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Model multiple antigenic and homopolymeric peptides from non-repetitive sequences of malaria merozoite proteins elicit biologically irrelevant antibodies

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Abstract

Three model peptides containing B-epitopes from conserved, non-repetitive regions of the merozoite surface antigens, MSA2 and MSA1, and the erythrocyte binding protein EBP of *Plasmodium falciparum* were synthesised. The peptides incorporated GPG spacers and C residues at the N and C termini, and were polymerised by oxidation to form cystine bridges. Multiple copies of essentially the same peptide sequences were also synthesised on a branching lysyl matrix to form a tetrameric multiple antigen peptide. Rabbits were immunised with the polymerised and multiple antigen peptides, in alum followed by Freund's adjuvant, and the antibody responses examined by IFA and ELISA. Reproducible antibody responses were obtained against the MSA1 and EBP but not MSA2 peptides. IgG antibody levels detected by ELISA after three injections of antigen in alum, increased significantly after further immunisation in Freund's adjuvant. IgG levels were largely maintained for at least 23 weeks after the final immunisation. IgM antibodies, generally detectable only after immunisation in Freund's adjuvant, were absent 23 weeks later. Antibody titres against the native protein on fixed parasites, assayed by IFA, were three to five orders of magnitude lower than the corresponding ELISA titres against the peptides. Antibody-dependent inhibition of *P. falciparum* growth in vitro could not be demonstrated with the immune rabbit sera. The MSA1 and EBP peptides elicited cross-reactive antibodies. The results suggest that the selected non-repetitive sequences are conformationally constrained in the native proteins and only a small proportion of the anti-peptide antibodies bind to the native proteins. The significance of the findings for the development of peptide vaccines and the use of peptides in immunoassays is discussed.

Keywords: Antigenicity; Immunogenicity; Malaria; Merozoite; Multiple antigen peptide; Peptide polymer; Vaccine; (*Plasmodium falciparum*)

Abbreviations: aa, amino acid; Boc, butoxycarbonyl; EBP, erythrocyte binding protein; FCA, Freund's complete adjuvant; FICA, Freund's incomplete adjuvant; IFA, immunofluorescence assay; mab, monoclonal antibody; MAP, multiple antigen peptide; MHC, major histocompatibility complex; MSA, merozoite surface antigen; PBS, 0.01 M phosphate-buffered saline, pH 7.2; *Pf*, *Plasmodium falciparum*; *Pv*, *Plasmodium vivax*; rbc, red blood cells; T_h, T-helper

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1. Introduction

Synthetic peptides, which are easily and inexpensively produced in a pure form in bulk, can be used to generate site specific immune responses to protein antigens [1,2]. One disadvantage of short peptides for vaccination is that antibody formation against haptenic peptides requires covalently linked sequences that bind to class II major histocompatibility complex (MHC) molecules for recognition by T-helper (T_h) cells. The first synthetic malaria peptide to be used in a human vaccination trial was based on the repetitive $(NANP)_n$ B-cell epitope of the *Plasmodium falciparum* (*Pf*) circumsporozoite protein [3]. $(NANP)_3$, coupled to tetanus toxoid as a carrier molecule for providing T_h-epitopes, elicited repeat-specific antibodies and a degree of protection against challenge with sporozoites. Immunisation with synthetic peptides containing non-repetitive B-cell epitopes from protective *Pf* merozoite surface antigens MSA1 [4–10] and MSA2 [11–14] coupled to diphtheria toxoid as a carrier in a phase I clinical trial, showed good antibody formation [15]. However, an unacceptable level of type III (Arthus) hypersensitivity to the toxoid was observed in the immunised individuals [15]. A disulphide-linked polymer of peptides, termed SPf66, derived from MSA1 (11 aa with the sequence YSLFQKEKMVL), the *Pf* circumsporozoite protein repeat (NANP), and two M_r 55 and 35 kDa proteins, has been reported to protect volunteers against *Pf* malaria after immunisation in alum [16]. SPf66 has since been widely tested in the field but its efficacy and mode of action is controversial [17,18]. Antibodies perform a critical role in the immune effector mechanisms against blood stages of the *Pf*. Antibodies agglutinate merozoites, block red blood cell (rbc) recognition, cause complement mediated lysis of merozoites, promote antibody-dependent killing by neutrophils, monocyte/macrophages and natural killer cells, generate parasite-toxic cytokines and nitric oxide by immune complex-mediated macrophage/monocyte activation, reverse cytoadherence to endothelium and neutralise parasite toxins (reviewed in [19]). Monoclonal antibodies (mabs) to the 45 kDa MSA2 (alternatively termed GYMSSA or PfMSP2) inhibit parasite growth in culture [11–13]. Humoral immune responses to MSA2 in endemic area populations are associated with protection against malaria [14]. Similarly mabs against the 185–200 kDa MSA1 (alternatively PMMSA or PfMSP1) inhibit parasite growth in culture [4]. Immunisation of monkeys with MSA1 protects against *Pf* malaria [5–8] and antibodies in persons living in endemic areas are associated with protection [9,10]. A 175-kDa erythrocyte binding protein (EBP) is a *Pf* merozoite receptor for glycophorin A [20,21]. The EBP domain mediating the initial binding to glycophorin has been characterised and a 19-residue peptide located elsewhere on the EBP molecule which reportedly elicits *Pf* growth inhibiting antibodies has been identified [22].

The repetitive sequences that constitute dominant B-cell epitopes are found in some *Pf* proteins, including MSA1 and MSA2, and may constitute a parasite strategy for evading protective immunity (reviewed in [19]). We therefore examined the immunogenicity of synthetic peptides containing non-repetitive EBP, MSA1 and MSA2 sequences as preliminary models for developing peptide-based vaccines against the asexual blood stages of *Pf*. The sequences selected contained known B-cell epitopes that were accessible to antibodies in immunofluorescence assays. The two MSA sequences also contained human T-cell epitopes.

The immunogenicity of peptides can often be increased or sometimes induced when presented as multiple copies in the immunogen [23,24]. Three peptides, one from each antigen, were therefore synthesised on a branching lysyl matrix to form tetrameric multiple antigen peptides (MAP) [23]. Essentially the same peptide sequences were also synthesised with terminal GPG spacers and C-residues for homopolymerisation through cystine bridges. Antibody responses to the peptide constructs were then determined in rabbits.

2. Materials and methods

2.1. Peptide and MAP synthesis

Peptides were manually synthesised by standard t-Boc solid phase chemistry using *p*-methyl benzhydrylamine resin, as modified for multiple synthesis in polypropylene mesh bags [25]. MAPs were also synthesised using t-Boc chemistry except that t-Boc-

βAla-OCH_2-phenylacetamidomethyl resin (Bachem, Switzerland) was used to initiate synthesis and N^{α},N^{ε}-Boc_2-Lys (Bachem) was used for the first and second couplings to provide the lysine scaffolding as described [23]. The efficiency of every coupling reaction was followed by a quantitative ninhydrin procedure on an aliquot of the resin [23]. Repeated coupling with t-Boc aa was performed where necessary. On completion of synthesis, dinitrophenyl side chain protecting groups on the histidine in MSA1 and MSA2 peptides were removed with thiophenol [23]. Peptides and MAPs were then cleaved from the resin by the low–high HF procedure [26]. They were purified by reverse phase HPLC using a C18 column and a Shimadzu 8A liquid chromatograph (Shimadzu, Japan) [27], and the aa composition checked after hydrolysis using the Picotag system (Waters, MA) as described [27]. The sequences of the peptides and MAPs and the residues of the parent protein from which they were derived are shown in Table 1.

The selection of the model non-repetitive MSA1 and MSA2 sequences was based on protein structure prediction [28], the peptides being recognised by antibodies that vary with malaria transmission rates in endemic area populations [28], sequence conservation in different parasite isolates, ability to induce merozoite surface reactive antibodies in volunteers when injected as diphtheria toxoid conjugates [15] and possession of epitopes recognised by human T-cells in cell proliferation assays [29,30]. The replacement of the native C by an A in P204 and MAP104 does not detectably affect the specificity of the elicited antibodies [15,27]. The EBP sequence was chosen for its reported ability to induce antibodies that inhibit *Pf* growth and show reactivity with merozoites in an immunofluorescence assay and its presence in different *Pf* isolates except for a change in residue 1074 from D to G in the 3D7 isolate [22].

2.2. Polymerisation of peptides

Peptides P204, P209 and P230 lyophilised after purification were dissolved in 0.01 M phosphate buffered saline, pH 7.2 (PBS) at 4–10 mg ml^{-1}, the pH adjusted to 7.6 with 0.1 M Tris/HCl and then oxidised by vigorously bubbling oxygen for 6 h.

2.3. Immunisation of rabbits with peptides

Each MAP and peptide was injected into two New Zealand white rabbits (5–7 months old) for determining immunogenicity. Rabbits received 1 mg peptide or MAP adsorbed onto 10 mg sterile aluminium hydroxide gel (alum) and injected intramuscularly at two sites. The animals were injected with antigen at 3-week intervals. Control rabbits were injected in parallel with PBS and adjuvant in an identical manner. Rabbits were ear bled before commencing immunisation to obtain prebleed sera (PI) and then 2 weeks after each injection of antigen. Because of the relatively poor antibody response after three injections in alum (yielding bleeds 1, 2 and 3, respectively), the rabbits were further injected intramuscularly with the same amounts of peptide and MAP emulsified in Freund's complete adjuvant (FCA) (to give bleed 4) and subsequently twice at 3-week intervals with antigen in incomplete Freund's adjuvant (FICA) (to give bleeds 5 and 6) and then finally with antigen in PBS (to give bleed 7). The animals were then allowed to rest for 23 weeks without further immunisations before bleed 8 was obtained. The immunisation and bleeding schedule is summarised in Fig. 1. Sera were stored at −20°C until use. The

Table 1
Sequences of peptides and MAPs and residues of the parent protein from which they were derived

Peptide	Protein (*Pf* isolate)	Residues	Sequence
P204	MSA2 (FC27)	207–223 with C in residue 221 replaced by an A [27]	CGPGRNNHPQNTSDSQKEATDGPGC
P209	MSA1 (K1)	20–38	CGPGVTHESYQELVKKLEALEDAGPGC
P230	EBP (Camp)	1069–1087	CGPGNEREDERTLTKEYEDIVLKGPGC
MAP 104	MSA2 (FC27)	207–226 with C in residue 221 replaced by an A [27]	RNNHPQNTSDSQKEATDGNK
MAP 109	MSA1 (K1)	20–38	VTHESYQELVKKLEALEDA
MAP 130	EBP (Camp)	1069–1087	NEREDERTLTKEYEDIVLK

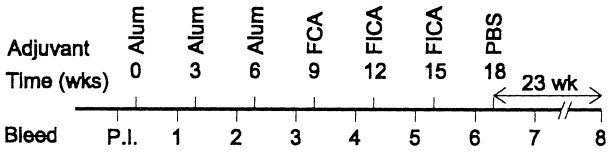

Fig. 1. Schedule of immunisation and serum collection.

care and use of animals were according to WHO guidelines (WHO88.1).

2.4. ELISA

ELISA was carried out on peptides P104 (sequence RNNHPQNTSDSQKEATDGNKC), P109 (sequence VTHESYQELVKKLEALEDAC) and MAP130 as target antigens for the antisera to the corresponding peptides, essentially as described previously [27]. Briefly, polystyrene microtitre plate wells (Costar, USA) were coated with 50 μl of peptide dissolved at 10 μg ml^{-1} in 0.05 M bicarbonate/carbonate buffer, pH 9.6 overnight at 37°C. The wells were then treated with 50 μl of methanol to fix the peptides and subsequently blocked for 3 h at room temperature in 5% w/v non-fat milk powder dissolved in PBS (Blotto). Ten-fold dilutions of rabbit anti-peptide sera in Blotto were used as first antibodies in the ELISA. The assays were performed in duplicate. For detecting IgM rabbit antibodies, a peroxidase conjugated IgG fraction of a goat antiserum to rabbit IgM μ-chains, with specificity for rabbit μ-chains (ICN, USA), was used as the second antibody. For detecting IgG rabbit antibodies, a peroxidase-conjugated IgG fraction of a sheep antiserum to rabbit IgG Fc, with specificity for rabbit γ-chains (ICN, USA), was utilised as the second antibody. For detecting all antibody classes, a peroxidase-conjugated IgG fraction of a sheep antirabbit IgG serum with H- and L-chain specificity (Silenius, Melbourne, Australia) was employed as the second antibody. The substrates 2,2′-azino-bis (3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) and H_2O_2 were used for colour development, which was quantitated in a Flow Multiskan Plus II ELISA reader at 405 nm. The reciprocal of the highest dilution of the antiserum, giving an average absorbance twice that of the corresponding preimmune serum at the same dilution, was taken as the antibody titre.

2.5. Immunofluorescence assay

Indirect immunofluorescence assays (IFA) were performed as described previously [12,13] on late stages of 3D7 isolate *Pf* using a fluorescein-conjugated IgG fraction of sheep antirabbit IgG serum with H- and L-chain specificity (Silenius, Melbourne, Australia) as the second antibody. IFA was done at 1:25, 1:100 and 1:500 dilutions of rabbit sera as first antibodies. Preimmune sera and sera from rabbits immunised with PBS were used as negative controls. A rabbit antiserum to recombinant MSA2 protein was used as a positive control for the IFA.

2.6. Pf growth inhibition assay

[^{3}H]Hypoxanthine incorporation growth inhibition assays were performed as described previously [31], except that human serum was replaced with 10% test rabbit serum in the culture medium. All rabbit sera were preabsorbed with human rbc before use in the assays.

3. Results

3.1. Characterisation of polymerised peptides by SDS-PAGE

SDS-PAGE analysis on 12.5% gels revealed that the polymerised peptides migrated as polydisperse material of up to M_r 24 kDa, indicating the presence of nonamers and smaller polymers of the peptides.

3.2. Antibody response in rabbits to immunisation with MAPs and peptide polymers

The results of ELISA to determine IgG and IgM antibody responses to the MAPs and homopolymeric peptides are given in Figs. 2 and 3, respectively. Significant antibody responses were detected in all rabbits except one (P204b) immunised with homopolymeric P204. Antibody responses to the MSA2 peptides (MAP104 and P204) were in general poorer than against the other peptides. Immunisation with antigen in alum generally yielded lower antibody titres than after subsequent immunisations with the peptides in Freund's adjuvant. This was particularly

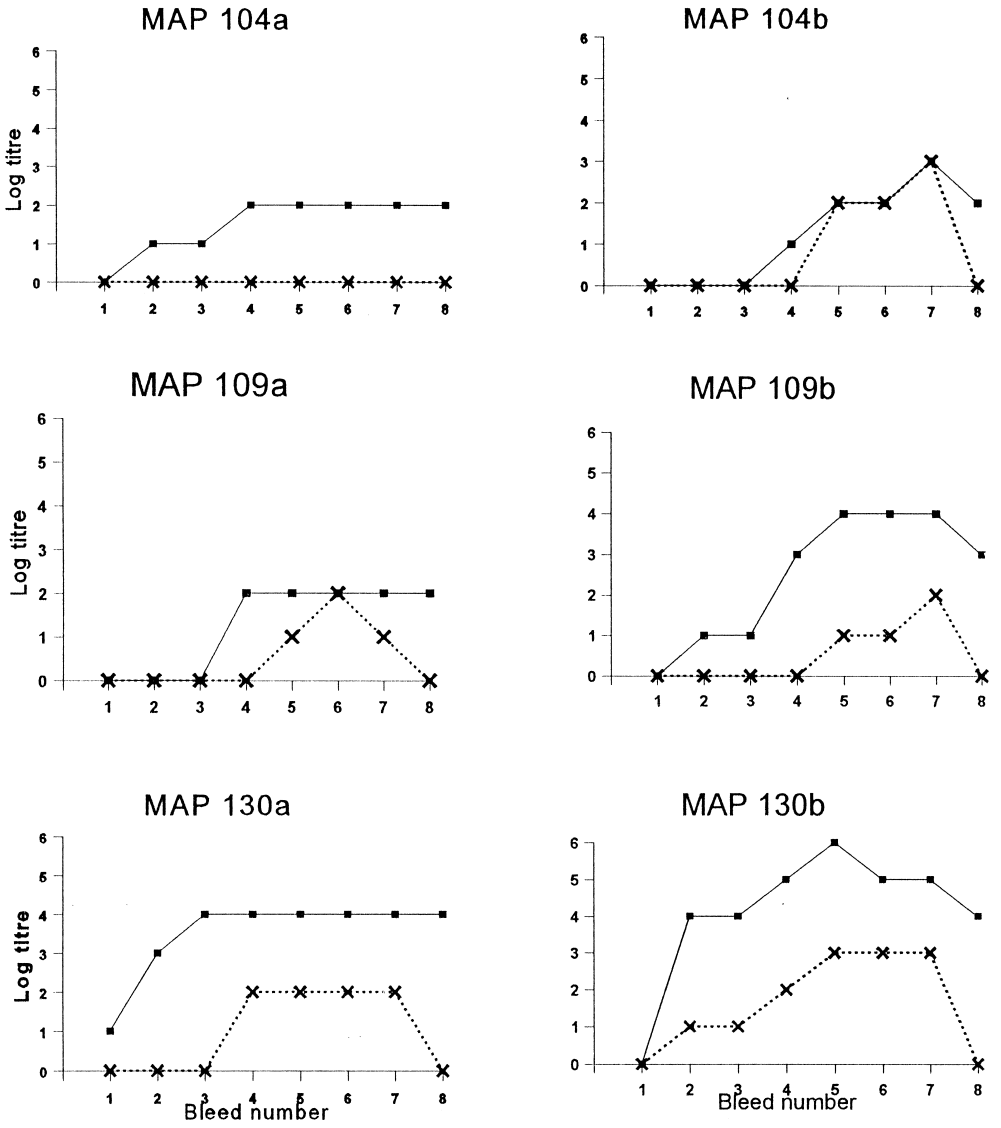

Fig. 2. IgG (■) and IgM (×) rabbit antibody responses to immunisation with multiple antigen peptides (MAPs). Values of antibody titres are expressed as logarithms to the base ten.

clear with IgM antibodies. The increase in antibody titres with multiple immunisations, particularly with the MSA1 and EBP peptides, suggests that memory responses were generated. While IgM antibodies decreased to preimmune concentrations 23 weeks after the last immunisation (bleed 8), the IgG antibody titres were either maintained or diminished by one order of magnitude during the same period.

When a peroxidase-conjugated sheep antibody recognising all rabbit immunoglobulin classes was used as the second antibody in ELISA, changes in total antibody concentrations similar to the changes in IgG antibody concentrations were observed, except that the titres were frequently one or two orders of magnitude greater than the titres of IgG antibodies. Essentially similar results were obtained when unpolymerised P204 replaced P104, unpolymerised P209 replaced P109 and unpolymerised P230 replaced MAP130 as antigens in the respective ELISAs. This shows that basically the same epitopes are being recognised in homologous peptides.

Cross-reactions, scored when reactions with peptides from a different antigen were observed at serum dilutions greater than 10^{-2}, were significant between

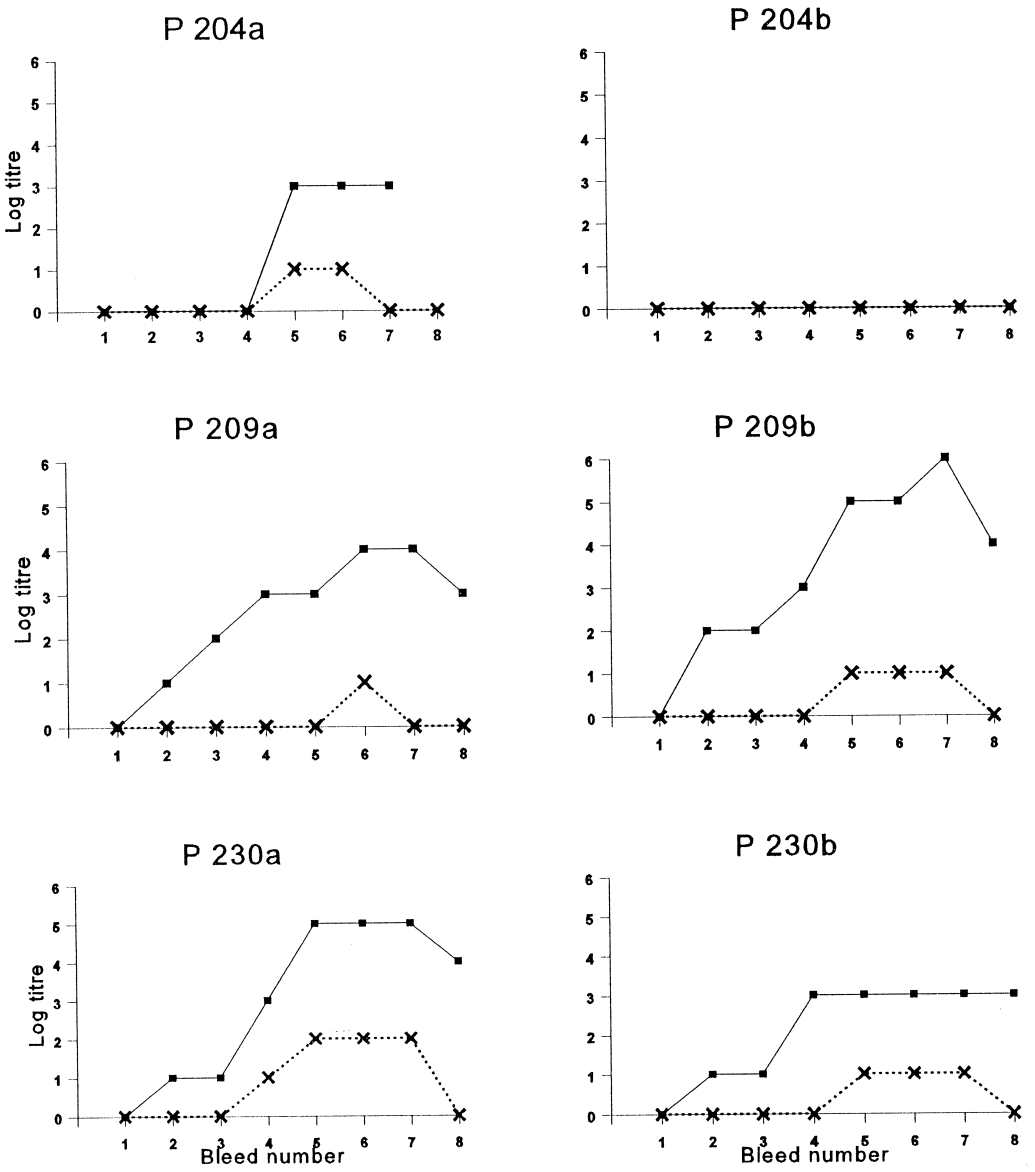

Fig. 3. IgG (■) and IgM (×) rabbit antibody responses to immunisation with homopolymeric peptides. Values of antibody titres are expressed as logarithms to the base ten.

the EBP and MSA1 MAPs and homopolymers. Antisera from rabbits immunised with MAP104 or P204 did not cross-react with the MSA1 and EBP peptides. ELISA antibody titres observed with the 7th bleed antisera to the MSA1 and EBP peptides, using peroxidase conjugated sheep IgG recognising all rabbit immunoglobulin classes as a second antibody and P109 and unpolymerised P230 as target antigen are shown in Table 2. The results demonstrate that the reactions of the antisera with the immunising peptides were greater than the reactions with cross-reacting peptides.

Antibody titres detected by an IFA reaction with fixed parasites reached a maximum with the 7th bleed sera. However, the IFA titres were several orders of magnitude lower than the ELISA titres, except with rabbit P204b where a weak IFA reaction was observed in the absence of detectable reaction of the antiserum with peptide in an ELISA. The IFA titres of the 7th bleed sera, determined with fluores-

Table 2
Cross-reactions between the MSA1 and EBP peptides

Antiserum	Log_{10} antibody titre against target antigen	
	P109	P230 (unpolymerised)
MAP109a	6	3
MAP109b	6	3
MAP130a	3	5
MAP130b	3	6
P209a	5	4
P209b	5	4
P230a	4	6
P230b	4	6

The 7th bleed antisera to the MSA1 and EBP peptides were used in conjunction with a peroxidase-conjugated sheep antibody that detects all rabbit immunoglobulin classes. The given values are the logarithms to the base ten of the ELISA titres.

cein-conjugated antibodies that detect all rabbit immunoglobulin classes, are shown in Table 3.

3.3. Activity of rabbit antibodies in the Pf growth inhibition assay

None of the antisera significantly inhibited parasite reinvasion and growth, by microscopy or in the [^{3}H]hypoxanthine incorporation assay, in comparison to the corresponding preimmune sera and serum from the control rabbit immunised with PBS.

Table 3
Characteristics of IFA reactions with the 7th bleed antisera on fixed parasites

Antiserum	IFA titre	IFA antigen location
MAP104a	0	–
MAP104b	0	–
MAP109a	25	merozoite surface
MAP109b	100	merozoite surface
MAP130a	25	merozoite
MAP130b	25	merozoite
P204a	25	merozoite surface
P204b	25	merozoite surface
P209a	100	merozoite surface
P209b	100	merozoite surface
P230a	0	–
P230b	25	merozoite

The IFA was performed with a fluorescein-conjugated sheep antibody that recognises all rabbit immunoglobulin classes. IFA was carried out at serum dilutions of 1:25, 1:100 and 1:500 and the values given are the reciprocal of the highest dilution giving a positive reaction.

4. Discussion

The potential of peptides as vaccines was first demonstrated when peptide fragments from the tobacco mosaic virus were shown to inhibit precipitation of the virus by an antiserum and when immunisation with a hexapeptide coupled to a carrier was shown to induce the formation of virus-neutralising antibodies [32]. This work has now been extended to produce neutralising antibodies against several viral pathogens and bacterial toxins [1]. The requirement for linked T_h-epitopes that are recognised by persons of varying MHC types is a drawback to the development of peptide vaccines for human use. For *Pf* peptide vaccines, this might be overcome by using T_h-epitopes from *Pf* proteins [33] or tetanus toxoid [34] that are recognised in the context of many human MHC class 2 molecules.

Polymerisation of peptides reported for SPf66 circumvents the need for a carrier for eliciting antibodies [16]. This may be due to T-independent antibody responses, the presence of uncharacterised T_h-epitopes in the SPf66 peptide sequences or the creation of new T_h-epitopes in the polymer. Linear homopolymerisation of 13 and 15 aa haptenic peptides by the carbodiimide method yielded polymers that on immunisation produced antibody titres of 10^4, a phenomenon ascribed to the generation of new T_h-cell epitopes as a result of polymerisation [24]. Our results show that the MSA1 and EBP peptides can elicit high-titre anti-peptide antibodies when presented in two different forms containing multiple copies of the epitopes viz. MAPs or polymers linked through cystine bridges. Similar antibody responses to the MAPs and polymers were also observed in mice (our unpublished observations). While there was no prior evidence for a T_h-epitope in the P230/MAP130 EBP sequence, good IgG antibody formation and memory responses, to both homopolymeric P230 and MAP130, suggests that an epitope recognised by rabbit T_h-cells is indeed present. There was previous evidence for the presence of human T-cell epitopes in the P209/MAP109 sequence [29,30]. The weak rabbit antibody response to the MSA2 peptides could involve deficient antigen presentation or T_h-cell recognition in rabbits, since P104-bovine serum albumin conjugates elicit good antibody responses to P104 in Balb/c mice [27], P104-diphtheria toxoid

yielded antibodies to P104 in human volunteers and the closely related P103 sequence was recognised by $>60\%$ of Solomon Islanders tested in T-cell proliferation assays [30]. Hence it is possible that the human antibody responses to at least the MSA1 and two peptides may be superior to that observed in the rabbits. The inclusion of stronger T_h-epitopes in the peptide sequences may, however, further improve immunogenicity in man. Apart from intrinsic differences between humans and rabbits in the immunological repertoire, prior exposure to *Pf* malaria in endemic areas would also be expected to influence the human antibody response to the peptides.

Homopolymers of peptides containing B- and T_h-epitopes produced by the carbodiimide method [24] or more recently by free radical induced polymerisation of acrolyl-derivatised peptides [35], and MAPs [23] yield good antibody producing immunogens. Twelve to sixteen haptenic groups are optimally required in a polymeric antigen for cross-linking B-cell receptors and generating a type 2 T-independent antibody response [36], which is typically dominated by IgM antibodies. This condition is not met with the homopolymers and MAPs used in these experiments. Memory antibody responses and the preponderance of IgG antibodies suggest that T_h-dependent antibody formation is a dominant feature of the observed antibody responses.

Significant levels of IgG, but not IgM, antibodies were detectable in the sera of rabbits, 23 weeks after the final immunisation. This is consistent with the shorter half life of IgM compared to IgG in blood. Antibody titres after three injections in alum were considerably lower (10^2 in all but rabbits MAP130a and b, where a titre of 10^4 was obtained) than the titres obtained after subsequent injections in Freund's adjuvant. It is possible that some of the IgM antibodies observed after immunisation in Freund's adjuvant are cross-reacting IgM antibodies produced in response to this adjuvant. The increase in non-specific antibodies, commonly observed after immunisation in Freund's adjuvant, may explain the weak merozoite-surface specific IFA reaction seen with 7th bleed P204b sera in the absence of an ELISA reaction with the immunising peptide. It is clear, however. that human immunisation with peptides will require stronger adjuvants than alum that, unlike Freund's, will be suitable for human use. Several such adjuvants have recently undergone successful clinical safety trials [37].

Reciprocal cross-reactions were significant between the MSA1 and EBP peptides. Cross-reactions are also seen between anti-P230 sera and P109 excluding a role for the terminal lysine residues or the linker sequences in generating cross-reactions. Both peptides carry net negative charges at pH 7. Maximum homology analysis of P109 and P130 showed that 10 aa matched in a 12 aa stretch as follows:

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. . . . . VTH ES YQ EL VKKL EALED A
          . : . : : . . . :
    :
N ER ED ER TLT KE ? Y ED I VL K. . . . . .
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The observed cross-reactivity may therefore reflect the importance of charge in the antibody–peptide interaction and/or the structural homology between the two peptides. Hence caution is needed in estimating antibody levels to native proteins by measuring antibodies binding to immobilised peptides in ELISA or RIA. Reactions of the different anti-peptide sera with several *Pf* proteins other than parent antigen, indicating extensive cross-reactivity, were also observed in Western blots (R. Ramasamy, unpublished observations). Extensive cross-reactions of antibodies reacting with repetitive and non-repetitive pepide epitopes in *Pf* with other *Pf* proteins containing structurally related sequences have been previously reported [38,39].

The replacement of residue 1074 G in the native 3D7 EBP protein by a D in the immunising peptide does not prevent elicitation of antibodies reacting with the protein by IFA. Anti-peptide ELISA titres obtained by immunisation with MAPs and polymeric peptides in the present study were three to five orders of magnitude greater than the corresponding IFA titres against merozoites. Hence only a small proportion of the anti-peptide antibodies elicited by immunisation with the MAPs or peptide polymers recognise the conformation of the peptides in the three native proteins. This is not desirable for human vaccination since formation of a large quantity of irrelevant antibodies may mediate pathological effects. The probable explanation for this phenomenon is that peptides generally assume a more relaxed con-

formation in solution when compared to their structure in the native protein and this influences the specificity of the antibodies elicited by the peptides [40]. Constraining peptides to resemble their conformation in the native proteins where this is known or predictable, by using covalent bond mimics [41] or cyclisation [42], for example, may improve the specificity of antibodies elicited against peptides.

Previous observations have shown that P104 and P109 conjugated to diphtheria toxoid as a carrier and then used to immunise volunteers in alum as an adjuvant, elicit anti-P104 and anti-P109 antibodies with merozoite surface-specific IFA titres, in the 640–2500 range, that were generally higher than the corresponding ELISA titres against the peptides [15]. In that study [15], identical ELISA and IFA methods to those used here were employed, so that the difference cannot be attributed to the relative sensitivities of the assays. Also, anti-peptide antibody titres in man to an $(NANP)_3$-tetanus toxoid conjugate injected in alum were generally only one order of magnitude greater than the IFA titres against *Pf* sporozoites by IFA [43]. Anti-peptide titres to *P. vivax* (*Pv*) circumsporozoite repeats after immunisation of Saimiri monkeys with a MAP construct in four different adjuvants were of the same order of magnitude as the IFA titres against *Pv* sporozoites [44]. Mice immunised with MAPs containing the *Pf* circumsporozoite protein repeat epitope together with a T_h-cell epitope, in Freund's adjuvant elicited antibodies with similar ELISA and IFA titres [45].

One possible explanation for the different results is that repetitive sequences in malaria parasite proteins, which are likely to be surface located on native proteins [19], assume a more similar range of conformations to the corresponding peptides in solution. Since the repetitive sequences in malaria parasite proteins may function in the evasion of protective immunity [19], this could present a conundrum for peptide vaccine development in malaria. It is also possible that P104 and P109 coupled to a carrier protein through the C-terminus assume a more restricted conformation, resembling the sequence in the native proteins, than in the corresponding MAPs or homopolymers. The orientation of a peptide coupled to a carrier for example is one factor that influences the formation of antibodies that react with the native parent protein [46]. Another factor to be considered is that multiple immunisations and the use of Freund's adjuvant, that are features of the study reported here, may increase the proportion of antibodies against non-native peptide conformations. There is clearly much that remains unknown about developing peptide reagents that yield antibodies reacting with native proteins.

The P109/MAP109/P209 sequence, which constitutes the N-terminus of the mature MSA1 protein, is located 4 aa away from the 11-aa MSA1 sequence present in the SPf66 malaria vaccine [16]. ELISA titres of up to 25 600 are observed in persons receiving three doses of SPf66 in alum [47] but the IFA titres against blood stage parasites are much lower, being 160 in the first reported vaccination of man [16]. Up to 98% inhibition of parasite growth over a 72-h period was reported with SPf66-immune human sera [16]. The reason for the lack of inhibition with the rabbit anti-MSA1 sera with higher ELISA and similar IFA titres against an adjacent MSA1 sequence is therefore not clear. However, our result is consistent with the report that only the C-terminal 19-kDa fragment of MSA1 is carried into rbc by invading merozoites and this region of MSA1 is the major target of invasion inhibiting antibodies [4]. While antisera raised to the 19 residue EBA peptide conjugated to a carrier protein reportedly inhibit *Pf* growth by 50% at 1:5 dilution [22], antisera produced against P230/MAP130 containing the same peptide sequence failed to do so. It is possible that differences in the specificities of the antisera are responsible for the discrepancy. Our results suggest that inhibition of the growth of *Pf* asexual blood stages may require a higher concentrations of antibodies that react with native proteins than those obtained by immunisation with the homopolymers and MAPs. In addition, antibodies may need to be directed towards different epitopes on the target proteins. The two anti-MSA2 monoclonal antibodies that react with a linear STNS epitope, bind to the native protein in an IFA, and produce 90% growth inhibition at 121–125 μg ml^{-1} [11], may illustrate the type of antibody response required for inhibiting the growth of *Pf* asexual blood stages. However it is relevant to note that additional antibody-dependent cellular mechanisms, involving monocyte/macrophages and neutrophils, promote the inactivation of blood stage *Pf* in vivo (reviewed in [19]) and that T_h1

and cytotoxic lymphocyte responses elicited by peptide immunisation against merozoites within infected hepatocytes may also conceivably protect in vivo. The in vitro invasion and growth inhibition assay could therefore underestimate the vaccine potential of the peptide constructs described here.

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References

[1] M.W. Steward, C.R. Howard, Immunol. Today 8 (1987) 51–58.
[2] R. Ramasamy, Pept. Res. 4 (1991) 210–219.
[3] D.A. Herrington, D.F. Clyde, G. Losonsky, M. Cortesia, J.R. Murphy, J. Davis, S. Baqar, A.M. Felix, E.P. Heimer, D. Gillessen, E. Nardin, R.S. Nussensweig, V. Nussensweig, M.M. Levine, Nature 328 (1987) 257–259.
[4] M.J. Blackman, H.G. Heidrich, S. Donachie, J.S. McBride, A.A. Holder, J. Exp. Med. 172 (1990) 379–382.
[5] S.P. Chang, S.E. Case, W.L. Gosnell, A. Hashimoto, K.J. Kramer, L.Q. Tam, C.Q. Hashiro, C.M. Nikaido, H.L. Gibson, C.T. Lee-Ng, P.J. Barr, B.T. Yokato, G.S.N. Hui, Infect. Immun. 64 (1996) 253–261.
[6] A. Cheung, J. Leban, A.R. Shaw, B. Merkli, J. Stocker, C. Chizzolini, C. Sander, L.H. Perrin, Proc. Natl. Acad. Sci. USA 83 (1986) 8328–8332.
[7] H.M. Etlinger, P. Capers, H. Matile, H.J. Schoenfeld, D. Stueber, B. Takacs, Infect. Immun. 59 (1991) 3498–3503.
[8] W.A. Siddiqui, L.Q. Tam, K. Kramer, G.S.N. Hui, S.E. Case, K.M. Yamaga, S.P. Chang, E.B.T. Chan, S.C. Kan, Proc. Natl. Acad. Sci. USA 84 (1987) 3014–3018.
[9] H.M. Muller, K. Fruh, A. von Brunn, F. Esposito, S. Lombardi, A. Crisanti, H. Bujard, Infect. Immun. 57 (1989) 3765–3769.
[10] E.M. Riley, S.J. Allen, J.G. Wheeler, M.J. Blackman, S. Bennett, B. Takacs, H.J. Schonfeld, A.A. Holder, B.M. Greenwood, Parasite Immunol. 14 (1992) 321–337.
[11] R.J. Epping, S.D. Goldstone, L.T. Ingram, J.A. Upcroft, R. Ramasamy, J.A. Cooper, G.R. Bushell, H.M. Geysen, Mol. Biochem. Parasitol. 28 (1988) 1–10.
[12] R. Ramasamy, G. Jones, R. Lord, Immunol. Lett. 23 (1990) 305–310.
[13] R. Ramasamy, Immunol. Cell Biol. 65 (1987) 419–424.
[14] F. Al-Yaman, B. Genton, R. Anders, J. Taraika, M. Ginney, S. Mellor, M.P. Alpers, Parasite Immunol. 17 (1995) 493–501.
[15] R. Ramasamy, D.A. Wijesundere, K. Nagendran, M.S. Ramasamy, Clin. Exp. Immunol. 99 (1995) 168–174.
[16] M.E. Patarroyo, R. Amador, P. Clavijo, A. Moreno, F. Guzman, P. Romero, R. Tascon, A. Franco, L.A. Murillo, G. Ponton, G. Trujillo, Nature 332 (1988) 158–161.
[17] P. Graves, H. Gelband, P. Garner, Parasitol. Today 14 (1998) 218–220.
[18] S.C. Gilbert, A.V.S. Hill, Parasitol. Today 14 (1998) 251.
[19] R. Ramasamy, Biochim. Biophys. Acta 1406 (1998) 10–27.
[20] D. Camus, T.J. Hadley, Science 230 (1985) 553–556.
[21] B.K. Sim L, C.E. Chitnis, K. Wasniowska, T.J. Hadley, L.H. Miller, Science 264 (1994) 1941–1944.
[22] B.K.L. Sim, Parasitol. Today 11 (1995) 213–216.
[23] J.P. Tam, Proc. Natl. Acad. Sci. USA 85 (1988) 5409–5413.
[24] F. Borras-Cuesta, Y. Fedon, A. Petit-Camurdan, Eur. J. Immunol. 18 (1988) 199–202.
[25] R.A. Houghten, Proc. Natl. Acad. Sci. USA 82 (1985) 5131–5135.
[26] J.P. Tam, W.F. Heath, R.B. Merrifield, J. Am. Chem. Soc. 105 (1983) 6442–6445.
[27] R. Ramasamy, C. Wickremaratne, Ind. J. Med. Res. 99 (1994) 21–26.
[28] R. Ramasamy, K. Nagendran, M.S. Ramasamy, Am. J. Trop. Med. Hyg. 50 (1994) 537–547.
[29] I.A. Quakyi, J. Currier, A. Fell, D.W. Taylor, T. Roberts, R.A. Houghten, R.D. England, J.A. Berzovsky, L.H. Miller, M.F. Good, J. Immunol. 153 (1994) 2082–2092.
[30] C.M. Rzepczyk, R. Ramasamy, D.A. Mutch, P.C.L. Ho, D. Battistutta, K.L. Anderson, D. Parkinson, T.J. Doran, M. Honeyman, Eur. J. Immunol. 19 (1989) 1797–1802.
[31] R. Ramasamy, R. Rajakaruna, Biochim. Biophys. Acta 1360 (1997) 241–246.
[32] F.A. Anderer, Biochim. Biophys. Acta 71 (1963) 245–248.
[33] J. Calvo-Calle, J. Hammer, F. Sinigaglia, P. Clavijo, R. Moya-Castro, E.H. Nardin, J. Immunol. 159 (1997) 1362–1373.
[34] P. Panina-Bordignon, A. Tan, A. Termijtelen, S. Demotz, G.P. Corradin, A. Lanzavecchia, Eur. J. Immunol. 19 (1989) 2237–2242.
[35] N.M. O'Brien-Simpson, N.J. Ede, L.E. Brown, J. Swan, D.C. Jackson, J. Am. Chem. Soc. 119 (1997) 1183–1188.
[36] H.M. Dintzis, R.Z. Dintzis, B. Vogelstein, Proc. Natl. Acad. Sci. USA 73 (1976) 3651–3675.
[37] J.C. Cox, A.R. Coulter, Vaccine 15 (1997) 248–256.
[38] R.F. Anders, Parasite Immunol. 8 (1986) 529–539.
[39] R. Ramasamy, H.M. Geysen, Parasite Immunol. 12 (1990) 457–471.
[40] J.A. Tainer, E.D. Getzoff, Y. Patterson, A.J. Olson, R.A. Lerner, Annu. Rev. Immunol. 3 (1985) 501–535.
[41] A.C. Satterthwait, T. Arrhenius, R.A. Hagopian, F. Zavala, R.S. Nussensweig, R.A. Lerner, Vaccine 6 (1988) 99–103.
[42] H.M. Etlinger, A. Trzeciak, Phil. Trans. R. Soc. Lond. B 340 (1993) 69–72.
[43] H.M. Etlinger, A.M. Felix, D. Gillesen, E.P. Heimer, M.

Just, R.L. Pink, F. Sinigaglia, D. Sturchler, B. Takacs, A. Trzeciak, H. Matile, J. Immunol. 140 (1988) 626–633.
[44] C. Yang, W.E. Collins, L. Xiao, A.E. Saekhou, R.C. Reed, C.O. Nelson, R.L. Hunter, D.L. Jue, S. Fang, R.M. Wohlhuetter, V. Udhayakumar, A.A. Lal, Vaccine 15 (1997) 377–386.
[45] D.Y. Munesinghe, P. Clavijo, M.C. Calle, R.S. Nussensweig, E. Nardin, Eur. J. Immunol. 21 (1991) 3015–3020.
[46] T. Dyrberg, M.B.A. Oldstone, J. Exp. Med. 164 (1986) 1344–1349.
[47] M. Salcedo, L. Barreto, M. Rojas, M. Moya, J. Cote, M.E. Patarroyo, Clin. Exp. Immunol. 84 (1991) 122–128.