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SAFETY AND EFFICACY OF A RECOMBINANT DNA PLASMODIUM FALCIPARUM SPOROZOITE VACCINE

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Summary A recombinant DNA *Plasmodium falciparum* sporozoite vaccine produced in *Escherichia coli* (FSV-1) was tested in doses of 10 µg to 800 µg protein in fifteen volunteers. No serious adverse reactions occurred. Antibodies that reacted with *P falciparum* sporozoite antigens by enzyme-linked immunoassay developed in twelve of the volunteers. The highest antibody titres induced were similar to those resulting from lifelong natural exposure to sporozoite-infected mosquitoes. Postimmunisation serum samples from a majority of volunteers mediated the circumsporozoite (CS) precipitation reaction and inhibited sporozoite invasion of hepatoma cells in vitro. Serum from the three volunteers who received 800 µg doses reacted with the surface of sporozoites in an immunofluorescence assay. Six immunised volunteers receiving a fourth dose of FSV-1 and two non-immunised controls were challenged by bites of mosquitoes infected from cultured *P falciparum* gametocytes. Parasitaemia did not develop in the volunteer with the highest titre of CS antibodies, and parasitaemia was delayed in two other immunised volunteers. This study confirms that human beings can be protected by CS protein subunit vaccines and provides a framework for the further development and testing of more immunogenic sporozoite vaccines.

Introduction

CONTROL of falciparum malaria remains one of the world's greatest health challenges. Global malaria eradication and control programmes carried out over the past 50 years have been seriously hampered by the widespread development of drug resistance by the parasite and insecticide resistance by the mosquito vector. There is, therefore, great interest in developing vaccines to prevent malaria. One of several potential vaccine targets is the sporozoite, the stage that is injected into the blood by mosquitoes and is exposed to serum antibodies for a brief time before it invades hepatocytes. Previous studies indicate

the feasibility of immunisation with radiation-attenuated sporozoites in human beings and animals,[1-3] but limited availability of purified sporozoites makes such an approach impractical for vaccine development. Sporozoite-immunised individuals show development of antibodies that are almost exclusively directed against a circumsporozoite (CS) protein which covers the parasite surface.[4] The general structure of CS proteins is remarkably similar among all malaria species studied and contains multiple tandem repeats of short aminoacid sequences.[5] Protection against sporozoite challenge in the *Plasmodium berghei* rodent malaria model can be passively transferred by means of monoclonal antibodies to intact *P berghei* sporozoites[6,7] or polyclonal antiserum to synthetic *P berghei* CS repeat peptides.[7] Rapid progress towards a human malaria vaccine was made by the cloning of the CS gene of *P falciparum*[8] and identification of its CS repeat epitopes as targets for functional antibodies.[9] A series of recombinant DNA *P falciparum* CS proteins expressed in *Escherichia coli*[10] have been identified as potential sporozoite vaccine candidates.[11] We now report the results of a human phase I safety and immunogenicity study and a preliminary efficacy study with one of these alum-adsorbed recombinant proteins, designated falciparum sporozoite vaccine 1 (FSV-1).

Subjects and Methods

Volunteers were recruited under protocols approved by an institutional human use review committee. Healthy men aged 22–50 years underwent baseline physical and laboratory examination including complete blood count, serum chemistry (blood urea nitrogen, creatinine, alanine and aspartate aminotransferases, and bilirubin), antibodies to both sporozoite and blood-stage parasites of *P falciparum*, antibodies to human immunodeficiency virus (HIV), and routine urinalysis. Volunteers were excluded if they gave a history of malaria; had evidence of cardiac, haematological, renal, hepatic, or immunological illness; were taking immunosuppressive medication; or had pre-existing antibodies to blood-stage *P falciparum* parasites by immunofluorescent assay or to $R32tet_{32}$ by enzyme-linked immunosorbent assay (ELISA). Fifteen volunteers meeting the enrolment criteria gave informed consent and entered the phase I study. Two volunteers consented to serve as non-immunised controls for the challenge part of the study.

The aminoacid sequence of $R32tet_{32}$ is $MDP(NANP)_{15}NVDP(NANP)_{15}NVDPtet_{32}$, where M = methionine, D = aspartic acid, P = proline, N = asparagine, A = alanine, V = valine, and tet_{32} = the first 32 aminoacids encoded by a tetracycline resistance gene, read out of frame.[10] FSV-1 consisted of single-dose ampules of sterile $R32tet_{32}$ in aqueous saline containing 0·5 mg Al^{3+} (as aluminum hydroxide gel) per 0·5 ml dose. Thiomersal was added as a preservative. FSV-1 was stored at 4°C and protected from light before administration. The vaccine was prepared at five different concentrations (10 μg, 30 μg, 100 μg, 300 μg, and 800 μg $R32tet_{32}$ per 0·5 ml dose).

The vaccine was given intramuscularly to three volunteers for each of the five doses. Volunteers received primary immunisation at week 0 and were boosted with identical doses at weeks 4 and 8 according to an immunisation schedule that had been used successfully in preclinical studies with small animals.[11] 50 weeks after primary immunisation, six of these volunteers received a fourth identical dose. Volunteers were observed for immediate toxic effects for 20 min after immunisation. 24 h and 48 h later they were examined for evidence of fever, local tenderness, erythema, warmth, induration, and lymphadenopathy and were asked about complaints of headache, fever, chills, malaise, local pain, nausea, and joint pain. Before each vaccine dose, blood and urine samples were taken for full laboratory examination. Complete blood count and serum chemistry profiles were rechecked 2 days after each vaccine dose.

Serum samples were taken 1 week after the first dose, then every 2 weeks for 16 weeks, and at the time of sporozoite challenge. Previously characterised human sera from malaria-endemic regions of Indonesia (4076) and western Kenya (60–2) were also studied. Serum was separated from blood that had clotted overnight at 4°C and stored at −70°C until assay. Samples were randomly coded before testing in all assays except ELISA.

The ELISA for CS antibodies was carried out as previously described.[11] As the test antigen we used R32LR—$MDP(NANP)_{15}NVDP(NANP)_{15}NVDPLR$; this is a purified recombinant construct that contains only the first 2 aminoacids encoded by the tetracycline resistance gene. Horseradish peroxidase conjugated to rabbit anti-human-IgG (heavy and light chains) was used to detect antibodies. Assays were run in triplicate and the mean absorbance and standard deviation were calculated for each dilution. Background values at a given dilution were determined with preimmunisation samples and defined as an optical density less than the mean plus 2 standard deviations of the week 0 serum sample's optical density for all volunteers. All SD were less than 0·1 units.

Serum samples were assayed for IgG, IgM, and IgA antibodies to R32LR by ELISA as described above, except that peroxidase-conjugated rabbit anti-human-heavy-chain (α, γ, μ) specific secondary antisera were used. For selected serum samples the concentrations of IgG subclasses reactive with R32LR were determined by ELISA[12] with murine anti-human-subclass-specific monoclonal antibodies as second antibody followed by horseradish peroxidase conjugated to goat anti-mouse-IgG (heavy and light chains). Estimates of IgG subclass concentrations were calculated from standard curves prepared by coating wells with serial dilutions of IgG1, IgG2, IgG3, or IgG4 human myeloma proteins. IgE antibodies reactive with $R32tet_{32}$ or R32LR were measured by ELISA with biotin-conjugated goat anti-human-IgE as second antibody and detected with streptavidin/horseradish-peroxidase complex. Human IgE myeloma protein was used to prepare a standard curve. The limits of detection in these assays were 0·01 μg/ml for all IgG subclasses and 0·1 μg/ml for IgE.

Immunofluorescence assays were carried out as previously described.[10] Salivary-gland sporozoites in 'Medium 199' containing bovine serum albumin were spread onto multiwell printed immunofluorescence assay slides, air-dried at room temperature, and stored at −80°C. Slides were incubated in serum diluted 1/100 or 1/400 for 20 min then washed. Antibodies reactive with sporozoites were detected with fluorescein-labelled anti-human-IgG under ultraviolet light at ×500 magnification. Fluorescence was graded from 0 to 4+, where 0 indicates no fluorescence detectable and 4+ intense fluorescence over the entire sporozoite.

Serum samples were assayed for circumsporozoite precipitation activity.[4] Salivary-gland sporozoites from *P falciparum* strain NF 54 (500 to 1000 sporozoites in 5 μl medium 199), were mixed with 5 μl serum on a microscope slide, sealed under a cover slip rimmed with petroleum jelly, and incubated at 37°C for 30 min. Reactions were evaluated by phase-contrast microscopy at ×400 magnification. 25 random sporozoites were examined for each serum sample and the number of CS-precipitation-positive organisms was recorded.

To assay inhibition of sporozoite invasion,[13] strain NF54 *P falciparum* sporozoites were added to replicate hepatoma (HepG2-A16) cell cultures containing serum (1/20 dilution) and incubated at 37°C. Intracellular sporozoites were visualised by a monoclonal antibody immunoperoxidase assay. Week 10 and week 0 serum samples were assayed simultaneously. Non-specific inhibitory activity was calculated as the mean minus 2SD of triplicate counts of internalised sporozoites incubated with week 0 (control) serum samples. The percentage inhibition of sporozoite invasion was calculated as ([control-test] control) × 100.

Before the first immunisation and 4 weeks after each dose in the primary series of immunisations, and before and 10 days after the fourth dose, peripheral-blood mononuclear cells were separated from heparinised venous blood by centrifugation on 'Ficoll-Hypaque', washed, and suspended in culture medium containing heat-inactivated pooled human AB or autologous serum obtained and frozen before the first immunisation. Peripheral-blood mononuclear cells (5×10^5 cells well) were incubated with antigen

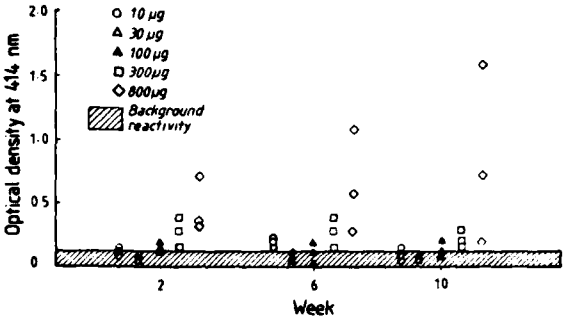

Fig 1—Humoral responses to FSV-1.

Mean ELISA absorbance for triplicate assays of serum diluted 1/50 for each volunteer 2 weeks after each dose of FSV-1.

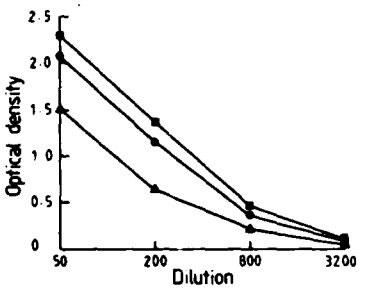

Fig 2—Comparison of antibodies to *P falciparum* CS protein repeat epitopes after immunisation with FSV-1 or natural exposure to infected mosquitoes.

Week 10 sera from volunteer 14 receiving 800 g doses of FSV-1 (▲) and sera from a hyperendemic region of Indonesia (■) and a holoendemic region of Kenya (●) were assayed by ELISA for antibodies against R32LR.

(R32tet$_{32}$ or R32LR) or culture medium in 96-well round-bottom tissue-culture plates for 7 days at 37°C in 5% carbon dioxide; 0·4 μCi tritiated thymidine was then added to each well. 7 h later, cultures were harvested and counted in a liquid scintillation counter. Results were expressed as the stimulation index (mean counts per minute incorporated by triplicate cultures of antigen-stimulated cells divided by the mean cpm incorporated by triplicate cultures of non-stimulated cells) and the difference in mean cpm between stimulated and non-stimulated cells. For each antigen concentration a positive lymphocyte proliferative response was defined as a stimulation index that exceeded the mean plus 2 SD of the baseline (pre-immunisation) stimulation index for all thirteen volunteers.

3 weeks after they had received a fourth dose of FSV-1, six immunised and two non-immunised control volunteers were challenged with the chloroquine-sensitive NF54 strain of *P falciparum* by allowing 5 mosquitoes with a mean salivary gland infection rate of 3·3 to feed as previously described.[14] Beginning on day 5 after mosquito feeding, daily thick blood films (200 fields = 0·25 μl blood) were examined for parasites. Infected volunteers were treated with oral chloroquine.[14]

TABLE I—IgG SUBCLASS RESPONSES IN FOUR VOLUNTEERS IMMUNISED WITH FSV-1

Subject	Dose (μg)	Subclass concentration (μg/ml)			
		IgG1	IgG2	IgG3	IgG4
10	300	2·10	0·38	0·35	<0·10
11	300	1·83	<0·10	<0·10	<0·10
13	800	7·50	0·72	<0·10	<0·10
14	800	50·5	1·50	1·25	0·37

Results

The vaccine was well tolerated at all five doses. There were no episodes of fever, chills, malaise, headache, nausea, or joint pain. Minor pain associated with the injection of vaccine occurred in seven of nine volunteers receiving doses of 100 μg or greater. The injection site was slightly tender in eight of fifteen volunteers after at least one dose of vaccine, including all those receiving 300 μg or 800 μg doses. In no case did these complaints limit function or persist more than 48 h.

One volunteer who received 800 μg doses suffered repeated sneezing followed by a general urticarial eruption without associated hypotension or dyspnoea within 5 min of a third dose of FSV-1. These symptoms resolved spontaneously within 30 min and did not recur. He had CS-repeat-epitope-specific IgE (1 μg/ml) in serum obtained 4 weeks after the second dose; IgE levels were higher (10 μg/ml) 2 weeks after the third dose but were undetectable 6 weeks later. Antigen-specific IgE did not develop in any other volunteer. Prick and intradermal inoculation tests with R32tet$_{32}$, R32LR, or thiomersal were negative in the allergic volunteer 3 months after the third dose and he had no toxic effects or IgE response after a fourth dose. There were no abnormalities in complete blood count, serum chemistry profiles, or urinalysis during the study.

To exclude the measurement of antibodies directed against the tet$_{32}$ portion of R32tet$_{32}$, a related recombinant protein containing the identical CS-repeat portion but only the first two aminoacids of the tet$_{32}$ tail (R32LR) was used as antigen in the ELISA. Antigen-specific IgG was detected 2 weeks after the primary immunisation and was dose-dependent between 100 μg and 800 μg R32tet$_{32}$ (fig 1). Twelve of fifteen (80%) volunteers had antibody titres of 1/50 or greater. Maximum antibody responses were sustained for 2 to 3 weeks and disappeared with a half-life of about 28 days. The titre rose significantly after repeated doses in only one volunteer, who received the 800 μg dose. His antibody levels were similar to those of the highest-titre sera we have yet observed from malaria-endemic populations (fig 2). Immunoglobulin class determinations revealed IgM, IgA, and IgG antibodies to the antigen in all positive serum samples, with IgG antibodies predominating

TABLE II—CS PRECIPITATION (CSP) AND INHIBITION OF SPOROZOITE INVASION (ISI) OF HEPG2-16A HEPATOMA CELLS ACTIVITIES IN WEEK 10 SERUM SAMPLES

Subject	Dose (μg)	CSP*			ISI (%)
		0	2+	4+	
1	10	25	0	0	36
2	10	18	7	0	0
3	10	25	0	0	0
4	30	25	0	0	62
5	30	25	0	0	51
6	30	21	4	0	0
7	100	16	9	0	45
8	100	23	2	0	48
9	100	23	2	0	0
10	300	25	0	0	85
11	300	9	16	0	50
12	300	5	20	0	50
13	800	25	0	0	24
14	800	21	4	0	12
15	800	25	0	0	55

*On 25 sporozoites. 0 = no detectable reaction; 2+ = fine granular precipitate around sporozoite; 4+ = filamentous tail of precipitate streaming from sporozoite.

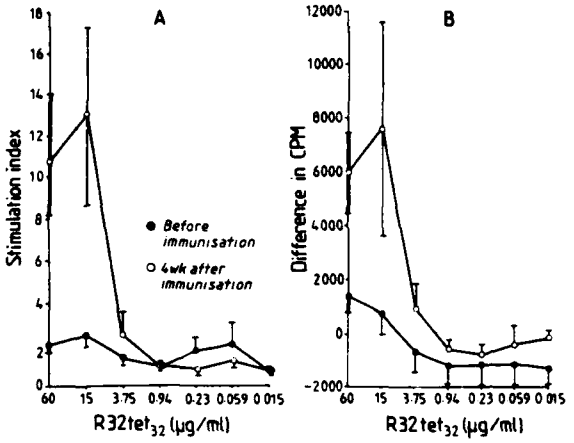

Fig 3—Lymphocyte blastogenic responses to FSV-1.

Bars indicate standard error of mean (n = 13).

(data not shown). IgG subclass concentrations were determined in samples from four volunteers with total IgG titres of 1/200 or greater (two each receiving 300 μg and 800 μg doses, table I). IgG1 was the major isotype found (69–94% of total antigen-specific IgG). Total antigen-specific IgG estimated by summing isotype concentrations ranged from 1 μg/ml to more than 50 μg/ml.

Only week 10 serum samples from the three volunteers receiving 800 μg doses of FSV-1 reacted with CS protein by immunofluorescence; two had 2+ reactions at 1/100 and the third had a 3+ reaction at 1/400.

CS precipitation activity developed after two or more doses of FSV-1 in nine of the volunteers, including at least one individual at each dose; reactions were limited to 2+ (table II). Inhibition of sporozoite invasion developed in eleven volunteers. There was no apparent correlation between ELISA titres and the results of either CS precipitation or inhibition of sporozoite invasion.

Baseline (week 0) lymphocyte proliferation assays in two volunteers (100 μg and 300 μg doses) had high background activities preventing adequate analysis of postimmunisation responses. The mean stimulation index (fig 3a) and difference in cpm (fig 3b) for the other thirteen volunteers at week 4 were significantly higher ($p < 0.05$, Student's t test) than baseline responses to antigen concentrations of 15 μg/ml and 60 μg/ml. Nine of thirteen volunteers responded to $R32tet_{32}$ in this assay 4 weeks after the first dose of FSV-1, and two others responded after the second dose. Despite variable individual responses, no differences in either mean stimulation index or difference in cpm were detected in studies carried out after the first, second, or third dose of FSV-1. There was no apparent correlation between the size of proliferative responses and vaccine dose or antibody levels.

TABLE III—IMMUNOGENICITY AND EFFICACY OF A FOURTH DOSE OF FSV-1

Subject	OD* (1:50)	Dose (μg)	SI†	IFA (1:100)	Prepatent period (days)	Incubation period (days)
C1	0·195	..	ND	–	9	9
C2	0·137	..	ND	–	10	9
11	0·122	300	1·08	–	10	10
7	0·147	100	1·40	–	10	9
10	0·264	300	2·95	–	10	9
8	0·294	100	9·88	–	12	11
13	0·613	800	7·07	2+	13	12
14	1·058	800	4·04	2+	>30	>30

*ELISA absorbance.
†Lymphocyte blastogenesis to R32LR; mean SI of four non-immunised controls was 1·71. ND = not done.

50 weeks after the first immunisation, six volunteers received a fourth dose of FSV-1. Antibody to CS epitopes increased above baseline in four subjects, but all titres were below the maximum titres achieved during the primary immunisation. In a preliminary study of efficacy, these volunteers and two non-immunised control subjects were challenged by the bite of *P falciparum*-infected mosquitoes 3 weeks after the booster dose. Protection appeared to correlate with antibody levels. Parasitaemia was not detected in the volunteer with the highest antibody response and among the subjects who became parasitaemic the incubation and prepatent periods were long in the two subjects with higher antibody titres (table III). Lymphocyte proliferative responses to R32LR generally paralleled the antibody response, indicating the presence of a human T-cell epitope on the repeat region of the molecule. Antibody titres did not increase after sporozoite challenge. The clinical manifestations of malaria were not modified in the two volunteers who had delayed parasitaemia.

Discussion

This study confirms that human beings can be protected from sporozoite-induced malaria by immunisation with a highly purified recombinant CS protein subunit vaccine. The vaccine we used was safe and well tolerated at all five doses but was not optimally immunogenic. Each volunteer developed an immune response to the vaccine as determined by at least one assay of cellular or humoral immunity, but high doses of vaccine were required to elicit antibody levels similar to those reported for other highly purified subunit protein or protein-polysaccharide vaccines.[15-17] At doses above 300 μg, antigen-specific IgG developed. The antibodies elicited were predominantly IgG1, the pattern of IgG response that is most commonly reported for protein antigens.[18] The role of specific IgG subclass antibodies in protection against sporozoites is unknown.

The 800 μg dose of FSV-1 elicited antibody levels similar to the highest titres of naturally occurring CS antibodies we have yet measured and exceeded those required for passive protection of mice against experimental challenge with small numbers of sporozoites.[7] Protective antibodies that react with immunodominant epitopes on CS proteins are detected by immunofluorescence assay with intact sporozoites and mediate CS precipitation and inhibition of sporozoite invasion.[6,7] No specific level of these two activities is correlated with protective immunity in animals or naturally exposed human subjects. The levels that developed in our volunteers were, however, generally lower than those found in animals and human beings immunised with irradiated sporozoites or in adults living in malaria-endemic regions.[2,6,12]

Higher and more sustained antibody titres would be needed before CS subunit vaccines could be useful. Although higher doses of FSV-1 could be used, the recombinant protein itself may need to be modified. Our data and studies of antibody responses and lymphocyte blastogenesis with $R32tet_{32}$ in mice have identified both T-cell and B-cell epitopes on the repeat portion of the

molecule, and antibody levels in mice primed with these CS epitopes can be boosted by intact sporozoites.[19] 80% of the recombinant CS protein in FSV-1 consists of multiply repeated units of only four aminoacids. This region of the molecule contains but a single murine T-cell epitope and so may be inefficient in eliciting boosting responses in human beings under natural exposure to sporozoites.[20] The identification of additional T epitopes on the CS gene and their incorporation into new vaccines[21] or the addition of non-sporozoite T epitopes by means of other carrier proteins or fusion products may enhance CS antibody production.

We recognise the fundamental role of cellular immune responses in the protection against intense sporozoite challenge afforded by immunisation with irradiated sporozoites,[7,22,23] but target antigens for cell-mediated immunity are unknown and may be independent of the CS protein. Antibody-mediated immunity may, however, be adequate to protect some individuals against natural mosquito-transmitted malaria. Although this preliminary study has demonstrated that human beings can be protected with a subunit CS protein vaccine and provides a framework for the further development of sporozoite vaccines, it is clear that FSV-1 as formulated is not a suitable candidate for field trials.

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EXCLUSION TESTING FOR HUNTINGTON'S DISEASE IN PREGNANCY WITH A CLOSELY LINKED DNA MARKER

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Summary 55 couples where one partner was at 50% risk of Huntington's disease (HD) were investigated with a DNA probe closely linked to HD, with a view to exclusion testing in a future pregnancy. In 3 of 9 pregnancies so far, HD was excluded in the absence of recombination. In 3 the risk was raised to around 50%, and in 2 exclusion tests were uninformative. The remaining couple changed their minds about termination of the pregnancy and the test was therefore judged inappropriate.

Introduction

HUNTINGTON'S disease (HD) is a progressive dominantly inherited neurodegenerative disorder. The mutation rate is low, penetrance is complete, and symptoms usually begin between age 30 and 50, so that most individuals at risk have had children before they realise they have inherited the disorder. Until lately it has not been possible to distinguish presymptomatic gene carriers from their unaffected sibs, so preventive measures have relied upon non-directive genetic counselling and a voluntary reduction in family size by those at high risk.[1,2]

In 1983 a DNA probe (G8) was found[3] which was localised to the short arm of chromosome 4 and assigned the locus D4S10.[4] Close genetic linkage was reported between D4S10 (G8) and the locus for the HD gene in two large kindreds.[3] This study has now been extended to include 52 families from various parts of the world, including our own region (South Wales); the maximum total lod score proved to be 75·3 at a recombination fraction of about 5 centimorgans (cM).[5] The 95% confidence intervals for the recombination fraction were 2·4 cM and 6·5 cM, with no evidence to support multilocus heterogeneity.[5]

A DNA marker tightly linked to the HD locus has two possible clinical applications: presymptomatic predictive tests for an individual at risk for the disorder (about which there has been much debate) or exclusion tests in pregnancy, where the estimated risk to the parent is not altered. We

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