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Protective Immunity against *Plasmodium yoelii* Malaria Induced by Immunization with Particulate Blood-Stage Antigens

JAMES M. BURNS, JR.,* PATRICIA D. DUNN, AND DONNA M. RUSSO

Department of Microbiology, Meharry Medical College, Nashville, Tennessee 37208

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The *Plasmodium yoelii* murine model was used to test several combinations of blood-stage antigens and adjuvants for the ability to induce immunity to blood-stage malaria. Upon fractionation of whole blood-stage antigen into soluble and insoluble components, only the particulate antigens (pAg) induced protective immune responses. Of a number of adjuvants tested, Quil A was the most effective. Immunization with pAg plus Quil A induced solid protection against nonlethal and lethal *P. yoelii* challenge infection. Analysis of cytokine production revealed mRNA for Th1-type cytokines (interleukin 2 [IL-2] and gamma interferon) as well as Th2-type cytokines (IL-4 and IL-10) in the spleens of both protected and susceptible animals. The data suggested that the protective pAg response was associated with the earlier production of cytokine mRNA with a Th2 phenotype somewhat favored. Immunization of B-cell-deficient J_HD mice indicated that the protection against *P. yoelii* induced by pAg immunization was B cell dependent. Although immunization with pAg plus Quil A increased the levels of antigen-specific antibodies of all four immunoglobulin G (IgG) isotypes, protection correlated most closely with the presence of IgG1 and IgG2b antibodies. Sera from pAg-plus-Quil A-immunized animals recognized only a limited subset of six to eight distinct *P. yoelii* antigens, primarily associated with the pAg fraction. These results provide the basis for the identification and characterization of potential vaccine antigens, selected solely for their ability to immunize against blood-stage malaria.

Estimates of the global incidence of malaria range from 300 to 500 million cases per year, with 2 to 3 million deaths (5). The protozoan parasite *Plasmodium falciparum* is the most significant cause of this disease. Despite many intervention efforts, morbidity and mortality estimates continue to rise. Chemotherapy and vector control programs have been largely ineffective, due to the emergence and spread of insecticide-resistant mosquito vectors and drug-resistant plasmodia. Thus, an effective malaria vaccine is urgently needed.

The key to vaccine development efforts is a clear understanding of the complexity of the immune responses against plasmodia. In contrast to many infectious agents, immunity to reinfection with *P. falciparum* does not develop in individuals following resolution of blood-stage malaria. Nevertheless, resistance to malaria, especially the severe complications of the disease, develops with age. In fact, some adults living in areas where malaria is endemic may not exhibit clinical manifestations of disease even when circulating blood parasites are present (8, 24). Sustained antigenic stimulation and antibody production may be important in maintaining this antidisease state. Studies of *Plasmodium yoelii* in mice support the notion that the resolution of blood-stage infection can be achieved by antibody-mediated mechanisms (25). However, antibody-independent, cell-mediated mechanisms of immunity may, in certain instances, be effective against other species of *Plasmodium* (25).

The immune response during malaria is not limited to responses ultimately leading to the resolution of disease. As much as 90% of the antibodies produced during infection are not parasite specific (6). Besides polyclonal activation, infection may activate autoimmune and/or immunosuppressive responses (45). Nevertheless, studies of *P. yoelii* and *Plasmodium chabaudi* indicate that sterilizing immunity and resistance to reinfection can develop (25, 45). The question remains as to which infection-induced protective responses can be induced by immunization with a subunit vaccine. Furthermore, there may be additional protective immune responses that do not arise during the normal course of infection that can be primed by vaccination and boosted by infection.

By using animal models, a number of laboratories have attempted to induce protective immune responses by immunization with crude preparations of whole blood-stage antigens. Various levels of protection have been reported against *Plasmodium gallinaceum* (31), *P. yoelii* (15, 23, 28, 29, 41), *Plasmodium berghei* (9, 27, 28), *Plasmodium vinckei* (18), *Plasmodium knowlesi* (2, 26, 38), and *P. falciparum* (30, 34, 35). Adjuvants used in these immunization studies included Freund's adjuvant, muramyl dipeptide, saponin, alhydrogel, gamma interferon (IFN-γ), nonionic block polymers, and detoxified lipopolysaccharide (LPS) as well as bacteria, including *Bordetella pertussis*, *Corynebacterium parvum*, and *Salmonella typhimurium*. These studies, however, focused primarily on the efficacy of the adjuvant and provided only a limited characterization of immunization-induced protective responses. Furthermore, the ability to readily identify and characterize protective antigens based on these immunization-induced responses was not possible. This limitation was largely due to the complexity of both the antigen preparations employed and the immune responses induced.

In the present study, we utilized the *P. yoelii* murine model to investigate immunization-induced protective immune responses to identify potential vaccine antigens. Upon immunization with a subset of blood-stage antigens, we induced solid protection against nonlethal and lethal *P. yoelii* infection. With the adjuvants tested, protective immune responses were driven primarily by immunization with antigens present in the insoluble or particulate fraction of a blood-stage antigen preparation. While both Th1 (interleukin 2 [IL-2] and IFN-γ) and Th2 (IL-4 and IL-10) type cytokines were produced, a bias toward

* Corresponding author. Mailing address: Meharry Medical College, Department of Microbiology, 1005 D. B. Todd Blvd., Nashville, TN 37208. Phone: (615) 327-5726. Fax: (615) 327-6072.

the Th2 phenotype was evident. In addition, protection was B cell dependent and associated with the production of immunoglobulin G1 (IgG1) and IgG2b antibodies. Of potential importance for vaccine development efforts, protective immunization induced the production of antibodies which were reactive with a discrete subset of only six to eight *P. yoelii* blood-stage antigens.

MATERIALS AND METHODS

Experimental infections. Male C57BL/6 or CByB6F1/J (BALB/cByJ × C57BL/6J) mice, 5 to 6 weeks of age, were purchased from the Jackson Laboratories (Bar Harbor, Maine) and housed in the AAALAC-approved Animal Care Facility of Meharry Medical College. B-cell-deficient J_HD and C57BL/6 mice were maintained and bred at the University of Wisconsin Gnotobiote Laboratory. B-cell-deficient J_HD mice (*H-2*^{b}) fail to produce functional immunoglobulin heavy chains due the targeted deletion of J_H gene segments. As such, B-cell differentiation is blocked and surface immunoglobulin-positive cells are completely lacking (3, 43). The lethal 17XL and nonlethal 17X strains of *P. yoelii* were originally obtained from William P. Weidanz (University of Wisconsin, Madison) and maintained as cryopreserved stabilates. Blood-stage infections were initiated by intraperitoneal injection of parasitized erythrocytes (RBCs) obtained from donor mice. The resulting parasitemias were monitored by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa (14).

Antigen preparation. When parasitemias averaged 35 to 40%, blood was collected from C57BL/6 mice infected with *P. yoelii* 17XL. The blood obtained contained a mixture of ring, trophozoite, and schizont-stage parasites. Mouse leukocytes were removed by passage of blood over microcrystalline cellulose columns (10). RBCs were collected and lysed with 0.01% saponin. Intact parasites were collected by centrifugation and washed to remove soluble RBC proteins. Parasite pellets were resuspended in 10 volumes of 50 mM KCl–5 mM $MgCl_2$. Following 10 cycles of rapid freezing and thawing, soluble antigen (sAg) and particulate antigen (pAg) fractions were recovered following centrifugation for 15 min at 2,000 × *g* (4°C). Of the 70 to 75 mg of total protein obtained in each of three preparations, 70 to 75% was recovered in the soluble fraction and the remaining 25 to 30% was recovered in the particulate fraction.

Immunizations. Groups of mice (four to six per group) were immunized subcutaneously with 100 μg of *P. yoelii* sAg or pAg plus adjuvant or with adjuvant alone. Based on previous studies (2, 9, 15, 18, 23, 26–31, 34, 35, 38, 41), the adjuvants selected included IFN-γ (5,000 U; Life Technologies, Grand Island, N.Y.), complete Freund's adjuvant (CFA; 1:1 emulsion; Life Technologies), RAS (50 μg of monophosphoryl lipid A plus 50 μg of synthetic trehalose dicorynomycolate; Ribi ImmunoChem Research Inc., Hamilton, Mont.), and Quil A (25 μg; Accurate Chemical and Scientific Corporation, Westbury, N.Y.). Three weeks following the primary immunization, the animals were again immunized subcutaneously with the same doses of antigen and adjuvant. CFA was used only in the primary immunization and was replaced with incomplete Freund's adjuvant (IFA; Life Technologies) for the boost. Seven to 10 days later, the animals were infected intraperitoneally with 10^5 or 10^6 *P. yoelii* parasitized RBCs and blood parasitemias were monitored. The statistical significance of differences in the mean peak parasitemia between groups was calculated with Student's *t* test.

Reverse transcriptase (RT)-PCR cytokine assays. Total RNA was isolated from the spleens of animals immunized with pAg plus Quil A or Quil A alone. Spleens were harvested from groups of mice ($n = 3$) sacrificed 2, 4, 6, 8, and 10 days following challenge infection with 10^5 *P. yoelii* parasitized RBCs. Total spleen cell RNA was isolated with the TRIzol Reagent (Life Technologies) by the method of Chomczynski and Sacchi (4) as modified by the manufacturer.

Specific cytokine mRNA was detected by use of a previously described reverse transcriptase PCR protocol (37) as modified by Wynn et al. (47). The assay permitted the comparison of the relative quantities of mRNA for a given cytokine present in a total RNA sample. Briefly, 1 μg of total RNA was reverse transcribed into cDNA with random hexamers as primers and Superscript II reverse transcriptase as described in the manufacturer's protocol (Superscript Preamplification System; Life Technologies). Following cDNA synthesis, the reaction mixture was diluted 10-fold with distilled water to a final volume of 200 μl. cDNA for IFN-γ, IL-2, IL-4, IL-10, and hypoxanthine-guanine phosphoribosyltransferase (HPRT) was PCR amplified in a 50-μl reaction mixture containing 10 μl of cDNA, 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, 15 mM $MgCl_2$), 0.2 μM sense and antisense primers, and 1 U of *Taq* DNA polymerase (Life Technologies). The specific primers used were as described previously and designed to permit the distinction of products amplified from cDNA (minus introns) and genomic DNA (plus introns) based on size (37). Following the initial denaturation, the amplification proceeded at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. As previously established (47), the number of cycles for each amplification was as follows: 29 for IFN-γ, 35 for IL-2, 33 for IL-4, 33 for IL-10, and 23 for HPRT. The amplification of mRNA for the constitutively expressed HPRT gene served as a control to ensure that all reactions contained an equal quantity of input RNA. Negative controls lacking input cDNA were included in each assay. Quantitative amplification of each cytokine mRNA was verified by using 5, 1, and 0.2 μg of input total RNA of selected samples.

Amplified PCR products (1/10 reaction mix) were analyzed by Southern blotting following agarose gel electrophoresis on 1% gels. Oligonucleotide probes were end labeled with T4 polynucleotide kinase (Life Technologies) in the presence of [γ-^{32}P]ATP (3,000 Ci/mmol; NEN Research Products, Boston, Mass.). The sequence of all oligonucleotide probes and conditions for hybridization were as described previously (37), with bound probe detected by autoradiography. All RNA samples were assayed for a given cytokine simultaneously.

Isotype ELISA. Following isolation as described above, *P. yoelii* 17XL blood-stage parasites were solubilized in 10 volumes of 20 mM Tris-HCl (pH 8.0)–50 mM NaCl–5 mM EDTA–1% sodium deoxycholate. After incubation at 4°C overnight, the antigen preparation was dialyzed extensively to remove the sodium deoxycholate. High-binding enzyme-linked immunosorbent assay (ELISA) plates (Easy-Wash; Corning Costar Corporation, Cambridge, Mass.) were coated with 2.5 μg of total *P. yoelii* 17XL antigen per well. Antigen was diluted in 100 mM Na_2HCO_3-NaH_2CO_3 buffer (pH 9.6), and plates were incubated overnight at 4°C. The next day, the wells were washed and blocked by incubation for 1 h in TBS (25 mM Tris-HCl [pH 8], 150 mM NaCl) containing 5% nonfat dry milk. Sera were diluted 1:100, 1:1,000, and 1:10,000 in TBS–0.1% Tween 20 containing 1% bovine serum albumin. Fifty microliters of diluted serum was added to each well and incubated at room temperature for 1 h. The sera assayed were collected from pAg-immunized and adjuvant control mice 7 to 9 days postclearance of *P. yoelii* 17X parasites. Five animals were assayed per group. Antigen-specific antibodies were detected with horseradish peroxidase-conjugated rabbit antibody (Zymed Laboratories, South San Francisco, Calif.) specific for mouse IgG1, IgG2a, IgG2b, or IgG3 and with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] as the substrate. Serum samples were run in duplicate, and absorbances were read at 405 nm. In each assay, wells coated with purified IgG1, IgG2a, IgG2b, or IgG3 myeloma proteins (16 ng/ml to 1 μg/ml) were used to generate a standard curve. The isotype standard curves were used to quantitate the antigen-specific antibody present in each serum sample, by using the dilution of serum that yielded an optical density at 405 nm between 0.1 and 1.0. The concentration of IgG isotype was expressed in units per milliliter, where 1 U/ml was equivalent to 1 μg of myeloma standard per ml. In each ELISA, no detectable *P. yoelii* antigen-specific antibody was demonstrated with a pool of normal mouse sera. The statistical significance of differences in the mean IgG concentration between groups was calculated by Student's *t* test.

Immunoblot assays. Total *P. yoelii* 17XL antigen was prepared as described above and solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2.5% SDS. Preparations of total blood-stage antigen, sAg, or pAg (10 μg/lane) were separated by SDS-PAGE on 10% polyacrylamide gels as described by Laemmli (19) and transferred electrophoretically to nitrocellulose membranes (42). Immunoblots were blocked for 1 h with TBS containing 5% nonfat dried milk. After the blots were washed with TBS–0.1% Tween 20, they were incubated for 2 h with *P. yoelii* infection sera or immunization sera diluted in TBS–0.1% Tween 20 containing 1% bovine serum albumin. Sera were collected from pAg-immunized and adjuvant control mice on day 0 prior to *P. yoelii* 17X infection or 7 to 9 days postclearance of the blood-stage parasites. Five animals per group were sacrificed at each time point. A pool of normal mouse sera served as the negative control. Antigen-specific antibodies were detected with horseradish peroxidase-conjugated rabbit antibody (Zymed Laboratories) specific for mouse IgG1, IgG2a, IgG2b, or IgG3 with 3,3′-diaminobenzidine tetrahydrochloride plus cobalt chloride (Sigma Chemical Co., St. Louis, Mo.) as the substrate. Alternatively, bound antibody was detected with [^{125}I]protein A (>30 μCi/μg; ICN Biomedicals, Inc., Irvine, Calif.) followed by autoradiography.

RESULTS

Immunization-induced protection against nonlethal *P. yoelii* 17X blood-stage infection. Several combinations of blood-stage antigens and adjuvants were tested for the ability to induce protection upon immunization against nonlethal *P. yoelii* 17X challenge infection. A preparation of whole *P. yoelii* 17XL blood-stage antigen was fractionated into sAg and pAg fractions. The sAg fraction represented 70 to 75% of the total antigen preparation, while the pAg fraction contained the remaining 25 to 30%. Groups of CByB6F1/J mice were immunized subcutaneously with *P. yoelii* sAg or pAg in adjuvant. Several adjuvants were tested, including Quil A (a semipurified component of saponin), IFN-γ, Freund's adjuvant, and RAS. Three weeks following the primary immunization, the animals were boosted with the same doses of antigen and adjuvant and challenged 7 to 10 days later with nonlethal *P. yoelii* 17X.

The level of protection afforded to mice immunized with *P. yoelii* pAg varied depending on the adjuvant selected. As shown in Fig. 1, mice immunized with *P. yoelii* pAg with either Freund's adjuvant, RAS, or Quil A showed a high level of

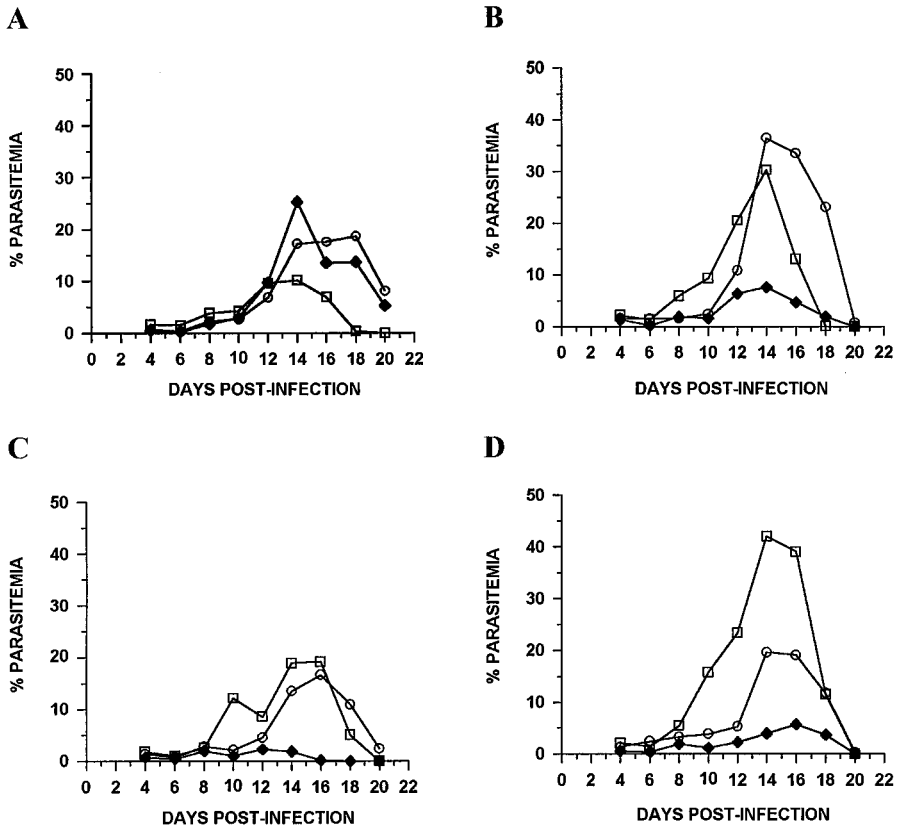

FIG. 1. Vaccine-induced immunity to nonlethal *P. yoelii* 17X infection. Groups of CByB6F1/J mice ($n = 5$) were immunized with *P. yoelii* sAg (○) or pAg (◆) in adjuvant or with adjuvant alone (□). Adjuvants included IFN-γ (A), CFA-IFA (B), RAS (C), or Quil A (D). Animals were boosted and challenged with 10^5 *P. yoelii* 17X parasitized RBCs. Resulting parasitemias were monitored by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa.

protection. In pAg-immunized mice, the mean peak parasitemia was reduced over threefold when Freund's adjuvant was used (Fig. 1B) (9.01% ± 5.19% versus 30.60% ± 8.86%, $P < 0.01$), approximately sevenfold when RAS was used as the adjuvant (Fig. 1C) (3.57% ± 3.53% versus 25.24% ± 19.27%, $P < 0.05$), and approximately sevenfold when Quil A was used as the adjuvant (Fig. 1D) (6.40% ± 12.33% versus 43.45% ± 5.65%, $P < 0.01$). No protection was observed in mice immunized with pAg and IFN-γ as the adjuvant (Fig. 1A).

In marked contrast, mice immunized with *P. yoelii* sAg developed patent parasitemia similar to control animals when the adjuvants were IFN-γ, Freund's adjuvant, and RAS (Fig. 1A to C). A partial reduction in mean peak parasitemia was noted upon immunization with sAg plus Quil A (Fig. 1D) (23.64% ± 14.36% versus 43.45% ± 5.65%, $P < 0.05$). Peak parasitemias varied between adjuvant control groups, possibly due to nonspecific effects of certain adjuvants.

In subsequent experiments, immunization with *P. yoelii* pAg plus Quil A reproducibly provided the maximal reduction in blood parasitemia. The specificity of the pAg plus Quil A response was confirmed since no protection was afforded to animals immunized with Quil A and a pAg fraction derived from uninfected RBC (data not shown). Accordingly, immunization with *P. yoelii* pAg plus Quil A was selected as the antigen-adjuvant combination to be used for additional studies.

Immunization-induced protection against lethal *P. yoelii* 17XL blood-stage infection. Immunization with *P. yoelii* pAg plus Quil A was tested for the ability to protect against infection with a lethal variant of *P. yoelii*. As with nonlethal infection (Fig. 2A), pAg plus Quil A immunization induced solid protection against *P. yoelii* 17XL, with a mean peak parasitemia of 7.1% ± 15.5% (Fig. 2B). Of the immunized animals, four of five developed a peak parasitemia of <0.5%. One animal was partially protected, developing a peak parasitemia of approximately 35%, which subsequently resolved. None of the pAg-immunized animals died. In contrast, animals immunized with Quil A alone developed a high-level parasitemia and became moribund, at which time they were euthanized. Thus, immunization with *P. yoelii* pAg plus Quil A induced a solid level of protection against nonlethal as well as lethal *P. yoelii* challenge infection.

Cytokine production associated with immunization-induced immunity to *P. yoelii*. The production of cytokine mRNA in the spleens of immunized and control mice upon challenge infection was assessed. Total spleen cell RNA was isolated from pAg-plus-Quil A-immunized and adjuvant control mice on days 2, 4, 6, 8, and 10 following challenge with *P. yoelii* 17X. Relative quantities of mRNA for IFN-γ, IL-2, IL-4, and IL-10 were detected by use of an RT-PCR-based protocol. Cytokine mRNA levels peaked in both the immunized and control groups during the first 6 days following nonlethal *P. yoelii* 17X challenge. In both groups, cytokine mRNA levels returned to baseline by day 10 of infection. Significant levels of cytokine mRNA were not detected in the spleens of mice following secondary immunization but prior to infection.

On days 2, 4, and 6 postinfection, mRNA for selected Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines was

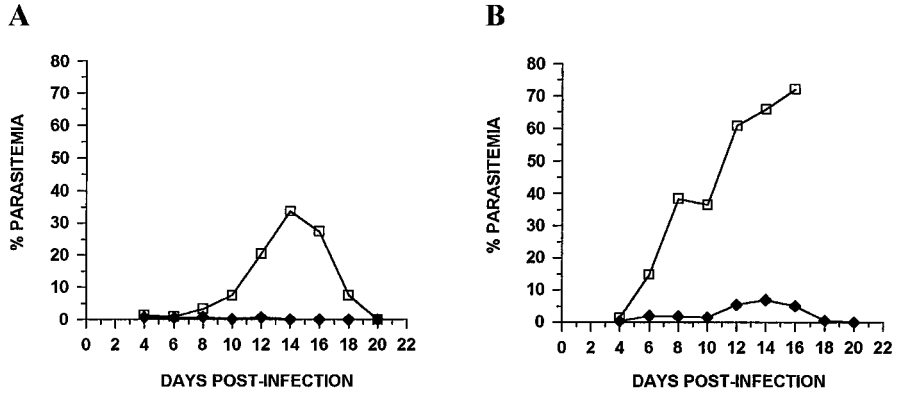

FIG. 2. Vaccine-induced immunity to lethal *P. yoelii* 17XL infection. Groups of CByB6F1/J mice ($n = 5$) were immunized with *P. yoelii* pAg plus Quil A (◆) or with Quil A alone (□). Animals were boosted and challenged with 10^5 parasitized RBCs of nonlethal *P. yoelii* 17X (A) or lethal *P. yoelii* 17XL (B). Resulting parasitemias were monitored by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa.

detected in groups immunized with pAg plus Quil A (Fig. 3B) and Quil A alone (Fig. 3A). mRNA for IFN-γ, IL-2, IL-4, and IL-10 was readily detected by day 2 following parasite challenge in pAg-plus-Quil A-immunized and protected mice. In comparison, cytokine mRNA was not detected until day 4 in susceptible adjuvant control mice. Neither Th1 nor Th2 type responses clearly dominated in either group. Differences between protected and susceptible groups were observed, however, considering the total amount of each cytokine mRNA produced during the period assayed. In particular, IFN-γ mRNA levels were reduced in pAg-plus-Quil A-immunized animals (Fig. 3B) as compared to that in adjuvant controls (Fig. 3A). Similarly, the production of IL-2 in protected mice was sharply reduced by day 6 of infection. In contrast, mRNA levels for IL-4 and IL-10 were somewhat higher in these pAg-immunized animals than in the controls. These data suggest that protective pAg immunization was associated with the earlier production of cytokine mRNA, with a Th2 phenotype somewhat favored.

Immunization-induced immunity to *P. yoelii* is B cell dependent. To assess the contribution of B cells to the protective pAg response, B-cell-deficient J_HD mice (*H-2*^{b}) were immunized with pAg plus Quil A and challenged with nonlethal *P. yoelii* 17X. As shown in Fig. 4A, B-cell-deficient J_HD mice immunized with either pAg plus Quil A or Quil A alone failed to control their *P. yoelii* infections. In contrast, pAg plus Quil A immunization induced solid protection against *P. yoelii* infection in genetically similar but immunologically intact C57BL/6 mice (*H-2*^{b}) as compared to that in controls (Fig. 4B). Thus, the protective immunity against *P. yoelii* blood-stage malaria induced by pAg plus Quil A immunization was B cell dependent.

Antibody production associated with immunization-induced immunity to *P. yoelii*. To investigate the nature of the protective B-cell response, the isotype profile of *P. yoelii*-specific antibodies present in the sera of immunized and control mice was assessed. Sera were collected from pAg-plus-Quil A-immunized and adjuvant control mice 7 to 9 days following complete clearance of blood-stage parasites. The concentration of antigen-specific antibodies of each IgG isotype was quantitated by ELISA. As shown in Table 1, levels of all four IgG isotypes were elevated in pAg-plus-Quil A-immunized animals compared to that in controls. Most notable in animals protected by pAg plus Quil A immunization, the levels of IgG1 and IgG2b antibodies were increased over 25-fold and 10-fold, respectively.

In addition to a quantitative assessment of antibody response, the specificity of antibodies present in these sera was also investigated. Seroreactivities were assayed on immunoblots of total *P. yoelii* blood-stage antigen, with bound antibod-

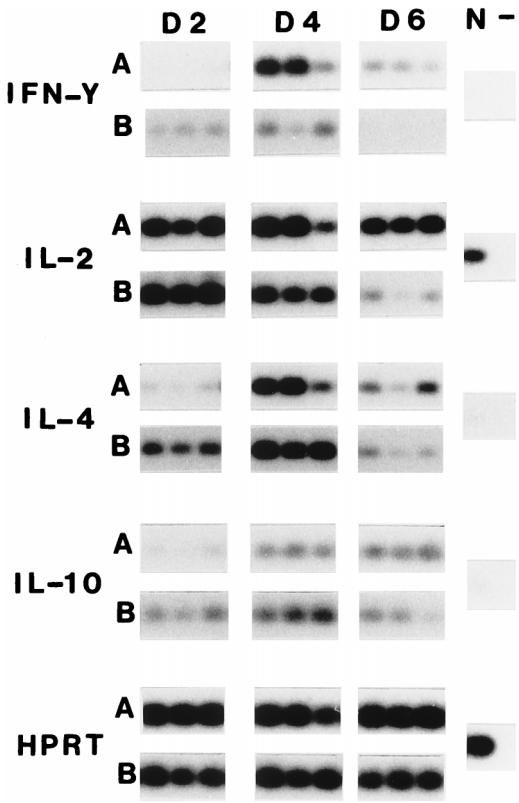

FIG. 3. Cytokine production associated with immunization-induced immunity to *P. yoelii*. Following *P. yoelii* 17X challenge infection, total RNA was isolated from the spleens of animals immunized with Quil A (A) alone or pAg plus Quil A (B). By use of a RT-PCR assay, relative quantities of mRNA for IFN-γ, IL-2, IL-4, IL-10, and HPRT were determined. Following agarose gel electrophoresis on 1% gels, amplified PCR products were analyzed by Southern blotting, with radiolabeled oligonucleotides specific for each cDNA sequence used as probes. Bound probes were detected by autoradiography. The results from each of three animals at 2 days (D2), 4 days (D4), and 6 days (D6) following challenge infection are shown. Products amplified from normal spleen cell RNA (N, unimmunized and uninfected) or with no input cDNA (−) are shown.

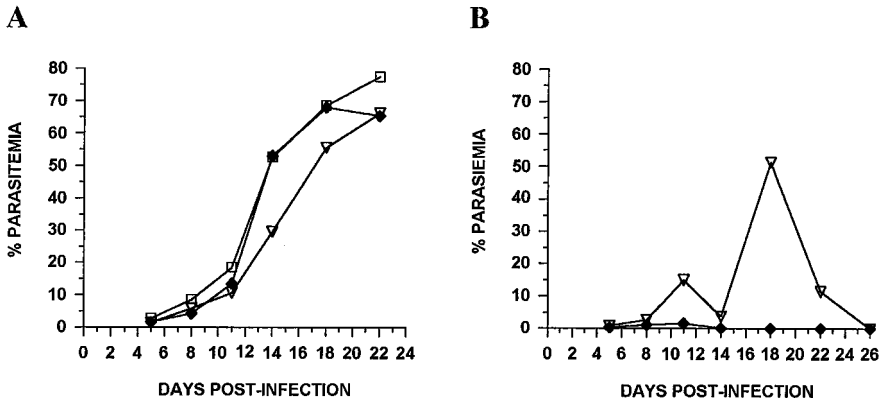

FIG. 4. pAg immunization of B-cell-deficient J_HD mice. B-cell-deficient J_HD mice (A) ($n = 6$) or immunologically intact C57BL/6 controls (B) ($n = 4$) were immunized and boosted with *P. yoelii* pAg plus Quil A (◆), Quil A alone (□), or PBS alone (▽) and challenged with 10^6 nonlethal *P. yoelii* 17X parasitized RBCs. Resulting parasitemias were monitored by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa.

ies detected by use of isotype-specific reagents. As shown in Fig. 5, a heterogeneous set of antigens was recognized by sera obtained from both pAg-plus-Quil A-immunized animals and adjuvant controls. While their blood parasitemias were much lower, pAg-plus-Quil A-immunized animals produced antibody to most of the same antigens as did adjuvant controls. In addition to these infection-induced specificities, seroreactivity to an additional set of antigens was associated with the protective pAg response. This was most clearly shown by a comparison of the specificities of antibodies of the IgG1 isotype present in the two groups (Fig. 5A, lanes 1 and 2).

To clearly identify antibody responses induced by pAg plus Quil A immunization, sera obtained from animals 7 to 10 days following secondary immunization, but prior to infection, were analyzed. The reactivities of these sera on immunoblots of preparations of *P. yoelii* total antigen, sAg, and pAg are shown in Fig. 6. Sera from pAg-plus-Quil A-immunized animals recognized six to eight distinct *P. yoelii* antigens of approximately 25 to 110 kDa (Fig. 6, lanes 4 to 6). These antigens were not recognized by sera from adjuvant controls (Fig. 6, lanes 1 to 3). Furthermore, these antigens were present predominately in the pAg fraction.

DISCUSSION

Continued efforts to identify and characterize protective antigens of *Plasmodium* will increase our ability to develop the most effective malaria vaccine. The present study focused on the induction of protective responses by immunization with selected antigen-adjuvant combinations. We attempted to understand the nature of the induced protective response while focusing on the identification of the antigens involved. We developed a system by which we can immunize mice against nonlethal and lethal *P. yoelii* blood-stage infection with a limited subset of *P. yoelii* antigens in combination with the adjuvant Quil A. The protective response was B cell dependent and appeared to be associated primarily with production of IgG1 and IgG2b antibodies. Furthermore, pAg plus Quil A immunization was associated with the production of antibody against six to eight distinct *P. yoelii* antigens.

Various crude blood-stage antigen immunization studies involving a number of plasmodial species have been conducted over the years with mixed results (2, 9, 15, 18, 23, 26–31, 34, 35, 38, 41). Some of the earliest successful immunizations were with formalin-fixed *P. yoelii* parasites, administered with *B. pertussis* (28). More recently, protective responses were induced by immunization with whole blood-stage *P. yoelii* antigen in combination with the nonionic block copolymer P1004 and detoxified LPS (15, 41). We attempted to extend these studies, considering it likely that soluble and insoluble antigens would have different processing requirements that could influence the developing immune response. The fractionation of

TABLE 1. Isotype profile of *P. yoelii*-specific antibodies in pAg-immunized and control mice

Immunization group	IgG concn (U/ml)[a]			
	IgG1	IgG2a	IgG2b	IgG3
Quil A	7.2 ± 4.9[b]	10.1 ± 1.9	22.6 ± 15.4[c]	19.2 ± 12.1[d]
pAg plus Quil A	204.6 ± 52[b]	72.3 ± 74.7	238.9 ± 140.1[c]	42.0 ± 13.4[d]

[a] Values are mean ± standard deviations ($n = 5$) of the concentration of *P. yoelii*-specific IgG isotypes in sera collected from pAg-immunized and adjuvant control mice following resolution of *P. yoelii* 17X infection.
[b] Significance, $P < 0.01$.
[c] Significance, $P < 0.05$.
[d] Significance, $P < 0.05$.

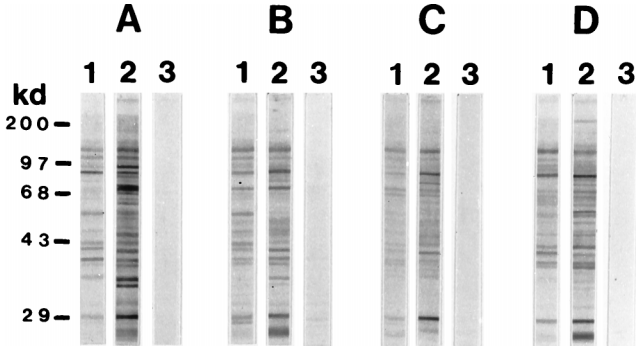

FIG. 5. Immunoblot analysis of antibodies associated with immunization-induced immunity to *P. yoelii*. The reactivities of *P. yoelii* 17X infection sera obtained from mice previously immunized with Quil A (lane 1) ($n = 5$) or pAg plus Quil A (lane 2) ($n = 5$) were assayed on immunoblots of total *P. yoelii* blood-stage antigen (10 μg/lane) separated by SDS-PAGE on 10% gels. A normal mouse serum pool (lane 3) served as the negative control. Antigen-specific antibodies were detected enzymatically with rabbit antibody specific for mouse IgG1 (A), IgG2a (B), IgG2b (C), or IgG3 (D) antibody isotypes. The molecular sizes of protein standards are indicated in kilodaltons.

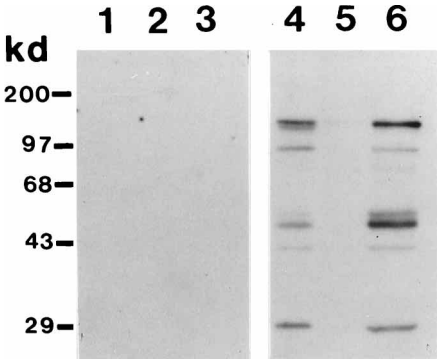

FIG. 6. Seroreactivity of pAg immunization sera with *P. yoelii* antigens. Immunoblots of *P. yoelii* total blood-stage antigen (lanes 1 and 4), sAg (lanes 2 and 5), and pAg (lanes 3 and 6) were probed with sera from Quil A-immunized (lanes 1 to 3) ($n = 5$) or pAg-plus-Quil A-immunized (lanes 4 to 6) ($n = 5$) mice. Blots containing 10 μg of the indicated antigen preparation per lane were separated by SDS-PAGE on 10% gels. Sera were collected 7 to 10 days following secondary immunization but prior to infection. Bound antibody was detected with ^{125}I-protein A followed by autoradiography. The molecular sizes of protein standards are indicated in kilodaltons.

whole blood-stage antigens into soluble and particulate components prior to immunization had a significant impact on our results. Immunization with *P. yoelii* pAg but not sAg induced protective immunity against subsequent challenge infection. The reduction in blood parasitemia was comparable to that achieved in previous studies which used unfractionated preparations of blood-stage antigens. As such, the antigens relevant to protective immunization were found primarily in an insoluble fraction which comprised only 25 to 30% of the total antigen preparation.

The diversity of costimulatory signals provided by different types of antigen-presenting cells (APC) combined with the varied ability of APC to process particulate antigens may also have had a significant bearing on our results (16). It is possible that our pAg immunization targeted a population of phagocytic APC necessary to drive the protective response. It is also likely that soluble and particulate antigens interacted differently with each adjuvant. Of the four adjuvants tested, Quil A was the preferred adjuvant for use with pAg but was minimally effective with sAg. Of interest, IFN-γ and CFA-IFA, adjuvants previously shown to be effective with whole blood-stage antigens, were ineffective when used in combination with pAg. Our sAg fraction, which represented 70 to 75% of the total antigen preparation, likely contained protective antigens which dominated the response in studies which involved whole blood-stage antigen immunizations. Combined, these results suggested that following pAg plus Quil A immunization, the protective response induced and/or the specific antigens involved were different from those previously observed.

In looking at the immune responses of protected versus susceptible mice, we observed the production of mRNA for selected Th1 (IFN-γ and IL-2) as well as Th2 (IL-4 and IL-10) cytokines in the spleens of both groups. In the spleens of mice immunized with pAg plus Quil A, cytokine production occurred earlier, with a Th2 phenotype somewhat favored. In particular, this was due to a reduction in IFN-γ mRNA in pAg-plus-Quil A-immunized mice compared to that in adjuvant controls. These results are in contrast to those of many murine studies, which in general have demonstrated a clear dichotomy in the activation and regulation of Th1 and Th2 $CD4^+$ T cells (32, 33). Depending on the infection or disease state, one subset usually dominates the other and correlates with either beneficial or detrimental immune responses. Our present data for *P. yoelii*, along with data from some studies of *P. chabaudi*, suggest that this may not be the situation in malaria. Complete resolution of a primary *P. chabaudi* blood-stage infection appears to involve an early Th1 $CD4^+$ T-cell response that subsequently shifts to a Th2-dependent response (20). While a Th2 type response was favored in our *P. yoelii* pAg-immunized and -protected mice, the contribution of the Th1 response to protection cannot be ruled out. In fact, both may be necessary for complete protection against blood-stage malaria.

To detect cytokine responses, we used an RT-PCR-based assay of in vivo mRNA production by spleen cells to avoid any selection or bias during an in vitro culture period. This may have allowed us to more easily detect the simultaneous presence of Th1 and Th2 type responses. In addition, other factors may have contributed to this observation. First, cytokine production may have been due to activated Th0 cells, prior to their differentiation into Th1 or Th2 T cells (33). This is less likely since the protected animals had been immunized and boosted previously with pAg, presumably driving the response past the Th0 step. A second possibility for the lack of a polarized T-cell response may be related to our choice of mouse strain. The use of (BALB/cByJ × C57BL/6J) F_1 mice may have minimized any potential genetic bias to develop the Th1 or Th2 response present in the parental strains (31, 36). A third possibility may simply be that plasmodial parasites drive an overwhelming, systemic immune response during blood-stage malaria. As a consequence, specific and/or nonspecific responses may alter the expected immunoregulatory circuits.

The inability of pAg-immunized B-cell-deficient J_HD mice to resist *P. yoelii* challenge indicated a dependence of the protective response on the presence of B cells and presumably specific antibody. For malaria, both human (1, 13) and murine (15, 39, 41, 46) studies indicate that an antibody's protective capacity depends on a combination of specificity and isotype. Quantitation of antigen-specific antibodies revealed a marked increase in IgG1 and IgG2b production in pAg-plus-Quil A-immunized mice. On the basis of our data examining cytokine production in the spleens of these animals, this may have been due to the influence of IL-4 over IFN-γ (11). It may also have been related to the use of Quil A as the adjuvant, which has previously been shown to preferentially drive the production of IgG1 antibodies (17). Immunoblot analysis indicated that these antibodies recognized a heterogeneous set of blood-stage antigens. In comparison with adjuvant controls, many of these specificities appeared to arise during the normal course of *P. yoelii* 17X infection. However, additional antigen specificities were observed with sera of immunized and protected mice, particularly with IgG1 antibodies. Using sera obtained following pAg plus Quil A immunization but prior to infection, we detected antibodies to six to eight distinct antigens (25 to 110 kDa). Furthermore, these antigens were associated with *P. yoelii* pAg, but not sAg, fractions. Immune responsiveness to these antigens was attributed specifically to pAg immunization and likely identified antigens important for protective immunization.

In studies of *P. yoelii* infection-induced polyclonal antibodies, Taylor et al. and White et al. demonstrated the importance of protective IgG2a antibodies which recognized a diverse set of antigens (39, 46). In these studies, a significant increase in the production of IgG1 antibodies was noted only after secondary *P. yoelii* challenge. Considering our data, the IgG1 response induced by protective pAg immunization may not normally arise during a primary *P. yoelii* infection but may be driven by repeated exposures. In some respects, a similar ob-

servation was made with *P. chabaudi*. In this model, a Th2-driven antibody response arising late during *P. chabaudi* infection may contribute to final parasite clearance (44) and may involve an IgG1-dependent mechanism (40). Protection in our *P. yoelii* studies may simply be due to the earlier induction of a protective IgG1 response by prior pAg plus Quil A immunization.

In recent immunization studies, Hunter et al. and ten Hagen et al. tested a variety of blood-stage antigen and adjuvant formulations for their ability to immunize against *P. yoelii* (15, 41). The nonionic block copolymer P1004 and detoxified LPS were the most successful adjuvants. In these studies, immune sera recognized a heterogeneous antigen set, since mice were immunized with a preparation of whole blood-stage antigens. The data also suggested that protection correlated more closely with the production of IgG2a antibodies. This protective response appeared to be directed toward labile, conformational epitopes of antigens on the surface of *P. yoelii* blood-stage parasites, which could be detected only in immunofluorescence assays. The authors suggested that only certain antigen-adjuvant formulations preserved these labile epitopes, allowing presentation in a more native form. Considering this possibility, it is not surprising that our results differed. In all likelihood, our pAg required more substantial degradation by phagocytic APC before presentation, such that native conformation was not preserved. As such, the antigens and protective responses involved in these two studies differed, emphasizing the importance of the consideration of both antibody isotype and specificity in vaccine development efforts.

Other studies of *P. yoelii* blood-stage malaria provide evidence supporting the importance of isotypes of IgG other than IgG2a in protective immunity. In passive protection experiments, monoclonal antibodies of the IgG1, IgG2a, and IgG3 isotypes have all been shown to be effective against *P. yoelii* (12, 22). Additional studies of *P. yoelii* merozoite surface protein-1 (MSP-1) are of particular interest. Monoclonal antibody 302, an IgG3 antibody against a MSP-1 C-terminal epitope, can passively protect against *P. yoelii* challenge infection (22). On the other hand, immunization of BALB/c mice with a recombinant MSP-1 antigen, GST-PYC2, in alum, induced protective PYC2-specific antibodies exclusively of the IgG1 isotype (7). GST-PYC2 is only 11 kDa and contains the epitope recognized by monoclonal antibody 302, suggesting that IgGs of similar specificities but different isotypes can be protective. While the importance of antibody in protective immunity to *P. yoelii* blood-stage malaria is clear, the combined data suggest that multiple antigens and IgG isotypes are involved.

The mechanism(s) by which *P. yoelii*-specific antibodies mediate a protective response in our system needs further investigation. The involvement of other elements of the immune system in the protective response must also be considered. Important to these studies as well as vaccine development efforts will be the characterization of the antigens involved in this protective response. In this regard, we have observed that protective pAg immunization induces the increased production of antibodies against a limited subset of *P. yoelii* antigens, which can be identified with a single pool of sera. Since multiple antigens appear to be involved, the crucial factor may be that a common protective response is being driven by several distinct antigens. In fact, it may be difficult to achieve that protective response by immunization with only a single antigen. Nevertheless, the results obtained provide the necessary information and means to characterize additional vaccine candidate antigens, selected solely on their ability to immunize against blood-stage malaria.

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