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Influence of Adjuvants on Protection Induced by a Recombinant Fusion Protein against Malarial Infection

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Previously, we described a protective immune response induced by the carboxyl-terminal region of the merozoite surface protein-1 (MSP-1) from the rodent malarial parasite *Plasmodium yoelii yoelii* 17XL, expressed as a fusion protein and designated glutathione *S*-transferase (GST)-PYC2. We also demonstrated that the humoral response induced by GST-PYC2 was the primary mechanism by which immunized animals controlled their blood-stage infections. We have now examined the influence of several adjuvants on the immune response to the GST-PYC2 fusion protein. While alum, Freund's adjuvant, Ribi adjuvant system, and TiterMax were efficacious in eliciting a protective response with GST-PYC2 in BALB/c mice, saponin failed to induce protection, although significant levels of PYC2-specific antibodies were produced in all immunized animals. This protection depended on the mouse strain since immunization of Swiss Webster mice with GST-PYC2 in alum did not produce levels of PYC2-specific antibodies comparable to those in BALB/c mice nor did it induce any demonstrable level of protection against parasite challenge. Swiss Webster mice were protected, however, when immunized with GST-PYC2 in other adjuvants. Immunization with PYC2, isolated free of GST induced lower levels of antigen-specific antibody; only those animals given PYC2 in Freund's adjuvant demonstrated a significant degree of protection, suggesting the possibility of additional cellular effector mechanisms. These findings demonstrate that adjuvant, host genotype, and the fine specificity of the response significantly influence the protection induced by the carboxyl terminus of MSP-1 in vivo and illustrate the need to consider these factors in evaluating MSP-1 as a vaccine component.

Recent efforts to develop a malaria vaccine have focused on several stage-specific antigens, including the merozoite surface protein MSP-1 (30, 32, 33), which is expressed during the erythrocytic phase of malarial infection within the vertebrate host. Previously, it was shown that immunization with intact MSP-1, affinity purified from parasites, could induce a protective response against challenge infection in nonhuman primate and rodent animal models of malarial infection (12, 14, 16, 34). Work of others with the MSP-1 of *Plasmodium falciparum* established that the molecule undergoes extensive processing during parasite maturation and that only a 19-kDa carboxyl-terminal fragment of the MSP-1 remains associated with the parasite membrane following erythrocyte invasion (2). It has also been shown that immunization with affinity-purified *P. falciparum* MSP-1 or carboxyl-terminal fragments of the *P. falciparum* MSP-1 could induce antibodies capable of inhibiting parasite growth in vitro (6, 8, 17, 19–21). The type of adjuvant used to induce a humoral response to this portion of the molecule significantly influenced the efficacy of elicited antibodies in invasion inhibition assays (7, 18, 21).

Studies done by this laboratory using a rodent model of plasmodial infection demonstrated that transfer of a monoclonal antibody specific for MSP-1 of *Plasmodium yoelii yoelii* 17XL could impart dramatic protection to naive recipients against a homologous, lethal challenge infection (31). It was further established that the epitope bound by this MAb was located within the first epidermal growth factor-like domain of the cysteine-rich, carboxyl-terminal region of the molecule (4, 5, 13).

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We have previously demonstrated that a 15-kDa carboxyl-terminal fragment of the *P. yoelii yoelii* 17XL MSP-1, fused to glutathione *S*-transferase (GST) of *Schistosoma japonicum* and expressed by recombinant *Escherichia coli*, could elicit antibodies capable of recognizing native MSP-1 when administered in Ribi adjuvant system (RAS) and that mice immunized with this fusion protein in RAS were protected against an otherwise lethal challenge infection with homologous parasites (10). These results were subsequently confirmed by others (29). In addition, we have determined that immunization with a truncated form of the original antigen, including only 11 kDa of the strain 17XL MSP-1 molecule and designated GST-PYC2, resulted in a significant protective response when administered in RAS. Moreover, we have shown that this effector function was predominantly mediated by antibodies as judged by the transfer of passive immunity with immune globulin (11). We now describe the influence of several adjuvants on the humoral response to the GST-PYC2 fusion protein and on the degree of protection induced by this antigen in a rodent model system.

MATERIALS AND METHODS

Experimental animals and parasites. Six- to 8-week-old, male, BALB/cByJ (BALB/c) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Swiss Webster/BR (SW) mice of similar age and sex were obtained from Taconic Farms, Inc. (Germantown, N.Y.). All animals were maintained in our American Association for the Accreditation of Laboratory Animal Care-approved facility and screened serologically on a regular basis to confirm the absence of murine pathogens. In compliance with a directive from Hahnemann University's Animal Welfare Committee, all animals deemed in distress or with parasitemias in excess of 50% were removed from the study and euthanized. *P. yoelii yoelii* 17XL, a lethal variant, was originally obtained from John Finerty (National Institutes of Health, Bethesda, Md.) and was maintained as a cloned, cryopreserved stabilate. Challenge inoculum was prepared from parasitized erythrocytes taken from a donor mouse infected with stabilate material.

Recombinant construct and fusion protein. The recombinant construct and

resultant fusion protein were 2described previously (11). Briefly, the carboxyl-terminal region of the *P. yoelii yoelii* 17XL MSP-1 gene (nucleotides 1699 to 1995 [4] or 5164 to 5460 [28]) was amplified by the PCR and joined in frame to the 3′ end of the *S. japonicum* GST gene within the pGEX/2T vector (35). The resultant fusion protein, designated GST-PYC2, was expressed in *E. coli* cells (HB101) and isolated by affinity chromatography as described previously (35). The PYC2 portion of the fusion protein was isolated from GST-PYC2 while bound to glutathione-agarose (Sigma Chemical Co., St. Louis, Mo.) with thrombin (ICN Biochemicals, Inc., Costa Mesa, Calif.) as described previously (10). GST was isolated by following the same procedures from *E. coli* cells transformed with the pGEX/2T vector containing no insert.

Immunizations and challenge infections. Eight- to 10-week-old BALB/c mice were immunized with 60 μg of GST-PYC2 (providing 20 μg of PYC2) or 40 μg of GST in various adjuvants. Fusion protein or GST control antigen was first administered subcutaneously (s.c.) in 200 μl (two sites, 100 μl each site) of RAS (MPL plus trehalose dimycolate [TDM] emulsion; Ribi Immunochem Research, Inc., Hamilton, Mont.), suspended in 20 mM NaH_2PO_4/Na_2HPO_4–150 mM NaCl (pH 7.4; PBS). This dose was repeated s.c. after 3 weeks and again at 6 weeks after the initial injection but administered intraperitoneally. Immunizations with similar amounts of antigen were begun s.c. with protein emulsified in 200 μl (two sites, 100 μl each site) of complete Freund's adjuvant (Sigma), followed by two injections in incomplete Freund's adjuvant (Sigma) as described above. Antigens were also prepared in TiterMax (Vaxcel Corp., Norcross, Ga.) and alum (Imject Alum; Pierce, Rockford, Ill.) as described in the manufacturer's instructions and administered in three s.c. injections by the schedule described above. Intraperitoneal injection of fusion protein in TiterMax was not performed as a result of an earlier observation that this seemed to cause some bowel irritation as indicated by loose stools. Injections containing alum were restricted to the s.c. route to simulate its use in humans. Food-grade saponin (Bell Flavors and Fragrances Inc., Northbrook, Ill.) was used at a concentration of 125 μg/ml in PBS with the proteins described above and given in three intraperitoneal injections of 200 μl as described above. Previous studies with intact MSP-1 in saponin demonstrated that this route was most efficacious for this adjuvant (14). Immunizations with PYC2 were performed as described above with 20 μg of antigen following isolation from GST-PYC2 bound to glutathione-agarose. SW mice were immunized as indicated by protocols described above. Challenge infections were initiated by intravenous injection of 10^4 erythrocytes infected with the lethal variant *P. yoelii yoelii* 17XL. The course of infection was monitored by microscopic examination of stained blood films as described previously (31).

ELISA. Enzyme-linked immunosorbent assays (ELISAs) were performed as described previously (10). Briefly, wells of Maxi-sorb immunoplates (Nunc, Naperville, Ill.) were coated with PYC2 or GST at 0.5 μg/ml in carbonate buffer (pH 9.6). Wells were blocked with 0.2% Tween 20 (Sigma) in 25 mM Tris-HCl (pH 8.0)–150 mM NaCl (Tris-buffered saline). Serum was isolated from small blood samples (20 μl) taken from immunized animals prior to parasite challenge and analyzed individually for quantification of antibodies or pooled for isotype analysis. Dilutions were made as indicated in 0.1% Tween 20–Tris-buffered saline and added to wells in duplicate or triplicate. For isotype analysis, bound antibodies were detected with affinity-purified, biotinylated, rabbit anti-mouse (immunoglobulin G1 [IgG1], IgG2a, IgG2b, IgG3, or IgM) antibodies (Zymed Laboratories, South San Francisco, Calif.), avidin-alkaline phosphatase (Zymed), and *p*-nitrophenyl phosphate (Sigma 104; Sigma). These isotype-specific reagents were shown to possess minimal cross-reactivity, and their relative concentrations were adjusted to provide equivalent levels of reactivity prior to analysis of immune sera. Biotinylated rabbit anti-mouse (IgG, IgA, and IgM, heavy and light chain) antibody (Zymed) was used as described above for the quantification of parasite-specific antibodies. Isotype distribution assays were terminated by addition of 5 M NaOH (50 μl per well) when the optical density had reached 1.0 to 1.5 O.D. units in wells with the greatest reactivity. This end point minimized the potential for overdevelopment in wells with lesser reactivity. Assays comparing relative levels of PYC2-specific antibodies were similarly terminated after some enzymatic activity was evident in the most weakly reacting wells incubated with serum from immunized animals. Hyperimmune serum (PyHIS) was prepared as described previously following subsequent infections with the nonlethal and lethal variants of *P. yoelii yoelii* 17X (5) and was used as a positive control.

Statistical analysis. To analyze the broad range of parasitemias within some of the experimental groups, the area under the curve, defined by the percent parasitemia versus day of observation over a 21-day period, was determined for each individual within a group and referred to as the relative parasite burden. If an individual animal attained a parasitemia in excess of 50%, the animal was removed from the study and assigned a value of 50% for each succeeding day of observation. These individual parasite burden values were then used to compare experimental groups with a Mann-Whitney U test. Where comparisons did not involve such disparity among individuals, comparisons were done with an unpaired *t* test. In experiment 2, an overall Kruskal-Wallis rank analysis of variance was significant with a *P* of 0.0054. Further analysis with a Mann-Whitney U test between these groups was performed but, because of the large number of comparisons (10), a *P* of <0.005 would be required for significance.

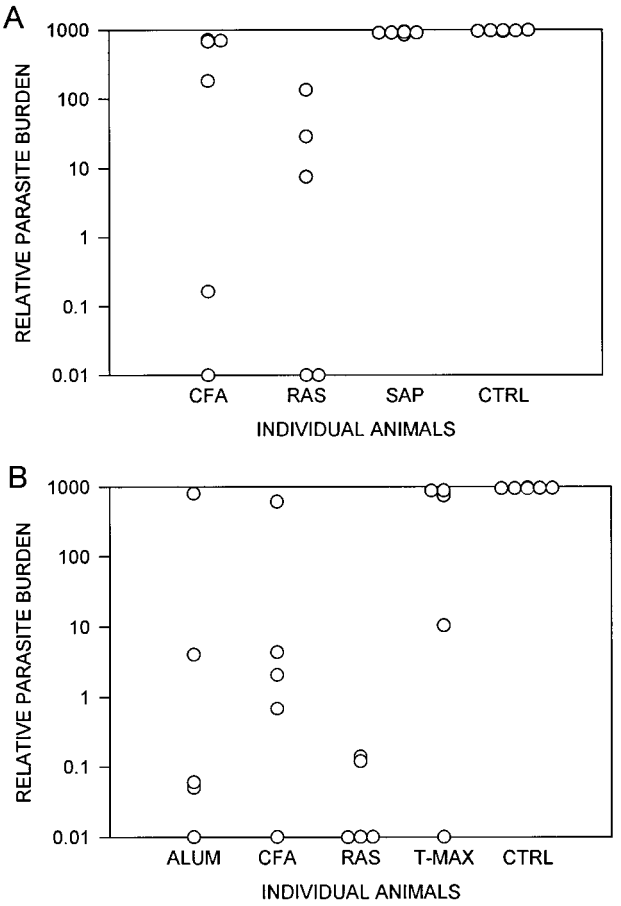

FIG. 1. Influence of adjuvants on protection induced by immunization with GST-PYC2 in BALB/c mice. (A) Groups of five BALB/c mice were immunized with fusion protein in FA, RAS, or saponin (SAP) and subsequently given a lethal challenge of 10^4 *P. yoelii yoelii* 17XL-infected erythrocytes. The graph displays the relative parasite burden, defined as the area under the curve formed by plotting the percent parasitemia versus time during the 21-day period of observation, for each animal within the various groups. A group of animals, immunized with GST in FA as described above, was also challenged and served as a control (CTRL). (B) A further comparison of the effect of adjuvants on protection was made for BALB/c mice immunized with GST-PYC2 in alum, FA, RAS, or TiterMax (T-MAX) and given a lethal challenge as described for panel A. Mice immunized with GST in alum were similarly challenged (CTRL). Again, points represent the relative parasite burden for each animal within the various groups.

RESULTS

Influence of adjuvants on protection induced by GST-PYC2. Groups of five BALB/c mice were immunized three times with 60 μg of GST-PYC2 in Freund's adjuvant (FA), RAS, or saponin by the protocols described above. Two weeks after the last inoculation, all animals were given a lethal challenge infection of 10^4 *P. yoelii yoelii* 17XL-parasitized erythrocytes. The parasite burden plots shown in Fig. 1A demonstrate that mice immunized with this fusion protein in RAS or FA exhibited various degrees of protection. In this experiment, two of five mice immunized with the fusion protein in RAS and one of five mice immunized with FA were completely protected as judged by the absence of any patent parasites during 28 days of observation after administration of the challenge infection. The two partially protected animals immunized with GST-PYC2 in FA experienced peak parasitemias of 0.1 and 32.1%, while those immunized with RAS had peak parasitemias of 1.9, 9.8,

TABLE 1. Protection induced by immunization with GST-PYC2 in various adjuvants

Expt	Mouse strain	Adjuvant	Antigen	Complete protection[a,b]	Partial protection[b]	% Survival[c]
1	BALB/c	FA	GST	0/5	0/5	0
			GST-PYC2	1/5	2/5	60
	BALB/c	RAS	GST	0/5	0/5	0
			GST-PYC2	2/5	3/5	100
	BALB/c	Saponin	GST	0/5	0/5	0
			GST-PYC2	0/5	0/5	0
2	BALB/c	Alum	GST	0/5	0/5	0
			GST-PYC2	2/5	2/5	80
	BALB/c	FA	GST-PYC2	1/5	3/5	80
	BALB/c	RAS	GST-PYC2	3/5	2/5	100
	BALB/c	TiterMax	GST-PYC2	1/5	1/5	40
3	SW	TiterMax	GST-PYC2	2/5	3/5	100
4	BALB/c	Alum	GST-PYC2	2/5	3/5	100
	SW	Alum	GST-PYC2	0/5	0/5	0
5	SW	RAS	GST-PYC2	1/5	4/5	100

[a] No patent parasites detected.
[b] Number of mice provided protection/total number of mice.
[c] Includes mice which were either completely or partially protected.

and 23.3% prior to the resolution of their infections. Two of five animals immunized with fusion protein in FA did not survive the challenge infection. These data are summarized in Table 1 (experiment 1). In contrast to these results, mice given the fusion protein with saponin were afforded no protection. All animals in this group experienced fulminating infections and were removed from the study by day 9 with parasitemias in excess of 50%. Statistical analysis of the relative parasite burden within these groups revealed that the parasitemias of mice immunized with FA and RAS were significantly different from those of the saponin group and a control group immunized as described above with 40 μg of GST in FA ($P < 0.01$, one-tailed, Mann-Whitney U test). Additional control groups immunized with GST in RAS and saponin experienced parasite burdens which were indistinguishable from that of the FA control, and all were removed from the study with parasitemias in excess of 50% ($P < 0.01$, data not shown). Regardless of the level of protection attained, all of the animals immunized with GST-PYC2 made a significant humoral response capable of recognizing native MSP-1, as demonstrated by the ability of antibodies in their sera to precipitate metabolically labeled parasite antigen (data not shown).

To confirm our initial findings with FA and RAS, and to extend these results to other adjuvants, immunization with GST-PYC2 in FA or RAS was compared with administration of fusion protein in alum or TiterMax. As can be seen by comparing the relative parasite burden plots of Fig. 1B, alum produced results similar to those of RAS, FA, and TiterMax, which were all significantly different from the control group immunized with GST in alum ($P < 0.005$, one-tailed, Mann-Whitney U test). Two of five mice immunized with the fusion protein in alum and three of five immunized with GST-PYC2 in RAS were completely protected, while one of five from the groups immunized with fusion protein in FA or TiterMax demonstrated no patent parasites. However, all of the partially protected animals immunized with GST-PYC2 experienced low peak parasitemias of 0.04 and 1.4% in alum, 0.2, 0.9, and 1.0% in FA, 0.4 and 0.3% in RAS, and 2.6% in TiterMax. These low-level parasitemias resolved rapidly. The protection data are summarized in Table 1 (experiment 2).

Influence of adjuvants on isotypes of PYC2-specific antibodies. Having achieved a significant level of protection by immunization with GST-PYC2 in several adjuvants, we sought to characterize the humoral response to the fusion protein. The isotype distribution of antibodies specific for the PYC2 portion of the fusion protein, induced by immunization with the adjuvants used in experiment 1, was determined by ELISA. As can be seen in Fig. 2A, the response patterns among all groups of animals immunized with GST-PYC2 appear to be similar, with a predominance of IgG1 and IgG2a isotypes. The levels of PYC2-specific antibody are comparable among the experimental groups at this dilution (1:500). These results contrast with the isotype distribution of PYC2-specific antibodies in *P. yoelii yoelii* 17XL hyperimmune serum (PyHIS), which is predominantly IgG2a, with lesser amounts of IgG1 and IgG3.

In experiment 2, the distribution of PYC2-specific antibody isotypes following immunization with the fusion protein in FA and RAS was found to be similar to that of the previous experiment. As can be seen in Fig. 2B, the response elicited with RAS was quite broad, as indicated by relatively high levels of IgG1, IgG2a, and IgG2b isotypes. Antibodies induced with FA and TiterMax were predominantly IgG1, while the response with alum was almost exclusively IgG1. The reactivity to PYC2

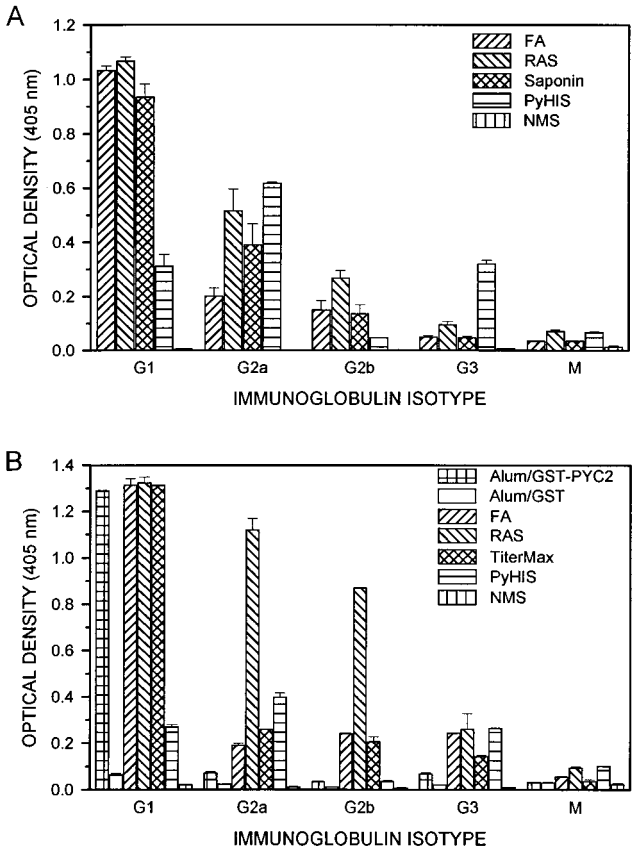

FIG. 2. Isotype distribution of PYC2-specific antibodies induced by immunization with GST-PYC2 in various adjuvants. (A) Sera from animals immunized with the same adjuvant were pooled and diluted 1:500, and the relative levels of isotypes among antibodies specific for PYC2 were determined by ELISA. Adjuvants used for immunization included FA, RAS, and saponin as indicated in the graph. PyHIS and normal mouse serum (NMS) served as controls. Optical densities represent the mean ± standard error of the mean. (B) A further comparison was done of sera (1:1,000 dilution) from groups of animals immunized with fusion protein in alum, FA, RAS, or TiterMax as indicated in the graph. Sera from immunized mice were pooled and assayed for isotype distribution as described for panel A. Optical densities represent the means ± standard errors of the mean.

in a pool of serum from animals immunized only with GST in alum was not significant.

Immunization of outbred SW mice with GST-PYC2. Studies done by others with fragments of the *P. falciparum* MSP-1 revealed that the genetic background of the mouse strain used for immunization had a significant effect on the humoral response to this parasite antigen. To test the efficacy of GST-PYC2 in a more outbred strain, SW mice were immunized as described above with this fusion protein in TiterMax and given a lethal challenge of *P. yoelii yoelii* 17XL-infected erythrocytes. All five animals were completely or partially protected, as shown in Table 1 (experiment 3), with two of five mice demonstrating no patent parasites for a period of 28 days. The remaining three mice experienced peak parasitemias of 0.08, 0.9, and 18.7% but resolved their infections by day 18. The distribution of isotypes among antibodies specific for PYC2 was similar to that for RAS in Fig. 2B, with a predominance of IgG1, IgG2a, and IgG2b (data not shown).

Having demonstrated the efficacy of GST-PYC2 in SW mice, we sought to determine whether immunization with the fusion protein in alum would be as effective in this outbred strain as it had been in BALB/c animals. As shown in Fig. 3A, SW mice given GST-PYC2 in alum experienced parasitemias similar to the infection controls, while BALB/c mice given the fusion protein in the same adjuvant showed a significant degree of protection. Two of five BALB/c mice were completely protected, and the remaining three animals experienced peak parasitemias of 0.2, 2.7, and 22.2%. In contrast, none of the SW mice immunized with GST-PYC2 in alum demonstrated any degree of resistance to parasite challenge, and these data are summarized in Table 1 (experiment 4). The lack of protection in the SW mice correlated with their failure to produce levels of PYC2-specific antibodies comparable to those in the BALB/c animals as determined by ELISA ($P < 0.001$) and shown in Fig. 3B. The relatively poor humoral response to the PYC2 region of the fusion protein by the SW mice did not reflect a general failure to respond to this antigen in alum. As shown in Fig. 3C, SW mice developed levels of GST-specific antibodies that were not significantly different from those of the BALB/c animals ($P = 0.05$, Mann-Whitney U test). The isotype distribution of antigen-specific antibodies from both BALB/c and SW mice was almost exclusively IgG1 for both GST and PYC2 (data not shown).

In an additional trial, SW mice were immunized with GST-PYC2 in RAS as described above and given a lethal challenge of *P. yoelii yoelii* 17XL-infected erythrocytes. As summarized in Table 1 (experiment 5), all of the immunized animals were protected, with one of five mice exhibiting no patent parasites during the 28-day period following challenge infection. The partially protected animals experienced very low peak parasitemias of 0.1, 0.15, 0.25, and 3.0%, which quickly resolved. As seen previously, the five unimmunized control animals developed fulminating infections and were removed from the study by day 7 (data not shown).

Protection induced by immunization with PYC2. Previously, we had reported that immunization of BALB/c mice with only the carboxyl-terminal region of MSP-1 from our original construct (PYC1), isolated free of GST and administered in RAS, failed to induce a significant humoral response or to provide any protection against challenge infection. However, it was later reported that this region of MSP-1, expressed as a similar construct, could induce a protective response in BALB/c mice when given intraperitoneally in FA (29). To determine whether PYC2, isolated free of GST, could induce a protective immune response when administered in different adjuvants, groups of BALB/c mice were immunized with 20 μg of PYC2 in FA, RAS, or TiterMax as described above. As shown in Fig. 4A, only those mice given the carboxyl terminus of the *P. yoelii yoelii* 17XL MSP-1 in FA were able to generate a protective immune response ($P < 0.02$, two-tailed, Mann-Whitney U test), with the exception of one animal in the TiterMax group. Two mice in the FA group were completely protected; those animals which were partially protected experienced peak parasitemias of 2.0, 5.4, and 20.6%. In contrast, four of five mice immunized with PYC2 in TiterMax were not protected, with only one surviving after developing a peak parasitemia of 5.6%. None of the animals immunized with the carboxyl-terminal region in RAS were able to control the challenge infection. These data are summarized in Table 2.

As shown in Fig. 4B, levels of antigen-specific antibodies in

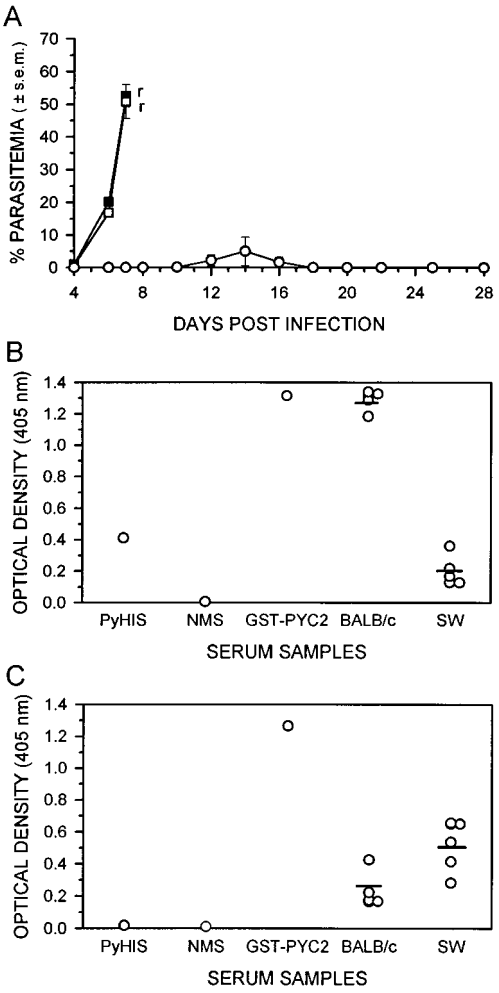

FIG. 3. Immunological response induced in BALB/c and SW mice following immunization with GST-PYC2 in alum. (A) Groups of BALB/c (○) and SW (□) mice were immunized with the fusion protein in alum and subsequently challenged with *P. yoelii yoelii* 17XL-infected erythrocytes. SW infection control animals (■) were similarly challenged. Curves represent the mean percent parasitemia ± standard error of the mean of all animals in each group. r, removed from study. (B) Relative levels of PYC2-specific antibodies in serum from individual BALB/c or SW mice (1:1,000 dilution) were determined by ELISA and compared with the levels in PyHIS, normal mouse serum (NMS), and a pool of serum from SW mice immunized with GST-PYC2 in RAS. Horizontal bars represent the mean optical density for each group. (C) Relative levels of GST-specific antibodies in serum from individual BALB/c or SW mice were determined and compared with the levels in PyHIS, NMS, and a serum pool from SW mice immunized with fusion protein in RAS. Horizontal bars represent the mean optical density for each group.

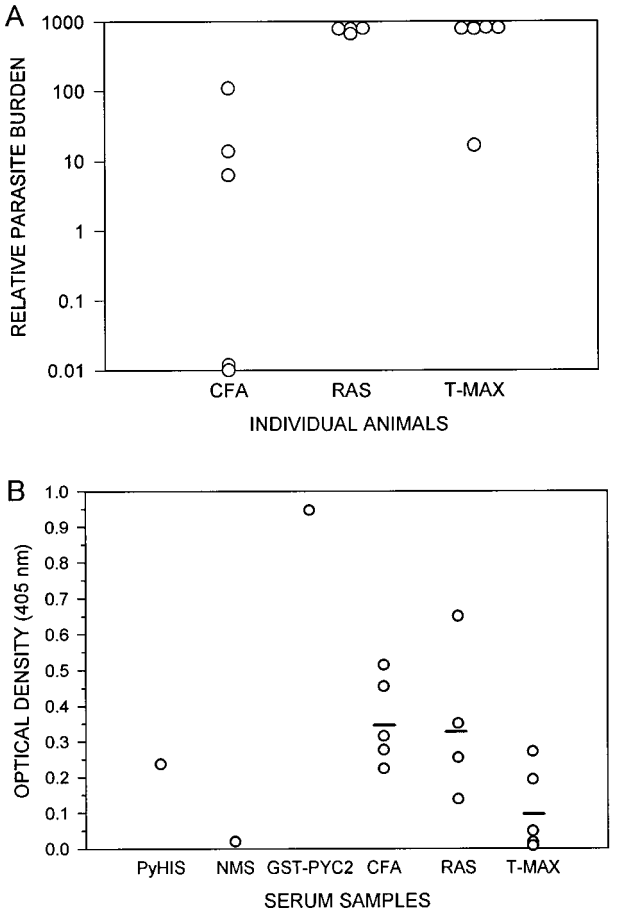

FIG. 4. Influence of adjuvants on immunization with PYC2 in BALB/c mice. (A) Groups of five mice were immunized with PYC2 in FA, RAS, or TiterMax and given a lethal challenge infection of *P. yoelii yoelii* 17XL-infected erythrocytes. Points represent the relative parasite burden of each animal from the various groups. (B) Relative levels of PYC2-specific antibodies in individual serum samples (1:1,000 dilution) from mice immunized with PYC2 in FA, RAS, and TiterMax were determined by ELISA prior to challenge infection. The specific reactivities of these sera were compared with that of anti-GST-PYC2, PyHIS, and normal mouse serum (NMS). Horizontal lines represent the mean optical density for each group.

individual serum samples of mice immunized only with PYC2 in different adjuvants were not significantly different, as assessed by ELISA with PYC2 prior to challenge infection ($P < 0.05$, analysis of variance. Further analysis by serial dilution revealed that levels of PYC2-specific antibodies in sera from the FA and RAS groups were comparable to levels in PyHIS but approximately 10% of that in a serum pool from SW mice, which is typical of animals immunized with GST-PYC2 (data not shown). Levels of plasmodium-specific antibodies were slightly lower in the group of mice receiving PYC2 in TiterMax (Tukey test). In spite of these moderate humoral responses, immunoprecipitation of metabolically labeled parasite protein with these sera revealed that only 1 of 14 mice had developed antibodies capable of recognizing native MSP-1 (data not shown). This single animal, from the FA group, was partially protected, having experienced a peak parasitemia of 2.0%. Overall, although the FA and RAS groups developed similar levels of PYC2-specific antibodies, none of the animals immunized with RAS survived the challenge infection, while all of those receiving FA were able to control their infections.

DISCUSSION

We have now extended our previous studies with the GST-PYC2 fusion protein to demonstrate that this molecule, including only 11 kDa of the *P. yoelii yoelii* 17XL MSP-1 carboxyl terminus, is an effective immunogen against an otherwise lethal challenge infection when administered in alum, FA, RAS, or TiterMax adjuvant. The data presented here clearly demonstrate that a significant protective immune response can be induced in BALB/c mice by this region of the MSP-1 molecule as a fusion protein administered in a low-toxicity adjuvant such as alum, the only one broadly approved for use in humans, and that FA was not required. However, successful immunization of mice was adjuvant dependent, as illustrated by the use of saponin. Animals immunized with GST-PYC2 in the presence of saponin developed significant humoral responses to the malarial portion of the fusion protein, but none of the animals demonstrated any degree of protection. The failure of saponin to promote a protective response with GST-PYC2 contrasts with earlier studies showing it to be very effective in rodents immunized with intact MSP-1 (14).

Another determinant of successful immunization was the genotype of the responding host. An example of this is illustrated by the comparison of protection induced in BALB/c and SW mice by GST-PYC2 administered in alum. While the BALB/c group was effectively protected, all SW mice experienced fulminating infections. This lack of protection among the SW animals was not due to a failure to respond to the immunogen as a whole, since the antibody response of the SW mice to the GST portion of the fusion protein was comparable to that of the BALB/c mice. It was also not due to their inability to mount a protective response, since they were shown to be protected by antigen administered in RAS (10) (Table 1, experiment 5) and in TiterMax (Table 1, experiment 3). This finding that the host genotype is an important determinant of a protective response is consistent with the observations of others, who have established that the induction of antibodies capable of inhibiting the growth of *P. falciparum* in vitro after immunization of mice with *P. falciparum* MSP-1 is significantly influenced by host major histocompatibility haplotype and by adjuvant (7, 18, 21).

Previously, we have established the predominant role of antibodies in the protective response induced by immunization with GST-PYC2 in RAS (11). Since the influence of adjuvant on the isotype of induced antibodies has been well documented (1, 25, 26), we sought to determine whether the differences between adjuvants seen in our studies could be explained by the pattern of isotypes produced. As we have now shown, immunization of BALB/c mice with GST-PYC2 in RAS and saponin resulted in PYC2-specific antibodies with a broad isotype distribution. The subclasses of these antibodies were primarily IgG1, IgG2a, and IgG2b, while those induced by the fusion protein in FA or in TiterMax were predominantly IgG1.

TABLE 2. Protection induced by immunization with PYC2 in various adjuvants

Adjuvant	Antigen	Complete protection[a,b]	Partial protection[b]	% Survival[c]
FA	PYC2	2/5	3/5	100
RAS	PYC2	0/5	0/5	0
TiterMax	PYC2	0/5	1/5	20

[a] No patent parasites detected.
[b] Number of mice provided protection/total number of mice.
[c] Includes mice which were either completely or partially protected.

In the case of BALB/c mice immunized with GST-PYC2 in alum, 80 to 100% of these animals were completely or partially protected from parasite challenge in two experiments and the induced PYC2-specific antibodies were exclusively of the IgG1 isotype. This restriction to the IgG1 isotype presumably reflects the Th2-type response frequently associated with antigen administered in alum (15). It also suggests that this isotype alone is capable of mediating protection. In contrast, others have demonstrated the importance of the IgG2a subclass in the transfer of passive immunity with anti-*P. yoelii yoelii* 17XL antibodies from hyperimmune mouse plasma (38) and in protection induced by whole *P. yoelii* parasite antigen (22, 36). The isotype distribution of PYC2-specific antibodies from SW mice immunized with GST-PYC2 in RAS or TiterMax was comparable to that of BALB/c mice (data not shown). This was also true of SW animals immunized with the fusion protein in alum, although the titers were dramatically lower. Therefore, no correlation could be made between IgG subclass of specific antiplasmodial antibodies and protective efficacy of various adjuvants in vivo.

It is of interest to note that this fusion protein elicited significant levels of PYC2-specific antibodies with all five of the adjuvants used in BALB/c mice, as assessed by ELISA. It was somewhat surprising, therefore, that mice immunized with GST-PYC2 in saponin failed to demonstrate any degree of protection, particularly since others have shown that immunization of BALB/c mice with intact MSP-1 in saponin induced a significant level of protection (14). In those studies, transfer of serum from immunized animals, shown to contain high-titer antibodies specific for MSP-1, failed to provide passive immunity to naive recipients. This result was interpreted as being indicative of a cellular rather than a humoral protective response (14). It is possible that the intraperitoneal route of inoculation used in our studies with GST-PYC2 and the possible effect of saponin on the fusion protein or the antigen-presenting cells may have directed the humoral response to less efficacious epitopes. This is consistent with previous results of others who demonstrated that the choice of adjuvant had a profound effect on the fine specificity of the antibodies elicited to *P. falciparum* MSP-1 (7, 18). In addition, others have shown that monoclonal antibodies, specific for the carboxyl terminus of the *P. falciparum* MSP-1 and able to inhibit competitively the binding of each other, have dramatically differing abilities to inhibit parasite growth in vitro (3). From these findings and from the data that we present here, it appears that the fine specificity of the induced antibodies, rather than immunoglobulin subclass, is more critical for the protective immune response produced by the GST-PYC2 fusion protein.

During our original experiments with a carboxyl-terminal segment of the *P. yoelii yoelii* 17XL MSP-1, isolated free of GST and designated PYC1, we found that immunization of BALB/c mice with this fragment in RAS failed to induce a humoral response (10). This was illustrated by the inability of antibodies from immunized animals to immunoprecipitate metabolically labeled parasite antigens. These findings were suggested to reflect limited T-cell epitopes in the MSP-1 C terminus. Moreover, these animals demonstrated no degree of protection upon parasite challenge. In contrast, others have subsequently reported that it was possible to induce a protective response in BALB/c mice with a similar MSP-1 antigen from *P. yoelii yoelii* (29). However, this result was achieved by administration of the antigen in FA. We have therefore compared immunization with PYC2 lacking the GST component of the fusion protein in other adjuvants. In confirmation of earlier reports, animals immunized with antigen in FA produced a significant protective response, while those immunized with PYC2 in RAS or TiterMax were afforded no protection, with one exception in the TiterMax group. These results again demonstrate the influence of adjuvant in induction of protective responses.

Although moderate levels of PYC2-specific antibodies were elicited as assessed by ELISA, it is of interest to note that immunoprecipitation of metabolically labeled parasite proteins with individual serum samples demonstrated that only 1 of 14 mice immunized with PYC2 developed detectable levels of antibodies able to recognize MSP-1. This animal had been immunized with FA but was not completely protected, having experienced a peak parasitemia of 2.0%. In addition, while the FA and RAS groups produced comparable levels of PYC2-specific antibodies, the RAS group was afforded no protection. One possible explanation for the efficacy of FA in inducing protective responses to PYC2 is that the antibody specificities elicited with FA are highly efficacious in protection. Another possibility is that ancillary cellular effector mechanisms may be induced by FA but not by the other adjuvants tested.

It has been reported by others that responses to GST fusion proteins can be profoundly influenced by the carrier portion of the protein and the adjuvant employed (37). These variables appear to determine whether the response is limited to either one or both regions of the fusion protein. It has been suggested that the carrier portion of the protein may be involved in major histocompatibility complex restriction or may determine the type of immune response elicited. These factors may play a role in the variable results obtained with GST-PYC2 and PYC2 administered in the various adjuvants used in this study.

As we and others have demonstrated with rodent models, the C terminus of the MSP-1 molecule holds potential as a component of a malaria vaccine (10, 11, 29). This has recently been extended to primate models of malaria since others have reported that immunization of several *Aotus* monkeys with $yMSP1_{19}$ in FA produced a significant level of protection against parasite challenge with *P. falciparum* (27). The highly conserved nature of the MSP-1 carboxyl-terminal region among strains of *P. falciparum* (32) as well as other species of plasmodia (9) and the fact that it remains associated with the parasite membrane following erythrocyte invasion (2) suggest that this segment has important functional significance. Therefore, it would seem likely that a concerted immune response to this region could provide a significant level of protection against the erythrocytic phase of infection and possibly provide necessary cross-protection between variant strains which exist in the field (23, 24). However, it is apparent that conditions must be identified to maximize elicitation of protective responses, particularly in view of the evidence presented here that host genotype, adjuvant, and the fine specificity of the immune response are important determinants of protection in vivo.

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