Human studies with synthetic peptide sporozoite vaccine $(NANP)_3$–TT and immunization with irradiated sporozoites

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The synthetic peptide Plasmodium falciparum *circumsporozoite (CS) protein conjugate vaccine $(NANP)_3$–TT was safe when given parenterally to 202 volunteers. However, with a few notable exceptions, antibody responses were low and could not be boosted. Vaccinees' lymphocytes did not proliferate when exposed* in vitro *to $(NANP)_3$. The tetanus toxoid (TT) carrier immunomodulated the response to the CS peptide in that both epitopic suppression and immune enhancement were demonstrated during the course of the clinical trials. During efficacy challenge studies, 1 of 7 vaccinees was protected against sporozoite challenge and in other vaccinees the prepatent period was significantly delayed.*

P. falciparum*-infected mosquitos were irradiated with 20 000 rad (200 Gy). Five volunteers were immunized with 54, 55, 224, 663, and 715 total infective bites of irradiated mosquitos in an attempt to immunize with attenuated sporozoites. Four of these volunteers had significant humoral and cellular immune responses. Two volunteers (who received the largest immunizing doses) were challenged by the bites of infective mosquitos and both developed parasitaemia. In the volunteer with the highest antibody titre there was a marked delay in patency as determined by serial plasmodial cultures. T-cell clones are being obtained and characterized.*

Introduction

One of the first candidate malaria vaccines to be evaluated, and the first synthetic peptide malaria vaccine to be given to humans was the $(NANP)_3$–tetanus toxoid (TT) conjugate designed by Nussenzweig and coworkers at New York University and manufactured by the Hoffmann-La Roche Co. Clinical trials with this vaccine, which was given to 202 volunteers over a 3-year period, are reviewed.

The immunologic responses to the synthetic peptide sporozoite vaccine were compared to those of 5 volunteers immunized with irradiated sporozoites. The irradiated sporozoite studies were carried out to generate human T-cell populations for use in mapping the T-cell epitopes of the *Plasmodium falciparum* CS (circumsporozoite) protein; these laboratory investigations are the subject of other reports (Nardin et al., Murphy et al., unpublished data).

Clinical trials

The preparation and composition of $(NANP)_3$–TT has been described (*1*, *2*). The final formulation contained 160 μg of conjugate protein per ml, of which 80% by weight was tetanus toxoid. All studies were conducted with the same lot of vaccine, which was stored at 4 °C as recommended.

The volunteers who participated in these trials were students at the University of Maryland and, for the studies carried out in Venezuela, healthy adult men and women from rural, non-malarious areas in and around the villages of Altamira and Capeton, about 150 km from Caracas. Methods of medical screening, care of the volunteers, and informed consent have been reported (*1*, *3*). All studies were approved by the Human Volunteers Research Committee of the University of Maryland, and followed established guidelines. Studies in Venezuela

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were conducted with the consent of the Venezuelan Ministry of Health.

Vaccination schedules and intramuscular dosages are listed in Table 1. Results of the Phase 1 safety and immunogenicity study (Study 1) have been reported (*1*). To address some possible reasons for the relatively poor immunogenicity of the vaccine, additional studies were undertaken to determine if a larger vaccine dose or an increase in the dosing interval would significantly improve antibody responses. In Study 2, conducted 1 year after the Phase 1 study, 320 μg of vaccine were administered to 26 volunteers on days 0 and 56. Study 3 was conducted concomitantly with Study 2 to measure vaccine stability after storage; the serological response elicited by a single 160 μg dose of vaccine was determined in 47 volunteers.

A study was also conducted to determine whether previous exposure to tetanus toxoid modulated the immune response to the NANP hapten. Study 3 was conducted in Venezuelan villages where volunteers with and without previous immunity to tetanus toxoid were identified. Study 4 was conducted at the same time as Study 3 in a US student population as a vaccine stability control.

Vaccine doses up to 160 μg were well tolerated. However, fevers were observed in 5 of 47 (11%) vaccinees when the dose was increased to 320 μg. Mild to moderate tenderness or soreness at the vaccination site was a common finding at all dosages.

Antibody responses against NANP and intact sporozoites were determined by ELISA (*1*) and indirect immunofluorescence (IFA) (*4*), respectively. Overall, 105 of 178 (59%) of volunteers immunized with one or more doses of 160 or 320 μg of $(NANP)_3$–TT seroconverted as defined by a 4-fold or greater rise in IgG or IgM antibody (Table 2). Six percent and 13% of vaccinees had high-titre IgG and IgM responses, respectively (defined as reciprocal ELISA titres $\geqslant 800$), but the majority of antibody responses were only moderate as reflected in the low peak geometric mean reciprocal titres (Table 2). There was excellent correlation between ELISA and IFA, indicating that the anti-peptide antibodies recognized the native CS protein on the intact sporozoite (*1*).

The conjugate vaccine lost potency during the first year of storage. A comparison of the antibody response of volunteers given a single vaccination of 160 μg 1 year or more after Study 1 demonstrated significantly lower IgG seroconversion rates (Table 2). A similar decrease in vaccine potency was not observed in independent trials conducted by Swiss investigators (Sturchler, personal communication).

During the course of the Phase 1 and 2 trials with $(NANP)_3$–TT, it was determined that background immunity to the protein carrier, tetanus toxoid, immunomodulated the response to the $(NANP)_3$ hapten (*22*). In North American volunteers, in whom there were very high prevaccination tetanus antitoxin titres, there was a significant negative correlation ($P < 0.02$) between the prevaccination tetanus antitoxin titres and the post-vaccination anti-(NANP) titres for vaccinees receiving 100 μg of vaccine. In contrast, in Venezuelan volunteers, who had low prevaccination tetanus antitoxin titres, there was a positive correlation between preimmunization tetanus antitoxin and subsequent IgM antibody response to the NANP hapten ($P = 0.001$). These results raise the possibility that, in humans, prior immunity to the carrier protein can immunomodulate the response to the peptide hapten, resulting in either epitopic suppression (*5–7*) or immune enhancement.

The development of cell-mediated immunity after vaccination was assessed for volunteers in Studies 1 and 2 by measuring the replication of lymphocytes *in vitro* in the presence of $(NANP)_3$, $(NANP)_{50}$ and

Table 1: **Vaccination schedule and dosages of $(NANP)_3$–TT**

Study	Vaccine dose (μg)	No. of vaccinees	No. of controls	Booster doses
1[a]	20	3	1	days +28, +56
	50	3	1	days +28, +56
	100	15	4	days +28, +56
	160	14	4	days +28, +56
2[b]	320	26	0	day +56
3[b]	160	47	0	None
4[c]	160	72	0	None
5[d]	160	22	0	None

[a] Results from study 1 have previously been reported (*1*).
[b] Studies 2 and 3 were carried out one year after study 1, using the same lot of vaccine which had been stored at 4 °C.
[c] Study 4 was carried out 2 years after study 1 in Venezuela.
[d] Study 5 was carried out 2 years after study 1 in North America to serve as a vaccine stability control for Study 4.

Table 2: **Serological results (IgG and IgM ELISA) of volunteers immunized with $(NANP)_3$–TT**

Study (description)	No. of seroconverters			Peak geometric mean titre		No. with titres ⩾ 800	
	IgG	IgM	IgG or IgM	IgG	IgM	IgG	IgM
1 (160 μg, 1986)	12/14 (86)[a]	10/14 (71)	14/14 (100)	110	86	2/14 (14)	2/14 (14)
2 (320 μg, 1987)	21/26 (81)	19/26 (73)	24/26 (92)	105	268	4/26 (15)	8/26 (31)
3 (160 μg, 1987)	11/47 (23)	20/47 (43)	25/47 (53)	26	74	2/47 (4)	9/47 (19)
4[b] (160 μg, 1988)	12/69 (17)	22/69 (32)	28/69 (41)	22	33	1/69 (1.4)	3/69 (4)
5 (160 μg, 1988)	5/22 (23)	12/22 (55)	14/22 (64)	28	59	2/22 (9)	3/22 (14)
Total (all studies)	62/178 (35)	83/178 (47)	105/178 (59)			11/178 (6)	24/178 (13)

[a] Figures in parentheses are percentages.
[b] Study 4 took place in Venezuela.

control antigens using described microculture techniques (*8*, *9*). Lymphocyte replication responses to NANP were not detected.

The characteristics of the immune response to the $(NANP)_3$–TT vaccine were typical of a T-cell independent antigen. These included a failure of a subset of vaccinees to manifest seroconversions, significant rises in IgM class antibody that were not followed by seroconversions in IgG class antibody in some vaccinees, and overall absence of significant increases in antibody titre following booster inoculations. Finally, the lymphocyte proliferation studies reported here, and work by other investigators (*10–12*) demonstrate that $(NANP)_3$ does not function as a T-cell epitope.

Two vaccine efficacy studies were carried out. Infection of *Anopheles stephensi* and *A. freeborni* mosquitos with *P. falciparum* strain NF54 was carried out as described (*1*, *3*). Enumeration of sporozoites in challenge mosquitos was performed by immunoradiometric assay (*13*, *14*) and by salivary gland grading by microscopic dissection (*15*, *16*).

In the first efficacy study with $(NANP)_3$–TT vaccine, three volunteers with the highest anti-NANP titres and four unimmunized control volunteers were challenged with *P. falciparum* strain NF54 sporozoites delivered via the bites of 5 infective mosquitos. The four control volunteers all developed parasitaemia between days 7 and 10 post-exposure. One of three vaccinees did not develop patent infection and the remaining two vaccinees who developed patent infection did so after 11 days, a significant delay in comparison with the control volunteers (*1*, *17*).

In the second vaccine efficacy study, four recipients of 320 μg of $(NANP)_3$–TT who had pre-challenge anti-NANP titres similar to the vaccinees in the first study, and four unimmunized controls were each exposed to five heavily infected mosquitos. The vaccinees all developed parasitaemia. In this study, in comparison with the first challenge efficacy study, the sporozoite inoculum was heavier based on both the mean sporozoite gland densities of the mosquitos used for challenge (*13*) and upon the difference in the average prepatent periods of the two control groups (8.5 vs. 7.4 days). Despite the larger sporozoite challenge in the second study, the significant delay in onset of patent infection in vaccinees vs. controls again demonstrated the biological activity of the vaccine-induced immune response.

Immunizations with irradiated sporozoites

Recognition of the potential importance of incorporating parasite-specific T epitopes in future candidate sporozoite vaccines led us to attempt to vaccinate volunteers with attenuated sporozoites using methods similar to those which had proved successful in the 1970s (*18–20*). The aims of these studies were to identify responder T cells, clone these cells to map the T-cell epitopes, identify the phenotype of the effector T cells, and acquire sera and lymphocytes for use as positive controls during immunological studies of candidate sporozoite vaccines. Immunizations were carried out in a group of 5 volunteers exposed to infected mosquitos which had been irradiated with a minimum of 20 000 rad (200 Gy). The immunizing schedule was largely determined by the availability of mosquitos. The total number of infective bites of the

volunteers was 54, 55, 224, 625, and 715. The two volunteers with the largest number of exposures received the majority of these in three sessions separated by 3-week intervals.

Although the primary aim of these studies was to investigate cell-mediated immunity to intact sporozoite antigen, the humoral immune responses were also of interest. Four of the five volunteers made anti-NANP antibody as detected by ELISA or IFA. One volunteer who was immunized with a total of 55 infective mosquitos made no detectable immune response. In contrast, a second volunteer immunized concurrently with 54 mosquitos exhibited antibody titres of 1:400 by ELISA and 1:1024 by IFA. The remaining three volunteers developed peak ELISA antibody titres of 1:800, 1:800, and 1:12 800 after 224, 625 and 715 total infective bites, respectively. IFA titres correlated closely with those of the ELISA. The volunteer with the highest titre demonstrated a CS precipitin reaction when sera were mixed with viable sporozoites. For comparison, the IgG and IgM seroconversion rates and geometric mean peak titre for volunteers immunized with the $(NANP)_3$–TT subunit vaccine are compared with the results with the five volunteers immunized with irradiated sporozoite (Table 3). The seroconversion rates are not significantly different, although the levels of antibody are higher for volunteers immunized with irradiated sporozoites.

The two volunteers with the greatest number of exposures to irradiated mosquitos and two malaria naive controls were challenged with *P. falciparum* infected mosquitos. Neither immunized volunteer was protected against developing parasitaemia. However, the volunteer with the highest prechallenge titres had a significantly delayed prepatent period.

Based on the very high antibody levels to NANP and to intact sporozoites, solid protection against sporozoite challenge was expected in the volunteer with the highest immunizing dose. That this was not the case underscores some of the difficulties with correlating protection with currently available *in vitro* tests. Possible explanations for the lack of protection include: the immunizing dose was not sufficiently high; the irradiating dose of 20 000 rad (200 Gy) (chosen because occasional breakthroughs were seen when 15 000 rad (150 Gy) was used during previous studies (*19*)) may have destroyed a critical epitope or hyperattenuated the sporozoites; the use of prophylactic weekly chloroquine phosphate during the immunization period may have interfered with the immune response.

The lessons learned from the clinical trials with $(NANP)_3$–TT are relevant not only to sporozoite malaria vaccines but to the use of synthetic peptides for vaccines in general. Our findings support the idea that synthetic peptides can be safely used in vaccines for humans. As a malaria vaccine, $(NANP)_3$–TT was partly successful. It stimulated antibody production in most humans tested; the antibodies produced against the peptide also recognize the native sporozoite; the vaccination generated measurable immunity against sporozoite challenge although only 1 of 7 vaccinees was totally protected; and the dose of antigen was important since larger vaccine dosages produced greater antibody responses (*1*).

The measurement of vaccine efficacy in human subjects is complicated by difficulties in standardizing the sporozoite inoculum (*13*). At the present time, for technical reasons, sporozoites must be delivered to volunteers via mosquito bite, and the size of the inoculum can only be inferred (*17*). Despite these constraints, this study and another recent sporozoite vaccine trial in human subjects (*21*) have nevertheless demonstrated that subunit vaccines are capable of inducing antibody which, even at low levels, confers antisporozoite activity measurable during experimental challenges (*1*, *17*).

Table 3: **Comparison of antibody responses against the immunodominant epitope of the *P. falciparum* CS protein (NANP) in volunteers immunized with synthetic peptide conjugate vaccine $(NANP)_3$–TT or with irradiated sporozoites**

	No. of seroconverters			Peak geometric mean titre		No. with titres ⩾ 800	
Study (description)	IgG	IgM	IgG or IgM	IgG	IgM	IgG	IgM
$(NANP)_3$–TT[a]	33/40 (83)[b]	29/40 (73)	38/40 (95)	107	180	6/40 (15)	8/40 (20)
Irradiated sporozoites[c]	4/5 (80)	4/5 (80)	4/5 (80)	528	115	3/5 (60)	1/5 (20)

[a] Includes volunteers receiving 2 doses of 160 or 320 μg of vaccine.
[b] Figures in parentheses are percentages.
[c] Range of total number of infected bites from 54 to 715 with mean of 336.

Acknowledgements

These studies were supported by the National Institute of Allergy and Infectious Diseases (research contract NO1 AI62553), the U.S. Agency for International Development, and the American Institute for Biological Sciences. We are grateful to Dr Hugh Eddy for irradiating the mosquitos and Dr Steven Wasserman for the statistical analyses.

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