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Antibody and clinical responses in volunteers to immunization with malaria peptide–diphtheria toxoid conjugates

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SUMMARY

Twenty residue peptides from the 185–200-kD and 45-kD merozoite surface antigens of the malaria parasite *Plasmodium falciparum* were covalently linked to diphtheria toxoid as a carrier and used to immunize human volunteers with aluminium hydroxide as an adjuvant. Significant antibody levels were elicited by two boosting injections. The antibodies reacted with acetone–methanol fixed merozoite membranes in an immunofluorescence assay, but no inhibition of merozoite reinvasion could be detected in *in vitro* cultures containing the antibodies. Antibody levels against the immunizing peptides declined markedly within 77 days after the third injection. No hypersensitivity was observed against the peptides. However, the volunteers developed hypersensitivity against diphtheria toxoid, and in particular a pronounced type III (Arthus) hypersensitivity after three injections with the toxoid. This effect might appear to limit the use of peptide–diphtheria toxoid conjugates for human immunization. Several biochemical, haematological and immunological tests done on the volunteers showed no other adverse effects from the immunizations.

Keywords diphtheria toxoid hypersensitivity malaria peptides vaccination

INTRODUCTION

Synthetic vaccines based on peptide–tetanus toxoid conjugates [1,2], peptide polymers [3,4] or recombinant proteins [5–7] directed against sporozoites [1,2,5–7] and asexual blood stages [3,4] of the malaria parasite *Plasmodium falciparum* have undergone phase I and II clinical trials in man. Partial protection against malaria was reported with sporozoite vaccines based on the repetitive region of the circumsporozoite protein [1,5]. A synthetic disulphide linked polymer based on peptides derived from 35- and 55-kD *P. falciparum* antigens, the *P. falciparum* circumsporozoite protein repeat and a 185–200-kD precursor glycoprotein to the major *P. falciparum* merozoite surface antigen or MSA-1, was reported to protect four out of five volunteers after immunization using alum as an adjuvant [3]. In subsequent field trials with this vaccine, termed SPf66, significant protection against *P. falciparum* was reported [8,9].

MSA-1 (alternatively termed PMMSA or PfMSP-1) [10,11] and a 45-kD glycosylated and myristilated smaller surface antigen on *P. falciparum* merozoites (termed GYMSSA, MSA-2 or PfMSP-2) [12,13] are potential candidate molecules for designing an asexual blood stage vaccine. Immunization with MSA-1 in Freund's adjuvant completely protects *Aotus* monkey against falciparum malaria [11]. Antibodies to MSA-1 [14] and MSA-2 [15] inhibit *P. falciparum* growth in *in vitro* culture, presumably by inhibiting merozoite invasion. Because of its widespread use in vaccination, diphtheria toxoid (DT) is a possible alternative to tetanus toxoid as a carrier molecule in synthetic peptide vaccines for human use. DT was used as a carrier in a phase 1 clinical trial of a birth control vaccine based on the β subunit of human chorionic gonadotropin (β-hCG) [16]. We report here on the immunological and clinical outcome of immunizing volunteers with two peptides derived from MSA-1 and MSA-2 that were linked to DT as a carrier molecule.

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MATERIALS AND METHODS

Peptides

Based on the sequence of MSA-2 in the FC27 isolate [13] and MSA-1 in the K1 isolate of *P. falciparum* [17], two peptides were synthesized by standard t Boc chemistry [18] using a *p*-methyl benzhydrylamine resin. The peptides were cleaved from the resin with HF containing 2·5% thiocresol and 7·5%

p-cresol to yield molecules with C-terminal amides. The sequences of the peptides and the residues they were derived from were as follows:

Antigen	*Peptide*	*Residues*	
MSA-2	P104	207–226	*Sequence*: RNNHPQNTSDSQKEATDGNKC
MSA-1	P109	21–39	*Sequence*: VTHESYQELVKKLEALEDAC

In P104, the cysteine in residue 221 of the native protein was replaced by an alanine to avoid polymerization. Both P104 and P109 contained an additional C terminal cysteine that was introduced to facilitate coupling. For P104, conjugation through a C-terminal cysteine was found to be preferable to coupling through the naturally occurring internal cysteine [19].

Antibody binding sites or B cell epitopes are located in the more hydrophilic regions [20] and β-turns [21]. Hydrophilicity and protein conformation analysis may therefore be used for predicting immunogenic peptide sequences. P104 and P109 were originally selected in this way, and their immunogenicity was confirmed in mice [19,22] and their antigenicity demonstrated with sera from endemic area populations [23]. Mouse anti-P104 [19] and anti-P109 antibodies reacted with merozoites in an immunofluorescence assay (unpublished data), showing that the corresponding epitopes were accessible to antibodies on merozoite surfaces. P104 and P109 were derived from sequences that are conserved between different allelic forms of the two antigens. Conserved sequences generate an immune response likely to be effective against different parasite strains. P109 is located close to, but does not overlap, the region of MSA-1 used by Patarroyo *et al.* in SPf66 [3], and is part of an N-terminal, 83-kD processed fragment of MSA-1 that can be detected after surface radio-iodination of intact merozoites [24]. Although growth inhibitory antibodies can be elicited by the cysteine-rich C-terminus of MSA-1, these are reportedly directed against conformational epitopes dependent on correct disulphide bonding and not linear (or peptide) epitopes [25].

Peptides were purified by reverse phase high performance liquid chromatography (HPLC) [19]. The amino acid compositions determined after hydrolysis in HCl/propionic acid at 130°C for 6 h, were consistent with the expected sequence of the two peptides.

Peptide–DT conjugates

Peptides were conjugated individually to DT with the heterobifunctional reagent, 6-maleimido caproic acyl N-hydroxysuccinimide ester (MCS) essentially as described [22,26]. Briefly, DT suitable for human immunization (Commonwealth Serum Laboratories, Melbourne, Australia) was dialysed against 0·1 M sodium phosphate pH 6·5. To 30 mg protein in 2·7 ml was added 300 μl of 50 mM MCS in dry dimethylformamide. After mixing gently for 30 min a further addition of 300 μl MCS solution was made and the stirring continued for an additional 30 min. The maleimidated protein was separated from MCS by dialysis. Peptide (30 mg) dissolved in 3 ml 0·1 M sodium phosphate pH 6·5, 1 mM EDTA was then added to the maleimidated DT, the mixture equilibrated with N_2 and the reaction allowed to proceed for 18 h at room temperature in the dark. The peptide–carrier conjugate was then purified by dialysis against 0·01 M PBS pH 7·2. The number of mols of peptide conjugated to one mol of DT determined from a comparison of the mol. wt of the peptide–protein conjugate with maleimidated protein by gel electrophoresis [20] was 4 and 1–2 for P104 and P109, respectively. The conjugates were sterilized by filtration through 0·22-μm millipore filters and stored in aliquots at −20 °C until use.

Subjects

The subjects were healthy males aged 18–30 years who gave their written and informed consent for participation in the trial. The protocol for the trial was approved by the Institute Committee on ethics of experiments involving human subjects. The volunteers, who had lived in non-malarial areas of Sri Lanka, were screened to ensure that they had no history of allergies or previous infection with malaria. Given their age, it is likely that all the volunteers were vaccinated against diphtheria during childhood, but this could not be definitively established in the absence of immunization records. They were randomly assigned numbers and divided into groups of four for the trial. Group I (volunteers 1–4), group II (volunteers 5–8) and group III (volunteers 9–12) were immunized with P104-DT, P109-DT and alum/saline, respectively. All immunizations were carried out in hospital, and the subjects were kept under regular observation for 72 h after the injections.

Immunizations

A pre-immune sample of blood was withdrawn for serological, biochemical and haematological tests. The first injection consisted of 1 mg peptide–DT conjugate (containing 140 μg of P104 or 45 μg P109) in a volume of 0·4 ml mixed with 0·1 ml sterile aluminum hydroxide gel. Following skin testing with DT, the injection was given in the gluteal muscle. Group I received P104-DT, group II P109-DT, and group III alum only in saline. Twelve days after the injection, a 5-ml sample of blood was withdrawn for serology. A second injection of antigen or saline mixed with alum was given on day 21 in a similar manner in the alternate gluteal muscle, and a blood sample for serology withdrawn 12 days later. A third injection of 1 mg conjugate in saline without alum, or saline alone for controls, was given on day 42 after the first injection, and a blood sample obtained 8 days later for serological, biochemical and haematological tests. A further blood sample was withdrawn on day 119 after the first injection, i.e. 77 days after the third and final immunization. Sera for antibody studies were stored at −20°C until use. The blood pressure, pulse and temperature of the subjects were monitored at 6-h intervals after each immunization for 48 h.

Skin tests

Skin tests were performed by injecting 1 μg peptide or DT in 0·1 ml sterile saline intradermally in the forearm. Readings for immediate, Arthus and DTH reactions were done by measuring skin induration and erythema at 15 min, 4–14 h and 24 h, respectively, after the injection. Volunteers were tested for hypersensitivity against diphtheria toxoid immediately before each of the injections and 119 days after the first injection. They were also tested for hypersensitivity against the immunizing peptides on day 119.

Biochemical and haematological tests

Tests were performed in the Pathology Laboratory of the

Table 1. Antibody titres after three immunizations (3°) in the vaccinees measured by immunofluorescence (IFA) on merozoite surfaces and ELISA. The titres of the corresponding pre-immune sera are given in parentheses

Immunization	Volunteer number	IFA titre 3° (pre-immune)	Anti-P104 ELISA titre 3° (pre-immune)	Anti-P109 ELISA titre 3° (pre-immune)
Group I	1	2500 (80)	5000 (640)	320 (80)
(P104-DT)	2	1250 (160)	2500 (320)	320 (80)
	3	640 (160)	640 (1250)	320 (80)
	4	2500 (160)	2500 (320)	640 (320)
Group II	5	640 (160)	640 (320)	320 (40)
(P109-DT)	6	2500 (80)	640 (640)	320 (40)
	7	640 (160)	160 (160)	1250 (80)
	8	640 (80)	320 (320)	640 (80)
Group III	9	320 (40)	320 (160)	80 (80)
(alum/saline)	10	1250 (40)	320 (320)	160 (80)
	11	160 (40)	160 (160)	80 (40)
	12	160 (40)	320 (160)	160 (80)

North Colombo General Hospital, Ragama, by routine procedures. The biochemical tests included measurement of serum glutamate-oxaloacetate transaminase, serum glutamate-pyruvate transaminase, serum alkaline phosphatase, blood urea and blood glucose levels. The haematological tests done were the erythrocyte sedimentation rate, haemoglobin concentration, total leucocytes and a differential count of leucocytes.

Tests for autoimmunity

Sera collected on day 119 were tested for anti-nuclear antibodies and rheumatoid factor by routine procedures at the Medical Research Institute, Colombo.

ELISA to measure antibody levels

For ELISA, 96-well polystyrene plates were coated with 50 μl of a 10 μg/ml solution of peptide or DT in PBS overnight at 4°C, and then blocked with 5% w/v non-fat milk (Blotto) for 2 h at 37 °C. The wells were washed and then reacted with serial dilutions of sera in Blotto with a starting dilution of 1 : 20. After reacting for 1 h at 26°C, the wells were washed and reacted with peroxidase-conjugated anti-human IgG with specificity for all immunoglobulin classes (Silenius, Melbourne, Australia). 2,2[1]-azino-bis (3-ethyl benz thiazoline-6-sulphonic acid) and H_2O_2 were used as substrates to develop the colour reaction. After 30 min, the intensity of the colour was read in a Multiskan Plus II ELISA reader (Flow Labs, Irvine, UK) at 405 nm. Tests for comparing different bleeds were performed at the same time under identical conditions. The titre was determined as the lowest dilution of serum giving an optical density of 0·1 above control with no added antibodies.

Immunofluorescence assay

Sera were diluted serially in 1% bovine serum albumin (BSA)/PBS starting with a 1 : 40 dilution. These were used to stain late stage 3D7 isolate parasites fixed on glass slides with acetone/methanol 9 : 1 at −20°C, essentially as described previously [12,15]. The titre was determined as the highest dilution giving a clear grape-like pattern indicative of binding to the merozoite surface [15].

Inhibition of merozoite invasion assay

This was performed as previously described [27], except that the numbers of parasites in the cultures were monitored after Giemsa staining and not by measuring ^{3}H-hypoxanthine incorporation. Test sera were individually added at 10% v/v to schizont stage parasites cultured in O+ erythrocytes.

RESULTS

Antibodies to peptides

All subjects injected with peptide–DT conjugates produced antibodies specifically against the immunizing peptide (Table 1 and Fig. 1). Higher antibody titres were produced against P104 than against P109. An exact correlation between immunofluorescence assay (IFA) titres against the merozoite surface and ELISA titres against P104 and P109 was not observed. The significant background IFA at up to 1:160 dilution with normal human sera is consistent with previous observations [28]. Increases in specific antibody levels were seen after the second and third injections of the peptide–DT conjugates (Fig. 1). However, the total antibody level against P104 fell, almost to background levels, 77 days after the third injection (Fig. 2). A similar rapid decline of antibody levels was observed in P109-immunized volunteers (data not shown).

No significant inhibition of reinvasion could be demonstrated with sera containing specific anti-P104 or anti-P109 antibodies added at 10% v/v to *in vitro* parasite cultures. Starting with 0·7% schizont stage parasitaemia in a typical experiment, 3·5% (range 3·4–3·6%), 3·3% (range 2·7–4·1%) and 2·8% (range 2·2–3·4%) mean ring stage parasitaemias were obtained after 24 h with anti-P104 (group I), anti-P109 (group II) and control (group III) sera, respectively.

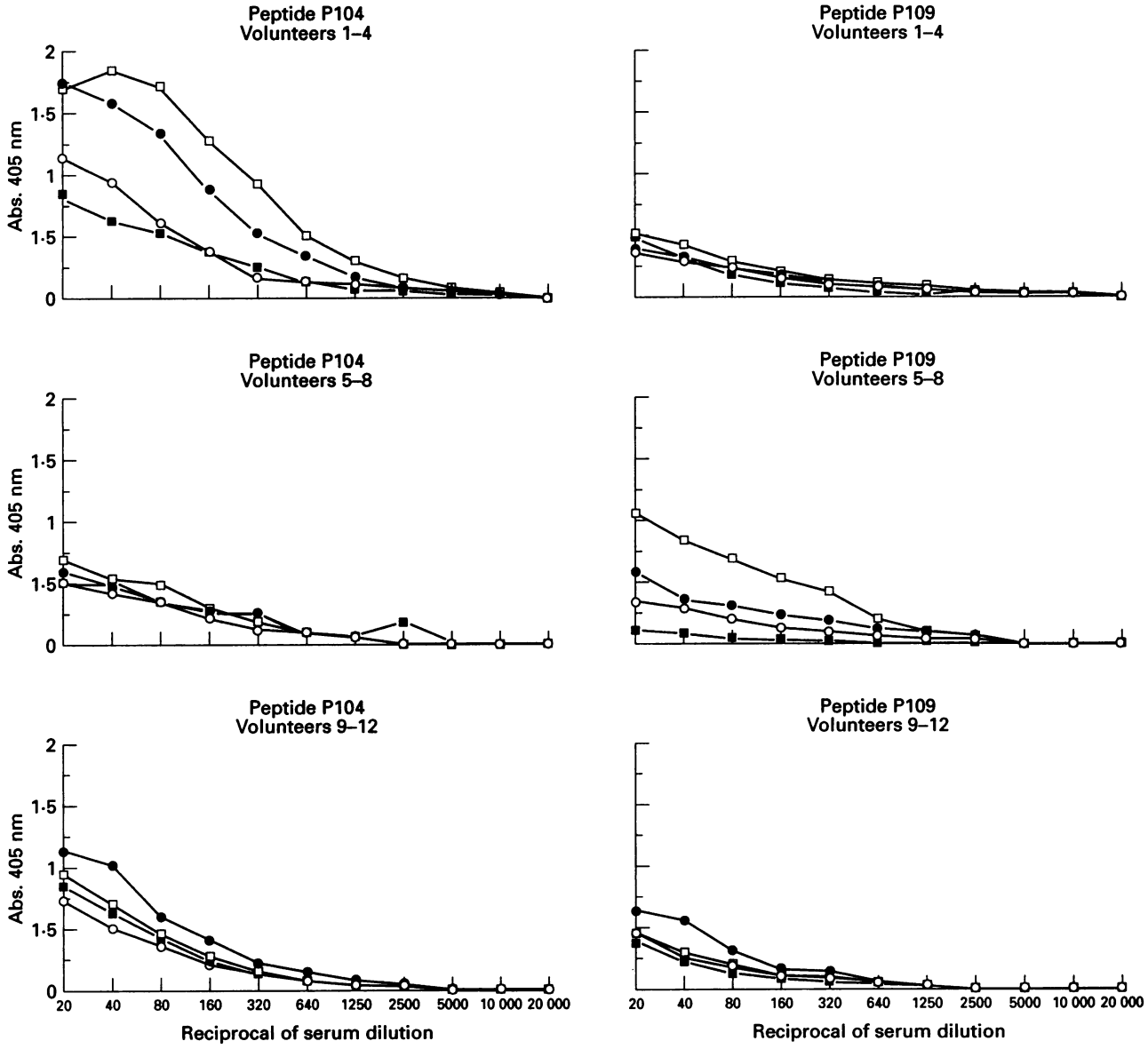

Fig. 1. Antibody response to P104 and P109 in vaccinees determined by ELISA. ■, Pre-immune; ○, bleed 1 (12 days after first injection); ●, bleed 2 (12 days after second injection); □, bleed 3 (8 days after third injection). The results are mean of the absorbtion at 405 nm of the sera from four volunteers within a group.

Antibodies to diphtheria toxoid

All subjects had an ELISA antibody titre of 10^{-4} against DT at the commencement of the trial. After two injections, the titres in the three groups receiving the vaccine, including those receiving injections of alum and saline only, increased to $\geqslant 10^{-6}$. Increasing titres in the control group were probably due to sensitization with DT during the skin tests performed before each immunization during the study.

Hypersensitivity responses

The volunteers did not experience any serious adverse effects consequent to the immunization. No immediate hypersensitivity to diphtheria toxoid was observed in the subjects before the three injections. After the first injection, local tenderness at the injection site was reported by the volunteers. After the second injection, local tenderness was reported by group III volunteers receiving the placebo. Apart from tenderness at the injection

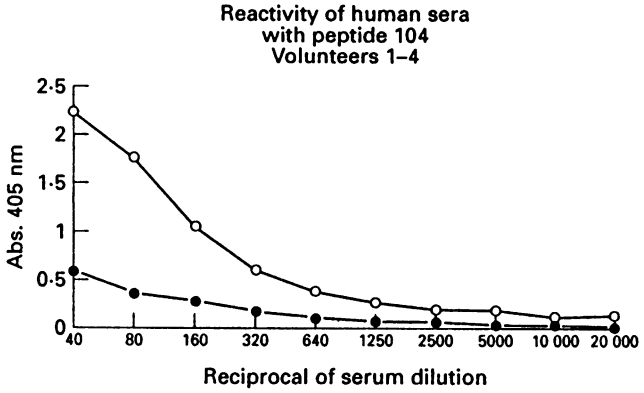

Fig. 2. Decline of antibody levels after the final injection in group I vaccinees. ○, Bleed 3 (8 days after third injection); ●, bleed 4 (77 days after third injection). The results are the mean of the absorbtion at 405 nm of the sera from four volunteers in an ELISA.

site, three out of four volunteers receiving the second P104-DT injection had a temperature < 39°C that resolved within 24 h. Myalgia was reported in two out of three of the persons experiencing fever. Only one of the four volunteers receiving the second P109-DT injection had a temperature < 39°C that also resolved within 24 h. After the third injection, no adverse effects were seen apart from tenderness at the injection site. However, Arthus type hypersensitivity to DT was seen in volunteers 1, 2 and 4 (group I receiving P104-DT) at the site of skin tests. A marginal DTH response to DT was seen in volunteers 3, 4, 5, 6, 9 and 10 at the site of skin tests.

By day 77 after the third and final injection, however, the Arthus type hypersensitivity to DT was very pronounced in all but two of the vaccinees, and a noticeable but weak DTH reaction was seen in seven of the 12 subjects, including two of the control group III (Table 2). A marginal immediate hypersensitivity reaction was also seen in 11 of the 12 volunteers (Table 2). No hypersensitivity to peptides was observed in any of the volunteers.

All biochemical and haematological tests done after three injections yielded normal results (data not shown). The 12 subjects were also found to be negative for anti-nuclear antibodies and rheumatoid factor.

DISCUSSION

There is considerable potential for developing peptide-based vaccines against malaria [29]. Ideally small portions of candidate molecules that are capable of generating growth inhibitory antibodies or conferring protection in *Aotus* or *Saimiri* monkey need to be identified before use in man. Apart from their restricted availability outside South America, the monkeys may differ from humans in their immune response to *P. falciparum* antigens and also develop widely varying parasitaemias on infection [30]. The N-terminus of the 83-kD processed fragment of MSA-1 [3] as well as carboxyl terminal fragments [14,25,31] have, however, been implicated in protective immunity in monkeys. Similar evidence is not available for MSA-2.

It was previously shown that immunizing mice with peptides P104 and P109 conjugated to BSA or DT and injecting in either saline alone or with alum led to the production of high titre antibodies that react with merozoite surfaces ([22], unpublished observations). The present results show that human anti-P104 and anti-P109 antibodies binding to merozoite surface proteins can be produced by immunization with peptide–DT conjugates. An exact correlation between ELISA and IFA titres in different volunteer sera was not observed, and this may be because of slightly varying specificities [19], so that only a proportion of the anti-peptide antibodies detected by ELISA can bind to the native antigen on merozoite surfaces in an IFA.

Table 2. Hypersensitivity reaction to peptides P104 (volunteers 1–4) and P109 (volunteers 7–11) and diphtheria toxoid (DT) in vaccinees 77 days after third injection. ±, Marginal reaction; +, < 1 cm^2; ++, < 4 cm^2; +++, < 9 cm^2 swelling

	Skin test 15 min		Skin test 4 h		Skin test 24 h	
Volunteer	Peptide	DT	Peptide	DT	Peptide	DT
1	–	±	–	+++	–	±
2	–	±	–	++	–	±
3	–	–	–	–	–	–
4	–	±	–	+++	–	±
5	–	±	–	+++	–	±
6	–	±	–	+++	–	±
7	–	±	–	++	–	–
8	–	±	–	+++	–	–
9	–	±	–	–	–	±
10	–	±	–	++	–	±
11	–	±	–	+	–	–
12	–	±	–	++	–	–

A similar lack of correlation between ELISA and IFA titres was seen after immunization with SPf66 [3].

Typically $\geqslant 7$ mol of peptide were coupled to each mol of BSA under the same conditions used for conjugation with DT [19,22]. The lower conjugation efficiency with DT may be due to the amino groups in the protein reacting with formaldehyde during detoxification, a factor that may also vary with the source of DT. Despite the low peptide-to-carrier ratio, significant boosting of antibody levels was observed with P104 and P109 after the second and third injections. The higher peptide to DT ratio in P104-DT results in more peptide being injected, and this was probably responsible for the higher antibody production in group I volunteers compared with group II volunteers. Although the antibodies reacted with the merozoite membrane by IFA, no inhibition of reinvasion could be demonstrated in *in vitro* culture at 10% serum concentration. The lack of inhibition may be due to low antibody concentration, or the absence of effector cells that promote antibody-dependent cell-mediated cytotoxicity [32]. The possibility also exists that P104 and P109 may not correspond to regions of MSA-2 and MSA-1 where antibody binding inhibits merozoite function. In this context it is worth noting that SPf66, which contains an epitope from MSA-1 close to P109, is protective despite being a weak immunogen [3] and also induces inhibitory antibodies in man [33]. The antibody response to peptides had a short half life, decreasing markedly within 77 days. The half lives of the anti-P104 and anti-P109 antibodies in the volunteers are similar to that observed in persons naturally exposed to malaria in Sri Lanka [23]. This effect has to be taken into consideration in any mass vaccination strategy. Vaccine formulations that prolong serum antibody levels may be helpful in this respect.

The pronounced type III (Arthus) hypersensitivity to DT that developed after three injections is a drawback to the use of DT–toxoid conjugates as human vaccines. If three or more boosting injections of peptide–DT conjugates are required for effective immunization (e.g. in a malaria eradication campaign), then high antibody levels against DT can result in a more severe Arthus type reaction at the injection site, accompanied by glomerular nephritis, polyarthritis, arthralgia and other symptoms of serum sickness. It is unlikely that the use of smaller quantities of toxoid for immunizations would significantly reduce the hypersensitivity reaction, since skin testing with 1 μg DT appears to boost antibody levels to the toxoid. The high serum antibody levels in vaccinees against DT are consistent with the development of type III hypersensitivity. It is interesting to note that significant hypersensitivity reactions were not observed after three immunizations with the SPf66 polymer [3,8,9]. In the β-hCG-DT phase 1 trial [16], one out of 20 subjects became skin test-positive to DT. However, there were several differences between the β-hCG trial and the present study that may have influenced the extent of hypersensitivity reactions. In the β-hCG trial, a muramyl dipeptide derivative was used as an adjuvant and squalene/mannide mono-oleate as an emulsifier. Up to approximately 500 μg DT can be estimated to have been given in each of two injections with an approximate conjugation ratio of 12–14 mols peptide per mol carrier in the β-hCG trial. Toxic, presumably hypersensitivity, reactions have been reported also with tetanus toxoid as a carrier [34]. An alternative strategy for vaccination involving the use of synthetic peptides with T helper activity derived from diphtheria or tetanus toxoid molecules as carriers [35] may reduce the incidence of type III but possibly not type I hypersensitivity in vaccinees. Carriers that do not elicit significant hypersensitivity involving possibly malaria T cell epitope-containing peptides, capable of being recognized in the context of many different MHC class II molecules [36], may have advantages for use in man. The results also highlight the need for developing alternative forms of synthetic vaccines, e.g. peptide polymers [3], multiple antigen peptides containing T and B epitopes [37], or recombinant proteins for human use.

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