

Cytokines and Antibody Subclass Associated with Protective Immunity against Blood-Stage Malaria in Mice Vaccinated with the C Terminus of Merozoite Surface Protein 1 plus a Novel Adjuvant

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A blood-stage malaria antigen comprising the C terminus of merozoite surface protein 1 fused to glutathione S-transferase, combined with an adjuvant formulation containing squalane, Tween 80, and pluronic L121 (AF), administered subcutaneously protected mice against death from a lethal *Plasmodium yoelii* infection. The protection induced by this antigen-adjuvant combination was compared with that induced by the antigen plus saponin in terms of survival from the lethal infection and clearance of parasitemia. The levels of gamma interferon and interleukin-4 in spleens were measured as indicators of Th1 and Th2 cell activation, and antibody classes and subclasses were determined by immunofluorescence. With a 10- μ g dose of antigen and AF as adjuvant, all mice recovered, but with saponin as the adjuvant, there were only a few survivors. With 30 μ g of antigen plus AF, the peak parasitemias were 10-fold lower than those with 10 μ g; with saponin, survival was slightly improved. The levels of both gamma interferon and interleukin-4 rose more rapidly and to higher levels with AF as the adjuvant than with saponin, and the same was true for immunoglobulin G1 (IgG1), IgG2a, and IgG2b subclasses. Thus, in terms of both cytokine production and antibody levels, AF is a more potent adjuvant for a malaria vaccine than is saponin.

The search for an effective vaccine against human malaria continues despite the progress made in antigen identification over the last 15 years. A number of antigens of *Plasmodium falciparum* together with various adjuvants have been tested in several systems, including alum-based vaccines which were poorly immunogenic in humans (7, 15), while in monkeys the most effective vaccines required the use of Freund's complete adjuvant (16), which is not acceptable for use in humans.

The choice of adjuvant therefore appears to be crucial in determining the immunogenicity of a blood-stage malaria vaccine. In mice, freeze-thawed preparations of blood-stage parasites of *P. yoelii* were effective when given subcutaneously with a variety of adjuvants, including saponin, *Bordetella pertussis*, copolymer P1004, or a detoxified preparation of lipopolysaccharide (9, 25). Our own earlier findings showed that a Triton X-100 lysate of blood-stage *P. yoelii* with saponin as the adjuvant was very effective when given intraperitoneally but less effective given subcutaneously (19). Recently, we have shown that strong protection against death from a lethal *P. yoelii* infection could be achieved by vaccination with a semipurified blood-stage antigen combined with the novel adjuvant formulation AF and that protective immunity is associated with an elevation of both Th1 and Th2 cell subset activities (3). To see if this adjuvant was also more effective with a single recombinant antigen, we immunized mice with a fusion protein containing the C-terminal fragment of merozoite surface protein 1 (MSP-1), a candidate vaccine antigen, and saponin was used for comparison because it has a strong adjuvant effect when

given with MSP-1 (5). The C-terminal fragment of MSP-1 has been shown previously to induce strong protection when used with Freund's complete adjuvant (11). We investigated the induction of cytokine and antibody responses in the two groups to see if these could account for the greater potency of AF. Our data suggest that both types of T-helper-cell subsets are involved in mediating protective immunity, together with immunoglobulin G (IgG) antibodies of the G1, G2a, and G2b subclasses.

MATERIALS AND METHODS

Mice. (BALB/c \times C57/BL)F₁ mice were bred at University College London Medical School, and both sexes were used at 12 to 15 weeks of age.

Parasites. The lethal YM strain of *P. yoelii* was obtained from D. Walliker (University of Edinburgh) and maintained by weekly blood passage. This parasite causes a rapid infection, killing nonimmune mice by day 8. Mice were infected intravenously with 10⁴ parasitized erythrocytes, and parasitemias were estimated on Giemsa-stained blood films from day 3 onwards.

Antigen. The C terminus of *P. yoelii* MSP-1, containing two epidermal growth factor-like motifs, was expressed as a recombinant protein fused with glutathione S-transferase (GST) (GST-MSP1EGF) as described previously (11).

Adjuvants. The AF adjuvant formulation was kindly supplied by IDEC Pharmaceuticals, San Diego, Calif. It consists of an oil-in-water emulsion of 15% (wt/vol) squalane (Aldrich), 0.6% Tween 80 (Aldrich), and 3.75% pluronic L121 (BASF, Parsippany, N.J.) (20). A volume of 67 μ l of AF was mixed with 10 or 30 μ g of antigen in 133 μ l to give a final volume of 200 μ l and vortexed for 45 s before injection.

Twenty-five micrograms of HP3 saponin (a gift from R. Bomford, Wellcome Research Laboratories, Beckenham, United Kingdom) was mixed with 10 or 30 μ g of antigen just before injection.

Vaccination and infection. Mice were injected subcutaneously twice, 2 weeks apart, with either 30 or 10 μ g of antigen plus adjuvant. Three weeks later, they were challenged intravenously with 10⁴ parasites, and their parasitemias were monitored daily from day 3. Control mice received antigen or GST alone or the AF adjuvant alone in phosphate-buffered saline.

Cytokine extractions. Endogenous levels of gamma interferon (IFN- γ) and interleukin-4 (IL-4) in the spleen were determined by a modification of the method of Nakane et al. (14) described previously (3). Individual spleens were weighed and homogenized in 1% 3-[(cholamidopropyl)-dimethyl-ammonio]-1-

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propanesulfonate (CHAPS; Sigma) in a Dounce tissue homogenizer, and 10% (wt/vol) homogenates prepared. Homogenates were left on ice for 1 h, and insoluble debris was then removed by centrifugation at $2,000 \times g$ for 20 min. The clear supernatants were stored at -70°C .

Cytokine assays. Standard capture enzyme-linked immunosorbent assays were used with monoclonal antibody pairs and Maxisorp (NUNC) plates. Primary monoclonal antibodies against IFN- γ (R46A2) and IL-4 (11B11) and secondary biotinylated anti-mouse IL-4 (BVD6-24G2) and anti-mouse IFN- γ (XMG1.2) monoclonal antibodies (PharMingen) were used with streptavidin peroxidase (Dako) and *o*-phenylenediamine (Sigma) as substrates. Recombinant IFN- γ and IL-4 standards came from PharMingen. The results shown are the means (in nanograms) per spleen from at least eight mice.

Antibody assays. Pre- and postchallenge antibody titers were measured by indirect fluorescence assay using schizont-coated slides as previously described (26). Antibody subclasses were detected with a 1:300 dilution of an isotype-specific antibody raised in rabbits (Dako) followed by a 1:500 dilution of a fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin (Dako).

Statistics. Significance levels were determined by Student's *t* test for unpaired observations.

RESULTS

Protection against infection. Groups of mice immunized with either 10 or 30 μg of GST-MSP1EGF antigen plus AF or saponin were compared with groups of control mice immunized either with antigen alone, adjuvant alone, GST alone, or GST plus adjuvant or not at all. All control animals died between 8 and 10 days after infection. The parasitemias of mice immunized with GST-MSP1EGF alone without adjuvant were indistinguishable from those of controls, and the infection was fatal (mean \pm standard deviation] day of death, 7.71 ± 0.99).

Immunization with low-dose antigen plus adjuvant. Ten micrograms of GST-MSP1EGF given with saponin protected only 2 of 10 vaccinated mice against death. In the survivors, the parasitemia peaked at 75% between days 8 and 12 and thereafter decreased steadily until eventually cleared at day 25; the remaining eight mice died on the same day as did controls. In contrast, all (10 of 10) of the animals vaccinated with GST-MSP1EGF plus AF were protected against death. Their parasitemias peaked at 10 to 12% (Fig. 1a); six animals were clear of detectable parasites on day 11, and the remaining four were clear on day 17.

Immunization with high-dose antigen plus adjuvant. Vaccination with the high dose (30 μg) of GST-MSP1EGF without adjuvant did not protect mice against death, and their parasitemias were no different from those of unvaccinated controls (data not shown). When saponin was used as the adjuvant (Fig. 1b), 60% (6 of 10) of the animals survived, with 4 of these survivors clearing their parasitemias on day 10 and 2 clearing their parasitemias on day 15, while 40% (4 of 10) died on day 8. However, when AF was the adjuvant, 100% (18 of 18) of the animals cleared their parasitemias and 4 were totally resistant to challenge infection. The protection induced by this dose of antigen with the AF adjuvant was markedly stronger than that induced by the low dose of antigen. Peak parasitemias were typically around 1%, i.e., 10-fold lower, and all the mice cleared their parasitemias, with a mean (\pm standard deviation) day of clearance of 8.51 ± 1.04 .

Cytokine production. To see if differences in protection were reflected in higher cytokine levels, total concentrations of IFN- γ and IL-4, calculated in nanograms per spleen, were measured during the course of infection. Spleens from four to five mice per group were assayed individually at various times after infection. Although spleen weights varied between groups receiving different treatments, variations within groups were minimal. Normal (\pm standard deviation) spleen weights were 0.103 ± 0.008 g. Spleen weights from both vaccinated and unvaccinated mice were threefold higher than normal 5 days after infection. While spleen weights from unvaccinated mice

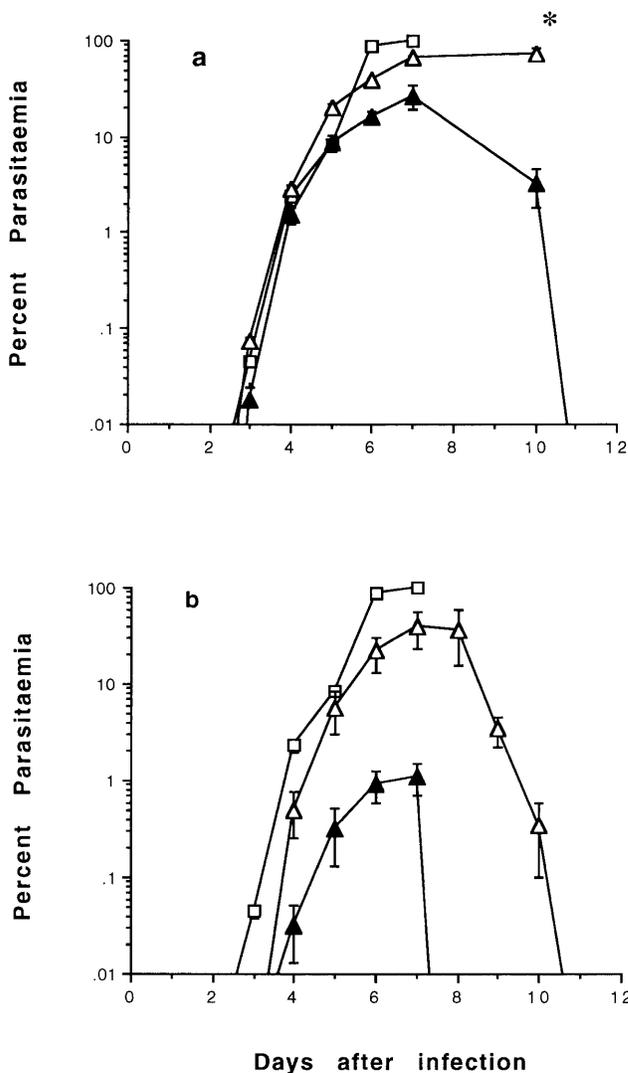


FIG. 1. Effects of vaccination with 10 (a) or 30 (b) μg of GST-MSP1EGF with AF or saponin on the course of infection with lethal *P. yoelii*. Mice were immunized twice subcutaneously with antigen plus adjuvant and challenged with 10^4 viable parasites as described in the text. Representative parasitemias (means \pm standard errors) from groups of 10 to 18 mice from two separate experiments are shown. \square , normal unvaccinated mice; \blacktriangle , mice vaccinated with antigen plus AF; \triangle , mice vaccinated with antigen plus saponin. The asterisk indicates that 2 of 10 mice recovered on day 25 (data not shown) and that the others died between days 8 and 10 as described in the text.

remained the same on day 7, those from vaccinated mice were 6-fold higher and increased to 12-fold higher than those from controls on day 10. Control groups vaccinated with either GST-MSP1EGF alone, GST plus AF, or GST plus saponin had spleen weights and cytokine levels similar to those of infected unvaccinated mice (data not shown).

Mice vaccinated with 10 μg of antigen plus adjuvant. The IFN- γ levels in age-matched normal mice ranged between 4.0 and 10.0 ng per spleen (Fig. 2a). The levels in infected but unvaccinated mice remained below 20 ng per spleen, except on day 5 after infection when they peaked at 37.5 ng per spleen. The levels in mice vaccinated with antigen plus adjuvant were not significantly different from those of unvaccinated controls on days 3 and 5 of infection. However, on day 7 the response in the group given antigen plus AF was significantly higher

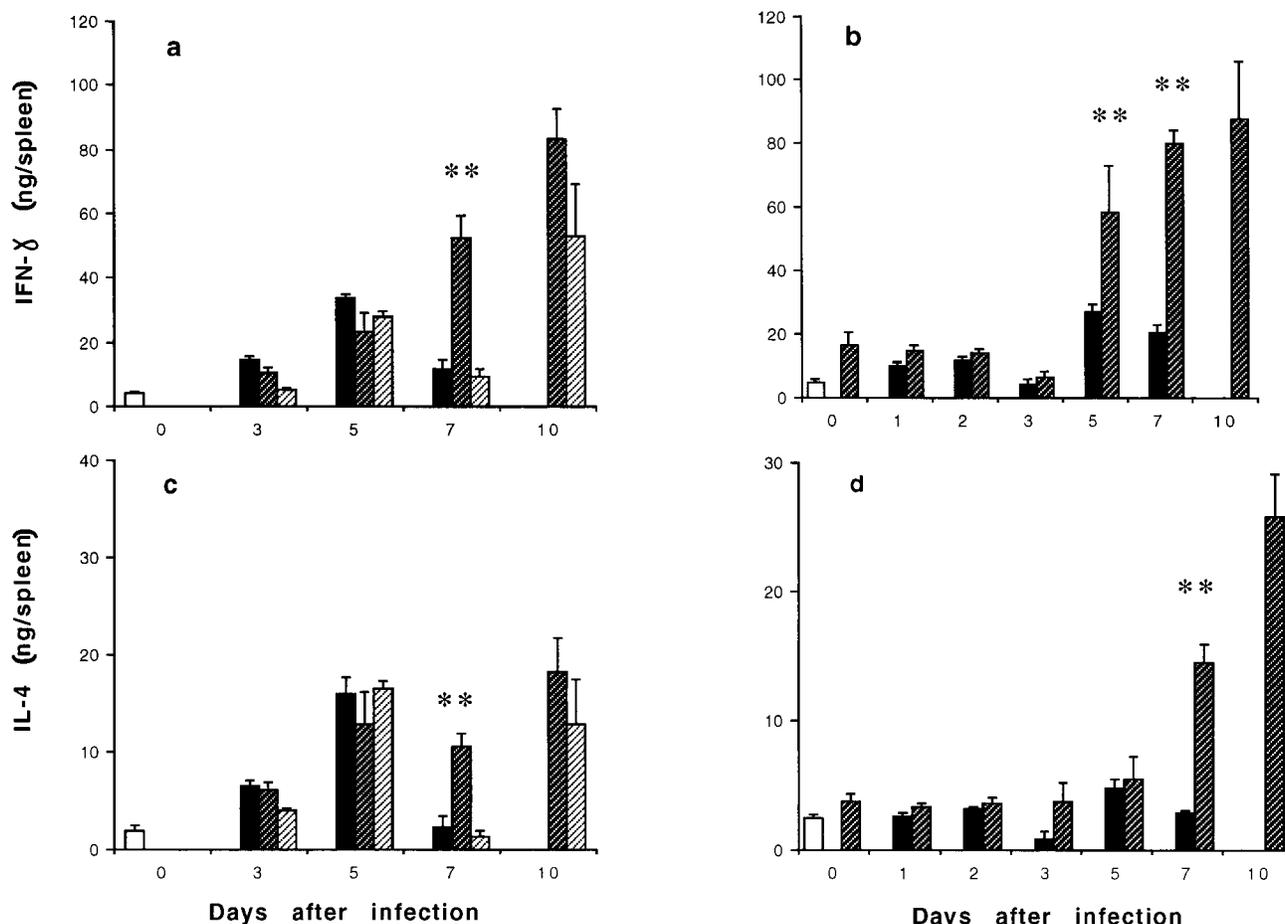


FIG. 2. Cytokine levels in the spleens of mice vaccinated with 10 (a and c) or 30 (b and d) μg of GST-MSP1EGF. Vaccination and challenge were done as described in the legend to Fig. 1. IFN- γ (a and b) and IL-4 (c and d) responses after *P. yoelii* infection in unvaccinated mice (■) or mice vaccinated with antigen plus AF (▨) or saponin (▩). Each bar represents the mean (\pm standard error) cytokine concentration of groups of 8 to 10 mice from two separate experiments. An open bar represents the response of normal unvaccinated and uninfected mice. **, $P < 0.003$, compared with the results with saponin (a and c) or with unvaccinated mice (b and d).

($P < 0.003$) than that in the group given antigen plus saponin, which decreased to the same level as that of controls. On day 10, the levels in the survivors were similar in both adjuvant groups, suggesting that survival was associated with high levels of IFN- γ .

The IL-4 levels in normal mice were between 0.5 and 3.5 ng per spleen (Fig. 2c). In infected, unvaccinated mice, the levels remained below 10.0 ng per spleen, except on day 5. Significant differences between the antigen-plus-AF group and the antigen-plus-saponin group were noted on day 7 ($P < 0.003$), the day recovery began. The IL-4 levels in controls and the saponin group decreased from days 5 to 7. The lower level of protection in the saponin group may be due to inadequate IL-4 activation on day 7. By day 10, the differences between the two adjuvants were not significant, suggesting that the earlier activation on day 7 was associated with better protection.

Mice vaccinated with 30 μg of antigen plus AF adjuvant.

The prechallenge IFN- γ levels in the vaccinated group were slightly higher than those of the normal unvaccinated control group, but 1 to 3 days later, there were no differences between the two groups (Fig. 2b). However, the differences were significant on days 5 ($P < 0.003$) and 7 ($P < 0.003$). By day 10, all unvaccinated mice had died.

The prechallenge and 1- to 5-day levels of IL-4 were not

significantly different between the vaccinated and unvaccinated groups (Fig. 2d). However, on day 7 the difference between the two groups was highly significant ($P < 0.003$).

The IFN- γ responses in the group given the high dose of antigen were significantly higher ($P < 0.0009$) on days 5 and 7 when compared with those in the group given the low dose of antigen with AF (compare Fig. 2b with a), while the IL-4 responses were higher only on day 7 (compare Fig. 2d with c). This is the day at which parasitemias reached a peak in mice given the high dose of antigen, suggesting that both IFN- γ and IL-4 levels contribute to the subsequent disappearance of parasites from the blood. Parasite clearance occurred after day 10 with the low dose of antigen. With AF as the adjuvant, higher doses of antigen stimulated a faster increase in IFN- γ production, particularly at day 5.

Antibody titers. To see if the greater protection induced by AF compared with that induced by saponin was associated with the induction of higher antibody titers or the production of different isotypes, measurements were made of the sera of mice whose spleens were taken for cytokine analysis. Pooled sera from three mice were tested in all assays. Control unvaccinated mice and mice vaccinated with antigen or adjuvant alone had prechallenge parasite-specific titers of all subclasses of 1:4, which never rose above 1:128 to 1:256 during the course

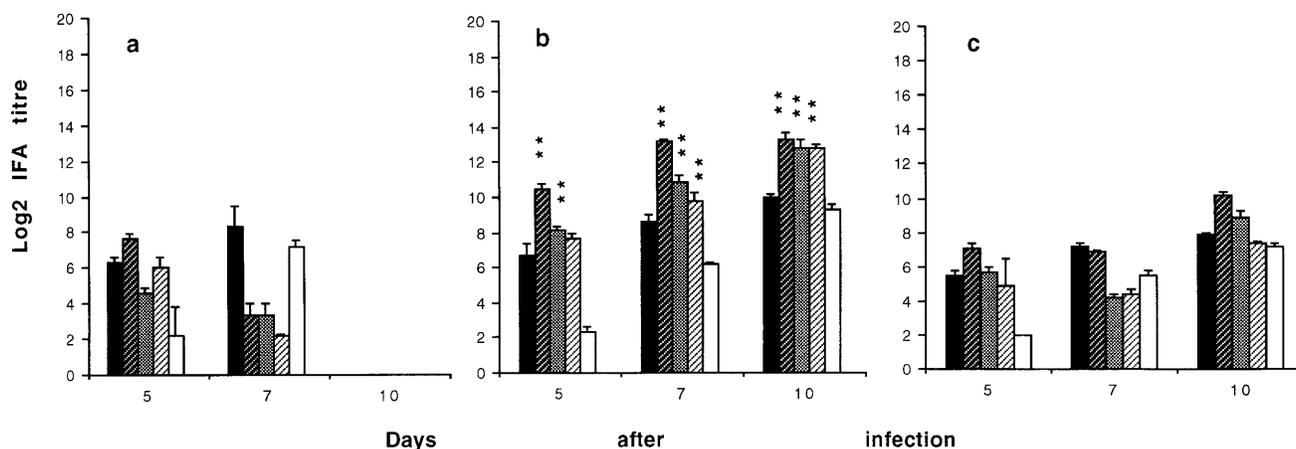


FIG. 3. Development of antibody subclasses in mice vaccinated with 10 μ g of antigen with and without adjuvant. The details of vaccination and challenge were the same as those described in the legend to Fig. 1. IgM (■), IgG1 (▨), IgG2a (▤), IgG2b (▥), and IgG3 (□) titers during *P. yoelii* infection in mice vaccinated with antigen alone (a), antigen plus AF (b), or antigen plus saponin (c). Each bar represents the mean indirect fluorescence assay (IFA) titer \pm standard error of pooled sera from six mice from two separate experiments. **, $P < 0.003$, compared with results without adjuvant or with saponin.

of infection. Animals vaccinated with the high dose of antigen had titers that were initially one- to twofold higher than those of animals vaccinated with the low dose, but there were no differences at the time when the parasitemia was resolving. Animals which were vaccinated with the low dose of antigen plus saponin and failed to resolve their parasitemias had antibody titers similar to those of controls. This association suggests that antibody plays a role in protective immunity. Figure 3 shows the effects of adjuvants with the low dose of antigen on the development of antibody classes and subclasses. All IgG responses increased more rapidly in mice given AF (Fig. 3b) than in those receiving saponin (Fig. 3c), and all except IgG3 were significantly higher by day 7 ($P < 0.003$) and remained higher at day 10. It is noteworthy that the titers in the group given antigen with AF rose more rapidly and were higher around the time when parasitemias started to diminish.

DISCUSSION

The cysteine-rich domain at the C terminus of MSP-1 is considered to be a promising antigen for a protective blood-stage malaria vaccine. Three doses of this antigen given intraperitoneally with either Freund's (11), Ribi (2), with Hunter's TiterMax (1) adjuvant subcutaneously induce strong protective immunity in mice. However, neither adjuvants nor the intraperitoneal route of administration is clinically acceptable. In this study, we have shown that just two doses of the antigen, in the form of a recombinant fusion protein (GST-MSP1EGF), administered subcutaneously with AF as the adjuvant were fully protective in terms of both survival against a lethal infection and elimination of parasitemia. This adjuvant and the route of inoculation are both clinically permissible, as the adjuvant has been tested in clinical trials and is known to be safe and well tolerated (12).

The AF adjuvant, when used with GST-MSP1EGF, induced excellent cytokine and antibody responses and greatly enhanced protective immunity against this lethal *P. yoelii* line. This was best seen with a discriminating dose of 10 μ g of protein, which failed to induce protective immunity with our reference adjuvant, saponin. This would appear to be due to the inability of saponin to switch on cytokines rapidly. In contrast, this dose of antigen induced good protective immunity when given with AF, which accelerated cytokine and antibody

responses. While partial protection was achieved with the high dose (30 μ g) of antigen with saponin, with AF strong protection marked by greatly reduced parasitemias occurred and 4 of 18 mice remained totally aparasitemic after challenge; protection was associated with increases in both cytokine and IgG antibody responses. This superior protective effect correlates with the induction of early increases in IFN- γ , IL-4, and antibody. The high-level IFN- γ and IL-4 responses in vaccinated mice corresponded well with increases in IgG2a and IgG1 antibody subclasses, respectively (4, 22); elevated IgG2b levels may also have contributed to protective activity, although their production has not yet been positively associated with a particular T-helper-cell subset. Differences in protection induced by the high and low doses of antigen with AF may be explained by higher IFN- γ levels on days 5 and 7 (Fig. 2a and b) and fourfold-higher titers of IgG1 on day 7 (data not shown). In addition, macrophage activation may be involved, as our current experiments suggest that tumor necrosis factor alpha levels are higher in animals which clear their parasitemias rapidly (unpublished data). Besides other factors, IFN- γ and tumor necrosis factor alpha are also required for protection against blood-stage *P. chabaudi* infections (24).

It has been suggested that the choice of adjuvant is important in inducing the most effective subclass of antibody required for protective immunity to *P. falciparum* in vivo (8). Studies of murine malaria indicate that the type of eliciting antigen used for immunization is equally important. In this study, GST-MSP1EGF plus AF boosted the IgG1, IgG2a, and IgG2b subclasses. Formalin-fixed blood-stage parasites given with *B. pertussis* as the adjuvant stimulate the production of all IgG subclasses (18), while freeze-thawed lysates of parasites given with copolymer adjuvants induce high IgG2a titers (9, 25). However, our semipurified antigens plus AF, which were associated with the rapid elimination of parasitemia, induced high titers of all IgG subclasses, including G3 (unpublished data). A monoclonal IgG3 antibody has been shown to be protective against this lethal parasite (13). However, in the experiments described here, the titers of IgG3 began to rise only after recovery in mice vaccinated with GST-MSP1EGF plus AF, suggesting that the induction of this antibody subclass depends on the antigen.

The findings reported here confirm our previous observations that when a semipurified blood-stage antigen was injected

subcutaneously with AF, both Th1 and Th2 T-helper-cell subsets were activated at the time of resolving parasitemia. Alternatively, it is possible that both cytokines were produced by activated Th0 cells, although increased IFN- γ responses preceded those of IL-4 by 2 days (3). In the case of the self-resolving *P. chabaudi* infection, it has been reported that an initial Th1 response is replaced by the Th2 response, which is thought to be responsible for the final elimination of parasitemia (10, 23). However, IL-4 itself may not be the only cytokine required and IL-5, IL-6, and IL-10 may take over in its absence, as shown in knockout mice (27). Recent evidence suggests that IFN- γ and tumor necrosis factor alpha are required for protection against a *P. chabaudi* infection (24). IFN- γ was important in controlling parasitemia in drug-cured *P. vinckei*-infected mice (17). It was also an important protective factor against an attenuated nonlethal variant of the lethal malaria parasite *P. berghei* (28). The induction of protective immunity against the latter two lethal malaria parasites, whether by attenuation or drug cure, appears to be mediated mainly by activation of the Th1 T-helper-cell subset. In *Aotus* monkeys vaccinated with recombinant blood-stage antigens, protective immunity after *P. falciparum* challenge is associated with high levels of IFN- γ in serum (6). Studies of human malaria indicate that high levels of both antibody and IFN- γ in response to the C terminus of MSP-1 are associated with the ability of partially immune children to resist episodes of fever associated with high-level parasitemia (21). Our studies with mice indicate that a successful vaccine can stimulate both Th1 and Th2 T-helper-cell subsets, as judged by increased levels of cytokines and antibodies of the IgG subclasses.

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