INFECTION AND IMMUNITY, Oct. 1991, p. 3547–3554
0019-9567/91/103547-08$02.00/0

Vol. 59, No. 10

Antimalarial Antibodies of the Immunoglobulin G2a Isotype Modulate Parasitemias in Mice Infected with *Plasmodium yoelii*

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Received 9 April 1991/Accepted 31 July 1991

Previous studies have demonstrated the importance of antibodies in mediating immunity to malaria, but the relative contribution of the different immunoglobulin isotypes has not been assessed. In this study, hyperimmune plasma was generated against *Plasmodium yoelii* and separated by protein A-Sepharose chromatography into fractions containing immunoglobulin G1 (IgG1), IgG2a, IgG2b, or IgG3 antibodies and the remaining nonbinding plasma proteins, including IgM. Following concentration, the antimalarial titer of each isotypic fraction was approximately equivalent to the corresponding isotype in hyperimmune plasma. The isotypic fractions were passively transferred to BALB/c and outbred ICR mice prior to challenge with virulent *P. yoelii* 17XL and to CBA/CaJ mice challenged with avirulent *P. yoelii* 17XNL. Only mice receiving IgG2a antibodies experienced an altered course of infection. Immunoprecipitation studies showed that all four IgG isotypes appear to recognize a similar set of antigens. These results suggest that antimalarial antibodies of the IgG2a isotype play a dominant role in modulating *P. yoelii* parasitemias.

Nonlethal *Plasmodium yoelii* 17X (17XNL) produces a self-resolving infection in mice, with parasitemias becoming subpatent in approximately 3 weeks (22). Immunity to this parasite is largely humoral in nature. Jayawardena et al. (9) demonstrated that the passive transfer of hyperimmune serum to CBA mice completely protected them against challenge with 10^4 *P. yoelii* 17XNL-parasitized erythrocytes (PRBC). In addition, passively transferred antibodies (Ab) delayed the onset of infection when larger inocula (5×10^4 or 1×10^5 PRBC) were used (9). Similar results have been reported for BALB/c mice (5).

A heterogenous Ab response is induced by *P. yoelii*. Langhorne et al. investigated the antimalarial Ab response to *P. yoelii* 17XNL in C3H mice and found that immunoglobulin M (IgM), IgG1, IgG2, and IgG3 Ab were produced, with the highest titers being of the IgG2 isotype (13). Recently, Taylor et al. investigated the anti-*P. yoelii* 17XNL response in 11 inbred strains of mice and determined that the strains varied in their isotypic response to the parasite (21). The largest difference was seen with Ab of the IgG1 isotype, in that only 3 of 11 strains produced detectable antimalarial Ab of this isotype during primary infection. All strains produced IgM, IgG2, and IgG3 Ab, although the kinetics of these responses varied among the strains.

Different strains of inbred mice also produce antimalarial Ab of various antigenic specificities (19). When sera collected on days 11 and 21 of primary infection were used to immunoprecipitate metabolically labeled *P. yoelii* proteins, different patterns of reactivity were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

The ability of mice to produce anti-*P. yoelii* 17XNL Ab of different isotypes and specificities raises two key questions: (i) is the protective capacity of hyperimmune plasma (HIP) found predominantly in a particular isotype, and (ii) do Ab of different isotypes recognize different antigens (Ag)? In this paper, we sought to answer these questions by transferring Ab of a particular isotype (IgM, IgG1, IgG2a, IgG2b, and IgG3) to mice infected with either *P. yoelii* 17XNL or its lethal variant, 17XL. In addition, the antigenic specificities of anti-*P. yoelii* Ab of the different IgG subclasses were investigated.

MATERIALS AND METHODS

Mice. Female BALB/cByJ and BALB/cCVF mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and Cumberland View Farms (Clinton, Tenn.), respectively. Female ICR mice were purchased from either Dominion Labs (Dublin, Va.) or from Harlan Sprague Dawley (Frederick, Md.). Female CBA/CaJ mice were purchased from the Jackson Laboratory. The mice were 2 to 4 months of age when they were used in passive transfer studies.

Parasites. The nonlethal (17XNL) and lethal (17XL) strains of *P. yoelii* were used. These strains, originally obtained from the Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, were maintained by repeated blood passage or by cryopreservation in liquid nitrogen. They were passaged once before use in an experiment. The percentage of parasitemia was determined by counting the number of PRBC per 500 erythrocytes (RBC) in thin smears of tail blood stained with Diff-quick (American Scientific Products, McGaw Park, Ill.).

Generation of HIP. Several different protocols were used to generate HIP. Groups of ICR, CBA/CaJ, and BALB/c mice (20 to 40 mice per group) were injected intraperitoneally with 10^5 *P. yoelii* 17XNL PRBC. Mice recovered spontaneously from infection within 21 days. One to three months later, ICR and CBA/CaJ mice were injected with 10^6 *P. yoelii* 17XNL PRBC and BALB/c mice were injected with 10^6 *P. yoelii* 17XL PRBC. Mice developed less than 1% parasitemias for a short duration after secondary infection. Two weeks later, the ICR mice received a third injection of 10^6 17XNL PRBC. In initial BALB/c studies, mice were

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boosted up to four additional times with *P. yoelii* 17XL. Plasma was collected and pooled for each group of mice 7 to 10 days after the final injection and stored at −20°C.

Protein A chromatography. The protocols for protein A chromatography described by Ey et al. (2) and Seppälä et al. (15) were essentially followed. Aliquots of 1 to 3 ml of HIP were adjusted to pH 8.0 with an equal volume of Tris-HCl buffer, pH 8.0 (56 mM Tris-HCl, 0.15 M NaCl), and applied to a column containing protein A-Sepharose (CL-4B; Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) with a bed volume of 13 ml. Protein A-agarose (Life Technologies, Inc., Gaithersburg, Md.) was found to work equally well. Nonbinding material including IgM was washed through the column and is referred to as the nonbinding-IgM fraction. Bound IgG Ab were eluted from the column by using a linear pH gradient from pH 7.0 to 3.0 formed by mixing equal volumes (50 ml) of Tris-HCl buffer, pH 7 (0.1 M Tris-HCl, 0.15 M NaCl), and acetate buffer, pH 3 (3 mM sodium acetate, 0.15 M NaCl). Fractions (2 ml) were collected, appropriately pooled, and dialyzed against 0.1 M phosphate-buffered saline, pH 7.4 (PBS). Pooled isotypic fractions were concentrated by using an Amicon Minicell-Ultrafiltration system equipped with a YM-10 filter, such that fractions contained an antimalarial Ab titer approximately equivalent to that of the corresponding isotype in unseparated HIP (see below). The isotypic fractions were stored with 0.01% sodium azide at 4°C until used.

Heavy chain isotype-specific assays. In initial studies, HIP and the concentrated isotypic fractions were assayed for the presence of antimalarial Ab by the direct isotype-specific, solid-phase radioimmunoassay previously described by Kim et al. (11). In this assay, antisera specific for heavy chain IgM, IgG1, IgG2 (both IgG2a and IgG2b), and IgG3 were employed. The specificities and specific activities of these reagents have been described previously (20). Ab titers were determined by using an endpoint of 25% maximum specific binding.

In subsequent studies, a "triple-sandwich" isotype-specific enzyme immunoassay (EIA) was used. Ag for the EIA was prepared by collecting blood from ICR mice infected with *P. yoelii* 17XL. The blood cells were suspended in PBS and passed over a CF-11 cellulose column to remove the leukocytes (8). Then the RBC were layered on a one-step 55% Percoll (Sigma, St. Louis, Mo.) gradient (nine parts of Percoll and one part of 10× RPMI 1640 [GIBCO, Grand Island, N.Y.] diluted to 55% with PBS) and centrifuged for 20 min at 650 × *g*, 25°C. The resulting band of late-stage parasites was removed, washed in PBS, and stored in 200-μl aliquots at −70°C.

For the isotype-specific EIA, an aliquot of PRBC was freeze-thawed three times and the protein concentration was determined spectrophotometrically (11). Then, 100 μl of the Ag solution containing 30 μg of parasite protein was added to polystyrene microtiter wells (Nunc-Immuno II; Nunc, Kamstrup, Denmark) and incubated overnight at 4°C. Plates were washed three times with PBS–0.1% Tween 20 and blocked with 10% milk in PBS for 2 h at room temperature. Serial 10-fold dilutions (100 μl) of mouse plasma in 1% milk–PBS were added in duplicate, and the plates were incubated overnight (4°C). Plates were washed as before, and 100 μl of a 1:1,000 dilution of goat anti-mouse IgM (kindly provided by R. Asofsky, National Institutes of Health); IgG1, IgG2a, or IgG3 (Southern Biotechnology Associates, Inc., Birmingham, Ala.); or IgG2b (Sigma) per well was added. The specificity of each of these reagents was confirmed prior to use. After an overnight incubation (4°C), the plates were washed and horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (Southern Biotechnology) diluted 1:8,000 in 1% milk in PBS was added (100 μl per well). The plates were incubated (3 h at room temperature) and washed, and the color was developed by adding 100 μl of azino-di[3-ethyl-benzthiazoline sulfonate] (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) per well. Ab titers were determined by using an endpoint of 25% maximum specific binding.

Passive transfer studies. Details for individual passive transfer experiments are provided below or in figure legends. In general, groups of three to five mice were injected intraperitoneally with 0.45 to 0.75 ml of one of the isotypic fractions, saline, normal mouse plasma (NMP), or HIP and challenged 2 to 5 h later with 10^4 to 10^6 PRBC. In some experiments, the mice received a second injection of Ab.

Metabolic labeling of *P. yoelii* 17XL parasites. The method for metabolic labeling of *P. yoelii* parasites has been described previously (13). The culture medium consisted of 90% SELECTAMINE (GIBCO) and 10% complete medium. The directions for preparing SELECTAMINE were followed when labeling with [^{3}H]Leu was performed. When labeling with a ^{14}C-amino acid mixture, 10% of the recommended concentration of the contained amino acids was used. Complete medium (100 ml) consisted of 0.34 ml of L-glutamine (29 mg/ml of stock), 0.2 g of glucose, 0.6 g of *N*-2-hydroxyethylpiperazinc-N'-2-ethanesulfonic acid (HEPES), 3 ml of 7.5% sodium bicarbonate, 1 ml of hypoxanthine (0.5 mg/ml), 1.5% fetal bovine serum, and 94.2 ml of RPMI 1640 (GIBCO). For labeling, 250 μCi of [^{3}H]Leu (Amersham, Arlington Heights, Ill.) or 50 μCi of ^{14}C-protein hydrolysate (Amersham) was added to 5 ml of culture medium. The ^{14}C-protein hydrolysate contained a mixture of all 20 essential amino acids except asparagine, cysteine, glutamine, and tryptophan. *P. yoelii* 17XL parasites obtained from BALB/c mice with parasitemias of 10 to 20% were cultured at approximately 10^8 parasites per ml for 14 h at 37°C in the presence of 90% nitrogen, 7% CO_2, and 3% O_2. The cells were washed with PBS and solubilized in NETN (0.15 M NaCl, 50 mM Tris, 5 mM EDTA, 0.5% Nonidet P-40, pH 8) containing 2 mM phenylmethylsulfonyl fluoride and 0.54 mM iodoacetamide.

Immunoprecipitation and SDS-PAGE. The metabolically labeled extracts of *P. yoelii* were preabsorbed with protein A-Sepharose CL-4B for 30 min at room temperature. Then aliquots of [^{3}H]Leu-labeled extracts were incubated with 6 μl of NMP or HIP, 10 μl of the nonbinding-IgM fraction, or 25 μg of the IgG isotypic fractions. Aliquots of ^{14}C-amino acid-labeled extracts were incubated with 5 to 7 μl of NMP or HIP and 13 to 15 μg of the IgG isotypic fractions. Next, 90 μl of a 50% slurry of protein A-Sepharose in NETN was added. Samples containing the nonbinding-IgM fraction received an equivalent amount of Sepharose coupled with anti-IgM Ab. The tubes were incubated at room temperature for 2.5 to 3 h. Following centrifugation at 800 × *g* for 1 min, pelleted Ag-Ab-Sepharose complexes were washed twice with NETN, twice with NETN plus 10% fetal bovine serum, twice with NETN plus 0.50 M NaCl, and two more times with NETN. Following complete removal of the supernatant, 50 μl of reducing SDS-sample buffer was added and the samples were electrophoresed on SDS–10% polyacrylamide gels according to the method of Laemmli (12). Gels were prepared for fluorography with Resolution Autoradiography Enhancer (EM Corp., Chestnut Hill, Mass.) and incubated with Kodak X-OMAT AR film at −70°C for 1 to 2 weeks.

Statistics. All statistical analyses were performed by using

TABLE 1. Estimated antimalarial Ab titers in BALB/c HIP and the corresponding isotypic fractions

HIP or fraction[a]	Radioimmunoassay Ab titer (log_{10}) in[b]:			
	IgM	IgG1	IgG2	IgG3
HIP	**2.6**	**4.0**	**4.6**	**3.7**
Isotypic fractions				
Nonbinding IgM	**2.6**	<1.0	<1.0	<1.0
IgG1	<1.0	**3.4**	<1.0	<1.0
IgG2a	<1.0	1.1	**4.5**	1.7
IgG2b	<1.0	<1.0	**2.6**	<1.0
IgG3	<1.0	<1.0	3.6[c]	**4.4**

[a] HIP used in this study was from BALB/c mice that had been injected with 17XNL once and 17XL five times. The fractions were transferred to mice as indicated in the legend to Fig. 1A.

[b] Boldface type indicates the Ab titer in HIP and the corresponding isotypic fractions. The lowest dilution tested was 1:10.

[c] On the basis of elution profiles, the majority of the contaminating IgG2 in the IgG3 fraction appears to be of the IgG2a isotype.

an unpaired, one-sided Student's t test. Data for survival times are expressed as mean ± standard deviation.

RESULTS

Initial studies using HIP from BALB/c mice. BALB/c HIP with a titer of >1:10,000,000 by radioimmunoassay was separated by protein A chromatography and used in passive transfer studies (Table 1 and Fig. 1). Radioimmunoassay analysis of the isotypic fractions showed that only antimalarial IgM was detected in the nonbinding-IgM fraction and that the IgG1, IgG2a, and IgG2b fractions were essentially pure, i.e., free of other IgG isotypes. However, there was a significant amount of contaminating IgG2a in the IgG3 fraction. An attempt was made to concentrate each IgG isotypic fraction such that it had a titer equivalent to that of its corresponding isotype in HIP. Results showed that desired titers were obtained for the IgM and IgG2 fractions, the IgG1 fraction titer was slightly low, and the IgG3 fraction titer was slightly high (Table 1).

To test the protective potential of the fractions, mice received 0.75 ml of either one of the above isotypic fractions or HIP intraperitoneally 2 h prior to challenge with 10^6 *P. yoelii* 17XL PRBC (Fig. 1A). Results show that all mice receiving the IgG1, IgG2b, or IgG3 fraction developed courses of infection similar to those of saline controls and had a similar mean day of death (8.1 ± 2.1 days, n = 5, versus saline control, 6.4 ± 0.5 days, n = 16). Mice receiving the nonbinding-IgM fraction had an altered course of infection with parasitemia and survival time of 12.2 ± 3.0 days. The mice receiving the IgG2a fraction survived still longer (17.4 ± 3.3 days, n = 5, $P < 0.005$) than the saline controls, and their mean survival time was comparable to that of the HIP controls (mean survival time, 20.8 ± 2.0 days). Thus, the IgG2a fraction was the most effective serum component in modulating parasitemia.

In the experiment described above, mice received a single injection of Ab. To determine whether the additional transfer of IgG2a Ab would more closely mimic HIP, mice received 0.5 ml of purified IgG2a or HIP at the time of infection and on day 2. Mice were challenged with 10^6 *P. yoelii* 17XL PRBC. Results showed that two injections of IgG2a resulted in protection equivalent to that produced by HIP (Fig. 1B).

HIP from additional sources. HIP was produced in ICR and CBA/CaJ mice as described in Materials and Methods and then fractionated, concentrated, and analyzed by the isotype-specific EIA. Reagents specific for IgG2a and IgG2b were included to more accurately evaluate Ab titers and purity. In the four separate fractionation experiments (two ICR and two CBA) no obvious differences in either purity or titer of ICR and CBA HIP were observed, so the data have been averaged (Table 2). In Table 2, the isotypic fractions have been arranged in the sequence they elute from the protein A column. As can be seen, the mean Ab titer of each IgG isotypic fraction was essentially identical to that of the corresponding isotype present in HIP. The nonbinding-IgM fraction and IgG1 and IgG2b fractions contained only minimal contamination from adjacent peaks. However, there was considerable cross contamination between the IgG2a and IgG3 fractions. The isotypic fractions were used in the studies discussed below (see Fig. 2 to 4).

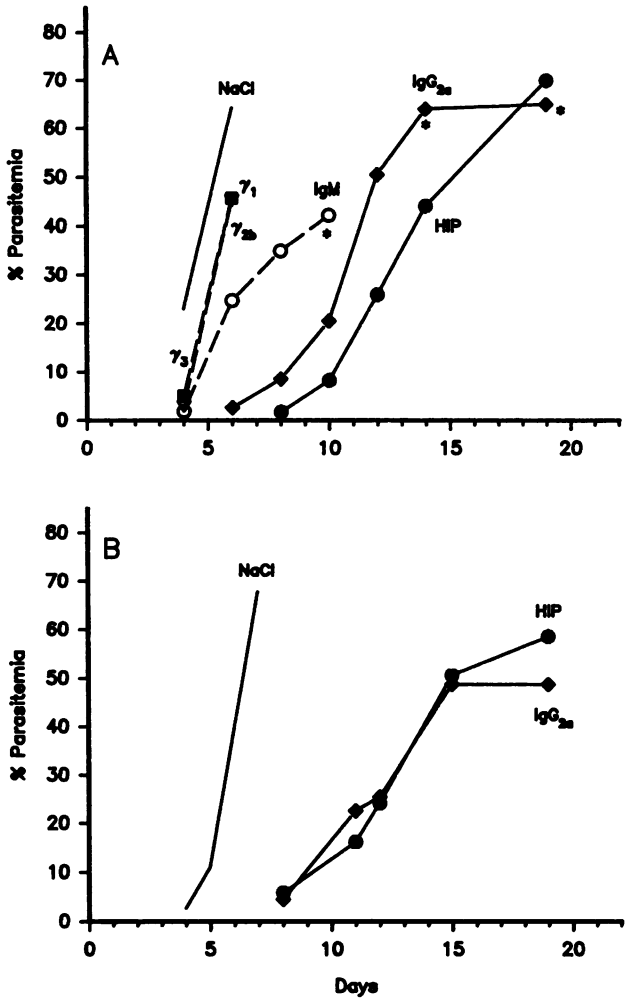

FIG. 1. Parasitemias in BALB/c mice receiving BALB/c HIP or isotypic fractions. (A) Mice received 0.75 ml of HIP (n = 5), saline (NaCl; n = 16), one of the isotypic fractions (n = 5), or the nonbinding-IgM fraction (n = 5) (Table 1) 2 h prior to challenge with 10^6 *P. yoelii* 17XL PRBC. Data are mean percent parasitemias. Asterisks denote parasitemias for surviving mice; i.e., some mice within the group had died. γ_1, IgG1; γ_{2b}, IgG2b; γ_3, IgG3. (B) Mice received 0.5 ml of HIP (n = 5) or the IgG2a fraction (n = 3) 2 h prior to challenge with 10^6 PRBC and a second injection of 0.5 ml 2 days later.

Passive transfer of isotypic fractions from ICR mice. Isotypic fractions from ICR HIP were adjusted such that they

TABLE 2. Purity of the isotypic fractions derived from ICR and CBA HIP

HIP or fraction	Isotype-specific EIA titer (mean $\log_{10}$ ± 1 SD)[a] in:				
	IgM	IgG1	IgG2a	IgG3	IgG2b
HIP	**4.0 ± 0.2**	**5.2 ± 0.3**	**5.6 ± 0.6**	**4.6 ± 0.2**	**4.8 ± 0.5**
Fractions					
Nonbinding IgM	**3.3 ± 0.4**	≤2.0[b]	≤2.0	≤2.0	≤2.0
IgG1	2.6 ± 0.4	**5.3 ± 0.8**	2.2 ± 0.3	<2.0	<2.0
IgG2a	ND	3.6 ± 0.3	**5.5 ± 0.5**	3.7 ± 0.5	<2.0
IgG3	ND	<2.0	4.2 ± 0.4	**4.3 ± 0.5**	≤2.5 ± 0.6
IgG2b	ND	<2.0	<2.0	2.7 ± 0.5	**4.7 ± 0.5**

[a] Boldface type indicates Ab titer in HIP and corresponding isotypic fractions. $n = 4$. ND, not determined.
[b] 1:100 was the lowest dilution tested.

had antimalarial Ab titers approximately equivalent to those present in ICR HIP (Table 3). In this experiment, titers in the IgG1 and IgG2b fractions were higher than in HIP, the IgG3 titers were equivalent, but the titer of the IgG2a fraction was low (Table 3). For comparison, BALB/c HIP and the IgG2a fraction from BALB/c HIP were also used. The titer of the BALB/c IgG2a fraction was adjusted to that of the IgG2a level in ICR HIP (i.e., titer of 1:500,000 [$\log_{10}$ 5.7]; Table 3). BALB/c mice were injected with 0.45 ml of one of the isotypic fractions, NMP, ICR HIP, or BALB/c HIP and challenged 5 h later with 10^4 *P. yoelii* 17XL PRBC. Each mouse received a second injection of either plasma or the isotypic fraction 2 days later. The results, summarized in Fig. 2, show that mice receiving NMP, IgG1, IgG2b, IgG3, and the nonbinding-IgM fraction experienced a course of infection similar to that of untreated control mice. The mean survival time of the mice in these groups was 10.2 ± 1.5 days. Mice which received IgG2a Ab from either the ICR or BALB/c HIP had a delay in the onset of the infection and lived significantly longer (18.7 ± 3.6 days, $P \leq 0.005$) than mice in the above groups. The mice which received the ICR HIP had a mean survival time of 19 ± 5.0 days. In contrast, mice which received BALB/c HIP had a mean survival time of 28 ± 4.4 days. It should be noted that the IgG2a titer in BALB/c HIP was 1:2,500,000, compared with 1:500,000 in ICR HIP. Thus, two injections of purified IgG1, IgG2b, and IgG3 Ab that had titers equal to or greater than those present in ICR HIP did not significantly modulate *P. yoelii* 17XL infection. On the other hand, IgG2a from ICR and BALB/c HIP significantly prolonged survival.

TABLE 3. Summary of Ab titers present in the isotypic fractions used in studies shown in Fig. 2 and 3

Source and fraction[a]	Antimalarial Ab titer by isotype-specific EIA ($\log_{10}$) of:	
	HIP	Isotypic fraction
ICR plasma		
IgM	3.9	3.0
IgG1	5.2	6.2
IgG2a	5.7	5.3
IgG2b	4.3	4.8
IgG3	4.6	4.6
BALB/c IgG2a	6.4	5.7
CBA plasma		
IgM	4.2	3.9
IgG1	5.6	5.3
IgG2a	6.3	6.2
IgG2b	5.6	5.4
IgG3	4.9	4.9

[a] Antibodies used in the passive transfer studies shown in Fig. 2 (ICR plasma and BALB/c IgG2a) and 3 (CBA plasma).

Effect of isotype on nonlethal (17XNL) *P. yoelii* infection. To determine whether IgG2a Ab were also effective in controlling nonlethal malaria, the *P. yoelii* 17XNL-CBA/CaJ model was used, as a uniformly nonlethal infection is produced in this mouse strain. CBA HIP was fractionated, the purity of the fractions was assessed (Table 2), and specific Ab titers were determined (Table 3). As can be seen in Table 3, the Ab titers in each fraction closely approximated those found in the CBA HIP from which they were obtained. CBA mice were injected with either one of the isotypic fractions or CBA HIP and then challenged 5 h later with *P. yoelii* 17XNL PRBC. The mice received a second injection of the same fraction or HIP 2 days later. Results are shown in Fig. 3. Mice receiving IgG1, IgG2b, IgG3, or the nonbinding-IgM fraction experienced a course of infection very similar to that of the untreated control mice. All mice in these groups developed patent infections by day 4 (parasitemias ranging from 0.2 to 4.4%). They developed mean peak parasitemias of 10 to 12% and cleared the infection by day 16 to 17. In contrast, all three mice receiving the IgG2a fraction did not develop patent infections until day 10 to 12, suggesting control of the infection by the passively transferred IgG2a Ab. Thereafter, parasitemias continued to climb and the mice developed a mean peak parasitemia that was significantly higher than that of mice receiving the other isotypic fractions ($P \leq 0.005$). Mice receiving IgG2a cleared the infection by day 27. Thus, the length of patent parasitemia was similar in all mice (range, 11 to 14 days). CBA mice receiving CBA HIP were completely protected; i.e., they did not develop patent infections. Thus, again, the only purified isotypic fraction that modulated parasitemia was the IgG2a fraction.

Immunoprecipitation studies. To determine whether Ab of the IgG2a isotype recognized unique *P. yoelii* antigens, aliquots of isotypic fractions from BALB/c and ICR HIP were incubated with extracts of *P. yoelii* 17XL parasites metabolically labeled in vitro with [^{3}H]Leu or a mixture of ^{14}C-amino acids. The isotypic fractions used are listed in Tables 1 and 2. Results show that Ab of each of the different IgG subclasses precipitated a similar series of bands (Fig. 4). There were no unique bands present in the IgG2a fractions obtained from BALB/c or ICR mice. In additional experiments, several different concentrations of ICR IgG2a Ab were used (including twice the concentration shown here), but unique reactivities were never observed (data not

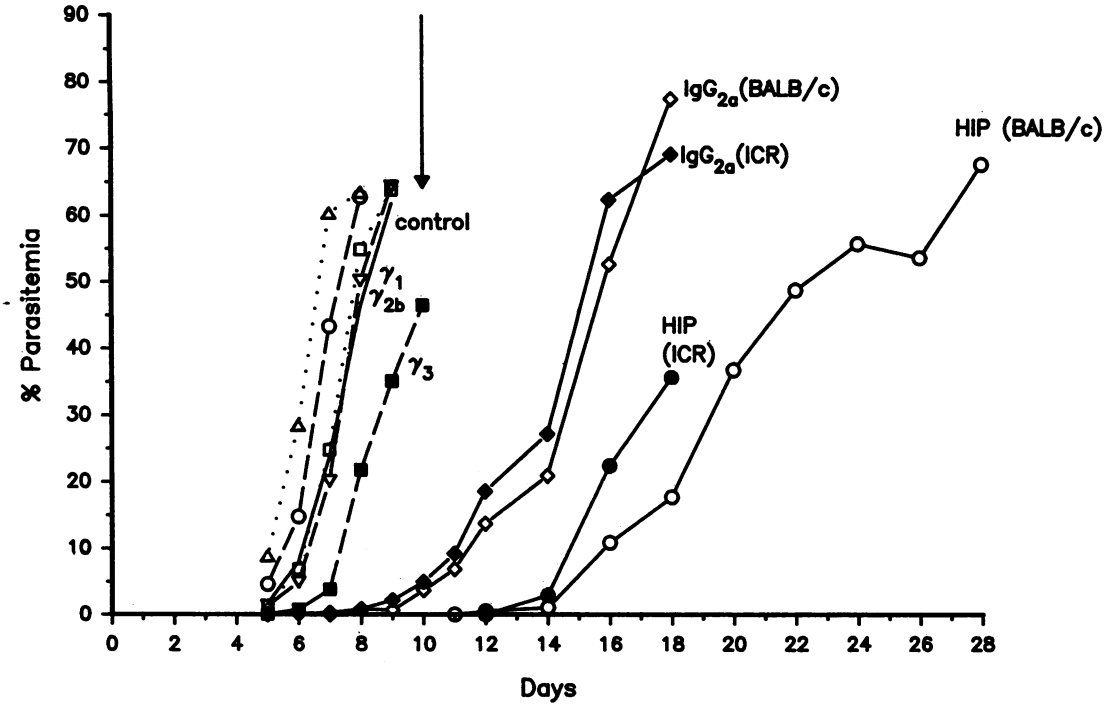

FIG. 2. Passive transfer of Ab obtained from ICR HIP: mean parasitemias of BALB/c mice injected on days 0 and 2 with 0.45 ml of one of the isotypic fractions from ICR HIP, the IgG2a fraction from BALB/c HIP, NMP, ICR HIP, or BALB/c HIP. There were five mice per group except for the BALB/c HIP group (three mice). Each data point represents the mean for at least three mice (i.e., data were not included when more than two of five mice within a group had died). The arrow indicates the mean survival time of mice in the IgG1, IgG2b, IgG3, IgM, NMP, and control (untreated) groups. O----O, IgM; □, IgG1; ▽, IgG2b; ■, γ_3, IgG3; —, control; △, NMP.

shown). This suggests that the partially protective effect of IgG2a Ab observed in passive transfer studies was not due to the recognition of one or more isotype-restricted *P. yoelii* antigens. When the IgG1 fraction of BALB/c HIP was used, an apparent unique band with an M_r of approximately 45,000 was seen. This suggests that the isotypic fractions were probably pure enough to detect unique reactivities had they been present.

DISCUSSION

Previous studies have shown that the passive transfer of hyperimmune serum to mice significantly modulates the course of *P. yoelii* infections (5, 9), but little is known about the nature of the protective component within immune sera. Results of the current study show that HIP contains anti-*P. yoelii* Ab of all the isotypes examined. This finding confirms earlier reports (13, 21) and extends them by demonstrating that both IgG2a and IgG2b are present.

The HIP used in these studies was from mice that had been injected with viable *P. yoelii* PRBC two to six times. Previous studies have shown that anti-*P. yoelii* Ab titers do not increase beyond the second or third exposure (18). Thus, maximal amounts of each of the isotypes were present in the HIP used. On the basis of the data from Table 2, the highest titer of antimalarial Ab was found in the IgG2a subclass ($\log_{10}$ 5.6), with slightly lower titers in IgG1 ($\log_{10}$ 5.2), IgG2b ($\log_{10}$ 4.8), and IgG3 ($\log_{10}$ 4.6), respectively. Thus, the titers of the four IgG isotypes differed by only a mean of 1 $\log_{10}$. This is interesting on the basis of the observations that lymphokines released by T_H1 and T_H2 cells regulate IgG isotype expression. Gamma interferon produced by T_H1 cells enhances IgG2a production and suppresses IgG1, whereas interleukin-4 produced by T_H2 cells enhances IgG1 synthesis and suppresses other IgG isotypes (17). Thus, there is no direct evidence that one of the T helper subsets predominates over the other in mice that have been infected several times.

Since HIP modulates parasitemias, two questions arose: (i) are protective Ab equally distributed among all the isotypes studied, and (ii) would a single isotype be capable of modulating infection, or would several isotypes or classes be required? Results of this study demonstrate that at physiological levels, Ab of the IgG2a isotype are predominantly responsible for modulating parasitemias in passive transfer studies. The nonbinding-IgM fraction had a minor effect in some but not all experiments. This fraction should contain IgM antimalarial Ab and possibly cytokines. Although IgG1 titers are high in HIP, it is uncertain whether IgG1 Ab play a key role in protection. For example, when isotypic fractions of ICR HIP were transferred to BALB/c mice (Table 3 and Fig. 2), the antimalarial titer in the IgG1 fraction was greater than in the IgG2a fraction (IgG1 fraction, $\log_{10}$ 6.2; IgG2a fraction, $\log_{10}$ 5.3), yet IgG1 Ab did not alter the course of infection (Fig. 2). The titer in the IgG1 fraction in this particular experiment was greater than that normally found in HIP (IgG1 fraction, $\log_{10}$ 6.2; HIP, $\log_{10}$ 5.2); thus, even artificially elevated levels of IgG1 were not effective. IgG2b and IgG3 Ab, which are not effective when used at physiological levels, might alter parasitemias if they were present in higher concentrations; however, they do not reach levels beyond those present in the HIP used in this study. The combined data highly suggest that antimalarial Ab of the IgG2a isotype are predominantly responsible for the protection observed in passive Ab transfer studies.

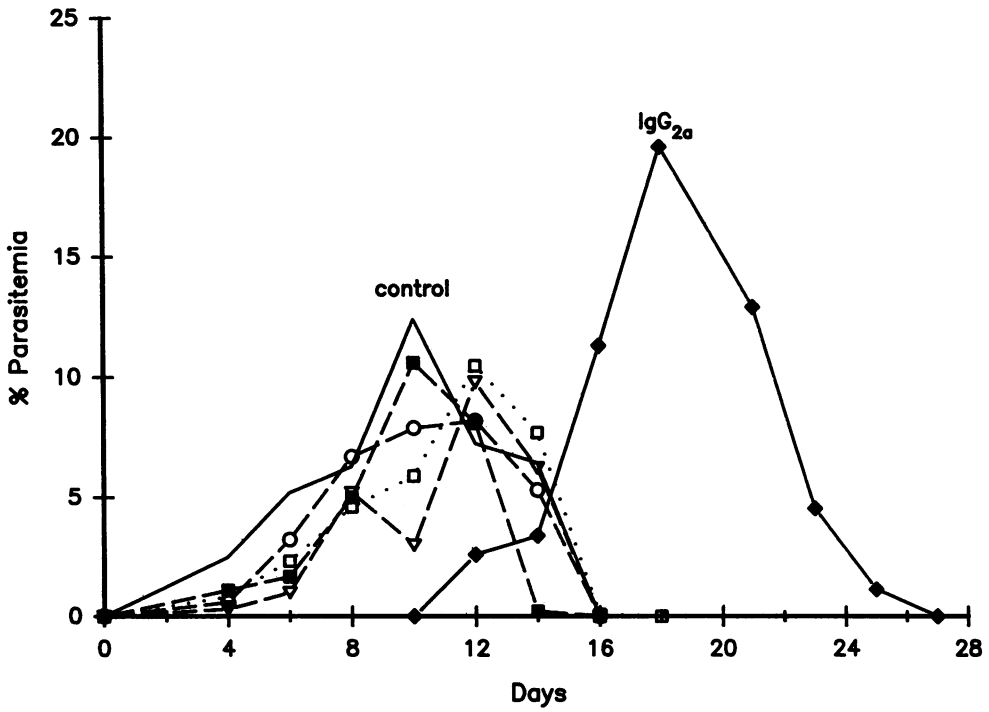

FIG. 3. Effect of isotypic fractions on *P. yoelii* 17XNL infection. The course of parasitemia in CBA mice (three per group) treated with syngeneic isotypic fractions (Table 3) and challenged 5 h later with 10^5 *P. yoelii* 17XNL PRBC is shown. The mice received 0.45 ml of the isotypic fractions or HIP on days 0 and 2; control mice received no treatment. Mice which received HIP did not develop a patent parasitemia. ○, IgM; □, IgG1; ▽, IgG2b; ■, IgG3; —, control.

The IgG3 fraction contained sizable amounts of IgG2a Ab but was not effective in passive transfer studies. In experiments not shown, amounts of IgG2a Ab similar to those found in the IgG3 fraction were passively transferred to mice with *P. yoelii* 17XL and 17XNL and were found to provide a low level of protection. It is possible that the protective effect of the IgG2a Ab in the IgG3 fraction was inhibited by the presence of antimalarial IgG3 Ab. In an in vitro study using Ab isolated from the sera of humans immune to malaria, Groux and Gysin showed that only IgG1 and IgG3 Ab were able to mediate opsonization and that this effect was inhibited by Ab of the other IgG isotypes (7). To our knowledge, similar experiments have not been conducted in the murine system.

Ab of the IgG2a isotype have been shown to be important in other parasitic infections. For example, Wechsler and Kongshaven showed that Ab of the IgG2a isotype transferred protection to animals infected with *Trypanosoma musculi* (25, 26, 27). In addition, these authors found a positive correlation between the ability of sera from different strains of mice to kill *T. musculi* in vitro (27) and IgG2a trypanosome-specific Ab titers. They suggest that IgG2a Ab mediates its effect against this parasite by complement-mediated lysis (25, 27).

It is not clear why Ab of the IgG2a isotype are best at conferring passive protection to *P. yoelii*-infected mice. Immunoprecipitation studies suggest that they do not react with unique *P. yoelii* Ag (Fig. 4); however, additional biochemical studies are needed before this possibility can be ruled out. In the passive transfer studies, there was a long lag phase in detection of patent parasitemias with both the 17XL and 17XNL infections (Fig. 1 to 3). These results suggest that passively transferred Ab helped to eliminate parasites within the initial inoculum. There are two known mechanisms by which antimalarial Ab mediate their effects, namely, by blocking an important receptor-ligand interaction (e.g., blocking freed merozoites from entering RBC) and enhancing Fc-mediated phagocytosis (1). Passive protection against *P. yoelii* has been achieved with monoclonal Ab of the IgG1, IgG2a, and IgG3 isotypes (6, 14). These monoclonal Ab are directed against merozoites and may prevent merozoite interaction with the RBC. Such an action would not require the effector functions of a particular isotype but would require appropriate idiotypic specificity. Several investigators have shown that during the early stages of rodent malaria, Ig-coated RBC are ingested by activated macrophages (16, 23). Since activated macrophages have enhanced expression of Fc receptors for IgG2a Ab (3), it is possible that macrophages preferentially phagocytize PRBC or merozoites that have been opsonized by Ab of the IgG2a isotype. Studies of human malaria demonstrate a correlation between protective immunity and the presence of opsonic Ab (7). Although both mechanisms could be operative in the current study, it is likely that IgG2a Ab are mediating their effects primarily by Fc-mediated phagocytosis, since there is no evidence that IgG isotypes differ in antigenic specificity.

IgG isotypes are thought to be important in human immunity to malarial parasites. Wahlgren and colleagues found in indirect immunofluorescence Ab studies that Ab of all isotypes give strong parasite reactivity but IgG2 Ab were unique in giving staining restricted to the surface of schizonts (24). Recent studies by Groux and Gysin reported that all four isotypes of human IgG bind to the surface of *Plasmodium falciparum*-infected RBC but only IgG1 and IgG3 were able to mediate opsonization (7). This result is not surprising, as human monocytes express receptors for only

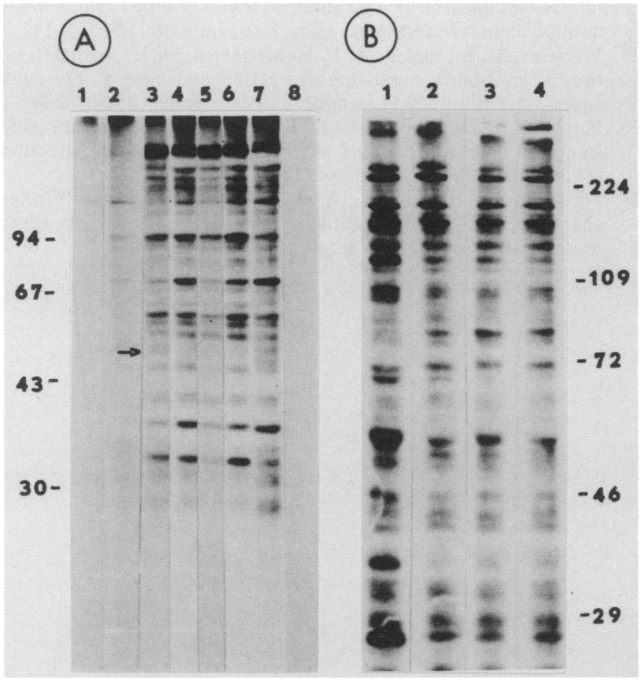

FIG. 4. Results of immunoprecipitation studies. (A) Extracts of *P. yoelii* parasites metabolically labeled with [^{3}H]Leu were immunoprecipitated with isotypic fractions from BALB/c HIP (Table 1 and Fig. 1A). Lane 1, 5 μl of NMP plus Sepharose–anti-μ; lane 2, 10 μl of nonbinding IgM plus Sepharose–anti-μ; lanes 3, 4, 5, and 6, 25 μg of IgG1, IgG2a, IgG2b, and IgG3, respectively, plus protein A-Sepharose; lanes 7 and 8, 5 μl of BALB/c HIP and NMP, respectively, plus protein A-Sepharose. The arrow indicates a unique band in the IgG1 fraction. (B) *P. yoelii* parasites were metabolically labeled with a mixture of ^{14}C-amino acids, and aliquots of the extract were immunoprecipitated with isotypic fractions from ICR HIP (Table 2). Results in lanes 1 to 4 were produced by immunoprecipitation using 13 to 15 μg of IgG1, IgG2a, IgG2b, and IgG3, respectively, plus protein A-Sepharose. Molecular sizes (in kilodaltons) are indicated on the sides.

IgG1 and IgG3. Although IgG1 and IgG3 Ab may be good at eliminating infected RBC, data by Facer suggest that autoanti-RBC Ab of these isotypes may also be involved in producing hemolytic anemia (4). Thus, there is much to be learned about the role of Ab isotype in both protection from and the pathogenesis of malaria.

Results of this study suggest that Ab of the IgG2a isotype are important in immunity to *P. yoelii* in mice, and results with humans (4, 7, 24) suggest that Ig-isotype expression is likely to be important in immunity to *P. falciparum*. Today, there is interest in the production of a vaccine for malaria which will probably need to be administered with an adjuvant. If so, care should be taken in selection of the adjuvant, since different adjuvants stimulate the expression of different isotypes (10). Thus, in developing a vaccine, investigators may need to consider both isotype expression and the induction of high titers of high-affinity Ab.

ACKNOWLEDGMENT

We thank T. Roberts for his help in the preparation of the manuscript.

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