Identification of Plasmodium falciparum histidine-rich protein 2 in the plasma of humans with malaria.

M E Parra, C B Evans and D W Taylor

Identification of *Plasmodium falciparum* Histidine-Rich Protein 2 in the Plasma of Humans with Malaria

MARCELA E. PARRA,* CHARLES B. EVANS, AND DIANE W. TAYLOR

Department of Biology, Georgetown University, 37th and O Streets, N.W., Washington, D.C. 20057

Received 31 December 1990/Accepted 15 May 1991

*Plasmodium falciparum* histidine-rich protein 2 (PfHRP-2) is a water-soluble protein released from parasitized erythrocytes into in vitro culture supernatants. This study sought to determine whether PfHRP-2 could be detected in the plasma of humans with *P. falciparum* malaria. A monoclonal antibody (1E1) is described that binds to PfHRP-2. By using monoclonal antibody 1E1, PfHRP-2 was identified by Western blot (immunoblot) analysis in the plasma of 37 of 39 (95%) patients experiencing either a first or repeat episodes of *P. falciparum* malaria and by dot blot analysis in the plasma of 40 of 41 patients tested. PfHRP-2 was not detected in 30 control, uninfected subjects. The current demonstration of PfHRP-2 in plasma, plus the fact that it is a structurally well-characterized molecule present in all natural isolates of *P. falciparum* tested, makes PfHRP-2 of interest for its potential effects on the host immune system and as an antigen for specific diagnosis of malaria.

**MATERIALS AND METHODS**

Production of MAb 1E1. The antigen used for immunization was prepared with blood from an *Aotus* monkey infected with the K*+* Malayan Camp strain of *P. falciparum* (11). Cryopreserved 1-ml aliquots of infected blood, kindly provided by R. J. Howard (National Institute of Allergy and Infectious Diseases), were defrosted by the method of Meryman and Hornblower (13) and then cultured in vitro by the method of Trager and Jensen (20). Cultured cells were harvested approximately 18 h later when the parasites were in the late trophozoite stage of development. Cells were washed twice with 5 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2. Then the cells were resuspended in 1 ml of 1% Triton X-100 in 0.15 M PBS (pH 7.2)–1 mM iodoacetamide (Sigma Chemical Co., St. Louis, Mo.)–1 mM phenylmethylsulfonyl fluoride (Sigma) for 15 min at 4°C. The extract was centrifuged at 12,100 × g for 20 min, the supernatant was discarded, and the insoluble cytoskeletal pellet was washed twice with 5 ml of 0.15 M PBS (pH 7.2) plus 1 mM iodoacetamide–1 mM phenylmethylsulfonyl fluoride.

A portion of the cytoskeletal preparation (equal to cytoskeletons from approximately 1.2 × 10⁹ parasitized erythrocytes) was emulsified in complete Freund adjuvant and injected in equal proportions intraperitoneally into each of three BALB/c mice (Cumberland View Farms, Clinton, Tenn.). Mice received a second immunization consisting of the same amount of antigen in incomplete Freund adjuvant 2 weeks later. Thus, each mouse received a total of ~0.8 × 10⁹ parasitized erythrocyte cytoskeletons. Mice were test bled 2 weeks later, and plasma was tested for the presence of antiparasite antibody by indirect immunofluorescence antibody (IFA) assay (see below). The mouse with the highest titer was boosted with approximately 0.4 × 10⁹ parasitized erythrocyte cytoskeletons intraperitoneally without adjuvant, and splenocytes were used for lymphocyte hybridization 4 days later.

Hybridomas were produced by fusing immune spleen cells with the P3-X63-Ag.8-U1 (PU) myeloma cell line, using the procedure of Margulies et al. (12). Culture supernatants were screened for reactivity with asexual-stage *P. falciparum* antigens, using the IFA assay described below. One of the MAb from this fusion was MAb 1E1. MAb 1E1

---

* Corresponding author.
was cloned twice by limiting dilution and cryopreserved, and ascites was produced in mice primed with 2, 6, 10, 14-tetramethyl-pentadecane (Sigma) as described previously (18).

The isotype of MAb 1E1 was determined by immunoelectrophoresis. Immunoelectrophoresis plates were prepared by coating glass slides (3.25 by 4 in. [8.26 by 10 cm]) with 1.5% agarose (Sigma) in barbital buffer (0.05 M sodium barbital, 0.01 M barbital, pH 8.6 [Sigma Diagnostics, St. Louis, Mo.]). Approximately 5 μl of ascites fluid was added to the center wells, and plates were electrophoresed in an E-C Immuno Cell (E-C Apparatus Corp., Philadelphia, Pa.) for 1.5 h, using 0.05 M barbital buffer (pH 8.6). Antiserum specific for mouse immunoglobulin M (IgM), IgG1, IgG2a, IgG2b, and IgG3 (Fisher Scientific Co., Birmingham, Ala.) were added to the troughs, and the plates were incubated overnight in the refrigerator. Plates were soaked in 0.15 M PBS (pH 7.2) for 2 days, dried, and stained with 0.2% Coomassie brilliant blue dissolved in 50% ethanol–7% acetic acid, and data were recorded.

IFA assay. Uncloned K+ and K− Malayan Camp (MC-Southeast Asia) and St. Lucia (SL-Ecuador) parasites in Aotus erythrocytes were grown in vitro as described above. P. falciparum parasites of the NF54 (The Netherlands), uncloned ITG2F2 (Brazil), 7G8 (a clone derived from ITG2F2), and W2.MEF (PHHRP-2 minus) (16, 23) lines were cultured in human erythrocytes as described by Trager and Jensen (20). Blood smears of these parasites were prepared and used in IFA studies. The IFA method of Voller (21) was followed. In brief, smears of parasitized erythrocytes were fixed with cold acetone for 5 min. Then, approximately 5 μl of ascites fluid diluted in 0.15 M PBS (pH 7.2) was applied to the slide for 20 min at room temperature. Slides were washed three times with 30-ml changes of 0.15 M PBS (pH 7.2); then 5 μl of a 1:40 dilution of goat anti-mouse immunoglobulin (polyvalent) labeled with fluorescein isothiocyanate (Sigma) was added, and slides were incubated at room temperature for 20 min. The slides were again washed in three changes of 0.15 M PBS (pH 7.2) and mounted in 50% glycerol in 0.05 M barbital buffer (pH 8.6). Slides were examined with a Laborlux epifluorescence microscope (Leitz, Wetzlar, Germany) equipped with a pass band filter (450 to 490 nm).

Metabolic labeling and immunoprecipitation. Aliquots of cryopreserved Aotus blood infected with the K− Malayan Camp strain of P. falciparum (10% parasitemia) were defrosted as described by Meryman and Hornblower (13), and the parasites were cultured in vitro in histidine-deficient medium (Selectamine; GIBCO Laboratories, Grand Island, N.Y.) as described previously (11). Cultures had a packed-cell volume of 5% and contained 100 μCi of [3H]histidine (2.22 TBq/mmol; Amersham Corp., Arlington Heights, Ill.) per ml. Parasites were cultured for 22 h until they reached the late trophozoite stage of development. The cells were harvested, washed three times with 5 ml of 0.15 M PBS (pH 7.2), and then solubilized in 2 ml of 1% Triton X-100 in 0.15 M PBS (pH 7.2) for 1 h as described previously (6). The extract was centrifuged at 1,000 × g for 5 min, and the soluble portion of the extract was used in the immunoprecipitation studies described below.

Aliquots of 5 μl of unpurified ascites containing MAb 1E1, MAb 2G12 specific for PHHRP-2 (16), and TEPC 183 IgM myeloma protein (isotype matched, irrelevant, antibody-negative) were combined with 50 μl of the above [3H]histidine-labeled parasite extract and incubated overnight at 4°C. Then 3 μl of rabbit anti-mouse IgM antiserum was added. One hour later, 150 μl of a 50% slurry of protein A-Sepharose (Pharmacia, LKB Biotechnology Inc., Piscataway, N.J.) in NET buffer (0.15 M sodium chloride, 5 mM EDTA, 50 mM Tris base [pH 7.4], 0.5% Nonidet P-40) was added, and samples were incubated for an additional hour. The precipitate was washed twice with 4 ml of NETN buffer, twice with NETN buffer plus 0.85 M NaCl, twice with NETN buffer plus 10% fetal bovine serum, and once again with NETN buffer. The complexes were solubilized in 100 μl of SDS sample buffer and separated on an SDS–10% polyacrylamide gel by the method of Laemmli (10). Gels were soaked in enhancing fluor in accordance with the manufacturer’s recommendations (En3'Hance; Dupont NEN, Boston, Mass.), dried on a Bio-Rad gel drier (Bio-Rad Laboratories, Richmond, Calif.), and exposed to X-Omat AR film (Eastman Kodak, Rochester, N.Y.) at −60°C for 6 days.

Plasma samples. Plasma samples were obtained from 54 Colombian adults (age range, 16 to 60 years) reporting at the Center for Malaria Eradication in Villavicencio, Colombia, who were infected with P. falciparum malaria. These individuals reported experiencing either a primary infection or two, three, or more previous infections. After obtaining malarial histories, 2 to 4 ml of heparinized blood was collected by venipuncture. Both thin and thick blood smears were prepared and examined for the presence of parasites. Percent parasitemias were determined from thin blood films. Blood samples were centrifuged at 1,000 × g for 20 min, and plasma was collected and frozen until used. As negative controls, plasma from 30 Colombian adult volunteers (ages, 18 to 21 years) residing in Bogota who had not been exposed to malaria were studied.

Western blot studies. In Western blot (immunoblot) studies, 39 of the 54 samples from infected patients and all 30 control plasma samples were diluted 1:10 in 0.15 M PBS (pH 7.2) and mixed with an equal volume (50 μl) of reducing SDS sample buffer (10). Then, 15 μl was applied to lanes of an SDS–10% polyacrylamide resolving gel with a 5% stacking gel. Gels were electrophoresed at room temperature for 1.5 to 2 h at 100 V in a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories) by the method of Laemmli (10). After electrophoresis, the gels were soaked for 30 min in a transfer buffer (0.003 g of Tris base, 15.14 g of glycine, 200 ml of methanol per liter) and proteins were transferred onto nitrocellulose paper (Trans-Blot; Bio-Rad) at 100 V for 1 h, using the protocol described by Towbin et al. (19). Nitrocellulose membranes were blocked for 60 min with 10% (wt/vol) nonfat dry milk–PBS for 4 h at 4°C, washed with three 25-ml changes of 0.15 M PBS (pH 7.2) supplemented with 0.1% Tween 20 (Sigma) at 4°C, and then incubated with a 1:400 dilution of affinity-purified goat anti-mouse immunoglobulin (polyvalent) coupled with horseradish peroxidase (Kirkgaard and Perry Laboratories, Gaithersburg, Md.). Bands were visualized by using HRP-substrate prepared by combining 12.5 mg of HRP color development reagent (Bio-Rad) in 7 ml of methanol with 37.5 ml of 0.15 M PBS (pH 7.2) containing 25 μl of 30% hydrogen peroxide.

Dot blot studies. To partially purify MAb 1E1, 5 ml of ascites fluid was combined with 5 ml of saturated ammonium sulfate (pH 7.0) and the mixture was incubated overnight at 4°C. The precipitated material was collected by centrifugation at 10,000 × g for 20 min, were resuspended in 5 ml of distilled water and dialyzed against approximately 4 liters of 0.15 M PBS (pH 7.2). The protein concentration in the partially purified immunoglobulin preparation was determined spectrophotometrically at optical density at 280 nm.
Then 1 mg of MAb 1E1 was coupled with 1 mg of biotin by using glutaraldehyde (1). The conjugate was dialyzed against 0.15 M PBS (pH 7.2) and used at a working dilution of 1:1,000.

Plasma samples from 41 of 54 patients and all 30 of the normal controls were diluted 1:10 in 0.15 M PBS (pH 7.2), and 5 µl was applied to nitrocellulose membranes (pore size, 0.45 µm; Schleicher and Schuell, Inc., Keene, N.H.). After drying, membranes were blocked for 1 h in 25 ml of quench buffer (0.3% bovine serum albumin, 0.3% Tween 20 in 0.15 M PBS [pH 7.2]) at room temperature and then incubated overnight in 15 ml of MAb 1E1-biotin (diluted 1:1,000 in 0.15 M PBS, pH 7.2) at 4°C. Membranes were washed three times (5 min each) in 25 ml of 0.05% Tween 20 in 0.15 M PBS (pH 7.2) and then incubated in 30 ml of a 1:3,000 dilution of streptavidin-horseradish peroxidase type V (Sigma) for 1.5 h at 4°C. Blots were washed in three 25-ml changes of 0.05% Tween 20 in 0.15 M PBS (pH 7.2), and antibody binding was visualized by using HRP-substrate (Bio-Rad) (see above).

RESULTS

MAb 1E1 binds to PHRP-2. MAb 1E1 was found to be of the IgM isotype and produced a pattern of immunofluorescence consistent with reactivity for PHRP-2 (Fig. 1) (6). Ring-stage parasites stained only weakly with a diffuse pattern of reactivity. In trophozoites, two patterns of fluorescence were routinely observed: spots of fluorescence within the erythrocyte cytosol (Fig. 1A) and rimmed fluorescence around the periphery of infected erythrocytes (Fig. 1B). In general, both patterns could be seen in the same smear, but the parasite itself was usually negative. All strains and isolates of P. falciparum tested consistently produced the above patterns except W2.MEF, the HRP-2-deficient strain, which was negative.

Results of immunoprecipitation studies are shown in Fig. 2. As can be seen (lane A), MAb 1E1 recognized predominately the 70-kDa species of PHRP-2. In other gels, MAb 1E1 also produced a weak reaction with the series of lower-molecular-weight bands. MAb 2G12 (lane B) always showed strong reactivity with both the 70-kDa and lower-molecular-weight moieties. This pattern is characteristic of PHRP-2 (6, 16). No histidine-labeled proteins were detected with control TEPC 183 myeloma protein (lane C). Thus, on the basis of fluorescence patterns, failure to react with PHRP-2-deficient parasites, and immunoprecipitation of [3H]histidine-labeled 70-kDa protein, MAb 1E1 was considered to react with PHRP-2.

Detection of PHRP-2 in plasma of individuals with P. falciparum malaria. A summary of the age, sex, and percent parasitemias of 13 patients who reported having primary P. falciparum infections is shown in Table 1. All had parasites present in their blood (as detected by blood smear) when the blood samples were obtained. Plasma from these patients was analyzed by Western blot analysis for the presence of PHRP-2. Plasma from an equal number of negative controls who did not have malaria were also examined. Results are shown in Fig. 3. A 70-kDa band was seen in all (13 of 13) of the samples from patients with malaria, but not in the negative controls. In addition to the 70-kDa band, the series of lower-Mr bands was observed in infected patients. There was not a clear-cut, direct correlation between the intensity or number of bands and the percent parasitemia in the

![FIG. 1. IFA pattern of MAb-1E1 on P. falciparum-infected erythrocytes. MAb-1E1 produces two characteristic patterns: (A) fluorescence within "vesicles" in the erythrocyte cytosol; (B) rimmed fluorescence around the periphery of infected erythrocytes.](image)

![FIG. 2. Results of immunoprecipitation of [3H]histidine-labeled parasitic proteins. Lane A, MAb 1E1; lane B, MAb 2G12, which is known to react with PHRP-2; lane C, control myeloma protein TEPC 183.](image)

### TABLE 1. Clinical history of Colombians with primary *P. falciparum* infections

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Parasitemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>M</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>37</td>
<td>F</td>
<td>0.2</td>
</tr>
<tr>
<td>D</td>
<td>41</td>
<td>M</td>
<td>0.2</td>
</tr>
<tr>
<td>E</td>
<td>26</td>
<td>M</td>
<td>0.2</td>
</tr>
<tr>
<td>F</td>
<td>52</td>
<td>F</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td>H</td>
<td>43</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>57</td>
<td>M</td>
<td>0.5</td>
</tr>
<tr>
<td>J</td>
<td>43</td>
<td>F</td>
<td>0.2</td>
</tr>
<tr>
<td>K</td>
<td>32</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td>L</td>
<td>16</td>
<td>M</td>
<td>0.5</td>
</tr>
<tr>
<td>M</td>
<td>24</td>
<td>M</td>
<td>2</td>
</tr>
</tbody>
</table>

* M, male; F, female.
patients (Table 1). A nonspecific, weak cross-reaction was also seen with a 72-kDa band in all plasma samples (Fig. 3).

Samples from 26 additional patients who had *P. falciparum* malaria as determined by blood smears and who reported having had malaria one or more times were also evaluated. The parasitemias in these individuals ranged from 0.2 to 2.5%. By Western blot analysis, PfHRP-2 was detected in 24 of 26 of the patients tested. Plasma samples from the two false-negative patients were retested by Western blotting and were again found to be negative. Plasma samples from 17 additional controls were evaluated and were found to be negative. In summary, PfHRP-2 was detected in 37 of the 39 patients studied who were infected with *P. falciparum* malaria, but not in any of the 30 control adults. Plasma samples from 41 of the 54 infected patients and from the 30 normal controls were also assessed by the dot blot assay. As shown in Fig. 4, a clear reactivity was shown with 40 of 41 samples from infected individuals. A weak borderline reactivity was seen with 2 of 30 of the normal control samples; the remainder were completely negative. The dot blots were repeated several times, and the same number of samples, 40 of 41, from infected individuals remained positive, but all 30 samples from negative controls tested negative. Identical reactivities were obtained with MAb 2G12, but the intensity of the reaction was considerably greater with MAb 1E1. Thus, by dot blot analysis, PfHRP-2 could be detected routinely in the plasma of patients with malaria, but not in the control samples.

**FIG. 3.** Results of Western blotting studies. Samples A to M are from individuals with acute *P. falciparum* malaria (Table 1), and samples 1 to 7 are from normal control subjects. Data for the other six control samples are not shown. Arrow denotes reactivity with the 70-kDa band of HRP-2. A weak reactivity with a 72-kDa band was seen in all serum samples.

**FIG. 4.** Results of dot blot assay. Serum samples from 41 acutely infected individuals were spotted in every other row of the top half of the paper (+), and 30 samples from control subjects were spotted on the bottom half (–).

**DISCUSSION**

Results of this study demonstrate that the malarial antigen PfHRP-2 circulates in the plasma of humans infected with *P. falciparum* and that its presence can be demonstrated by using MAb 1E1. Earlier studies showed that PfHRP-2 was secreted from infected erythrocytes into the supernatants of cultures of *P. falciparum* (6). Other malarial proteins have also been identified in culture supernatants: for example, a variety of exoantigens, including (i) the 83- and 100-kDa antigens described previously (4, 7, 17), (ii) a 96-kDa thermostable polypeptide reported by Jouin et al. (8), (iii) the 101- and 112-kDa merozoite antigens identified by Chulay et al. (2), (iv) a 126-kDa antigen localized in the parasitophorous vacuole (3), and (v) a 130-kDa protein that binds to glycoprotein (15). In addition, processed products of a number of antigens have been found in sera, including breakdown products of the major surface merozoite antigen gp195 (5). However, to date, PfHRP-2 is the only one of these *P. falciparum* proteins clearly shown to be released from intact, infected erythrocytes during parasite development (6). The others appear to be either shed or released when schizont segmenters rupture and merozoites are released. Thus, while *P. falciparum*-infected erythrocytes are sequestered in the deep vasculature, it is possible that PfHRP-2 could be actively secreted into the blood.

The above antigens have been identified in culture supernatants, but it is unclear how many of these may actually circulate in sera. Their presence in culture supernatants does not guarantee that they would be found in detectable levels in infected humans. In in vitro cultures, parasitemias are frequently higher than found in vivo, and malarial antigens accumulate with time. In addition, antigens may be rapidly cleared in vivo by direct phagocytosis, Fc-mediated phago-
cytosis, proteolytic degradation, or filtration through the kidneys. Thus, it could not be assumed that PfHRP-2 would circulate in detectable levels in human plasma.

In the current study, MAb 1E1 detected a 70-kDa protein in extracts of *P. falciparum* (Fig. 2), in culture supernatants (data not shown), and in acute-phase plasma of infected individuals (Fig. 3). It also detected a "ladder" of lower- Mr species in some experiments, which are presumed to contain PfHRP-2 with lesser numbers of the repeat sequences (6). Throughout the study, the 70-kDa band was always seen in positive plasma samples, but the lower-Mr species were occasionally weak or absent (e.g., Fig. 3, lanes G and K).

The 70,000-Mr band and lower-Mr species were identified in 13 of 13 plasma samples from people with primary malaria. The antigen was also detected in 24 of 26 patients experiencing repeated infections. If antibodies had been produced during previous infections, they did not interfere with the detection of PfHRP-2 in subsequent infections. In this study, plasma from 2 of 39 infected patients gave negative results by Western blotting. One of these patients had received passive transfer of serum from an individual immune to malaria prior to collection of the blood sample. Plasma samples from these two patients, however, were positive in the dot blot assay. Thus, immune responses in susceptible individuals do not appear to be sufficient to remove PfHRP-2 from the peripheral circulation.

MAb 1E1 recognized not only PfHRP-2 but also a 72-kDa band which was present in all plasma samples evaluated by Western blot analysis (Fig. 3). That is, the 72-kDa reactivity was detected in the plasma of negative control Colombians as well as in plasma of those infected with malaria. The nature of this cross-reactive protein is unknown, but it may be one of the histidine-rich proteins normally present in human sera (14). In the dot blot assay, each plasma sample was diluted 1:10 before it was applied to the nitrocellulose sheets. As a result, the 72-kDa protein was not detected in the majority of samples from normal, uninfected individuals (Fig. 4). It is possible, however, that an occasional background or plus/minus reaction may be observed in dot blots due to detection of this protein. Thus, Western blots are preferential to dot blots since they can distinguish between PfHRP-2 and the 72-kDa molecule.

The immunologic importance of PfHRP-2 remains uncertain. It has been shown that polycationic polypeptides such as polyhistidine increase the invasion rate of *Toxoplasma gondii* in a concentration-dependent manner (24). In addition, it has been shown that molecules of polyhistidine bind free radicals. Perhaps *P. falciparum* parasites secrete PfHRP-2 while bound to endothelial cells in order to inactivate oxidative products produced by macrophages and reticuloendothelial cells. Recent studies have also shown that *Aotus* monkeys can be partially protected against challenge with *P. falciparum* if they are immunized with a 165-amino acid fusion protein that contains the repeat Ala-His-His and has high homology with PfHRP-2 (9). Monkeys immunized with the fusion proteins developed low parasitemias (<2%) after challenge with *P. falciparum* compared with control monkeys which were not protected. Thus, PfHRP-2 remains an interesting molecule of potential immunologic relevance.

Detection of PfHRP-2 by using MAb 1E1 appears to have potential for serodiagnosis of malaria. MAb 1E1 was originally produced against the Malayan Camp strain of *P. falciparum*. In this study, MAb 1E1 detected PfHRP-2 (i) by IFA in parasites obtained from Africa, Brazil, and Southeast Asia, (ii) in immunoprecipitation studies with the K+ Malayan Camp strain, and (iii) in plasma of patients in Colom-

bria. Thus, MAb 1E1 detects PfHRP-2 in parasites from geographically different areas and overcomes one major problem in serodiagnosis, namely, antigenic variation. The ability of MAb 1E1 to detect PfHRP-2 in dot blot assays is encouraging, suggesting that one should be able to convert this assay to a "dipstick" format to be tested in the field. Clearly, basic questions need to be answered before the feasibility of this approach can be ascertained; nevertheless, on the basis of our knowledge of circulating soluble antigens, PfHRP-2 is currently the best candidate on which to base an antigen detection assay for the diagnosis of malaria.

ACKNOWLEDGMENTS

We thank the Center for Malaria Eradication, Villavicencio, Colombia, for helping us obtain the samples and I. A. Quayk and R. J. Howard for reviewing the manuscript.

This work was supported by DiaTech, P.A.T.H.

REFERENCES


