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CONSTRUCTION OF IMMUNOGENS FOR SYNTHETIC MALARIA VACCINES

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The immunogenicity of a peptide consisting of eight repeats of the tetrapeptide sequence NANP (Asn-Ala-Asn-Pro) contained in the circumsporozoite protein of Plasmodium falciparum was investigated in mice under different modes of presentation. This peptide was able to produce biologically active antibodies when administered with adjuvant and linked to a protein carrier. However, a (NANP) peptide polymerized by carbodiimide was found to be immunogenic in the absence of protein carrier in H-2^b mice. In contrast, the $(NANP)_8$ peptide polymerized by glutaraldehyde was not immunogenic in the same strain. Furthermore, the efficacy of murabutide in saline, as an immunological adjuvant, was compared to the efficacy of Freund's complete adjuvant.

Protective antibodies against Malaria have been induced in several animal species and in man by immunization with irradiated sporozoites (1,2). In the latter case, these antibodies recognize a single immunodominant region of the circumsporozoite (CS) protein of Plasmodium falciparum containing 37 repeats of four amino acids NANP (Asn-Ala-Asn-Pro) interspaced with 4 repeats of four other amino acids NVDP (Asn-Val-Asp-Pro) (3,4). Currently, antisporozoite vaccines containing (NANP) repeats obtained either by chemical synthesis or by recombinant DNA technology, have been shown to produce biologically active antibodies in animals (5,6). Moreover, anti NANP antibodies recognizing the sporozoite were produced in recent human trials performed with both vaccines

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(7,8). Nevertheless, the antibody titers were low indicating that more potent immunomodulators should be added to enhance the antibody response. In previous reports, an MDP derivative, murabutide (9) which has been used in clinical trials with tetanus toxoid vaccine (10), was shown to have an adjuvant activity in animals using a P. knowlesi synthetic vaccine (11). In the present report, the influence of murabutide on the production of biologically active antibodies was compared to that of Freund's complete adjuvant (FCA). The immunogenicity of P. falciparum peptides free, polymerized or coupled under different conditions has been investigated in outbred mice. Also the ability of some of these constructs to induce an antipeptide antibody response in inbred mice has been examined.

MATERIALS AND METHODS

Antigens and adjuvants

Synthetic peptides representing one and eight repeats of the (NANP) tetrapeptide sequence found in the CS protein of P. falciparum and a synthetic peptide of 34 amino acids named S34 representing a repetitive fragment of type 24 streptococcal M protein (12) were used in these experiments. These peptides were synthesized by a solid phase method by using method already described (13).

Tetanus toxoid (TT) was provided by Dr. D. Labert (Institut Pasteur Production, Paris, France). Freund's complet adjuvant (FCA) was purchased from Difco Laboratories Detroit Michigan. N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP, muramyl dipeptide) was obtained from GIRPI-Choay Chimie Reactifs (Paris, France). The two following compounds were synthesized by Lefrancier et al. (Institut Choay, Paris, France): MDP-Lys (14) and N-acetylmuramyl-L-alanyl-D-glutamine-n-butyl-ester, a butyl-ester derivative of MDP (Murabutide) (9).

Conjugates of peptide-TT were prepared by a method described previously (15). Each peptide has been coupled by glutaraldehyde via its NH_2 group to TT. Homopolymerization of $(NANP)_8$, copolymerization of $(NANP)_8$ peptide with S34 peptide and copolymerization of $(NANP)_8$ peptide with MDP-Lys were performed using glutaraldehyde as coupling agent under conditions previously described (16).

Homopolymerization of (NANP) was performed with water soluble carbodiimide as follows: 44 mM of (NANP) peptide dissolved in 0.4 ml of dimethylformamide (DMF) was added to 440 mM of water soluble carbodiimide (3,3' diisopropyl carbodiimide). The reaction was allowed to proceed for 2 days with constant stirring, followed by filtration on a Sephadex G-50 column.

The synthesis of $(NANP)_8$MDP was performed as follows: the peptide was reacted with the N-hydroxysuccinimide ester of MDP (MDP-OSu) in the presence of N-methylmorpholine in dimethylformamide. The reaction was monitored by reverse phase HPLC. $(NANP)_8$MDP was then purified by chromatography on Biogel P2 (ultrafine) column.

Chemical analysis

Protein content in each conjugate was measured by Folin reaction and MDP content by a colorometric method (17).
Amino acid analysis of peptide-TT conjugates after acid hydrolysis was

performed by using a Beckman amino acid analyzer to determine the ratio of peptide to TT carrier.

Immunization

Female mice were used at 8 to 12 weeks of age; they were purchased from Iffa Credo (St-Germain-sur L' Arbresle, France) and Harlan (Madison, Wisconsin, USA). Mice (eight per group) were immunized subcutaneously with 50 µg of protein of each conjugate with adjuvant. Aluminum hydroxide ($Al(OH)_3$) was used at a dose of 40 µg per mouse; murabutide was used at 100 ug per mouse. Mice were boosted 30 days later in absence of additional adjuvant. Sera were collected by retroorbital bleeding at weekly intervals after the first injection of conjugate and stored at -20 °C before titration.

Antibody titration

Antibody titers against the synthetic peptide or against TT was determined by enzyme linked immunosorbent assay (ELISA) according to experimental conditions previously described (16). Plates were coated with 10 µg of $(NANP)_8$, S34 or 4 µg of TT per ml. Readings were performed at 492 nm in a Titerteck Multiskan ELISA reader (Flow Laboratories) 10 minutes after the addition of the substrate. ELISA titers were expressed as the maximal dilution giving a twofold-higher absorbance than the negative control serum diluted at 1:100. Recognition of the P. falciparum sporozoite surface antigen was assessed by an immunofluorescence antibody assay with, as previously described (18), wet preparations of sporozoites attached to poly-L-treated glass slides. Biological activities of the antipeptide antibodies were measured by inhibition of P. falciparum sporozoite penetration into cultured human hepatocytes as previously described (6). Percent of inhibition was estimated by counting the number of intracellular trophozoites at 48 h compared with the number in the corresponding control culture.

RESULTS

It has previously been demonstrated that $(NANP)_3$ or $(NANP)_4$ coupled to TT can induce biologically active antibodies if administered with FCA or $Al(OH)_3$ (19,20). The following experiments were performed to evaluate the influence of murabutide on the immunogenicity of $(NANP)_8$ compared to FCA. $(NANP)_8$ was administered free, polymerized or coupled to a carrier. Six groups of eight mice were treated as follows: Two control groups received either 50 µg of $(NANP)_8$TT alone in saline solution or $(NANP)_8$ with FCA (a and b); group c) received FCA containing 50 µg of $(NANP)_8$ polymerized by glutaraldehyde; group d) received FCA containing 50 µg of the copolymer $(NANP)_8$S34 ; group e) received FCA containing 50 µg of $(NANP)_8$TT conjugate, whereas group f) received this conjugate administered in saline with 100 µg of murabutide. The molecular ratio of peptide to TT carrier, determined by amino acid analysis, gave 10 moles of peptide per mole of TT carrier. Thus, the mice of groups a, e, and f received only 8 µg of $(NANP)_8$ in 50 µg of TT conjugate. All mice were boosted with the different immunogens in saline solution 30 days later. Results obtained in Table 1, show that in groups a,b,c,and d, no antipeptide antibodies could be detected after two injections. Futhermore, immunization of mice with two other conjugates obtained either by coupling of $(NANP)_8$ to MDP-

Table 1. Antibody titers to $(NANP)_8$ and Carrier, as assessed by ElISA

Group	Treatment on Day 0	Antibody responses to: $(NANP)_8$ Primary	$(NANP)_8$ Secondary	Carrier Primary	Carrier Secondary
a	$(NANP)_8$TT	< 100	< 100	< 100	9,000
b	$(NANP)_8$ + FCA	< 100	< 100	-	-
c	Polymer $(NANP)_8$ + FCA	< 100	< 100	-	-
d	$(NANP)_8$S34 + FCA	< 100	< 100	< 100	900
e	$(NANP)_8$TT + FCA	2,000	54,000	20,000	141,000
f	$(NANP)_8$TT + Murabutide	500	15,000	15,000	30,000

Swiss mice (eight per group) were immunized with 50 ug of protein of each conjugate with adjuvant. Mice were boosted 30 days later in absence of adjuvant. Anti $(NANP)_8$ and anti Carrier antibody responses were measured on day 28 and day 37 by ELISA. Results are given as the maximal dilution giving an absorbance twice as high as the normal serum diluted 100 times.

FCA: Freund's complete adjuvant.

Lys ($(NANP)_8$-lys-MDP) or by total synthesis ($(NANP)_8$MDP) did not give rise to antibodies (data not shown). However, mice immunized with $(NANP)_8$TT and adjuvant produced a strong antipeptide antibody response. Antibodies obtained against $(NANP)_8$ were measured by immunofluorescence (IFAT) on sporozoites and inhibition of P. falciparum sporozoite penetration into human hepatocytes in culture. The data obtained (Table 2) confirmed the previous ELISA and showed that these antibodies were biologically active.

In a second set of experiments, $(NANP)_8$ polymerized by glutaraldehyde, (NANP) polymerized by carbodiimide and $(NANP)_8$ conjugated to TT were administered with adjuvant in three strains of inbred mice. As can be seen in Table 3, when $(NANP)_8$ was coupled to TT and administered with $Al(OH)_3$, antibodies were produced in all strains. In contrast, even with FCA, glutaraldehyde polymerized $(NANP)_8$ could not induce a detectable antibody response in any of the three strains, whereas (NANP) polymerized by carbodiimide elicited a antibody response but only in C57Bl/6.

DISCUSSION

It is generally agreed that an effective antisporozoite vaccine will require the addition of a safe adjuvant capable of enhancing the antibody level and

Table 2. Antibody antisporozoite responses of mice immunized with $(NANP)_8$TT conjugate

Treatment	ELISA titer	IFAT titer	% Inhibition
$(NANP)_8$TT	< 100	< 100	negative
$(NANP)_8$TT + Murabutide	15 000	16 000	65
$(NANP)_8$TT + FCA	54 000	32 000	87

Swiss mice (eight mice) were immunized as described in Table 1. Pooled sera on Day 37 were titrated by ELISA, immunofluorescence (IFAT), and inhibition of sporozoite penetration into cultured hepatocytes.

eliciting cell mediated immunity (CMI). Two recent reports of clinical trials (5,6) in which aluminum hydroxide was used, indicate that the titers of neutralizing antibodies against the sporozoite which were obtained, were low even after several booster injections. Therefore, the association of adjuvants such as muramyl peptides which, under certain experimental conditions has been

Table 3. Secondary antibody responses in mice immunized by different preparations of $(NANP)_8$

Treatment	Mouse strains		
	DBA/2 ($H\text{-}2^d$)	BALB/c ($H\text{-}2^d$)	C57BL/6 ($H\text{-}2^b$)
Polymer $(NANP)_8$[a] + FCA	< 100	< 100	< 100
Polymer $(NANP)$[b] + FCA	< 100	< 100	17,000
$(NANP)_8$TT + $Al(OH)_3$	9,000	4,300	2,000

Mice (eight per group) were immunized as described in Table 1. Sera on day 37 were tested in ELISA for the presence of anti-$(NANP)_8$ antibodies.
a) Polymerization of $(NANP)_8$ was performed by using glutaraldehyde.
b) Polymerization of $(NANP)_8$ was performed by carbodiimide.

shown to enhance CMI, should be considered (21,22). The present experiments were performed with murabutide in saline since this adjuvant is acceptable for human use (10). These data have shown an enhancing effect on the antipeptide antibody response as measured by ELISA, immunofluorescence and inhibition of sporozoite penetration into cultured hepatocytes. A previous report has indicated that a suppression of the antipeptide antibody response can occur when mice pretreated with TT are subsequently immunized with a P. knowlesi sporozoite peptide linked to TT (20). In contrast to this effect, we have demonstrated that a preimmunization with the carrier induced an enhancement instead of a suppression of the antibody response against $(NANP)_4$ coupled to TT (20). The use of a TT carrier can present other disadvantages, such as epitopic-specific suppression, and it remains desirable to substitute synthetic constructs for peptide-TT conjugates. Therefore, experiments were performed with the view of enhancing the immunogenicity of (NANP) peptides in the absence of a protein carrier. Previous reports have shown that biologically active antibodies have been obtained by immunizing animals with a synthetic LHRH peptide coupled to MDP (22) or by polymerizing a foot-and-mouth disease virus (FMDV) peptide with glutaraldehyde (24). It has also been demonstrated that peptides can be rendered immunogenic by copolymerization with a synthetic streptococcal peptide (S34) bearing T cell epitopes (25). The peptide $(NANP)_8$ was not rendered immunogenic by any of these procedures, but did become immunogenic after coupling to TT. However, it has been indicated recently that an antibody response against a $(NANP)_{40}$ peptide in the absence of the carrier was obtained, but exclusively in H-2^b mice (26). Our experiments have shown that the tetrapeptide (NANP) polymerized by carbodiimide was also immunogenic in absence of carrier in h-2^b mice. In contrast, $(NANP)_8$ polymerized by glutaraldehyde was unable to induce antibodies in the three strains tested. Since, these two polymers were eluted as a single peak corresponding to an apparent molecular weight of 20,000 when analyzed by high performance size exclusion chromatography, the results obtained suggest that polymerization by glutaraldehyde could affect the helper T-cell epitope of the malaria peptide necessary for its immunogenicity in C57Bl/6 (h-2^b mice). Therefore, studies are being conducted in responder mice utilizing smaller (NANP) repeats copolymerized or mixed with synthetic immunomodulators in view of eliciting a efficient antipeptide antibody response in the absence of FCA and protein carrier. Our data have demonstrated the importance of the presentation of the repetitive epitope (NANP) to the immune system. Results reported here also show that murabutide produced an efficient protective antipeptide response to the $(NANP)_8$ peptide coupled to a toxoid carrier. It is hoped that a better understanding of the relationship between the conjugates and their immunological properties in inbred and outbred populations will help to design synthetic immunogens in new vaccine preparations.

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