BUTTERWORTH HEINEMANN

0264-410X(94)00052-2

Vaccine, Vol. 13, No. 15, pp. 1474–1481, 1995

0264–410X/95 $10+0.00

Fine specificity of immune responses to epitopic sequences in synthetic peptides containing B and T epitopes from the conserved *Plasmodium falciparum* blood-stage antigens

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Immunisation with two chemically synthesised, linear, multiple epitope peptides (MEP) containing B and T cell epitopes from two conserved blood-stage antigens of the human malaria parasite, Plasmodium falciparum, *induced high levels of circulating antibodies without the use of a carrier protein. Immunisation of BALB/c mice with MEP constructs (P1 and P2) induced antibodies against the various epitope sequences included in their structures, although the immune response was focused more towards the N terminal and the middle portion of the peptides.* In vitro *T cell proliferation assays indicated that only one of the two Th epitopes included in P1 and P2 are functional. Both P1 and P2, based on* P. falciparum *sequences, cross-reacted with sera from* P. yoelii-*infected mice. Immunisation with P1 in CFA, but not with P2, provided partial protection to BALB/c mice against* P. yoelii *challenge infection. Peptide P1 was highly immunogenic in alum also, and a somewhat higher level of protection was observed as compared to CFA immunisation. We found that immunisation with P1 induced antibody responses in different strains of mice, although to different extents. These results suggest that linear, multiple epitope peptides may offer attractive alternatives as subunit vaccine candidate molecules, but at the same time highlight the fact that the design principles are far from being clear and have yet to be worked out.*

Keywords: *Plasmodium falciparum*; subunit vaccine; malaria; B and T epitopes; synthetic peptides

Widespread multidrug resistance in malaria infection is fast narrowing the options for treatment. An effective vaccine would not only save about 2–3 million lives, but also bring relief to many millions living in the developing world. The impracticality of large-scale parasite culture, together with the risk of contaminations from blood- and serum-derived products in a human vaccine, means that a malaria candidate vaccine will probably have to be synthesised chemically or be based on recombinant DNA technology[1].

In nature, acquired immunity to malaria in humans takes several years to develop and is usually short-lasting. A major reason for this appears to be considerable antigenic diversity of most malaria proteins occurring in different strains of the human malaria parasite, *Plasmodium falciparum*[2,3]. However, in spite of the extensive antigenic polymorphism, it may be argued that many parasite molecules that are critical for biological function are probably conserved and may serve as attractive targets for vaccine development. For example, immunodominant B- and T-cell epitopes of the circumsporozoite protein of *P. falciparum* are highly conserved[4,5]. Likewise, the merozoite surface antigens, MSA-1, MSA-2 and AMA-1, have highly conserved regions in their sequences, not only in different strains but also in different species of plasmodia[6–8]. The ring-infected erythrocyte surface antigen (RESA) of the blood stages also appears to be invariant[9].

However, immunisation with recombinant malaria antigens has not been as successful as expected[10]. Synthetic peptides representing protective epitopes from malaria antigens have shown more promise[11–14], and in general offer an attractive strategy to produce specific

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Table 1 Amino acid sequence of the synthetic peptides

Peptide Code	Amino Acid Sequence	Source Protein
B1	Y S L F Q K E K M V L	MSP-1 (Ref 31)
T1	Y K L N F Y F D L L R A K L	MSP-1 (Ref 26)
BT1	L D N I K G N V G K N E D Y I K K N K K	MSP-1 (Ref 18)
BT2	E E N V E H D A E E N V E E N V	RESA (Ref 9)
P1	BT1-P-B1-GPG-BT2	
P2	BT1-P-T1-P-B1	

immune responses. On the other hand, synthetic peptides are mostly poor immunogens and although carrier proteins may be used to enhance the antibody production, certain problems such as hypersensitivity and epitope-specific immunosuppression exist[15]. It is becoming clear that a putative synthetic peptide vaccine must contain relevant B cell epitopes as well as Th epitopes so as to obviate the need for an extraneous carrier protein. Several successful peptide immunogens have been developed using this strategy[16–18], even though the details of how the T-epitope sequences confer immunogenicity to a hapten are not yet clearly understood.

It is generally believed that a future malarial vaccine needs to contain a combination of different antigens to combine their protective potential and to minimise the risk of parasite escape by mutation under immunological pressure. Several peptide constructs containing immunodominant regions from malaria antigens have been developed[19–21]. We have recently described, in a brief report[22], two linear multiple epitope peptides (MEP) based on B and T epitopes from conserved regions of the major surface antigen-1 (MSA-1) and the ring-infected erythrocyte surface antigen (RESA), the two major vaccine target *P. falciparum* antigens. While both constructs were highly immunogenic in mice, only one of them provided protection against a murine malaria challenge infection. We have now tried to analyse various immune responses to the MEP constructs. Our results indicate that the arrangement of B and T epitopes in linear multiple epitope peptides may be crucial for providing appropriate immune responses. It is clear that while peptides based on conserved B and T epitopes may form the basis for vaccine development, there exist no rules yet for the design of multiple epitope constructs and that each case may have to be studied separately for its immunological potential.

MATERIALS AND METHODS

Peptides

Sequences of the peptides P1, P2 and their constituent peptide fragments representing B1, T1, BT1 and BT2 are shown in *Table 1*. All peptides were synthesised on 0.1 mmol scale using small-scale rapid t-Boc cycles on preloaded PAM resins, using an Applied Biosystems peptide synthesiser, model 430A. N-Boc protected amino acids were coupled as their symmetrical anhydrides (five times excess). Arginine, aspargine and glutamine were coupled using N,N′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT). The peptides were cleaved and deprotected by treatment with trifluoromethanesulphonic acid using thioanisole and ethanedithiol as scavengers. Deprotected peptides were placed in reducing conditions, subjected to gel filtration on a Sephadex G-25 column and purified by HPLC on reverse-phase column using acetonitrile in 0.1% trifluoroacetic acid as the eluent (0–60% acetonitrile in 30 min). Peptides were characterised by protein sequencing (model 477A Applied Biosystems) and amino acid analysis.

Immunisation of mice

Four to six-week-old mice from different inbred strains, namely BALB/c (H-2:d), C3H/He (H-2:k), C57BL/6 (H-2:b) and SJL (H-2:s), were purchased from the small animal facility of The National Institute of Immunology, New Delhi, India. To find out whether P1 and P2 were immunogenic on their own, two groups of mice (BALB/c, six per group) were immunised with 50 μg of the peptide emulsified in CFA and injected via the intraperitoneal (i.p.) route. Animals were boosted on days 32 and 60 with the same inoculum emulsified in IFA. Animals were bled on days 0 (pre-immune), 14, 28, 40, 50, 70, 100 and 120 from the retro-orbital plexus and sera separated for immunoassays. Similarly, to check whether the immune response to P1 was genetically restricted, mice belonging to different haplotypes were immunised as described above and their sera collected. Further, to compare the immune response to P1 administered with different adjuvants, two groups of BALB/c mice (four/group) were immunised with 50 mg of P1 emulsified 1:1 in CFA or adsorbed to alum. Animals were boosted twice with a similar dose of respective immunogen (in IFA or alum) at intervals of 3 weeks each. A week after the second boost, mice were bled and

sera separated for immunoassays. Sera were also collected from mice infected with *P. yoelii nigeriensis*. All sera were stored at −20°C until used.

Immunoassay

Antibody levels in peptide immunised mice sera were assayed by ELISA, using appropriate synthetic peptides as capture antigens. Briefly, wells of flat bottommed 96-well microtitre plates (Greiner, Nurtingen, Germany) were coated with the relevant antigen. Uncoated reactive sites in the wells were blocked by incubation with 5% solution of a non-fat dried milk powder in PBS, pH 7.2. All sera samples were diluted in PBS, pH 7.2, containing 0.5% milk powder and incubated in antigen-coated wells for 2 h at room temperature in a humid chamber. The horse-radish peroxidase conjugated goat anti-mouse IgG or goat anti-human IgG (Sigma) were used as the second antibodies in the respective assays. The enzyme reaction was developed using *o*-phenylenediamine dihydrochloride (Sigma) as substrate. Reaction was stopped with 8 N H_2SO_4 and O.D. of the reaction product obtained with the microplate reader (Biotek 2000) at 490 nm. The last dilution of a test serum giving an O.D. value greater than twice the O.D. value obtained with the respective pre-immune serum diluted 1/100 was taken as the end-point titre.

Indirect immunofluorescence assay was performed with sera from mice immunised with P1 or P2. Briefly, wells of immunofluorescence slides were coated with *P. yoelii*- or *P. falciparum*-infected erythrocytes at a concentration of 10^4 cells 20 μl^{-1}. Cells were fixed onto slides by immersing in cold acetone at −20°C for 2 h. Slides were incubated with dilutions of sera in individual wells. After extensive washing with PBS, slides were incubated with 1:40 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate for 1 h in the dark. After washing, the slides were observed under fluorescence microscope (Nikon) using transmitted and UV light alternately to see specific binding to infected RBC.

Lymphocyte proliferation assays

Two groups of four mice each were primed with 50 μg of P1 or P2, emulsified 1:1 in CFA, via foot pad inoculations. A group of control mice was immunised with CFA alone. Eight days after immunisation, the popliteal lymph nodes were extracted and were crushed to release cells, which were washed twice with RPMI 1640 medium (Sigma). The viability of the cells was assesed by the trypan blue dye exclusion, and cells were plated at 4×10^5 cells $well^{-1}$ in 96-well tissue culture plates (Greiner, Germany) in RPMI medium, supplemented with 15 mM HEPES, 0.3% sodium bicarbonate, 50 μM β-mercaptoethanol (Biorad, Richmond, CA), 2 mM glutamine, 50 units ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 10% foetal calf serum (Sigma). Appropriate peptides were incubated with the seeded lymphocytes at concentrations varying from 200 to 0.2 μg ml^{-1}. All cultures were set up in triplicate and the final volume in each well was 200 μl. Plates were incubated at 37°C with 5% CO_2 (Becton Dickinson, USA). Two days later 0.5 mCurie of tritiated thymidine (NEN, UK) was added to each well and incubation terminated after 18 h. Cells were harvested and the tritiated thymidine incorporation was determined using a liquid scintillation counter (Betaplate, Pharmacia, Sweden). Counts were derived from the mean of four separate experiments and expressed as Δ c.p.m. (Δ c.p.m.=counts per minute of stimulated culture − counts per minute of control culture). As a positive control, the T cell mitogen concanavalin A (Sigma) was added at a dilution series of 200–0.04 μg ml^{-1}.

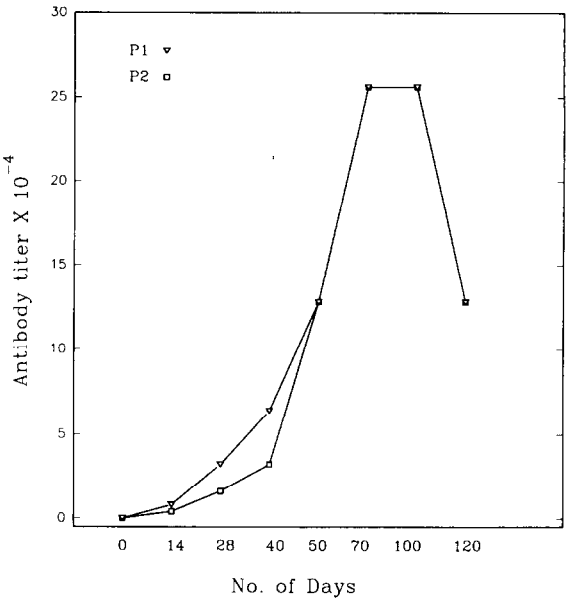

Figure 1 Evolution of the antibody response induced by immunisation with MEP, P1 or P2 in BALB/c mice. Sera were drawn at different intervals from the immunised mice and antibody titres were measured by ELISA at different dilutions (see Materials and Methods). Each data point represents the ELISA titres obtained in a pool of sera from six mice

Protection in mice

Two groups of 10 inbred mice (BALB/c) were immunised intraperitoneally with 50 μg of P1 or P2, emulsified in CFA. Control mice received only PBS in the adjuvant. All mice received boosts on days 21 and 42. On day 49, all mice were bled and sera were collected. A week later, the immunised mice were challenged with an inoculum of 1×10^4 *Plasmodium yoelii nigeriensis* (lethal strain)-infected erythrocytes. From the third day after challenge, thin blood smears were collected from the cut tail tip of each mouse, air-dried and fixed in methanol. Slides were stained with Giemsa stain and the percentage parasitaemia of each mouse was determined.

RESULTS

Antibody responses in mice immunised with the MEP constructs, P1 and P2

Sera from the BALB/c mice immunised with P1 were tested for the presence of antibodies by peptide ELISA. In the experiment, sera from six mice were pooled. The antibodies titres reached a peak at day 50, 18 days after boosting (1/256 000) and essentially remained at the same level after the second boost (*Figure 1*). Antibody level decreased at day 120 (1/128 000). Immunisation with P2 also gave similar results; ELISA titres reached a peak at day 65 (1/256 000) and declined at day 120 (1/128 000).

Table 2 Humoral response to the constituent epitope peptides on immunisation with MEP, P1 and P2

No.	Immunogen	Peptides					
		BT1	B1	BT2	T1	P1	P2
1	P1	16 000	128 000	8000	ND	256 000	8000
2	P2	16 000	8000	ND	32 000	8000	256 000

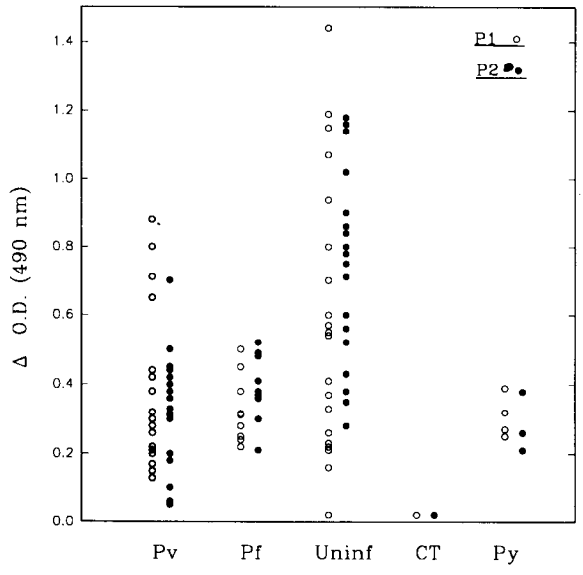

Figure 2 Antibody levels obtained by ELISA in sera from the individuals exposed to malaria infection to the MEP constructs. Pv and Pf indicate clinical cases of *P. vivax* and *P. falciparum* infection; Uninf. represents individuals living in *P. falciparum* endemic area but with no current malaria infection; CT indicates three individuals never exposed to malaria; Py represents *P. yoelii nigeriensis*-infected mice. Each point represents the mean of duplicates

In order to determine the fine specificity of the humoral response, ELISAs were performed with peptides representing different constituent epitopes as the capture antigens. In the case of P1 immunisation, in BALB/c mice, the highest antibody titres were observed against the B epitope, B1 (1/128 000), followed by BT1 (1/16 000). Antibody titres against the sequence BT2, which forms the C-terminal of P1, were the lowest (1/8000). As seen in *Table 2*, variable antibody titres against the individual B and T epitopes were also observed in P2 immunised mice. Since P1 and P2 share two common B-epitope sequences, their antibody cross-reactivity was also probed. However, only a weak cross-reactivity was observed in ELISA (*Table 2*).

Serology

Serum samples collected from *P. falciparum* and *P. vivax* patients and from individuals living in malaria endemic areas with no recent history of malaria were analysed for the presence of antibodies reactive with the MEP constructs, P1 and P2. Both *P. falciparum* and *P. vivax* infected human sera, at 1/400 dilution, reacted with P1 and P2. However, sera from uninfected individuals from the same also reacted with these peptides; in fact, the response was somewhat higher in these cases (*Figure 2*). Sera collected from three European donors with no previous exposure to malaria did not react with the peptides and served as controls. Further, sera obtained from BALB/c mice infected with rodent malaria parasite, *P. yoelii nigeriensis*, also reacted with P1 and P2 in an peptide ELISA at 1/400 dilution (*Figure 2*).

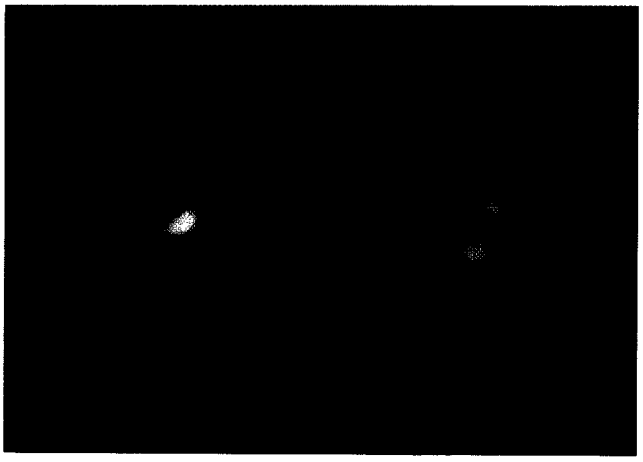

Figure 3 Reaction of anti-P1 sera with *P. yoelii* by immunofluorescence. Acetone-fixed parasitised cells were incubated with a 1:400 dilution of antiserum followed by a 1:40 dilution of fluoresceinated goat anti-mouse antibodies

To determine whether antipeptide (P1 and P2) antibodies specifically recognised surface antigens from the blood stages of *P. falciparum* and *P. yoelii*, indirect immunofluorescence antibody test was carried out with sera from mice immunised with P1 and P2. Anti-P1 antibodies bound to both *P. yoelii* (*Figure 3*) and *P. falciparum* blood-stage parasites at a sera dilution of 1/400. A pool of anti-P1 sera yielded bright, positive immunofluorescence with both *P. yoelii* and *P. falciparum*, even at a dilution of 1/1000; however, we did not determine the end-point titre. On the other hand, sera from mice immunised with P2 showed only a weak reactivity with the parasites even at a sera dilution of 1/20. These results suggest that immunisation with P1 induces a memory (IgG) response which is cross-reactive with the surface antigens of the parasite, whereas anti-P2 antibodies do not show such cross-reactivity with native proteins of the parasite. Furthermore, we also found that sera from mice immunised with P1 also exerted a strong growth inhibitory effect on *P. falciparum in vitro*. When we cultured the asexual erythrocytic stages of *P. falciparum* in the presence of immune or preimmune serum (10% final concentration), the parasitaemias we obtained at termination of the experiment were $0.15 \pm 0.03\%$ and $2.36 \pm 10.03\%$, respectively, indicating nearly 93% inhibition of the parasite growth in the presence of anti P1 serum. Clearly, the MEP construct P1 was not only immunogenic but also potentially protective against the parasite.

Fine specificity of the cellular immune response to P1 and P2

To determine whether the T-epitope sequences incorporated in P1 and P2 induced helper T-cell functions, groups of mice (BALB/c, 4/group) were immunised separately with either P1 or P2. Eight days later, lymph node cells from P1-primed mice were cultured *in vitro* with P1 itself, P2 or the constituent epitope peptides B1, BT1 and BT2. As shown (*Figure 4A*), strong proliferative responses were induced by P1 and BT2 although different concentrations of these peptides were required

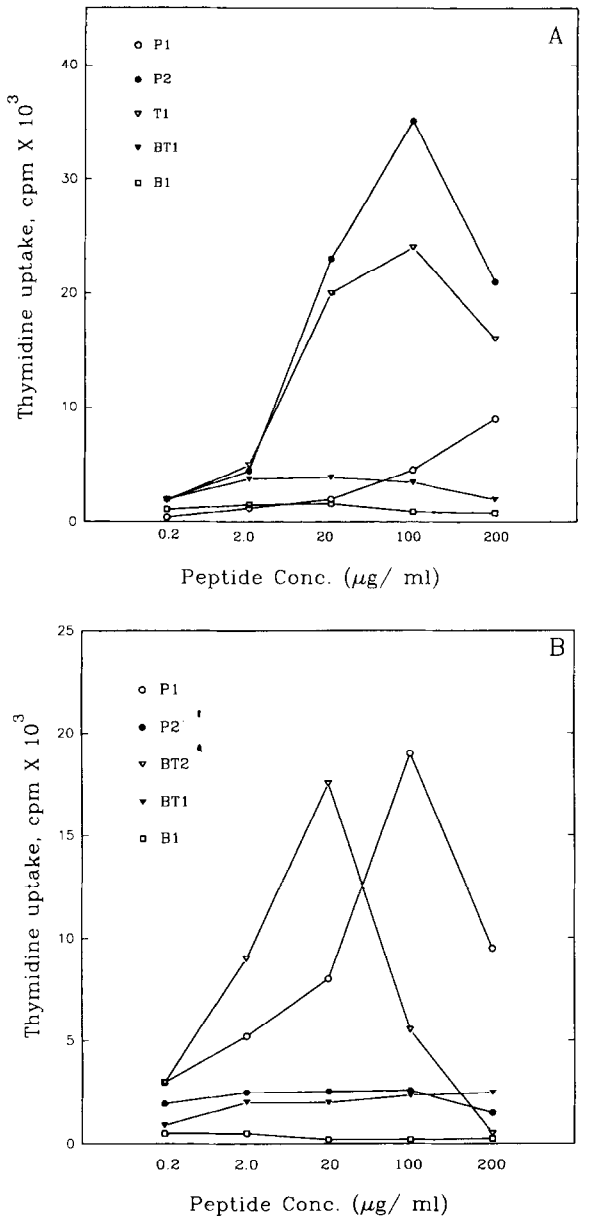

Figure 4 *In vitro* proliferative responses to various peptides. BALB/c mice were immunised with either P1 (A) or P2 (B) and the lymph node cells were challenged with the indicated peptides. Concanavalin A (1 μg ml^{-1}) was used as a positive test antigen. Each data point represents a mean of values obtained in four mice. Δc.p.m.=c.p.m. with Ag − c.p.m. without Ag. Maximum background incorporation in the absence of antigen was 4980.5 c.p.m. for panel A and 4203.2 c.p.m. for panel B. Concanavalin A incorporation was 120 000 c.p.m. for A and 109 000 c.p.m. for B

to produce the peak response. The B epitope peptide, B1, did not cause any stimulation of the cells. Surprisingly, both P2 and BT1 also induced only a weak proliferation of P1 sensitised cells. Similarly, in the case of P2-primed mice, strong proliferative responses were observed on stimulation with P2 itself, and the T epitope peptide, T1. Peptides BT1 and P1 were able to induce only a weak proliferative response, whereas B1 did not cause any stimulation of P2-sensitised cells (*Figure 4B*). In order to ascertain that the synthetic peptides used in this study were not mitogenic on their own, lymph node cells from mice immunised with CFA alone were co-cultured with various concentrations of P1, P2 or the different constituent epitope peptides. None of the

Table 3 Murine humoral response on immunisation with P1[a]

No.	Mouse strain	H-2 haplotype	I/Antibody titer against peptide P1	
			Day 40	Day 70
1	BALB/c	d	128 000	256 000
2	SJL	s	256 000	512 000
3	C3H/H3	k	128 000	128 000
4	C57 BL/6	b	4000	8000

[a]Each value represents the titre obtained in a pool of sera from at least four animals. End-point titres were designated to be the serum dilution producing an ELISA O.D. value greater than twice the O.D. obtained with respective pre-immune serum (1/1000 dilution)

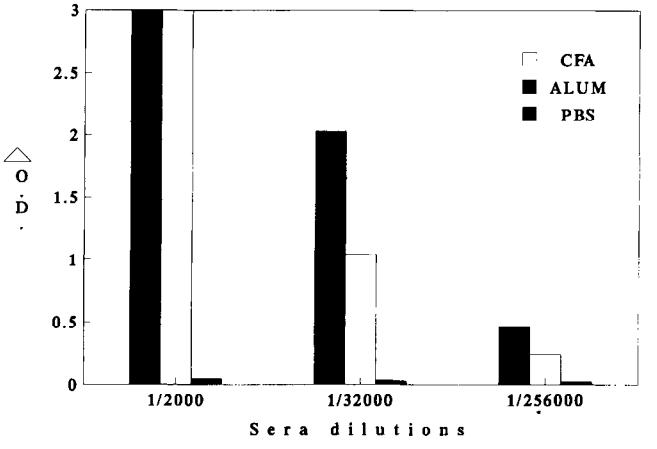

Figure 5 Comparison of antibody response in BALB/c mice immunised with P1 emulsified in CFA, alum or in PBS. Individual sera obtained from immunised mice (see Materials and Methods) were assayed by ELISA at different dilutions. Results are expressed as mean response of 10 mice in each group. Values are the absorbance obtained at 490 nm after subtracting absorbance obtained for pre-immune serum at each dilution

synthetic peptides caused any noticeable stimulation of cells from the CFA primed mice (data not shown).

Immunogenicity of P1

To determine whether immune response to P1 was genetically restricted, mice of three other different inbred strains representing C3H/He (H-2:k), C57 BL/6 (H-2:b) and SJL (H-2:s) haplotypes were primed and boosted with P1. All the mice generated an antibody response but significant differences were observed (*Table 3*). The highest response was seen in SJL mice (1/512 000), followed by BALB/c mice (1/256 000). A significantly lower response (1/8000) was seen in C57BL/6 mice. Intermediate levels of antibody titres (1/128 000) were found in C3H/He mice.

We also compared the antibody response induced by immunisation with P1 in CFA and alum, the latter being the only adjuvant acceptable for routine use in human. Groups of 10 BALB/c mice were primed and boosted with 50 μg of P1, emulsified in CFA, alum, or PBS. Control mice were given PBS in CFA or alum. An almost similar, high antibody response was observed with both adjuvants. As evidenced by peptide ELISA, mice immunised with P1 in either of the two adjuvants reached titres of 1/256 000. Mice immunised with P1 in PBS alone had negligible levels of antibodies (*Figure 5*).

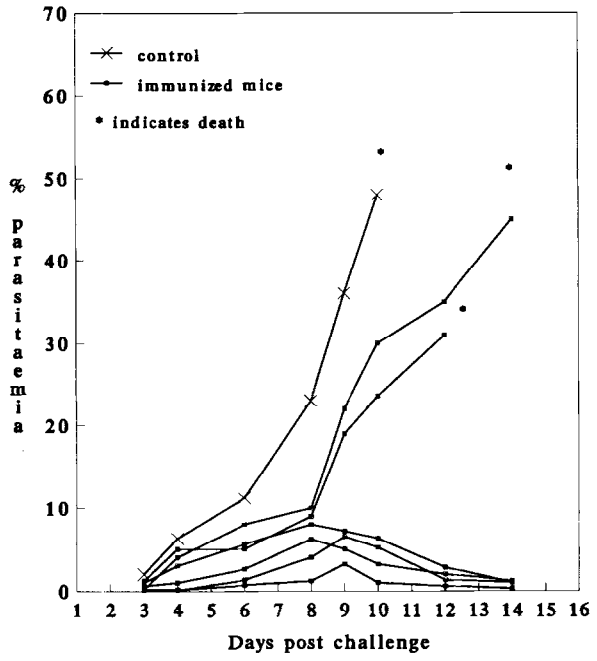

Figure 6 Development of parasitaemia after lethal *P. yoelii* challenge infection of BALB/c mice immunised with P1 in alum (see Materials and Methods). Parasitaemia is expressed as the percentage of infected erythrocytes. Each line represents a different mouse except for the control (x) which is representative of four control mice. Four of ten immunised mice did not develop any parasitaemia after the challenge infection and are not shown in the figure

Protection in mice immunised with synthetic peptides

To determine if immunisation with P1 in CFA or alum could provide protection in mice against murine malaria, the above groups of immunised mice were challenged with a lethal dose of *P. yoelii nigeriensis* blood-stage parasites. Five of the ten immunised mice in the CFA group survived the challenge. The remaining five mice also showed a delayed onset of parasitaemia in comparison with the control mice, but all died by day 14 (data not shown). In the other group in which alum was used as the adjuvant, 8 of the 10 immunised mice were protected; 4 did not develop any parasitaemia, while the remaining 4 cleared it by day 14 and lived on (*Figure 6*). All the control mice died within 9 days after the challenge. Mice immunised with P2, BT1 or BT2 in CFA were not protected and all died within 12 days of the challenge (data not shown).

DISCUSSION

As briefly reported earlier[22], the two multiple epitope peptides, P1 and P2, were highly immunogenic in mice. However, even though the two peptides shared common epitopes, only P1 immunisation provided protection against a heterologous challenge infection[22]. In the present study we have analysed fine specificity of the immune responses to these peptides and have further characterised P1 as an immunogen for the development of potential blood-stage vaccine peptide.

We found that both the peptides produced an anamnestic antibody response and that significant levels of antibodies were observed for an extended period of time. Peptides representing epitope sequences from malaria antigens are mostly poor immunogens and although several methods to enhance their immunogenicity, including the use of carrier proteins[11,23], multiple antigenic peptides systems[25] (MAPS) and polymeric constructs using terminal cysteine residues[24] have been described, they all have inherent limitations. Our results support the view that linear peptides containing B- and T-cell determinants can be highly immunogenic[17,18] and therefore may be considered as suitable alternatives in subunit vaccine design.

The high antipeptide antibody responses to the MEP peptides, without any carrier protein, suggest that the T epitopes included in P1 and P2 are able to provide appropriate T helper functions. This was further confirmed by lymphocyte proliferation assays *in vitro*. As expected we found that P1 primed lymph node cells recognised P1 and the constituent peptide BT2, and the P2 primed cells proliferated in response to stimulation by P2 itself and T1. However, the lymph node cells from the BALB/c mice primed with either P1 or P2 showed no response to BT1 stimulation even though this peptide sequence contained a human malaria T epitope[26]. In a separate study[18], we were able to show that the T epitope in BT1 was genetically restricted to the H-2:s haplotype. Therefore, since BT1 was the only common sequence containing a T-cell determinant, it was not surprising that lymph node cells from the P1-primed BALB/c mice did not react with P2, and vice versa. Although there are examples of malaria T epitopes reactive in both humans and mice[27], our results indicate that the patterns of genetic restriction of Th epitopes may be different, and therefore findings from animal studies may not always be extendable to humans.

An important issue in multiple epitope peptide design strategy is the number of T epitopes one should include in a given construct to ensure high immunogenicity. It might appear logical to include more than one T epitope in a given MEP construct, particularly to address the problem of genetic restriction of the immune response in synthetic peptides. On the other hand, existence of a hierarchy between covalently linked T epitopes has been demonstrated[28]. Our finding that the T epitope in BT1 sequence is not functional in BALB/c (H-2:d) mice, which is a high responder strain, may have interesting implications. Although both P1 and P2 were designed to contain at least two T epitopes each, our results suggest that a single functional T epitope may be sufficient to invoke antibody response against several B epitopes present in the molecule. However, since immunisation with individual epitope peptides or with a physical mixture of these peptides did not produce any significant antibody titres (data not shown), our results support the view that B-and T-cell epitopes need to be covalently linked in order for the Th cells to provide cognate help for B-cell activation and antibody production[29]. Successful construction of effective synthetic immunogens by combination of T and B epitopes has been reported by several groups[16,18]. On the other hand, there have been reports that immunisation with a physical mixture of B-and T-epitope peptides can also produce antibody responses to the B-cell determinants[30,31]. Since the mechanisms involved in the processing of peptide immunogens in relation with its structure are not yet clearly understood, each synthetic immunogen will have to be evaluated individually.

We also found that the antibody response to the B epitope, B1, which is common to the both MEP constructs, was remarkably higher in the case of P1- than P2-immunised mice. Also, in an indirect immunofluorescence assay only anti-P1 serum reacted with blood-stage parasites. Together with the finding that only P1 immunisation provides protection against *P. yoelii* challenge infection, these results suggest that B1 may be one of the crucial protective epitopes and should be considered to be a part of any peptide based blood-stage vaccine construct. Indeed this epitope is a consituent of the first synthetic peptide vaccine, SPf 66, which is currently undergoing human trials[19]. Moreover, antibodies against a tripeptide motif within the B1 sequence have been shown to correlate with protection[31]. We also found that both the MEP constructs reacted with sera collected from individuals exposed to malaria infection[22,32].

Our observation that the antibody response against the B epitope B1, upon P2 immunisation, is remarkably poor, even though P2 itself is a good immunogen, may have relevance to MEP design. While it may no longer be difficult to identify B and T epitopes from antigenic proteins, several questions regarding the design of multiple epitope peptides, such as the optimal number of B and T epitopes to be included, the polarity of synthetic constructs with respect to B and T epitopes, the possibility of generating new epitopes, etc., remain unanswered. In the case of P2 immunisation, the humoral responses seem to be focused more towards the N terminus and the middle of the molecule; the antibody titres against the C-terminus, which is represented by the B epitope B1, are the lowest. There is already some evidence that synthetic immunogens consisting of B-and T-cell determinants are polar[33] but there is no consensus yet on their orientation in MEP constructs. It was found necessary to place haptenic peptide sequences in the N-terminal position with respect to T epitopes for optimal humoral response in some studies[34,35]. On the other hand, reverse placement of B and T epitopes was found to be the appropriate orientation in some cases[36,37]. Since the molecular mechanism to explain the immunogen polarity are not clear, the most appropriate position of T epitopes to induce high B epitope antibody titres in peptide constructs will have to be assessed experimentally.

Since P1 alone was protective in mice, its immune responses were further characterised. We found that P1 was immunogenic not only in CFA but also when used in conjunction with alum as adjuvant. In fact, we observed that immunisation with alum provided a higher degree of protection in mice against *P. yoelii* challenge compared to CFA. There is some evidence that in humans an antibody isotype imbalance may be responsible for the delayed acquisition of protective immunity[38]. Adjuvants have been shown to modulate the nature of immune responses of malaria antigens[39] but we did not find any noticeable differences in the distribution of the IgG subclasses in alum and CFA immunisation in BALB/c mice (data not shown). The reasons for somewhat better efficacy of P1 in alum are not clear from our present data.

We also found that immunisation, with P1, of different strains of mice produced an antibody response in all the strains tested, although to different extents. The highest antibody titres were seen in SJL mice. This appears to be consistent with our earlier observation that the T epitope included in the BT1 sequence is genetically restricted to the SJL (H-2:s) haplotype[18]. It is possible that the high antibody response in SJL mice is due to the fact that there are two functional T epitopes available to provide T-cell help instead of one, as is the case in other strains of mice. Immunodominance resulting from intramolecular competition between T-cell epitopes which are covalently linked together may be a valid concern in designing relatively short synthetic peptides as immunogens[28]. At the same time it is also recognised that immunodominance is not simply a function of the primary amino acid sequence, but is a function of peptide conformation which may determine the context of the epitope within the polypeptide molecule[40]. Although we did not investigate the fine specificity of the immune responses in this strain (SJL), our results indicate that it is not necessarily detrimental to include more than one T epitope in a synthetic peptide immunogen. In fact, the poor antibody response in one of the strains tested in this study (C57 BL/6) suggests that it might be necessary to include more than one T epitope in a MEP construct to overcome the problem of genetic restriction of the immune response.

As mentioned earlier[22], an important observation was that P1, based on *P. falciparum* conserved sequences, was protective against murine malaria *P. yoelii*. Conserved peptide sequences from another merozoite antigen, MSA-2, of *P. falciparum* were found to be protective in mice against rodent malaria, *P. chabaudi*[11]. These results indicate that at least some neutralisation-sensitive epitopes on blood-stage malaria Ags are conserved not only in different isolates but also in different species, and could form the basis of a synthetic malaria vaccine which could, in principle, circumvent the problem of antigenic diversity in malaria antigens.

The implication of the data presented here may be significant for the peptide subunit vaccine design in general. The results support the view that relatively short peptides containing B- and T-cell determinants can be highly immunogenic. However, it seems that polarity of synthetic immunogens *vis-à-vis* placement of the epitopes can be crucial for obtaining appropriate immune responses. The feasibility of using MEP constructs as vaccines for humans will depend, to a large extent, on the identification of Th epitopes and a knowledge of their pattern of genetic restriction. This will allow one to assess the number of different T-cell epitopes that will be needed to cover most of the population. The findings of this study underscore the fact that there are no clear-cut rules for the design of MEP constructs and each case will have to be evaluated separately.

ACKNOWLEDGEMENTS

We thank Dr V. P. Sharma and his colleagues at the Malaria Research Centre, Delhi for their help in obtaining the human sera used in this work. We wish to thank Ms Ashima Bharadwaj and Ms R. Radha for helping in the preparation of the manuscript.

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