Seroprevalence and Specificity of Human Responses to the *Plasmodium falciparum* Rhoptry Protein Rhop-3 Determined by Using a C-Terminal Recombinant Protein

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Rhoptry proteins participate in invasion of erythrocytes by malaria parasites. Antibodies to some of these proteins can inhibit invasion and partially protect monkeys from disease. To examine human serological responses to the 110-kDa component (Rhop-3) of the high-molecular-weight rophtry protein complex, two cDNA clones corresponding to Rhop-3 were identified by immunologic screening. A recombinant protein representing the C-terminal one-third of the Rhop-3 was used to assess the seroprevalence to this protein in geographically isolated populations with different patterns of malaria transmission. The immunoglobulin G (IgG) positivity rate for the recombinant Rhop-3 in an enzyme-linked immunosorbent assay was 30% in an area of Papua New Guinea where malaria is holoendemic. In Kenya, the prevalence rates were 43 and 36%, respectively, in an area of hyperendemicity and an area of seasonal transmission. By contrast, rates of IgG seroprevalence to an extract of Gambian strain of *Plasmodium falciparum* were 48, 90, and 97% respectively, in these populations. In these areas, the pattern of antibody recognition of Rhop-3 is more similar (1.7-fold maximum difference) than the parasite extract (5-fold difference). The difference in seroresponses may represent antigenic polymorphism in different parasite strains, while their similarity for the Rhop-3 fragment may represent conservation of this protein. Recombinant- and parasite extract-specific IgG was not found in individuals infected only with *Plasmodium vivax*. Cross-reactivity was seen in the IgM assay. In Mombasa (Kenya), maternal and cord Rhop-3-specific IgG activities were similar. Fetal antigen-specific IgM reactivity was generally undetectable for all antigens.

Plasmodium falciparum rophtries are paired organelles that participate in the events leading to successful merozoite entry into the erythrocyte. Each pair of rophtries discharges its contents into the erythrocyte membrane during parasite invasion. Two of the important constituents of these organelles are non-covalently associated high- and low-molecular-weight protein complexes designated Rhop-H and Rhop-L (21). Rhop-H proteins are perhaps the less studied of the two complexes. The Rhop-H complex consists of three components, Rhop-1 (140 kDa), Rhop-2 (130 kDa), and Rhop-3 (110 kDa) (9). This complex is known to bind to erythrocyte membranes, inside-out vesicles, and liposomes, yet none of its components are integral membrane proteins (24), and it is unlikely that they contain glycosylphosphatidylinositol anchors (23). Brown and Coppel determined the complete genomic sequence for Rhop-3 and found that, in contrast to most other genes of *P. falciparum*, the Rhop-3 gene encoded no internal amino acid repeats, had a complex organization with seven exons, cross-hybridized to seven geographic strains, and was recognized by some human immune sera (6).

Both the Rhop-H and Rhop-L complexes are precipitated by antisera directed against their individual components, and studies indicate that antibodies against either complex are associated with resistance to infection in vivo (19) and inhibition of parasite invasion and growth in vitro (9, 24). Some humans in the course of *P. falciparum* infection develop antibodies to the rophtry complexes (6, 12). In animal models, immunization with the Rhop-H complex confers partial protection (25). These studies indicate that the rophtry proteins are involved in merozoite invasion; thus, the various rophtry proteins have been considered for use in vaccines (21).

In malaria, antibodies have an important role in resistance to infection and disease (17), and individuals in areas where malaria is endemic acquire humoral immunity directed against different asexual-stage antigens of *P. falciparum*. Significant antigenic diversity, however, has been observed in experimental primate or natural human infections (11, 14). Serological responses against different isolates of malaria parasite from the same area differ among individuals (32). The basis for some of this antigenic variation has been explained by the differential expression of members of a large gene family encoding an erythrocyte membrane protein, pEMP1 (5). Other parasite antigens, including EBA 175 and MSP1, also show a high degree of variability (17). Polymorphism of all important antigens of *P. falciparum*, therefore, should be examined.

Since the Rhop-H proteins are potentially important for parasite survival and host defense, we investigated the range of

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antibody responses following *P. falciparum* infection to the Rhop-3 protein in different epidemiologic, geographic, and clinical settings. It is important to determine whether *P. falciparum* antigens are immunologically conserved between different geographic areas and areas with different patterns of transmission, as well as to evaluate responses in groups most at risk for severe disease.

MATERIALS AND METHODS

Parasites. *P. falciparum* FCR3 (The Gambia), 7G8 (Brazil), HB3 (Honduras), and K1 (Thailand) isolates were maintained in type A⁺ human erythrocytes (obtained from the Cleveland Branch of the American Red Cross) at 5% hematocrit and 10% type A⁺ human serum (Sera-Tek Biologicals Inc., North Brunswick, N.J.) as described by Trager and Jensen (30). Synchronization of schizont-infected erythrocytes and parasite protein extraction in 1% Nonidet P-40–0.1% sodium deoxycholate were performed as described previously (22). Protein concentration was determined by the Bio-Rad assay (Bio-Rad Inc., Hercules, Calif.).

Antisera. Collections of sera were made with the informed consent of the subjects or guardians of the children and were approved by the appropriate authorities of the local government and the Institutional Review Board at Case Western Reserve University.

(i) Holoendemic transmission. In the Sepik River area of Papua New Guinea (PNG), 64 and 57 sera were collected from two areas where transmission of *P. falciparum*, *P. vivax*, and *P. malariae* occurs year round (26) and splenomegaly occurs in >75% of children. The PNG sera were collected in April and May 1995. Thick blood films were prepared for 64 samples from a village in Wosera District. The numbers of malaria-infected erythrocytes and the percent parasitemia were determined for each individual. Blood smears were positive for malaria in 33% of those examined. Samples were collected at random from 57 of 243 villagers in Dreikikir District. Blood smears from these villagers were not examined.

(ii) Hyperendemic transmission. Paired maternal and cord sera were collected from March to May 1995 from 131 Kenyan mothers and newborns; in addition, 40 unpaired sera were collected from pregnant women from the area of Mombasa where transmission is hyperendemic. In this area, *P. falciparum* is transmitted year round and splenomegaly rates are between 10 and 50%. Eight of the mothers were positive for *P. falciparum* at the time of labor. Histories of malaria and maternal parity were not available for most women in this sample. Of 300 maternal sera examined during the same period in the previous year, 10% were smear positive for malaria (12a). During the high-transmission season, 40 to 100% of schoolchildren have been found to have positive smears (27, 28).

(iii) Seasonal transmission. As part of a survey for hydatid disease, sera were collected in June 1994 from 33 individuals living in Turkana Province, Kenya, where malaria is transmitted only during 3 months of the rainy season. In this region, less than 40 inches (ca. 102 cm) of rain falls in a year, primarily during a 3-month period. The sera were collected immediately following the rainy season during the peak of malaria transmission.

(iv) Other parasitic infections. Sera were collected from four individuals infected only with *P. vivax*. Two were from India (courtesy of Deep C. Kaushal, Central Drug Research Institute, Lucknow, India), and two were from travelers to Laos and PNG. Twelve sera were obtained from schoolchildren in Bahia (Brazil) in 1993. These children were infected with *Schistosoma mansoni* and geohelminths (i.e., *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm) but no malaria parasites. Malaria is not transmitted in this region (31), which is more than 1,000 km away from areas where malaria exists. Since public transportation is undeveloped in the Bahia region, these schoolchildren are highly unlikely to be exposed to malaria parasites through travel.

(v) North American controls. Negative controls consisted of individual sera from seven healthy North American adults who had no history of travel to areas where malaria is endemic and a pool of normal North American human sera from the Cleveland Branch, American Red Cross.

The Brazilian sera and the North American controls sera were tested for *P. falciparum*-specific antibodies in an indirect immunofluorescence assay (IFA).

Rabbit polyclonal Rhop-H antisera 671 and 680 have been described previously (22). Rabbit antisera K15 and RAP3 were generously provided by George S. Hui and Christopher Locher, respectively (University of Hawaii, Honolulu). Both K15 and RAP3 antibodies recognize the Rhop-H complex in the immunoblotting assay.

Immunoscreening, hybridization, and nucleotide sequence analysis. A *P. falciparum* (strain 3D7) λ gt11 cDNA library was a gift from Naomi Lang-Unnasch (University of Alabama at Birmingham). Immunologic screening of the library was performed with combined polyclonal antisera K15 and RAP3 as described by Young and Davis (33). Before use in screening, the sera were affinity purified with Rhop-H antigens. The Rhop-H antigens in the parasite extract were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The nitrocellulose strips containing the Rhop-H antigens, bound to Rhop-H-specific antibodies, were cut out for the affinity purification of the specific rabbit antibodies. The

Rhop-H-specific immunoglobulin G (IgG) antibodies were eluted as described previously (4). The cDNA inserts were amplified by PCR and gel purified for subcloning.

To obtain a larger insert, cDNAs from a positive clone were radioactively labeled with [³²P]dATP by oligonucleotide random priming and used to further screen the library under high-stringency hybridization conditions (8). The blots were washed at 60°C in 0.1× standard saline citrate (SSC; 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. The clone with the largest insert was selected for further analysis.

Nucleotide sequences of the cDNA inserts were determined for both strands directly from phage DNA, using an fMol sequencing kit (Promega Inc., Madison, Wis.), on the basis of dideoxynucleotide termination of the PCR (7, 18). Initial sequencing primers consisted of λ gt11-specific oligomers, and subsequent primers were synthesized on the basis of the cDNA sequence. Oligonucleotides for sequencing were purchased from Gibco BRL (Grand Island, N.Y.). The BLAST program (3) was used to search the GenBank database. Sequence comparison and alignment were performed with GeneWorks version 2.0 computer software (IntelliGenetics Inc., Mountain View, Calif.).

Construction of recombinant plasmid; expression and purification of the RH3 3-5 recombinant protein. EcoRI-restricted and phosphorylated phage cDNA inserts were ligated into the multiple cloning site of the pRSET A, B, and C vectors (Invitrogen Inc., San Diego, Calif.) by T4 DNA ligase. The internal HindIII site of the insert was used to determine orientation of the constructs. In the pRSET vectors, transcription of the insert is controlled by a T7 promoter. The recombinant plasmids were transformed into *Escherichia coli* BL-21(DE3) (29). This bacterial strain contains a bacteriophage T7 RNA polymerase gene which is integrated into its chromosome downstream of a *lacZ* promoter. The T7 RNA polymerase is expressed under isopropyl- β -D-thiogalactopyranoside (IPTG) induction. In the recombinant, the vector contributes an N-terminal fusion peptide including a tag of six histidine residues for protein purification by nickel chelate chromatography (13).

The recombinant protein from clone RH3 3-5 was expressed in transformed bacteria for 2 h under 1.5 mM IPTG induction before harvesting. The activity of the expressed recombinant was verified by immunoblotting assay using Rhop-H-specific rabbit antiserum 680 (22), human serum from a patient with malaria from Zambia, pooled sera from 12 individuals living in an area with seasonal malaria transmission (Turkana, Kenya), and normal rabbit serum. The recombinant protein was then purified by pH step gradient on a Ni²⁺-nitrilotriacetic acid-agarose column (Qiagen Inc., Chatsworth, Calif.) under denaturing conditions (8 M urea, 0.1 M NaPO₄) as instructed by the manufacturer. The size and purity of the recombinant protein were determined by silver staining and Coomassie blue staining after SDS-PAGE and immunoblotting assay.

Generation of the recombinant-specific rabbit antiserum. One New Zealand White rabbit was immunized with 75 μ g of RH3 3-5 recombinant protein emulsified 1:1 with Titermax adjuvant (Cytrx Corp., Norcross, Ga.) and administered intramuscularly and subcutaneously. Thereafter, a total of three boosts were administered intramuscularly at 2-week intervals. Blood was collected before administration of immunogen and after each boost. The resultant RH3 3-5 recombinant-specific rabbit antiserum (antiserum 686) was screened for antibody reactivity by immunoblotting of the *P. falciparum* extract.

Immunoblotting, immunoprecipitation, IFA, and immunoelectron microscopy (IEM) assays. For immunoblotting assay, protein antigens were separated by SDS-PAGE on 5 to 15% gradient polyacrylamide gels and then transferred onto nitrocellulose membranes. The blots were incubated with antisera diluted 1:100 and then with horseradish peroxidase-conjugated protein A, and color was developed with 4-chloro-1-naphthol substrate. Identification of the expressed recombinant was based on the immunoblotting assay with Rhop-H-specific rabbit antiserum 680 and sera from *P. falciparum*-infected individuals which were preadsorbed against a lysate of induced *E. coli* BL-21(DE3) containing a pRSET vector without insert.

To determine the relationship between Rhop-3 and other components of the Rhop-H complex, immunoprecipitation was performed as described previously (20). [³⁵S]methionine-labeled schizont extracts were incubated with protein A-Sepharose previously complexed with rabbit antisera diluted 1:5 or 1:10 in precipitation buffer. Precipitation was carried out for 1 h at room temperature, and the beads were washed with precipitation buffer. Immune complexes were solubilized in electrophoresis sample buffer with dithiothreitol and analyzed by SDS-PAGE and fluorography.

IFA was performed on cold acetone-fixed segmented schizonts as described previously (22). The bound antibodies were visualized by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, Ind.) or fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Sigma Chemical Company, St. Louis, Mo.) under a Nikon Labophot microscope.

For IEM, mature segmented schizonts from strain FCR3 were fixed for 30 min at 4°C with 1% formaldehyde–0.1% glutaraldehyde in 0.1 M NaPO₄ (pH 7.4). The fixed samples were washed, dehydrated, and embedded in LR White resin (Polysciences Inc., Warrington, Pa.) as described previously (2). Thin sections were incubated for 30 min in phosphate-buffered saline (PBS) containing 1% bovine serum albumin fraction V (BSA) and 0.01% Tween 20. Grids were then incubated for 2 h at room temperature with recombinant-specific antiserum 686 in PBS-BSA-Tween 20 (PBT). Negative controls included normal rabbit serum and PBT applied as the primary antibody. After being washed in PBT, the grids

were incubated for 1 h with 15-nm gold-labeled goat anti-rabbit IgG (Amersham Life Science, Arlington, Ill.) diluted 1:20 in PBT, rinsed with PBT, fixed with glutaraldehyde to stabilize the gold particles, and then stained with uranyl acetate and lead citrate. Samples were examined in a Zeiss CEM902 electron microscope (Zeiss, Oberkochen, Germany).

Antigen-specific human IgG and IgM antibody assays. Recombinant-specific and parasite protein-specific total IgG and IgM antibodies in human sera were determined by enzyme-linked immunosorbent assay (ELISA). Pooled sera from three *P. falciparum*-infected individuals served as the positive control in the assay. The pooled positive control sera were tested for *P. falciparum*-specific antibodies by IFA. For the ELISA, the optimal concentration of antigens and dilution of human sera were selected by checkerboard titration using the positive control sera. The purified RH3 3-5 recombinant protein solubilized in 8 M urea-0.1 M NaPO₄ (pH 9.4) was diluted 1:250 with the coating buffer (0.2 M NaHCO₃ [pH 9.4]), as was the parasite protein extract from strain FCR-3 in 1% Nonidet P-40-0.1% deoxycholate. Flat-bottomed Immulon-4 ELISA plates (Dynatech Laboratories Inc., Chantilly, Va.) were coated with 50 µl of the recombinant or parasite extract (1 µg/ml) overnight at 4°C. After coating, the plates were washed six times in PBS with 0.05% Tween 20, blocked in 3% BSA-0.05% Tween 20 in PBS for 2 h at 37°C, and again washed extensively. Human sera were diluted 1:3,200 and 1:800 in PBS with 1% BSA-0.05% Tween 20 for the IgG and IgM assays, respectively, and incubated in the coated wells for 2 h at 37°C. The plates were washed and incubated with alkaline phosphatase-conjugated goat anti-human IgG (1:1,000) or IgM (1:3,000) (Jackson ImmunoResearch Laboratory, West Grove, Pa.) at room temperature for 1 h. After washing, the plates were developed with 10% diethanolamine (pH 9.6) and analyzed at 405 nm with an ELISA reader. The blank and standards were run in duplicate on each plate, while the test samples were run in triplicate. The blank represented the complete reaction without incubation with human antibodies. The standard was the reaction with positive control sera serially diluted (1:2). Mean optical density (OD) of the blank was subtracted from OD of the standard and the sample reactions on the same plate. Samples with OD lower than the blank were given an ELISA value of 0.000. One ELISA unit was defined as the mean OD of 3 dilutions of the positive control sera. ELISA unit values were calculated by using the following formula: (OD sample - OD blank)/(OD positive control - OD blank). The mean ELISA unit value is the arithmetic mean of the individual ELISA units of a population. The cutoff for a positive result was defined as an ELISA value >3 standard deviations above the mean of the North American negative controls.

Statistics. For comparing antigen-specific antibody activity, the ELISA units were log transformed, and a two-tailed Student *t* test was used to compare the transformed ELISA units between populations. Significance was defined as a *P* value of <0.05. Correlations between antigen-specific maternal and cord antibodies were analyzed by linear regression, and Pearson's correlation coefficient (*r*) was calculated.

RESULTS

Nucleotide sequence. Initial screening of the *P. falciparum* cDNA library with native Rhop-H affinity-purified rabbit antibodies identified one clone, RH3 2-1, whose insert was 0.5 kb by agarose gel electrophoresis. Another 21 clones were identified by hybridization with labeled RH3 2-1 cDNA. Clone RH3 3-5 was found to have the largest insert (1.2 kb) and therefore was used in the subsequent analyses. After nucleotide sequence determination, both RH3 2-1 and RH3 3-5 cDNAs were found to have more than 98% identity to the 3' region of the Rhop-3 genomic DNA (GenBank accession number M65059). Both cDNA sequences have a single long open reading frame; by DNA sequence analysis, RH3 2-1 and RH3 3-5 are 554 and 1,164 bp long, with the capacity to encode 184 and 296 amino acid residues, respectively. RH3 2-1 and RH3 3-5 began at nucleotides 3288 and 3078, respectively, of the genomic DNA. The RH3 3-5 cDNA extended over the last two exons of the genomic sequence and included the complete 3' noncoding region (Fig. 1). Six bases differed between the published genomic sequence and both the RH3 2-1 and RH3 3-5 cDNAs. Of these sequence differences, two, a cytosine-thymine transition at position 3285 and a thymine-adenosine transversion at 3824, were in the coding region. The C-T transition did not affect the encoded amino acid, while T-A transversion produced a conservative amino acid substitution of leucine for isoleucine. The other differences were in the 3' noncoding region, where there was a deletion of two bases in a stretch of 20 adenines (4186 to 4205) and a two-base deletion from a stretch of 17 thymines (4228 to 4244) relative to the previously

FIG. 1. Maps and sequencing strategies for RH3 2-1 and RH3 3-5. Open boxes represent exons 6 and 7 of the genomic DNA (6); shaded boxes are the coding regions of the RH3 2-1 and RH3 3-5 cDNAs. The RH3 3-5 cDNA is aligned to the genomic DNA by the dashed lines. Numeration is based on the full-length genomic DNA sequence. The locations of the C-T transition and the T-A transversion are shown, and the two-base deletions in the noncoding region of the RH3 3-5 cDNA are indicated in parentheses.

published genomic sequence. No poly(A) tail was found at the 3' end of the RH3 3-5 sequence. Only one 4-kb mRNA hybridized with the labeled RH3 3-5 probes upon Northern (RNA) blotting of poly(A)⁺ RNA isolated from *P. falciparum* FCR-3 (data not shown).

Expression of recombinant proteins. Recombinant proteins were expressed from both the pRSET C/RH3 2-1- and pRSET B/RH3 3-5-transformed bacteria under IPTG induction. The recombinants were recognized by pooled sera from an area where malaria is endemic as well as by individual sera from a smear-positive malaria patient and Rhop-H-specific rabbit serum 680 (data not shown). The sizes of the expressed RH3 2-1 and RH3 3-5 fusion proteins were 26 and 39 kDa, respectively. They represented only the C-terminal one-third of the Rhop-3 protein. Recombinant RH3 3-5 protein was purified by using nickel affinity chromatography under denaturing conditions. The recombinant protein eluted in 8 M urea precipitated even when dialyzed against gradually increasing volumes of PBS. After precipitation, only partial solubilization occurred in SDS-PAGE sample buffer.

Specificity of anti-recombinant RH3 3-5 antibodies. Recombinant-specific antiserum 686 was obtained from a New Zealand White rabbit immunized with the purified RH3 3-5 protein. Antibodies from this serum recognized the native Rhop-3 proteins on immunoblots of parasite extracts from strains 7G8 (Brazil), FCR3 (The Gambia), HB3 (Honduras), and K1 (Thailand) as well as excretory-secretory proteins in spent culture supernatants (data not shown). Preimmune serum nonspecifically bound to a protein 40 kDa in size (Fig. 2A, lane 1). Following immunization with the purified recombinant protein, this serum specifically bound only to a broad band of 100 to 110 kDa on an immunoblot of crude parasite extract (Fig. 2A, lane 2). By immunoprecipitation, the recombinant-specific antiserum 686 brought down the three constituents of the Rhop-H complex of 110-, 130-, and 140-kDa proteins (Fig. 2B), as do the Rhop-H-specific antisera 671 and 680 (22). By IFA, the antibodies recognized the punctate apical structures typical of rhoptries in segmented schizonts (data not shown). By IEM, the antibodies were distinctly localized to the matrix of the rhoptry bodies (Fig. 3). The RH3 3-5 protein is also immunogenic in Swiss mice. The ELISA titers of the rabbit and mouse antisera against the Rhop-3 recombinant were 2×10^5 and 6.5×10^4 , respectively.

ELISA with urea-solubilized recombinant antigen. Since the RH3 3-5 recombinant was insoluble in PBS, coating the insol-

FIG. 2. Immunoblotting and immunoprecipitation of *P. falciparum* Rhop-3 and Rhop-H complex, using rabbit anti-recombinant RH3 3-5 sera. (A) Immunoblot of *P. falciparum* (Brazilian strain 7G8) protein extract incubated with preimmune rabbit serum 686 (lane 1) or rabbit serum 686 collected 6 weeks following immunization with recombinant RH3 3-5 (lane 2). (B) Immunoprecipitation of the 140-130-110-kDa rophtry protein complex (Rhop-H) from the [³⁵S]methionine-labeled *P. falciparum* extract (Gambian strain FCR-3) by IgG antibodies from preimmune rabbit serum 686 diluted 1:5 (lane 1), rabbit serum 686 collected 6 weeks following immunization with recombinant RH3 3-5 diluted 1:5 (lane 2) or 1:10 (lane 3), and Rhop-H-specific rabbit antisera 671 diluted 1:10 (lane 4) and 680 diluted 1:10 (lane 5). Lane 6 represents the starting material.

uble proteins on the plates gave a poor signal on the ELISA. To optimize sensitivity of the reaction, various solubilization conditions, including different concentrations of SDS, Nonidet P-40, and Tween 20, were investigated. Only urea was able to satisfactorily dissolve the expressed and purified protein. Solubilizing the recombinant protein in urea increased the sensi-

tivity of the IgG assay with sera from *P. falciparum*-infected individuals, while the responses of sera from *P. vivax*-infected individuals and the North America healthy adults were unchanged (Fig. 4). By contrast, the IgM ELISA with or without urea-solubilized recombinant protein produced variable responses for *P. vivax*-infected individuals and decreased specificity relative to the IgG assay.

Specificity of Rhop-3 serologic responses. The Brazilian sera, the North American control sera, and the pooled sera from three *P. falciparum*-infected individuals were tested for *P. falciparum*-specific antibodies by IFA. The IFA titers for the Brazilian sera and the North American control sera were less than 50, while the titer of the pooled sera from *P. falciparum*-infected individuals was higher than 2,000.

In this study, we evaluated the ELISAs based on the recombinant Rhop-3 fragment and *P. falciparum* extracts for sensitivity and specificity, using sera from 29 smear-positive individuals from two areas where *P. falciparum* is endemic (PNG and Kenya), sera from 4 individuals exposed only to *P. vivax* and from 12 *S. mansoni*- and geohelminth-infected individuals from an area of Brazil with no malaria, and 8 North American negative control sera (Fig. 5). No statistical difference was observed in Rhop-3 IgG values between the two *P. falciparum*-infected groups, but responses to the *P. falciparum* extract were markedly higher for infected individuals in Mombasa ($P < 0.001$). IgG from *P. vivax* infections cross-reacted with neither antigen preparation. For the smear-positive individuals from Mombasa, IgM antibodies to the Rhop-3 antigen were significantly associated with malaria infection compared with the North American control sera ($P < 0.001$), but IgM response to the parasite extract was not. In general, IgM responses were not specific for *P. falciparum* infection. In fact, the highest

FIG. 3. Localization of rabbit anti-recombinant RH3 3-5 antibodies in *P. falciparum* merozoites by IEM. Gold particles (15 nm) label the electron-dense compartment of the rophtries (R). PV, parasitophorus vacuole membrane. Panel A shows one rophtry from two merozoites developing within a schizont. Panel B shows the paired rophtries within a single developing merozoite. Bar = 0.5 μ m.

FIG. 4. Sensitivity of urea-solubilized recombinant RH3 3-5 in IgG and IgM ELISAs. The purified recombinant RH3 3-5 (1 μ g/ml) in PBS or 8 M urea was incubated with sera from individuals with *P. falciparum* ($\blacktriangle$) and *P. vivax* ($\square$) infections and with sera from healthy North American adults ($\circ$) diluted 1:3,200. Each symbol represents the mean OD (405 nm) of triplicate measurements of individual samples.

mean responses were observed in the group with multiple parasitic infections and no malaria.

Intensity and seroprevalence of responses to Rhop-3 and *P. falciparum* extract by transmission pattern. Areas with three different patterns of *P. falciparum* transmission were compared by ELISA for serologic responses to crude parasite proteins or the Rhop-3 antigen. The frequencies of IgG seropositivity to the Rhop-3 antigens were similar in areas where *P. falciparum* transmission is holoendemic (30% positive), hyperendemic (43%), and seasonal (36% positive), but against the parasite extract, the frequency of a positive response in the population living in the area where transmission is holoendemic (48%) was approximately twofold lower than in the other two populations (90 and 97%) (Table 1). Comparing the Kenyan and PNG populations, we found that the mean Rhop-3 IgG values were similar though slightly greater by a factor of 1.7 for the Mombasa and Turkana populations compared with the PNG sample ($P = 0.048$ and 0.002 , respectively). By contrast, the magnitude of parasite-specific IgG responses was fivefold greater in both Kenyan populations than in the PNG population.

To assess whether differences in the magnitude of ELISA values correlated with current or past infection, the mean of ELISA units from smear-positive individuals was compared with mean values from individuals in populations from areas where transmission is endemic, selected at random or for conditions other than malaria. The infection status of the majority of sera from Mombasa and PNG was unknown, though at the time of year the sera were collected, less than 50% of adults had parasitemia. Mean Rhop-3 recombinant ELISA values (0.675) for the smear-positive individuals were significantly greater than values (0.341) for the rest of the sample from Mombasa ($P = 0.013$). This was not the case for PNG: values were 0.393 and 0.221 for the smear-positive individuals and the rest of the population, respectively. Mean values for the IgG ELISA using *P. falciparum* extract, however, did not differ between the smear-positive individuals and the rest of the sample from either Mombasa or PNG. In Mombasa, the values were 1.027 for the smear-positive individuals and 0.902 for the others. In the sera from PNG, these values were 0.214 and 0.165, respectively.

Antigen-specific antibodies in paired maternal and cord sera. Recombinant- and parasite-specific IgG and IgM anti-

body activities were analyzed in 131 paired maternal-cord sera from Mombasa, Kenya. At the same time in the previous year, 10% of mothers were found to be smear positive for *P. falciparum*. Positive IgG responses to the Rhop-3 antigen were detected in both maternal and cord sera, with mean values of 0.334 and 0.194 ELISA units, respectively (Fig. 6). Parasite-specific IgG activities in both maternal and cord sera were high, with mean values of 0.885 and 0.788 ELISA units, respectively. Maternal mean values for both anti-Rhop3 and anti-parasite extract antibodies were higher than those for newborns ($P < 0.001$ and 0.002 , respectively). The frequencies of Rhop-3 IgG ELISAs above the cutoff value were 38% for maternal and 18% for cord sera. Frequencies of positive parasite extract IgG values were similar for maternal (91%) and cord (88%) sera.

Although the IgM antibodies were not specific for *P. falciparum* infections in adults and children, they still can serve as an indicator of infection in the fetus, since they do not cross the placenta, nor do common helminthic infections. IgM in cord blood, therefore, is produced only by the fetus in response to in utero exposure to antigens. Specific IgM responses to Rhop-3 were higher in the maternal sera than in the cord sera, with mean values of 0.956 and 0.078 ELISA units, respectively, giving frequencies of seropositivity of 78 and 4% (Fig. 6). The mean value of maternal parasite antigen IgM was 1.431 ELISA units, compared with a mean value of 0.115 for cord sera. The frequencies of seropositive mothers and newborns were 20 and

FIG. 5. Specificity of IgG and IgM recombinant RH3 3-5 and *P. falciparum* extract ELISAs. Urea-solubilized recombinant RH3 3-5 and parasite extract (1 μ g/ml) were incubated with human sera diluted 1:3,200 and 1:800 for IgG and IgM, respectively. P.f.(+), sera from *P. falciparum* smear-positive individuals from PNG or Mombasa, Kenya; P.v.(+), *P. vivax* smear-positive individuals from residents of India or travelers to Laos and PNG; P.f.(−), sera from individuals with no past or present malaria exposure, represented either by schoolchildren with *S. mansoni* and geohelminth infection from Brazil or by healthy North American adults (N.A.). Each point represents the mean of triplicate measurements. The bar marks the arithmetic mean of the group. The shaded area represents the cutoff value (mean value of North American control sera plus 3 standard deviations).

TABLE 1. IgG responses to Rhop-3 recombinant and *P. falciparum* extract by transmission pattern and geographic distribution

Malaria transmission (geographic area)	n	Recombinant specific (RH3 3-5)		Parasite specific (strain FCR-3)	
		Mean ELISA units $\pm$ SD	Seroprevalence (% positive) ^a	Mean ELISA units $\pm$ SD	Seroprevalence (% positive)
Holoendemic (PNG)	121	0.251 $\pm$ 0.399	30	0.174 $\pm$ 0.244	48
Hyperendemic (Mombasa, Kenya)	171	0.357 $\pm$ 0.387	43	0.906 $\pm$ 0.635	90
Seasonal (Turkana, Kenya)	33	0.365 $\pm$ 0.434	36	0.883 $\pm$ 0.373	97

^a Percentage of the population with an ELISA unit value greater than 3 standard deviations above the mean of the North American negative controls.

1%. The rates of IgM seropositivity in the parasite antigen-based ELISA were lower than seropositivity for Rhop-3 because of the very high background for IgM against native antigens.

To compare individual mother and infant paired antibody responses, linear regression analysis was performed (Fig. 7). A significant linear correlation was observed for the Rhop-3- and parasite-specific IgG responses from maternal-cord pairs (Pearson's correlation coefficient; $r = 0.711$ and 0.850 , respectively). There was no correlation between maternal and cord sera for parasite- or recombinant-specific IgM responses. Six cord samples had high parasite- and recombinant-specific IgM responses, while the corresponding maternal IgM responses were very low.

DISCUSSION

Two different cDNA clones encoding the C-terminal one-third of the Rhop-3 protein were identified, isolated, and expressed. The identity of each of these cDNAs was confirmed by nucleotide sequence similarity with the Rhop-3 genomic DNA sequence and binding of Rhop-H-specific antibodies to the expressed proteins from these clones. In addition, the purified recombinant protein produced an antiserum that recognized the rhoptry organelles and a 110-kDa protein upon immunoblotting and coprecipitated the other two Rhop-H components. Nucleotide sequences of the two identified cDNAs demonstrated a small degree of heterogeneity relative to the previous sequence of a Rhop-3 gene. One of the two differences in the coding region is silent, and the other results in a

conservative amino acid substitution of leucine for isoleucine. Neither the two cDNAs that we identified nor a cDNA isolated by Brown and Coppel (6) contained terminal poly(A) tracts. None of the cDNAs identified thus far have consensus sequences for polyadenylation in an appropriate position in the 3' untranslated region. Rhop-3 may be encoded by a nonpolyadenylated mRNA that binds to oligo(dT) columns by virtue of long adenine tracts within the untranslated region.

An ELISA based on the recombinant protein was developed to survey the prevalence of human seropositivity to the 110-kDa Rhop-3 antigen in areas of different patterns of *P. falciparum* transmission. Although the recombinant protein was insoluble in PBS, urea solubilization was found to increase the sensitivity of IgG ELISA responses without a loss of specificity. IgG responses show a high degree of specificity and indicate that at least the expressed fragment of Rhop-3 is species specific relative to *P. vivax*. For the recombinant Rhop-3 fragment as well as the *P. falciparum* extract, the mean IgG ELISA values of sera from smear-positive individuals were significantly greater than for all other groups. While the mean Rhop-3 IgG ELISA values for the PNG and Mombasa sera differed by a factor of 1.7 (0.393 versus 0.674 units), there was nearly a fivefold difference between these two groups for the parasite extract IgG ELISA (0.214 versus 1.026 units). Only for the population in the area of Mombasa where transmission is hyperendemic and only in the IgG Rhop-3 ELISA were values

FIG. 6. Serologic responses in maternal and cord blood to Rhop-3 and *P. falciparum* extract. The ELISA used urea-solubilized recombinant RH3 3-5 and parasite extract (1 μ g/ml) incubated with maternal and cord sera ($n = 131$) diluted 1:3,200 and 1:800 for IgG and IgM, respectively. The bar represents the arithmetic mean of the group. Each point represents the mean of triplicate measurements. The number in parentheses indicates the highest value of points not indicated by the scale.

FIG. 7. Linear correlation between paired maternal and cord blood for recombinant RH3 3-5-specific and *P. falciparum* extract-specific IgG and IgM reactivity. The assay was as described for Fig. 6. Each point plots the maternal ELISA unit value on the x axis and the corresponding cord value on the y axis. The linear regression equation for the recombinant IgG is $y = 0.405x + 0.059$; that for the parasite-specific IgG is $y = 0.808x + 0.065$.

for smear-positive individuals significantly higher than for a random population, most of whom were probably smear-negative. Strong responses to the Rhop-3 under certain circumstances may correlate with current rather than past infection. In contrast to IgG responses, IgM responses did not correlate with infection, and probably IgM cross-reacts with antigens from even unrelated parasites. While the IgG ELISAs were 100% specific, the highest IgM responses were actually found in children infected with *S. mansoni* and geohelminths. Use of a full-length Rhop-3 recombinant may increase the sensitivity of the IgG assay and specificity of IgM by permitting a higher dilution of serum. The positive serological responses were correlated with individuals living in areas where malaria is endemic.

The serologic assays were developed to survey the prevalence of antibodies to the Rhop-3 protein in populations and not as diagnostic tools. To provide a context within which to interpret Rhop-3-specific reactivity, the seroprevalence of Rhop-3 antibodies was compared with responses to native parasite extract. The comparison uncovered an interesting aspect of the geographic distribution of immune responses to malaria. We observed that the Rhop-3 antigen is recognized in the populations from three widely separated areas where malaria is endemic, and responses to this antigen are similar in magnitude despite either geographic isolation or differences in the prevalent transmission pattern. Since the recombinant represents only one-third of the Rhop-3 protein, it is expected there will be a higher seroprevalence rate for the whole molecule. IgG reactivity against the Rhop-3 protein is in sharp contrast to responses to native parasite antigens, for which there was a fivefold difference in mean parasite-specific IgG ELISA values between the Kenyan and PNG populations. The overall seroprevalence is also significantly higher for the Kenyan samples than for the PNG sample (97 and 90% versus 48%). This differences in seroprevalence between random samples from populations from areas of endemicity whose infection status is unknown (Table 1) parallels the differences observed in sera from smear-positive individuals whose infection status is known (Fig. 5).

It is possible that parasite strain differences account for this disparity. When 26 different *P. falciparum* isolates were reacted with a battery of monoclonal antibodies to two *P. falciparum* strains, a high degree of variability in binding was observed even between isolates obtained from the same area (15). Further, isolates from the same individual can show marked antigenic variability (14, 32). Some antigens must be conserved between *P. falciparum* strains, but the relative conservation of responses to Rhop-3 highlights the variability in crude preparations of antigens from different sources.

Alternative or contributing causes for the geographically divergent responses to the parasite extract may involve host differences. Since more intense and unvarying transmission occurs in the area of PNG studied compared with either of the areas of Kenya, tolerance or immune suppression to malarial antigens could also explain these differences. Further, depressed responses to immunodominant parasite antigens may be linked to genetically restricted traits more common in one population than in another (16).

Immune responses were also studied in pregnant women and their newborns, populations at greatest risk for morbidity and mortality due to malaria. For this study, IgM antibodies were an unreliable measure of exposure to *P. falciparum* in adults or children, but they should reflect fetal exposure to antigens in newborns, since few parasites, their antigens, or IgM antibodies cross the placenta. IgM responses to the recombinant Rhop-3 fragment, however, were a better assay for

fetal exposure than the ELISA based on native parasite proteins, since they demonstrated higher specificity. A similar approach was used in a recent study of ring-infected erythrocyte surface antigen- and circumsporozoite protein-specific antibodies in paired maternal-cord sera from Nigerian mothers (1).

In this study, mean IgM values against either the purified recombinant protein or the extract were over 10-fold higher for mothers than for their newborn children. Thus, the IgM ELISA shows little evidence of congenital infection or transplacental transfer of Rhop-3 proteins from mother to fetus. When individual maternal and cord blood values were matched, the high IgM responses observed in some cord sera appeared to be spurious, since in no case was antigen-specific IgM elevated in the corresponding maternal serum. On the other hand, the IgG responses were the result of passively transferred IgG, as evidenced by the positive correlation between values for mothers and newborns. Similar results were obtained by Desowitz et al. (10), who examined maternal and cord sera from PNG and found that an IgG reactivity correlated between mother and newborn for parasite antigens, that parasite-specific IgG was slightly less in the newborn than in the mother, and that parasite-specific IgM was absent from cord blood (10). In the light of our findings, a positive cord blood IgM response in their study would have been difficult to interpret.

We find that the C-terminal fragment of Rhop-3 examined is antigenically species specific. Although there are minor nucleotide and amino acid differences between isolates or strains in this Rhop-3 fragment, there is antigenic conservation relative to a whole parasite extract. The Rhop-3 antigen is recognized by individuals from different geographic areas with various patterns of transmission, as well as by pregnant women.

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