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Induction of Humoral Immune Response against *Plasmodium falciparum* Sporozoites by Immunization with a Synthetic Peptide Mimotope Whose Sequence Was Derived from Screening a Filamentous Phage Epitope Library

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The mouse monoclonal antibody 2A10 (immunoglobulin G), which recognizes the $(NANP)_n$ repeat of *Plasmodium falciparum* circumsporozoite surface protein, was used to screen a filamentous phage epitope library expressing random amino acid hexamers. The sequences obtained were TNRNPQ, SNRNPQ, NNDNPQ, SNYNPQ, and QNDNPQ (single-letter amino acid designation). These peptides showed 50% homology with the native epitope (PNANPN) and therefore were considered to mimic its structure (mimotopes). Two of these mimotopes (TNRNPQ and NNDNPQ) inhibited the binding of monoclonal antibody 2A10 to the recombinant protein R32LR, which contains the amino acid sequence $[(NANP)_{15}NVDP]_2$. Immunization of mice and rabbits using the peptide $(TNRNPQ)_4$ induced a humoral response that recognized R32LR by an enzyme-linked immunosorbent assay and *P. falciparum* sporozoites by an immunofluorescence assay. These results suggest that phage epitope libraries can be exploited to screen for mimotopes in the design of subunit vaccines against infectious agents.

Several groups have independently reported the construction and use of filamentous phage epitope libraries (7, 8, 23) to obtain peptide sequences (ligands) that bind antibodies of interest. The filamentous phage in these libraries contained as a binding motif a random 6- to 15-amino-acid sequence expressed as part of the gene III surface protein. The gene III protein is presented as 5 molecules at one end of the phage and is felt to be involved in the initial attachment of the phage to the F pilus (13). Epitope sequences derived by screening these libraries with antibodies sometimes show significant sequence divergence from the native antigens and are felt to mimic their conformation. Therefore, these epitopes are also referred to as mimotopes.

Peptide mimotopes are of considerable interest because of their potential for use in vaccine development. Peptide mimotopes can now be derived from phage libraries (7, 8, 23) or by chemical synthesis (11, 14, 18). However, there are limited data on whether mimotopes so derived can induce a humoral response that recognizes native antigens. To this end, we constructed a phage epitope library and screened it with a mouse monoclonal antibody (MAb), 2A10 (immunoglobulin G), which recognizes the $(NANP)_n$ repeat of *Plasmodium falciparum* circumsporozoite surface protein (CSP) (5, 19, 29, 30). We report here the isolation and characterization of mimotopes of $(NANP)_n$ that stimulate the production of antibodies that recognize native *P. falciparum* CSP.

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MATERIALS AND METHODS

Library construction. The vector used for the construction of the random hexapeptide library is the filamentous phage fAFF1, kindly provided by William J. Dower, Affymax Research Institute, Palo Alto, Calif. Vector fAFF1 (7) contains a tetracycline resistance gene which allows for antibiotic selection. The vector also contains a frameshift mutation in gene III which greatly reduces its ability to infect *Escherichia coli* cells. The reading frame can be restored by digestion with the restriction enzyme *Bst*XI, which removes a short DNA fragment, and insertion of a synthetic DNA sequence with the proper number of bases which codes for a random amino acid hexamer. Once the correct reading frame is restored, random hexamers are expressed at the amino terminus of the gene III surface coat protein of the phage. DH5α *E. coli* cells (*supE44 hsdR17 deoR recA13 endA1 lacZ*ΔM15 F′) transformed with fAFF1 DNA were grown at 37°C overnight in 2 to 3 liters of Luria-Bertani (LB) broth supplemented with 25 μg of tetracycline per ml. The double-stranded replicative phage DNA was isolated by Qiagen column purification (Qiagen, Chatsworth, Calif.). fAFF1 vector DNA was digested to completion with *Bst*XI as assessed by 0.7% agarose gel electrophoresis (23). After digestion, the vector DNA was subjected to potassium acetate gradient centrifugation to separate the larger phage DNA from the small piece of DNA removed by restriction enzyme digestion (2). A partially double-stranded DNA insert which contained the degenerate sequence $(NNK)_6$, where N equals A, G, C, or T and K equals G or T, was constructed and ligated to approximately 13 μg of purified digested phage DNA. The remainder of the procedure was carried out as described previously (7). The insert-containing phage was isolated by double polyethylene glycol precipitation of the supernatant from 1 liter of transformed *E. coli* MC1061 [*araD139* Δ(*ara-leu*)*7696* (*lac*)*I*74in *galU galK hsdR2*(r_K^- m_K^+) *mcrB1 rpsL*(Str^r)] grown overnight at 37°C in LB broth supplemented with tetracycline as described previously (22). The final phage pellets were dissolved in 2 ml of 50 mM Tris-HCl–150 mM NaCl (pH 7.5; TBS) containing 1 mg of bovine serum albumin (BSA) and 0.05% thimerosal.

Amplification and determination of titer of phage. The phage to be amplified was added to an equal volume of *E. coli* K91Kan (HfrC *lacZ*::mTn*10*Kan *thi*) grown to an optical density of 0.5 in LB broth supplemented with 25 μg of kanamycin per ml. The phage-cell mixture was incubated for 30 min at 37°C and then plated on LB agar containing 25 μg of tetracycline per ml. The plates were then incubated for 24 h at 37°C. The colonies were scraped off the agar surface and resuspended in TBS. The cell suspension was cleared by centrifugation in Oakridge tubes at 15,000 × *g* for 30 min, and the phage was isolated by double polyethylene glycol precipitation as described before. The titer of the phage was determined by essentially the same procedure except that serial dilutions of

phage in TBS were made and added to *E. coli* K91Kan as described above. The titer was derived by counting colonies of tetracycline-resistant transductants.

Library screening and clone selection. The library was screened twice with biotinylated MAb 2A10 by a modification of the method of Parmley and Smith (21). A fresh aliquot of the library was used each time. Two strategies were used for screening. The initial concentrations of biotinylated MAb 2A10 were 20 μg/ml for strategy 1 and 100 μg/ml for strategy 2. Falcon petri dishes (60 by 15 mm) were coated overnight with 1 ml of a 1-mg/ml concentration of streptavidin in 0.1 M $NaHCO_3$ buffer (pH 9) at 4°C. At the same time, biotinylated MAb 2A10 (2 or 10 μg) was added to 100 μl of a library stock containing approximately 10^{11} transducing units (TU)/ml. The samples were allowed to react overnight at 4°C in 1.5-ml microcentrifuge tubes. The following day, the streptavidin solution was removed from the plates. The plates were then blocked by adding 5 ml of 0.1 M $NaHCO_3$ (pH 9) containing 3% BSA and 3 μg of streptavidin per ml, rotated for 1 h on an orbital shaker at room temperature, and then washed three times with TBS containing 0.5% Tween. The phage-antibody mixture from the previous night was placed on the streptavidin-coated plates and allowed to react for 10 min at room temperature. Unbound phage was removed by washing 10 times with 5 ml of TBS containing 0.5% Tween. Finally, the bound phage was eluted by washing for 10 min with 800 μl of 0.1 M HCl adjusted to a pH of 2.2 with glycine. The eluate was neutralized by the addition of 38 μl of 2 M Tris base. After each round of panning, the phage was amplified as described above. The phage was then used for the subsequent round of selection. Three rounds of selection were carried out in strategy 1, and two rounds were carried out in strategy 2. The amount of antibody used in the subsequent round was always 1/10 of that used in the previous round of selection. The phage obtained after the last round was used to infect *E. coli* K91Kan, and the cells were plated at low density on LB agar containing 25 μg of tetracycline per ml. Colony lifts were performed on nitrocellulose filters as described previously (22). The filters were incubated in horseradish peroxidase-conjugated MAb 2A10 (5 μg/ml) for 2 to 4 h. Colorimetric detection was done by the addition of the substrate 4-chloro-1-naphthol (500 μg/ml) in TBS-methanol (5:1) containing 0.015% hydrogen peroxide. Positive colonies were picked and grown in 2 ml of LB broth containing 25 μg of tetracycline per ml overnight at 37°C, and the phage was isolated by double polyethylene glycol precipitation as described before for further analysis and DNA sequencing.

DNA sequencing. The amino acid sequence of the insert in the isolated clones was deduced by single-stranded DNA sequencing using the *fmol* (Promega, Madison, Wis.) sequencing kit with the primer CGCCTGTAGCATTCCACA GACAGCCC as described in the manufacturer's recommendations. Typically, 1 μl of phage obtained from a 2-ml culture was used for each reaction mixture without further DNA extraction. The samples were subjected to 30 cycles of denaturation (95°C for 30 s) and elongation (70°C for 1.5 min). Sequencing reactions were separated on 6% acrylamide gels as described previously (22).

Peptide synthesis and conjugation. All peptides were synthesized by standard solid-phase methods (3). For immunizations, the peptide $(TNRNPQ)_4C$ was synthesized and conjugated to ovalbumin via the carboxy terminus as described previously [$(TNRNPQ)_4$C-ovalbumin)] (16). The synthetic amino-terminal portion of the lipoprotein of *E. coli*, *N*-palmitoyl-*S*-[2,3(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam3Cys), was purchased from Bachem, Torrance, Calif. The lipopeptide antigen Pam3Cys-SS$(TNRNPQ)_4$-Th, where Th is the universal T-helper epitope sequence of *P. falciparum* CSP (IEKKIAKMEKASSVFNVV) (24), was synthesized by solid-phase synthesis as described previously (26).

Animal immunizations. All animals were treated in accordance with institutional guidelines. New Zealand White rabbits were given a primary immunization intramuscularly (i.m.) with 100 μg of $(TNRNPQ)_4$C-ovalbumin, or with ovalbumin for controls, emulsified 1:1 in Freund's complete adjuvant and then boosts with 150 μg of antigen emulsified 1:1 in Freund's incomplete adjuvant at biweekly intervals. Rabbits R56795 and R56487 were bled at week 5, 5 days after the second boost. The remaining rabbits were boosted three times and bled 3 days after the last boost at week 7 (see Table 2). Six- to 8-week-old female BALB/c and C57BL/6 mice were also immunized intraperitoneally (i.p.) with 75 μg of $(TNRNPQ)_4$C-ovalbumin, or with ovalbumin for controls, emulsified 1:1 in Freund complete adjuvant. The animals were boosted twice at biweekly intervals with 75 μg in Freund incomplete adjuvant and bled at week 6. For immunizations with Pam3Cys-SS$(TNRNPQ)_4$-Th, 6- to 8-week-old female BALB/c and C57BL/6 mice were given a primary immunization i.m. with 50 μg of antigen or Pam3Cys for controls. The antigen was sonicated with 10 bursts in phosphate-buffered saline (PBS) and emulsified 1:1 in 20% intralipid. Subsequent boosts were carried out with the same amount of antigen at biweekly intervals. For the last boost, the antigen was emulsified in Freund incomplete adjuvant instead of intralipid, and the mice were bled at week 6. Blood samples from mice of the same group were pooled and used in an enzyme-linked immunosorbent assay (ELISA) and an immunofluorescence assay (IFA). Serum samples from rabbits were tested individually.

ELISA. ELISAs of serum were performed as described previously (27). The recombinant protein R32LR containing the sequence $[(NANP)_{15}NVDP]_2$ (28) was used as capture antigen (0.1 μg per well). The peptide $(TNRNPQ)_4C$ was also used as capture antigen at a concentration of 0.01 μg per well. For inhibition ELISA, peptides were dissolved in 0.01 M phosphate–0.15 M NaCl (pH 7.4; PBS) at different concentrations and then diluted 1:1 in PBS containing 0.5% boiled casein, 0.05% Tween, and 10 μg of phenol red per ml. The pH was corrected to 6 to 7 by the addition of 0.1 to 2 μl of 2 M Tris base. Horseradish peroxidase-conjugated MAb 2A10 was added to a final concentration of 50 ng/ml. This concentration of antibody had been determined in previous experiments to be below saturation for the amount of capture antigen used. The solutions were incubated at 4°C for 3 h, and 50 μl was transferred to triplicate wells of a microtiter plate previously coated overnight with R32LR (0.1 μg per 50 μl per well) in PBS and blocked with PBS containing 0.5% boiled casein and 1% Tween. After this step, the plates were treated as described previously (27). All ELISA experiments were done at least twice with triplicate points.

IFA. Salivary gland sporozoites for the IFA were produced by feeding laboratory-reared *Anopheles stephensi* mosquitoes on *P. falciparum* NF54- or 7G8-infected cell cultures. The CSP of strain NF54 differs from that of strain 7G8 in that the former contains a 19-amino-acid deletion in the amino-terminal nonrepeat portion of the molecule (6). Salivary gland sporozoite infection rates were monitored, and sporozoites from infected glands were harvested in medium 199 (Sigma Chemical Co., St. Louis, Mo.), counted with a hemocytometer, and immediately transferred to multiwell (2,000 to 4,000 sporozoites per spot) printed slides, air dried at room temperature, and stored at −20°C until used. IFAs were initiated by spreading 30 μl of serum diluted in Dulbecco's PBS (pH 7.4; Sigma) onto the slide well containing the dried sporozoites. The slide was incubated in a moist chamber at room temperature for 30 min. The well was then aspirated dry and washed once with 30 μl of PBS; the addition of a 30-μl aliquot of fluorescein-labeled anti-mouse or anti-rabbit immunoglobulin G (heavy plus light chain; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.; 1:100 in PBS) followed. After 30 min, the well was washed as described above before being mounted in glycerol-PBS (10:1). Slides were examined under UV light at ×500 magnification for fluorescence and graded from 0 to 4+, with 0 equal to no fluorescence and 4+ equal to fluorescence seen uniformly over the sporozoite. The titer was determined as the last dilution to give a fluorescence of 1+. The location of the sporozoites on the slide was confirmed by phase microscopy. The visible light and fluorescent outline of the sporozoites always coincided. All IFAs were performed and read at least twice by one person (R.A.W.) who was blinded to the experimental treatment of the animals.

Electrophoresis and Western blotting immunoblotting. Sporozoites were harvested from salivary glands of infected mosquitoes as described previously (4). Protein from 5×10^4 sporozoites was solubilized in Laemmli's sample buffer (17) by heating at 95°C for 10 min. The proteins were separated on 4 to 20% gradient polyacrylamide gels containing sodium dodecyl sulfate (17). The proteins were transferred to a nitrocellulose membrane (25). Membranes were incubated for 1 h in blocking buffer (3% BSA, 0.5% boiled casein, 10 mM Tris, 150 mM NaCl, 0.5% Tween [pH 8.0]) and then incubated for 2 h with pre- or postimmune sera from rabbits R56795, R56487, R01159, and R97990. Several dilutions of serum in blocking buffer were used (1:100, 1:200, 1:400, and 1:500) in preliminary experiments to determine the one dilution that gave the lowest possible background. The diluted serum was preabsorbed overnight with a blank piece of nitrocellulose filter paper. The membranes were then washed three times with 10 mM Tris (pH 8.0)–150 mM NaCl–0.5% Tween (TNT) and incubated for 1 h in a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) in TNT. The membranes were washed three times in TNT, and antibody binding was detected by chemiluminescence (Amersham, Arlington Heights, Ill.) by exposing X-ray film for 5 s.

RESULTS

Library construction and screening. An estimate of the total number of phage-containing hexapeptide inserts was determined by multiplying the number of tetracycline-resistant transformants by the percentage of clones producing infectious phage since insertion of the hexapeptide into the amino terminus of gene III protein restores infectivity. By this estimate, we recovered 2×10^7 clones with inserts. Since this number is less than the theoretical maximum number of hexamers possible ($20^6 = 6.4 \times 10^7$), a majority of these clones probably had unique sequences. Three distinct sequences were recovered among 36 clones analyzed after the first screen (strategy 1): TNRNPQ, SNRNPQ, and NNDNPQ (Table 1). The successive use of smaller concentrations of antibody in screening the library should theoretically select for higher-affinity ligands as a result of competition for fewer antibody-binding sites. Therefore, to obtain a wider range of ligands, the library was screened a second time with higher concentrations of antibody (strategy 2). Ten clones were analyzed after two rounds of selection. In addition to all of the previously observed sequences, two new sequences were found: SNYNPQ and QND-

TABLE 1. Amino acid sequences obtained after screening a phage epitope library with MAb 2A10

Source	Sequence(s)[a]
Native epitope	PNANPN
Strategy 1	TNRNPQ (44)
	SNRNPQ (39)
	NNDNPQ (17)
Strategy 2	SNRNPQ (10)
	TNRNPQ (20)
	NNDNPQ (50)
	SNYNPQ (10)
	QNDNPQ (10)

[a] All values in parentheses indicate the percentages of clones found bearing that sequence.

NPQ. However, all clones had the same consensus sequence detected previously, XNXNPQ (Table 1).

Inhibition ELISA. Competitive ELISAs were performed to demonstrate that the new mimotopes bind to MAb 2A10 at the antigen-binding site. Two of the mimotopes (TNRNPQ and NNDNPQ) were compared with the native epitope (PNANPN) for their ability to inhibit the binding of MAb 2A10 to the recombinant protein R32LR in this system. Since the phage expresses the mimotopes as only one copy per molecule of gene III, we compared levels of peptide inhibition on the basis of monomeric units rather than repeats. To enhance the efficiency of binding to the MAb, each mimotope was synthesized as N-terminal to the sequence GGTVES, the 6-amino-acid sequence immediately following the random hexapeptide sequence in the phage vector, thereby retaining their monovalency (7). The sequence NTPRNQ was synthesized as a negative control "scramble" of the mimotope TNRNPQ (Table 2). We also compared the inhibition produced by the sequence $(TNRNPQ)_4C$ with that of the native repeat $(NANP)_6C$. Although the concentration of monovalent mimotopes required for 50% inhibition of MAb 2A10 binding to R32LR was less than half that of the monovalent native sequence, the CSP repeat peptide $(NANP)_6C$ was far superior to either multiple copies of the mimotope $(TNRNPQ)_4C$ or the monovalent CSP peptide PNANPNGGTVES (Table 2).

Animal immunizations. Peptides containing one and three repeats of TNRNPQ conjugated to ovalbumin were used in preliminary experiments to immunize mice. These peptides failed to induce a cross-reactive humoral response that recognized R32LR by ELISA despite the presence of adequate antipeptide titers (data not shown). We therefore synthesized a longer repeat of TNRNPQ for immunizations in the form of the conjugate [$(TNRNPQ)_4C$-ovalbumin] and the lipopeptide Pam3Cys-SS$(TNRNPQ)_4$-Th. Sera from immunized and control animals were tested by ELISA for the presence of antibodies against the capture antigens $(TNRNPQ)_4C$ or R32LR and by IFA against *P. falciparum* sporozoites.

TABLE 2. Fifty percent inhibitory concentrations (IC_{50}s) of different peptides derived by inhibition ELISA

Peptide	IC_{50} (mM)
PNANPNGGTVES	>8
NTPRNQGGTVES[a]	∞[b]
TNRNPQGGTVES	~2
NNDNPQGGTVES	~2
$(QEETHK)_4C$[a]	∞
$(TNRNPQ)_4C$	~0.1
$(NANP)_6C$	~0.01

[a] Peptides used as negative controls.
[b] ∞, too large to measure.

Antisera obtained from rabbits immunized with the antigen $(TNRNPQ)_4C$-ovalbumin showed significant recognition of the peptide $(TNRNPQ)_4C$ and R32LR by ELISA and against *P. falciparum* sporozoites by IFA (Table 3). One of three rabbits (R56487) also recognized *P. falciparum* CSP by Western blot (Fig. 1).

Both strains of inbred mice responded vigorously to immunization with the $(TNRNPQ)_4C$-ovalbumin conjugate as measured by ELISA responses to the immunizing peptide (Table 3). C57BL/6 mice also developed high antipeptide titers following immunization with the Pam3Cys-SS$(TNRNPQ)_4$-Th antigen. Interestingly, whereas BALB/c mouse responses to this antigen were low but definitely positive, these were the only mice that developed antibodies reactive with R32LR by ELISA. BALB/c mice immunized with the antigen Pam3Cys-SS$(TNRNPQ)_4$-Th and C57BL/6 mice immunized with the $(TNRNPQ)_4C$-ovalbumin conjugate also developed antibodies reactive with intact sporozoites by IFA, albeit at low titers. This IFA reactivity could be inhibited by incubation of sera with the antigen R32LR but not by incubation with a peptide containing the sequence of the T-helper epitope (Th) prior to performing the IFA, thus confirming the specificity of these reactivities (data not shown).

DISCUSSION

It is clear from the work of other investigators that peptide mimotopes can be obtained from phage epitope libraries (7, 8, 23) or by chemical synthesis (11, 14, 18) and that these sequences bind antibodies raised against native structures. The larger issue of whether such mimotopes can be exploited to elicit functional antibodies against pathogenic organisms has yet to be fully resolved. In this study, we selected a well-characterized functional MAb and its target epitope from the *P. falciparum* CSP system to ask two relevant questions. (i) Could mimotopes be obtained from a phage library that elicits a relevant CSP antibody response? (ii) Would these mimotopes provide clues regarding the nature of the binding sites of neutralizing MAb against *P. falciparum* sporozoites?

MAb 2A10 is a mouse MAb (immunoglobulin G) that was raised by immunization of mice with *P. falciparum* sporozoites (19). The linear epitope to which it maps, PNANPN from the central repeat region of the CSP (5), is the principal B-cell epitope included in nearly all CSP-based malaria subunit vaccines tested to date in humans. These studies have suggested that while antibody-mediated protection against malaria sporozoites can be achieved, these epitopes are poorly immunogenic for many humans, and responses directed against this linear sequence have not always correlated with antibody titers as measured against intact sporozoites. Thus, we and others have speculated that the preferred target of MAb 2A10 on sporozoites may be conformationally constrained through interactions of distant binding sites on the same or adjacent CSP molecules.

Although we expected to find mimotopes, we were surprised that none of the clones obtained by screening the phage library with MAb 2A10 showed exact homology with the native linear epitope PNANPN. This discrepancy might have been due to the incomplete repertoire of the library and, thus, the absence of any phage bearing this sequence. Alternatively, our inhibition ELISA data support the possibility that phage bearing sequences closely resembling the native epitope was excluded by competition with sequences more avidly bound by the an-

TABLE 3. Immunogenicity of mimotope peptide repeat $(TNRNPQ)_4$ in rabbits and mice

Animal	Immunogen	Route	ELISA[a] results for:		IFA results[b]
			$(TNRNPQ)_4C$	R32LR	
Rabbits					
R56795	Ovalbumin	i.m.	<1	<1	Neg[c]
CR32	Ovalbumin	i.m.	<1	<1	Neg
R01159	$(TNRNPQ)_4C$-ovalbumin	i.m.	>6,400	131	1:400
R97990	$(TNRNPQ)_4C$-ovalbumin	i.m.	>6,400	25	1:100
R56487	$(TNRNPQ)_4C$-ovalbumin	i.m.	>6,400	584	1:1,600
Mice[d]					
BALB/c	Ovalbumin	i.p.	<1	<1	Neg
BALB/c	$(TNRNPQ)_4C$-ovalbumin	i.p.	>6,400	<1	Neg
BALB/c	Pam3Cys	i.m.	<1	<1	Neg
BALB/c	Pam3Cys-SS$(TNRNPQ)_4$-Th[e]	i.m.	10	35	1:25
C57BL/6	Ovalbumin	i.p.	<1	<1	Neg
C57BL/6	$(TNRNPQ)_4C$-ovalbumin	i.p.	>6,400	<1	1:100
C57BL/6	Pam3Cys	i.m.	<1	<1	Neg
C57BL/6	Pam3Cys-SS$(TNRNPQ)_4$-Th	i.m.	>6,400	<1	Neg

[a] Results are expressed in optical density units, with the dilution giving an optical density of 1.00 against the indicated capture antigen.
[b] Immunofluorescence results against *P. falciparum* sporozoites (NF54) are expressed as the greatest dilution giving a positive signal.
[c] Neg, negative.
[d] Five mice per group.
[e] Th, T-helper epitope from *P. falciparum* CSP (IEKKIAKMEKASSVFNVV).

tibody. Whatever the reason for the failure to recover the PNANPN sequence from the library, the technique clearly was successful in revealing a series mimotopes and thus validating this screening concept in a relevant model of parasite immunity.

An analysis of the amino acid sequence of the *P. falciparum* CSP reveals the presence of several areas of homology with the mimotopes outside of the main repeat region. Specifically, the sequence NNDNPQ has 50% homology with amino acids 82 to 87 (NGDNGR) and amino acids 99 to 103 (NEDNEK) (5). The existence of other areas of homology outside the main repeat region of *P. falciparum* CSP is further supported by the finding that MAb 2A10 weakly binds the region of *P. falciparum* CSP between amino acids 301 and 310, as determined by Geysen-pin analysis (5). It is interesting that amino acids 301 to 310 contain the sequence PNRNVD, which has 50% homology with T/SNRNPQ (5). Therefore, MAb 2A10 may recognize several regions within the same molecule that may represent cross-reactive epitopes. This is of interest since the existence of cross-reactive epitopes within a molecule or between different molecules has been proposed as a mechanism by which malaria parasites may avoid induction of high-affinity antibodies in the host (1).

The poor inhibition demonstrated by the monovalent mimotope PNANPNGGTVES is in agreement with previous observations that peptides containing less than three repeats of NANP poorly inhibit the binding of MAb 2A10 (20). The >100-fold increase in inhibition obtained when using the peptide $(NANP)_6C$ instead of the peptide PNANPNGGTVES is also in agreement with previous studies (20) and cannot be explained by an increase in the number of binding sites in the former molecule. These observations, and the fact that IFA responses in BALB/c mice were detected in the absence of high antipeptide titers, suggest that the full-length CSP molecule or synthetic fragments containing multiple NANP repeats may adopt a conformational structure that is recognized preferentially by MAb 2A10. These data imply that antibody responses to subunit vaccines based on the CSP may be critically affected by the conformational structure adopted by their repeat sequences during antigen presentation and processing.

All rabbits immunized with the antigen $(TNRNPQ)_4$-

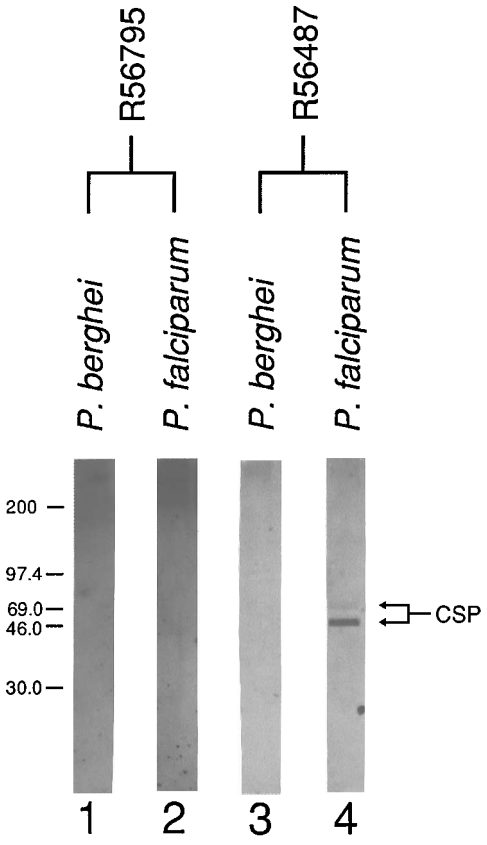

FIG. 1. Western blot of *Plasmodium berghei* (lanes 1 and 3) and *P. falciparum* (lanes 2 and 4) sporozoite extracts using postimmunization sera from rabbits R56795 (ovalbumin immunized) and R56487 [$(TNRNPQ)_4$-ovalbumin immunized] at a 1:500 dilution. There are 5×10^4 sporozoites per lane. Numbers on the left indicate molecular masses in kilodaltons. The molecular mass of the immunoreactive protein in lane 4 is in agreement with that previously reported for *P. falciparum* CSP (30).

ovalbumin recognized *P. falciparum* sporozoites by IFA and R32LR by ELISA (Table 3). One rabbit (R56487) also recognized *P. falciparum* CSP by Western blot (Fig. 1). The lack of Western blot recognition in the other two rabbits may be due to low titers or, alternatively, to the loss of an important conformational determinant upon denaturation. In contrast to the response in rabbits, only half of the groups of mice recognized *P. falciparum* sporozoites by IFA (Table 3). In all animals, the presence of anti-R32LR antibodies was predictive of a positive IFA against *P. falciparum* sporozoites. The presence of antibodies against the peptide $(TNRNPQ)_4C$ was a poor predictor of the recognition of *P. falciparum* sporozoites by IFA, especially in mice. This suggests that in most cases only a fraction of the antibodies directed against the peptide $(TNRNPQ)_4C$ cross-react with R32LR or sporozoites. The exception is BALB/c mice immunized with the lipopeptide antigen which paradoxically have a weak response against the peptide $(TNRNPQ)_4C$ and yet have the highest amount of antibody against R32LR of all the mice. Therefore, despite having a weak humoral response against $(TNRNPQ)_4C$, most of the antibodies produced by BALB/c mice immunized with the lipopeptide antigen also recognized R32LR and sporozoites by IFA.

In contrast to previous studies of the immunogenicity of the $(NANP)_n$ repeat in animals (4), the peptide $(TNRNPQ)_4C$, although immunogenic, seems to induce low antibody titers against R32LR in mice. The immunogenicity of CSP peptide repeats in mice has been found to be closely linked to the major histocompatibility complex haplotype of these animals (12). Therefore, lack of induction of the desired antibody response in some of the mice could then be attributed in part to their restricted major histocompatibility complex repertoire. Another possible explanation is that the peptide $(TNRNPQ)_4C$ may lack the optimal conformation to induce a cross-reactive immune response. Our failure to induce the appropriate cross-reactive humoral response with peptides containing less than four repeats, despite the presence of antipeptide antibodies, also suggests that a more complex conformational structure may be required to stimulate the desired B-cell clones.

Our successful use of mimotopes to induce an immune response against the native antigen of an important pathogen is supported by recent work by Folgori et al. (10). These investigators screened a phage library with polyclonal antisera against hepatitis B surface antigen to obtain mimotopes which induced an immune response against the native antigen. Keller et al. (15) also recovered sequences that mimic a V3 loop motif of human immunodeficiency virus type 1 and induced an immune response against the virus. However, in the latter example, the sequences recovered matched the native epitope so closely that they could be considered to be identical. Other studies have not been as successful in demonstrating the utility of mimotopes (9). Our results together with those cited above suggest that phage epitope libraries may have an application in the design of subunit vaccines. We and others (10) believe that this strategy may permit the design of immunogenic peptides without prior knowledge of the amino acid sequence of a target antigen. In addition, the induction of the appropriate humoral response in one species using a mimotope identified by antibodies from a second species suggests that the antibody used to screen the phage library may not need to be of the same species for which a vaccine is ultimately intended. This is important given the scarcity of human MAbs raised against pathogens.

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