Prediction of Merozoite Surface Protein 1 and Apical Membrane Antigen 1 Vaccine Efficacies against *Plasmodium chabaudi*

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For the development of blood-stage malaria vaccines, there is a clear need to establish in vitro measures of the antibody-mediated and the cell-mediated immune responses that correlate with protection. In this study, we focused on establishing correlates of antibody-mediated immunity induced by immunization with apical membrane antigen 1 (AMA1) and merozoite surface protein 142 (MSP142) subunit vaccines. To do so, we exploited the *Plasmodium chabaudi* rodent model, with which we can immunize animals with both protective and nonprotective vaccine formulations and allow the parasitemia in the challenged animals to peak. Vaccine formulations were varied with regard to the antigen dose, the antigen conformation, and the adjuvant used. Prechallenge antibody responses were evaluated by enzyme-linked immunosorbent assay and were tested for a correlation with protection against nonlethal *P. chabaudi* malaria, as measured by a reduction in the peak level of parasitemia. The analysis showed that neither the isotype profile nor the avidity of vaccine-induced antibodies correlated with protective efficacy. However, high titers of antibodies directed against conformation-independent epitopes were associated with poor vaccine performance and may limit the effectiveness of protective antibodies that recognize conformation-dependent epitopes. We were able to predict the efficacies of the *P. chabaudi* AMA1 (PcAMA1) and *P. chabaudi* MSP142 (PcMSP142) vaccines only when the prechallenge antibody titers to both refolded and reduced/alkylated antigens were considered in combination. The relative importance of these two measures of vaccine-induced responses as predictors of protection differed somewhat for the PcAMA1 and the PcMSP142 vaccines, a finding confirmed in our final immunization and challenge study. A similar approach to the evaluation of vaccine-induced antibody responses may be useful during clinical trials of *Plasmodium falciparum* AMA1 and MSP142 vaccines.

Infection with the protozoan parasites *Plasmodium falciparum* and *Plasmodium vivax* causes 300 million to 500 million clinical episodes of malaria annually (21). With at least 40% of the world’s population at risk for malaria, multiple strategies are being explored to reduce this global public health problem. Progress continues to be made in the development of malaria vaccines for potential use in areas where malaria is endemic (18, 28). It is encouraging that in a recent trial, the rate of severe malaria was significantly reduced in young children in Mozambique immunized with RTS,S, a *P. falciparum* preerythrocytic-stage vaccine (1); and it was found that RTS,S was safe, immunogenic, and efficacious in infants (1 to 3 months of age) (3). For blood-stage vaccines, the testing of vaccine safety, immunogenicity, and efficacy in human subjects has moved forward for two candidate antigens, namely, apical membrane antigen 1 (AMA1) (15, 31, 42) and merozoite surface protein 1 (MSP1) (30, 35, 47, 49, 56).

Both AMA1 and MSP1 are expressed on the surface of extracellular, invasive merozoites and are essential for blood-stage parasite growth (17, 36, 50). We do not fully understand the precise functions of AMA1 and MSP1 in this invasion process, but their roles do appear to be distinct and nonoverlapping. The basic strategy for AMA1- and MSP1-based vaccines is the induction of antibodies that neutralize the merozoites released upon schizont rupture. The mechanisms of action of such neutralizing antibodies may include the blocking of key receptor-ligand interactions, inhibition of the proteolytic processing steps required for the invasion of erythrocytes (RBCs), as well as the opsonization and/or agglutination of parasites. In both in vitro and in vivo studies, antibodies against MSP1 (4–8, 13, 14, 22, 25, 27, 29, 37, 45) and AMA1 (2, 4, 5, 11, 12, 48) effectively neutralized merozoites of homologous parasite strains and provided protection against blood-stage malaria.

As the development and testing of AMA1- and MSP1-based vaccines advanced, the need to identify measurable parameters of the vaccine-induced immune responses that predict protection became a priority. One obstacle to defining such correlates has been the lack of data for a cohort of human subjects who were immunized with AMA1- or MSP1-based vaccines and who were protected to some degree against *P. falciparum* malaria. Nevertheless, the use of the in vitro *P. falciparum* growth inhibition assay (GIA) did emerge as one surrogate assay that could be used to measure the parasite-neutralizing activities of vaccine-elicited antibodies in nonhuman primates (45). While the assay has been standardized and provides some useful information, it is still imperfect. The GIA measures immuno-
globulin G (IgG) activity in the absence of other components of the immune system, such as complement and Fc-bearing phagocytes, that may be important (33). As such, the GIA cannot mimic the complex host-parasite interactions that occur in the in vivo environment and that collectively influence vaccine efficacy and infection outcome.

The Plasmodium yoelii and Plasmodium chabaudi rodent models of malaria provide an opportunity to define correlates of AMA1 and/or MSP1 vaccine-induced protection (2, 4–6, 12, 22). With these models, we can effectively measure vaccine efficacy, as we can allow a blood-stage infection to progress to the peak level of parasitemia in the absence of antimalarial drug treatment. This is one clear advantage of the models over immunogenicity and efficacy studies involving Aotus monkeys or human subjects. Previously, we used the P. chabaudi model to investigate the mechanisms of P. chabaudi AMA1 (PcAMA1) and P. chabaudi MSP142 (PcMSP142) vaccine-induced protection (4, 5). We showed that blood stage infections were initiated by the intraperitoneal injection of 556KA, hereafter referred to as Quil A (Accurate Chemical and Scientific Corporation, Westbury, NY) as the adjuvant. In trial 1, the mice were immunized with 25 μg of recombinant antigen formulated with 25 μg of Quil A and were subsequently challenged with 106 washed, parasitized RBCs obtained from donor mice. The resulting peak level of parasitemia in the absence of antimalarial drug treatment was monitored for 3 to 4 weeks, until complete resolution.

In vitro studies of P. falciparum and in vivo studies with animal model systems indicate that antibodies against conformational epitopes of AMA1 and MSP1 are protective (8–10, 12, 13, 22, 25, 29, 37, 48). To evaluate whether the levels of prechallenge antibodies against linear and disulfide-dependent epitopes can be used as a correlate of protection, groups of C57BL/6 mice (n = 5) were immunized and boosted twice with 25 μg of RF or RA rPcAMA1 or with 25 μg of RF or RA rPcMSP142 formulated with Quil A (25 μg) as the adjuvant in trial 4. This would be predicted to induce antibody responses to a wide range of both protective and nonprotective epitopes. The control mice received Quil A alone.

Small volumes of prechallenge sera were collected from each animal 8 to 10 days following the third immunization. Two weeks following the third immunization, the mice were challenged with 25 μg of RF or RA rPcAMA1 or RF rPcMSP142 formulated with Quil A (25 μg) as the adjuvant prior to the P. chabaudi challenge to test the predictive value of the correlations identified in trial 4.

Quantitation of antigen-specific IgG responses. The titers of antibodies present in sera collected 2 weeks following the primary, secondary, and tertiary immunizations with RF, rPcAMA1, or rPcMSP142 that were selected based on the number of RBCs (>106) at the peak of the acute phase of infection, and the results are expressed as the percent parasitemia in the absence of antimalarial drug treatment. This is one clear advantage of the models over immunogenicity and efficacy studies involving Aotus monkeys or human subjects. Previously, we used the P. chabaudi model to investigate the mechanisms of P. chabaudi AMA1 (PcAMA1) and P. chabaudi MSP142 (PcMSP142) vaccine-induced protection (4, 5). We showed that blood stage infections were initiated by the intraperitoneal injection of 1 × 108 washed, parasitized RBCs obtained from donor mice. The resulting peak level of parasitemia in the absence of antimalarial drug treatment was monitored for 3 to 4 weeks, until complete resolution.

Recombinant antigens. Recombinant PcAMA1 (rPcAMA1) and recombinant rPcMSP142 (rPcMSP142) were produced by using a PET/T7 RNA polymerase bacterial expression system with the pET-15b plasmid vector and Escherichia coli BL21(DE3)(pLysS) as the host strain (Novagen, Madison, WI). The 54-kDa rPcAMA1 protein encompasses the large ectodomain of AMA1. The rPcMSP142 antigen contains the C-terminal portion of MSP1 minus the hydrophobic anchor sequence. Both recombinant proteins contain 20 plasmid-encoded N-terminal amino acids which include a 6-residue histidine tag to facilitate nickel chelate affinity chromatography. The expression, purification, and refolding of recombinant PCs were conducted to ensure that the colony remained specific pathogen free. The titer of antibodies present in sera collected 2 weeks following the primary, secondary, and tertiary immunizations with RF, rPcAMA1, or rPcMSP142 that were selected based on the number of RBCs (>106) at the peak of the acute phase of infection, and the results are expressed as the percent parasitemia in the absence of antimalarial drug treatment. This is one clear advantage of the models over immunogenicity and efficacy studies involving Aotus monkeys or human subjects. Previously, we used the P. chabaudi model to investigate the mechanisms of P. chabaudi AMA1 (PcAMA1) and P. chabaudi MSP142 (PcMSP142) vaccine-induced protection (4, 5). We showed that blood stage infections were initiated by the intraperitoneal injection of 1 × 108 washed, parasitized RBCs obtained from donor mice. The resulting peak level of parasitemia in the absence of antimalarial drug treatment was monitored for 3 to 4 weeks, until complete resolution.
significant associations were observed by using log-transformed data for analysis software; Dataxiom Software, Los Angeles, CA). In trial 4, the most cient for both nontransformed and log-transformed data (StatMost statistical evaluated by calculation of the Pearson product-movement correlation coeffi-

antibody responses and protection against malaria were initially determined by ELISA (Fig. 1). Relatively low titers of antigen-specific antibodies were detected following a single immunization with either antigen, with the response elicited by 1 μg of antigen formulated with Quil A being significantly lower than that induced by 25 μg of antigen formulated with either Quil A or alum (P < 0.05). For PcAMA1, all responses were significantly boosted by a second immunization (P < 0.002). Only the responses induced by immunization with rPcAMA1 formulated with alum (trial 1) were boosted further by the third and final immunization (P < 0.05). Following the third immunization, however, the PcAMA1-specific antibody levels induced by immunization with 1 μg or 25 μg of antigen formulated with Quil A were high and comparable to those induced by immunization with 25 μg of rPcAMA1 formulated with alum (P < 0.05) (Fig. 1A). The responses induced by rPcMSP142 immunization followed a similar pattern, with the antigen-specific IgG titers being significantly boosted by either a second immunization (trials 1 and 3; P < 0.01) or a third immunization (trial 2; P < 0.001). As was found with PcAMA1, the final titers of PcMSP142-specific antibodies induced by immunization with 1 μg or 25 μg of antigen formulated with Quil A were two- to threefold greater than those induced by immunization with 25 μg of rPcAMA1 formulated with alum (P < 0.05) (Fig. 1B). In the sera from mice that received a tertiary immunization, the antibody titers induced by rPcMSP142 were higher than those induced by rPcAMA1, irrespective of the adjuvant used (P < 0.05), but the differences were less than twofold.

Two weeks following the third immunization, the final group of rPcAMA1- and rPcMSP142-immunized and adjuvant-treated control mice were challenged with 1 × 10⁶ P. chabaudi-parasitized RBCs. The results from the challenge infection are shown in Fig. 2A. In trial 1, the mean peak level of parasitemia in mice immunized with RF rPcAMA1 (25 μg) was significantly reduced to 4.43% ± 1.89%, whereas it was 8.68% ± 2.66% in mice immunized with alum alone (P < 0.05). In contrast, the mean peak level of parasitemia in mice immunized with RF rPcAMA1 (25 μg) reached 9.73% ± 2.89%, which was comparable to that for the adjuvant-treated control

RESULTS

Induction of antigen-specific antibodies by protective immunization with rPcAMA1 or rPcMSP142. One approach that can be used to define the correlates of vaccine-induced protective B-cell responses is to elicit antibodies by immunization with distinct antigen-adjuvant vaccine formulations that provide various levels of protection against disease. Groups of C57BL/6 mice were immunized and boosted twice with RF rPcAMA1 or RF rPcMSP142 in three distinct formulations. The two antigen doses (1 μg and 25 μg) and two adjuvants (alum and Quil A) selected were previously reported to induce intermediate or high levels of protection in this vaccine model (4, 5). Mice were immunized with 25 μg of antigen formulated with alum in trial 1, with 1 μg of antigen formulated with Quil A in trial 2, or with 25 μg of antigen formulated with Quil A in trial 3. Sera were collected from sets of immunized animals that were sacrificed 2 weeks after the primary, secondary, and tertiary immunizations. The titers of antigen-specific antibodies were determined by ELISA (Fig. 1). The avidity index was calculated with a dilution of serum that yielded an 405 of 0.5 to 1.0 in the absence of ammonium thiocyanate.
group. The converse was true in trial 2. The mean peak level of parasitemia in mice immunized with RF rPcAMA1 (1 μg) was markedly reduced (to 0.49% ± 0.79%) compared with the level in the mice immunized with Quil A alone (9.48% ± 3.78%) (P < 0.01). However, the mean peak level of parasitemia in mice immunized with RF rPcMSP142 (1 μg) reached 8.99% ± 4.16% and was indistinguishable from that for the adjuvant-treated control group. Finally, in trial 3, the mean peak levels of parasitemia in mice immunized with RF rPcAMA1 (25 μg) or RF rPcMSP142 (25 μg) were significantly reduced (to 0.07% ± 0.03% and 0.46% ± 6.119%, respectively) compared to the level in mice immunized with Quil A alone (16.48% ± 3.25%) (P < 0.01). With the exception of these differences in the levels of parasitemia, the courses of infection in antigen-immunized mice relative to that in the adjuvant-treated control mice in each of the three trials were comparable. Infection typically peaked between days 8 and 10 postinfection, and there were no significant differences between the protected and the nonprotected groups (Fig. 2B). All animals survived and suppressed parasitemia to undetectable levels by day 18 to 20 postchallenge with P. chabaudi.

Despite the variation in the protective efficacy against P. chabaudi malaria induced by these distinct PcAMA1 and PcMSP142 vaccine formulations, the modest differences in the prechallenge IgG titers (tertiary sera) in antigen-immunized mice could not be correlated with the reduction in the mean peak level of parasitemia. In all three trials, the most dominant antibodies by far were those that recognized conformational epitopes of PcAMA1 and PcMSP142, that had comparable avidities, and that were of the IgG1 subclass (data not shown). As such, this approach did not induce antigen-specific antibodies of sufficient heterogeneity to allow measurable parameters of vaccine-induced antibody responses to be linked with the outcome of infection.

Alternative approach to identification of in vitro correlates of antibody-mediated immunity. In the next series of experiments, a different approach was taken to induce a broader array of protective and nonprotective PcAMA1- and PcMSP142-specific antibody responses to improve the chances of identifying correlates of vaccine-induced immunity. Instead of using various antigen doses and adjuvants in each vaccine formulation, the state of the antigen itself was altered. In trial 4, the mice were immunized three times with 25 μg of RF or RA rPcAMA1 or with RF or RA rPcMSP142 formulated with Quil A as the adjuvant or with adjuvant alone. Two weeks following the final immunization, prechallenge sera were obtained for analysis of the antibody responses, and the mice were then challenged with P. chabaudi-parasitized RBCs. As shown in Fig. 3A, mice immunized with RF rPcAMA1 or RF rPcMSP142 were solidly protected against P. chabaudi malaria and had mean peak levels of parasitemia of 0.36% ± 0.47% and 0.24% ± 0.40%, respectively, whereas the peak level of parasitemia in the adjuvant-treated control group was 13.13% ± 4.62% (P < 0.001). In contrast, mice immunized with RA antigens were partially protected against P. chabaudi challenge infection and had only an ~50% reduction in the mean peak level of parasitemia. The mean peak level of parasitemia in mice immunized with RA rPcAMA1 was 6.80% ± 3.55%, which was significantly lower than that in mice immunized with Quil A alone (P < 0.05) but higher than that in mice immunized with RF rPcAMA1 (P < 0.01) (Fig. 3A). Likewise, the mean peak level of parasitemia in mice immunized with

FIG. 2. PcAMA1- and PcMSP142-induced protection against P. chabaudi malaria in trials 1 to 3. Groups of C57BL/6 mice (n = 5) were immunized three times with purified RF rPcAMA1 (C) or RF rPcMSP142 (■) in three distinct formulations (trials 1 to 3). Control mice were immunized with adjuvant alone (□). Two weeks following the third immunization, the mice were challenged with 1 × 10⁸ P. chabaudi-parasitized erythrocytes. The resulting levels of parasitemia were monitored by enumerating the parasitized RBCs in thin smears of tail blood stained with Giemsa. The peak levels of parasitemia (mean ± standard deviation) (A) and the days of the peak levels of parasitemia (mean ± standard deviation) (B) are shown for each group.

FIG. 3. Antigen conformation influences protection against P. chabaudi malaria by PcAMA1 and PcMSP142 vaccines. In trial 4, groups of C57BL/6 mice (n = 5) were immunized three times with RF or RA rPcAMA1 (□) or with RF or RA rPcMSP142 (■) formulated with Quil A as the adjuvant. Control mice were immunized with adjuvant alone (□). Two weeks following the third immunization, the mice were challenged with 1 × 10⁸ P. chabaudi-parasitized erythrocytes. The resulting levels of parasitemia were monitored by enumerating the parasitized RBCs in thin smears of tail blood stained with Giemsa. The peak levels of parasitemia (mean ± standard deviation) (A) and the days of the peak levels of parasitemia (mean ± standard deviation) (B) are shown for each group.
RA rPcMSP142 was 5.80% ± 3.88%, which was significantly lower than that in mice immunized with Quil A alone (P < 0.03) but higher than that in mice immunized with RF rPcMSP142 (P < 0.02). As described above, the peak levels of parasitemia in all groups occurred between days 7 and 9 postchallenge (Fig. 3B), and 100% of the mice survived.

The titer and specificity of vaccine-induced antibodies for disulfide-dependent B-cell epitopes of PcAMA1 and PcMSP142 were measured by ELISA (Fig. 4). For RA rPcAMA1- and RA rPcMSP142-immunized mice, the overall titers of antibodies present in prechallenge sera were not significantly different when the titers against the immunizing antigen were measured (P = 0.63). However, mice immunized with RF rPcAMA1 did produce high titers of antibodies that preferentially recognized the RF antigen over the RA antigen (P < 0.001). The converse was true for mice immunized with RA rPcAMA1 (P < 0.001). For PcMSP142, immunization with RF rPcMSP142 induced a threefold higher titer of antibodies than immunization with RA rPcMSP142 when the responses against the immunizing antigen were measured (P = 0.005). As expected, mice immunized with RF rPcMSP142 had high titers of antibodies that preferentially recognized the RF antigen over the RA antigen (P < 0.001). Again, the converse was true for mice immunized with RA rPcMSP142 (P = 0.02).

The isotypic profiles and avidities of the antibodies present in prechallenge sera in trial 4 were also measured by ELISA (Table 1). Isotyping assays with RF rPcAMA1-coated wells showed a dominant IgG1 response in mice immunized with either RF rPcAMA1 or RA rPcAMA1 (P < 0.001) compared to the responses achieved with each of the other IgG isotypes. Assays with RF rPcMSP142-coated plates also revealed a dominant IgG1 response in mice immunized with RF rPcMSP142 (P < 0.001). Mice immunized with RA rPcMSP142 produced similar quantities of IgG1 and IgG2a/c. The antigen-specific IgM levels in mice immunized with PcAMA1 or PcMSP142 were not significantly different from those observed in adjuvant-treated control mice (data not shown). For both PcAMA1 and PcMSP142, the avidities of antibodies induced by immunization with the RF and RA antigens were comparable when binding was assayed with plates coated with RF PCAMA1 or RF PcMSP142 (P > 0.3).

The data on the immunization-induced PcAMA1- and PcMSP142-specific antibody responses were tested to determine if they correlated with protection against P. chabaudi malaria. For this analysis, mice immunized with the RF and RA versions of each antigen were considered to be a single group, and correlations were evaluated by using paired antibody and parasitemia data from individual animals. As shown in Table 2, the titer of prechallenge antibodies that specifically recognized RF PcAMA1 or PcMSP142 was inversely correlated with the peak level of P. chabaudi parasitemia, indicative of a protective role for these antibodies. In light of these data and considering the dominance of the IgG1 response, it was not surprising to see a similar inverse correlation between IgG1 levels and parasitemia levels. Conversely, increasing titers of prechallenge antibodies that bound to linear epitopes associated with RA PcAMA1 or RA PcMSP142 were detrimental, as they directly correlated with protection against P. chabaudi malaria.

No significant correlation between the level of IgG2a/c, IgG2b, or IgG3 antibodies specific for PcAMA1 or PcMSP142 and the level of parasitemia was apparent. The avidities of the

### Table 1. Isotype profiles and avidity indices for prechallenge sera in trial 4

| Immunization group | IgG1 concn (U/ml [10^6]) | IgG2a/c | IgG2b | IgG3 | Avidity index |
|--------------------|--------------------------|---------|-------|------|--------------|
| RF PcAMA1          | 791 ± 216                | 26 ± 20 | 23 ± 16 | 0.2 ± 0.04 | 1.53 ± 0.09  |
| RA PcAMA1          | 221 ± 56                 | 8 ± 6   | 12 ± 10 | 0.5 ± 0.4  | 1.33 ± 0.40  |
| RF PcMSP142        | 1,831 ± 629              | 153 ± 54 | 93 ± 33 | 1 ± 0.4   | 1.61 ± 0.16  |
| RA PcMSP142        | 159 ± 112                | 53 ± 23 | 43 ± 20 | 15 ± 19   | 1.41 ± 1.32  |

* Isotype profiles and avidity indices were determined by ELISA with plates coated with RF PcAMA1 or RF PcMSP142.

### Table 2. Correlation of prechallenge antibodies that specifically recognized PcAMA1 or PcMSP142 with peak P. chabaudi parasitemia

| Antibody response | Statistical data for peak level of parasitemia |
|-------------------|-----------------------------------------------|
|                   | PcAMA1^a                                      | PcMSP142^b                                    |
|                   | r value | P value | r value | P value |
| RF antigen titer  | −0.90   | <0.001  | −0.66  | 0.04   |
| RA antigen titer  | 0.81    | 0.004   | 0.79   | 0.007  |
| IgG1              | −0.93   | <0.001  | −0.67  | 0.03   |
| IgG2a/c           | −0.36   | 0.31    | −0.60  | 0.064  |
| IgG2b             | −0.45   | 0.19    | −0.58  | 0.082  |
| IgG3              | 0.08    | 0.83    | 0.40   | 0.25   |
| Avidity           | −0.34   | 0.34    | −0.42  | 0.22   |

* Pearson product-movement correlation determined on log10-transformed data points.

* Pearson product-movement correlation determined on linear data points.
PcAMA1 and PcMSP142 antibodies were not a distinguishing feature of the vaccine-induced response relative to protection.

**Use of identified correlates of antibody-mediated immunity to predict PcAMA1 and PcMSP142 vaccine efficacies.** Figure 5 shows the results of a linear regression analysis comparing prechallenge antibody titers to RF and RA PcAMA1 (Fig. 5A and B) and to RF and RA PcMSP142 (Fig. 5C and D) individually with the outcome of *P. chabaudi* infection in trial 4. On the basis of these data, a multiple-regression analysis was utilized to generate equations that could be used to predict the peak level of *P. chabaudi* parasitemia on the basis of the prechallenge antibody titers against the RF antigen and the RA antigen considered in combination. To point out the relationship between these immune parameters and the expected impact on the outcome of infection, vaccine efficacy was predicted on the basis of a range of theoretical prechallenge antibody titers to both RF antigen and RA antigen (Table 3). For the rPcAMA1 formulation, a prechallenge antibody titer of 100,000 against RF rPcAMA1 and an associated titer of only 10,000 against RA rPcAMA1 were predicted to reduce the peak level of *P. chabaudi* parasitemia by approximately fourfold. A reduction in the peak level of parasitemia was expected to increase substantially to ∼25-fold, if these prechallenge antibody titers to RF and RA rPcAMA1 were proportionally doubled to 200,000 and 20,000, respectively. If a titer of 200,000 against RF rPcAMA1 was maintained, further increases in the level of antibodies that recognize linear epitopes of RA rPcAMA1 was maintained, further increases in the level of antibodies that recognize linear epitopes of RA rPcAMA1 were predicted to marginally influence protective efficacy. The situation with the rPcMSP142 formulation was somewhat different. A prechallenge titer of 100,000 against RF rPcMSP142 with a corresponding titer of 10,000 against RA rPcMSP142 was predicted to markedly reduce the peak level of *P. chabaudi* parasitemia by 9- to 10-fold. However, only a limited improvement would be anticipated by a doubling of these prechallenge titers. As important, further increases in the titer of prechallenge antibodies that recognize only RA rPcMSP142 would be expected to negatively affect vaccine efficacy even when high levels of antibodies that recognize RF rPcMSP142 are maintained.

To independently evaluate the ability of these in vitro correlates to predict vaccine efficacy, an additional immunization and challenge trial was completed. In trial 5, groups of mice were immunized three times with 25 μg of RF rPcAMA1 or RF rPcMSP142 formulated with Quil A as the adjuvant or with adjuvant alone. Two weeks following the final immunization, prechallenge sera were obtained for analysis of the antibody titer.

**TABLE 3. Relationship between prechallenge antibody titers and the expected impact on the outcome of infection**

| Vaccine antigen | Titer | Predicted peak parasitemia (%) | Fold reduction in level of parasitemia |
|-----------------|-------|--------------------------------|--------------------------------------|
|                 | RF    | RA                             |                                      |
| PcAMA1 RFa      | 100,000 | 10,000                         | 3.31                                 | 4.0 |
|                 | 200,000 | 20,000                         | 0.49                                 | 26.9 |
|                 | 200,000 | 100,000                        | 0.63                                 | 20.8 |
|                 | 200,000 | 200,000                        | 0.71                                 | 18.6 |
|                 | 200,000 | 400,000                        | 0.79                                 | 16.6 |
| PcMSP142 RFb    | 100,000 | 10,000                         | 1.41                                 | 9.3 |
|                 | 200,000 | 20,000                         | 1.27                                 | 10.3 |
|                 | 200,000 | 100,000                        | 2.12                                 | 6.2 |
|                 | 200,000 | 200,000                        | 3.19                                 | 4.1 |
|                 | 200,000 | 400,000                        | 5.31                                 | 2.5 |

*a For PcAMA1, log(peak parasitemia) = 14.48 – [2.92(log titer for RF form)] + [0.16(log titer for RA form)].

*b For PcMSP142, parasitemia = 1.54 – [2.42 × 10^-6(titer of RF form)] + [1.06 × 10^-7(titer of RA form)].

*c On the basis of a peak level of parasitemia of 13.13% in adjuvant-treated control mice (trial 4).
TABLE 4. Prechallenge antigen-specific titers against RF and RA PcAMA1 and PcMSP142 and predicted and observed peak levels of parasitemia

| Vaccine and animal no. | Antigen titer (10^6) | Level of parasitemia (%) |
|------------------------|----------------------|--------------------------|
|                        | RF       | RA       | Predicted | Observed |
| PcAMA1\(^a\)           |          |          |          |          |
| 1                      | 705      | 49       | 0.015    | 0.02     |
| 2                      | 809      | 337      | 0.014    | <0.01    |
| 3                      | 840      | 70       | 0.010    | <0.01    |
| 4                      | 853      | 212      | 0.011    | 0.02     |
| 5                      | 871      | 85       | 0.009    | 0.02     |
| 6                      | 918      | 119      | 0.008    | <0.01    |
| 7                      | 945      | 86       | 0.007    | <0.01    |
| 8                      | 967      | 283      | 0.008    | <0.01    |
| 9                      | 1,118    | 130      | 0.005    | <0.01    |
| 10                     | 1,634    | 206      | 0.002    | <0.01    |
| Mean                   | 966      | 158      | 0.009    | <0.01    |
| SD                     | 258      | 97       | 0.004    |          |
| PcMSP142\(^b\)         |          |          |          |          |
| 1                      | 767      | 91       | 0.661    | 1.59     |
| 2                      | 790      | 104      | 0.739    | 0.52     |
| 3                      | 797      | 208      | 1.826    | 1.87     |
| 4                      | 830      | 56       | 0.138    | 0.14     |
| 5                      | 877      | 185      | 1.392    | 2.10     |
| 6                      | 1,165    | 77       | 0.000    | 0.02     |
| 7                      | 1,277    | 126      | 0.000    | 4.78     |
| 8                      | 1,301    | 41       | 0.000    | 0.40     |
| 9                      | 1,506    | 109      | 0.000    | 2.49     |
| 10                     | 1,512    | 70       | 0.000    | 0.30     |
| Mean                   | 1,082    | 107      | 0.476    | 1.42     |
| SD                     | 303      | 54       | 0.668    | 1.48     |

\(^a\) For PcAMA1, \(\log\) (parasitemia) = 14.48 – [2.92(log titer for RF form) + 0.16(log titer for RA form)].

\(^b\) For PcMSP142, parasitemia = 1.54 – [2.42 \times 10^{-4}(titer for RF form) + 1.06 \times 10^{-3}(titer for RA form)].

The development of AMA1- and MSP1-based vaccines for \(P. falciparum\) has faced considerable challenges related to the production of recombinant antigens in the proper conformation, adjuvant selection, and the polymorphism of protective epitopes. Progress has been made in overcoming a number of these issues (18, 28). However, the clinical trial data needed to further guide these efforts are difficult to obtain quickly due to the need for testing in complex field trials in areas where malaria is endemic (15, 30, 31, 35, 42, 47, 49, 56). Confounding this situation is the current lack of an adequate body of data that defines measurable vaccine-induced AMA1- and MSP1-specific immune responses that are associated with protection in vivo. Recent studies involving \(Aotus\) monkeys immunized with \(P. falciparum\) MSP142 and challenged with \(P. falciparum\) noted some association between prechallenge antibody responses measured by ELISA and/or GIA and protection, generally for responses beyond a given threshold (27, 45). Further refinement of this type of analysis of protective and nonprotective antibody responses is needed to improve the ability to predict vaccine efficacy on the basis of diverse immunization-induced responses.

Studies of malaria parasites in rodents afford flexibility for the testing of the immunogenicities and the efficacies of blood-stage malaria vaccine formulations and the evaluation of in vitro immunoassays that might predict the outcome of infection. In the present study with a \(P. chabaudi\) model, the immunogenicities and efficacies of the rPcAMA1 and rPcMSP142 vaccine formulations tested were variable and consistent with those described in our previous reports (4, 5). As such, our expectation was that an in-depth analysis of the prechallenge antibody responses would allow us to identify immune correlates of protection. In our initial studies, we generally observed a marked boost in the antigen-specific antibody responses following a second immunization, regardless of the specific antigen, dose, or adjuvant used. We noted only marginal increases in antibody titers with a third immunization. These data suggest that more than three immunizations would not be expected to effectively boost the PcAMA1 or PcMSP142 antibody responses further nor result in an increase in protection upon challenge. Consideration of the efficacy data shows that the use of alum as the adjuvant was partially effective for the PcMSP142 vaccine but was unacceptable for use with the PcAMA1 vaccine. This may relate to the particulate nature and/or the strong Th2-biasing effects of alum. Alternatively, Quil A was suitable for use with both the PcAMA1 and the PcMSP142 vaccines, although the Quil A-based formulations...
of PcMSP1$_{42}$ required a higher dose of antigen (25 µg per immunization) to be effective.

In our initial analysis of antibody responses in animals immunized with various doses of PcAMA1 or PcMSP1$_{42}$ formulated in different adjuvants, we could not establish correlations between a reduction in the level of *P. chabaudi* parasitemia and the prechallenge antibody titer, isotype, avidity, or epitope specificity. The antigen-specific antibodies induced by three sequential immunizations in each of the first three trials were less variable than we anticipated, as they were primarily IgG1 and had comparable titers and avidities. Attempts to establish correlates of protection when dissimilar antigen-adjuvant formulations are compared may also be problematic. The antibodies elicited by each formulation may mediate protection by multiple, distinct mechanisms. Beyond B cells and antibodies, adjuvant-dependent influences on the balance of protective and nonprotective T-cell responses induced by immunization may have further masked our ability to establish correlates.

In our fourth trial, we evaluated the response and protective efficacy induced by immunization with the RF antigen compared with those induced by immunization with the RA antigen. By using the same antigen dose and adjuvant, we also reduced the effects of some of the confounding variables mentioned above. Again, we could not establish any relationship between antibody isotype or avidity and protection. We were, however, able to show that the titer of antibody against the RF antigen negatively correlated with the level of parasitemia and that the titer of antibody against the RA antigen positively correlated with the level of parasitemia. Somewhat unexpectedly, the relative importance of these two measures of vaccine-induced responses as predictors of protection differed for the PcAMA1 and the PcMSP1$_{42}$ vaccines. Consideration of these differences in clinical trials evaluating *P. falciparum* AMA1- and MSP1-based vaccines may be beneficial.

In vitro GIA data with antibodies that recognize *P. falciparum* AMA1 have indicated the importance of conformational B-cell epitopes as targets of neutralizing antibodies (9, 10, 23). Early reports also showed that proper disulfide bonds of the ectodomain of PcAMA1 are required for protection against lethal *P. chabaudi* challenge (2, 12). Through in vivo studies involving the immunization of B-cell-deficient J$_{k}$id mice, we previously showed that PcAMA1 vaccine-induced protection against nonlethal *P. chabaudi* malaria was largely, if not completely, antibody mediated (4). In the current study, we observed an ~50% reduction in the level of *P. chabaudi* parasitemia in mice immunized with RA rPcAMA1. We interpret these data in combination to indicate that antibodies against linear epitopes of PcAMA1 are partially effective against nonlethal *P. chabaudi* malaria but that the most effective protective response involves disulfide-dependent B-cell epitopes. This is reflected in the marked gain in efficacy that we predicted when the prechallenge titers of antibodies against RF PcAMA1 were simply doubled. If high titers of antibodies against conformational epitopes of RF PcAMA1 are induced, the presence of an additional population of antibodies specific for linear determinants of RA PcAMA1 is not predicted to greatly influence efficacy against homologous challenge.

Protective epitopes associated with the disulfide-bonded epidermal growth factor-like domains of MSP1$_{42}$ have largely been defined by using antibodies that passively protect mice in vivo (6, 29, 46) or that inhibit the growth of *P. falciparum* in vitro (8, 37, 52). In the present study, we observed a partial reduction in the rate of *P. chabaudi* malaria following immunization with RA PcMSP1$_{42}$. This may reflect some protective role for antibodies that recognize linear determinants of MSP1, as has been suggested on the basis of GIA data for epitopes associated with MSP1$_{33}$ (57). However, we believe that this partial protection more likely reflects the contribution of antibody-independent, cell-mediated immune responses to PcMSP1$_{42}$ that we previously demonstrated in immunization and challenge studies with rPcMSP1$_{42}$-based vaccines and B-cell-deficient mice (4). Of particular interest and unlike the results that we observed for PcAMA1, our data further indicate that antibodies against nonconformational epitopes of MSP1 negatively affect protection even in the presence of high titers of otherwise protective antibodies. Some of these antibodies may be related to the MSP1-specific, blocking antibodies that have previously been demonstrated to be functional in vitro in *P. falciparum* growth inhibition assays (20, 52).

In our final trial (trial 5), we were able to confirm our findings and show that the titers of prechallenge antibodies against the RA and RF antigens induced by prior immunization with PcAMA1 and PcMSP1$_{42}$ could be used to predict vaccine efficacy. It is of interest that sufficiently high titers of antibodies to RF PcAMA1 are predicted to and in fact do completely neutralize *P. chabaudi* parasites, such that detectable parasitemia is not observed. We have noted such potent protection with PcAMA1-based vaccines in our earlier studies (4, 5). This is generally not the case for PcMSP1$_{42}$. While immunization with PcMSP1$_{42}$ induces solid protection against challenge infection, most animals do develop some low level of parasitemia. In MSP1 vaccine studies with actively or passively immunized mice, this has been attributed to the need to mount an immune response to other blood-stage antigens before parasitemia can be fully suppressed (13, 22). In trial 5, blood-stage parasites were detected in all PcMSP1$_{42}$-immunized mice, and we noted a reasonable correlation between the predicted and the observed peak levels of parasitemia, with one caveat. The correlation dropped off in the PcMSP1$_{42}$-immunized animals that mounted the strongest responses upon immunization and that developed the highest antibody titers. These data again suggest that MSP1$_{42}$-specific antibodies alone cannot completely protect against blood-stage malaria. Furthermore, efforts to induce antibodies beyond a certain level will not improve efficacy and may in fact be counterproductive. Such a strong, dominant, and ongoing immune response to a single antigen may delay the development of responses to other parasite antigens required to ultimately clear the infection. Further investigation will be necessary to confirm this possibility.

The studies with a rodent malaria model described here gave us an opportunity to complete in vivo studies of the correlates of vaccine-induced immunity that would not be feasible with human subjects for ethical reasons. Establishing the relevance of our findings for *P. falciparum* AMA1 and MSP1 vaccines will require additional work. As clinical testing with human subjects proceeds, however, we feel that several pieces of information are relevant and should be considered. First, the results again confirm that AMA1- and MSP1$_{42}$-based vaccines need to induce high titers of antibodies to maximally suppress blood-stage parasitemia. This has been a critical issue in trials of *P.
additional P. falciparum MSP142 and/or AMA1 allele that must be added to a vaccine cocktail to counter issues of polymorphisms. The question of whether or not this can be achieved should continue to be discussed. Second, in P. falciparum AMA1 and MSP142 vaccine trials, it will be informative to measure the titers against the RF and RA antigens in an effort to establish associations with protection. In doing so, these values should also be considered in combination when an attempt to predict efficacy is made. The data can be obtained from simple ELISA-based assays. This approach is distinct from the current practice of measuring the titers of antibodies to conformational epitopes of selected subdomains of the immunizing antigen. Third, immunization with P. falciparum MSP142 can clearly induce protective antibodies but will also likely induce some level of blocking antibodies that are predicted to reduce efficacy. Work will need to continue to determine if immunization with monomeric or dimeric MSP142 (44) or with MSP142 complexed with MSP143, MSP-6, MSP-7, and/or MSP-9 (24, 26, 32, 34, 40, 51) more effectively induces merozoite-neutralizing antibodies. Finally, focusing on the induction of a sufficiently strong and sustained B-cell response to a single antigen in order to control blood-stage malaria is unwise. We need to better understand if other responses, including cell-mediated responses, elicited by infection are synergistic or antagonistic to vaccine-induced responses (16, 19, 28, 39, 41, 53–55). In addition, the targets of these protective responses will need to be identified.

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