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Monoclonal Antibodies of Three Different Immunoglobulin G Isotypes Produced by Immunization with a Synthetic Peptide or Native Protein Protect Mice against Challenge with *Plasmodium yoelii* Sporozoites

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Passive transfer of monoclonal antibodies (MAbs) against malaria circumsporozoite (CS) proteins protects animals against malaria. Active immunization with synthetic or recombinant peptides induces a level of polyclonal antibodies to sporozoites comparable to those found after passive immunization but does not provide comparable protection. In the *Plasmodium yoelii* system, synthetic or recombinant peptide-induced antibodies have never been shown to protect. The current studies were designed to determine whether immunogen structure (native protein versus synthetic peptide) or immunoglobulin G (IgG) subclass of antibodies was responsible for the absolute differences between protective, passively transferred MAbs and nonprotective, actively induced polyclonal antibodies. In this study we produced two MAbs, QGP-S1 (IgG1) and QGP-S2 (IgG2b), by immunization with a synthetic peptide based on the *P. yoelii* CS major repeat, $(QGPGAP)_4$, conjugated to keyhole limpet hemocyanin. These MAbs were compared to NYS1 (IgG3), an anti-CS protein MAb previously produced by immunization with irradiated *P. yoelii* sporozoites, which recognizes $(QGPGAP)_2$. QGP-S1 and QGP-S2 passively transferred protection. However, when compared with NYS1, there was a hierarchy of protection, NYS1 > QGP-S1 > QGP-S2. There was no correlation between antibody level at challenge as determined by immunofluorescent antibody test against sporozoites or enzyme-linked immunosorbent assay against $(QGPGAP)_2$ or apparent antibody avidity for $(QGPGAP)_2$ by sodium thiocyanate elution assay. The data demonstrate that a synthetic peptide can induce protective antibodies and that a specific antibody subclass is not required for protection. Work to determine whether antibody affinity or fine specificity can explain the hierarchy of protection among the MAbs is under way.

Passive transfer of NYS1, a monoclonal antibody (MAb) against the *Plasmodium yoelii* circumsporozoite (CS) protein completely protects against sporozoite challenge (3). The binding of NYS1 to sporozoites is inhibited by incubation with $(QGPGAP)_2$, a peptide based on the repeat region of the *P. yoelii* CS protein (3). However, apparently similar levels of antibodies to $(QGPGAP)_2$ or sporozoites induced by immunization with subunit synthetic and recombinant vaccines against the CS protein were not protective (3, 4, 9, 14–16). A number of explanations have been offered for these findings. The protective MAb was made by immunizing with live, attenuated sporozoites, a native protein, while the polyclonal antibodies were produced by immunization with peptides. Perhaps the native structure of the immunogen is critical for induction of protective antibodies. Another possibility is that antibody subclass may be a critical factor; the protective MAb is an immunoglobulin G3 (IgG3), and the vaccine-induced antibodies are predominantly IgG1 (3). To study these possibilities, we produced MAbs of multiple subclasses by immunizing mice with a synthetic peptide containing four copies of QGPGAP and tested these MAbs for protective capacity in passive transfer.

MATERIALS AND METHODS

Mice. Female, 6- to 10-week-old, BALB/c ByJ mice (The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. The experiments reported here were conducted according to the principles set forth by the National Research Council (10a).

Sporozoites. *P. yoelii* sporozoites (17XNL, nonlethal strain) were dissected from *Anopheles stephensi* salivary glands into medium 199 containing 5% normal mouse serum and were used for the induction of infections. Sporozoites isolated by a discontinuous gradient (11) in medium 199 without serum were used in the preparation of antigen slides for an immunofluorescent antibody test (IFAT) (2).

Peptides. Two synthetic peptides based on the predominant repeat of the *P. yoelii* CS protein, glutamine-glycine-proline-glycine-alanine-proline (QGPGAP), were used. $(QGPGAP)_4$ was conjugated to keyhole limpet hemocyanin through an amino-terminal cysteine (Peninsula Laboratories, Belmont, Calif.) and used to immunize mice. $C(QGPGAP)_2$ was synthesized by a solid-phase method (10) on an Applied Biosystems model 430A synthesizer with single coupling of

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each amino acid. Cleavage and deblocking were performed with 10% trifluoromethanesulfonic acid and subsequent precipitation in diethyl ether. The peptide was finally redissolved and lyophilized. Analytical high-pressure liquid chromatography and quantitative amino acid analyses confirmed the identity and purity of the peptide product. This peptide was used in enzyme-linked immunosorbent assay (ELISA) and thiocyanate elution experiments.

Production and characterization of MAbs. Mice were immunized with the synthetic peptide $(QGPGAP)_4$, conjugated to keyhole limpet hemocyanin, by the intraperitoneal (first two doses) and intravenous (third dose) routes. For the first dose, antigen was emulsified in Freund's complete adjuvant; for the second, antigen was emulsified in Freund's incomplete adjuvant; and for the third dose, antigen was suspended in phosphate-buffered saline PBS without adjuvant. Three days after the third immunization, spleens were removed from each mouse for fusion with the mouse myeloma cell line X63.Ag8.653 by a technique previously described (6). Culture supernatants were screened for antibodies by IFAT and ELISA. Positive hybridoma lines were cloned by limiting dilution, and a series of hybridoma clones secreting MAbs to sporozoites or to $(QGPGAP)_2$ were obtained. On the basis of their reactivities with sporozoites, two MAbs, QGP-S1 (IgG1) and QGP-S2 (IgG2b) were selected for further study. The MAbs were purified from ascitic fluid on a *Staphylococcus* protein A (Sigma Chemical Co., St. Louis, Mo.) affinity column by a method previously described (8).

Passive transfer experiments. All three passive transfer experiments were performed by injecting various concentrations of the MAbs into tail veins of BALB/c ByJ mice. Controls used included an anti-*P. falciparum* CS protein MAb, NFS1 (IgG1); an anti-*P. vivax* CS protein MAb, NVS3 (IgG3); and an antitrypanosomal MAb, 31.4G8.1 (IgG2b). The MAb 31.4G8.1 was kindly provided by Ted Hall from the Walter Reed Army Institute of Research. Thirty minutes after injection of the MAbs, sporozoites were injected into the tail veins of the mice. Immediately before injection of the sporozoites, blood samples were collected, and the sera were separated for the determination of antibody levels by IFAT and ELISA. Parasitemia was determined by examining Giemsa-stained thin and thick smears on days 5, 7, 9, 11, and 14 after challenge.

Antibody assays. (i) ELISA. Fifty microliters of $C(QGPGAP)_2$ in PBS (4 μg/ml) was added to each well of 96-well ELISA plates (Corning Glass Works, Corning, N.Y.), and the plates were incubated overnight at 4°C. The antigen wells were blocked for 2 h with 5% nonfat dry milk in PBS, pH 7.4 (blocking buffer). Fifty microliters of the serial dilutions of the sera or MAbs was added to each antigen well, and the plates were incubated for 1 h at 37°C. The wells were washed five times with PBS containing 0.05% Tween 20 (washing buffer) and incubated for 1 h with horseradish peroxidase-labeled goat anti-mouse IgG (heavy plus light chains) (Bio-Rad Laboratories, Richmond, Calif.). The plates were washed five times with washing buffer after incubation, and 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) (ABTS) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added. Color reaction was measured in a Dynatech MR5000 Microplate reader by determining optical density at 410 nm (OD_{410}). The results were calculated as mean OD readings of triplicate assays ± standard deviations.

(ii) IFAT. Serial dilutions of sera or MAbs to be tested were added to each well of antigen slides containing 2,000 air-dried *P. yoelii* sporozoites per well. The slides were incubated in a moist chamber for 30 min at 37°C and washed twice in PBS. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, San Jose, Calif.) was added, and the slides were incubated for an additional 30 min. Slides were then washed with PBS to remove the excess conjugate, mounted in 10% glycerol in PBS, and examined with an American Optical fluorescence microscope.

(iii) Avidity assay. The avidity of MAbs for the repeat region of the *P. yoelii* CS protein was estimated by the sodium thiocyanate elution method, as previously described (3, 13). Briefly, sera or MAbs to be tested were titrated against the peptide $C(QGPGAP)_2$ in an ELISA. Sera or MAbs at the dilution at which the absorbance (OD_{410}) in the standard ELISA was 1.0 were incubated for 1 h at 37°C in peptide-coated plates. The plates were washed, and various concentrations (0.1 to 6.0 M) of sodium thiocyanate (NaSCN) were added to each well. The plates were incubated for 15 min at room temperature to allow disruption of antigen-antibody binding, washed, and further incubated for 1 h at 37°C with horseradish peroxidase-labeled goat anti-mouse IgG. The plates were washed, ABTS substrate was added, and color reaction was measured as OD_{410}. Results were depicted by plotting the log percent initial OD (absorbance when no NaSCN was added) against the molarity of NaSCN used. The avidity index was calculated as the molarity of NaSCN that resulted in a 50% reduction in absorbance from the reading when no NaSCN was added (i.e., log 50% = 1.699).

TABLE 1. Protection of mice against challenge with 200 *P. yoelii* sporozoites[a] after passive transfer of MAbs produced by immunization with KLH-$C(QGPGAP)_4$

MAb	Dose (μg)	IFAT titer[b]	No. infected/ no. tested	% Protection
Tested				
QGP-S1 (IgG1)	1,000	16,384	0/5	100
	500	8,192	3/6	50
	250	8,192	5/6	17
	125	4,096	5/6	17
QGP-S2 (IgG2b)	1,000	16,384	3/6	50
	500	8,192	4/6	33
	250	4,096	5/6	17
	125	2,048	6/6	0
Control, NFS1[c] (IgG1)	1,000	<8	6/6	0

[a] Five of five naive mice challenged with 200 or 40 sporozoites became infected, and three of five challenged with 8 sporozoites became infected.
[b] Titer of antibodies to air-dried sporozoites by IFAT in sera taken from the mice at the time of sporozoite challenge.
[c] Anti-*P. falciparum* CS protein MAb.

RESULTS

Protection after passive immunization. Both synthetic peptide-induced MAbs, QGP-S1 (IgG1) and QGP-S2 (IgG2b), protected against sporozoite challenge. However, QGP-S1 was consistently more effective than QGP-S2 in preventing infection (Table 1).

Having established that QGP-S1 was superior to QGP-S2, we compared QGP-S1, a synthetic peptide-induced MAb, with NYS1, an irradiated sporozoite-induced MAb. Mice that received 1,000 μg NYS1 or QGP-S1 were challenged with increasing numbers of *P. yoelii* sporozoites. Both MAbs were protective, but NYS1 was consistently more protective

TABLE 2. Protection of mice against challenge with increasing numbers of *P. yoelii* sporozoites[a] after passive transfer of MAbs

MAb[b]	No. of sporozoites	No. infected/ no. tested	% Protection
QGP-S1 (IgG1)	200	0/6	100
	1,000	2/6	67
	5,000	2/6	67
	25,000	5/6	17
NYS1 (IgG3)	200	0/5	100
	1,000	0/6	100
	5,000	1/6	83
	25,000	1/6	83

[a] Six of six naive mice challenged with 200 or 40 sporozoites and two of six challenged with 8 sporozoites became infected.
[b] At the time of challenge, the mice that received 1,000 μg of QGP-S1 had an IFAT titer of 16,384 against *P. yoelii* sporozoites, while those that received 1,000 μg of NYS1 had a titer of 8,192.

than QGP-S1 (Table 2). In the QGP-S1 group, all mice challenged with 200 sporozoites and only 17% of those challenged with 25,000 sporozoites were protected. In the NYS1 group, all mice challenged with 200 and 1,000 sporozoites and 83% of those challenged with 25,000 sporozoites were protected (Table 2).

With new batches of all three MAbs, we then performed an experiment to compare protective capacities of all three MAbs simultaneously at two fixed antibody concentrations against 200 sporozoites. We used three unrelated antibodies as controls for the three different subclasses. Again, there was a hierarchy of protection: NYS1 > QGP-S1 > QGP-S2. All control mice became infected. These results are summarized in Table 3.

Lack of correlation between antibody level, avidity, and protection. Sera taken after passive transfer (immediately before challenge) were tested by IFAT with air-dried sporozoites and by ELISA with $(QGPGAP)_2$ as antigens. Each MAb protected mice in a dose-dependent manner. However, when different MAbs were compared, there was no correlation between antibody level and protection (Tables 1 to 4 and Fig. 1), nor was there a correlation between the avidity index

TABLE 3. Protective capacities of MAbs against challenge with 200 *P. yoelii* sporozoites[a]

MAb	Dose (μg)	No. infected/ no. tested	% Protection
Tested			
NYS1 (IgG3)	500	0/10	100
	250	1/10	90
QGP-S1 (IgG1)	500	2/10	80
	250	9/10	10
QGP-S2 (IgG2b)	500	6/10	40
	250	9/10	10
Control			
NVS3[b] (IgG3)	500	6/6	0
NFS1[c] (IgG1)	500	6/6	0
31.4G8.1[d] (IgG2b)	500	6/6	0

[a] Six of six naive control mice became infected with injection of 200 sporozoites.
[b] Anti-*P. vivax* CS protein MAb.
[c] Anti-*P. falciparum* CS protein MAb.
[d] Anti-*Trypanosoma rhodesiense* MAb.

TABLE 4. Protective capacities, serum levels, and avidity indices of MAbs

MAb[a]	% Protection at:		Antibody against:		Avidity index[d] for $(QGP\ GAP)_2$
	500 μg	250 μg	$(QGPGAP)_2$ (OD units[b])	Sporozoites (IFAT titer[c])	
NYS1 (IgG3)	100	90	90	4,096	0.22
QGP-S1 (IgG1)	80	10	42	4,096	0.31
QGP-S2 (IgG2b)	40	10	4,096	4,096	0.55

[a] MAbs (500 or 250 μg) were passively transferred into mice, and 30 min later sera were obtained and mice were challenged with 200 *P. yoelii* sporozoites.
[b] OD units of sera collected 30 min after passive transfer of 500 μg of MAbs (immediately before challenge). One OD unit is the serum dilution at which the optical density in the ELISA was 1.0.
[c] IFAT titer of sera collected 30 min after passive transfer of 500 μg of MAbs (immediately before challenge).
[d] Avidity indices are calculated from the means of three experiments.

and protection (Table 4). In fact, QGP-S2, the least protective MAb, had the highest avidity index (0.55) for $(QGP\ GAP)_2$, and NYS1, the most protective MAb, had the lowest avidity index (0.22) (Fig. 2 and Table 4).

DISCUSSION

Previous work has shown that passive transfer of MAbs against the *Plasmodium berghei* (7, 12), *P. yoelii* (3), and *P. vivax* (1) CS proteins can protect against sporozoite-induced

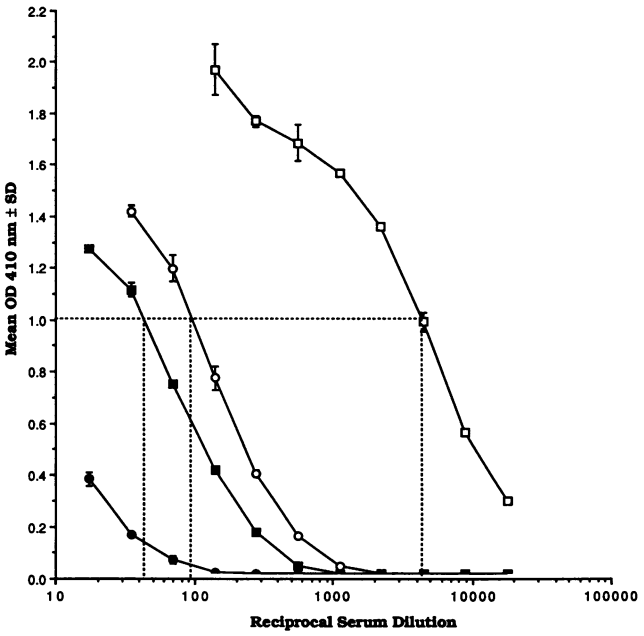

FIG. 1. Levels of antibodies against the repeat region of the *P. yoelii* CS protein after passive transfer of MAbs. Circulating antibodies against $C(QGPGAP)_2$ were measured by ELISA in sera obtained from BALB/c mice 30 min after administration of 500 μg of the MAb QGP-S1 (■), QGP-S2 (□), or NYS1 (○). Sera from mice that received NVS3 (●), an anti-*P. vivax* MAb, were used as negative controls. Mean OD readings ± standard deviations of triplicate wells are plotted against reciprocal serum dilution. One OD unit is defined as the serum dilution at which the OD reading is 1.0. The OD units are 90 for NYS1, 42 for QGP-S1, and 4,096 for QGP-S2.

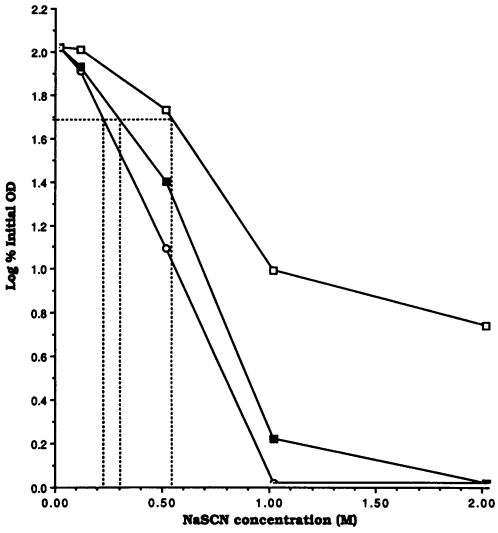

FIG. 2. Avidity indices of MAbs for the repeat region of the *P. yoelii* CS protein. QGP-S1 (■), QGP-S2 (□), and NYS1 (○) MAbs were incubated with plate-bound $C(QGPGAP)_2$, the antigen-antibody complex was exposed to increasing concentrations of sodium thiocyanate (NaSCN), and absorbance was determined by ELISA. The avidity index was arbitrarily considered to be the molarity of NaSCN required to reduce the initial absorbance by 50% (log 50% initial absorbance = 1.699) and is designated by the point where the dotted line crosses the ELISA curves. As shown in this figure, avidity indices are 0.22 for NYS1, 0.31 for QGP-S1, and 0.55 for QGP-S2.

malaria. In the *P. berghei* system, vaccine-induced polyclonal antibodies have provided as much as 80% protection against moderate sporozoite challenges (17). In the *P. yoelii* (3, 4, 9, 14–16) and *P. vivax* (1, 5) systems, vaccine-induced antibodies have provided little, if any, protection. Two possible explanations for this difference in the level of protection between MAbs and polyclonal antibodies are the following: (i) the structure of the subunit vaccine may not be appropriate to induce adequate immunity, or the essential epitope may not be included in the peptide, and (ii) the antibody subclass may be important for protection, since in the *P. yoelii* and *P. vivax* systems, all the protective MAbs were IgG3 (1, 3). To examine these potential explanations, we produced MAbs of different IgG subclasses by immunization with synthetic peptides, tested these MAbs for their protective efficacies in passive transfer experiments, and tried to correlate their protective abilities with antibody levels and apparent antibody avidity for the predominant CS protein repeat unit, QGPGAP.

The data indicate that antibodies of at least three different IgG subclasses can protect against sporozoite challenge and that immunization with synthetic peptides as well as native protein can lead to induction of protective MAbs. As in our previous studies, neither the antibody level at the time of challenge nor the apparent avidity for what is thought to be the target of these MAbs, $(QGPGAP)_n$, correlates with protection (3).

In previous work with *P. vivax*, we established that a protective MAb, NVS3, recognized only four of the nine amino acids of the *P. vivax* CS protein repeat region (1). As in the case of *P. vivax*, we believe that the fine specificity of the antibodies may be critical for mediation of protection. QGPGAP is the predominant repeat of the *P. yoelii* CS protein. The peptide $(QGPGAP)_2$ inhibits the binding of the protective MAbs to sporozoites, and the targets for each of the MAbs studied are almost certainly included within this peptide either as linear or assembled epitopes on the basis of conformation. However, appropriate discrimination between the MAbs may be impossible by using this peptide, since it may include cross-reacting and overlapping epitopes for each of the MAbs and therefore may not be the appropriate target for assays to compare antibody level and apparent avidity. Other possibilities are that the assays for antibody level and avidity that we have used are inappropriate and that antibody affinity may more faithfully predict protective capacity. Current work is aimed at determining the true affinities of these MAbs for QGPGAP and the fine specificities of these MAbs.

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