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Assessment in mice of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, *P. falciparum*

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SUMMARY

The anti-*P. falciparum* sporozoite vaccine consisting of the synthetic peptide, Ac-Cys-$(NANP)_3$, conjugated to the protein tetanus toxoid (TT), [Ac-Cys-$(NANP)_3]_{25}$–TT, is currently undergoing human trials. The purpose of the present study was to assess various immunological parameters of this vaccine in mice, which have practical implications in humans. Two injections of [Ac-Cys-$(NANP)_3]_{25}$–TT adsorbed to $Al(OH)_3$ were required to elicit a high antibody response against both Ac-Cys-$(NANP)_3$ and TT. The vaccine initiated equivalent Ac-Cys-$(NANP)_3$ priming for a secondary IgG response in 1-week-old and adult mice. Immunization of female mice with TT or [Ac-Cys-$(NANP)_3]_{23}$–TT prior to mating resulted in offspring that passively received anti-Ac-Cys-$(NANP)_3$ and/or anti-TT antibody and that had reduced secondary responses to Ac-Cys-$(NANP)_3$ and TT. Tertiary challenge with vaccine could substantially overcome such inhibition. Pre-immunization of adult mice with TT resulted in a specific inhibition of the anti-Ac-Cys-$(NANP)_3$ antibody response that disappeared following tertiary challenge with the vaccine. The conjugate initiated an antibody response against Ac-Cys-$(NANP)_3$ and TT in mice of 16 different genotypes; only very low T-cell proliferative responses to $(NANP)_3$ were observed for some of these strains. Mice injected with $(NANP)_3$ coupled to protein demonstrated a secondary response to Ac-Cys-$(NANP)_3$ when challenged with $(NANP)_3$ on a heterologous carrier, indicating that B-cell priming alone may be sufficient for a secondary antibody response. These results demonstrate that the vaccine has favourable and unfavourable characteristics in mice; the potential for both exists in humans.

INTRODUCTION

Antibody can prevent infection by the malarial sporozoite (Nussenzweig, Vanderberg & Most, 1969; Potoenjak *et al.*, 1980; Gysin *et al.*, 1984). The specificity of such antibody is the circumsporozoite protein and, in the case of *Plasmodium falciparum*, the main antigenic determinant carried by sporozoites from diverse areas of the world has the amino acid sequence [Asp Ala Asp Pro)$_3$(NANP)$_3$] (Dame *et al.*, 1984; Zavala *et al.*, 1985). Recombinant proteins containing the (NANP) sequence, or synthetic $(NANP)_3$ that has been conjugated to carrier protein, and injected into animals initiates the production of anti-$(NANP)_3$ antibodies that bind to circumsporozoite protein as well as sporozoites, and prevent infection of human hepatoma or normal liver cells (Zavala *et al.*, 1985; Ballou *et al.*, 1985; Young *et al.*, 1985; Mazier *et al.*, 1986). It has also been found that $(NANP)_3$ inhibits the reaction of essentially all human antibodies with sporozoites (Zavala *et al.*, 1985). The above observations served as a basis for the construction of a synthetic peptide-based vaccine against the sporozoite stage of *P. falciparum* malaria. This vaccine, which consists of Acetyl-Cys-$(NANP)_3$, [Ac-Cys-$(NANP)_3$], coupled to tetanus toxoid, [Ac-Cys-$(NANP)_3]_{25}$–TT, has been used in clinical trials, and evidence for protection has been recorded (Herrington *et al.*, 1987). In addition, protection has also been obtained with a recombinant DNA vaccine containing NANP repeats, R32tet32 (Ballou *et al.*, 1987). The intent of the present study was to establish the immunogenicity in mice of the human formulation as well as examine facets of the vaccine in mice that have practical implications in humans. Since most of the 10^6 or more deaths that are caused by *P. falciparum* malaria each year occur in very young children, it is essential that the vaccine works well in this age group. This is particularly important to stress in as

Abbreviations: Ac, acetyl; [Ac-Cys-$(NANP)_3]_{25}$-TT, Acetyl-Cys (Asp Ala Asp Pro)$_3$-tetanus toxoid; BSA, bovine serum albumin; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; G.M., geometric mean; i.p., intraperitoneal; KLH, keyhold limpet haemocyanin; $(NANP)_3$, (Asp Ala Asp Pro)$_3$; $[(NANP)_3]_2$ BSA, $[(NANP)_3]_2$ bovine serum albumin; s.c., subcutaneous; TNP, trinitrophenyl; TNP_{26} KLH, trinitrophenyl $_{26}$ keyhole limpet haemocyanin; TT; tetanus toxoid.

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much as the immune system of infants is immunologically immature. Because of this aspect, we have assessed the ontogeny of the response to [Ac-Cys-$(NANP)_3]_{25}$–TT in mice.

Moreover, since anti-$(NANP)_3$ or anti-TT antibodies present in mothers in endemic areas are passively transfered *in utero* or after birth to nursing children (Higgins-Nardin *et al.*, 1981), the anti-[Ac-Cys-$(NANP)_3]_{25}$–TT response of mice born to TT- or [Ac-Cys-$(NANP)_3]_{23}$–TT-immunized mothers has also been evaluated. A further parameter that has been studied concerns the effect of prior immunization with TT on the subsequent response to [Ac-Cys-$(NANP)_3]_{25}$–TT. This point is especially pertinent since many potential vaccinees will have been immunized with TT and there are several reports demonstrating that pre-immunization with protein inhibits antibody responses to new determinants that are conjugated to the protein (Rajewsky *et al.*, 1969; Mitchison, 1971; Herzenberg & Tokuhisa, 1982; Herzenberg *et al.*, 1982; Schutze *et al.*, 1985; Jacob, Arnon & Sela, 1985; Schutze *et al.*, 1987). In addition, since it is essential to employ a vaccine to which the genetically diverse human population will be responsive, the antibody responses of a variety of mouse strains have been analysed.

T cells, themselves, have also been implicated in providing protection in malaria (Chen, Tigellar & Weinbaum, 1977; Ferreira *et al.*, 1986; Egan *et al.*, 1987). Furthermore, antibody responses to most proteins require antigen-specific B- and T-helper cells and, since the ideal vaccine should both initiate a protective response and prime the immune system to respond to subsequent exposure with *P. falciparum* sporozoites, a further point to be analysed was whether [Ac-Cys-$(NANP)_3]_{25}$–TT not only elicited anti-Ac-Cys-$(NANP)_3$ antibody production but was also capable of sensitizing $(NANP)_3$-specific T cells. Finally, to test for the possibility that B-cell priming alone to $(NANP)_3$ in the absence of T-cell sensitization could be a useful activity of the vaccine, the last and related question to be examined was the capacity of [Ac-Cys-$(NANP)_3]_{25}$–TT to prime mice subsequently challenged with $(NANP)_3$ conjugated to a heterologous carrier.

MATERIALS AND METHODS

Animals

Mice were obtained from the Institute for Biomedical Research, Füllinsdorf, Switzerland; strains utilized were: DBA/2, NZB, CBA.M523, CSW, CWB/13, fu albino hr/hr, C57Bl-H2k, fu albino, C57Bl/6 × DBA/2, C57Bl/6, CBA, A/J, ICR, C3H.Q, C3H.HeJ and BALB/c of haplotypes: d, d, kml, b, b, outbred, k, outbred, b × d, b, k, a, outbred, q, k and d, respectively. Unless otherwise indicated, 6–8-week-old female animals were utilized.

Preparation of peptides and antigens

TT was obtained and treated as described elsewhere (Zavala *et al.*, 1985). $(NANP)_3$ and Ac-Cys-$(NANP)_3$ were prepared by solid-phase synthesis (Barany & Merrifield, 1980). Ac-Cys-$(NANP)_3$ was coupled to TT by the SH-function of the Cys residue with a 3-maleimidoproprionate moiety, as previously described (Keller & Rudinger, 1975). Three preparations of [Ac-Cys-$(NANP)_3]_n$–TT differing in molar ratios of Ac-Cys-$(NANP)_3$ to TT, as determined by amino acid analysis, were prepared. Trinitrophenyl$_{26}$ keyhold limpet haemocyanin (TNP_{26} KLH) was prepared as described elsewhere (Little & Eisen, 1967). [$(NANP)_3]_4$–TT was prepared using glutaraldehyde conjunction, as described elsewhere (Zavala *et al.*, 1985). Bovine serum albumin (BSA) was purchased from SERVA, Heidelberg, FRG, as Cohn fraction V, passed over a Sephadex G25 column and conjugated to $(NANP)_3$ using glutaraldehyde yielding, [$(NANP)_3]_2$–BSA, as described previously (Briand, Muller & van Regenmortel, 1985).

Immunizations

[Ac-Cys-$(NANP)_3]_{19}$–TT and [Ac-Cys-$(NANP)BI_3]_{23}$–TT were adsorbed to $Al(OH)_3$ (Alhydrogel, Superfos Biosector, Denmark) by adding 50 μg protein to 100 μg of Al in the form of $Al(OH)_3$ in 0·15 M NaCl, pH 6·8, in a total volume of 0·2 ml; where indicated, mice received 0·2 ml of this material s.c. The preparation containing [Ac-Cys-$(NANP)_3]_{25}$–TT was adsorbed to $Al(OH)_3$ (Alhydrogel) and bottled under the direction of Professor Edgar Relyveld, Pasteur Foundation; this is the material utilized for clinical trials. This vaccine contains 5·3 μg Al/μg conjugate. Where described in the text, mice received a total of 0·1 ml s.c. containing antigen emulsified with an equal volume of Freund's incomplete (FIA) or complete adjuvant (FCA; Difco, Detroit, MI). To analyse the ontogeny of the response to [Ac-Cys-$(NANP)_3]_{25}$–TT/$Al(OH)_3$, mice received indicated doses of this vaccine i.p. To test for T-cell sensitization, mice received 50 μg of $(NANP)_3$ or 50 μg of [Ac-Cys-$(NANP)_3]_{19}$–TT in FCA or $Al(OH)_3$ s.c.; 10 days later draining lymph nodes were removed.

Antibody analysis

ELISA were done in microtitre plates (Nunc, cat. no. 4-39459, Kamstrup, Denmark) whose wells had contained 50 μl of Ac-Cys-$(NANP)_3$, 2 μg/ml, or TT (300 Lf/ml obtained from Swiss Serum and Vaccine Institute, Berne, Switzerland) 16 μg/ml, diluted in 0·05 M $NaHCO_3$, pH 8, during an overnight incubation. After washing with cold tap water, each well was then blocked by the addition of 200 μl of BSA, 5 g/l in 0·1 M Tris-HCl (Fluka, Bucks, CH), pH 7·5. In experiments where the anti-Ac-Cys-$(NANP)_3$ response of mice that received [$(NANP)_3]_2$–BSA was monitored, 200 μl of 3% gelatin in phosphate-buffered saline (Merck, Darmstadt, FRG), pH 7·4, were added to each well. After washing, 50 μl of a dilution of plasma in 0·1 M sodium phosphate buffer (Merck), pH 6·8, containing 20% fetal calf serum (diluent C) (Amimed, Basel, CH) were added, followed by incubation for 4 hr/37°. After washing, 50 μl of peroxidase coupled sheep anti-mouse Ig or rabbit anti-mouse μ or mouse γ, from Bio-Science Products A.G., Emmenbrücke, Switzerland, in diluent C were added and incubation proceeded for 2 hr/37°.

After washing, the presence of antibody in the plate was revealed by colour following the addition of 200 μl 5 mM H_2O_2 and 20 mM 3,3′,5,5′-tetramethylbenzidine in 0·2 M citric acid/KOH (Fluaka), pH 3·95; the incubation period was 30 min at room temperature, at which time colour development was stopped by the addition of 100 μl 1 M H_2SO_4. Titres were the last dilution of plasma with $OD_{455} \geqq 0{\cdot}1$ and $\geqq 2$ times OD_{455} of plasma from mice injected with saline.

Plasma that did not exhibit activity were assigned titres of 150 for Ac-Cys-$(NANP)_3$ or 1000 or 1500 for TT, the reciprocals of the lowest dilutions of plasma tested.

T-cell proliferation assay

This assay was performed as described previously (Corradin, Etlinger & Chiller, 1977; Ratcliffe & Julius, 1982). Single cell suspensions were prepared from lymph nodes and 5×10^5 cells/

Table 1. Antibody responses of mice to [Ac-Cys-(NANP)$_3$]$_n$–TT conjugates

Group*	Immunization	Antigen	Antibody titre: Anti-Ac-Cys-(NANP)$_3$ × 10^3 Day 51–95†	Day 64	Day 89	Day 119	Day 336	Antibody titre: Anti-TT × 10^5 (Day 29–101†)
1	50 μg on Al(OH)$_3$ Days 1, 27	[Ac-Cys-(NANP)$_3$]$_{23}$–TT	53‡					2·6
2	50 μg on Al(OH)$_3$ Days 1, 29	[Ac-Cys-(NANP)$_3$]$_{19}$–TT	92					7·9
3	50 μg on Al(OH)$_3$ Days 1, 39	[Ac-Cys-(NANP)$_3$]$_{25}$–TT	110					11
4	40 μg on Al(OH)$_3$ Day 1	[Ac-Cys-(NANP)$_3$]$_{25}$–TT		1·6	1·4	2·3	2·3	8·7
5	40 μg on Al(OH)$_3$ Days 1, 33	[Ac-Cys-(NANP)$_3$]$_{25}$–TT		66	132	132	44	10·9
6	40 μg on Al(OH)$_3$ Days 1, 33, 60	[Ac-Cys-(NANP)$_3$]$_{25}$–TT			159	159	121	15·2

* BALB/c mice, 5–20 per group, received antigen s.c. Mice in Groups 4–6 received the preparation used in clinical trials. Each injection of vaccine contained 100 μg of Al(OH)$_3$ and 212 μg of Al(OH)$_3$ for Groups 1–3 and 4–6, respectively.

† Values shown between Days 51 and 95 and 29 and 101 are peak titres.

‡ Values are geometric means; coefficient of variance of G.M. were 1·2–1·6 in all cases.

Table 2. Ontogeny of the response of BALB/c mice to [Ac-Cys-(NANP)$_3$]$_{25}$–TT

Age at initial injection (weeks)	Antigen: Amount (μg)	Antigen: Day given	Antibody titre*: Anti-Ac-Cys-(NANP)$_3$ × 10^3 Total	IgM	IgG	Anti-TT × 10^4 (Total)
1	25	1	182 (1·1)	2·9 (2·8)	115 (1·3)	28 (1·3)
	50	34				
2	37·5	1	311 (1·3)	11 (1·3)	124 (1·5)	36 (1·4)
	50	35				
3	50	1	115 (1·3)	3·7 (1·2)	65 (1·4)	37 (1·3)
	50	30				
4	50	1	58 (1·3)	1·1 (1·2)	53 (1·2)	83 (1·6)
	50	29				
4	50	1	0·45 (1·4)			

* Titres are geometric means (cofficient of variance G.M.) and are peak responses recorded 6–20 days following the second injection. Each group had 5–10 mice. For 4-week-old mice that received a single injection, the value is the peak response recorded 14–29 days later.

well in duplicate were admixed with (NANP)$_3$, 20 μg/ml, TT, 10 μg/ml or, as a control, saline. Proliferation was measured during the last 24 hr of a 4-day incubation period by the incorporation of [^{3}H]thymidine.

RESULTS

Antibody responses to [Ac-Cys-(NANP)$_3$]$_n$-TT conjugates

Each of the 3 [Ac-Cys-(NANP)$_3$]$_n$-TT conjugates tested initiated a good antibody response against Ac-Cys-(NANP)$_3$ and TT (Table 1). To insure that the specificity of binding of anti-Ac-Cys-(NANP)$_3$ antibody was dependent on recognition of (NANP)$_3$, anti-Ac-Cys-(NANP)$_3$–TT plasma was preincubated with (NANP)$_3$ prior to introduction into Ac-Cys-(NANP)$_3$-coated plates. The specific inhibition of binding to Ac-Cys-(NANP)$_3$- but not TT-coated plates (data not shown) demonstrated that Ac-Cys was not essential for binding of the antibody to the peptide.

The mice in Groups 4–6 (Table 1) received the vaccine formulation utilized in clinical trials. A single injection of this material was sufficient to initiate the production of high levels of anti-TT but not anti-Ac-Cys-(NANP)$_3$ antibody (Group 4, Table 1). A second injection of vaccine produced a marked increase in the level of anti-Ac-Cys-(NANP)$_3$ antibody (Table 1, Group 5), which, in terms of peak titre, was not substantially increased by a third immunization (Table 1, Group 6). The persistence of higher levels of anti-Ac-Cys-(NANP)$_3$ antibody was, however, increased as a result of the third immunization.

Ontogeny of the response of BALB/c mice to [Ac-Cys-(NANP)$_3$]$_{25}$–TT

One- to 4-week-old mice received a single i.p. injection of

Table 3. Antibody responses of BALB/c mice born to mothers injected with TT

		Anti-Ac-Cys-$(NANP)_3$ titre $\times 10^3$			Anti-TT titre $\times 10^4$				Anti-TNP titre $\times 10^4$		
		Day			Day				Day		
Exp.	Mother	112	134	153	77	112	134	153			
1a*	TT	0·5 (1·4)	6·3 (1·4)	6·6 (1·3)	2·6 (1·1)	12 (1·2)	26 (1·2)	25 (1·2)			
	Non-TT	0·8 (1·6)	13 (1·2)	11 (1·3)	0·1 (0)	76 (1·6)	63 (1·3)	75 (1·2)			
					80	115			115	137	164
1b†	TT				1·6 (0)	0·5 (0·3)			109 (1·3)	63 (1·3)	63 (1·3)
	Non-TT				0·1 (0)	0·1 (0)			157 (1·4)	91 (1·2)	63 (1·3)
		107	167		57	107	167				
2‡	TT	0·2 (1·2)	3·8 (1·7)		13 (1·2)	13 (1·5)	109 (0)				
	Non-TT	2·3 (1·3)	46 (1·2)		0·1 (0)	89 (1·4)	409 (1·2)				

* Mice whose average age was 78 days received 50 μg [Ac-Cys-$(NANP)_3]_{23}$–TT in FIA s.c. Titres are geometric means (coefficient of variance G.M.). TT group had six mice and non-TT group had 22.

† Mice whose average was 78 days received 50 μg TNP_{26}KLH in FIA s.c. Titres are geometric means (cofficient of variance G.M.). Each group had five mice.

‡ Mice received 50 μg $[(NANP)_3]_4$–TT and 40 μg [Ac-Cys-$(NANP)_3]_{19}$–TT in FIA s.c., on Days 58 and 133 of life, respectively. Each group had five mice.

Table 4. Antibody responses of mice born to mothers immunized with [Ac-Cys-$(NANP)_3]_{23}$–TT

		Antibody titre								
		Anti-Ac-Cys-$(NANP)_3 \times 10^3$			Anti-TT $\times 10^4$			Anti-TNP $\times 10^4$		
Mother	Antigen injected into offspring	Day			Day			Day		
Immunized	[Ac-Cys-$(NANP)_3]_{25}$–TT	20	71	112	20	71	112			
		96	1	155	68	2·3	109			
Non-immunized		0·15	241	345	0·15	52	219			
		27	49	64	27	49	64	49	64	71
Immunized	TNP_{26}KLH†	110	9·3	2	111	17	1	1·4	48	109
Non-immunized		0·15	0·15	0·15	0·15	0·15	0·15	1·4	45	88

* Mice received 50 μg [Ac-Cys-$(NANP)_3]_{25}$–TT/$Al(OH)_3$ i.p. on Days 23, 52 and 101 of life. Titres are geometric means; coefficient of variance of G.M. were 0–1·6. Each group had 18 or 23 mice.

† Mice received 50 μg TNP_{26}KLH/$Al(OH)_3$ i.p. on Days 28 and 57 of life; each group had five mice.

$Al(OH)_3$-adsorbed [Ac-Cys-$(NANP)_3]_{25}$–TT followed by a second immunization approximately 4 weeks later. The antibody responses shown in Table 2 represent the peak levels recorded during the 3-week period following the boost. The vaccine was effective in priming mice in each age group for anti-Ac-Cys-$(NANP)_3$ and anti-TT responses. The majority of anti-Ac-Cys-$(NANP)_3$ antibody produced in each case was IgG.

Antibody response in offspring from TT- or [Ac-Cys-$(NANP)_3]_{23}$–TT-immunized mothers

The effect of maternal transfer of antibody reactive with Ac-Cys-$(NANP)_3$ and/or TT on the response of offspring to conjugates was analysed next. Animals born to mothers immunized with TT produced lower amounts of anti-Ac-Cys-$(NANP)_3$ or –TT antibody than did offspring born to non-immunized mothers (Table 3). Inhibition was specific in as much as the anti-TNP response to TNP_{26} KLH was not affected in mice derived from TT-immunized mothers. The degree of inhibition of the anti-Ac-Cys-$(NANP)_3$ response appeared to be related to the level of circulating anti-TT antibody present at the time of injection with [Ac-Cys-$(NANP)_3]_{25}$–TT, since inhibition was greater in offspring that had an average anti-TT titre of 130,000 (Table 3, Exp. 2) compared with 26,000 (Table 3, Exp. 1a); in contrast to the anti-Ac-Cys-$(NANP)_3$ response, the anti-TT response was inhibited to a similar degree in both groups of offspring and inhibition was not as great.

Animals born to mothers immunized with [Ac-Cys-$(NANP)_3]_{23}$–TT demonstrated a marked (>90%) specific reduction in both anti-Ac-Cys-$(NANP)_3$ and anti-TT antibody production when challenged twice with [Ac-Cys-$(NANP)_3]_{25}$–TT (Table 4). Upon tertiary challenge with [Ac-Cys-

Table 5. Effect of pre-immunization of BALB/c mice with carrier protein

Immunization*				Antibody titre†					
				Anti-Ac-Cys-$(NANP)_3 \times 10^3$			Anti-TT $\times 10^5$		
TT		[Ac-Cys-$(NANP)_3]_{25}$–TT		Day			Day		
Day	Amount (μg)	Day	Amount/ injection (μg)	76	99	144	76	99	144
1	0·6	33, 61, 102	50	228 (1·4)	119 (1·3)	229 (1.3)	5 (·2)	4·3 (1·3)	5·8 (·2)
1	6·0	33, 61, 102	50	190 (1·4)	64 (1·5)	25 (1·3)	5·7 (1·2)	12 (1·2)	17 (1·2)
1	60	33, 61, 102	50	58 (1·5)	40 (1·5)	152 (1·2)	7 (1·2)	21 (1·2)	15 (1·2)
1	Nothing	33, 61, 102	50	169 (1·3)	92 (1·4)	169 (1·2)	2·9 (1·2)	5·3 (1·5)	3·6 (1·3)

* Mice, 10 per group, received TT/$Al(OH)_3$, i.p. All subsequent injections were s.c.
† Values are geometric means (coeficient of variance G.M.).

Table 6. Antibody responses of various mouse strains to $[(NANP)_3]_4$–TT

Strain*	H-2 haplotype	Antibody titres: Anti-Ac-Cys-$(NANP)_3 \times 10^3$	Antibody titres: Anti-TT $\times 10^4$
DBA/2J	d	4·3 (1·3)	163 (1·2)
NZB	d	5·9 (1·6)	81 (1·2)
CBA.M523	kml	5·9 (1·1)	158 (1·4)
CSW	b	20 (1·3)	228 (1·4)
CWB/13	b	8·0 (1·3)	158 (1·4)
fu albino hr/hr	Outbred	5·9 (1·6)	129 (1·2)
C57Bl-H-2k	k	1·1 (1·7)	65 (1·5)
fu albino	Outbred	5·9 (1·7)	172 (1·2)
C57Bl/6J	b	4·2 (1·9)	473 (1·4)
CBA/J	k	5·9 (1·6)	228 (1·4)
A/J	a	68 (1·3)	473 (1·4)
ICR	Outbred	20 (1·9)	328 (0)
C57Bl/6J × DBA/2J	b × d	2·3 (3·3)	163 (1·2)
C3H.Q	q	5·9 (1·4)	227 (1·4)
C3H.HeJ	k	11 (1·7)	228 (1·4)
BALB/c J	d	15 (0)	145 (1·2)

* Mice, three per strain, received 50 μg $[(NANP)_3]_4$–TT in FIA s.c. values shown are geometric means coefficient of variance G.M.) of peak responses recorded 34–60 days post-injection.

$(NANP)_3]_{25}$–TT, high titres of both anti-Ac-Cys-$(NANP)_3$ and anti-TT antibody, $> 10^5$ and $> 10^6$, respectively, were produced by all animals, although the responses of mice from immune mothers were half of those from normal mothers (Table 4). Finally, the persistence of maternally derived anti-TT and -Ac-Cys-$(NANP)_3$ antibody is indicated by the plasma analysis of offspring immunized with TNP_{26} KLH. The bulk of each antibody had disappeared by Day 49 and was essentially absent by 2 months after birth.

Effect of pre-immunization with TT on subsequent anti-[Ac-Cys-$(NANP)_3]_{25}$–TT response

Mice were pre-immunized with 0·6–60 μg of TT prior to injection with [Ac-Cys-$(NANP)_3]_{25}$–TT. After two challenges with the conjugate, the anti-Ac-Cys-$(NANP)_3$ response was found to be not significantly altered in animals that received 0·6 μg TT and reduced up to 65% in mice that were pretreated with 60 μg of TT (Table 5).

However, upon tertiary challenge, no inhibition of the anti-Ac-Cys-$(NANP)_3$ response in 60 μg TT-pre-immunized mice was observed. The specificity of inhibition of the anti-Ac-Cys-$(NANP)_3$ response was demonstrated by the anti-TT response which was elevated in animals pre-injected with 60 μg of TT.

Antibody responses of mice from various strains to $[(NANP)_3]_4$–TT

In order to obtain some information on genetic restrictions associated with anti-$[(NANP)_3]_4$–TT responsiveness, mice from a variety of genetic backgrounds were challenged with this antigen in FIA s.c. Animals of each of the 16 different genotypes produced anti-Ac-Cys-$(NANP)_3$ and anti-TT antibody as a result of a single injection of $[(NANP)_3]_4$–TT (Table 6).

T-cell sensitization

Various mouse strains were tested for T-cell sensitization after receiving a single s.c. injection of $(NANP)_3$ or [Ac-Cys-$(NANP)_3]_{19}$–TT in $Al(OH)_3$ or FCA. $(NANP)_3$, 20 μg/ml, or TT, 10 μg/ml, were utilized in *in vitro* secondary responses. This concentration of TT has been found to yield optimal T-cell proliferation (H. Etlinger, unpublished results), while optimal proliferative responses to $(NANP)_4$ are found between 10 and 100 μg/ml (Togna *et al.*, 1986).

Proliferation to TT was recorded for each mouse strain when [Ac-Cys-$(NANP)_3]_{19}$–TT/FCA was utilized; [Ac-Cys-$(NANP)_3]_{19}$–TT/$Al(OH)_3$ failed to sensitize for a secondary *in vitro* response to TT, with the exceptions of NZB and C3H.Q mice (Table 7). In general, regardless of the immunization

Table 7. T-cell proliferactive responses of mice injected with $(NANP)_3$ or $[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT

	Antigen*											
	$(NANP)_3$						Ac-Cys-$(NANP)_{19}$–TT					
	$Al(OH)_3$			FCA			$Al(OH)_3$			FCA		
Mouse strain	Saline	TT	$(NANP)_3$	Saline	TT	$(NANP)_3$	Saline	TT	$(NANP)_3$	Saline	TT	$(NANP)_3$
Fu albino	25†	15	7·7	73	153	47	6·8	11	12	170	380	120
A/J	5·6	5·8	3·9	42	62	37	4·2	17	4	78	420	54
CSW	3	5·2	7·6	12	12	19	37	7	3	180	480	320
C57Bl-H-2k	6·7	2·9	5	17	16	14	4·1	9·9	4·6	107	372	110
C3H.HeJ	7·6	8·5	8·7	18	11	13	4	3·1	2·9	21	44	11
NZB	4·3	2·9	4·7	44	66	38	14	230	12	67	420	57
C57Bl/6J	19	11	4·7	39	22	60	13	6·4	11	43	270	43
CBA/J	23	6·4	14	67	35	68	6·4	1·5	2·2	16	182	19
DBA/2J	3·9	3·4	9·4	37	56	39	4·8	2·4	16	61	417	94
ICR	9·6	16	4·4	7·2	1·9	4	8	14	6·4	197	563	187
CBA.M523	12	9·1	11	54	18	50	18	9·6	18	16	570	15
C3H.Q	7·8	2·6	7·8	160	78	130	97	220	90	180	380	190
CWB/13	5·1	13	0·8	24	23	6·4	14	12	3·2	85	280	170
C57Bl/65 × DBA/2J	13	8·9	1·2	44	42	11	1·8	5·3	2·2	9·9	190	180

* Mice, three per strain, received $(NANP)_3$ or $[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT in $Al(OH)_3$ or FCA. Single cell suspensions from draining lymph nodes were incubated *in vitro* with saline, TT (10 μg/ml) or $(NANP)_3$ (20 μg/ml).

† Arithmetic mean c.p.m./culture × 10^{-2}.

Table 8. Responses of mice primed with $(NANP)_3$ on heterologous carriers

Immunization*		Anti-Ac-Cys-$(NANP)_3$
First	Second	(Titre × 10^3)†
$[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT	None	0·3 (1·8)
$[(NANP)_3]_2$–BSA	None	0·4 (1·1)
$[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT	$[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$ TT	53 (1·4)
$[(NANP)_3]_2$–BSA	$[(NANP)_3]_2$–BSA	2 (1·1)
$[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT	$[(NANP)_3]_2$–BSA	12 (1·4)
$[(NANP)_3]_2$–BSA	$[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT	7 (1·5)

* BALB/c mice, five/group, received 50 μg $[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT/$Al(OH)_3$ or 50 μg $[(NANP)_3]_2$–BSA/FIA s.c. Forty-two days later mice received 50 μg of antigen s.c. as indicated.

† Titres are geometric means (coefficient of variance G.M.) from bleedings made 51 days after the first injection.

protocol, mice did not present secondary proliferative responses to $(NANP)_3$. Low responses to $(NANP)_3$ were occasionally seen for CSW and CWB/13 mice immunized with the conjugate in FCA, and for DBA/2J mice using the conjugate in $Al(OH)_3$.

Capacity of $(NANP)_3$ conjugates to prime for anti-$(NANP)_3$ antibody responses to $(NANP)_3$ coupled to a heterologous carrier

In order to test for the possibility that B-cell priming to $(NANP)_3$ alone could serve as the basis for a secondary response, mice were primed with $[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT and challenged with $(NANP)_3$ conjugated to the heterologous carrier, BSA. BSA was chosen because there is no evidence that pre-immunization of mice with TT before challenge with haptenated BSA has either a positive or negative effect on subsequent responsiveness to the hapten (Schutze *et al.*, 1985). Mice were injected with either $[Ac\text{-}Cys\text{-}(NANP)_3]_{19}$–TT or $[(NANP)_3]_2$–BSA, prepared using two different conjugation methods to minimize the possibility of T cells specific for bridge determinants providing help, and challenged with $(NANP)_3$ on the heterolgous carrier.

As can be seen in Table 8 antibody responses to Ac-Cys-$(NANP)_3$ in animals pre-injected with this hapten coupled to a heterologous carrier were at least 10-fold greater than primary anti-Ac-Cys-$(NANP)_3$ responses. These increased anti-peptide responses are evidence for a secondary antibody response independent of T-cell priming that occurred during the primary response.

DISCUSSION

The present study has assessed the immunogenicity in mice of a potential anti-falciparum sporozoite vaccine that is currently undergoing human trials. Two injections of $[Ac\text{-}Cys\text{-}(NANP)_3]_{25}$–TT were required for high antibody responses to both Ac-Cys-$(NANP)_3$ and TT in adult mice. Priming with a single injection of $[Ac\text{-}Cys\text{-}(NANP)_3]_{25}$–TT was equally effective in adult and 1-week-old mice, since secondary challenge yielded similar anti-Ac-Cys-$(NANP)_3$ responses composed primarily of IgG antibody. Since both new-born mice and humans are immunologically immature, the present results suggest that $[Ac\text{-}Cys\text{-}(NANP)_3]_{25}$–TT could effectively prime the latter for an anti-$(NANP)_3$ antibody response to homologous antigen. At the same time it was noted that each age group also responded

well to TT, suggesting that [Ac-Cys-$(NANP)_3]_{25}$–TT might also serve as a tetanus vaccine. The marked boosting effect and magnitude of the responses in mice contrasts with the variable and generally low responses seen in human volunteers (Herrington *et al.*, 1987).

Immunization of female mice with TT or [Ac-Cys-$(NANP)_3]_{25}$–TT prior to mating resulted in maternal transfer of antibody and the specific inhibition of the response of offspring to [Ac-Cys-$(NANP)_3]_n$–TT. Since inhibition of the antibody response by maternal transfer of anti-$(NANP)_3$ or –TT antibody could similarly occur in humans, the present observations are contradicative for injecting new-borns in endemic areas where the transfer of anti-$(NANP)_3$ and –TT antibodies exist (Higgins Nardin *et al.*, 1981). It is interesting to note that the marked inhibition of the response of offspring from anti-[Ac-Cys-$(NANP)_3]_{19}$–TT-immunized mothers could be substantially overcome by a second boost of [Ac-Cys-$(NANP)_3]_{25}$–TT. This finding is similar in practice to the ability of multiple injections of antigen to overcome the immunosuppression associated with Chagas' disease (Choromanski & Kuhn, 1986).

Pre-immunization of mice with carrier (TT) protein resulted in a dose-dependent inhibition of the anti-Cys-$(NANP)_3$ antibody response to secondary challenge with [Ac-Cys-$(NANP)_3]_{25}$–TT. Results from previous studies indicate that the cellular bases for inhibition is carrier–specific T-suppressor cells as well as defects at the B-cell level (Herzenberg & Tokuhisa, 1982; Herzenberg *et al.*, 1982; Schutze *et al.*, 1987).

The observation that offspring from TT-immunized mothers present reduced responses to [Ac-Cys-$(NANP)_3]_{25}$–TT suggests that anti-TT antibody, itself, may also play a role in the inhibition associated with carrier priming seen in the present study, although the report that some maternal lymphocytes are transferred to offspring (Weiler & Sprenger, 1981) introduces a caveat to this point. The capacity of tertiary challenge with [Ac-Cys-$(NANP)_3]_{25}$–TT to overcome inhibition is similar to the results described above for offspring from pre-immunized mothers as well as the earlier finding that repeated immunization of carrier-primed mice with haptenized carrier eventually restored anti-hapten responsiveness (Herzenberg *et al.*, 1982). Since volunteers who received [Ac-Cys-$(NANP)_3]_{25}$–TT were pre-immunized with TT, the low responses to the Ac-Cys-$(NANP)_3$ component of the vaccine could be related to such pre-immunization and an optimal choice for a carrier should, minimally, exclude proteins that already are utilized as vaccines. It should be noted, however, that a vaccine composed of diphtheria toxoid or the related protein CRM197 conjugated to oligosaccharides from *H. influenzae*, which contains a carrier previously used for immunization, has been used successfully (Insel & Anderson, 1986). Moreover, the failure of the recombinant DNA vaccine, R32tet32, which contains no component to which volunteers have been injected, to stimulate high levels of antibody in humans indicates that factors in addition to pre-immunization with carrier protein may be responsible for low antibody responses; this point does require the qualification that the response to R32tet32 is under genetic restriction in mice (Good *et al.*, 1986).

Previous reports have documented the genetic restriction associated with the murine immune response to a polymer of (NANP) as well as a fusion protein containing this sequence (Good *et al.*, 1986; Del Giudice *et al.*, 1986). As was the case, the present results utilizing Ac-Cys-$(NANP)_3$ conjugated to a protein carrier highlight the loss of such genetic restriction, since each strain tested produced anti-Ac-Cys-$(NANP)_3$ antibody. Attempts to demonstrate good T-cell priming to the $(NANP)_3$ determinant on [Ac-Cys-$(NANP)_3]_{25}$–TT failed, suggesting that such a conjugate may not effectively sensitize parasite-specific [i.e. $(NANP)_3$-reactive] T cells, although T-cell sensitization to $(NANP)_n$ has been recorded in clinical trials (Ballou *et al.*, 1987; Etlinger *et al.*, 1988). The utility of a vaccine that primarily sensitizes B cells would seem to be less than that of one which sensitizes T and B cells. However, it should be noted that the anti-oligosaccharide responses of children vaccinated with diphtheria toxoid conjugated to oligosaccharides from *H. influenzae* are boosted, presumably through subsequent natural exposure to organisms containing such oligosaccharide determinants (Insel & Anderson, 1986). Furthermore, the current finding of priming for a secondary response with Ac-Cys-$(NANP)_3$ conjugated to a heterologous carrier indicates that [Ac-Cys-$(NANP)_3]_{25}$–TT may also prime for a response to sporozoites or may boost the anti-$(NANP)_3$ response of individuals primed with sporozoites.

It should be noted that the use of Freund's adjuvant for some experiments reflected the intent to (i) analyse a primary anti-Ac-Cys-$(NANP)_3$ response that was too low for suitable analysis when $Al(OH)_3$ was employed (Table 3, Exp. 1, and 7); (ii) provide a potent adjuvant to test whether the observed inhibition could be overcome (Table 3, Exp. 2); and/or (iii) provide the best adjuvanticity for $[(NANP)_3]_2$ BSA that, even in a secondary response with Freund's, elicits low levels of anti-Ac-Cys-$(NANP)_3$ antibody (Table 8).

Finally, it should be stressed that the utility of a vaccine depends on its ability to sensitize or initiate effector activity with the correct specificity in those populations of lymphocytes that are important for protection against the pathogen. In the case of the malaria sporozoite, based on *in vitro* and animal experiments, it appears that T- or B-cell effector cells can play a decisive role and, therefore, the optimal vaccine should contain the appropriate determinants recognized by both populations of lymphocytes.

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