Protection against *Plasmodium chabaudi* Malaria Induced by Immunization with Apical Membrane Antigen 1 and Merozoite Surface Protein 1 in the Absence of Gamma Interferon or Interleukin-4

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Received 22 April 2004/Returned for modification 1 June 2004/Accepted 24 June 2004

Strategies to optimize formulations of multicomponent malaria vaccines require a basic knowledge of underlying protective immune mechanisms induced by each vaccine component. In the present study, we evaluated the contribution of antibody-mediated and cell-mediated immune mechanisms to the protection induced by immunization with two blood-stage malaria vaccine candidate antigens, apical membrane antigen 1 (AMA-1) and merozoite surface protein 1 (MSP-1). Immunologically intact or selected immunologic knockout mice were immunized with purified recombinant *Plasmodium chabaudi* AMA-1 (PcAMA-1) and/or the 42-kDa C-terminal processing fragment of *P. chabaudi* MSP-1 (MSP-142). The efficacy of immunization in each animal model was measured as protection against blood-stage *P. chabaudi* malaria. Immunization of B-cell-deficient JH<sup>−/−</sup> mice indicated that PcAMA-1 vaccine-induced immunity is largely antibody dependent. In contrast, JH<sup>−/−</sup> mice immunized with PcMSP-1<sub>42</sub> were partially protected against *P. chabaudi* malaria, indicating a role for protective antigen-dependent and antibody-independent mechanisms of immunity. The involvement of γδ T cells in vaccine-induced PcAMA-1 and/or PcMSP-1<sub>42</sub> protection was minor. Analysis of the isotypic profile of antigen-specific antibodies induced by immunization of immunologically intact mice revealed a dominant IgG1 response. However, neither interleukin-4 and the production of IgG1 antibodies nor gamma interferon and the production of IgG2a/c antibodies were essential for PcAMA-1 and/or PcMSP-1<sub>42</sub> vaccine-induced protection. Therefore, for protective antibody-mediated immunity, vaccine adjuvants and delivery systems for AMA-1 and MSP-1-based vaccines can be selected for their ability to maximize responses irrespective of IgG isotype or any Th1 versus Th2 bias in the CD4<sup>+</sup>-T-cell response.

In spite of the efforts of many governments and in the face of tremendous scientific advancement, the global burden of malaria is as great as it has ever been. It is estimated that as many as 500 million clinical cases of malaria result in 2.5 to 3.0 million deaths each year (6). The most realistic approach to reduce morbidity and mortality due to *Plasmodium falciparum* malaria is to develop safe and effective drugs and vaccines to treat and/or prevent malaria. Extensive and ongoing studies using humans and animal models indicate that protective immunity against malaria parasites develops (35, 36). The challenge is to develop multicomponent vaccines that induce protective immune responses that are broadly effective against geographically distinct strains of the malarial parasite.

A clear understanding of the immune responses that cooperate to suppress malaria parasite growth in the infected host is critical for the vaccine development effort. Parasite-specific antibodies, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and γδ T cells also contribute to protection against infective sporozoites and parasites that initially develop in hepatocytes (27). Cell-mediated immune responses against these liver-stage parasites are particularly important. Parasite-specific antibodies, CD4<sup>+</sup> T cells, and γδ T cells also contribute to protection against blood-stage malaria parasites (35, 40, 46). In this case, antibody-mediated immune responses may play the predominant role in protection. Of importance, the production of Th1-type cytokines appears to play a central role in the protective response to both pre-erythrocytic-stage and blood-stage malaria parasites and may involve the synthesis of gamma interferon (IFN-γ) by NK cells, γδ T cells, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells. To some extent, the beneficial influence of IFN-γ on antibody production has also been observed, as a number of studies have correlated protection with elevated levels of parasite-specific immunoglobulin G1 (IgG1) and IgG3 in humans (4, 20) and IgG2a/c in mice (53).

By utilizing various screening strategies, a number of plasmodial antigens have been identified as targets of protective immune responses and may be potentially useful as vaccine components (37). This number will likely increase over time as a result of the sequence analysis of the *P. falciparum* (18) and *Plasmodium yoelii* genomes (8). Concurrently, extensive efforts have been made to develop and test a variety of platforms, delivery systems, adjuvants, and immunization protocols for malaria subunit vaccines. Clinical trials of candidate malaria vaccines have met with some limited success (19). A continued effort is needed, and additional vaccine trials are ongoing.

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impediment in this effort has been the inability to define suitable immune correlates of protection that can be measured and used to optimize vaccine formulations and immunization protocols.

The rodent malarial parasite *Plasmodium chabaudi* is a useful tool in this effort to develop and test blood-stage malaria vaccines. In previous work, the *P. chabaudi* model was used extensively to characterize infection-induced immune mechanisms effective against blood-stage parasites (35, 46). From these studies, it became clear that it is possible to separately measure the protective antibody-mediated immune response (AMI) or the protective γδ and CD4+ T-cell-mediated immune response (CMI) against *P. chabaudi* by using mice made to be deficient in immunity by antibody depletion and/or targeted gene knockout (21, 46–48, 50, 52). Furthermore, it was also shown that in naïve mice, the production of IFN-γ is critical for the suppression of *P. chabaudi* malaria by either AMI or CMI (3, 42, 49). The synthesis of interleukin-4 (IL-4) late during *P. chabaudi* malaria has also been reported and is believed to contribute to the production of parasite-specific antibodies necessary for final parasite clearance (31, 41).

The two leading *P. falciparum* blood-stage vaccine candidate antigens are apical membrane antigen 1 (AMA-1) and merozoite surface protein 1 (MSP-1). These two merozoite surface proteins have been extensively studied using several species of *Plasmodium*, and their vaccine potential has been mainly demonstrated (26, 29). Well-characterized orthologues of *P. falciparum* AMA-1 (PIAMA-1) and MSP-1 (PMSP-1) are present in *P. chabaudi* (PCAMA-1 and PCMP-1) (11, 15, 32, 34). Furthermore, we and others have shown that immunization with recombinant PCAMA-1 and/or PCMP-1 protects against *P. chabaudi* malaria (2, 7, 11, 39). In the present study, we extend previous efforts to evaluate the efficacy of immunization with vaccines based on PCAMA-1 and the 42-kDa C-terminal processing fragment of PCMP-1 (PCMP-142) (7) to further define immune correlates of protection. Herein, we utilize gene-targeted knockout mice to evaluate the importance of AMI and CMI to immunity induced by immunization with PCAMA-1 and/or PCMP-142 and the dependence of the protective response on IFN-γ, IL-4, and IgG subclasses.

**MATERIALS AND METHODS**

Mice, malaria parasites, and experimental infections. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Likewise, γδ T-cell-receptor-deficient (TCR-γδ−/−), IFN-γ−/−, and IL-4−/− mice, backcrossed to C57BL/6 mice for at least 10 generations, were purchased from Jackson Laboratories. TCR-γδ−/− mice have a targeted deletion of the genes for the β chain of the T-cell receptor and fail to develop populations of T cells bearing γδ receptors (25). The IFN-γ−/− and IL-4−/− mice are unable to produce IFN-γ and IL-4, respectively, due to targeted disruptions of the corresponding cytokine genes (12, 28). B-cell-deficient Jμ−/− mice on a C57BL/6 × 129 background were kindly provided by Dennis Huszar (GenPharm International, Mountain View, Calif.). These Jμ−/− mice fail to produce mature surface Ig-positive B cells due to the targeted deletion of Ig heavy chain J gene segments (9). TCR-γδ−/− and IL-4−/− mice were bred at the University of Wisconsin Animal Care Facilities (Madison, Wis.) in microisolator cages and provided with sterile food and water ad libitum. Routine screenings of sentinel mice were conducted throughout these studies to ensure that animals remained free of infection with common viral and bacterial pathogens.

Mice of both sexes, 6 to 12 weeks of age, were used in all immunization and challenge experiments. *P. chabaudi* adami 556KA, hereafter referred to as *P. chabaudi*, was maintained as cryopreserved stablilates. Blood-stage infections were initiated by intraperitoneal injection of 10^6 washed, parasitized erythrocytes obtained from BALB/c donor mice. Resulting parasitemias were monitored by enumerating parasitized red blood cells in thin tail blood smears stained with Giemsa stain.

Expression and purification of recombinant PcAMA-1 and PcMP-142. The expression and purification of recombinant PCAMA-1 and PCMP-142 from *P. chabaudi* have been previously described in detail (7). Briefly, recombinant antigens were produced by using a petT/T7 RNA polymerase bacterial expression system with the petET-15b plasmid vector and *Escherichia coli* BL21(DE3) (pLYS5) as the host strain (Novagen, Madison, Wis.). A 54-kDa recombinant PCAMA-1 protein that represented the large ectodomain of *P. chabaudi* AMA-1 (amino acids 26 to 479) was expressed. The recombinant PCMP-142 antigen contained the C-terminal portion of MSP-1 but lacked the hydrophobic anchor sequence. Both recombinant antigens contained 20 plasmid-encoding N-terminal amino acids which included a six-histidine tag to facilitate purification. Following isolation and solubilization of the inclusion body fraction, PCAMA-1 and PCMP-142 were purified by ammonium sulfate fractionation and nickel chelate affinity chromatography under denaturing conditions. PCAMA-1 and PCMP-142 were refolded by gradual removal of guanidine-HCl by dialysis in the presence of reduced and oxidized glutathione. Protein concentrations were determined by using the bichinchoninic acid protein assay ( Pierce Chemical Company, Rockford, Ill.). The purity of recombinant PCAMA-1 and PCMP-142 preparations was assessed by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions.

Immunization and challenge protocols. A single immunization protocol was used for all immunogenicity and efficacy studies. Groups (n = 5) of immunologically intact C57BL/6 mice or immunodeficient knockout mice were immunized subcutaneously with 25 μg of purified recombinant PCAMA-1 or PCMP-142, with 25 μg of Quil A (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) as adjuvant. An additional group of mice was immunized with a combination of PCAMA-1 (25 μg) and PCMP-142 (25 μg) similarly formulated with Quil A. Control animals were immunized with Quil A alone or saline. Three weeks following the primary immunization, animals were boosted with the same doses of antigen and adjuvant. Eight to 10 days later, small volumes of prechallenge sera for antibody assays were collected. Approximately 10 to 14 days following the booster immunization, mice were infected intraperitoneally with 10^6 *P. chabaudi*-parasitized erythrocytes, and blood parasitemias were monitored. The statistical significance of differences in mean peak parasitemia between groups was calculated by analysis of variance using the StatMost statistical analysis software package (Data Most Corp., Salt Lake City, Utah). For comparison purposes, note that the peak mean parasitemias in saline and adjuvant control TCR-γδ−/−, IFN-γ−/−, and IL-4−/− mice are not statistically different than those obtained with intact C57BL/6 mice. The data presented on the responses of C57BL/6, TCR-γδ−/−, IFN-γ−/−, and IL-4−/− mice were obtained from concurrently immunized and challenged groups of mice.

IgG isotype ELISA. The quantity and isotypic profile of antigen-specific antibodies in prechallenge sera were determined by enzyme-linked immunosorbent assay (ELISA) as previously described using recombinant PCAMA-1- or PCMP-142-coated wells (7). Serum from each animal per group (n = 5) was assayed on antigen-coated wells at dilutions of 1:200, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. Antigen-specific antibodies were detected with horseradish peroxidase-conjugated rabbit antibody specific for mouse IgG1, IgG2b, or IgG3 (Zymed Laboratories, San Francisco, Calif.) or with horseradish peroxidase-conjugated goat anti-mouse IgG2c (IgG2a b allotype: Southern Biotechnology Associates, Inc., Birmingham, Ala.) (33) and ABTS (2,2′-azinobis(3-ethylbenz-thiazolesulfonic acid)] as the substrate. Serum samples were run in duplicate, and absorbance was read at 405 nm. In each assay, wells coated with purified IgG1, IgG2b, or IgG3 myeloma proteins (16 ng/ml to 1 μg/ml; Zymed Laboratories) were used to generate a standard curve. The IgG2c standard curve was generated by using a purified monoclonal IgG2c (IgG2a b allotype) antibody (BD Biosciences Pharmingen). The isotype standard curves were used to quantify antigen-specific antibody present in each serum sample with the dilution of serum that yielded an optical density at 405 nm of 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/ml. Values obtained with adjuvant control sera (n = 5) were comparable to background values obtained with normal mouse sera (n = 5) and have been subtracted. The statistical significance of differences in mean IgG concentrations between groups was calculated by analysis of variance using the StatMost statistical analysis software package (Data Most Corp.).
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c response to both antigens was three- to sixfold higher than the 

IgG1, IgG2a/c, IgG2b, and IgG3 antibodies specific for PcAMA-1 (white bars) or PcMSP-1 42 (black bars) present in prechallenge sera were determined by ELISA. Background values obtained when adjuvant control sera (n = 5) were used have been subtracted. (B) Groups of C57BL/6 mice (n = 5) immunized with purified recombinant PcAMA-1 ( ), PcMSP-1 42 ( ), or PcAMA-1 plus PcMSP-1 (* ) with Quil A as adjuvant were challenged with 10^6 P. chabaudi-parasitized erythrocytes. Mice immunized with Quil A alone ( □ ) or phosphate-buffered saline (PBS) alone ( ▼ ) served as controls. Resulting parasitemias were monitored by enumerating parasitized 

erythrocytes in thin tail blood smears stained with Giemsa stain. The results obtained are similar to previously reported data (7).

RESULTS

Isotypic profile of antigen-specific IgG antibodies induced by protective immunization with PcAMA-1 and PcMSP-1 42. To begin to characterize PcAMA-1- and PcMSP-1 42-induced protective responses, immunologically intact C57BL/6 mice were immunized with purified recombinant PcAMA-1, PcMSP-1 42, or a combination of PcAMA-1 and PcMSP-1 42 by using Quil A as adjuvant. All groups of mice received one booster immunization 3 weeks later with the same doses of antigen and adjuvant. Approximately 2 weeks later, a small volume of prechallenge serum was collected from each animal to determine the quantity and isotypic profile of PcAMA-1- or PcMSP-1 42-specific antibodies induced by immunization. The efficacy of immunization was evaluated following challenge infection with P. chabaudi-parasitized erythrocytes.

A dominant IgG1 response to PcAMA-1 and PcMSP-1 42 was observed following immunization with each antigen alone or with the antigen combination (Fig. 1A). Overall, the IgG1 response to both antigens was three- to sixfold higher than the IgG2a/c (P < 0.05) and the IgG2b (P < 0.05) responses. The IgG2a/c and IgG2b antibody levels were comparable. Very little antigen-specific IgG3 was induced by PcAMA-1 or PcMSP-1 42 immunization. In mice immunized with PcAMA-1 or PcMSP-1 42 alone, the anti-PcMSP-1 42 antibody concentrations were consistently greater than the concentrations of anti-PcAMA-1 antibodies with significant differences in antigen-specific total IgG (P < 0.05) and IgG1 (P < 0.05) production. For the combined antigen immunization, the PcAMA-1 antibody responses were essentially the same as those observed in mice immunized with PcAMA-1 alone. In contrast, there was a small but consistent drop in the anti-PcMSP-1 42 antibody response in the combined antigen immunization group. As a result, no significant differences were noted when the overall anti-PcAMA-1 and anti-PcMSP-1 42 antibody levels induced by immunization with the combined antigen formulation were compared.

Significant protection against P. chabaudi challenge infec-
tion was induced by PcMSP-1 42 immunization, with a fourfold reduction in mean peak parasitemia compared to those of the Quil A (P < 0.01) and saline (P < 0.01) control groups (Fig. 1B). The mean peak parasitemia in PcMSP-1 42-immunized mice was reduced to 3.05% ± 2.03% compared to 12.99% ± 6.32% and 12.48% ± 4.75% in adjuvant and saline control groups, respectively. Solid protection was also induced by immunization with PcAMA-1 compared to Quil A (P < 0.002) and saline (P < 0.001) control groups (Fig. 1B). Parasitemia levels remained below 0.01% in PcAMA-1-immunized mice throughout the course of infection. While the overall prechallenge anti-PcMSP-1 42 antibody levels were higher than anti-PcAMA-1 antibody levels, the reduction in parasitemia was significantly greater in PcAMA-1-immunized versus PcMSP-1 42-immunized mice (P < 0.01). This high level of protection was also evident in mice immunized with the combination of PcAMA-1 and PcMSP-1 42. Mean peak parasitemia in the combined antigen immunization group was 0.14% ± 0.26% and not significantly different than that induced by immunization with PcAMA-1 alone (Fig. 1B).

Protective AMI and CMI induced by PcAMA-1 and PcMSP-

1 42 immunization. To assess the contribution of γδ T-cell-dependent cell-mediated immunity to PcAMA-1 and PcMSP-

1 42 immunization-induced protection, TCR-δ^-/- mice lacking 

γδ T cells were immunized with PcAMA-1 and/or PcMSP-1 42 as described above. Analysis of prechallenge sera reflected a strong B-cell response induced by PcAMA-1 and PcMSP-1 42 immunization in TCR-δ^-/- mice (Fig. 2A), again characterized by the production of high levels of IgG1 antibodies. Considering both the quantity and isotypic profile of PcAMA-1 and PcMSP-1 42 antigen-specific antibodies, no significant differences were noted in the responses of TCR-δ^-/- mice (Fig. 2A) and immunologically intact C57BL/6 mice (Fig. 1A).

Upon P. chabaudi challenge infection, TCR-δ^-/- mice im-

munized with PcAMA-1, PcMSP-1 42, or the combination of 
PcAMA-1 and PcMSP-1 42 were protected against P. chabaudi malaria (Fig. 2B). Overall, mean peak parasitemia in TCR-
\( \delta^{-/-} \) mice immunized with single or combined antigen formulations was \( \leq 2\% \) and significantly reduced from 11.67\% \( \pm 4.13\% \) in Quil A controls \( (P < 0.01) \) and 10.65\% \( \pm 5.60\% \) in saline controls \( (P < 0.02) \). TCR-\( \delta^{-/-} \) mice immunized with PcAMA-1 developed a slightly higher mean peak parasitemia of 1.54\% \( (\pm 1.86\% \) compared to PcAMA-1-immunized C57BL/6 mice in which parasitemia remained below 0.01\%. In contrast to immunologically intact C57BL/6 mice, TCR-\( \delta^{-/-} \) mice immunized with PcAMA-1 and/or PcMSP-142 also developed a low level of parasitemia that persisted into the third week of \( P. \) chabaudi infection but was subsequently cleared \( (Fig. 2B) \). This low level of persistent parasitemia was also apparent in control TCR-\( \delta^{-/-} \) mice immunized with Quil A or saline alone. These data suggest that \( \gamma \delta \) T cells contribute to \( P. \) chabaudi parasite clearance in naïve and immunized mice. However, the contribution of \( \gamma \delta \) T cells specifically to PcAMA-1 and/or PcMSP-142 vaccine-induced protection using Quil A-based formulations appears to be minor.

To further evaluate the contribution of antibody-independent, cell-mediated immune mechanisms to PcAMA-1 and PcMSP-142 vaccine-induced protection, B-cell-deficient \( J_{H^{-/-}} \) mice were immunized with PcAMA-1 and/or PcMSP-142 as described above. As shown in Fig. 2C, protection induced by PcAMA-1 immunization was essentially lost in B-cell-deficient \( J_{H^{-/-}} \) mice. Mean peak parasitemia in PcAMA-1-immunized \( J_{H^{-/-}} \) mice was 8.10\% \( \pm 3.50\% \) and was not statistically different \( (P > 0.05) \) from the mean peak parasitemia of 9.59\% \( \pm 3.82\% \) for \( J_{H^{-/-}} \) control mice. In contrast, partial protection against \( P. \) chabaudi malaria was induced by PcMSP-142 immunization in B-cell-deficient mice. PcMSP-142-immunized \( J_{H^{-/-}} \) mice developed a mean peak parasitemia approximately 2.5-fold lower than that observed in control mice \( (3.91\% \pm 1.35\% \) versus 9.59\% \( \pm 3.82\%; P < 0.02) \). A comparable reduction in mean peak parasitemia was also observed in \( J_{H^{-/-}} \) mice immunized with both PcAMA-1 and PcMSP-142, with the mean peak parasitemia reaching 4.12\% \( \pm 1.74\% \) \( (P < 0.02) \). The partial protection in \( J_{H^{-/-}} \) mice immunized with both PcAMA-1 and PcMSP-142 was not significantly different that induced by immunization with PcMSP-142 alone. As such, these data suggest that both antibody-mediated and cell-mediated mechanisms of immunity contribute to protection against \( P. \) chabaudi malaria induced by PcMSP-142 and Quil A immunization. Conversely, protection induced by immunization with PcAMA-1 and Quil A is largely, if not completely, antibody mediated.

**IFN-γ is not required for PcAMA-1 and PcMSP-142 vaccine-induced protection.** Studies of \( P. \) chabaudi infection using naïve mice indicate that IFN-γ is an important cytokine in the development of protective AMI and CMI against blood-stage parasites. Accordingly, IFN-γ-deficient mice were utilized to
assess the importance of this cytokine for protection induced by PcAMA-1 and/or PcMSP-142 immunization. IFN-γ−/− knockout mice were immunized with PcAMA-1 and/or PcMSP-142 formulated with Quil A as adjuvant as described above. Analysis of prechallenge sera showed that the lack of IFN-γ significantly altered the isotypic profile of antibodies induced by PcAMA-1 and PcMSP-142 immunization. Following single or combined antigen immunization, the PcAMA-1- and PcMSP-142-specific IgG2a/c response was markedly reduced in IFN-γ−/− mice (Fig. 3A). However, with the decrease in IgG2a/c antibody levels, compensatory increases in IgG1 and/or IgG2b production were apparent. Consequently, the total level of PcAMA-1- and PcMSP-142-specific IgG induced by immunization in IFN-γ−/− mice was equal to or slightly greater than that induced in immunologically intact C57BL/6 mice (Fig. 1A).

Upon *P. chabaudi* challenge infection of IFN-γ−/− mice, a more severe and prolonged infection in Quil A and saline controls developed, with mean peak parasitemias of 27.25% ± 3.44% and 27.76% ± 3.37%, respectively (Fig. 3B). Compared to this exacerbated *P. chabaudi* infection in naïve controls, parasitemia in immunized IFN-γ−/− mice was markedly reduced (*P < 0.01) with mean peak parasitemias of 0.02% ± 0.04% in PcAMA-1 immunized mice, 8.29% ± 10.81% in PcMSP-142-immunized mice, and 0.61% ± 1.37% in mice immunized with PcAMA-1 plus PcMSP-142. Similar to previous observations of prolonged *P. chabaudi* infection in IFN-γ−/− mice (3, 49), a high level of parasitemia persisted in IFN-γ−/− control mice into the third and fourth week of *P. chabaudi* infection. During this same time period, parasitemia was controlled at low levels in PcAMA-1- and/or PcMSP-142-immunized IFN-γ−/− mice prior to final parasite clearance. These combined data indicated that neither the IFN-γ deficiency nor the impaired IgG2a/c antibody response significantly altered the efficacy of immunization with PcAMA-1 and/or PcMSP-142 formulated with Quil A as adjuvant.

IL-4 is not required for PcAMA-1 and PcMSP-142 vaccine-induced protection. The dominant IgG1 response induced by PcAMA-1 and PcMSP-142 immunization suggested that IL-4 and the production of antigen-specific antibodies of this isotype may be necessary for vaccine-induced protection. To address this possibility, IL-4 knockout (IL-4−/−) mice were immunized with PcAMA-1 and/or PcMSP-142 formulated with Quil A as adjuvant and subsequently challenged with *P. chabaudi*-parasitized erythrocytes. Mice immunized with Quil A alone (○) or PBS alone (□) served as controls. Resulting parasitemias were monitored by enumerating parasitized erythrocytes in thin tail blood smears stained with Giemsa stain. Mean peak parasitemia in IFN-γ−/− mice immunized with Quil A or saline is significantly higher than that observed in immunologically intact C57BL/6 controls (*P < 0.01). This finding is consistent with previously reported data (3, 49).

FIG. 3. IFN-γ is not required for PcAMA-1 and PcMSP-142 vaccine-induced protection. (A) Groups of IFN-γ−/− mice (n = 5) were immunized with recombinant PcAMA-1 (A), PcMSP-142 (M), or the combination of PcAMA-1 and PcMSP-142 (A+M) with Quil A as adjuvant. The concentration of IgG1, IgG2a/c, IgG2b, and IgG3 antibodies specific for PcAMA-1 (white bars) or PcMSP-142 (black bars) present in prechallenge sera were determined by ELISA. Background values obtained by using adjuvant control sera (n = 5) have been subtracted. (B) Groups of IFN-γ−/− mice (n = 5) immunized with purified recombinant PcAMA-1 (●), PcMSP-142 (○), or PcAMA-1 plus PcMSP-142 (▲) with Quil A as adjuvant were challenged with 105 *P. chabaudi*-parasitized erythrocytes. Mice immunized with Quil A alone (□) or PBS alone (◇) served as controls. Resulting parasitemias were monitored by enumerating parasitized erythrocytes in thin tail blood smears stained with Giemsa stain. Mean peak parasitemia in IFN-γ−/− mice immunized with Quil A or saline is significantly higher than that observed in immunologically intact C57BL/6 controls (*P < 0.01).
merozoites (i.e., AMA-1 and MSP-1) that are antibody accessible (26, 29, 37). There is consensus in the field that for these vaccines, the adjuvants and delivery systems employed must promote the development of high titers of antibodies to achieve some measure of efficacy. There is also evidence that the isotype of parasite-specific IgG may be important in *P. falciparum* malaria. The production of cytophilic IgG1 and IgG3 (4, 20) and the contribution of these IgG isotypes to antibody-dependent cellular inhibition of *P. falciparum* blood-stage growth have been correlated with protection (5). Similar supporting data are evident from studies of *P. yoelii* and *P. chabaudi* which suggest that the production of cytophilic IgG2a/c is important for infection and/or immunization-induced protective responses (43, 44, 53). Clearly, it is expected that the relative importance of IgG isotype for protection will vary depending on the specific antigen and protective immune effector mechanisms involved. In conjunction with AMI, vaccine-induced CMI, the activation of γδ and CD4+ T cells, and the synthesis of IFN-γ may further enhance protective efficacy (35, 40, 46).

In the present study, we have experimentally manipulated responses induced by immunization with PcAMA-1 and PcMSP-142 and then measured protective efficacy against *P. chabaudi* malaria. In naive mice, the resolution of acute *P. chabaudi* malaria requires IFN-γ and the expansion of parasite-specific CD4+ T cells. Downstream immune effector mechanisms may involve either γδ T cells or B-cell activation and the secretion of parasite-specific antibodies. The lack of protection following immunization of B-cell-deficient J11d-/- mice with PcAMA-1 indicated that AMA-1 vaccine-induced immunity is antibody dependent. This finding is consistent with studies by Anders and colleagues which highlighted the importance of antibodies specific for disulfide-dependent epitopes of PcAMA-1 for protection (2). We did not see any evidence for an antibody-independent contribution of CD4+ T cells to PcAMA-1-induced protection as was previously suggested (55).

Extensive studies using humans and animal models have established the protective role of antibodies that recognize conformation-dependent B-cell epitopes associated with the C-terminal epidermal growth factor-like domains of MSP-119 (13, 17, 22, 26, 30). However, there appears to be a paucity of CD4+-T-cell epitopes associated with MSP-119 (16, 45). Undoubtedly, there are CD4+ T-cell epitopes associated with the 33-kDa fragment that is proteolytically cleaved from MSP-142 to yield MSP-119. The contribution of these T-cell epitopes to antibody-independent protective responses induced by MSP-142 immunization has been uncertain (1, 54). Our present data, however, clearly show that PcMSP-142-immunized B-cell-deficient mice are partially protected against *P. chabaudi* malaria compared to PcMSP-142-immunized C57BL/6 controls (2.45-fold versus 4.25-fold reduction in mean peak parasitemia). These data support the conclusion that T-cell epitopes contained within PcMSP-142 can induce protective CMI in the context of recombinant antigen and Quil A immunization.

The production of IFN-γ during *P. falciparum*, *P. chabaudi*, and *P. yoelii* malaria has been shown to be important in the development of responses leading to the suppression of blood-stage parasitemia in nonimmunized hosts (27, 35, 36, 40, 46). In naive animals infected with *P. chabaudi*, IFN-γ appears to be necessary for the development of both AMI and CMI and the suppression of acute blood-stage infection (3, 42, 49). The ability to completely clear *P. chabaudi* blood-stage parasites from circulation has been attributed to the production of IL-4 late during *P. chabaudi* malaria and enhanced AMI (31, 41). The importance of IFN-γ and IL-4 for immunization with blood-stage malaria vaccines has not been adequately addressed. Using immunologic knockout mice, we have shown that protection induced by immunization with PcAMA-1 and PcMSP-142, alone or in combination, is not strictly dependent on either IFN-γ or IL-4. While AMI is clearly important, protective antibody responses can be induced in the presence or absence of IFN-γ or IL-4. Because of this redundancy, vaccine adjuvants and delivery systems for AMA-1- and MSP-1-based vaccines should primarily be selected for their ability to maximize antibody responses irrespective of a Th1 or Th2 bias. This may be particularly important in order to minimize potentially pathological Th1-type proinflammatory responses (10). Furthermore, this ability to induce protective AMI by multiple pathways may also permit the development of MSP-
142-based vaccines that effectively induce both antibody-dependent and antibody-independent immune mechanisms.

In general, immunization with PcMSP-142 induced higher antibody levels than those observed following immunization with PcAMA-1. In spite of this result, PcAMA-1-induced protection was consistently better than that induced by PcMSP-142 immunization. The protective PcAMA-1 response potentially involves antibodies specific for B-cell epitopes distributed across the three domains (domains I, II, and III) that define the conformationally constrained 54-kDa ectodomain of PcAMA-1 (24). In contrast, protective B-cell epitopes of MSP-142 are primarily restricted to the C-terminal epidermal growth factor-like domains (MSP-143). The advantage of using the larger PcMSP-142 recombinant antigen for immunization is that the additional parasite-specific T-cell epitopes included may (i) promote the boosting of PcMSP-1-specific antibody responses upon exposure to blood-stage parasites during infection and (ii) elicit protective cell-mediated responses. Unfortunately, the use of the larger PcMSP-142 antigen for immunization may induce high overall IgG levels but may not adequately focus the B-cell responses on the most protective C-terminal epitopes. Thus, the challenge will be to design MSP-1 vaccine constructs that more effectively promote the development and boosting of protective AMI and CMI to maximize efficacy.

A dominant antigen-specific IgG1 response in immunologically intact animals immunized with PcAMA-1 and/or PcMSP-142 was not unexpected considering previous data with Quil A as adjuvant (7). High IgG1 titers have also been observed in animals protectively immunized with Plasmodium yoelii MSP-19 (PyMSP-19; PyMSP-144) (14). However, it was not clear from these previous data if protection induced by PcAMA-1 and/or PcMSP-142 immunization required IgG1 or could be enhanced with increased production of IgG bearing other isotypes with different associated effector functions (i.e., IgG2a/c). Our data obtained from the immunization of IFN-γ−/− and IL-4−/− mice indicate that neither IgG1 nor IgG2a/c is essential for PcAMA-1- or PcMSP-142-induced protection. Furthermore, increased production of IgG2a/c and/or IgG2b antibodies can compensate for a lack of antigen-specific IgG1 but does not appear to significantly improve vaccine efficacy. Combined, these data suggest that protective AMI induced by immunization with PcAMA-1 and PcMSP-142 is IgG isotype independent and therefore does not likely require complement activation or Fc receptor-bearing cells. This is in agreement with previous data demonstrating that anti-PyMSP-143-mediated protection is unaffected by FcRγ or FcγRI receptor deletion (38, 51).

Considering the overall antibody data, no major competition between the two antigens was noted for animals immunized with the combination of PcAMA-1 and PcMSP-142. This finding is encouraging, as multiallelic formulations of PIAMA-1 and PIHMSP-142 vaccines will be required. It also appears that the formulation of PIAMA-1 in combination with multiple antigens in various adjuvants should be reasonably straightforward as long as sufficient antibody responses are induced. On the other hand, immunization with PIHMSP-142-based vaccines may require additional effort to optimize the induction of antibodies to the most protective epitopes while concurrently stimulating potentially protective cell-mediated responses. Our present data also suggest that IFN-γ-dependent and γδ T-cell-dependent responses contribute to parasite clearance in naive as well as immunized mice. While the protection observed was statistically significant, we noted some variability in protection in PcMSP-142−/− immunized IFN-γ−/− mice that we could not clearly correlate with changes in antibody responses. This result may reflect an IFN-γ-dependent contribution to the CMI induced by PcMSP-142 immunization that is partially masked by the presence of antibody. However, it also is probable that other parasite antigens are targets of IFN-γ-dependent and γδ T-cell-dependent protective responses. In studies of PyMSP-145 Daly and Long (13) and Hirunpetcharat et al. (23) showed that additional infection-induced immune responses are necessary for final parasite clearance in actively or passively immunized animals. As such, it appears that it should be possible, and likely necessary, to formulate PIAMA-1- and PIHMSP-142-based vaccines with additional plasmoidal antigens in order to increase vaccine-induced protection against blood-stage malaria parasites.

ACKNOWLEDGMENT

This work was supported by NIH-NIAID grant AI49585.


