided data to indicate that synthetic CS protein constructs or recombinant TRAP constructs containing a conserved nine-residue motif indeed bind to hepatocytes and can competitively inhibit sporozoite invasion of HepG2 cells (4, 25). Recently, we have demonstrated that immunization of mice with a synthetic CS peptide, a 32-residue construct containing this conserved motif, could protect them against live challenge with a lethal inoculum of *Plasmodium berghei* sporozoites (6).

If a TRAP-like molecule is indeed involved in the recogni-

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tion of a receptor molecule on the erythrocyte surface, then it may well be an attractive target for interference with the asexual erythrocytic cycle of the parasite. It was thus appealing to probe whether the most conserved motif is in some way involved in the interaction of the parasite with the host erythrocyte. We have synthesized peptides corresponding to the conserved motifs (18-mer) and the most homologous nonapeptide sequence within these motifs and present evidence that antibodies to these peptides recognize predominantly a 78-kDa protein in the asexual erythrocytic stages (trophozoites) of *P. falciparum* and also inhibit merozoite invasion of erythrocytes in a dose-dependent manner.

MATERIALS AND METHODS

Parasite. The FID-3 strain of *P. falciparum*, isolated from a patient in New Delhi in the year 1987 and subsequently maintained in continuous in vitro culture by the candle jar method of Trager and Jensen (36), was used in this study. For making the whole parasite protein lysate, a preparation rich in trophozoite- and schizont-infected erythrocytes was first obtained by centrifugation of cultures showing high-level parasitemia (~7%) and a preponderance of trophozoites and schizonts on a Histopaque-1077(R) (Sigma Chemical Co., St. Louis, Mo.) density gradient. This parasite-rich preparation was washed with plain RPMI-1640 medium, i.e., medium without serum but containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 0.2% sodium bicarbonate, and subjected to hemolysis with 0.2% saponin in 0.85% NaCl solution at 4°C. The liberated parasites were extensively washed with chilled phosphate-buffered saline (PBS) (0.15 M, pH 7.2) to get rid of hemoglobin, and the erythrocyte membranes were separated from the lysate by centrifugation. Extraction of the sedimented parasites with 5 volumes of the chilled extraction buffer (50 mM Tris-HCl, 5 mM EDTA [pH 8.0], 0.5% Triton X-100, 10 μM chymostatin, 1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin, and 10 μM leupeptin) was followed by ultrasonication in an ice bath. Finally, the preparation was centrifuged at 14,000 rpm at 4°C, and the supernatant was used as the whole parasite lysate in the enzyme-linked immunosorbent assay (ELISA) or the immunoblotting assay.

Synthetic peptides. An 18-residue peptide representing the conserved motifs (^{249}EWSPCSVTCGKGTRSRKR^{266}) of TRAP was synthesized by stepwise solid-phase peptide synthesis with phenylacetamidomethyl resin on a manual synthesizer. The peptide was simultaneously deprotected and cleaved with trifluoromethanesulfonic acid in the presence of thioanisole and 1,2-ethanedithiol. The crude peptide was extracted in 20% acetic acid, lyophilized, and purified by high-pressure liquid chromatography (HPLC) on an aquapore C-8 column (100 by 10 mm; Applied Biosystems). Purified peptide was characterized by amino acid analysis and analytical reverse-phase HPLC on a C-18 column (Waters). This construct was readily soluble in water as well as in PBS, pH 7.2, and could be used in the parasite growth inhibition assays described below. An analog of the 18-mer, with the −SH side chain of cysteine residues kept protected with a tertiary butyl group, was synthesized by Fmoc (fluorenylmethoxyucarbonyl) chemistry.

A nonapeptide, representing the most conserved sequence (^{250}WSPCSVTCG^{258}) within the 18-mer, was also synthesized and purified in the same manner as described above. The nonapeptide, however, was only sparingly soluble in water and required 20% acetic acid for its dissolution, rendering it unsuitable for use in the parasite growth inhibition assays.

Another *P. falciparum* peptide, a 21-residue construct representing a conserved region of MSP-1 and described as P-8 in our previous work (21, 33), was also included in this study for comparison.

Recombinant TRAP constructs. Small amounts of a series of nested recombinant constructs representing portions of TRAP containing the conserved-motif sequence or its adjacent sequences were a kind gift from Andrea Crisanti of the University of Rome, Rome, Italy. A detailed description of the procedures employed to obtain and characterize these constructs has been published by Muller and colleagues (25). Specifically, these constructs spanned almost the entire length of the protein (TRAP 1), from residue 26 to 393 (TRAP 1.1), 26 to 376 (TRAP 1.2), 26 to 299 (TRAP 1.3), 26 to 273 (TRAP 1.4), and 26 to 204 (TRAP 1.5).

Immunization with peptides. Rabbits (New Zealand White) used in this study were procured from the Small Animal Facility of the National Institute of Immunology, New Delhi, India. The animals were housed, fed, and handled in experimental manipulations in accordance with the recommendations made by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals).

We found that immunization of rabbits with carrier-free 18-mer or nonapeptide did not induce any detectable specific antibody response, as monitored by ELISA (unpublished results). These peptides were, therefore, conjugated to a carrier protein for the purpose of immunizing animals. The 18-mer was coupled to tetanus toxoid (TT) by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the conjugating reagent (21), while conjugation of the nonapeptide with bovine serum albumin (BSA) was accomplished by using glutaraldehyde as the coupling reagent (19).

Rabbits were immunized with the 18-mer–TT conjugate (250 μg per animal) emulsified in complete Freund's adjuvant (CFA) and inoculated subcutaneously at multiple sites; they were boosted on day 28 with a similar amount of the same immunogen mixed with incomplete Freund's adjuvant (IFA). Samples of rabbit serum were obtained on days 0, 14, 28, 42, and 56 and assayed for the presence of peptide-specific antibody in an ELISA with carrier-free 18-mer as the capture antigen. The preimmune and test sera were adsorbed with fresh, normal human erythrocytes as well as with *Escherichia coli* bacterial lysate before they were used to test for cross-reactivity with the parasite lysate and various recombinant TRAP constructs in the ELISA and immunoblotting assays.

Another group of two rabbits was immunized and boosted with the nonapeptide-BSA conjugate in a similar fashion following an identical schedule. Sera collected on days 0, 14, 28, 42, and 56 were tested for the peptide-specific antibody in an ELISA with nonapeptide-TT conjugate as the capture antigen.

Serum samples from a rabbit making a high-level antibody response to the P-8 *P. falciparum* MSP-1 peptide and from a rabbit immunized with the whole parasite lysate were also included in certain experiments as described below. All sera were adsorbed with normal human erythrocytes and *E. coli* bacterial lysate, as mentioned above, to remove heterophile antibodies.

In some experiments, purified immunoglobulin G (IgG) fractions obtained from rabbit preimmune and rabbit antipeptide sera were used to corroborate findings first obtained with the rabbit sera. The purification of IgG fractions from rabbit sera was achieved by ammonium sulfate precipitation of serum to obtain the Ig fraction followed by ion-exchange chromatography on an Econo-Pac Ig purification column (Bio-Rad Laboratories, Richmond, Calif.) as per instructions supplied by the manufacturers. Briefly, the prepacked column was extensively washed with regeneration buffer (1.5 M sodium thiocyanate) and equilibrated with application buffer (0.02 M Tris-HCl, pH 8.0, containing 0.028 M NaCl). The Ig fraction of serum preequilibrated with the application buffer was applied to the column and IgG was eluted with the application buffer. The absorbance at 280 nm of each fraction was determined, and fractions with an optical density (OD) of ≥0.1 were pooled and dialyzed against 200 volumes of PBS (0.15 M, pH 7.2). The purity of this preparation was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

In still another experiment, immune IgG was isolated from the test sera by immuno-affinity chromatography. An immunoadsorbent column was prepared by coupling 18-mer (dissolved in 0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl) to the cyanogen bromide-activated Sepharose 4B matrix (Pharmacia Fine Chemicals, Uppsala, Sweden), and its uncovered reactive sites were blocked with ethanolamine. The purified IgG fraction obtained after ion-exchange chromatography of rabbit anti-18-mer serum was applied to the immunoadsorbent column, extensively pre-washed, and equilibrated with PBS containing Tween-20 (0.2%, vol/vol). The peptide-specific antibody was eluted with glycine-HCl buffer (0.1 M, pH 2.5), and the fractions were neutralized by addition of Tris base (2.0 M). The fractions were pooled and characterized as described above for the ion-exchange chromatography.

ELISA. The wells of flat-bottomed Immulon-2 plates (Dynatech) were coated with an appropriate concentration of the capture antigen (carrier-free 18-mer, nonapeptide-TT conjugate, the whole parasite lysate, or each of the six recombinant TRAP constructs) in 0.06 M carbonate-bicarbonate buffer, pH 9.6. The uncovered reactive sites in the wells were blocked with a 5% solution of milk powder in PBS. The antigen-coated wells were sequentially incubated with serial dilutions of test sera and an optimally diluted enzyme-labeled second antibody (horseradish peroxidase-labeled anti-rabbit IgG). In between these incubations, plates were washed with a 0.05% solution of Tween-20 in PBS. The enzyme reaction was developed biochemically with H_2O_2 as the enzyme substrate and *ortho*-phenylenediamine dihydrochloride as the chromogen; the reaction was stopped with 8 N H_2SO_4, and the OD_{490} of the reaction product in the wells was recorded with a Bio-Tek microplate reader.

Immunoblotting. Having ascertained the reactivity of the antipeptide antibody in ELISA, we used this antibody to probe the whole parasite lysate, which had been fractionated by SDS-PAGE and transferred onto nitrocellulose paper by standard procedures. The recombinant fragments of TRAP, viz., TRAP 1.1, 1.2, 1.3, 1.4 (all containing the conserved sequence motif), and 1.5 (lacking the conserved motif) (25), were also studied for their reactivity with the antipeptide sera by immunoblotting. The parasite proteins and the recombinant TRAP constructs, separated by SDS-PAGE and transferred onto a nitrocellulose paper, were incubated first with the rabbit antiparasite serum or antipeptide sera or with the affinity-purified immune IgG and then with the horseradish peroxidase-labeled anti-rabbit IgG antibodies. The final enzyme reaction was developed with 4-chloro-1-naphthol as the chromogen and H_2O_2 as the substrate.

Immunofluorescence assay. The rabbit antipeptide sera were also tested, in an immunofluorescence assay, for their reactivity with the authentic parasite protein(s). The assay was performed essentially as described earlier (34). Briefly, multispot antigen slides were made from a *P. falciparum* (FID-3 isolate)-infected erythrocyte suspension prepared from an asynchronous culture of the parasite. The antigen spots on the slides were air dried and fixed with an acetone-methanol (90:10, vol/vol) mixture at −20°C for 40 min. The antigen spots were sequentially incubated with serially diluted test sera and optimally diluted fluo-

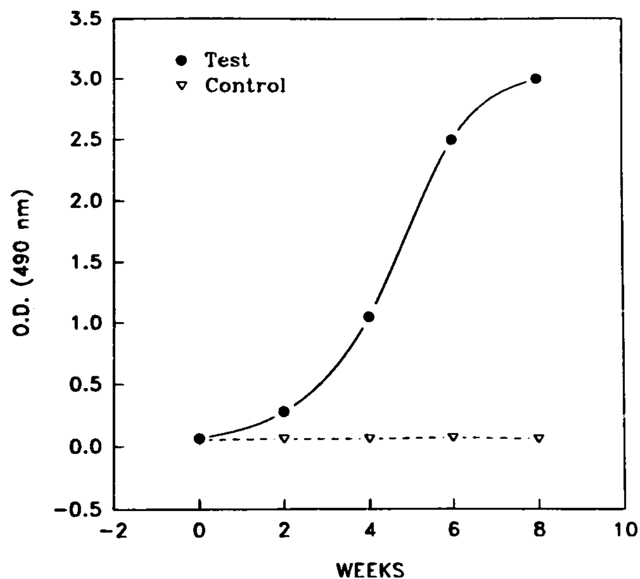

FIG. 1. Time course of anti-18-mer antibody response as monitored by ELISA, with 18-mer as the capture antigen. The rabbit was either immunized with 18-mer–TT conjugate emulsified with CFA and IFA (Test) or injected with the adjuvants alone (Control). Each serum sample was diluted 1/400 and each datum point is an average of OD values obtained in duplicate wells.

rescein isothiocyanate-labeled secondary antibody with the excess reactants washed off after each incubation. The slides were examined under a fluorescence microscope (Wild Leitz GmbH, Wetzlar, Germany) by alternately using visible and UV light to see the specific binding of antibody to the parasite.

Merozoite invasion inhibition and parasite growth inhibition assays. For the merozoite invasion inhibition assay, cultures of the FID-3 isolate of *P. falciparum* were synchronized by two treatments with 5% sorbitol (22) and incubated for about 30 h so that at the time the assay was set up, more than 95% of the parasites were late trophozoites. The assay cultures were then set up with normal or immune rabbit serum incorporated in the culture medium and incubated in a candle jar at 37°C for 20 h (the 20-h assay). At the end of the period, a smear from each culture well was drawn and stained with Giemsa for recording parasitemia by microscopy; only the ring-infected erythrocytes were counted as parasitized cells for calculating percent parasitemia. In a subsequent experiment, various concentrations of purified IgG fractions isolated from the preimmune or immune rabbit sera were incorporated in the test system.

For the parasite growth inhibition assay, cultures were grown in RPMI-1640 medium supplemented with 10% human blood group AB/Rh^+ serum plus 0.5 to 5.0% normal (preimmune) or immune rabbit serum or various concentrations of different synthetic peptide constructs. The cultures were incubated for 72 h (the 72-h assay) in a candle jar at 37°C.

Percent inhibition of growth or merozoite invasion was calculated as 100 − [(% parasitemia in test/% parasitemia in control) × 100]. The results of the parasite growth inhibition assays were analyzed by using Student's *t* test.

RESULTS

Immunogenicity of the synthetic peptides. Immunization of rabbits with the carrier-free 18-mer (100 μg per animal) or nonapeptide in CFA and two booster injections with the respective peptide in IFA did not induce any detectable level of the peptide-specific antibody response (data not presented). One of the two rabbits immunized with the 18-mer–TT conjugate mounted a boostable, IgG antibody response directed against the peptide, as measured in an ELISA. Figure 1 represents the kinetics of the peptide-specific antibody response elicited by the 18-mer–TT conjugate. The preimmune serum (1/400 dilution) yielded an ELISA OD_{490} of 0.06, while the test (day 42) serum from the same rabbit gave an OD_{490} of 2.5. This antiserum or its purified immune IgG fraction was used in subsequent assays, viz., immunoblotting of the parasite proteins or recombinant TRAP constructs, immunofluorescence, and parasite growth inhibition assays, etc. Rabbits immunized with the nonapeptide-BSA conjugate also made a boostable, IgG-type antibody response directed against the nonapeptide, as measured in an ELISA with the nonapeptide-TT conjugate as the capture antigen. The preimmune sera (1/100 dilution) of two rabbits, immunized with nonapeptide-BSA, yielded OD_{490} values of 0.08 and 0.09, respectively, while the test (day 42) sera from the same rabbits gave corresponding values of 0.78 and 1.123, respectively. The end point titers of the test sera were 1/10,000 or higher, at which dilution the ELISA OD value

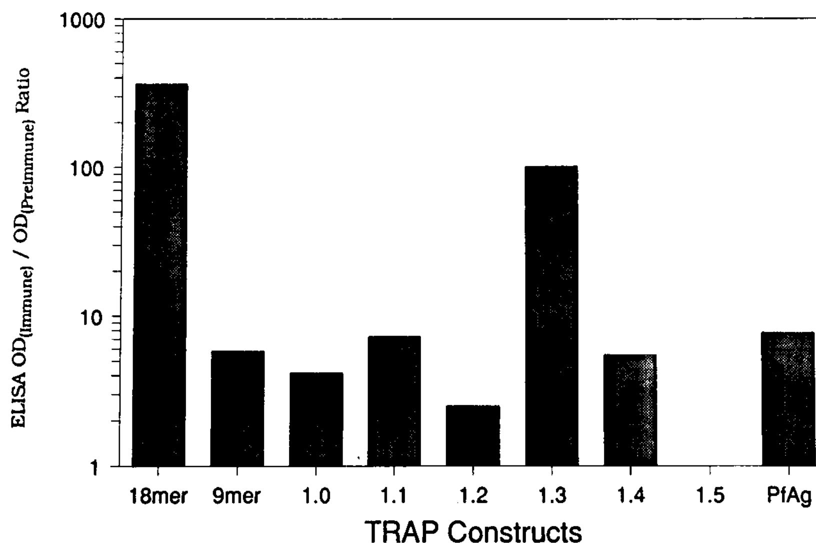

FIG. 2. Specificity of rabbit anti-18-mer antibody. The preimmune and test sera were diluted 1/1,000 and tested in an antibody-binding ELISA with various TRAP constructs as capture antigens. 9-mer and 18-mer, synthetic peptides; 1.0 to 1.5, recombinant TRAP constructs; PfAg, whole parasite lysate antigen.

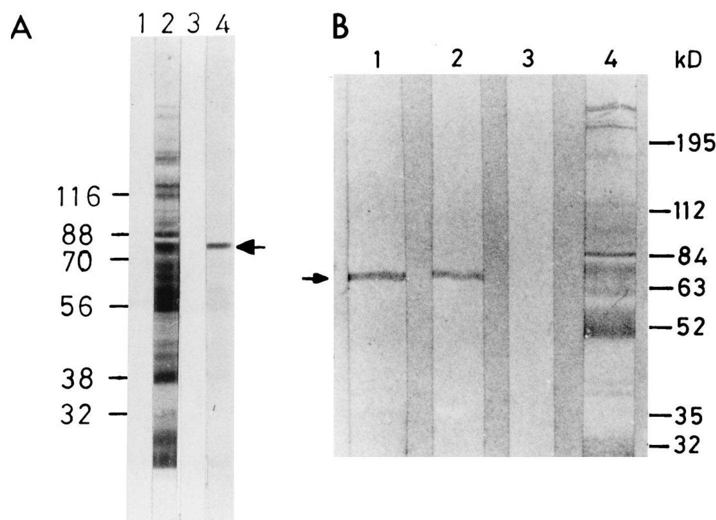

FIG. 3. (A) Immunoblot of *P. falciparum* lysate with the rabbit antipeptide serum. The parasite antigens were separated on an 8% acrylamide gel by SDS-PAGE and electroblotted onto nitrocellulose paper before being probed with preimmune sera (lanes 1 and 3), antiparasite serum (lane 2) and antipeptide (18-mer) serum (lane 4). Prestained molecular mass markers (SDS-7B; Sigma) to indicate electrophoretic migration are on the left. (B) Immunoblot of *P. falciparum* lysate probed with the affinity-purified, 18-mer-specific, rabbit IgG. The parasite proteins were separated as described above and probed with antipeptide (18-mer) serum (lane 1), affinity-purified IgG fraction from the same serum (lane 2), the same serum but depleted of antipeptide antibody (lane 3), and a rabbit antiparasite serum (lane 4). Prestained molecular mass markers (SDS-7B; Sigma) are on the right. Arrows indicate the 78-kDa protein.

was more than twice the OD obtained with the preimmune sera (diluted 1/100). Immune IgG purified from the test serum of one of these animals was used in the merozoite invasion inhibition assay as described below.

Cross-reactivity of the antipeptide serum with the recombinant TRAP constructs and the parasite protein(s). The results of an ELISA to characterize the specificity of the anti-18-mer antibody are presented in Fig. 2. We found that of the six recombinant constructs, namely, TRAP 1, TRAP 1.1, TRAP 1.2, TRAP 1.3, TRAP 1.4, and TRAP 1.5, as well as the whole parasite lysate used as an antigen, TRAP 1.3, which contains the entire 18-mer sequence and a stretch of 26 additional residues at the carboxyl terminus, bound the maximum amount of rabbit anti-18-mer antibody, as an apparent from the ratio of ELISA OD values obtained with the test serum and the corresponding preimmune serum (both diluted 1/1,000). In contrast, no detectable levels of the anti-18-mer antibody bound to TRAP 1.5, which completely lacks the conserved motif but has almost the entire amino-terminal sequence of the protein intact. Clearly, the rabbit anti-18-mer antibody is specifically directed against the conserved motif sequence of TRAP. Our results presented above indicated that the synthetic peptides could generate a specific antibody response, but in order to determine whether the response would be of any value against the pathogen, it would be important to ascertain if the anti-18-mer serum recognized the parasite protein, a positive result indicating that the synthetic construct faithfully represented the authentic parasite protein structure. We found that this antibody, indeed, cross-reacted with the parasite protein in different assays. In an immunoblot experiment, we found that this antibody reacted very strongly with a parasite protein with an apparent molecular mass of approximately 78 kDa (Fig. 3A). Similar reactivity was obtained with the affinity-purified antibody as well; more significantly, rabbit anti-18-mer serum depleted of antipeptide antibody by incubation with 18-mer immobilized on a Sepharose 4B matrix failed to reveal any

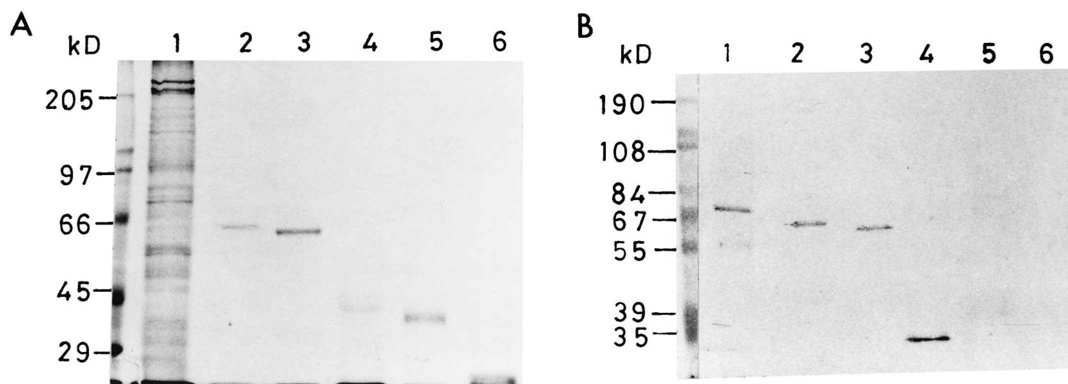

FIG. 4. Immunoblot showing cross-reactivity of the antipeptide serum with the recombinant TRAP constructs. The whole parasite lysate proteins (lane 1) and the recombinant TRAP constructs 1.1 to 1.5 (lanes 2 to 5, respectively) were separated on an 8% polyacrylamide gel by SDS-PAGE and either stained with Coomassie blue (A) or blotted with the antipeptide (18-mer) serum (B). Molecular mass markers (SDS-6H in panel A and SDS-7B in panel B; Sigma) are on the left side of each panel.

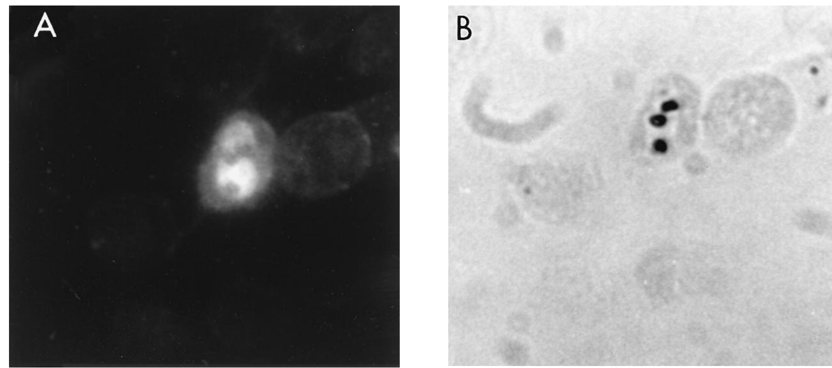

FIG. 5. Immunofluorescent staining of the trophozoites of *P. falciparum* with the antipeptide serum. (A) Parasites showing bright fluorescent staining under UV light. (B) The same field under visible light showing the parasite-infected erythrocyte with a late trophozoite and dark hemozoin pigment bodies; some uninfected erythrocytes can also be seen.

parasite protein in the immunoblots (Fig. 3B). Furthermore, the recombinant fragments of TRAP (TRAP 1.1, 1.2, and 1.3) containing the conserved motif were distinctly recognized by this antibody (Fig. 4, lanes 2, 3, and 4, respectively); in contrast, the same anti-peptide serum showed no reactivity with the recombinant fragment of TRAP (TRAP 1.5) which lacks the conserved motif (Fig. 4, lanes 6). Surprisingly, TRAP 1.4, which contains the conserved nonapeptide sequence at its carboxyl terminus, did not show any positive result in this immunoblot (Fig. 4, lanes 5). The preimmune serum from the same rabbit consistently gave a negative result in immunoblots of the whole parasite lysate and the recombinant constructs (results not presented). Both preimmune and test sera had been preadsorbed with fresh human erythrocytes and with *E. coli* bacterial lysate to obviate the possibility of nonspecific interactions in all of the assays described above.

Immunological reactivity of the antipeptide serum with the parasite protein was further corroborated by the results we obtained with the immunofluorescence assay (Fig. 5). We observed a generalized fluorescent staining of the late trophozoites of *P. falciparum* in this assay. The preimmune serum showed no reactivity with the parasite or with the parasitized erythrocyte.

Merozoite invasion inhibitory and parasite growth inhibitory activities of peptide-specific antibody. Our initial experiments to grow *P. falciparum* in the medium containing 10% normal rabbit serum, instead of human serum, were not successful (unpublished data). However, addition of up to 5.0% normal rabbit serum to the cultures growing normally in the medium with 10% human serum did not affect the rate of growth of parasites. Thus, when *P. falciparum* was cultured in complete medium supplemented with normal rabbit sera in concentrations ranging from 0.5 to 5.0%, the levels of parasitemia obtained after 72 h at various concentrations were comparable; from an initial concentration of 0.5% normal rabbit serum at 0 h, the parasitemia recorded at 72 h ranged from 6.97 to 7.20%, with an average of 7.1% ± 0.09% (mean ± standard deviation). However, incorporation of as little as 0.5% rabbit antinonapeptide (the most conserved sequence in TRAP) serum caused a 26% inhibition ($P < 0.01$) of the parasite growth in a span of 72 h. With the concentration of immune serum raised to 5.0%, we found that the inhibition of growth increased to almost 50 percent (Table 1). Comparable results were obtained with the rabbit anti-18-mer serum as well (Table 1). In contrast, antiserum from a rabbit immunized with the P-8 MSP-1 peptide exerted only a marginally adverse effect on the growth of parasites in this assay; at the end of a 72-h growth inhibition assay, levels of parasitemia obtained in the presence of 5.0% preimmune and immune sera from this rabbit were 7.1 and 6.12%, respectively. This amounted to merely a 14% inhibition of the parasite growth, which was statistically insignificant (Table 1).

In order to establish further that the inhibitory effect of the serum was due to antibodies, we tested the purified IgG fraction isolated from the anti-nonapeptide serum for its effect on the merozoite invasion of erythrocytes in the 20-h assay. Results of this assay, presented in Fig. 6, clearly indicated that the immune rabbit IgG could inhibit the merozoite invasion in a dose-dependent manner, while the normal (preimmune) rabbit IgG had no significant effect. Thus, a nearly 50% inhibition of invasion was obtained with a 300-μg/ml concentration of purified immune IgG while almost complete inhibition was achieved with a 1.2-mg/ml concentration of immune IgG.

The 18-mer, which readily dissolved in the culture medium, was also tested for its potential to inhibit the merozoite invasion of erythrocytes in vitro in the 20-h assay. This experiment was repeated at least five times with reproducible results. Results from one representative experiment are presented in Table 2. At the end of the 20-h assay, the parasitemia (only

TABLE 1. In vitro growth of *P. falciparum* in the presence of preimmune and immune sera from rabbits immunized with nonapeptide, 18-mer, or P-8[a]

Peptide	Serum concn (%)	% Parasitemia[b]		% Inhibition
		Preimmune serum	Immune serum[c]	
9-mer	0.5	6.97 ± 0.21	5.15 ± 0.15*	26.20
	1.0	7.10 ± 0.04	4.60 ± 0.10*	35.22
	2.5	7.20 ± 0.20	4.20 ± 0.30*	41.70
	5.0	7.20 ± 0.60	3.75 ± 0.02*	48.00
18-mer	0.5	ND[d]	4.65 ± 0.03*	23.18
	1.0	ND	3.65 ± 0.07*	39.70
	2.5	ND	3.52 ± 0.02*	41.85
	5.0	6.05 ± 0.02	2.87 ± 0.04*	52.59
P-8	5.0	7.10 ± 0.35	6.12 ± 0.58	13.80

[a] Parasitemia at 72 h is presented; initial parasitemia was 0.51%.
[b] Data are means ± standard deviations of the results obtained in triplicate wells for each category of serum concentration.
[c] The effect of P-8 was statistically insignificant; all other results were significant at $P < 0.01$ by Student's *t* test.
[d] ND, not determined.

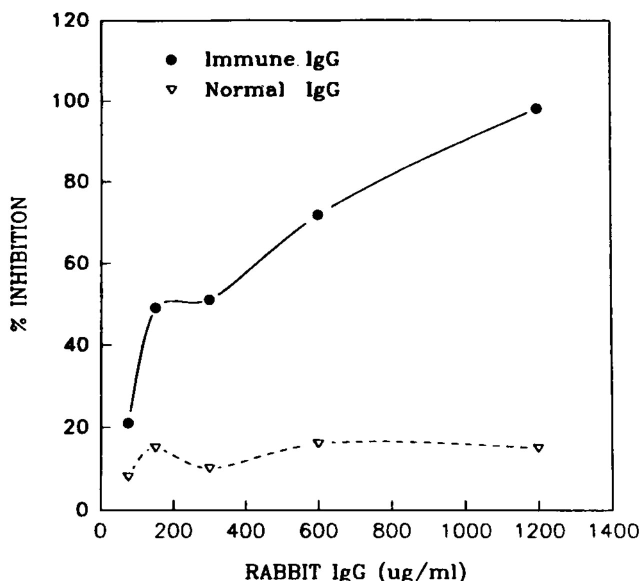

FIG. 6. Inhibition of the merozoite invasion of human erythrocytes by the rabbit antinonapeptide IgG. Data are percents inhibition of invasion observed in the 20-h assay calculated from parasitemias obtained in the presence of preimmune (Normal IgG) and the corresponding immune (Immune IgG) sera purified by ion-exchange chromatography. Each datum point is an average of triplicate values. % parasitemia at 0 h was 0.37; % parasitemia in control serum at 20 h was 1.53.

ring-infected cells) in the normal control wells was 3.6% ± 0.09% and was within the normal limits of growth. However, the 18-mer, when incorporated at concentrations ranging from 25 to 200 μM in the culture medium, caused an approximately 25 to 50% inhibition of the merozoite invasion, respectively, compared with the controls (Table 2). The levels of inhibition were considerably lower when we used the 18-mer analog in which the conserved cysteine residues were protected and, therefore, not available as free sulfhydryls as in the 18-mer (Table 2). Another peptide, P-8, exerted an inhibitory effect to a maximum of only 15.84% even when used at a concentration of 200 μM (Table 2).

DISCUSSION

The most important finding of this study is the demonstration of a TRAP-like protein in the asexual blood stages of the parasite, which was fractionated by SDS-PAGE and immunoblotted with the rabbit anti-18-mer serum. The apparent molecular mass of the parasite protein recognized in these immunoblots was estimated to be 78 kDa (Fig. 3), which is higher than that estimated from the deduced amino acid sequence of the *P. falciparum* TRAP (~63.3 kDa) (29). In fact, it is known for several malarial proteins that because of the preponderance of hydrophobic residues in these proteins, their apparent molecular weights do not conform to the predicted molecular weights (1). Further corroboration of our finding that the parasite protein seen in our immunoblot indeed represents a TRAP-like protein comes from our observation that the antipeptide serum bound specifically to the recombinant TRAP constructs (TRAP 1.1, TRAP 1.2, and TRAP 1.3) containing the conserved motif, WSPCSVTCG, and not to the construct that did not contain this motif (TRAP 1.5), clearly establishing the immunological specificity of the test serum (Fig. 4). In the immunofluorescence assay also, the antipeptide antibodies reacted strongly with the trophozoites of *P. falciparum* (Fig. 5), while the preimmune serum from the same experimental animal consistently yielded a negative reaction. Robson and colleagues (29) have demonstrated TRAP-specific mRNA in the parasitized erythrocytes, and they later demonstrated the presence of this protein in both sporozoites and trophozoites by immunofluorescence assay (12). Our findings presented in Fig. 3 to 5 provide evidence for the expression of a TRAP-like protein during the erythrocytic cycle of the parasite.

The results that we obtained in ELISAs regarding the reactivity of the anti-peptide (18-mer) serum with various recombinant TRAP constructs (Fig. 2) deserve further discussion, besides providing additional evidence for the specificity of the antibody. While, predictably, we observed no detectable reactivity with the construct TRAP 1.5 (residues 26 to 204), representing almost the entire region of the protein from the amino terminus to the conserved motif, we obtained the highest level of reactivity with TRAP 1.3 (residues 26 to 299), which includes the 18-residue conserved motif (residues 249 to 266) and, more importantly, a stretch of another 33 residues containing two cysteines, towards the carboxyl terminus. Interestingly, the levels of ELISA reactivity with the whole parasite lysate, as well as with other constructs containing the nonapeptide conserved motif (residues 250 to 258), were lower than that obtained with TRAP 1.3 by more than an order of magnitude (Fig. 2). Thus, of all the recombinant constructs used in this study, TRAP 1.3 (residues 26 to 299) seems to present the target epitope of this antibody in the most appropriate conformation. This observation was further supported by the results of the immunoblot experiment presented in Fig. 4. It is interesting that the construct TRAP 1.4 (residues 26 to 273), which has the most conserved nonapeptide sequence at close proximity to its carboxyl terminus ending with a cysteine, showed almost no reaction with the antipeptide antibody in the immunoblot, underlining the crucial role of amino acid residues 274 to 299 in providing appropriate conformation to the target epitope. It seems pertinent to recall that Ballou and colleagues (2) had also observed that antibodies raised against the region RII peptide, with the amino acid sequence TEWSPCS VTCGNGIQ (the conserved TRAP motif is indicated in bold letters), of the CS protein of *P. falciparum* did not recognize the intact CS protein in the immunofluorescence and immunoblot assays. In fact, in our studies, TRAP 1.3 has proved to be more immunoreactive than even the putative TRAP-like parasite protein. This might be something similar to what

TABLE 2. Inhibitory effect of 18-mer on *P. falciparum* merozoite invasion of human erythrocytes[a]

Peptide	Concn (μM)	% Parasitemia[b] (rings only)	% Inhibition
None	Nil	3.60 ± 0.09	0.00
18-mer	25	2.65 ± 0.05*	26.39
	50	2.40 ± 0.00*	33.34
	100	2.18 ± 0.01*	39.56
	200	1.77 ± 0.03*	50.84
18-mer (Cys protected)	25	3.36 ± 0.10	6.70
	50	3.22 ± 0.06	10.56
	100	2.95 ± 0.06	17.87
	200	2.91 ± 0.03	19.07
P-8	200	3.03 ± 0.39	15.84

[a] Parasitemia at 20 h is presented; initial parasitemia was 0.85%.
[b] See Table 1, footnote *b*. *, $P < 0.01$ by Student's *t* test.

Wrightsman and colleagues (37) have reported for the trypomastigote surface antigen-1 protein of *Trypanosoma cruzi*. The cleavage of trypomastigote surface antigen-1 protein in the form of recombinant amino-proximal and carboxyl-proximal constructs was found to unmask a protective epitope which otherwise remained cryptic and immunologically inaccessible in the intact protein. The observations of Ballou and colleagues (2) and the results of our present study provide a reasonable basis for further investigation of the importance of amino acid residues 274 to 299 in determining the antigenicity of TRAP and its possible role in the parasite-host cell interaction.

The second important finding of this study is that rabbit antibodies raised against as short a sequence as a nonapeptide present in an asexual blood stage protein of *P. falciparum* can exert a strong parasiticidal effect (Table 1; Fig. 6). As far as immunity to the asexual blood stages is concerned, Brown (3) has argued that antibodies appear to be important in providing specific protection to the host, as also demonstrated earlier by Cohen and colleagues in their classical studies of children in the Gambia (11). Others have shown that antibodies from immune monkeys and humans can inhibit in vitro growth of the parasite as well as protect naive recipients (10, 28, 35). There is no information in the literature about the putative protective potential of TRAP or TRAP-like protein against the asexual blood stages of the malaria parasite. However, in another system, namely, *P. yoelii* in mice, immunization of animals with a mixture of CS protein and sporozoite protein SSP-2, which is an analog of TRAP, was found to impart protection to mice against a sporozoite challenge (20). Interestingly, immunization with CS protein or SSP-2 alone induced protection in only a small proportion of animals. It was also established in that study that cytotoxic T lymphocyte responses played a critical role in protection against the sporozoite challenge. These results of Khusmith and colleagues (20) with SSP-2, and our results with the TRAP motif that antibodies to this motif can inhibit in vitro merozoite invasion of erythrocytes (Table 1; Fig. 6), indicate that an appropriate immune response to TRAP might prove effective against two different stages of the parasite. Even though it required a relatively high concentration (1.2 mg/ml) of immune IgG to achieve a nearly total inhibition of the merozoite invasion (Fig. 6), the dramatic decline in parasitemia obtained with this antibody implies that an appropriate immune response elicited with a synthetic immunogen might help to reduce the parasite load in vivo, thus alleviating the severity of infection.

Our observation that the synthetic construct, i.e., the 18-mer, which contains the nonapeptide sequence, caused significant inhibition of the merozoite invasion of erythrocytes suggests that this motif, along with some other such motifs, might be involved in some vital biological process(es) of the parasite. As mentioned earlier, this sequence is also present in the CS protein of all *Plasmodium* species sequenced so far as well as in SSP-2 of *P. falciparum* and *P. yoelii* (30, 31). Such widespread occurrence of this motif in these parasite proteins strengthens the contention about its potential role in the biology of the parasite. Since this motif is also present in thrombospondin and properdin, the cell adhesive molecules involved in cell-cell interaction, it is reasonable to speculate that in the malaria parasite, this molecule, and particularly the motif around the WSPCSVTCG sequence, may also play some important role in the parasite-host cell interaction, especially in the process of invasion of hepatocytes by sporozoites and of erythrocytes by merozoites. In fact, Cerami and colleagues (4) have demonstrated that recombinant constructs representing the CS protein of *P. falciparum* and containing the WSPCS VTCG motif can bind to human hepatocytes and specifically inhibit invasion by sporozoites, whereas a construct without this motif, but with all other features of the CS protein intact, does not do so. More recently, Muller and coworkers (25) have reported similar findings with recombinant fragments of TRAP of *P. falciparum*. Subsequently, Robson and colleagues (28a) have observed that the expression of TRAP in sporozoites is concomitant with the formation of micronemes and the development of sporozoite infectivity. These findings lend support to the suggestion that TRAP is one of the critical parasite molecules involved in the sporozoite invasion of hepatocytes. We believe that a similar binding of TRAP-like protein to erythrocytes is quite likely to occur as part of the process of invasion. We have obtained a significant level of inhibition of the merozoite invasion of erythrocytes with the 18-mer. In order to rule out the possibility of a nonspecific toxic effect of the synthetic construct, we preincubated normal erythrocytes with the 18-mer for 24 h and then washed off the excess of the peptide. These cells remained fully susceptible to merozoite invasion and supported normal parasite growth (data not presented). We also found that a modified analog of the 18-mer in which the cysteine side-chain sulfhydryl groups were protected and a 21-mer malaria peptide (P-8) containing a cysteine residue, both of which were synthesized and purified in our laboratory by essentially the same procedures, inhibited the merozoite invasion to almost insignificant levels (Table 2). This further lends support to our contention that the inhibition of merozoite invasion obtained with the 18-mer was specific in nature. From our observation that the 18-mer peptide inhibits the parasite growth in a dose-dependent manner, it may still be hypothesized that the conserved-motif peptide somehow competes with the blood stage parasite (merozoite) in the initial interaction with some receptor(s) on the erythrocyte.

The observation that carrier-free nonapeptide and 18-mer did not induce antibodies in rabbits or mice lends support to results of previous studies indicating poor immunogenicity of synthetic peptides (2, 7, 8, 21). In sera collected from clinical cases of *P. falciparum* malaria, we obtained much lower levels of antibodies reactive against the nonapeptide or the 18-mer (unpublished observations), although all of these sera harbored high levels of malaria antibodies as measured in an ELISA with the whole parasite lysate as well as two other synthetic antigens representing B-cell determinants of MSP-1 of *P. falciparum* (21). Once again, this underscores the problem of poor immunogenicity of malaria peptides in general and of putatively protective epitopes in particular. With the delineation of "universal" T_h cell determinants in the parasite protein, the problem of genetic restriction of immune response to peptide immunogens might be addressed with some measure of success (21). For enhancing the immunogenicity of synthetic peptides, it will be necessary to devise a strategy which facilitates appropriate presentation of these molecules to the immune system. It can be argued that the poor immunogenicity of the TRAP motif may partly be due to its being a part of self-molecules like thrombospondin and properdin, etc., and that induction of an immune response to this motif is potentially fraught with inducing autoimmune responses, as has been shown for the hsp-70 cognate parasite protein (23). On the other hand, several other *P. falciparum* proteins, viz., Pfs25, MSP-1, and p41 (aldolase), contain sequences homologous to those of host proteins such as the epidermal growth factor (18), the intermediate filament protein (8), and the human aldolase enzyme (5), respectively; significantly, none of these sequences have been shown to induce or be the target of any autoimmune response. Studies are under way to investigate these aspects of possible autoimmune response to the 18-mer motif as well as

the potential of this motif to impart protection to mice against challenge infection with *P. yoelii.*

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REFERENCES

1. **Anders, R. F., R. L. Coppel, G. V. Brown, and D. J. Kemp.** 1988. Antigens with repeated amino acid sequences from the asexual blood stages of *Plasmodium falciparum*. Prog. Allergy **41:**148–172.
2. **Ballou, W. R., J. Rothbard, R. A. Wirtz, D. M. Godson, J. S. Williams, R. W. Gore, I. Schneider, M. R. Hollingdale, R. L. Beaudoin, W. L. Maloy, L. H. Miller, and W. T. Hockmeyer.** 1985. Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum*. Science **228:**996–999.
3. **Brown, K. N.** 1991. Protective immune responses as indicators of antigenic diversity and stability. Acta Leiden. **60:**111–119.
4. **Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos, and V. Nussenzweig.** 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell **70:**1021–1023.
5. **Certa, U., P. Ghersa, H. Dobeli, H. Matile, H. P. Kocher, I. K. Shrivastava, A. R. Shaw, and L. H. Perrin.** 1988. Aldolase activity of a *Plasmodium falciparum* protein with protective properties. Science **240:**1036–1038.
6. **Chatterjee, S., M. Wery, P. Sharma, and V. S. Chauhan.** 1995. A conserved peptide sequence of the *Plasmodium falciparum* circumsporozoite protein and antipeptide antibodies inhibit *Plasmodium berghei* sporozoite invasion of Hep-G2 cells and protect immunized mice against *P. berghei* sporozoite challenge. Infect. Immun. **63:**4375–4381.
7. **Chauhan, V. S., S. Chatterjee, and P. K. Johar.** 1993. Synthetic peptides based on conserved *Plasmodium falciparum* antigens are immunogenic and protective against *Plasmodium yoelii* malaria. Parasite Immunol. **15:**239–242.
8. **Cheung, A., J. Leban, A. R. Shaw, B. Merkli, J. Stocker, C. Chizzolini, R. C. Sander, and L. H. Perrin.** 1986. Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. Proc. Natl. Acad. Sci. USA **83:**8328–8332.
9. **Clark, L. E., F. M. Tomley, M. H. Wisher, I. J. Foulds, and M. E. G. Boursnell.** 1990. Regions of an *Eimeria tenella* antigen contains sequences which are related to the thrombospondin gene family. Mol. Biochem. Parasitol. **41:**269–280.
10. **Coggeshall, L. T., and H. W. Kumm.** 1937. Demonstration of passive immunity in experimental monkey malaria. J. Exp. Med. **66:**177–190.
11. **Cohen, S., I. A. McGregor, and S. C. Carrington.** 1961. Gamma globulin and acquired immunity to malaria. Nature (London) **192:**733–737.
12. **Cowan, G., S. Krishna, A. Crisanti, and K. Robson.** 1992. Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. Lancet **339:**1412.
13. **Cox, F. E. G.** 1992. Malaria vaccines - progress and problems. Trends Biotechnol. **9:**389–394.
14. **Fenton, B. J., J. T. Clark, C. F. Wilson, J. S. McBride, and D. Walliker.** 1989. Polymorphism of a 35-48 kDa *Plasmodium falciparum* merozoite surface antigen. Mol. Biochem. Parasitol. **34:**79–86.
15. **Goundis, D., and K. B. M. Reid.** 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. Nature (London) **335:**82–85.
16. **Hadley, T. J., and L. H. Miller.** 1988. Invasion of erythrocytes by malaria parasites: erythrocyte ligands and parasite receptors. Prog. Allergy **41:**49–71.
17. **Holder, A. A.** 1988. The precursor to major merozoite surface antigen: structure and role in immunity. Prog. Allergy **41:**72–97.
18. **Kaslow, D. C., I. A. Quakyi, C. Syin, M. G. Raum, D. B. Keister, J. E. Coligan, T. F. McCutchan, and L. H. Miller.** 1988. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature (London) **333:**74–76.
19. **Kaur, P., P. Sharma, A. Kumar, and V. S. Chauhan.** 1990. Synthetic, immunological and structural studies on repeat unit peptides of *Plasmodium falciparum* antigens. Int. J. Pept. Protein Res. **36:**515–521.
20. **Khusmith, S., Y. Charoenvit, S. Kumar, M. Sedegah, R. L. Beaudoin, and S. L. Hoffman.** 1991. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. Science **252:**715–718.
21. **Kumar, A., R. K. Arora, P. Kaur, V. S. Chauhan, and P. Sharma.** 1992. "Universal" T helper cell determinants enhance immunogenicity of a *Plasmodium falciparum* merozoite surface antigen peptide. J. Immunol. **148:** 1499–1505.
22. **Lambros, C., and J. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65:**418–420.
23. **Mattei, D., A. Scherf, O. Bensaude, and L. Perreira da Silva.** 1989. A heat-shock protein from the human malaria parasite, *Plasmodium falciparum* induces autoantibodies. Eur. J. Immunol. **19:**1823–1828.
24. **McCutchan, T. F., V. F. de la Cruz, M. F. Good, and T. E. Williams.** 1988. Antigenic diversity in *Plasmodium falciparum*. Prog. Allergy **41:**173–192.
25. **Muller, H.-M., I. Reckmann, M. R. Hollingdale, H. Bujard, K. J. H. Robson, and A. Crisanti.** 1993. Thrombospondin-related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. EMBO J. **12:**2881–2889.
26. **Nussenzweig, V., and R. S. Nussenzweig.** 1985. Circumsporozoite proteins of malaria parasites. Cell **42:**401–403.
27. **Perkins, S. J., A. S. Nealis, P. I. Haris, D. Chapman, D. Goundis, and K. B. M. Reid.** 1989. Secondary structure in properdin of the complement cascade and related proteins: a study by Fourier transform infrared spectroscopy. Biochemistry **28:**7176–7182.
28. **Reese, R. T., and M. R. Motyl.** 1979. Inhibition of the in vitro growth of *Plasmodium falciparum*. I. The effects of immune serum and purified immunoglobulin from owl monkeys. J. Immunol. **123:**1894–1899.

28a. **Robson, K. J. H., U. Frevert, I. Reckmann, G. Cowan, J. Beier, I. G. Scragg, K. Takehara, D. H. L. Bishop, G. Pradel, R. Sinden, S. Saccheo, H.-M. Muller, and A. Crisanti.** 1995. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. EMBO J. **14:**3883–3894.

29. **Robson, K. J. H., J. R. S. Hall, M. W. Jennings, T. J. R. Harris, K. Marsh, C. I. Newbold, V. E. Tate, and D. J. Weatherall.** 1988. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood-stages of a human malaria parasite. Nature (London) **335:**79–82.
30. **Rogers, W. O., A. Malik, S. Mellouk, K. Nakamura, M. D. Rogers, A. Szarfman, D. M. Gordon, A. K. Nussler, M. Aikawa, and S. L. Hoffman.** 1992. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. Proc. Natl. Acad. Sci. USA **89:**9176–9180.
31. **Rogers, W. O., M. D. Rogers, R. C. Hedstrom, and S. L. Hoffman.** 1992. Characterization of the gene encoding sporozoite surface protein 2, a protective *Plasmodium yoelii* sporozoite antigen. Mol. Biochem. Parasitol. **53:** 45–52.
32. **Saul, A., G. L. Jones, and L. Spencer.** 1992. Protective immunization with invariant peptides of the *Plasmodium falciparum* antigen MSA2. J. Immunol. **148:**208–211.
33. **Sharma, P., A. Kumar, S. Batni, and V. S. Chauhan.** 1993. Co-dominant and reciprocal T helper cell activity of epitopic sequences and formation of junctional B cell determinants in synthetic T:B chimeric immunogens. Vaccine **11:**1321–1326.
34. **Sharma, P., T. K. Ruebush II, G. H. Cambell, S. J. Richman, P. P. Wilkins, J. R. Broderson, F. Ardeshir, M. Gross, C. Silverman, J. C. Skinner, V. Filipski, C. Wilson, J. M. Roberts, N. S.-F. Ma, P. S. Stanfill, R. T. Reese, and W. E. Collins.** 1992. Immunogenicity and efficacy trials in *Aotus nancymai* monkeys with model compounds representing parts of a 75-kDa merozoite antigen of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. **46:**691–707.
35. **Stanley, H. A., and R. T. Reese.** 1984. *In vitro* inhibition of intracellular growth of *Plasmodium falciparum* by immune sera. Am. J. Trop. Med. Hyg. **33:**12–16.
36. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
37. **Wrightsman, R. A., B. D. Dawson, D. L. Fouts, and J. E. Manning.** 1994. Identification of immunodominant epitopes in *Trypanosoma cruzi* trypomastigote surface antigen-1 protein that mask protective epitopes. J. Immunol. **153:**3148–3154.

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