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Immunology

Immunogenicity of an engineered internal image antibody

(molecular mimicry/engineered idiotype/malaria vaccine)

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ABSTRACT **We engineered an antibody expressing in the third complementarity-determining region of its heavy chain variable region a "foreign" epitope, the repetitive tetrapeptide Asn-Ala-Asn-Pro (NANP) of the circumsporozoite protein of *Plasmodium falciparum* parasite, one of the etiologic agents of malaria in humans. A monoclonal antibody to *P. falciparum* specific for the $(NANP)_n$ amino acid sequence bound to the engineered antibody, and a synthetic $(NANP)_3$ peptide blocked this interaction. Immunization of rabbits and mice with the engineered antibody resulted in the elicitation of a humoral response to $(NANP)_3$ synthetic peptide and *P. falciparum* parasite. In mice, in which immunity to the $(NANP)_n$ epitope is highly restricted by immune response genes, antibodies were induced in responder and nonresponder haplotypes of the major histocompatibility complex. Rabbit antibodies efficiently inhibited the *in vitro* invasion of cultured liver cells by *P. falciparum* parasite. Collectively, this study indicates that immunity to malaria in the absence of the parasite can be induced using antibody variable regions engineered to mimic the parasite's molecular structure. In general terms, the results suggest that antibody (idiotype) mimicry of an exogenous antigen is possible and may only require a discrete stretch of identity between the two molecules. The implication for the preparation of antibody-based vaccines and idiotype regulation of immunity are discussed.**

Immunoglobulins are the main effector of humoral immunity, a property linked with their ability to bind antigens. A second general property is the immunogenicity of their antigenic determinants (idiotypes) whereby they can regulate the immune system in a predictable way (1). The sequence of events antigen → idiotype → anti-idiotype (2–4) and the ability of the latter to mimic peptide (5) and carbohydrate epitopes (6, 7) (internal image anti-idiotypes) (8) constitute a framework for the specific and rational manipulation of the immune system. Structurally, the immunogenic property of immunoglobulins resides in the complementarity-determining regions (CDRs) of their variable (V) domains, sites where changes in sequence and conformation are tolerated with little, if any, effect on the framework of the molecule (9). For instance, the CDRs of one antibody grafted into another immunoglobulin molecule maintain in the new molecular environment the antigen-binding property of the donor antibody (10–12).

Mimicry of antigens by antibodies has been reported in many systems and internal image antibodies generated through a conventional idiotypic cascade served to immunize animals against bacteria, viruses, and parasites (13, 14). Whereas the structural basis for this phenomenon remains by and large unclear, few reports exist to suggest that functional mimicry can be associated with shared primary structure between an antibody's CDR and the respective nominal antigen (15–17).

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In the study presented here we used protein engineering techniques to verify whether an antibody expressing a "foreign" peptide epitope as an integral part of a V region could be used to induce immunity of predetermined specificity *in vivo*. We tested this hypothesis using a chimeric mouse/human antibody (γ1NANP) constructed to express three copies of the tetrapeptide Asn-Ala-Asn-Pro (NANP) of malaria's *Plasmodium falciparum* circumsporozoite (CS) protein in the CDR3 of the heavy (H) chain. The rationale to this experiment was 2-fold. On one hand, we chose to engineer the CDR3 of the H chain as studies from this laboratory indicated that the CDR3 loop of the murine V_H (18) used expresses an immunodominant idiotype at the surface of the molecule (19). On the other hand, we selected the NANP sequence, an oligopeptide that occurs as 37 tandem repeats interspersed with 4 repeats of the variant sequence Asn-Val-Asp-Pro (NVDP), because it is immunodominant in humans and correlates with protective immunity (20). Monoclonal antibodies (mAbs) that passively protect animals against sporozoite infection bind to the repeat region of the CS protein (10). Immunization of mice and rabbits with the dodecapeptide $(NANP)_3$ coupled to tetanus toxoid induced antibodies that neutralized *P. falciparum* sporozoite infectivity to cultured liver cells (21). A similar immunization protected a human volunteer to *P. falciparum* sporozoite challenge (22).

Our experiments show that rabbits and mice immunized with an engineered antibody bearing the *P. falciparum* sporozoite $(NANP)_3$ molecular structure in a loop of the V region produced antibodies specific for that loop, the corresponding synthetic peptide, and the parasite's antigen in its natural environment. These results imply that homology at the primary structure level between an antibody CDR(s) and an exogenous antigen could be sufficient in certain instances for the induction of immunity to that antigen by the antibody by means of the known rules of idiotype regulation of the immune response.

MATERIAL AND METHODS

Animals. Adult New Zealand White rabbits were purchased from a local breeder. Eight-week-old C57BL/6 (*H-2*^{b}), BALB/c (*H-2*^{d}), C3H/He (*H-2*^{k}), and SJL (*H-2*^{s}) mice were purchased from The Jackson Laboratory.

Protein Engineering Methods. The productively rearranged V_H gene used (V_H62) was isolated as described (18). The D region (KAYSHG; residues 93–98) was mutagenized by introducing a unique restriction site, *Kpn* I/*Asp*718, to yield the intermediate sequence (KVPYSHG; residues 93–99), in which 94A was deleted and substituted by the VP doublet.

Abbreviations: CDR, complementarity-determining region; MHC, major histocompatibility complex; C, constant; V, variable; H, heavy; L, light; CS, circumsporozoite; mAb, monoclonal antibody; HP, horseradish peroxidase; WT, wild-type; ISI, inhibition of sporozoite invasion.

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The complementary synthetic oligonucleotides 5′-GTACCCAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA-3′ and 5′-GTACTGGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTGG-3′ coding for three copies of the NANP tetramer were introduced between 94V and 95P of the modified V_H region. The 2.3-kilobase (kb) *Eco*RI fragment coding the engineered V_HNANP gene was cloned upstream from a human γ1 constant (C) region gene contained in the 12.8-kb vector pNγ1 (19). The final construct, pNγ1NANP (Fig. 1*a*), was electroporated in the murine J558L cell line that carries the rearrangement for a λ_1 L chain (23). G418-resistant clones secreting the engineered antibody, γ1NANP, were identified by enzyme-linked immunosorbent assay (ELISA) using a horseradish peroxidase (HP)-conjugated goat antibody to human immunoglobulin (Sigma). The construction of the wild-type (WT) chimeric antibody (γ162) has been described (18, 19).

Synthetic Peptides. The following synthetic peptides (p) served in this study: 14-mer KK(NANP)$_3$, 15-mer YYCARKAYSHGMDYW of CDR3 and 17-mer CAINSNGGSTYYPDTVK of CDR2 of the WT V_H62, 15-mer SHIPAVHPGSFRPKC and 10-mer DENGNYPLQC of the human invariant chain, and 15-mer YPQVTRGDVFTMPED derivative of vitronectin. They were prepared by a solid-phase method.

Immunochemical Reagents. Murine mAb Sp3-B4 (IgG2a, k) was generated by immunization with *P. falciparum* parasite, is specific for the repetitive epitope $(NANP)_n$ of the CS protein, and was the kind gift of H. Matile (Hoffman–La Roche). mAbs 62 (γ1, k; anti-thyroglobulin) and 10.12 (γ2b, λ; anti-2,4-dinitrophenol) have been described (18). Human gamma globulins, Cohn fraction II, were purchased from Miles. Transfectoma antibodies γ1NANP (γ1, λ), WT (γ1, λ), and γ1Vk (γ1, k) (19) were purified using a Sepharose 4B-protein A column and eluted with 0.2 M glycine hydrochloride buffer (pH 3.0). They were analyzed on 10% SDS/polyacrylamide gels under reducing and nonreducing conditions. Proteins were stained with Coomassie blue dye.

Immunological Methods. Antibodies to p(NANP)$_3$ were detected by ELISA on 96-well polyvinyl microtiter plates coated with p(NANP)$_3$ (5 μg/ml). The bound antibodies were revealed using HP-conjugated goat antibody to rabbit immunoglobulin absorbed with human gamma globulins (Tago). The binding of preimmune sera served as the background value. The titers were expressed as the geometric mean of reciprocal end-point serum dilution giving an A_{492} reading ≥0.200. Quantitation of antibodies was done by radioimmu-

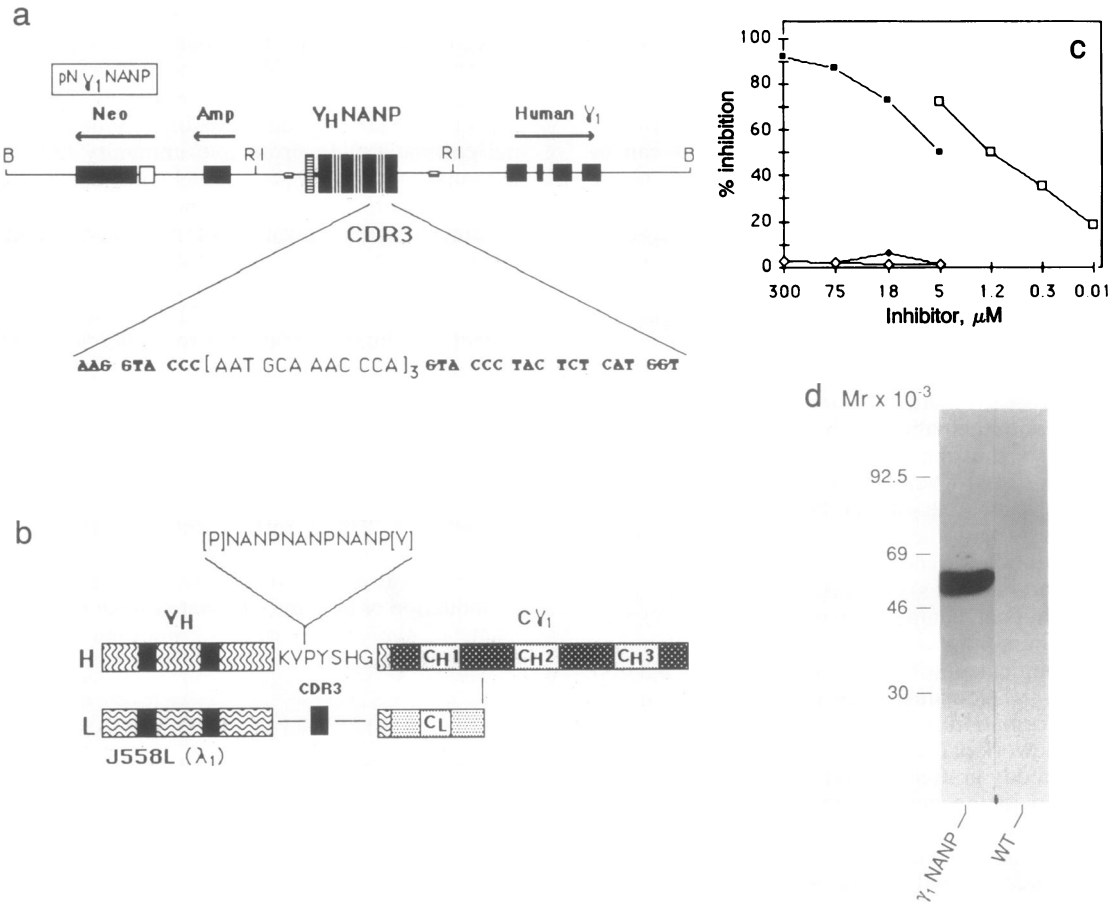

FIG. 1. (*a*) Diagram illustrating the pNγ1NANP expression vector. The CDR3 of V_H62 (residues 93–98) was mutagenized as described in the text. B, *Bam*HI; RI, *Eco*RI; Neo, neomycin (G418) resistance; Amp, ampicillin resistance. (*b*) Scheme depicting the H and light (L) chains of engineered mouse/human chimeric γ1NANP antibody (not to scale). In the H chain the human γ1 C region is fused with murine V_H62 gene encoding the $(NANP)_3$ repetitive epitope in CDR3. As indicated, the D segment was modified by inserting the amino acid residues $(NANP)_3$ between 94V and 95P. In the engineered V_H region the dodecapeptide $(NANP)_3$ is flanked at each side by V and P residues [VP $(NANP)_3$ VP]. These residues originate from the construction of the *Kpn* I/*Asp*718 restriction site in which A was substituted with a V and P, yielding the formation of two VP doublets at each end of the inserted $(NANP)_3$ peptide epitope. The L chain is murine λ_1 that is provided by the myeloma J558L cell. (*c*) Specific inhibition of the binding of mAb Sp3-B4 to synthetic p(NANP)$_3$ (5 μg/ml) by γ1NANP. ^{125}I-labeled mAb Sp3-B4 was mixed (vol/vol) with the following inhibitors: p(NANP)$_3$ (■); γ1NANP (□); WT (◆); and γ1Vk (◇). Tests were done in triplicate. Binding without inhibitor was ≈5.300 cpm. (*d*) ^{125}I-labeled mAb Sp3-B4 binds specifically the H chain of recombinant γ1NANP (left lane) but not WT (right lane). Exposure time: 18 hr at −70°C.

noassay (RIA) on microtiter plates coated with $F(ab')_2$ fragments of a goat antibody to rabbit immunoglobulin (2.5 μg/ml). ^{125}I-labeled (24) goat antibody to rabbit immunoglobulin served as the revealing antibody. The counts per minute (cpm) of experimental sera were plotted on a standard curve constructed from known amounts of rabbit immunoglobulin. Results were expressed as mean ± SEM of triplicate wells.

Specificity of binding was assessed in inhibition studies. A fixed amount of ^{125}I-labeled mAb Sp3-B4 or affinity-purified rabbit anti-$(NANP)_3$ antibodies was mixed (vol/vol) by rocking overnight at +4°C with decreasing amounts of inhibitor (mAb, transfectoma antibody or synthetic peptide) diluted in phosphate-buffered saline (PBS, pH 7.3) containing 1% bovine serum albumin (BSA) and 1% Tween 20 (PBSA). The mixture was then incubated (50 μl per well) on γ1NANP-coated wells (5 μg/ml) and bound antibodies were measured either in RIA or ELISA. Tests were done in triplicate. The percent inhibition was calculated as follows: [(average binding of antibody alone − average binding of antibody incubated in the presence of inhibitor)/average binding of antibody alone] × 100.

Western blots were performed according to Towbin *et al.* (25). Five micrograms of γ1NANP or WT was loaded onto a 10% SDS/PAGE gel, electrophoresed under reducing conditions, and then transferred to 0.45-μm nitrocellulose paper (Millipore). The nitrocellulose sheets were blocked with a 10% solution of dry milk and incubated overnight at +4°C with ^{125}I-labeled antibody Sp3-B4 (40×10^4 cpm/ml) or affinity-purified rabbit antibodies to γ1NANP subsequently revealed with ^{125}I-labeled goat antibody to rabbit immunoglobulin. After the filters were washed, they were dried and exposed to Kodak XAR-5 film at −70°C for 18 hr.

Indirect immunofluorescence staining was done as follows. *P. falciparum* sporozoites were isolated from mosquito salivary glands, diluted in medium 199 containing 0.5% BSA, air-dried on a glass slide (2000–5000 sporozoites per well), fixed in acetone for 5 min, and incubated for 30 min with 20 μl of serum diluted in PBS. After the slides were washed, they were incubated with fluorescein-conjugated goat antibody to rabbit or mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD), washed, mounted in 50% glycerol, and examined by fluorescence microscopy.

Immunizations. Rabbits were immunized subcutaneously in the back with 50 μg of γ1NANP or WT emulsified in complete Freund's adjuvant. Booster immunizations (50 μg) were given in incomplete adjuvant on day 30 and day 60. Sera were collected on days −1, 30, and 70 and tested by ELISA and RIA. The binding of WT-immunized rabbits served as a control. Eight-week-old mice were immunized intraperitoneally with 50 μg of γ1NANP or WT in alum on days 1, 30, and 120. Sera were collected on days −1, 30, and 130 and tested by ELISA.

RESULTS

The general structure of the engineered antibody used for immunization and the precise site of insertion of the CS oligopeptide are depicted in Fig. 1*b*. The $(NANP)_3$ oligopeptide was inserted between valine 94 and proline 95 of the mutagenized V_H62 CDR3. We used murine mAb Sp3-B4, generated against *P. falciparum* sporozoite and specific for the $(NANP)_n$ epitope, to assess the immunological accessibility of the $(NANP)_3$ epitope borne on γ1NANP. As shown in Fig. 1*c*, ^{125}I-labeled mAb Sp3-B4 bound specifically to synthetic $p(NANP)_3$. Only $p(NANP)_3$ and γ1NANP, but not other transfectoma antibodies lacking the $(NANP)_3$ epitope, inhibited binding. ^{125}I-labeled mAb Sp3-B4 also bound in Western blot to the H but not the L chain of γ1NANP (Fig. 1*d*).

Table 1. Immunogenicity of engineered antibody γ1NANP in rabbits

Immunogen	Rabbits, no. positive/total no.	Synthetic $p(NANP)_3$ Titer	Synthetic $p(NANP)_3$ μg/ml*	Control peptide titer
γ1NANP	6/6	2511	114 ± 54	<100
WT	0/4	<100	<1	<100

Titers shown refer to a day 70 bleed and are expressed as the geometric mean of reciprocal serum dilutions of ELISA binding to $p(NANP)_3$ giving an A_{492} reading ≥0.200. The control peptide is DENGNYPLQC.
*Values were calculated as described in the text.

A humoral immune response to the $(NANP)_n$ epitope of *P. falciparum* parasite was induced in all six rabbits immunized with γ1NANP but in none of four controls immunized with the WT—a recombinant molecule that differs from γ1NANP in CDR3 by lacking the $(NANP)_3$ epitope (Table 1). All animals also mounted an anti-human immunoglobulin response (not shown). In each instance antibodies to $p(NANP)_3$ became detectable 30 days after the first immunization. The rabbit sera also reacted with *P. falciparum* parasite by immunofluorescence (Fig. 2 *a* and *b*). These results suggest that $(NANP)_3$ within the engineered antibody closely mimics the CS epitope in its native configuration.

The murine response to a synthetic peptide corresponding to 40 NANP repeats or to a fusion protein containing 32 NANP repeats is restricted to the $H\text{-}2^b$ haplotype (26, 27). The γ1NANP was used to immunize mice of various *H-2* haplotypes. All mice immunized with γ1NANP, unlike those injected with WT, responded with a specific anti-$(NANP)_3$ response (Table 2). The antibody titer in the responder C57BL/6 mice ($H\text{-}2^b$) did not differ from that of BALB/c ($H\text{-}2^d$) or C3H/He ($H\text{-}2^k$) nonresponder mice. Sera from mice immunized with γ1NANP but not with WT also stained

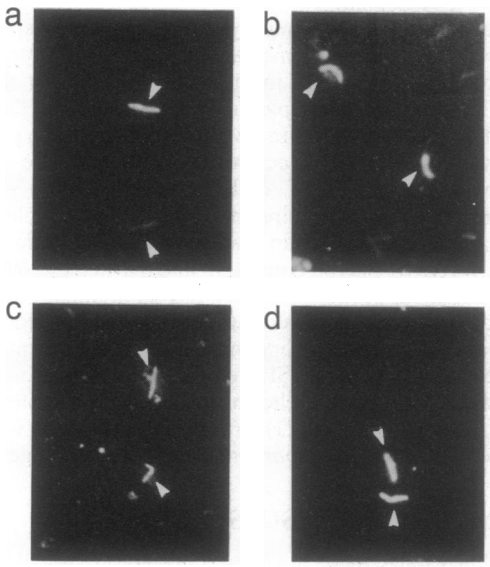

FIG. 2. Rabbit and mouse antibodies against γ1NANP bind *P. falciparum* sporozoites in indirect immunofluorescence. Fluorescent sporozoites are indicated by arrowheads. Photographs were taken at ×190 magnification using a Kodak Tri-X Pan film. The primary incubations were as follows. (*a*) Serum of a rabbit immunized with *P. falciparum* sporozoites that served as a positive control (1:500 dilution). (*b*) Serum of a rabbit immunized with γ1NANP (1:500 dilution). (*Inset*) View of a different field in the same preparation (same magnification). (*c*) mAb 2A10 (20 μg/ml) generated from a mouse immunized with *P. falciparum* sporozoites that served as a positive control. (*d*) Serum of an SJL mouse immunized with γ1NANP (1:500 dilution).

Table 2. Immunogenicity of engineered antibody γ1NANP in mice of various MHC haplotypes

Strain	*H-2*	Immunogen γ1NANP	Immunogen WT	No.	Serum antibody titer (NANP)$_3$	Serum antibody titer Control peptide
C57BL/6	*b*	+		4	1584	≤200
			+	5	≤200	≤200
BALB/c	*d*	+		5	1819	≤200
			+	4	234	≤200
C3H/He	*k*	+		3	1600	≤200
			+	3	234	≤200
SJL	*s*	+		5	9549	≤200
			+	5	398	≤200

MHC, major histocompatibility complex. Titers shown refer to a day 130 bleed and are expressed as the geometric mean of reciprocal serum dilutions of ELISA binding to p(NANP)$_3$ giving an A_{492} reading ≥0.200. The control peptide is DENGNYPLQC.

sporozoites in indirect immunofluorescence (Fig. 2 *c* and *d*). The highest titer measured in the nonresponder SJL (*H-2*^{s}) mice is not surprising as mice of this haplotype are a high responder to murine immunoglobulin allotypes (28).

We ascertained that the antibodies induced by immunization with γ1NANP were specific for the (NANP)$_3$ epitope. Rabbit antibodies purified on a p(NANP)$_3$/Sepharose-4B column reacted in ELISA with γ1NANP (Fig. 3*a*) but not with any control protein. In Western blot they bound the H chain of γ1NANP (Fig. 3*b*), a reactivity completely abrogated by preincubation with synthetic p(NANP)$_3$ (Fig. 3*c*). The ELISA binding to γ1NANP could be inhibited by γ1NANP and p(NANP)$_3$ but not by any of the various proteins and synthetic peptides used as controls, including pCDR3 and pCDR2 of the WT V_H primary structure (Fig. 3 *d* and *e*). Collectively, these results suggest that antibodies induced by immunization with γ1NANP (*i*) possess specificity for the primary-structure-dependent (NANP)$_3$ epitope engineered in CDR3 and (*ii*) were induced by means of an idiotypic mechanism.

Protective humoral immunity to *P. falciparum* sporozoite infection is thought to be mediated by anti-NANP antibodies that intercept sporozoites in the bloodstream after the mosquito's bite and block sporozoite invasion of liver cells (21). *In vitro* inhibition of sporozoite invasion (ISI) has been proposed as an assay for testing protective antibodies (29). We explored the biological activity of antibodies induced by γ1NANP immunization by determining their ISI activity (Table 3). The serum and immunoglobulin fraction of a rabbit immunized with γ1NANP displayed consistent and marked (>80%) levels of inhibition, suggesting that γ1NANP immunization induced anti-sporozoite antibodies that recognize a conformation-dependent protective epitope important in the mechanism of liver cell infection. The ISI titer was comparable to that of purified immunoglobulin elicited by (NANP)$_3$ coupled to tetanus toxoid in rabbits (21) or that of a human volunteer protected against *P. falciparum* sporozoite challenge (22).

DISCUSSION

To our knowledge it has not been reported previously that the V_H region of an antibody molecule engineered to express a 15-amino acid residue epitope of an unrelated exogenous antigen elicits a humoral response specific for that epitope on the native antigen. The ability of the engineered antibody expressing a malaria *P. falciparum* sporozoite epitope to initiate a molecularly specific and biologically active humoral response against the parasite is reminiscent of internal image anti-idiotypes reported to immunize against *Trypanosoma rhodesiense* (7), *Schistosoma mansoni* (30), and *Trypanosoma cruzi* (31). The novelty of our approach is that molecular mimicry and immunologic specificity of the antibody

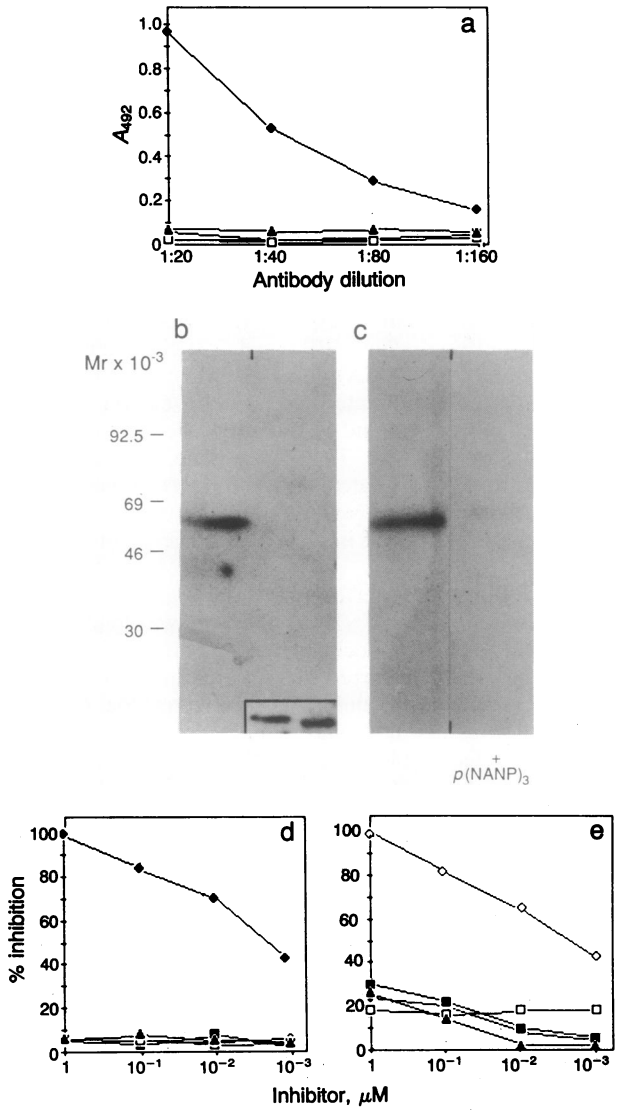

FIG. 3. Specificity of rabbit antibodies elicited by engineered γ1NANP for the peptide epitope (NANP)$_3$. (*a*) Binding of purified anti-γ1NANP antibodies (day 70 bleed) to polyvinyl microtiter wells coated with γ1NANP (◆). Antibodies were purified by affinity chromatography from a Sepharose 4B column coated with synthetic p(NANP)$_3$ from a serum aliquot depleted of any reactivity with human gamma globulins by appropriate absorption. The bound antibodies were revealed using HP-conjugated goat antibody to rabbit immunoglobulin. The following controls were used: WT (□); γ1Vk (▲); and mAb 62 (△). (*b*) Purified rabbit antibodies to γ1NANP react in a Western blot with the H chain of the engineered antibody. Rabbit antibodies were allowed to react with a nitrocellulose filter blotted with γ1NANP (left) or WT (right) and were revealed using ^{125}I-labeled goat antibody to rabbit immunoglobulin. (*c*) Binding of purified rabbit antibodies to the H chain of γ1NANP (left) is abrogated by preincubation with 100 μg of soluble p(NANP)$_3$ (right). (*d*) ELISA inhibition studies demonstrate the specificity of rabbit antibodies for γ1NANP. A fixed amount (≈0.4 μg/ml) of purified anti-NANP antibodies in PBSA was mixed for 1 hr at room temperature with the following inhibitors: synthetic p(NANP)$_3$ (◆); 15-mer YYCARKAY-SHGMDYW (□); 17-mer CAINSNGGSTYYPDTVK (▲); 15-mer SHIPAVHPGSFRPKC (■); and 10-mer DENGNYPLQC (△). The mixtures were transferred to microtiter wells coated with γ1NANP (5 μg/ml) and the assay was continued. [A_{492} binding without inhibitor (i.e., 100% binding) ≈0.900.] (*e*) Inhibitors were as follows: γ1NANP (◇); WT (◆); γ1Vk (□); mAb 62 (▲); and mAb 10.12 (■).

were programed *in vitro* at the primary structure level. Antibody mimicry of exogenous antigens based on defined amino acid sequence of the CDRs has been proposed for the

Table 3. Antibodies induced by immunization with γ1NANP prevent sporozoite invasion of hepatocytes

Immunogen	% ISI*	
	Immune serum†	Immune Ig
γ1NANP	82	88
WT	12	23

*Serum or immune immunoglobulin was diluted to 1:100 or 50 μg/ml, respectively, in culture medium and added to 1-cm² cultures of human hepatoma HepG2-A16 cells followed by addition of 30,000 *P. falciparum* sporozoites. Cultures were incubated at 37°C for 3 hr, fixed in methanol, and allowed to react with a mAb to *P. falciparum* CS protein in an immunoperoxidase antibody assay. Invaded sporozoites were counted, and ISI was calculated as the % reduction in invasion with immune serum or immunoglobulin as compared to preimmunization serum or immunoglobulin, respectively.

†Serum from a single rabbit donor bled on day 70 after the primary immunization with γ1NANP.

Glu-Ala-Tyr random polymer (15) and the mammalian reovirus type 3 hemagglutinin (16).

A second important aspect of our findings is that an anti-$(NANP)_3$ response could be induced in animals of different species and in strains of mice nonresponder to immunization with a synthetic $(NANP)_{40}$ peptide (26). Because anti-idiotype responses are T-cell-dependent phenomena (32), it will be important to elucidate the pathway of presentation and generation of T-cell help for peptide epitopes integrated within the immunoglobulin structure. Whatever the case may be, the results suggest that this approach may be valuable for immunization of individuals of different MHC haplotypes, an aspect of key importance for human vaccination against malaria *P. falciparum* sporozoite.

Owing to the fact that the CDR3 of the H chain naturally displays considerable sequence variability (33) and the atomic structure of the host V region can be resolved by x-ray crystallography and/or molecular modeling using data from known variants (34), our approach may allow one to gain insights into the possible three-dimensional structure of biologically relevant oligopeptide epitopes. Whereas the three-dimensional structure of the NANP oligopeptide could not be firmly predicted using computational models (35, 36), antibodies engineered to express $(NANP)_3$ or mutants of this epitope can be easily probed with human antibodies from protected individuals to assess the relative contribution of single residues in determining the immunogenic site required for protection. This could lead to design a sporozoite vaccine with enhanced protective immunogenicity.

Because the immune response induced by immunization with the engineered antibody was specific and biologically active, we propose this approach as a way to design and develop antibody vaccines against malaria and other infectious agents in those instances in which (*i*) the natural pathogen cannot be produced in large enough quantities, (*ii*) an alternative to synthetic peptides and recombinant subunit vaccines is required particularly when these are subject to strict immune response gene control, and (*iii*) a protective epitope needs to be separated from suppressive epitopes. The strategy described constitutes, therefore, a way to produce medically useful antibodies, including antibody vaccines.

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