INFECTION AND IMMUNITY, June 1998, p. 2895–2904 Vol. 66, No. 6
0019-9567/98/$04.00+0

Characterization of Protective Epitopes in a Highly Conserved *Plasmodium falciparum* Antigenic Protein Containing Repeats of Acidic and Basic Residues

PAWAN SHARMA,[1]* ANIL KUMAR,[1] BALWAN SINGH,[1] ASHIMA BHARADWAJ,[1] V. NAGA SAILAJA,[1] T. ADAK,[2] ASHIMA KUSHWAHA,[1] PAWAN MALHOTRA,[1] AND V. S. CHAUHAN[1]

International Centre for Genetic Engineering and Biotechnology, New Delhi 110067,[1] and Malaria Research Centre (Indian Council of Medical Research), Delhi 110009,[2] India

Received 23 October 1997/Returned for modification 28 January 1998/Accepted 27 March 1998

The delineation of putatively protective and immunogenic epitopes in vaccine candidate proteins constitutes a major research effort towards the development of an effective malaria vaccine. By virtue of its role in the formation of the immune clusters of merozoites, its location on the surface of merozoites, and its highly conserved nature both at the nucleotide sequence level and the amino acid sequence level, the antigen which contains repeats of acidic and basic residues (ABRA) of the human malaria parasite *Plasmodium falciparum* represents such an antigen. Based upon the predicted amino acid sequence of ABRA, we synthesized eight peptides, with six of these (AB-1 to AB-6) ranging from 12 to 18 residues covering the most hydrophilic regions of the protein, and two more peptides (AB-7 and AB-8) representing its repetitive sequences. We found that all eight constructs bound an appreciable amount of antibody in sera from a large proportion of *P. falciparum* malaria patients; two of these peptides (AB-1 and AB-3) also elicited a strong proliferation response in peripheral blood mononuclear cells from all 11 human subjects recovering from malaria. When used as carrier-free immunogens, six peptides induced a strong, boostable, immunoglobulin G-type antibody response in rabbits, indicating the presence of both B-cell determinants and T-helper-cell epitopes in these six constructs. These antibodies specifically cross-reacted with the parasite protein(s) in an immunoblot and in an immunofluorescence assay. In another immunoblot, rabbit antipeptide sera also recognized recombinant fragments of ABRA expressed in bacteria. More significantly, rabbit antibodies against two constructs (AB-1 and AB-5) inhibited the merozoite reinvasion of human erythrocytes in vitro up to ~90%. These results favor further studies so as to determine possible inclusion of these two constructs in a multicomponent subunit vaccine against asexual blood stages of *P. falciparum*.

Plasmodium falciparum causes the most virulent kind of malaria in humans and is almost exclusively responsible for all malaria-related deaths in the world. Several parasite antigens from the asexual erythrocytic stages, such as merozoite surface protein 1 (MSP-1), MSP-2, the apical membrane antigen 1, etc., which are targets of the potentially protective immune responses, are now being developed as candidates for vaccines (reviewed in reference 22). However, a major problem in developing an effective vaccine is the high degree of genetic diversity and antigenic variation found in the target antigens (5, 9, 27, 29, 35, 40). This problem is further aggravated by the fact that in several cases, these variant regions constitute immunodominant determinants with the potential to divert immune responses from critical epitopes and/or obstruct maturation of high-affinity antibodies to these epitopes (2, 3, 15). These critical epitopes might represent structures involved in some important processes, such as the merozoite invasion, which is a crucial event in the life cycle of the parasite and are, hence, rather conserved. For example, MSP-1 of *P. falciparum* has several blocks which display a high degree of polymorphism among various strains of the parasite (reviewed in reference 29). However, its C-terminal region, termed MSP-1_{19}, with its epidermal growth factor-like domains, is essentially conserved even across the species, and it is this region that has been shown to be critically implicated in the merozoite invasion of erythrocytes (6, 7, 20). Similarly, a highly conserved region II motif present in the circumsporozoite protein of all *Plasmodium* species sequenced so far seems to play an essential role in the sporozoite invasion of hepatocytes (11, 30). In fact, we (12), as well as others (36), have shown that immunization with synthetic peptides modeling highly conserved regions of the *P. falciparum* antigens can even protect mice against live challenge with the murine malaria parasites, viz., *P. berghei* or *P. yoelii*. Such conserved portions of malarial proteins are currently the subject of active investigation as putative vaccine molecules.

The antigen which contains repeats of acidic and basic residues (ABRA) of *P. falciparum* (41) seems to be another such highly conserved molecule. It is a 101-kDa protein located on the surface of merozoites as well as in the parasitophorous vacuole within the infected erythrocytes (14, 46). Significantly, this protein is also present in the immune clusters of merozoites which are formed at the time of rupture of mature schizonts in the presence of immune serum. Formation of such clusters prevents the dispersal of merozoites, resulting in a marked decrease in parasitemia, which is considered an indicator of protective immunity (17, 28). Furthermore, ABRA is almost fully conserved among various laboratory isolates of *P. falciparum* (46), possesses a chymotrypsin-like activity (33), and has a partial protein sequence homology with an extracellular cysteine protease of another protozoan, *Trichomonas*

* Corresponding author. Mailing address: Immunology Group, ICGEB, P.O. Box 10504, Aruna Asaf Ali Marg, New Delhi 110067, India. Phone: 91-11-6176680. Fax: 91-11-6162316. E-mail: pawans@icgebnd.ernet.in.

vaginalis (16). All these findings about ABRA seem to indicate its potential role in a protease-mediated process(es), such as merozoite invasion of erythrocytes, which is a critical event in the life cycle of the parasite. Because of its location on the merozoite surface, its presence in the immune clusters of merozoites, its highly conserved sequence, and its reported protease activity, ABRA represents an attractive molecule for development as a vaccine candidate.

In the present study, we have attempted to delineate the putative epitopic sequences of ABRA by using a battery of eight synthetic peptides based on its most hydrophilic regions and its repeat sequences. We found that these sequences represented target epitopes for the serum immunoglobulin G (IgG) antibodies in a large proportion of humans recovering from *P. falciparum* infection. They also stimulated the peripheral blood mononuclear cells from convalescing patients from an area of endemicity. Our results indicated that five out of six sequences from the nonrepetitive regions and one of the two repeat sequences elicited a boostable, IgG-type antibody response in rabbits immunized with the carrier-free peptides. We also found that antibodies against two of the peptides, both nonrepetitive, exerted a strong inhibitory effect on the merozoite invasion of erythrocytes, indicating the importance of these constructs in inducing a potentially protective antibody response.

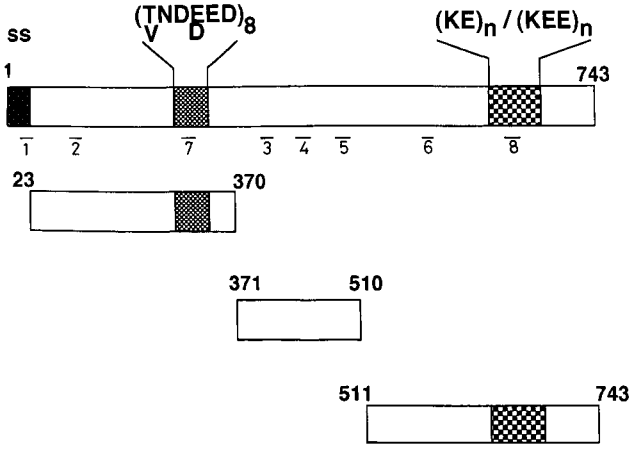

FIG. 1. Schematic representation of *P. falciparum* ABRA and its three fragments obtained by PCR amplification of the parasite genomic DNA and expressed as recombinant proteins as described in Materials and Methods. The small, numbered horizontal bars indicate the positions of the sequences chosen for synthetic peptides AB-1 to AB-8 as described in Materials and Methods. ss, signal sequence.

MATERIALS AND METHODS

Parasite. The FID-3 isolate of *P. falciparum* was maintained in continuous culture essentially according to the methods described by Trager and Jensen (42), and a detergent-soluble extract of the parasite proteins was prepared as described previously (38) for use as the antigen in the enzyme-linked immunosorbent assay (ELISA) or the immunoblotting assay. A schizont-rich preparation of the parasites was also used to obtain genomic DNA by phenol-chloroform extraction and ethanol precipitation following standard procedures. The quality and yield of genomic DNA was ascertained by agarose gel electrophoresis; this DNA was used to obtain the full-length ABRA-encoding gene and its various fragments by amplification using PCR as described below.

Synthetic peptides. Analysis of the predicted amino acid sequence of ABRA (46) according to the Chou-Fasman algorithm revealed six stretches, ranging from 12 to 18 residues, having a hydrophilicity score of 40% or more. These sequences are as follows (amino acid numbers are according to the numbering system of Weber and colleagues [46]): AB-1, ^{19}NIISCNKNDKNQ^{30}; AB-2, ^{99}ANNSANNGKKNNAEE^{113}; AB-3, ^{395}YKAYVSYKKRKAQEK^{409}; AB-4, ^{448}LKNKIFPKKKEDNQAVDT^{465}; AB-5, ^{518}VPPTQSKKKNKNET^{531}; and AB-6, ^{639}ENDVLNQETEEEMEK^{653}. In addition, two more constructs, representing the repetitive sequences in ABRA, were also synthesized: AB-7, TNDE EDTNDEEDTNDEED, and AB-8, KEEKE EKEEKEEKEKEKE. The procedures employed for synthesis, purification, and characterization of the synthetic constructs AB-1 to AB-8 were essentially the same as those described in our earlier work (24, 37, 38). Briefly, peptides were synthesized by stepwise solid-phase synthesis in an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, Calif.) and purified by gel filtration followed by reverse-phase high-performance liquid chromatography. The purity and authenticity of the synthetic peptides were ascertained by reverse-phase analytical high-performance liquid chromatography and amino acid analysis, respectively.

Recombinant ABRA constructs. The full-length ABRA gene (but lacking an N-terminal putative signal sequence) and its three major fragments were amplified from the parasite genomic DNA by PCR and cloned in *Escherichia coli* by standard molecular biology protocols. Briefly, the ABRA gene encoding amino acids (aa) 23 to 743, i.e., the full-length protein except for the putative signal sequence, was amplified by PCR using the *P. falciparum* genomic DNA as the template. A forward primer (5′-CGGGATCCCGATGAACATG-3′) representing the N-terminal region and incorporating a *Bam*HI restriction site and a reverse primer (5′-AACCCAAGCTTATTTTGATTCTTCAG-3′) representing the C terminus and incorporating a *Hin*dIII restriction site were used for this amplification reaction. The amplified DNA fragment (~2.1 kb) was cloned into pGEMT vector (Promega Corporation, Madison, Wis.), and the nucleotide sequence of the cloned gene was partially determined by the dideoxynucleotide chain termination method.

For recombinant protein expression in bacteria, *E. coli*, the ABRA gene was divided into three regions (Fig. 1), the 5′ region (AB-N) encoding the amino-terminal portion of the protein (aa 23 to 370), which contained the hexapeptide repeats; the middle region (AB-M) corresponding to the repeatless portion of the protein (aa 371 to 510); and the 3′ region (AB-C) encoding the carboxyl-terminal portion of the protein (aa 511 to 743), which contained the KE and KEE repeats (Fig. 1). These three fragments were amplified by using the high-fidelity *Pfu* DNA polymerase enzyme and the recombinant plasmid DNA isolated from the pGEMT clone of ABRA as the template and were subcloned as *Bam*HI-*Hin*dIII fragments in pMal vector (New England Biolabs, Inc., Beverly, Mass.) for expression as a fusion protein with the maltose-binding protein.

To study the protein expression from these clones, *E. coli* cells (strain TB-1) carrying the recombinant plasmids were grown overnight at 37°C in Luria broth containing ampicillin. The overnight cultures were diluted 10-fold and incubated at 37°C to an optical density at 600 nm (OD_{600}) of 0.7 to 0.8 when protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. The cells were harvested and resuspended in lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA). These cells were subjected to two cycles of freeze-thaw treatment, followed by ultrasonication. After centrifugation of the sonicated extract, an aliquot of the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with rabbit anti-peptide sera specific for each fragment.

Human samples. Serum samples were collected from eight normal, healthy individuals who had no known past history of malaria and were negative for malaria by slide examination at the time of drawing blood (24). With the *P. falciparum* lysate used as the capture antigen, these sera (each diluted 1/200) yielded an average OD_{490} of 0.35 with a standard deviation of 0.05 (0.35 ± 0.05) by ELISA. With the synthetic constructs in the same assay, these sera gave average OD_{490}s of 0.10 ± 0.02, 0.15 ± 0.02, 0.13 ± 0.03, 0.19 ± 0.05, 0.10 ± 0.02, 0.14 ± 0.04, 0.10 ± 0.01, and 0.2 ± 0.04 with AB-1 to AB-8, respectively.

Sera were also collected from 50 patients, positive for *P. falciparum* malaria by slide examination, admitted to the medical wards of Rabindra Nath Tagore Medical College and Associated Hospitals, Udaipur (Rajasthan), India (24). All these patients presented with characteristic symptoms of high fever, chill, and rigor; a majority of them also had a previous history of fever of undetermined etiology. All but one of these patients were successfully cured following treatment with the standard regimen of chloroquine (at 600, 600, and 300 mg on days 1, 2, and 3, respectively). One patient that did not respond to chloroquine was subsequently cured with a single dose of sulfalene (1,000 mg) plus pyrimethamine (50 mg). Blood for serum collection from these patients was generally obtained 1 day after the completion of drug treatment (24).

In addition, peripheral blood samples from 11 *P. falciparum*-infected patients who had recovered from their last malaria episodes about 4 to 5 weeks prior to the study and were malaria negative upon slide examination at the time of sample collection and five normal, healthy individuals malaria negative by slide examination were also collected for the lymphocyte transformation assay as described below.

Informed consent from all the human subjects was obtained after explaining to them the objectives of the present study in detail, particularly emphasizing the fact that the results of this study might not be of any direct benefit to them. The protocol for this study was approved by the institutional Human Volunteers Research Ethical Committees of the two participating institutes, viz., the International Center for Genetic Engineering and Biotechnology, New Delhi, India, and the Malaria Research Centre (Indian Council of Medical Research), Delhi, India.

Lymphocyte proliferation assay. Peripheral blood samples were collected from human volunteers who were living in an area of endemicity, had suffered from confirmed *P. falciparum* malaria infection several months prior to the study, and had been cured with the standard chloroquine regimen. They were malaria negative by slide examination at the time of sample collection and gave informed consent to participate in the study. Of 18 subjects approached, only 11 agreed to give a blood sample for this part of the study. The peripheral blood mononuclear cells (PBMC) were separated by centrifuging each of the blood samples on a density gradient (Histopaque-1077; Sigma Chemical Co., St. Louis, Mo.). The lymphocyte proliferation assay was set up in the 96-well tissue culture plates (catalog no. 3595; Costar Scientific Corp., Cambridge, Mass.) with PBMC cultured in RPM 1640 medium supplemented with 25 mM HEPES, 0.2% sodium bicarbonate, 50 μM β-mercaptoethanol, 1.0 mM pyruvic acid, and 10% pooled human serum (AB/Rh^+ group). Seven of eight ABRA constructs were used at three doses each, i.e., 10, 1.0, and 0.1 μg/well, with each well containing the cell suspension in a total volume of 200 μl. Each dose was tested in triplicate wells; concanavalin A, at a previously determined optimal concentration of 1 μg/well, was used as a nonspecific, polyclonal mitogen. On day 3 (concanavalin A) or day 5 (peptide), cultures were pulsed with [*methyl*-^{3}H]thymidine (0.5 μCi/well; Amersham International plc, Little Chalfont, Buckinghamshire, England) for 6 h. The cells were then harvested onto glass fiber filters by using the PHD cell harvester (Cambridge Technology, Inc., Watertown, Mass.), and the ^{3}H incorporation was determined by β-emission liquid scintillation spectroscopy. The results were expressed as stimulation indexes (SIs); the SI represents the ratio of counts per minute obtained in the presence of the peptide to those obtained in the absence of the peptide.

Animals and their immunization. Animals used in this study were procured from the Small Animal Facility of the National Institute of Immunology, New Delhi, India. Animals were housed, fed, and used in the experiments following guidelines set forth in the National Institutes of Health manual *Guide for the Care and Use of Laboratory Animals* (30a).

Rabbits (New Zealand White; about 2 kg each) were immunized with a dose of 200 μg of the carrier-free peptides emulsified in complete Freund's adjuvant and injected subcutaneously at multiple sites in the nuchal region, and on day 28 they received boosters containing a similar dose of the respective peptide emulsified in incomplete Freund's adjuvant. Sera were collected from these rabbits on days 0, 14, 28, 42, and 56, heat inactivated at 56°C, and stored at −20°C. Peptides which failed to elicit a significant antibody response after the booster injection, i.e., AB-2, AB-6, and AB-7, were inoculated once more on day 42 at a dose of 200 μg each. Sera were tested for the presence of antipeptide antibodies by an ELISA using respective carrier-free peptides as the capture antigens.

Both preimmune and immune rabbit sera were adsorbed with fresh, washed, normal human erythrocyte ghosts and then were dialyzed against chilled and sterile phosphate-buffered saline (PBS) (0.15 M; pH 7.2) before being tested for the presence of antiparasite antibodies in various assays.

For some experiments, IgG fractions were purified from preimmune and immune rabbit sera by ammonium sulfate precipitation of the sera to obtain the gamma globulin fraction followed by ion-exchange chromatography on an Econo-Pac IgG purification column (Bio-Rad Laboratories, Richmond, Calif.) as described previously (38). After their purity was ascertained by SDS-PAGE and immunoblotting, the purified IgG fractions were dialyzed against plain RPMI 1640 medium, i.e., the medium supplemented with 25 mM HEPES and 0.2% sodium bicarbonate but without serum, passed through sterile 0.22-μm-pore-size membrane filters, and used in the merozoite invasion inhibition assays.

Purified IgG fractions obtained from serum samples from a rabbit immunized with an 18-residue peptide sequence conserved in thrombospondin-related anonymous protein and circumsporozoite protein of the parasite, previously shown to exert a dose-dependent inhibitory effect on the *P. falciparum* merozoite reinvasion of human erythrocytes (38), were also included as positive controls in some experiments as described below. Serum samples from another rabbit immunized with P-8, a 21-mer synthetic peptide construct based on *P. falciparum* MSP-1 (24, 38), were also used as negative controls in some assays.

ELISA. Sera were tested for the presence of antibodies in an ELISA, using carrier-free peptides or parasite lysate as the capture antigen. Procedures employed for the preparation of the parasite lysate (FID-3 isolate of *P. falciparum*) and for performing the assay were essentially as described previously (24, 38). Briefly, wells of flat-bottom Immulon-2 plates (Dynatech Laboratories Inc., Chantilly, Va.) were coated with the previously determined optimal concentration of capture antigen (carrier-free synthetic peptides or parasite lysate); the uncovered reactive sites were blocked with 5% milk powder solution in PBS. The antigen-coated wells were then sequentially incubated with appropriate dilutions of the first antibody followed by optimally diluted, enzyme-labeled secondary antibody (horseradish peroxidase-labeled anti-human or anti-rabbit IgG), with thorough washing of plates in between the incubations. The enzyme reaction was developed with *o*-phenylenediamine dihydrochloride as the chromogen and hydrogen peroxide as the substrate. After stopping the reaction with sulfuric acid, the OD_{490} of the reaction product in the wells was recorded by using a Microplate Reader (Molecular Devices, Palo Alto, Calif.). In an ELISA using parasite lysate as the capture antigen, *P. falciparum* patient sera giving an OD_{490} of 0.2 or more than the average mean OD_{490} obtained with the normal sera (i.e., an OD of ≥0.55) were defined as positive sera. We realize that this arbitrary cutoff OD value of 0.55 is rather high, but it ensures stringent specificity against background noise in the sera from a region of endemicity like India. The same criterion of the difference between OD values (ΔOD) being ≥0.2 was applied to determine the specific positivity of clinical sera against the individual ABRA peptides. In the end point titrations, the last dilution of a test serum yielding an OD_{490} twice or more than twice that obtained with the respective preimmune serum (diluted 1/100) was taken as the end point titer.

Immunoblotting. The reactivity of the rabbit antipeptide sera with the parasite protein(s) was further ascertained by immunoblotting. The whole parasite lysate was fractionated on a 10% gel by SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane following standard procedures. After the uncovered reactive sites of the nitrocellulose membrane were blocked by saturation with 5% nonfat milk powder solution in PBS overnight, the membrane was probed with various preimmune and immune rabbit sera by using a Mini Protean II Multi-Screen apparatus (Bio-Rad Laboratories). Total lysates of bacteria expressing the recombinant fragments of ABRA, viz., AB-N, AB-M, and AB-C, were also similarly fractionated and probed with the respective region-specific rabbit antibody, i.e., anti-AB-1, anti-AB-3, and anti-AB-8 antisera, respectively. The parasite proteins and the recombinant ABRA fragments in the bacterial lysates, separated by SDS-PAGE and transferred onto nitrocellulose paper, were incubated first with rabbit antipeptide sera and then with the horseradish peroxidase-labeled anti-rabbit IgG antibodies. The final enzyme reaction was developed with H_2O_2 as the substrate and 4-chloro-1-naphthol as the chromogen.

Immunofluorescence assay. Sera from rabbits immunized with ABRA constructs were tested for their reactivity with the authentic parasite protein(s) in the immunofluorescence assay as well (38). All rabbit sera were preadsorbed with fresh human erythrocytes so as to get rid of any heterophile antibody possibly present in these sera (38). Multispot antigen slides were made from a parasite-infected erythrocyte suspension prepared from an asynchronous culture of *P. falciparum* (strain FID-3). The antigen spots, air dried and fixed with the acetone-methanol (9:1, vol/vol) mixture, were sequentially incubated with serial dilutions of the test sera and the optimally diluted fluorescein isothiocyanate-labeled anti-rabbit IgG solution. The slides were finally mounted in the buffered glycerol containing *p*-phenylenediamine dihydrochloride (1 mg/ml) as the antifading reagent and examined under a fluorescence microscope (Wild Leitz GmbH, Wetzlar, Germany), alternately in visible and UV light, to see specific binding of the antibody to the parasite.

Merozoite invasion inhibition assay. The in vitro cultures of the FID-3 strain of *P. falciparum* were synchronized at the ring stage by two treatments with 5% sorbitol solution (25) and incubated further for about 30 h, so that at the time of setting up of the assay, nearly 90% of the parasites were >4*N* segmenters. The cultures were grown in RPMI 1640 medium supplemented with 10% human serum plus 5% normal (preimmune) or immune rabbit serum and incubated in a candle jar at 37°C for 20 h. Additional controls included culture wells with no rabbit serum, wells with rabbit anti-$P\text{-}8_{(MSP\text{-}1)}$ serum (negative control [38]), and wells containing rabbit anti-$18\text{-}mer_{(conserved\ TRAP\ motif)}$ total immune IgG at a concentration known to cause 50% inhibition of the merozoite invasion in this assay (38). In each experiment was included a parallel set of culture wells with appropriate rabbit sera, monitored every 2 to 3 h by microscopy for any possible toxic effect of these sera on the parasite or parasitized erythrocytes. At the end of the assay, smears were drawn from aliquots taken from each well, stained with Giemsa stain, and examined under a microscope by two researchers; only the ring-infected cells were counted as parasitized cells for calculating percent parasitemia (number of parasitized erythrocytes out of a total of 100 erythrocytes); at least 10,000 cells were counted to determine the level of parasitemia in each smear. In a subsequent experiment, various concentrations of the purified IgG fractions isolated from preimmune and immune rabbit sera were also incorporated in the test system.

RESULTS

Immunogenicity of synthetic ABRA peptides. We synthesized six nonrepetitive sequence peptides based on their high hydrophilicity score, potentially facilitating their accessibility to the immune system and, in addition, two more constructs modeling the repeat regions of ABRA. In an ELISA for measuring levels of circulating IgG antibodies, we found that each of these eight constructs bound appreciable amounts of antiparasite antibodies in the sera from a large proportion of human subjects recovering from natural *P. falciparum* infection (Fig. 2). Of 50 such sera that we tested, 33 (66%) yielded a positive ELISA reaction with one or more ABRA constructs; of these 33 positive samples, we found that only 3 reacted with all eight of the constructs, 6 reacted with seven constructs, 5 reacted with six constructs, 3 reacted with five constructs, 2 each reacted with four, three, and two constructs, and 10 reacted with only one of the eight constructs. Synthetic construct AB-3 was

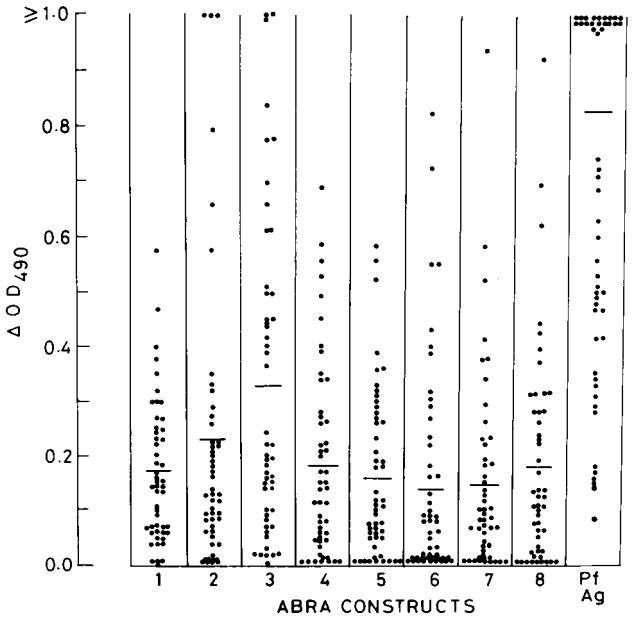

FIG. 2. Distribution profile of antibody levels obtained by ELISA using sera from 50 clinical malaria patients. Each serum was diluted 1/200 and tested in duplicate against each of the eight ABRA constructs. The ΔOD_{490} value was obtained by subtracting the OD_{490} value given by a normal human serum pool from that given by the respective clinical serum. Each data point represents a mean of duplicate values. A ΔOD_{490} value of 0.2 or more was defined as positive. The solid horizontal line in each column represents the mean ΔOD_{490} value. Pf Ag, *P. falciparum* antigen.

by far the most frequently recognized epitopic sequence, since as many as 50% of the clinical samples bound to this peptide, and as a group, the positive samples also yielded the highest average ΔOD_{490} value (0.56 ± 0.41) in the peptide ELISA, while AB-1, which was recognized by 36% of these sera in the same assay yielded the lowest average ΔOD_{490} value (0.31 ± 0.09). We found four samples to be negative for both parasite- and peptide-specific antibodies in this assay. Two other samples which were seronegative for the parasite antigen yielded a positive ELISA reaction with one (AB-3) and two peptides (AB-2 and AB-6), respectively.

In order to ascertain their putative potential to elicit T-cell reactivity in humans, we tested these peptides in a lymphocyte proliferation assay using PBMC obtained from humans who had recovered from a recent malaria infection. Results of this assay are summarized in Table 1. We have taken an SI of 2 or more to indicate a positive result; in Table 1, we have presented only the positive results obtained in this assay. The proliferative response made by PBMC from five normal, healthy controls was uniformly poor to negligible. For four of these patients, the values obtained in the unstimulated cultures were 283 ± 38, 652 ± 276, 299 ± 58, and 295 ± 36 cpm and the corresponding highest values following stimulation with any ABRA construct were 550 ± 111, 744 ± 172, 390 ± 72, and 296 ± 24 cpm; in terms of the SI, these values represented negative results (SI < 2). In the remaining one normal subject, the unstimulated cultures gave a value of 774 ± 48 cpm and the corresponding highest value obtained was 1,875 ± 488 cpm, with AB-3, yielding an SI of 2.4; with all other constructs, SIs below 2 were obtained. As evident from the data we have provided in Table 1, most of the peptides worked optimally at a dose of 1 μg/well in this assay. We found that AB-1 and AB-3 induced generally high levels of lymphoproliferation in almost all 11 subjects tested, with SIs ranging from <2 (in only one case) to 16.91 (Table 1); two more peptides, AB-4 and AB-5, were also found to elicit a strong proliferation response in 3 of 11 subjects each, with SIs varying from 2.4 to 19.76 (Table 1). The proliferative responses obtained with other peptide constructs were below the threshold of positivity; the AB-8 peptide, which represents a tandem repeat of KEE and KE, proved to be the most ineffective T epitope in this assay (data not presented).

Having ascertained that our synthetic constructs, indeed, represented targets of the human immune response generated during natural malaria infection, we proceeded to assess these as carrier-free immunogens in experimental laboratory animals. As evident from the results presented in Fig. 3, five of the six nonrepetitive peptides, viz., AB-1, AB-3, AB-4, AB-5, and AB-6, when injected into rabbits, elicited a boostable IgG antibody response with high titers persisting for several weeks after the last immunization. One of the two repetitive sequence peptides, i.e., AB-8, also induced a similar response. However, the remaining two constructs, namely, AB-2 and AB-7, did not stimulate any detectable antibody response even after another booster.

Cross-reactivity of antipeptide sera with parasite protein(s) and recombinant ABRA constructs. Furthermore, rabbit antibodies generated against synthetic peptides also cross-reacted with the native parasite protein in three different assays. Results of an ELISA using parasite lysate as the capture antigen are presented in Fig. 4. Although the general pattern of the time course of antibody reactivity with the parasite lysate antigen was comparable to that obtained with the peptides used as capture antigens (Fig. 3), the level of reactivity with the parasite antigen was predictably low but well within the range expected of antibodies raised with short, carrier-free peptide immunogens (38). A careful analysis of data obtained with the preimmune (week 0) and test (week 8) sera revealed that in the hierarchy of responses obtained with the peptide ELISA (Fig. 3), AB-6, with an OD of test serum/OD of preimmune serum ratio (T/P ratio) of 44.5, ranked at the top, followed by AB-4 (T/P ratio, 39.2), AB-8, AB-1, AB-5, and AB-3; as mentioned earlier, the remaining two peptides, AB-2 and AB-7, induced barely detectable levels of antibody response, their T/P ratios

TABLE 1. Lymphocyte proliferative responses in *P. falciparum* patients determined with ABRA peptides as stimulating antigens

Patient's age (yr)	Patient's sex[a]	Response in unstimulated cultures (cpm [mean ± SD])	Highest SI obtained[b]			
			AB-1	AB-3	AB-4	AB-5
28	F	241 ± 73	8.95	14.85	<2	<2
24	F	220 ± 66	<2	2.40[c]	<2	<2
35	M	283 ± 54	3.38	10.04	<2	<2
20	M	815 ± 123	7.14	5.20	3.52	5.36
15	M	459 ± 115	7.80	4.70[c]	<2	<2
16	M	364 ± 248	4.91	6.90[c]	7.19[c]	2.40[c]
11	F	425 ± 228	2.45	3.94	<2	<2
14	M	230 ± 26	14.25	14.47	<2	<2
50	M	207 ± 70	13.22[c]	16.91	11.80	19.76
22	F	272 ± 41	5.09	5.20[c]	<2	<2
12	F	363 ± 68	4.16	8.60[c]	<2	<2

[a] F, female; M, male.

[b] SI, counts per minute in stimulated cultures/counts per minute in unstimulated cultures.

[c] Dose of stimulating peptide, 0.1 μg/well; all other values were obtained at a 1-μg/well dose of the respective peptide; responses observed with the remaining ABRA peptides gave SIs which were all <2 and, therefore, are not shown in this table.

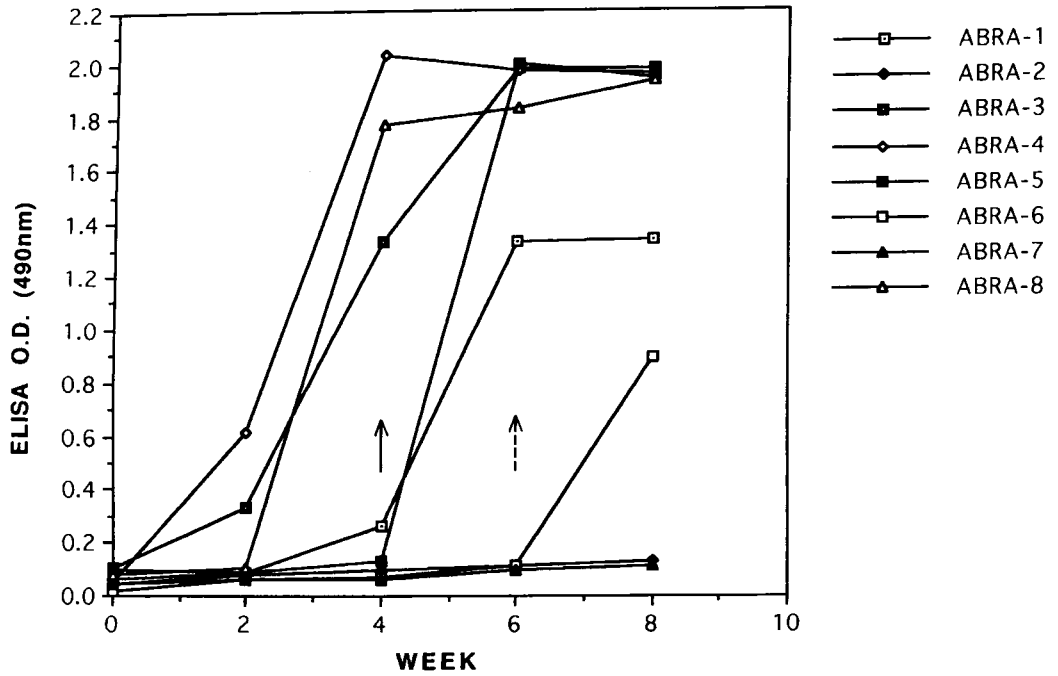

FIG. 3. Time course of IgG-type antibody responses generated in rabbits as monitored by an ELISA using homologous peptides as the capturing antigens. Rabbits immunized with the carrier-free ABRA peptides received boosters at week 4 (solid arrow); animals immunized with AB-2, AB-6, and AB-7 received boosters one more time at week 6 (broken arrow). Each serum was tested at a single dilution of 1/200 in duplicate wells.

being 3.25 and 2.75, respectively. With the parasite antigen (Fig. 4), on the other hand, the highest T/P ratio (15.8) was obtained with AB-3 followed by AB-1, AB-4, AB-6, and AB-5; the T/P ratios obtained for the remaining sera were indicative of marginal levels of antibody as detected in this assay. The most notable feature of the results obtained in this assay was the poor cross-reactivity of anti-AB-8 antibodies with the parasite antigen (T/P ratio, 1.9), in stark contrast to its very high peptide-specific reactivity (T/P ratio, 27.71). In the immunoblot assay, all six of the high-titer, antipeptide sera cross-reacted with the parasite protein(s); thus, the rabbit antisera, raised against AB-1, AB-3, AB-4, AB-5, AB-6, and AB-8, all reacted with a parasite protein of the expected size, i.e., ~101 kDa (Fig. 5, lanes 9, 11 to 14, and 16, respectively); furthermore, sera against AB-1, AB-3, AB-4, and AB-5 also recognized some additional protein bands of lower molecular weight

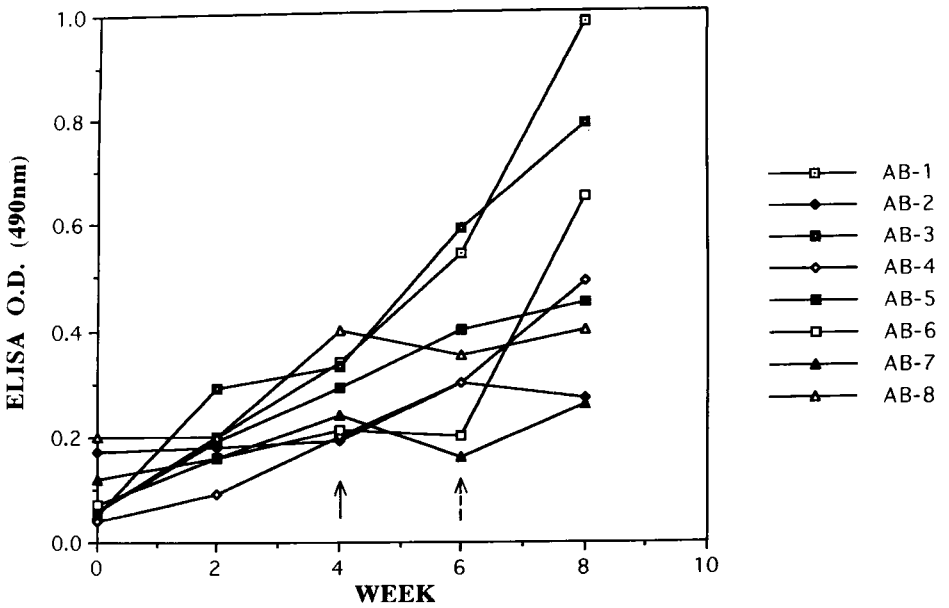

FIG. 4. Cross-reactivity of rabbit antipeptide antibodies with the parasite protein(s) as monitored in an ELISA using parasite lysate prepared from the asexual blood stages of *P. falciparum* as the capturing antigen. Each serum was tested at a single dilution of 1/200 in duplicate wells. See the legend to Fig. 3 for additional details.

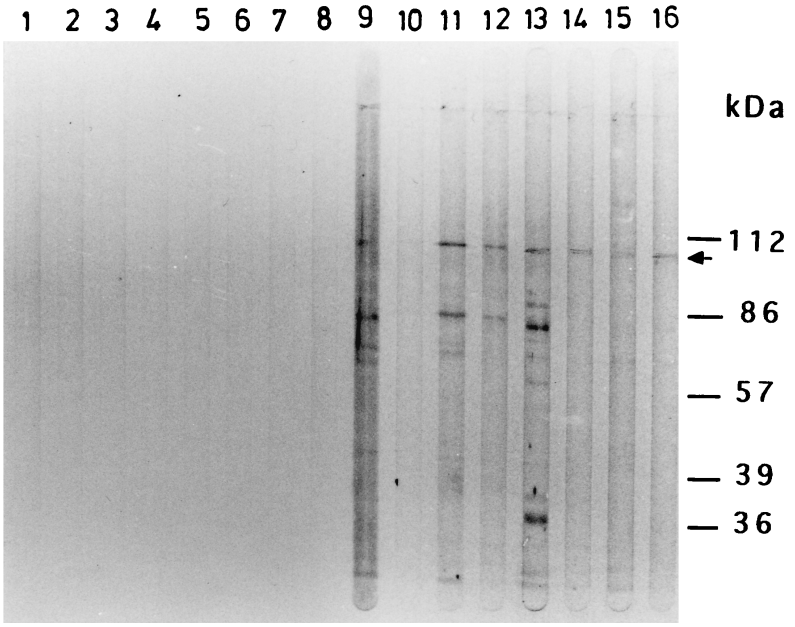

FIG. 5. Cross-reactivity of rabbit antipeptide sera with the parasite protein(s) in an immunoblot assay. The parasite proteins extracted from the asexual blood stages of *P. falciparum* were separated on an SDS–10% PAGE gel, transferred onto a nitrocellulose membrane, and probed with different antipeptide sera by using a Bio-Rad Mini Protean II Multi-Screen apparatus. Lanes 1 to 8 were probed with the preimmune sera, and lanes 9 to 16 were probed with the respective test sera. Thus, the lanes are for sera as follows: 1 and 9, AB-1 sera; 2 and 10, AB-2 sera; 3 and 11, AB-3 sera; 4 and 12, AB-4 sera; 5 and 13, AB-5 sera; 6 and 14, AB-6 sera; 7 and 15, AB-7 sera; and 8 and 16, AB-8 sera. Apart from the specific protein at approximately 101 kDa (arrow), some other bands were also detected, which may be degradation products of ABRA.

in the parasite lysate; we observed a rather weak recognition of several parasite proteins, including one at ~101 kDa, with the anti-AB-7 serum, which had shown virtually no antibody titer in ELISAs with the peptide or the parasite lysate. As expected, rabbit anti-AB-2 serum recognized no parasite protein in the immunoblot assay; none of the rabbit preimmune sera reacted with any parasite protein (Fig. 5, lanes 1 to 8).

In another immunoblot, rabbit sera against AB-1, AB-3, and AB-8 strongly recognized the recombinant ABRA fragments AB-N, AB-M, and AB-C, respectively, expressed as maltose-binding protein fusion proteins in the IPTG-induced bacterial cultures (Fig. 6, lanes I); these sera gave no such reaction with the uninduced bacterial cultures. Although, these sera were preadsorbed with bacterial lysates prepared from the host *E. coli* strain lacking the specific inserts, their cross-reactivity with some bacterial proteins persisted (Fig. 6).

The immunofluorescence assay (IFA) further established the cross-reactivity of antipeptide sera with the parasite antigen. The antibodies stained the trophozoites and the protein present in the parasitophorous vacuole or even in the tubovesicular membrane network (Fig. 7A and B). Furthermore, antibodies also seemed to stain merozoites within the mature schizonts (Fig. 7C and D). This range of reactivity was observed most notably with sera from rabbits immunized with AB-1, AB-5, and AB-8, although all ELISA-positive sera showed at least some reactivity in this assay, as apparent from the IFA titers given in Table 2. However, we found no strict correlation between the levels of seroreactivity we ob-

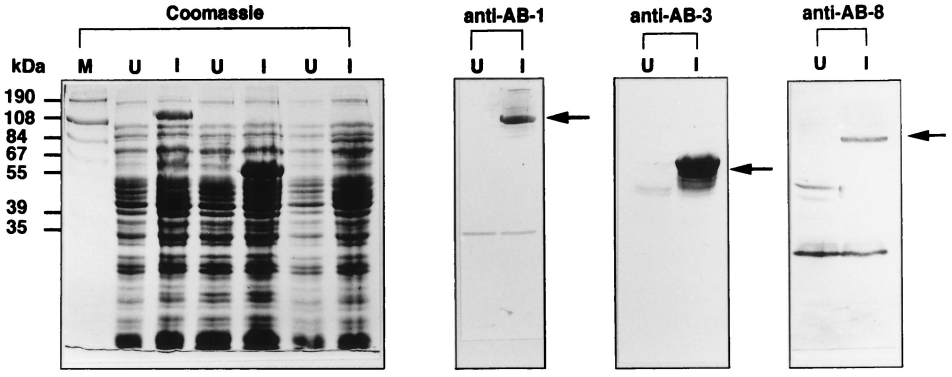

FIG. 6. Three recombinant constructs representing the N-terminal region (AB-N), the middle region (AB-M), and the C-terminal region (AB-C) of ABRA were expressed as recombinant fusion proteins in *E. coli* under conditions of IPTG induction (lanes I) and immunoblotted with rabbit anti-AB-1 (AB-1), anti-AB-3 (AB-3), and anti-AB-8 (AB-8) sera. ABRA constructs of the expected sizes (arrows) were specifically recognized by the respective sera; no such reactivity was noticed in the control, uninduced bacterial cultures (lanes U).

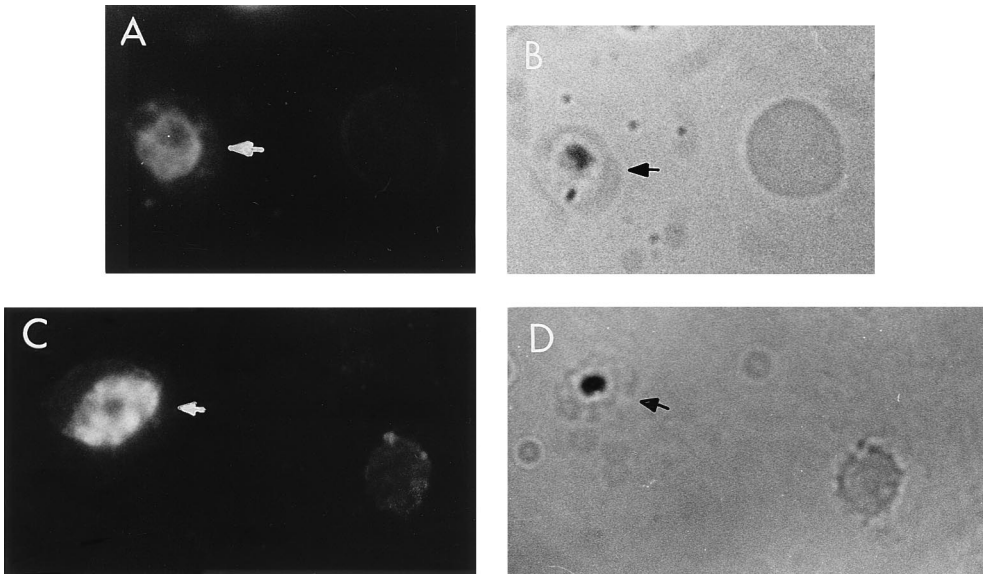

FIG. 7. Immunofluorescence on air-dried, acetone-fixed monolayers of *P. falciparum*-infected erythrocytes (arrows) probed with rabbit anti-AB-1 serum. Shown are a brightly fluorescent trophozoite (A and B) and merozoites (C and D) within a mature schizont seen under UV illumination (A and C) and visible light (B and D); uninfected erythrocytes which did not react with antibody are also seen.

tained by ELISA and by IFA. Thus, although the peptide ELISA end point titers of AB-1, AB-5, and AB-8 were 1/12,800, 1/25,600, and 1/51,200, respectively, their IFA titers (1/160) were similar (Table 2). It is pertinent to point out here that in ELISA, sera were tested at twofold serial dilutions. Therefore, although the three ELISA titers mentioned above may seem to vary over a wide range, they actually fall within merely three twofold serial dilutions.

Merozoite invasion inhibitory activity of the antipeptide sera. Having established the immunogenicity of ABRA constructs in rabbits directly by immunization and in humans as inferred from our immunological observations of the clinical samples, we considered it logical to ascertain the biological function, if any, of the peptide-specific antibodies. In order to accomplish this, we tested the rabbit anti-peptide sera for activity against the parasite in a growth inhibition assay. We tested sera from only six rabbits that had shown positive antibody responses to six different ABRA constructs, as shown in Fig. 3. The results of the merozoite invasion inhibition assay are shown in Table 2. Surprisingly, the two sera, anti-AB-4 and anti-AB-3, which had yielded the high parasite-specific ELISA titers had only a marginal effect (anti-AB-3) or virtually no effect (anti-AB-4) on the merozoite invasion of the erythrocytes; two other sera (anti-AB-6 and anti-AB-8) caused only about 50% inhibition of invasion, while the remaining two sera (anti-AB-1 and anti-AB-5), which had ELISA titers of only 1/12,800 and 1/25,600, respectively, inhibited the parasite invasion by 94.4 and 77.6%, respectively (Table 2). That this inhibition was, indeed, mediated through antibodies and not any other serum component was further corroborated by the results we obtained with purified IgG fractions from the pre-

TABLE 2. Antiparasite activity in rabbit antipeptide sera

Immunogen	Peptide ELISA titer^{-1}	IFA titer^{-1}	% Parasitemia[a]		% Inhibition[b]
			Preimmune serum (5%)	Immune serum (5%)	
AB-1	12,800	160	2.13 ± 0.11	0.12 ± 0.06	94.3
AB-3	25,600	80	2.45 ± 0.15	1.75 ± 0.25	28.5
AB-4	102,400	40	2.18 ± 0.07	2.18 ± 0.11	0
AB-5	25,600	160	2.42 ± 0.17	0.54 ± 0.21	77.6
AB-6	102,400	80	2.49 ± 0.16	1.16 ± 0.16	53.4
AB-8	51,200	160	2.85 ± 0.10	1.23 ± 0.14	56.8
18-mer$_{(TRAP)}$[c]	25,600	160	2.11 ± 0.06	0.97 ± 0.23	54.0
P-8$_{(MSP-1)}$[d]	16,000	ND	2.44 ± 0.22	2.14 ± 0.26	12.3

[a] Parasitemia at 20 h is presented; percent parasitemia at 0 h was 0.23; data are means ± standard deviations obtained in triplicate wells for each serum in the merozoite invasion inhibition assay.

[b] Percent inhibition in the merozoite invasion assay was calculated as [(% parasitemia in preimmune serum − % parasitemia in immune serum)/% parasitemia in preimmune serum] × 100.

[c] Positive control (38). Percent parasitemia is presented as that observed in the presence of total IgG (600 μg/ml) from preimmune and immune rabbit sera.

[d] Negative control (37, 38).

TABLE 3. Inhibition of merozoite reinvasion of human erythrocytes by rabbit antipeptide total IgG fractions

Immunogen	Total IgG (mg/ml)	% Parasitemia (rings only)[a]		% Inhibition
		Preimmune IgG	Immune IgG	
AB-1	0.5	5.07 ± 0.29	2.10 ± 0.30	58.57
	1.0	4.73 ± 0.11	0.63 ± 0.12	86.68
	2.0	4.65 ± 0.17	0.35 ± 0.05	92.47
	4.0	4.70 ± 0.21	0.28 ± 0.06	94.04
AB-5	0.5	4.65 ± 0.27[b]	1.87 ± 0.09	59.78
	1.0	4.65 ± 0.27[b]	1.07 ± 0.11	76.98
	2.0	4.65 ± 0.27[b]	0.43 ± 0.03	90.80
	4.0	4.65 ± 0.27[b]	0.28 ± 0.04	93.98
AB-4	4.0	4.53 ± 0.06	4.49 ± 0.53	0.88

[a] Parasitemia at 20 h is presented; percent parasitemia at 0 h was 0.87; data are means ± standard deviations obtained in triplicate wells for each serum.

[b] Parasitemia obtained in the presence of rabbit preimmune IgG (0.5 to 4 mg/ml) was similar; therefore, the data from each of these sera was pooled to obtain the best estimate of the mean.

immune and immune rabbit sera in this assay. As apparent from the data presented in Table 3, total immune IgG purified from the sera of rabbits immunized with AB-1 or AB-5 exerted an inhibitory effect on the in vitro merozoite invasion of erythrocytes in a dose-dependent manner. In contrast, a similarly purified IgG fraction from another rabbit, immunized with AB-4 and showing high levels of peptide-specific antibodies (Table 2), had no adverse effect on the merozoite invasion of erythrocytes (Table 3).

DISCUSSION

Of several secretory proteins, such as the S antigen, the serine-rich protein, the glycophorin binding protein, etc., which *P. falciparum* liberates during its asexual erythrocytic cycle, ABRA alone shows virtually no polymorphism (23). In fact, ABRA represents one of the most conserved antigenic proteins of *P. falciparum*; in a comparative study of three laboratory isolates of the parasite (namely, IMTM-22, FCR-3, and Camp), Chulay and colleagues (14) observed little difference in the size of this protein. Moreover, genes encoding this protein are nearly identical, with only four differences in the ABRA nucleotide sequences from the two isolates FCR-3 and Camp, which otherwise differ significantly in the sequence of the serine-rich, 126-kDa protein (14, 45) as well as in the molecular weights of several other proteins recognized by the growth-inhibitory antibodies (13, 43). At the same time, no protein analogous to ABRA from any other malarial parasite has been described so far, nor has any strong homology between ABRA and any other housekeeping proteins been found in the database search (45). These observations seem to underline the uniqueness of this protein and its potentially significant role in the biology of the parasite. In fact, there is immunological as well as biochemical evidence to suggest the possible involvement of ABRA in the processes of rupture of mature schizonts, release of merozoites, and invasion of fresh erythrocytes. In an elegant study, Nwagwu and colleagues (33) demonstrated chymotrypsin-like proteinase activity associated with the affinity-purified, parasite-derived ABRA protein. Such a proteinase(s) has been implicated in the release of mature merozoites from the parasitized erythrocytes and in the merozoite invasion of fresh erythrocytes (4, 19, 26). ABRA might well be one such proteinase. Thus, it represents an attractive target for chemotherapeutic and immunological intervention. However, there has not been any study on the mapping of epitopic sequences in this protein and their possible immunogenicity and protectivity against the parasite.

We have, therefore, concentrated mainly on the hydrophilic regions of the protein which, we reasoned, would be more accessible for generating an antibody response. In the present study, we have used eight synthetic peptides to delineate putatively protective epitopic sequences in ABRA. Our results with the human sera tend to support our contention, since we found that a majority of sera did contain appreciable levels of antibodies directed against these sequences. It is of interest that the synthetic construct AB-3 yielded the highest ELISA positivity (50%) as well as the highest OD values for serum samples tested. Furthermore, in a lymphocyte proliferation assay with PBMC of human subjects, we found that four of these constructs were recognized as T-helper-cell epitopes as well. Interestingly, AB-1 and AB-3 elicited strong proliferation responses in all 11 subjects tested (Table 1). Although we did not determine the HLA haplotype of these patients, it seems plausible that they represented more than one haplotype, and to that extent, AB-1 and AB-3 appear to be degenerate in their ability to bind to genetically restricted different HLA haplotypes and stimulate T-cell responses. If that indeed is the case, we would have two promiscuous T-helper-cell epitopes available from an asexual blood stage protein of the parasite. We are currently looking into this possibility in greater detail in both mice and humans.

It was encouraging to find that all eight constructs of ABRA we synthesized for the present study indeed represented epitopic targets of antibody responses generated during the natural infections with *P. falciparum* (Fig. 2). But we realize that the mere presence of circulating antibody in serum as measured in an ELISA provides little information about the possible protective potential of these sequences as immunogens. It seemed to us quite pertinent to assess the immunogenicity of these constructs in an experimental animal model and to ascertain the potential antiparasite activity of the experimentally raised, peptide-specific antibodies.

Interestingly, five of the six nonrepetitive sequences (AB-1 to AB-6) and one of the two repetitive sequences (AB-7 and AB-8), without the use of any carrier protein, induced boostable, IgG-type antibody responses in rabbits (Fig. 3); only AB-2 and AB-7 failed to generate any boostable antibody response. The peptide-specific antibodies cross-reacted with the parasite protein(s) in an ELISA (Fig. 4) and an IFA (Fig. 7). In an immunoblot also, rabbit anti-AB-5 serum recognized a protein band at about 101 kDa and two more bands with lower molecular masses, which could be the autoproteolytic products of ABRA or cross-reactive epitopes present in other proteins; a similar pattern of bands was seen with the rabbit sera against other immunogenic ABRA constructs (Fig. 5). To an extent defined by these results, the synthetic constructs seem to mimic the portions of native protein faithfully enough to induce antibodies which recognized the authentic parasite protein in three different immunoassays. More significantly, we found that antibodies to four synthetic constructs also exerted antiparasite activity, causing 40 to 90% inhibition of the in vitro merozoite invasion of erythrocytes with as little as 5% antiserum incorporated in the culture system (Table 2). Although it remains far from being fully established how antibodies exert their influence upon the intracellular parasite, several workers have used this assay as a suitable in vitro correlate of potentially protective antibody (13, 34, 39, 43, 44). In the case of ABRA, monoclonal antibodies have been demonstrated to agglutinate freshly released merozoites, thus preventing reinvasion of erythrocytes (14). Rabbit antibodies which we raised against synthetic peptides modeling parts of ABRA may also function in a similar way. However, it is also likely that antibodies to ABRA or its parts might be working by blocking its protease activity, which, along with other proteases, is possibly involved in the secondary processing of MSP-1, a process shown to be critical for producing invasive merozoites (19).

Notwithstanding the apparent immunodominance of AB-3 in humans, as evident from the serological results (Fig. 2), rabbit anti-AB-3 serum, though yielding a reasonably high ELISA titer, was found to be poor in its direct antiparasite activity; it caused only ~30% inhibition of the parasite in vitro; similarly, AB-4-specific rabbit antibodies had virtually no direct inhibitory effect on parasite growth. However, these negative results do not rule out the possibility of their growth-inhibitory potential in cooperation with monocytes. In fact, a number of studies investigating the importance and relevance of this mechanism of parasite clearance have provided convincing evidence for such a phenomenon occurring in malaria (8, 10, 17, 18).

Synthetic peptides such as those evaluated in the present study provide a promising alternative to conventional vaccines

or those being produced by recombinant DNA technology. A number of studies have demonstrated the feasibility of using such constructs as potentially protective immunogens (1, 12). In the first-ever human clinical trials of a synthetic peptide vaccine, a polymeric, multicomponent malaria vaccine, SPf66, has been found to be safe and immunogenic in both adults and children residing in widely distant geographical regions with different transmission rates (1, 31, 32), although its efficacy has become a subject of controversy and contradiction (31, 32). At the same time, results of these trials have underlined the need to continuously search for other molecules and for better ways to generate a protective immune response. Our present study, which delineates a couple of putatively protective epitopes in ABRA, represents only the first essential step in our ongoing efforts to evaluate this protein as a possible vaccine candidate. An important question would relate to the immunogenicity of these peptide constructs in the context of different major histocompatibility complex haplotypes on the one hand and various adjuvant formulations, other than Freund's, on the other hand. Studies have indicated that adjuvants could play a critical role in determining the magnitude and specificity of immune responses to a particular epitope(s) (21, 41a). We already have preliminary data to indicate that AB-1 and AB-5 are recognized in the context of several different major histocompatibility complex haplotypes of mice, although the titers of antibodies were not very high (unpublished observations). Using different adjuvant formulations, we are now in the process of establishing the repertoire of humoral responses to ABRA in terms of IgG subclasses of antibodies induced by various ABRA constructs, the pattern of their affinity maturation for the antigen, and their qualitative or functional features, such as their growth-inhibitory or merozoite invasion-inhibitory potential.

ACKNOWLEDGMENTS

We are grateful to P. P. Singh and Surendrra K. Rajpurohit of the Biochemistry Department, Rabindra Nath Tagore Medical College, and B. Shahi of the Malaria Research Centre (Indian Council of Medical Research), Field Station, Shankargarh, Allahabad (Uttar Pradesh), India, for invaluable help in obtaining human blood samples. Expert technical assistance rendered by Narendra Singh Negi in handling animals is gratefully acknowledged.

This investigation received partial financial support from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR Project ID no. 960578).

REFERENCES

1. **Alonso, P. L., T. Smith, J. R. M. Armstrong Schellenberg, H. Masanja, S. Mwankusye, H. Urassa, I. Bastos de Azevedo, J. Chongela, S. Kobero, C. Menendez, N. Hurt, M. C. Thomas, E. Lyimo, N. A. Weiss, R. Hayes, A. Y. Kitua, M. C. Lopez, W. L. Kilama, T. Teuscher, and M. Tanner.** 1994. Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania. Lancet **344:**1175–1181.
2. **Anders, R. F.** 1986. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. Parasite Immunol. **8:**529–539.
3. **Anders, R. F., and J. A. Smythe.** 1989. Polymorphic antigens in *Plasmodium falciparum*. Blood **74:**1865–1875.
4. **Banyal, H. S., G. C. Misra, C. M. Gupta, and G. P. Dutta.** 1980. Involvement of malaria proteases in the interaction between the parasite and the host erythrocyte in *Plasmodium knowlesi* infections. J. Parasitol. **67:**623–626.
5. **Biggs, B. A., L. Gooze, K. Wycherly, W. Wollish, B. Southwell, J. H. Leech, and G. V. Brown.** 1991. Antigenic variation in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **88:**9171–9174.
6. **Blackman, M. J., H. G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. **172:**379–382.
7. **Blackman, M. J., I. T. Ling, S. C. Nicholls, and A. A. Holder.** 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor like domains. Mol. Biochem. Parasitol. **49:**29–34.
8. **Bouharoun-Tayoun, H., P. Attanath, A. Sebchareon, T. Chongsuphajaisiddhi, and P. Druilhe.** 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. **172:** 1633–1641.
9. **Brannan, L. R., C. M. R. Turner, and R. S. Phillips.** 1994. Malaria parasites undergo antigenic variation at high rates *in vivo*. Proc. R. Soc. Lond. Biol. Sci. **256:**71–75.
10. **Celada, A., A. Cruchaud, and L. H. Perrin.** 1983. Phagocytosis of *Plasmodium falciparum* parasitized erythrocytes by human polymorphonuclear leukocytes. J. Parasitol. **69:**49–53.
11. **Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos, and V. Nussenzweig.** 1992. The basolateral domain of the hepatocyte membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell **70:**1021–1033.
12. **Chauhan, V. S., S. Chatterjee, and P. K. Johar.** 1993. Synthetic peptides based on conserved *Plasmodium falciparum* antigens are immunogenic and protective against *P. yoelii* malaria. Parasite Immunol. **15:**239–242.
13. **Chulay, J. D., J. D. Haynes, and C. L. Diggs.** 1985. Serotypes of *Plasmodium falciparum* defined by immune serum inhibition of *in vitro* growth. Bull. W. H. O. **63:**317–323.
14. **Chulay, J. D., J. A. Lyon, J. D. Haynes, A. I. Meierovics, C. T. Atkinson, and M. Aikawa.** 1987. Monoclonal antibody characterization of *Plasmodium falciparum* antigens in immune complexes formed when schizonts rupture in the presence of immune serum. J. Immunol. **139:**2768–2774.
15. **De La Cruz, V., W. L. Maloy, L. H. Miller, M. F. Good, and T. F. McCutchan.** 1989. The immunological significance of variation within malaria CSP sequences. J. Immunol. **142:**3568–3575.
16. **Garber, G. E., L. T. Lemchuk-Favel, K. C. Meysick, and K. Dimock.** 1993. A *Trichomonas vaginalis* cDNA with partial protein sequence homology with a *Plasmodium falciparum* excreted protein ABRA. Appl. Parasitol. **34:**245–249.
17. **Green, T. J., M. Morhardt, R. G. Brackett, and R. L. Jacobs.** 1981. Serum inhibition of merozoite dispersal from *Plasmodium falciparum* schizonts: indicator of immune status. Infect. Immun. **31:**1203–1208.
18. **Gysin, J., S. Gavoille, D. Mattei, A. Scherf, S. Bonnefoy, O. Mercereau-Puijalon, T. Feldman, B. Muller-Hill, and L. Pereira da Silva.** 1993. In vitro phagocytosis inhibition assay for the screening of potential candidate antigens for sub-unit vaccines against the asexual blood stage of *Plasmodium falciparum*. J. Immunol. Methods **159:**209–219.
19. **Hadley, T., M. Aikawa, and L. H. Miller.** 1983. *Plasmodium knowlesi*: studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. Exp. Parasitol. **55:**306–311.
20. **Holder, A. A., J. A. Chappel, and M. J. Blackman.** 1994. Malaria merozoite surface protein-1 structure and processing: targets to interfere with the parasite life-cycle, p. 99–106. *In* G. P. Talwar, K. V. S. Rao, and V. S. Chauhan (ed.), Recombinant and synthetic vaccines. Narosa Publishing House, New Delhi, India.
21. **Hui, G. S. N., S. P. Chang, H. Gibson, A. Hashimoto, C. Hashiro, P. J. Barr, and S. Kotani.** 1991. Influence of adjuvants on the antibody specificity to the *Plasmodium falciparum* major merozoite surface protein, gp195. J. Immunol. **147:**3935–3941.
22. **Jones, T. R., and S. L. Hoffman.** 1994. Malaria vaccine development. Clin. Microbiol. Rev. **7:**303–310.
23. **Kemp, D. J., A. F. Cowman, and D. Walliker.** 1990. Genetic diversity in *Plasmodium falciparum*. Adv. Parasitol. **29:**75–145.
24. **Kumar, A., R. Arora, P. Kaur, V. S. Chauhan, and P. Sharma.** 1992. 'Universal' T helper determinants enhance immunogenicity of a *Plasmodium falciparum* merozoite surface antigen peptide. J. Immunol. **148:**1499–1505.
25. **Lambros, C., and J. P. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65:**418–420.
26. **Lyon, J. A., and J. D. Haynes.** 1986. *Plasmodium falciparum* antigens synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of protease inhibitors. J. Immunol. **136:**2245–2251.
27. **Marshall, V. M., R. L. Anthony, M. J. Bangs, S. Purnomo, R. F. Anders, and R. L. Coppel.** 1994. Allelic variants of the *Plasmodium falciparum* merozoite surface antigen 2 (MSA-2) in geographically restricted area of Irian Jaya. Mol. Biochem. Parasitol. **63:**13–21.
28. **Miller, L. H., M. Aikawa, and J. A. Dvorak.** 1975. Malaria (*Plasmodium knowlesi*) merozoites: immunity and the surface coat. J. Immunol. **114:**1237–1242.
29. **Miller, L. H., T. Roberts, M. Shahabuddin, and T. F. McCutchan.** 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol. Biochem. Parasitol. **59:**1–14.
30. **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.

30a. **National Institutes of Health.** 1985. Guide for the care and use of laboratory animals. National Institutes of Health publication no. 86–23. U. S. Depart-

ment of Health and Human Services, Washington, D.C.
31. **Nosten, F., C. Luxemburger, D. E. Kyle, W. R. Ballou, J. Wittes, E. Wah, T. Chongsuphajaisiddhi, D. M. Gordon, J. C. Sadoff, and D. G. Heppener.** 1997. Randomized double-blind placebo-controlled trial of SPf66 malaria vaccine in children in southwestern Thailand. Lancet **348:**701–708.
32. **Nosten, F., C. Luxemburger, D. E. Kyle, D. M. Gordon, W. R. Ballou, J. C. Sadoff, A. Brockman, B. Permpanich, T. Chongsuphajaisiddhi, and D. G. Heppener.** 1997. Phase I trial of the SPf66 malaria vaccine in a malaria-experienced population in southeast Asia. Am. J. Trop. Med. Hyg. **56:** 526–532.
33. **Nwagwu, M., J. D. Haynes, P. A. Orlandi, and J. D. Chulay.** 1992. *Plasmodium falciparum*: chymotryptic-like proteolysis associated with a 101-kDa acidic-basic repeat antigen. Exp. Parasitol. **75:**399–414.
34. **Reese, R. T., and M. A. Motyl.** 1979. Inhibition of the in vitro growth of *Plasmodium falciparum*. I. Effects of immune serum and purified immunoglobulin from owl monkeys. J. Immunol. **123:**1894–1899.
35. **Roberts, D. J., A. G. Craig, A. R. Berendt, R. Pinches, G. Nash, K. Marsh, and C. I. Newbold.** 1992. Rapid switching to multiple antigenic and adhesive phenotype in malaria. Nature (London) **357:**689–692.
36. **Saul, A., R. Lord, G. L. Jones, and L. Spencer.** 1992. Protective immunization with invariant peptide of the *Plasmodium falciparum* antigen MSA-2. J. Immunol. **148:**208–211.
37. **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.
38. **Sharma, P., A. Bharadwaj, V. K. Bhasin, V. N. Sailaja, and V. S. Chauhan.** 1996. 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. Infect. Immun. **64:** 2172–2179.
39. **Sim, B. K. L., P. A. Orlandi, J. D. Haynes, F. W. Klotz, J. M. Carter, D. Camus, M. E. Zegans, and J. D. Chulay.** 1990. Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. J. Cell Biol. **111:**1877–1884.
40. **Snewin, V. A., M. Herrera, G. Sanchez, A. Scherf, G. Langsley, and S. Herrera.** 1991. Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. Mol. Biochem. Parasitol. **49:**265–276.
41. **Stahl, H. D., A. E. Bianco, P. E. Crewther, R. F. Anders, A. P. Kyne, R. L. Coppel, G. F. Mitchell, D. J. Kemp, and G. V. Brown.** 1986. Sorting large numbers of clones expressing *Plasmodium falciparum* antigens in *Escherichia coli* by differential antibody screening. Mol. Biol. Med. **3:**351–368.
41a. **Stoute, J. A., M. Slaoui, G. Heppner, P. Momin, K. E. Kester, P. Desmons, B. T. Wellde, N. Garcon, U. Krzych, M. Marchand, W. R. Ballou, and J. D. Cohen.** 1997. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. N. Engl. J. Med. **336:**86–91.
42. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
43. **Vernes, A., J. D. Haynes, P. Tapchaisri, J. J. L. Williams, E. Dutoit, and C. L. Diggs.** 1984. Strain-specific human antibody inhibits merozoite invasion of erythrocytes. Am. J. Trop. Med. Hyg. **33:**197–203.
44. **Wahlin, B., M. Wahlgren, H. Perlmann, K. Berzins, A. Bjorkman, M. E. Patarroyo, and P. Perlmann.** 1984. Human antibodies to a M_r 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. Proc. Natl. Acad. Sci. USA **81:**7912–7916.
45. **Weber, J. L., J. A. Lyon, and D. Camus.** 1987. Blood stage antigen genes of *Plasmodium falciparum*, p. 379–388. *In* N. Agabian, H. Goodman, and N. Nogueira (ed.), Molecular strategies of parasitic invasion. Alan R. Liss, Inc., New York, N.Y.
46. **Weber, J. L., J. A. Lyon, R. H. Wolff, T. Hall, G. H. Lowell, and J. D. Chulay.** 1988. Primary structure of a *Plasmodium falciparum* malaria antigen located at the merozoite surface and within the parsitophorous vacuole. J. Biol. Chem. **263:**11427–11431.

Editor: R. N. Moore