

The P Domain of the P0 Protein of *Plasmodium falciparum* Protects against Challenge with Malaria Parasites

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Monoclonal antibodies (MAbs) specific for the P domain of the *Plasmodium falciparum* P0 phosphoriboprotein (PfP0) blocked the invasion of RBCs by *P. falciparum*. Vaccination with this P-domain peptide protected mice upon malaria parasite challenge. The absolute specificity of the MAbs and the PfP0 P peptide makes them potential protective malaria reagents.

Ribosomal phosphoprotein P0 is a neutral protein that is found in all eukaryotes (17). P0 is related to the family of acidic ribosomal phosphoproteins P1 and P2, and these three proteins collectively constitute the ribosomal P proteins. The P proteins possess a highly homologous carboxy-terminal domain (the P domain) and also complex together as (P1)₂-P0-(P2)₂ to form the GTPase binding stalk region of the 60S ribosomal subunit of eukaryotes (18, 25, 26). Of the three ribosomal P proteins, P0, but not P1 or P2, is a vital protein as demonstrated by knockout studies in *Saccharomyces cerevisiae* (19).

P proteins have been shown to be immunogenic and have been studied extensively in humans because of their association with systemic lupus erythematosus (SLE), an autoimmune disorder. Approximately 10 to 15% of patients suffering from SLE possess autoantibodies against the conserved carboxy-terminal 22 amino acids of the P proteins (4). The reactive domain has been narrowed to 11 amino acids (11), and recently, it was narrowed to the 6 amino acid residues at the carboxy-terminal ends of the P proteins (14). The roles of these anti-P-protein antibodies in the pathogenesis of SLE disease are not clear. The ribosomal protein P0 can act as an immunogen, since P0 has been shown to be located on the surfaces of *Plasmodium*, *Toxoplasma gondii*, and *S. cerevisiae* as well as on the surfaces of neuronal, hepatic, and other cell lines using cross-reactive antibodies (9, 12, 23). In some of these studies, the surface reactivity was attributed to a P0-like determinant, since a cross-reactivity of anti-P0 polyclonal antibodies to other protein(s) could not be ruled out (23). By transfecting *T. gondii* cells with tagged *T. gondii* P0 phosphoriboprotein (TgP0), Sehgal et al. recently demonstrated that the cognate TgP0 protein translocates to the cell surface (20).

Specific antibody responses against the P0 protein of protozoan parasites have been shown to be prevalent among people with chronic parasitic infections (1, 2, 13, 24). Eighty-seven percent of adult residents in areas of eastern India where

malaria is endemic possess antibodies against *Plasmodium falciparum* P0 phosphoriboprotein (PfP0) (13). Approximately 60% of adults residing in Kenya showed T-cell responses to the PfP0 protein, and the magnitude of this response was comparable to the T-cell responses to MSP-1 protein, a candidate vaccine antigen (I. Malhotra, P. Mungai, J. Ouma, S. Sharma, J. W. Kazura, and C. L. King, unpublished data). In the case of *Trypanosoma cruzi*, it has been shown that antibodies against the P domain were present in a large proportion of cardiac chagasic patients (2). Seventy-four percent of *Toxoplasma* patients showed the presence of anti-*Toxoplasma* P0 antibodies (1). For dogs with visceral leishmaniasis, 78% of infected sera were shown to possess *Leishmania infantum* P0-specific antibodies (24). The *L. infantum* acidic ribosomal protein P0 has recently been shown to confer protective immunity to *Leishmania major* infection in BALB/c mice (10). Polyclonal antibodies against PfP0 have been shown to block the parasite invasion of red blood cells (RBCs) (3).

Cross-reactivity of parasite and human P0 (HuP0) proteins has been reported earlier (5, 11, 22). Although antibodies to ribosomal P proteins of *Trypanosoma cruzi* in Chagas' disease differed from anti-P-protein autoantibodies in lupus, these antibodies were found to possess functional autoreactivity with heart tissue (11). Antibodies found in SLE patients show distinct cross-reactivity and inhibit *P. falciparum* culture through specific reactivity with PfP0 protein (5, 22). Anti-PfP0 antibodies and other autoantibodies are prevalent in adult residents of areas where malaria is endemic, but there is no apparent linkage to SLE disease in these regions (13). The correlation of human anti-P-protein antibodies to SLE disease progression is not clear (14). However, since disease progression has been postulated to be correlated with anti-P-protein antibodies in some of the studies (7, 16), it is imperative to elucidate the parasite-specific protective immune response. To obtain parasite-specific reagents, monoclonal antibodies (MAbs) were raised against the recombinant PfP0 protein. In this paper, we show that three of these MAbs were protective and reacted very specifically to the parasite P0 protein. Using active and passive immunizations, we demonstrate that the 16-amino-acid C-terminal peptide sequence (P peptide) of PfP0 is protective and that the response is very specific for the parasite protein.

The MAbs were generated against the recombinant carboxy-

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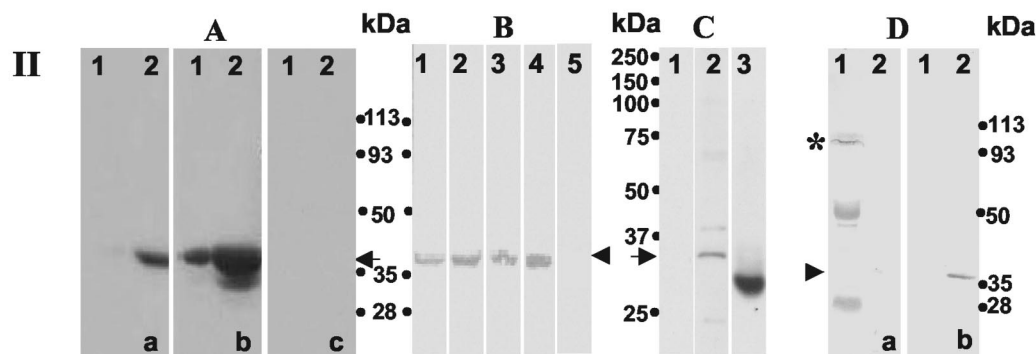
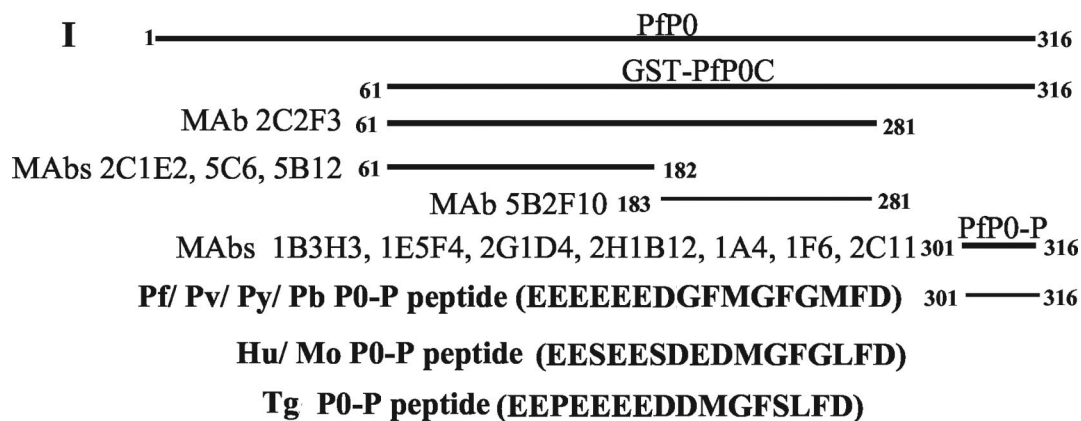


FIG. 1. (I) Schematic representation of PfP0 protein with domains recognized by the MAbs. The sequences of conserved P0 P peptides are shown. The P0 P peptides conserved in *P. falciparum*, *P. vivax*, *P. yoelii*, and *P. berghei* (Pf/Pv/Py/Pb), humans and mice (Hu/Mo), and *T. gondii* (Tg) are shown. (II) Immunoblot analysis. Western blots of total protein extracts of *P. falciparum* (A), *P. berghei* (B), *T. gondii* (C), and human lymphocytes (D) with various MAbs are shown. The extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gels) under reducing conditions. (A) *P. falciparum* particulate (lanes 1) and supernatant (lanes 2) fractions, probed with MAb 5B2F10 (a and b) and control Sp2/0 culture supernatants (c). A shorter (a) and longer (b) exposure of the enhanced chemiluminescence reaction for the same immunoblot is shown. (B) *P. berghei* protein extract probed with culture supernatants of different MAbs. Lanes: 1, MAb 2C1E2; 2, MAb 2C2F3; 3, MAb 5B2F10; 4, MAb 1E5F4; 5, control Sp2/0. (C) *T. gondii* total protein probed with pooled MAbs (lane 1), polyclonal anti-PfP0 antibodies (diluted 1:100) (lane 2), and anti-SAG1 antibodies (diluted 1:5,000) (lane 3). (D) Human lymphocyte extract (lanes 1) and *P. berghei* protein extract (lanes 2) using goat anti-human dynamin antibody (diluted 1:1,000) (a) and pooled MAbs (b). The positions of the respective P0 proteins (arrows and arrowheads) and the 100-kDa dynamin protein (asterisk) are indicated.

terminal region of P0 (PfPOC; amino acids 61 to 316), which was purified as described earlier (3). Approximately 50 μ g of this purified PfP0 protein emulsified with Freund's adjuvant was administered intraperitoneally into 6-week-old female BALB/c mice. After four weekly injections, the mice were immunized on a monthly basis. Five days before fusion of splenocytes with mouse myeloma Sp2/0 cells, the mice were immunized once with 250 μ g of immunogen in phosphate-buffered saline (PBS). Antibody-secreting clones were selected by an enzyme-linked immunosorbent assay (ELISA) using immobilized glutathione *S*-transferase (GST)-PfP0C protein. Of the 12 hybrid clones that showed exclusive reactivity to PfP0C, 7 were subcloned to monoclonality by limiting dilutions, yielding 7 pure clones. MAbs 2C1E2 and 2C2F3 mapped to amino acid residues 61 to 182 and 61 to 281, respectively, and MAb 5B2F10 mapped to residues 183 to 281 of PfP0, while MAbs 1B3H3, 1E5F4, 2G1D4 and 2H1B12 mapped to the linear P domain (Fig. 1). Thus, MAbs reactive to the extreme carboxy-terminal 16-amino-acid domain of PfP0 were prevalent among the MAbs generated, strengthening the enhanced immuno-

genic status of the extreme carboxy-terminal domain of P0 protein.

Specificity of reactivity of the MAbs to the parasite P0 protein. To test the binding of the MAbs to the PfP0 protein and to check the cross-reactivity of the MAbs with (i) the murine malaria parasite *Plasmodium berghei*, (ii) the closely related apicomplexan parasite *T. gondii*, and (iii) the HuP0 proteins, immunoblotting and immunofluorescence assay (IFA) were performed. Asexual stages of *P. falciparum* (3D7 strain) were cultured in vitro as described earlier (6). Parasites were maintained in 5% hematocrit in complete RPMI at 37°C in a humidified chamber containing 5% CO₂. The *P. berghei* ANKA strain was maintained by passaging asexual stages through BALB/c mice by intraperitoneal injections. Intracellular parasites were liberated from infected erythrocytes by treatment with 0.15% saponin (3). The parasite pellet was solubilized with 5 volumes of PBS buffer containing 1% Triton X-100, and the supernatant and the particulate fractions, normalized to equal volumes, were used for Western blot analysis. Parasite protein extract from approximately 10⁵ parasites/lane was elec-

trophoresed on a 10% polyacrylamide gel under reducing conditions. Proteins were transferred onto nitrocellulose paper and probed with the MAbs, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, and immunoreactive bands were visualized with 3,3'-diaminobenzidine (Sigma). *T. gondii* was cultured, and tachyzoites were harvested for immunoblotting and IFA as described earlier (20).

To determine whether the MAbs cross-reacted with HuP0, immunoblotting of protein extract from human lymphocytes was done. Human blood was collected with the anticoagulant EDTA, and the lymphocytes were separated by centrifugation at $600 \times g$ for 15 min at 4°C on Histopaque 1077 density gradient (Sigma Diagnostics, Inc.). The buffy coat was used as the source of human lymphocytes. Crude protein extract was obtained by sonication of the lymphocytes in PBS containing pepstatin (1 µg/ml) and leupeptin (1 µg/ml).

Immunoblot analysis of each of the seven MAbs with total *P. falciparum* protein extract showed a single band of reactivity to the 38-kDa P0 protein. Figure 1, panel IIA, shows a representative sample of such an immunoblot using MAb 5B2F10. Most of the PfP0 protein was found in the soluble supernatant fraction, but there was approximately 10% residual activity in the particulate fraction as well (Fig. 1, panel IIA). This is consistent with the surface localization property of this protein (3). Total crude protein extract from *P. berghei* showed distinct cross-reactivity with each MAb (Fig. 1, panel IIB). Neither the *T. gondii* tachyzoites nor the human lymphocytes showed any reactivity with the pooled MAbs (Fig. 1, panels IIC and D). However, the same preparation reacted with standard marker antibodies, ensuring proper protein preparation and immunoscreening procedure. The murine malaria parasites *P. berghei* and *Plasmodium yoelii* P0 proteins and the other human malaria parasite *Plasmodium vivax* P0 protein show >86% identity and >94% similarity to PfP0 (www.PlasmoDB.org), and the P-peptide domain is identical for each of these parasites (Fig. 1). TgP0 protein is also very similar to PfP0 (20), and the polyclonal antibodies to PfP0 show cross-reactivity with the TgP0 protein extract (Fig. 1, panel IIC). However, the non-reactivity of the MAbs with TgP0 indicates that there are distinct conformational differences in the *Plasmodium* and *Toxoplasma* P0 proteins. The P-peptide sequence of TgP0 is quite distinct from that of PfP0 and is identical to the other unicellular protozoan parasites, such as *Leishmania* and *Trypanosoma* (Fig. 1).

MAbs 2C1E2, 2C2F3, 5B2F10, and 2H1B12 (one MAb for each of the four classes reactive to different domains of PfP0) were used for IFA, flow cytometric analysis, and invasion-blocking assays. IFA was performed with *P. falciparum* and *T. gondii* as described earlier (20, 21). Briefly, slides coated with the parasites were air dried and fixed for 5 min with chilled methanol. After blocking of the slides with 100 µg of bovine serum albumin (BSA) per ml, the slides were incubated with the hybridoma culture supernatants for 1 h. This was followed by incubation with Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) (heavy plus light chains) (Molecular Probes) for 30 min. 4',6'-Diamidino-2-phenylindole (DAPI) (Boehringer Mannheim) (0.5 µg/ml) was used for 1 min for double staining of the slides. Confocal microscopy was performed using a Bio-Rad Radiance imaging system mounted on a Ni-

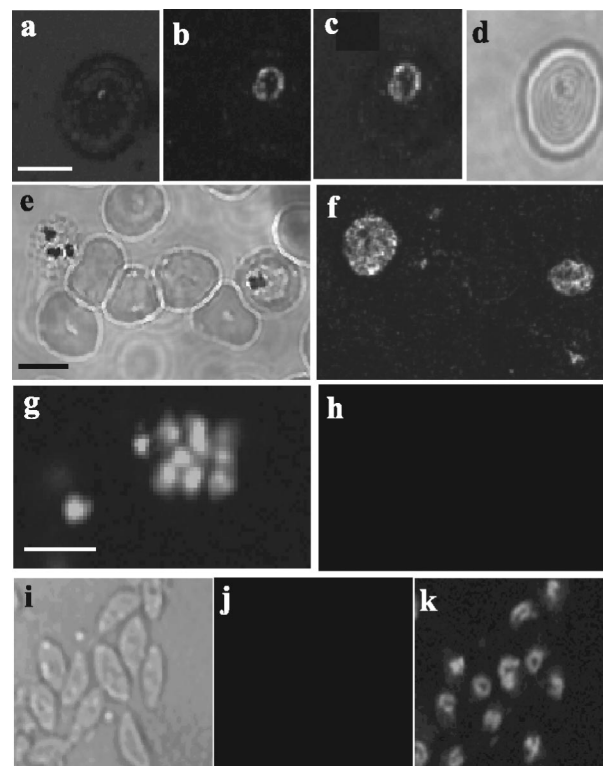


FIG. 2. Immunofluorescence of *P. falciparum* asexual stages and *T. gondii* tachyzoites. Ring (a to d) or schizont (e to h) stages of *P. falciparum* with the culture supernatants of 5B2F10 (a to f) and Sp2/0 (g and h). Panels a to d and panels e and f show the same views, respectively, with panels d and e representing the corresponding bright fields. *P. falciparum* ring stage was stained with DAPI (a) and MAb 5B2F10 (b). Panel c shows a merged view of panels a and b, with nonoverlapping staining. (i to k) *T. gondii* tachyzoites by bright-field microscopy (i), with pooled MAbs (j), and with DAPI stain (k). DAPI stain and Alexa Fluor 488 goat anti-mouse IgG stain were used. Bar, 5 µm.

kon inverted microscope. An argon laser with a wavelength of 488 nm was used as the excitation source. The samples were viewed using 100× oil immersion objective lens.

A representative IFA reactivity with MAb 5B2F10 is shown in Fig. 2. A ring stage is shown in Fig. 2a to d. In this early stage, the PfP0 protein appears in the thin cytoplasmic region. In later stages, the reactivity is observed all over the parasite (Fig. 2e and f). Maximum reactivity of the MAbs was observed with the trophozoite and schizont stages, which is consistent with the transcription profile of PfP0 (6) (<http://PlasmoDB.org>). No reactivity was seen with the control Sp2/0 culture supernatant (Fig. 2g and h), RBCs (Fig. 2e and f), or lymphocytes (data not shown). No reactivity was seen with *T. gondii* tachyzoites with any of these four MAbs (Fig. 2i to k).

To further rule out any cross-reactivity of the MAbs to native HuP0, flow cytometric analysis was performed using human lymphocytes (Fig. 3). Human lymphocytes, obtained from the buffy coat after Histopaque density gradient centrifugation, were fixed with 4% paraformaldehyde for 10 min, and permeabilized for 10 min using PBS containing 1.5% Triton X-100 and 2% fetal bovine serum. Aliquots containing 10^6 cells per 100 µl of PBS containing 2% fetal bovine serum were

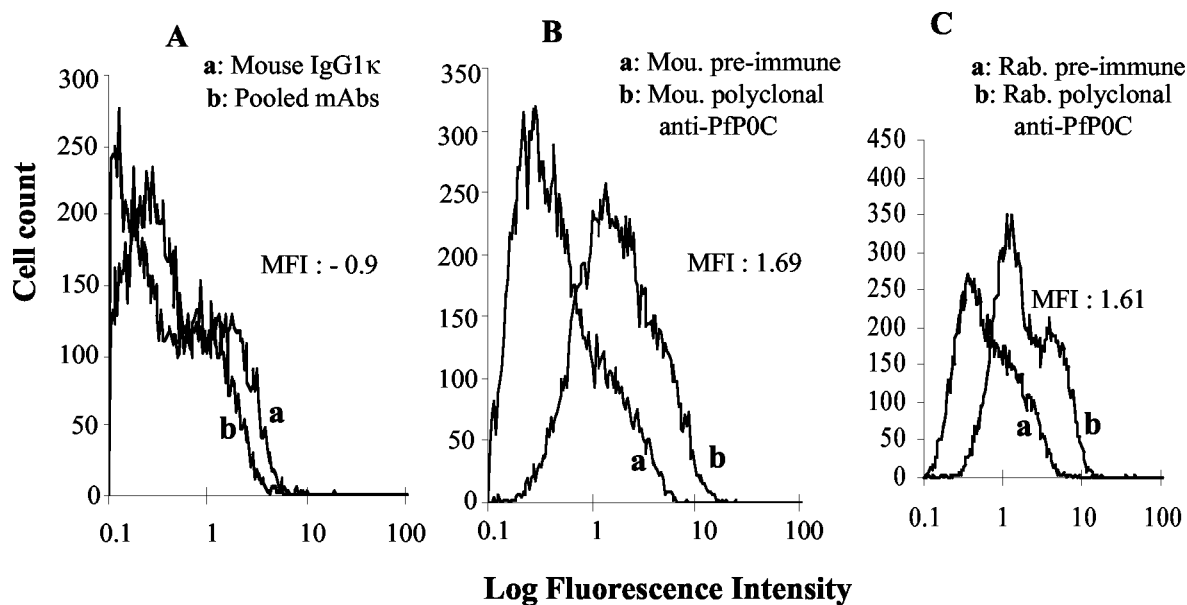


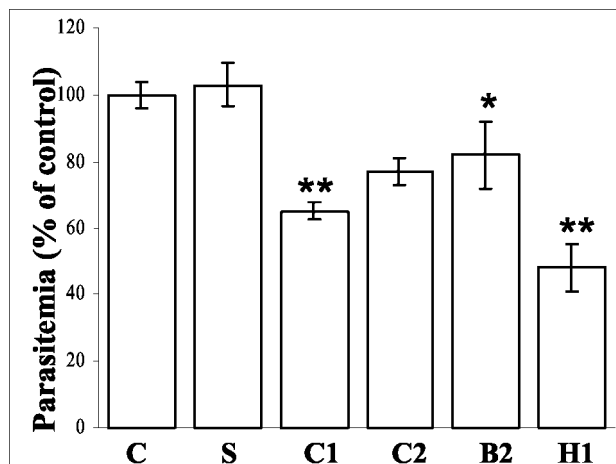
FIG. 3. Flow cytometric analysis of fixed and permeabilized human lymphocytes. (A) Purified mouse IgG1(κ) isotype control and pooled MAbs were used. (B and C) Mouse (Mou.) and rabbit (Rab.) preimmune serum (a) and mouse and rabbit polyclonal anti-GST-PfP0C antibodies (b) were used. The mean fluorescence intensity (MFI) for specific anti-Pf0 staining over control staining is indicated.

incubated for 30 min with purified mouse IgG1(κ) (BD Pharmingen) as an isotype control and with various anti-PfP0 antibodies. In each of the treatments, MAbs were used at a concentration of 1 μ g of protein per 10^6 cells. This was followed by incubation with Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG (heavy plus light chains) (Molecular Probes) for 45 min. All the steps were performed on ice. Labeled cells were analyzed in a flow cytometer (Partec PAS) using Partec Flowmax software. No reactivity was observed with the pooled MAbs (Fig. 3A). The cross-reactive mouse and rabbit polyclonal antisera against PfP0 protein (23) showed significant reactivity with the same lymphocyte population under the same conditions (Fig. 3B and C).

Invasion-blocking assay. Asexual stages of *P. falciparum* were cultured in vitro as described earlier (3). The invasion-blocking assay was performed in triplicate in 96-well sterile tissue culture plate (Nunc, Roskilde, Denmark) in a total culture volume of 200 μ l. *P. falciparum*, synchronized by repeated sorbitol treatment (21) at a parasitemia of 1 to 2% with 5% hematocrit, was incubated with MAbs 2C1E2, 2C2F3, 5B2F10, and 2H1B12 concentrated by 50% ammonium sulfate precipitation of ascitic fluid. With recombinant GST-PfP0C protein at a concentration of 50 μ g/ml, MAbs 2C1E2 and 2H1B12 bound to up to 30 ng of the protein, while MAbs 2C2F3 and 5B2F10 bound to as little as 7 ng of the protein (data not shown). Hence, MAbs 2C1E2 and 2H1B12 were used at a concentration of 400 μ g/ml, while the other two MAbs were used at 100 μ g/ml.

RBC smears were made at different time points and stained with Giemsa, and parasitemia was measured by microscopic counting. Three independent experiments were performed for each treatment, and at least two sets of 5,000 RBCs were counted for each experiment at every time point (Fig. 4). Results of invasion-blocking assay were subjected to statistical

analysis. The means of experimental groups were compared using one-way analysis of variance (GraphPad InStat, San Diego, Calif.). The means were considered statistically significantly different if the *P* values were <0.05 . RBC smears were counted at different stages, rings, trophozoites, and schizonts



**P* < 0.05

***P* < 0.01

FIG. 4. Invasion-blocking assay. Synchronized *P. falciparum* cultures were treated with different antibodies as described in the text. MAbs 2C1E2 (C1), 2H1B12 (H1), and Sp2/0 (S) were used at 400 μ g/ml, while MAbs 2C2F3 (C2) and 5B2F10 (B2) were used at 100 μ g/ml. An untreated control culture (C) is shown. Percent inhibition in parasitemia after 55 h is depicted. The values were considered statistically significantly different if the *P* values were <0.05 . Values are expressed as means \pm standard errors of the means (error bars).

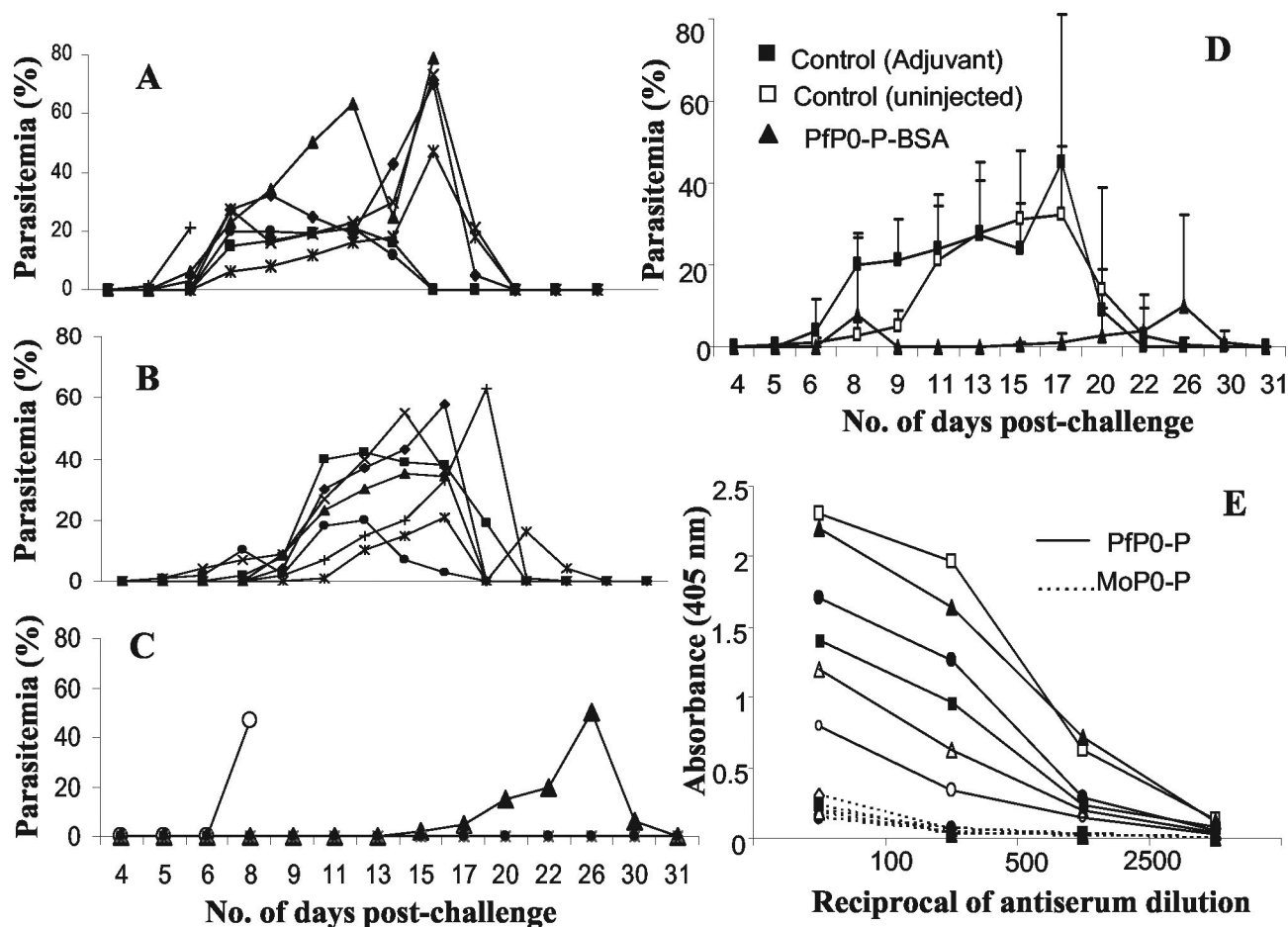


FIG. 5. Vaccination of mice with PfP0 P peptide. The parasitemia profile of each mouse over a period of 31 days is shown. Mice were injected with adjuvant (A) or not injected (B) for controls or injected with PfP0 P-BSA conjugate (C). (D) Average parasitemia values over a period of 31 days. The parasitemia values were considered statistically significantly different if the *P* values were <0.05. (E) Reactivity of antisera from mice immunized with PfP0 P-BSA to PfP0 P and MoP0 P peptides.

until the next ring stage. No significant differences in parasitemia were observed up to the schizont stage. However, there were significant differences in parasitemia in the control and antibody-treated wells in the smears made after 55 h, when most of the parasites were in the ring stage (Fig. 4). At a final concentration of 400 µg/ml, MAbs 2C1E2 and 2H1B12 inhibited parasitemia by approximately 35 and 52%, respectively. MAbs 2C2F3 and 5B2F10, used at 100 µg/ml, inhibited parasitemia by 23 and 18%, respectively. No inhibition was seen in the wells with Sp2/0 preparation (400 µg/ml). All the MAbs except 5B2F10 showed significant inhibition (*P* < 0.05).

Vaccination studies with PfP0 P peptide in mice. MAbs reactive to the extreme carboxy-terminal 16-amino-acid domain of PfP0 were prevalent among the MAbs generated. Moreover, the anti-PfP0 P-peptide MAb 2H1B12 was the most effective inhibitor of parasite invasion. It was also observed that all four MAbs as well as three other parent lines (7 of 12 total lines tested) reacted specifically with the linear PfP0 P-peptide epitope in ELISA, while they did not react with the equivalent P0 P-peptide epitope conserved in mice and humans (data not shown). Therefore, we decided to test the PfP0 P peptide in vaccination studies in mice.

The PfP0 P peptide was coupled to BSA (PfP0 P-BSA) using glutaraldehyde (15). Experiments were performed on three groups of 8-week-old female Swiss mice (six or seven mice per group). Mice were injected intraperitoneally with PfP0 P-BSA conjugate in Freund's adjuvant at 21-day intervals. In one control group, mice were injected in parallel with PBS emulsified in Freund's adjuvant. The other control group received no injections. After five immunizations, the titers of the anti-PfP0 antibodies were measured, and the mice were challenged with *P. yoelii* (10⁶ parasites per mouse), and parasitemia was monitored daily.

Figure 5 shows the parasitemia profile of each mouse over a period of 31 days after challenge in the three groups. All 14 control mice developed parasitemia by the sixth day postchallenge (Fig. 5A and B). Figure 5D shows the average parasitemia values for each treatment. The highest average parasitemia values were 45 and 32% on day 17 for mice injected with adjuvant and unimmunized mice, respectively. Of the seven adjuvant-treated mice, two mice died on day 6 and day 17. Of the seven control mice that received no immunization, one mouse died on day 20. However, of the six mice immunized with PfP0 P peptide, only two mice developed para-

sitemia that was detected throughout the study. One mouse showed parasitemia on day 7 and died on day 8 when parasitemia was approximately 47%. This mouse had the lowest titer of antibodies to PfP0 P peptide (Fig. 5E). The other mouse showed a delayed parasitemia from day 14 and showed a peak value of approximately 50% on day 26. However, this mouse recovered by day 31. None of these mice showed reactivity to MoP0 P peptide (Fig. 5E). Mean parasitemia levels of the three groups were compared using one-way analysis of variance (GraphPad InStat). The means were considered statistically significantly different if the *P* values were <0.05. Differences in the mean parasitemia levels were extremely significant on day 9 (*P* < 0.0001), day 11 (*P* = 0.0014), day 13 (*P* = 0.0007), day 15 (*P* = 0.003), and day 17 (*P* = 0.023).

To monitor possible long-term effects of immunization with the PfP0 P peptide, sera were collected from these mice 4 months after parasite challenge. While the sera continued to be positive for PfP0 P peptide (average titer, 1:2,000), they showed no reactivity to MoP0 P peptide (data not shown). Repeated vaccination studies with PfP0 P peptide showed similar results, with the extent of protection correlating with the titer of anti-P-peptide antibody. Initial vaccination studies were performed with mice after four immunizations of the antigen. The titers of the anti-PfP0 P antibodies were less than 1:2,000 (data not shown). Significant protection was not seen when the mice were subjected to parasite challenge. Hence, subsequent trials were performed after five immunizations with the antigen.

A comparison of the P-peptide sequence of the P0 protein conserved in humans and mice and the *Plasmodium* P0 protein reveals a difference of 5 amino acids in the last 16-amino-acid stretch (Fig. 1, panel I). In the last six amino acids, GFGLFD (HuP0) versus GFGMFD (PfP0), there is just one amino acid difference. The amino acids crucial for SLE serum reactivity are postulated to be the two phenylalanine residues (14). Although these two phenylalanine residues are conserved in the *Plasmodium* P0, neither the MAbs nor the polyclonal sera from vaccinated mice reacted with the mammalian P peptide. However, the SLE serum samples do react with the PfP0 16-amino-acid P peptide in an ELISA (A. B. Mannan and S. Sharma, unpublished data). Thus, although the postulated 6-amino-acid SLE target P domain is nearly identical between PfP0 and HuP0, there appears to be a significant difference between the antibodies produced in SLE patients compared to the immune response generated upon P-peptide immunization. The other four amino acids, which are different in the HuP0 and PfP0 16-amino-acid P peptides, are radical changes from uncharged to charged amino acids (Fig. 1); these changes are likely to confer considerable structural differences between the HuP0 and PfP0 peptides. A notable difference between PfP0 and the mammalian P peptide is the absence of the serine residues at positions 303 and 306. These residues have been shown to be phosphorylated in mammals and are postulated to play important target residues for regulatory structural changes (8). Thus, despite the 69% identity between the P peptides of *Plasmodium* and mammalian species, the P epitopes appear to be structurally and functionally different between these species.

The closely related apicomplexan and protozoal parasites appear to have other structures for the P peptide. In the *T.*

gondii P peptide, six amino acids are different compared to the *Plasmodium* P-peptide sequence, with a proline residue replacing a charged amino acid (Fig. 1). This proline residue is observed in other protozoan species as well (10, 11). The functional relevance (if any) for the differences for these three sets of P peptide is not clear as yet. However, with distinct differences in the P peptides of mammals and other protozoans, but with the conservation among all the *Plasmodium* P0 proteins, this linear epitope should be able to target all forms of malaria specifically. The invasion-blocking parasite-specific MAbs and vaccination data presented here demonstrate malaria-specific protective properties of the PfP0 P peptide. With the current scenario of emerging drug resistance to virtually every existing drug against malaria, it may be worthwhile to explore the PfP0 P epitope towards therapeutic and prophylactic control of malaria.

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