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Active and passive immunization against *Plasmodium yoelii* sporozoites

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Three subunit vaccines based on the major repeat, $(QGPGAP)_n$, and flanking regions of the Plasmodium yoelii *circumsporozoite protein were designed, produced, and tested. All were immunogenic, but none gave consistent protection against a 40–200 sporozoite challenge. To demonstrate that antibodies to* P. yoelii *CS protein could provide protection we established a passive transfer model. Passive transfer of NYS1, an IgG3 MAb against the* P. yoelii *CS protein, protected 100% of mice against challenge with 5000* P. yoelii *sporozoites. Binding of NYS1 to sporozoites was inhibited by incubation with $(QGPGAP)_2$, indicating that the epitope on sporozoites recognized by this MAb was included within this peptide. The levels of antibodies to $(QGPGAP)_2$ by ELISA, and to sporozoites by IFAT and CS precipitation reaction were similar in sera from mice that received NYS1 in passive transfer and were protected against challenge with 5000 sporozoites, and from mice that had been immunized with subunit vaccines containing QGPGAP but were not protected against challenge with 40–200 sporozoites. To determine if antibody avidity, not the absolute concentration, could explain the striking differences in protection, we established a thiocyanate elution assay. The results suggest that NYS1, the protective MAb, has a lower avidity for $(QGPGAP)_2$ and for sporozoites than do the vaccine-induced antibodies. The data clearly demonstrate that antibodies to the CS protein can protect against intense sporozoite infection. Improved understanding of the differences between protective MAbs and non-protective polyclonal antibodies will be important in the further development of malaria vaccines*

Introduction

During the past decade, the primary strategy for malaria sporozoite vaccine development has been to produce subunit vaccines that induce antibodies to the repeat region of the circumsporozoite (CS) protein, antibodies that prevent effective sporozoite invasion of hepatocytes (*1–4*). Thus far, protective immunity after immunization of humans (*5, 6*) and non-human primates (*7*) has been disappointing. Mice immunized with subunit *Plasmodium berghei* vaccines have been protected against moderate, but not against large sporozoite challenge doses (*8–11*). In contrast, mice immunized with irradiation-attenuated sporozoites have been protected against large doses of sporozoites (*8, 10*). In the *P. yoelii* model system, mice immunized with irradiation-attenuated sporozoites have also been shown to be protected against large dose of sporozoite challenge (*12*), and the protection was dependent on $CD8^+$ T cells (*12, 13*). Irradiated sporozoite immunized mice challenged with as few as 200 sporozoites were not protected after $CD8^+$ T-cell depletion (*13*), indicating that antibodies induced by such immunization could not protect on their own. To determine whether antibodies to CS protein could protect against *P. yoelii* sporozoite challenge we studied active and passive immunization against *P. yoelii* sporozoites.

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Materials and methods

Animals

Female, 6 to 10 week, BALB/c ByJ mice (Jackson Laboratory, Bar Harbor, ME), were used in all experiments.

Sporozoites

All experiments were conducted with sporozoites of the 17X NL (nonlethal) strain of *P. yoelii*, which were raised in *Anopheles stephensi*. Salivary gland dissected sporozoites suspended in Medium 199 containing 5% normal mouse serum were used for intravenous infection. Sporozoites isolated by discontinuous gradient (*14*) in Medium 199 without serum were used in an enzyme-linked immunosorbent assay (ELISA), as whole sporozoites or as an antigen extract in 1% sodium dodecyl sulfate (*15*), and in an immunofluorescent antibody test (IFAT) (*15*).

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Active immunization

Mice were immunized with four copies of a synthetic peptide derived from the predominant repeat of the *P. yoelii* CS protein (*16–18*) (Gln-Gly-Pro-Gly-Ala-Pro) $(QGPGAP)_4$ conjugated to KLH or proteosomes (*19*) (Sedegah et al., manuscript in preparation) or with PY CS.1 (Yuan et al., manuscript in preparation). PY CS.1 is a fusion protein produced in *E. coli* (*17*) including 258 amino acids from the CS protein fused to 81 amino acids derived from a non-structural protein of influenza A (Fig. 1B). PY CS.1 includes 19 copies of the major repeat (QGPGAP), 7 copies of the minor repeat (Gln-Gln-Pro-Pro) (QQPP), and 45 amino acids EEKKDDPP KDGNKDDLPK EEKKDDLPK EEKKDDPPK DPKKDDPPK, apparently comprising 5 copies of a degenerative sequence of 9 amino acids (repeat 3). Groups of mice were immunized i.m. or i.p. at 2–3-week intervals. Freund's complete adjuvant (FCA) was used in initial immunizations, and Freund's incomplete adjuvant (FICA) was used in subsequent immunizations. The control animals received adjuvant alone or received antigen without adjuvants. Two weeks after the fourth immunization, blood samples were collected from the mice via retro-orbital sinuses and sera were separated for determination of antibody levels. Mice were then challenged with 40–200 salivary gland dissected *P. yoelii* sporozoites. Parasitaemias were determined at 5, 7, 9, 11 and 14 days after sporozoite challenge, from Giemsa-stained blood films prepared from the blood collected from tail veins of these mice.

Monoclonal antibodies

NYS1, an IgG3 MAb used in this study, was produced and characterized as described previously (*15*), and purified from ascitic fluid using a staphylococcal protein A column (*20*). NFS1, a MAb directed against *P. falciparum*, served as unrelated antibody control.

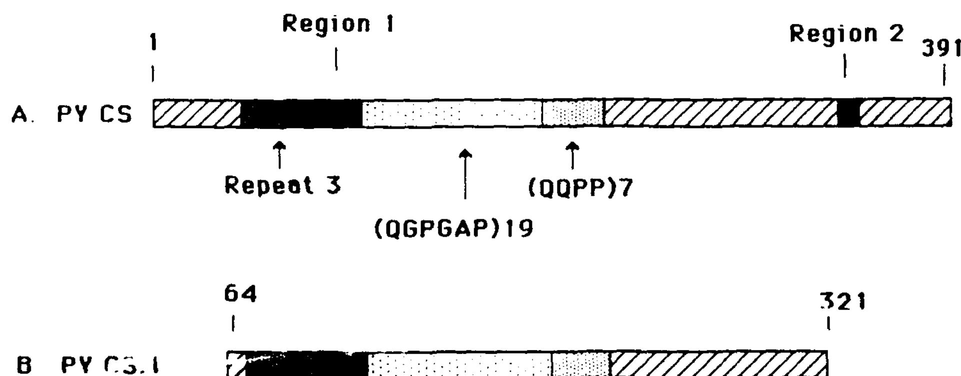
Passive immunization

Groups of mice were injected in the tail vein with MAb NYS1. Control mice received either PBS or NFS1, a MAb directed against the *P. falciparum* CS protein. Thirty minutes after injection of the MAb, blood samples were collected for determination of antibody levels, and mice were immediately challenged intravenously with sporozoites. Blood smears were prepared as described above.

ELISA

Antigens used in an ELISA were sporozoites and three *P. yoelii* CS protein derived antigens (Fig. 1A). The first was a synthetic peptide composed of two copies of Gln-Gly-Pro-Gly-Ala-Pro $(QGPGAP)_2$, the major repeat of the *P. yoelii* CS protein. The second included three copies of Gln-Gln-Pro-Pro $(QQPP)_3$, the minor repeat of the *P. yoelii* CS protein. The third was PY CS.1. One hundred microlitres of 10 μg/ml $(QGPGAP)_2$, $(QQPP)_3$, or PY CS.1 or 100 μl of whole sporozoite suspension in PBS (2×10^4 sporozoites/ml) or sporozoite antigen extracted in 1% SDS (2×10^4 sporozoites/ml) were incubated for 24 h at

Fig. 1. **Schematic diagrams of the *P. yoelii* CS protein and the recombinant fusion protein PY CS.1.** The *P. yoelii* CS protein (**A**) consists of 391 amino acids, including 19 copies of QGPGAP, 7 copies of QQPP, and a 45 amino acid sequence (EEKKDDPPK DGNKDDLPK EEKKDDLPK EEKKDDPPK DPKKDDPPK) (designated as repeat 3), which is apparently 5 copies of a degenerative sequence of EEKKDDPPK, and two hydrophobic stretches of amino acids called region 1 and region 2 that are highly conserved among *Plasmodium* species. The PY CS.1 protein (**B**) extends from amino acids 64 to 321, including all of the repeats and region 1.



37°C in Immunolon II flat-bottom microtitration plates (Dynatech Laboratories, Inc., Chantilly, VA). The plates were then blocked for 2 h with 1% BSA in PBS pH 7.2 (blocking buffer). The antigen wells were washed 3 times with PBS containing 0.05% Tween 20 (washing buffer). One hundred microlitres of serial dilutions of MAb NYS1 (4.0–0.3 μg/ml) or of the sera were added to each antigen well, and the plates were further incubated for 2 h. The wells were washed 3 times with washing buffer prior to incubating for 2 h with horse-radish peroxidase (HRP) labelled goat anti-mouse IgG or anti-mouse μ chain (TAGO, Inc., Burlingame, CA). The plates were washed again after incubation, and ABTS (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate]) substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added. Colour reaction was measured in a Dynatech Micro-ELISA auto reader, at OD 405 nm. The results were recorded as mean OD readings of triplicate assays ± SE.

Inhibition ELISA

In addition to a standard ELISA, an inhibition ELISA was used to characterize the NYS1 epitope. NYS1 (2 μg/ml) was incubated for 2 h at 37 °C with increasing concentrations (0–160 μg/ml) of $(QGPGAP)_2$, $(QQPP)_3$, or with a control peptide derived from the immunodominant repeat domain of the *P. vivax* CS protein (Gly-Asp-Arg-Ala-Asp-Gly-Gln-Pro-Ala (GDRADGQPA)) (*21*). The NYS1-peptide mixtures were then added to plates previously coated for 24 h at 37 °C with 2×10^3 *P. yoelii* sporozoites well. The plates were further incubated for 2 h at 37 °C, washed, and then incubated with HRP-labelled goat anti-mouse IgG and ABTS substrate.

IgG subclass antibodies $(QGPGAP)_2$

The subclass of IgG antibodies to $(QGPGAP)_2$ was determined by ELISA using the method previously described (*22*). Briefly, affinity-purified mouse myeloma IgG1, IgG2a, IgG2b, and G3 (Sigma Laboratories, St. Louis, MO) were tested in the ELISA assay against HRP rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Pharmingen Laboratories, San Diego, CA) and the standard curves of the IgG subclasses were plotted. Serial dilutions of sera from subunit vaccine immunized mice were incubated with $(QGPGAP)_2$ coated on the plates, followed by HRP rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 and ABTS substrate. The OD reading at 405 nm was measured, and the percentage $(QGPGAP)_2$-specific subclass antibodies calculated from the standard curves.

Indirect fluorescent antibody test (IFAT) and circumsporozoite (CS) precipitation test

IFAT and CS precipitation tests were performed as previously described (*15, 23*) using *P. yoelii* sporozoites.

Estimation of antibody avidity

The avidity of antibodies to CS protein and sporozoites was estimated using the method described by Pullen et al. (*24*) with a slight modification. Briefly, all sera to be tested were first titrated against the antigens using the standard ELISA. The antigens used were $(QGPGAP)_2$ and PY CS.1 at a concentration of 10 μg/ml, and whole sporozoites or sporozoite antigen extract at 2×10^4 sporozoites/ml. Sera at the dilutions at which the absorbance (405 nm) in the standard ELISA was 0.8 were incubated for 2 h at 37 °C in antigen coated plates. The plates were washed, and varying concentrations (0.1–4.0 mol) of Na thiocyanate were added to them. The plates were then incubated for 15 min at room temperature to allow disruption of antigen-antibody binding. The plates were washed. HRP-conjugated goat anti-mouse IgG was added to them and they were further incubated for 2 h at 37 °C. The plates were washed and HRP substrate added. Colour reaction was measured at OD 405 nm. The results were depicted by plotting the log % initial OD (absorbance when no thiocyanate [SCN] was added) against the molarity of SCN used. The avidity index was calculated as the molarity of SCN which resulted in a 50% reduction in absorbance from the reading when no SCN was added (i.e., log of 50% reduction = 1.699).

Results

Active immunization

Mice immunized four times with a synthetic peptide $(QGPGAP)_4$ coupled to either KLH or proteosome were not protected against challenge with 200 sporozoites. Mice immunized 4–5 times with PY CS.1 were protected against a 200 sporozoite challenge in an initial experiment. However, the results were never duplicated, and in subsequent experiments these mice were not protected against challenge with 40 sporozoites (Yuan et al., manuscript in preparation).

Passive immunization

Previous work in our laboratory (unpublished results) indicated that the number of *P. yoelii* sporozoites required to infect 50% of mice ranged from 1.6 to 40 sporozoites and that 100% of mice were always infected by the inoculation of 200 sporozoites. In the first experiment, varying amounts of NYS1 (15.1 μg to

Table 1: **Passive transfer of NYS1 to BALB/c ByJ mice and challenge with 200 *P. yoelii* sporozoites**

Groups	NYS1 (μg/mouse)	IFAT (titre)	No. infected/No. tested	% protection
1	1000	8000	0/6	100
2	500	4096	0/6	100
3	250	3000	1/6	83
4	125	2048	2/6	67
5	62.5	750	6/6	0
6	30.3	512	6/6	0
7	15.1	128	6/6	0
8	PBS	< 8	6/6	0
9	1000 μg NFS1[a]	< 8	6/6	0

[a] A MAb directed against the repeat region of the *P. falciparum* CS protein.

Table 2. **Passive transfer of 500 μg NYS1 to BALB/c ByJ mice and challenge with varying numbers of *P. yoelii* sporozoites**

Groups	No. of sporozoites for challenge	No. infected/No. tested	% protection
1	200	0/6	100
2	1000	0/6	100
3	5000	0/6	100
4	10 000	1/6	83
5	25 000	2/6	67
6	Control[a]	6/6	0

[a] Mice that received PBS instead of NYS1 and were challenged with 200 sporozoites.

1000 μg) were given to mice prior to challenge with 200 sporozoites. All mice that received 500 μg NYS1, and 67% of those that received 125 μg were protected (Table 1). In a subsequent experiment, mice that received 500 μg NYS1 were challenged with 200 to 25 000 sporozoites. All mice challenged with 5000 sporozoites, 83% of those challenged with 10 000 sporozoites, and 67% of those challenged with 25 000 sporozoites were protected (Table 2).

Epitope mapping

Having established that NYS1 was highly protective, we mapped its antigenic site on sporozoites. In a standard ELISA, NYS1 recognized (QGPGAP)$_2$, but did not recognize (QQPP)$_3$ (data not shown). In an inhibition ELISA, the binding of NYS1 to whole sporozoites was completely inhibited by prior incubation with (QGPGAP)$_2$ (Fig. 2), indicating that (QGPGAP)$_2$ included the epitope on sporozoites recognized by NYS1.

Antibody levels and CS precipitation reaction

Since passive transfer of NYS1 conferred such impressive protective immunity against challenge with 5000 sporozoites, while immunization with the subunit vaccines induced excellent antibody responses but no protection against 200 sporozoites, we studied the sera taken from the mice that received 500 μg NYS1 in passive transfer and mice that were immunized with the subunit vaccines. Since we had established that mice immunized with irradiated sporozoites were no longer protected against challenge with 200 *P. yoelii* sporozoites after *in vivo* depletion of CD8$^+$ T cells (*12, 13*), indicating that circulating antibodies were not responsible for the excellent protective immunity induced by immunization with irradiated sporozoites, sera from mice immunized with irradiated sporozoites were also included in these studies. All sera used were collected just prior to challenge. The results outlined in Table 3 indicated that there were no striking differences in the levels of antibodies to (QGPGAP)$_2$, the 66% of the CS protein represented by PY CS.1, and sporozoites in the sera of the protected and non-protected animals. IgM antibody to CS protein was not detectable in the sera of subunit vaccine immunized mice (data not shown).

Avidity assays

The above findings suggested the possibility that the protective MAb, NYS1, bound to sporozoites with higher affinity or avidity than did the non-protective polyclonal antibodies. Studies conducted using the thiocyanate elution assay suggest that this may not

Fig. 2. **Inhibition of binding of NYS1 to sporozoites.** *P. yoelii* synthetic peptides $(QGPGAP)_2$ (□), $(QQPP)_3$ (○), and a peptide reflecting the repeat region of the *P. vivax* CS protein $(GDRADGQPA)_2$ (●) were incubated with 2 μg/ml NYS1 prior to incubating with sporozoites. Binding of NYS1 to sporozoites was then determined by ELISA.

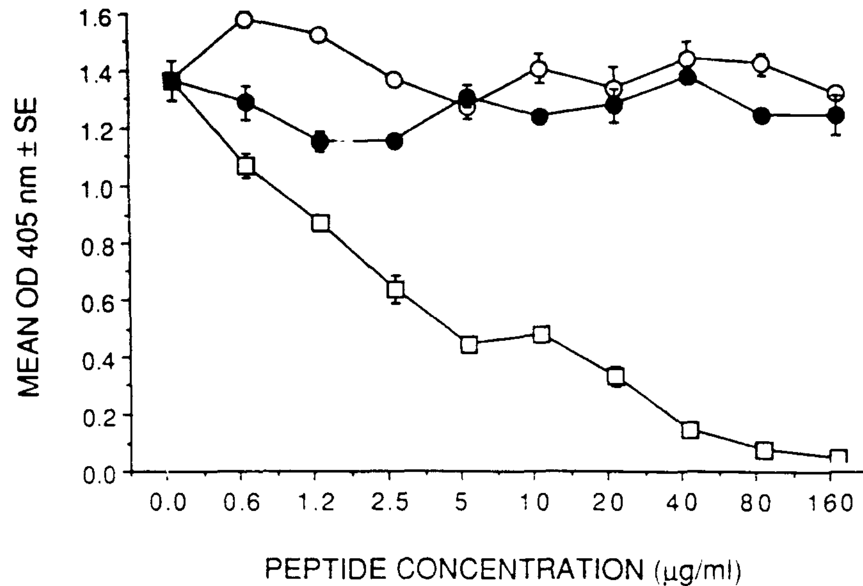


Table 3. **Antibodies to CS protein and sporozoites in mice that received NYS1 and in mice immunized with subunit vaccines**

Groups	Antibody levels[a] Sporozoites (IFAT)	PY CS.1 (ELISA)	$(QGPGAP)_2$ (ELISA)	Sporozoites (CSP)[b]
NYS1 passive transfer	4096	8000	16000	9/25
$(QGPGAP)_4$-KLH	8192	4000	8000	2/25
$(QGPGAP)_4$-proteosomes	8192	2000	6000	0/25
PY CS.1	4096	120 000	8000	7/25
Irradiated sporozoites	2048	2000	4000	2/25
Normal mouse serum	< 8	< 200	< 200	0/25

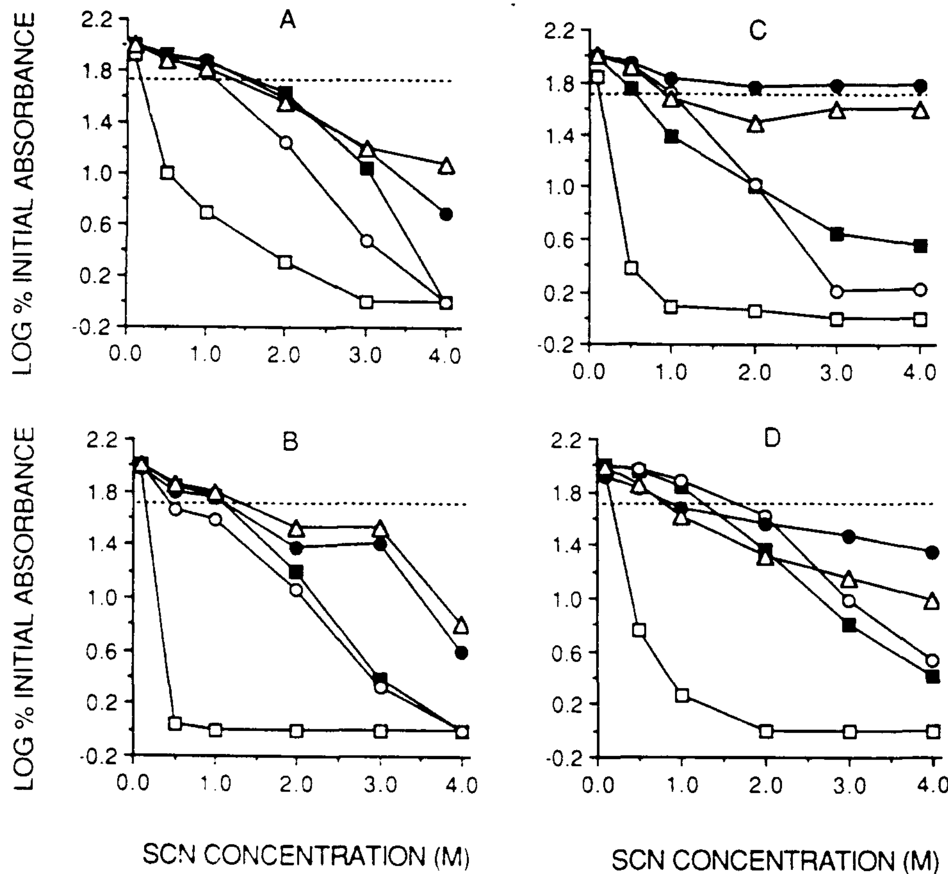
[a] Serum antibody level is recorded as the end-point titre for both IFAT and ELISA. In the IFAT, sporozoites were used as antigens; in the ELISA, PY CS.1 and $(QGPGAP)_2$ were used as antigens.
[b] CS precipitation results are recorded as no. positive/no. of sporozoites counted at a 1:2 dilution of serum.

be the case (Fig. 3, A–D). The avidity index of NYS1 for $(QGPGAP)_2$, PY CS.1, sporozoite extract, and for whole sporozoites is 5–10 times lower than that of the vaccine-induced IgG antibodies.

IgG subclass antibodies to $(QGPGAP)_2$

Since the protected mice had received an IgG3 monoclonal antibody in passive transfer, we addressed the possibility that a subclass of antibody may influence the protection. We measured the levels of IgG subclass antibodies against $(QGPGAP)_2$. All vaccines induced primarily IgG1 antibodies against $(QGPGAP)_2$. For the $(QGPGAP)_4$-proteosomes vaccine 92% were IgG1, 5% IgG2a, 3% IgG2b, and 0% IgG3. For the $(QGPGAP)_4$-KLH vaccine the respective percentages were 84, 10, 6 and 0, and for PY CS.1 they were 60, 4, 2 and 34.

Fig. 3. **Estimation of antibody avidity.** Sera from mice immunized with $(QGPGAP)_4$-KLH (○), $(QGPGAP)_4$-proteosomes (●), PY CS.1 (△) and irradiated sporozoites (■), and from mice that received NYS1 in passive transfer (□) were tested in the thiocyanate elution assay to estimate antibody avidity to: (**A**) $(QGPGAP)_2$, (**B**) PY CS 1, (**C**) sporozoite extract, and (**D**) whole sporozoites. The avidity index was arbitrarily considered to be the concentration of SCN required to reduce the initial absorbance by 50% (log % initial absorbance = 1.699), and is designated by the point where the dotted line crosses the elution curve.



Discussion

Immunization of mice with three subunit vaccines induced excellent antibody responses, but no consistent protection against sporozoite challenge. In contrast, these studies clearly demonstrate that a monoclonal antibody directed against the repeat region of the CS protein can protect animals against a very large *P. yoelii* sporozoite challenge. In the *P. yoelii* system the ID 50 (infectious dose 50%) is almost always less than 10 sporozoites, and often 1–2 sporozoites, yet NYS1 can protect 100% of mice against challenge with 5000 sporozoites (500–5000 times the ID 50). It is, however, still unclear why apparently comparable levels of vaccine-induced antibodies to the CS protein provide little, if any, protection against sporozoite challenge.

NYS1 was made by immunizing mice with irradiated sporozoites, the native protein. An initial hypothesis was that although the binding of NYS1 to sporozoites could be inhibited by $(QGPGAP)_2$, NYS1 was bound to sporozoites with highest efficiency at an assembled site that reflected the tertiary structure of the native protein, and not the primary structure reflected by the deduced amino acid sequence. Peptides are often disordered structures in

water; thus, if the synthetic peptide $(QGPGAP)_4$ or the *E. coli*-produced PY CS.1 did not assume the structure of the native CS protein, immunization with these subunit vaccines would not elicit the appropriate protective antibodies, antibodies with the high degree of complementarity for the native protein required to produce the highly efficient interaction that protects against challenge with live sporozoites. One cannot draw definitive conclusions from comparisons of monoclonal and polyclonal antibodies in affinity or avidity assays, but the results of the thiocyanate elution studies (Fig. 3) suggest that this initial interpretation is incorrect. The apparent avidity of NYS1 for peptides and native protein is 5–10 times lower than that of the vaccine-induced polyclonal antibodies. It is also possible that the vaccine-induced polyclonal antibodies contain subpopulations of non-protective antibodies that compete with the protective antibodies for binding to sporozoites, the ELISA and IFAT are too crude to distinguish between protective and non-protective antibodies to sporozoites, and the absolute concentration of the "correct" antibody is much lower in the sera from the actively immunized animals.

The results of the inhibition of liver-stage development assay (ILSDA) (25) indicate that despite the fact that the vaccine-induced antibodies were not adequate to provide sterile immunity, in fact some of the antibodies had significant activity. In this biologic assay that assesses the capacity of sera to inhibit sporozoite invasion and development *in vitro*, there is an enormous difference in the inhibitory activity of the NYS1 passive transfer sera as compared to the sera from the vaccine immunized animals at a serum dilution of 1:100, correlating with the protection against sporozoite-induced malaria. However, sera from mice immunized with the two QGPGAP-based vaccines had significant inhibitory activity at 1:10 and 1:20 dilutions. The vaccine-induced antibodies can clearly inhibit sporozoite invasion and development, but do not inhibit as well as the MAb. This may be due to their being primarily IgG1 antibodies, as opposed to NYS1, the IgG3 MAb. If this is the case, then we may have to engineer vaccines to induce specific subclasses of antibodies. However, it has been shown that passive transfer of an IgG1 MAb can protect against *P. berghei* infection (8), and we doubt that a subclass alone is responsible for the observed differences in protection against sporozoite challenge. Another possibility is that the protective epitope recognized by NYS1 does not include all of QGPGAP, but a smaller subunit included within this sequence, and that the vaccines did not induce antibodies to this subunit.

These data reemphasize that antibodies to the CS protein can be highly protective against sporozoite-induced malaria. The challenge is to produce subunit vaccines that induce antibodies comparable to protective monoclonal antibodies like NYS1.

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