Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites

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A 12 amino-acid synthetic peptide $(NANP)_3$ comprising the immunodominant epitope of *Plasmodium falciparum* circumsporozoite protein was conjugated to tetanus toxoid (TT), adjuvanted with aluminium hydroxide, and administered intramuscularly in three doses at monthly intervals to 35 healthy males as a malaria vaccine. No significant adverse reactions were noted, with mild soreness at the injection site the only common symptom. Seroconversions against NANP occurred in 53% and 71% of recipients of 100 or 160 µg, respectively, measured by enzyme-linked immunosorbent assay (ELISA). Most ELISA-positive sera reacted with sporozoites by indirect immunofluorescence (IFA). Three vaccinees with the highest ELISA and IFA titres and four unimmunized controls were challenged with *P. falciparum* sporozoites introduced via the bites of infective *Anopheles* mosquitoes. Blood stage parasites were detected in all controls by 10 days (mean 8.5 days, range 7–10). In contrast, the two vaccinees who became infected did not manifest parasitaemia until day 11 and the third vaccinee showed neither parasites nor symptoms during the 29 day observation period. This first synthetic peptide parenteral vaccine against a communicable disease tested in man is safe and stimulates biologically active antibodies. These observations encourage the development of improved vaccine formulations which, by enhancing immunogenicity, may lead to practical vaccines to assist in the control of falciparum malaria.

Plasmodium falciparum malaria is undergoing a worldwide resurgence because of the spread of drug resistant parasite strains and increasing resistance of mosquitoes to insecticides[1]. Malaria vaccines are being developed that may complement traditional malaria control measures[2].

In the early 1970s, complete protection against malaria in small numbers of volunteers was achieved by 'vaccination' with attenuated, X-irradiated sporozoites[3-5] and was correlated with antibodies directed against the circumsporozoite (CS) protein which covers the surface of the mature sporozoite[6]. Recognition that the immunodominant epitope of the *P. falciparum* CS protein is a repetitive four amino-acid sequence (asparagine-alanine-asparagine-proline, or NANP)[7-9] and that monoclonal antibodies directed against this sequence neutralize sporozoite infectivity *in vitro*[10], paved the way for the development of sporozoite vaccine candidates prepared by synthetic peptide and recombinant DNA techniques[2,10,11]. Synthetic NANP peptides conjugated to protein carriers generate specific anti-sporozoite antibodies in animals[10]. Accordingly, a vaccine suitable for testing in man was prepared by conjugating acetyl-cysteine-$(NANP)_3$-OH [Ac-Cys-$(NANP)_3$-OH] made by solid-phase peptide synthesis[12] to tetanus toxoid (see Table 1 for details). Amino acid analysis revealed 25 residues of peptide per mole of tetanus toxoid.

Table 1 Vaccination schedule and dosages of $(NANP)_3$-TT

Vaccine dose (µg)	No. of vaccinees	No. of controls
20	3	1
50	3	1
100	15	4
160	14	4

Immunizations were given into the triceps muscle on days 0, 28 and 56. Three volunteers in the 160 µg vaccine group received a third booster injection on day 133 in preparation for participation in the vaccine efficacy study. Ac-Cys $(NANP)_3$-OH was prepared by solid-phase peptide synthesis starting with proline-hydroxymethyl-resin (21.0 g, 0.36 nmol g^{-1}, 7.56 mmol) and 12 cycles of coupling reactions using 4 equivalents of *N*-α-*t*-butyloxycarbonyl (Boc)-amino acids with dicyclohexylcarbodiimide (DCC) couplings. Boc-asparagine was introduced as the *N*-hydroxybenzotriazole-ester. The final cycle was carried out with Boc-S-3,4-dimethyl-benzyl-L-cysteine by the DCC procedure. Acetylation was achieved with acetic anhydride-pyridine for 1 h. All couplings were monitored by the ninhydrin reaction[26] and repeated until a negative test was observed. A portion of the protected peptide-resin (10.5 g) was cleaved with anhydrous hydrogen fluoride (containing 10% dithioethane) and the crude product (1.98 g) purified by preparative high pressure liquid chromatography (HPLC) on a Nucleosil C_{18} reversed-phase column using a linear gradient of (A) H_2O (0.1% trifluoracetic acid (TFA) to (B) acetonitrile (0.1% TFA) from 5%-25% (B) in 120 min. The purified peptide (914 mg, 19.6% yield) was homogenous by analytical HPLC. TT(350 mg) in 80 ml of 0.1 M phosphate buffer (*p*H 7.5) was reacted with *N*-succinimidyl-3-maleimidopropionate (586 mg) in dimethylformamide, adjusted to *p*H 6 (HCl) and applied to a Sephadex G25S column. When eluted with 0.05 M phosphate buffer (*p*H 6.0), the modified TT appeared as the first peak. This was reacted with Ac-Cys-$(NANP)_3$-OH (148 mg) and re-chromatographed on a Sephadex G25S solumn as above. The resultant conjugate was dialysed against saline and concentrated (50 ml).

Male students from the University of Maryland Schools of Medicine and Dentistry were selected based on their expressed interest, good health, and lack of previous history of malaria. Medical screening included a history and physical examination, a complete blood count, urinalysis, and serology for hepatitis B, syphilis, and human immunodeficiency virus. Volunteers were excluded if they had received TT within the past 3 years, had known hypersensitivity to TT, or had previously experienced notable allergic reactions. All aspects of the studies were explained in detail, including potential risks and benefits, and informed consent was obtained. Studies were approved by ethical review committees of the University of Maryland Hospital and the National Institute of Allergy and Infectious Diseases.

Volunteers were randomized to four dosage groups to receive 20, 50, 100, or 160 µg of $(NANP)_3$-TT (Table 1). Vaccine was administered in double-blind fashion, with control volunteers within each group receiving a dosage of TT without peptide equal to the amount of TT in the conjugate vaccine for that dosage group. Booster vaccinations were administered 28, 56 and, in select volunteers, 133 days later.

Neither TT nor the conjugate vaccine elicited serious local or systemic untoward effects. The only common symptom was mild soreness at the vaccination site in 57% of vaccinees following the first injection; booster injections were simiarly well tolerated. No specific reactions could be attributed to the synthetic peptide component of the conjugate vaccine.

The frequency and magnitude of the antibody responses correlated with the dose of vaccine administered (Table 2). Peak

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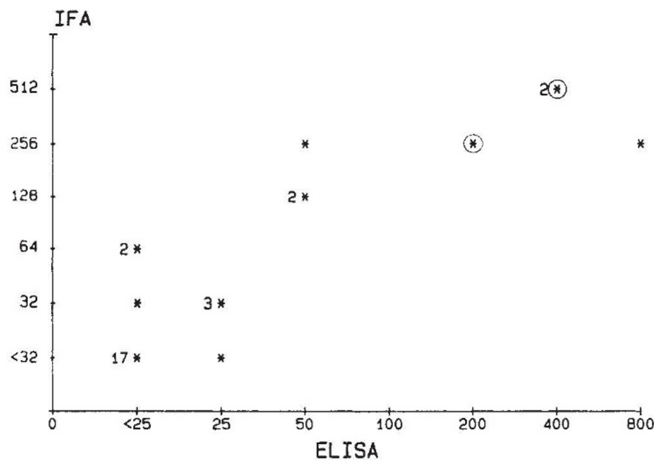

Fig. 1 Scatter plot of antipeptide IgG (ELISA) against anti-sporozoite IgG (IFA) for recipients of all doses of $(NANP)_3$-tested on day +72. Volunteers participating in the sporozoite challenge study are circled. IFA was carried out using multiple well slides containing sporozoites from the NF54 strain of *P. falciparum* fixed in 0.1% glutaraldehyde[27].

ELISA titres to NANP were detected after the first dose of vaccine and did not rise further following booster immunizations. Most sera with antibody to NANP detected by ELISA reacted with *P. falciparum* sporozoites and the correlation between ELISA and IFA titres (Fig. 1) was significant ($r_S = 0.858$, $P < 0.001$). Circumsporozoite (CSP) reactions[13], known to be poorly sensitive[14], were not seen.

Peripheral blood lymphocytes from all vaccinees and controls were reacted with $(NANP)_3$ and $(NANP)_{50}$ in a lymphocyte replication assay[15]. Lymphocyte proliferation in response to NANP was not detected in vaccine recipients. The fact that the titre of antibody to peptide did not increase after booster vaccinations also indicates that T-cell stimulation by $(NANP)_3$ was not a major effect of immunization with $(NANP)_3$-TT.

Vaccine efficacy was assessed by inoculating volunteers with infectious sporozoites. *P. falciparum* strain NF54 was selected on the basis of known chloroquine sensitivity and high infectivity for mosquitoes, and was cultured as previously described[16]. *Anopheles stephensi* and *Anopheles freeborni* mosquitoes were infected by membrane feeding[16] on a mixture of NF54 cultures and fresh normal blood with a final gametocytaemia of ~1-2%. Sporozoite inoculation was accomplished through the bites of infected mosquitoes. Each fully fed mosquito was immediately dissected to detect salivary gland sporozoites which were found in 50%. Salivary gland sporozoites were graded 1+ (1-10 per paired gland), 2+ (11-100 per paired gland), 3+ (101-1,000 per paired gland) or 4+ (>1,000 per paired gland)[17]. Each volunteer was exposed to five infected mosquitoes, at least one of which in each case had a 3+ or 4+ sporozoite density. This is a relatively high challenge dose since, except in areas of very high malaria endemicity, the infection rates of mosquitoes are generally much lower than 1%. Analysis of variance revealed no significant differences between individual volunteers in the mean sporozoite rates of the infected mosquitoes that bit them.

Giemsa-stained thick and thin blood films from each volunteer were examined every 12 hours, beginning 6 days after mosquito feeding. Parasitaemias were estimated by enumerating the number of parasites and leukocytes in a thick film until 500 leukocytes were counted. The number of parasites per microlitre of blood was determined based on the results of a white blood cell count made within 48 hours for each volunteer. A thick blood film was considered negative only after 15 or more minutes of examination, corresponding to at least 200 oil immersion fields. A course of oral chloroquine phosphate (600 mg base, followed by 300 mg 6, 24 and 48 hours later) was immediately initiated upon confirmation of parasitaemia.

Three vaccinees who had the highest rises in antisporozoite antibody by ELISA and IFA (Fig. 1) and four control volunteers with no history of prior malaria infection or vaccination participated in the efficacy study. As shown in Table 3, the four control volunteers developed parasitaemia 7, 8, 9 and 10 days following challenge (mean of 8.5 days). The two vaccinees who developed

Table 2 Summary of serological results (IgG and IgM-ELISA) of volunteers immunized with varying dosages of $(NANP)_3$-TT vaccine

Vaccine dose group	Number of vaccinees	Number (%) with significant rises in anti-(NANP) antibody: IgG	IgM	IgG or IgM	Geometric mean IgG titre: Day 0	Day 14	Day 44	Day 72
Controls	10	0	0	0	12.5	12.5	14.4	13.5
20 or 50 μg	6	0	0	0	12.5	12.5	12.5	14.9
100 μg	15	5 (33)	4 (27)	8* (53)	12.5	20.5	22.8	20.5
160 μg	14	8 (57)	3 (21)	10* (71)	13.1	35.4	41.0	42.6

Immunizations were given on days 0, 28 and 56 and sera were obtained at 2 week intervals following each vaccination. ELISA was carried out as follows: Wells of every other column of polystyrene Cooke ELISA plates (Dynatech Corporation) were coated with 100 ng per well of $(NANP)_{50}$ (synthetic peptide prepared in our laboratories) in carbonate coating buffer, *p*H 9.6. Plates were incubated overnight at 4 °C and washed 3 times with phosphate buffered saline (PBS)*p*H 7.3. PBS with 20% fetal bovine serum was added to each well. Plates were incubated for 1 h at 37 °C and washed three times with PBS containing 0.05% Tween. Serial twofold dilutions of sera were made starting at 1:25. Plates were incubated for 2 h at 37 °C, and washed 5 times with PBS containing 0.05% Tween. Goat anti-human IgG alkaline phosphatase conjugate (Kirkegaard Perry Laboratories) at a dilution of 1:600 in PBS containing 1% fetal bovine serum was added to each well. Plates were incubated for 1 h at 37 °C, washed 5 times as above and alkaline phosphatase substrate was added. Colour was permitted to develop for 30 min at 37 °C. The optical densities (OD) of the wells were read in a Titertek multiscan automated reader set at a wavelength of 450 nm. Net ODs were calculated by subtracting the OD of the corresponding background well from the antigen-containing test well. Titres were determined based on a cutoff OD corresponding to the mean OD of preimmunization sera at a 1:25 dilution plus 3 standard deviations. A four-fold rise in titre from pre- to post-immunization was considered significant. The procedure for determining IgM antibody was the same except that the conjugate used was goat anti-human IgM (Kirkegaard Perry Laboratories) at a dilution of 1:500.

* Sera from one volunteer contained both IgG and IgM antibodies to NANP.

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Table 3 Serological and parasitological results after *P. falciparum* sporozoite inoculation in vaccinees and controls

Volunteer	Prevaccination	Reciprocal IgG anti-NANP titres Prechallenge	Day +7	Day +28	Days to patency†	Peak parasitaemia (parasites mm^{-3})
1	ND*	<25	<25	<25	9	49
2	ND	<25	<25	<25	8	20
3	ND	<25	<25	<25	10	<10
4	ND	<25	<25	<25	7	21
5	<25	400	200	400	11	24
6	<25	400	400	200	‡	‡
7	<25	200	400	400	11	24

Volunteers 1-4 were unimmunized controls. Volunteers 5, 6 and 7 were vaccine recipients. Antibody titres were measured by ELISA as previously described. Parasites mm^{-3} of blood were enumerated microscopically (see text).

* ND, not done. Control volunteers received no vaccine.

† $P = 0.029$ when prepatent periods between vaccinees and controls are compared using 1-tailed Wilcoxon rank sum test.

‡ Volunteer 6 did not develop parasitaemia during the 29 day post challenge observation period.

malaria infection after challenge did not have asexual blood stage parasites detected until 11 days after challenge and the remaining vaccinee was still free of parasitaemia at 29 days; the difference in number of days until detection of parasitaemia between the controls and vaccinees is significant ($P = 0.029$, Wilcoxon rank sum test). All infected volunteers received curative doses of chloroquine and recovered uneventfully; the maximum parasitaemia recorded was 49 parasites per mm^3. The one non-infected volunteer received chloroquine after a 29-day period of observation. Red blood cells from this volunteer supported the growth of plasmodium strain NF54 in *in vitro* culture. Routine testing of urine[17] showed that no 4-aminoquinolines had been surreptitiously ingested by any volunteer during the study.

Studies in sporozoite-induced malaria in man have suggested an inverse relationship between the size of the sporozoite inoculum and the duration of the prepatent period[18,19]. This inverse relationship has been quantitaitvely documented in animal malaria[20,21]. In mice infected with known numbers of dissected sporozoites of *P. berghei*, the prepatant period was lengthened when the inoculum was reduced from 5×10^3 to 10 sporozoites[22]. Thus, the significantly increased prepatent period of 11 days seen in the two vaccinees who developed infection compared to a mean of 8.5 days in the controls probably represents a significant vaccine-induced reduction in the numbers of sporozoites which entered and developed in hepatocytes.

These data demonstrate that a synthetic peptide of the immunodominant epitope of *P. falciparum* circumsporozoite protein conjugated to tetanus toxoid as a protein carrier has resulted in a first generation malaria vaccine that is safe in man, stimulates a biologically active immune response that apparently diminishes the number of circulating sporozoites that reach the liver and, in one of three vaccinees, provided complete protection against malaria. The serological response in mice immunized with similar vaccines incorporated in Freund's adjuvant and containing the repeated epitope of the *P. berghei* CS protein has been more prominent and has demonstrated the effect of booster immunizations. Moreover, most mice were protected against challenge although cell-mediated responses against the synthetic peptide could not be demonstrated[23]. It is likely that improved formulations and new, more potent adjuvants, can markedly enhance the antibody response and perhaps also increase T-cell stimulation. It is also possible that tetanus toxoid, given to our volunteers in the past as childhood immunizations, dampened the immune response to the synthetic peptide component of the conjugate[24,25]; further studies are planned to resolve this issue. We are also exploring whether HLA or other genetic restrictions to immune responsiveness to this vaccine exist. A possible disadvantage of this particular peptide conjugate vaccine is that it may not prime parasite-specific T cells, and a boosting of immune response and lymphokine production may not occur during subsequent exposure to sporozoites under natural conditions[2]. Nevertheless, the results reported here represent a step forward on the road towards the development of practical malaria vaccines.

After this paper was submitted, a report appeared[28] describing the results of a trial in humans of a recombinant DNA *P. falciparum* vaccine containing multiple NANP repeats. With this vaccine, protection also appeared to correlate with antibody levels.

We thank the US Agency for International Development, the National Institute of Allergy and Infectious Diseases, the American Institute of Biological Sciences, the MacArthur Foundation and Hoffman-La Roche for support. Absorption of $(NANP)_3$-TT to aluminium hydroxide and subsequent bottling of the vaccine were carried out at the Pasteur Institute under the superivision of Dr E. H. Relyveld. We thank Pamela Leland for technical assistance, and the volunteers for participating in the study.

Received 1 June; accepted 19 June 1987.

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