PEPTIDES DERIVED FROM A MALARIA PARASITE SURFACE ANTIGEN - EFFECT OF ADJUVANTS AND NUMBER OF IMMUNIZATIONS ON THE TOTAL ANTIBODY RESPONSE IN MICE

R. RAMASAMY AND C. WICKREMARATNE

Malaria Laboratory, Division of Life Sciences, Institute of Fundamental Studies, Hantana Road, Kandy.

Abstract: Peptides corresponding to known B cell epitopes in a 45kDa merozoite surface glycoprotein of the human malaria parasite **Plasmodium falciparum** were conjugated via 6-maleimido caproic acyl N-succinimide ester to bovine serum albumin. The conjugates were used to immunize Balb/c mice in saline and with the following adjuvants: Freunds adjuvant, the muramyl dipeptide derivatives murametide and murabutide, and aluminium hydroxide. Three injections of antigen were given intra-muscularly at 21d intervals and sera obtained after each injection. Antibody levels against peptides were measured by ELISA. Freunds adjuvant produced the highest titre of antibodies (up to 10^{-5} after three injections). Antibody levels obtained with alum absorbed antigen reached titres of 10^{-3} - 10^{-4} while antigen administered in saline alone yielded titres of 10^{-3}. The soluble MDP derivatives did not produce higher antibody levels than antigen administered in saline. The results suggest that three injections of peptide-carrier conjugates in alum or saline may be an acceptable procedure for raising sufficiently high titre anti-peptide antibodies in animals and man.

Key words: Adjuvant, Immunization, Malaria, Merozoite surface antigen, **Plasmodium falciparum**, Vaccine.

INTRODUCTION

Antibodies produced against peptides are used in biological research for a variety of purposes e.g. as probes for determining protein structure, in immunodiagnosis, in identifying protein corresponding to open reading frames in nucleic acid sequences etc. Since anti-peptide antibodies are able to react specifically with the corresponding native protein in many instances,[1-3] immunization with chemically synthesized peptides is also being developed as a method of vaccinating against infectious diseases.[2-4] Peptides are often poorly immunogenic and need to be conjugated to carrier proteins or otherwise suitably modified in order to elicit antibodies.[4] An adjuvant is also regarded as being needed to augment the immune response to peptide-carrier conjugates.[5] The combination of an emulsion of mineral oil and killed mycobacteria known as Freunds complete adjuvant (FCA) is one of the most potent adjuvants known for stimulating cellular immunity and antibody production. However, it is toxic and produces lesions at the site of injection and is therefore considered to be unsuitable for human use and is not preferred for use with animals. The active component for adjuvant action in mycobacteria has been characterized as N-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP).[6] MDP

possesses pyrogenic activity. However, from screening a large number of MDP derivatives, two compounds, murametide (N-acetylmuramyl-L-Ala-D-Gln-OCH₃) MUM and murabutide (N-acetylmuramyl-L-Ala-D-Gln-n-butyl ester) MBU that lack pyrogenic activity, and therefore are candidate adjuvants for human use, were identified.[5]

Aluminium salts (alum) have been widely used in traditional human and veterinary vaccines as adjuvants.[7] Alum has also been used with a recombinant surface protein to induce immunity in man against hepatitis B.[8] Peptide-based vaccines from protein antigens of human malaria parasites are reported to induce partial protection in man when used with alum as an adjuvant.[9,10] In these studies with malaria peptides, the antibody levels developed were reported to be modest (titres of 1/20-1/400) and cell-mediated responses relatively poor. Conditions for the development of optimal immune responses to peptide antigens therefore require further investigation. A 45 kDa surface membrane glycoprotein on merozoites of the human malaria parasite *Plasmodium falciparum*[11,12] is one of the candidate molecules for developing a malaria vaccine, since antibodies against defined epitopes of the antigen inhibit parasite growth and reinvasion in cultures.[13,14] We have used two peptides derived from the sequence of this antigen in the FC 27 strain of *P. falciparum*[12] to investigate the effects of FCA, alum, MUM and MBU and the number of immunizations on antibody production in mice.

METHODS AND MATERIALS

Peptides

The sequences of synthetic peptides derived from the malaria parasite surface glycoprotein,[11,12] in the single letter code for amino acids, were as follows :

Peptide	Residues	Sequence
P102	122-138	TAADTPTATESISPSPPC
P103	207-226	RNNHPQNTSDSQKECTDGNK

P102 contains a B cell epitope that is present in one of the allelic forms of the 45kDa antigen and is the target of an inhibitory monoclonal antibody.[14] P103 is a relatively hydrophillic sequence that on independent structural analysis was shown to contain predicted B and T cell epitopes. Both P102 and P103 are recognized by antibodies present in up to 60% of the sera of persons living in a *P. falciparum* endemic area of Sri Lanka (submitted for publication).

A cysteine was incorporated into the C-terminus of P102 for coupling, while P103 contained a naturally occurring cysteine in position 221. Peptides were synthesized by

standard t-Boc solid phase chemistry as modified for multiple synthesis.[15] They were analysed and purified by reverse phase high performance liquid chromatography (HPLC) using a C18 column and a Shimadzu 8A liquid chromatograph (Shimadzu, Tokyo). A gradient of acetonitrile containing 0.1% trifluoroacetic acid was used for elution. The eluted peptides were lyophilised and stored desiccated at -20^{0}C until use. The HPLC profiles of peptide 102 and peptide 103 and the fraction used in the experiments are shown in Figure 1 and Figure 2 respectively. The amino acid composition, determined after complete hydrolysis of the peptides, using the Waters Picotag system (Waters, Bedford, MA), was consistent with the expected sequence of the peptides.
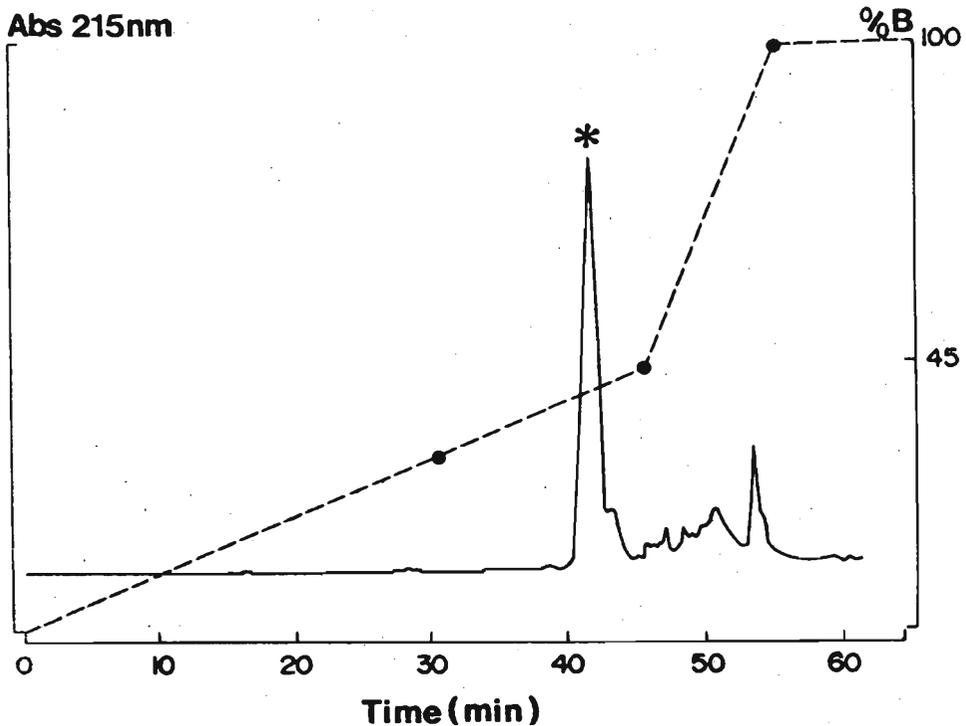

Figure 1: HPLC separation of peptide 102. The gradient of acetonitrile (solvent B) employed is indicated by the broken line. Absorbtion was measured at 215nm and is given in arbitrary units. Elution was performed at 10ml min^{-1}. The peak containing peptide is indicated by an asterisk.

Preparation of peptide carrier conjugates

The peptides were covalently linked to bovine serum albumin using 6-maleimido caproic acyl N-succinimide ester (MCS) essentially as described previously.[16] Briefly, to a solution of 5mg bovine serum albumin (BSA) in 0.45ml of 0.01M sodium phosphate buffer, pH 7.5, 0.05ml of 50mM MCS in dry dimethylformamide was added
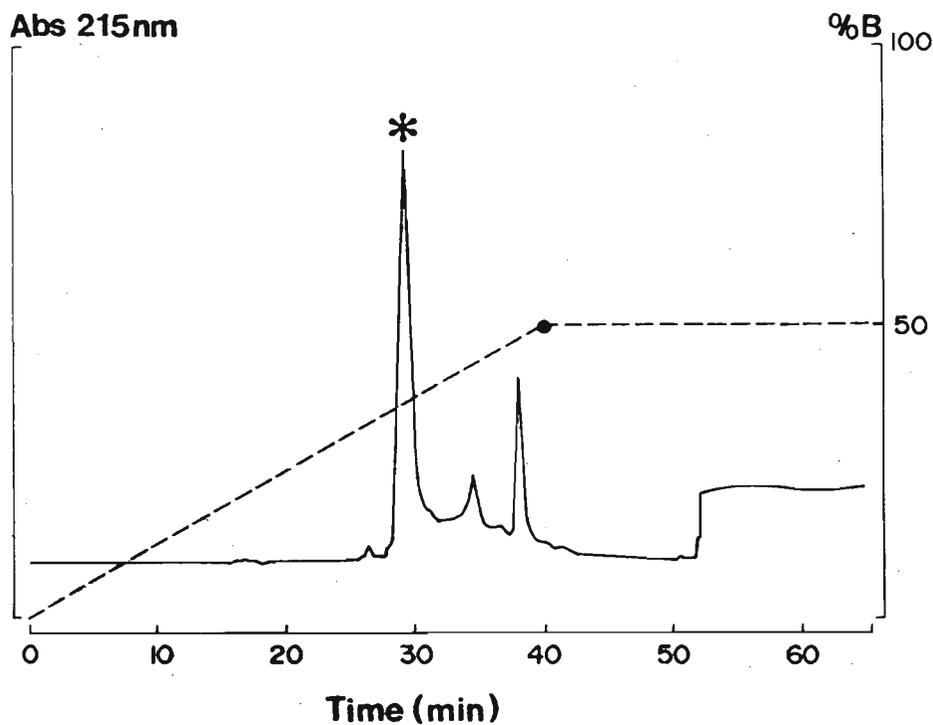
Figure 2: HPLC separation of peptide 103. The gradient of acetonitrile (solvent B) is indicated by the broken line. Absorbtion was measured at 215nm and is given in arbitary units. Elution was performed at 10ml min^{-1}. The peak containing peptide is indicated by an asterisk.

and the mixture gently shaken for 30min at 26^0C. 0.05ml of the MCS solution was again added and the incubation continued for a further 30min. The maleimidated protein was then dialysed against 0.1M sodium phosphate pH 6.5. 5mg of peptide was dissolved in 0.5ml of 0.1M sodium phosphate, pH 6.5, 1mM EDTA and added to 0.5ml of the maleimidated serum albumin. The mixture was equilibrated with nitrogen, sealed in a nitrogen atmosphere and incubated for 18h at 26^0C. The peptide-carrier conjugate was then dialysed against several changes of 0.01M phosphate buffered saline pH 7.2 (PBS).

The conjugation ratio determined by comparison of the M_r of the peptide-carrier conjugate with maleimidated carrier by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Production of antisera

Groups of 4 nine month old Balb/c mice were injected, for primary and boosting immunizations, with peptide - carrier conjugate equivalent to $50\mu g$ of peptide per

mouse intra-muscularly at two sites. Female mice were used for immunization with P102 and PBS control and male mice for immunization with P103. (a) For FCA immunization, the first injection was given in FCA followed by incomplete Freunds adjuvant for the boosting injections. (b) For immunization with the two MDP derivatives, 100μg of MBU and MUM in PBS were mixed with the conjugates for the primary and boosting injections. (c) For immunization with alum, aluminium hydroxide precipitate was washed in PBS and the conjugate absorbed onto it for injection. Both primary and boosting injections contained alum. (d) For immunization without adjuvant, mice were injected with the conjugate in PBS.

Boosting injections were given on day 21 and day 42 and the mice tail bled for sera 10d after each injection. After the third injection the mice were sacrificed and bled out. Pre-immune sera were also collected before commencing the immunizations.

Quantitation of antibodies

Anti-peptide antibody levels were determined by enzyme linked immunosorbent assay (ELISA) using peptides as antigens. Costar 96 well microtitre plates (Biorad, Richmond, CA) were coated with 0.05 ml of a 0.01mg ml^{-1} solution of peptide in PBS overnight at 37^0C. Unreacted absorption sites in the wells were blocked with a 5% wt vol^{-1} solution of non-fat milk powder (Blotto) for 2h at 26^0C. The wells were washed and then incubated with 0.05ml of tenfold serial dilutions of individual mouse sera for 2h at 26^0C. After washing four time in PBS, the wells were treated with 0.05ml of a 1:2000 dilution of sheep anti-mouse IgG - horse radish peroxidase conjugate with H and L chain specificity (Silenius, Melbourne, Australia) in Blotto for 1h. This reagent detects all murine immunoglobulin isotypes. The wells were then washed and the colour reaction developed with 2,2' -azino -bis (3-ethyl benz thiazoline - 6- sulphonic acid) and H_2O_2 for 30min. Optical density at 405nm was then measured in a Multiskan Plus II ELISA reader (Flow labs, Irvine, Scotland).

RESULTS

Molecular weight determination by SDS-PAGE analysis demonstrated that 12 mols of P102 and 16 of P103 were present per mol of BSA in the two conjugates (data not shown). The individual mice within a group yielded antisera with similar reactivity and specificity in the ELISA. Hence the mean absorption at 405nm obtained with sera within a group are presented below.

Anti-P103 antibodies raised with alum as adjuvant specifically reacted with P103 compared to P102 (Figure 3) and vice versa (data not shown). Similar specificity was observed with antisera produced using the other adjuvants and without adjuvant and
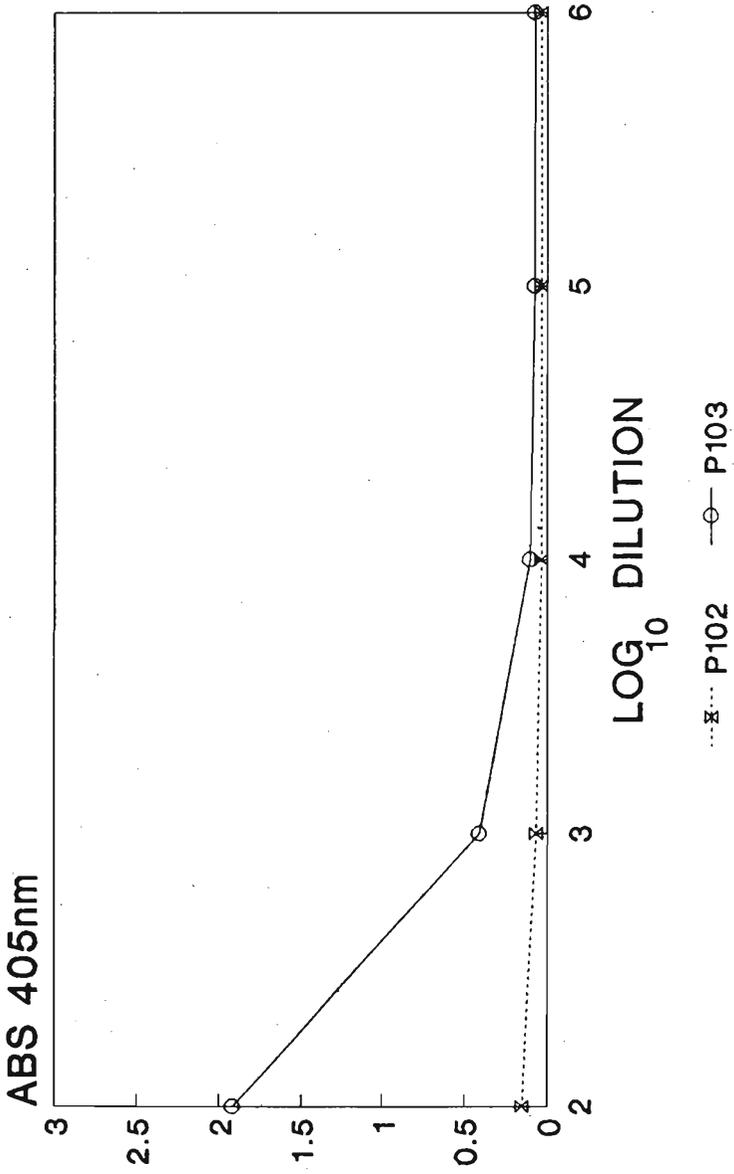
ABS 405nm

Figure 3: Reactivity of anti-P103 (Alum adjuvant) with P102 & P103.
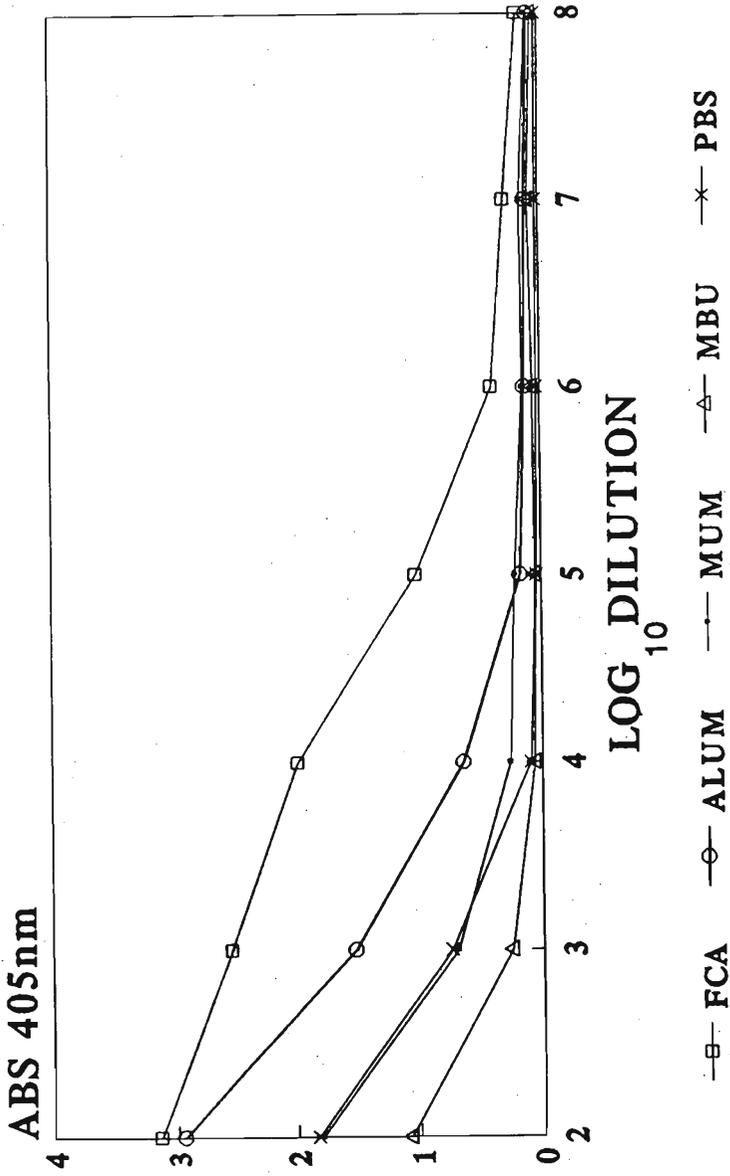
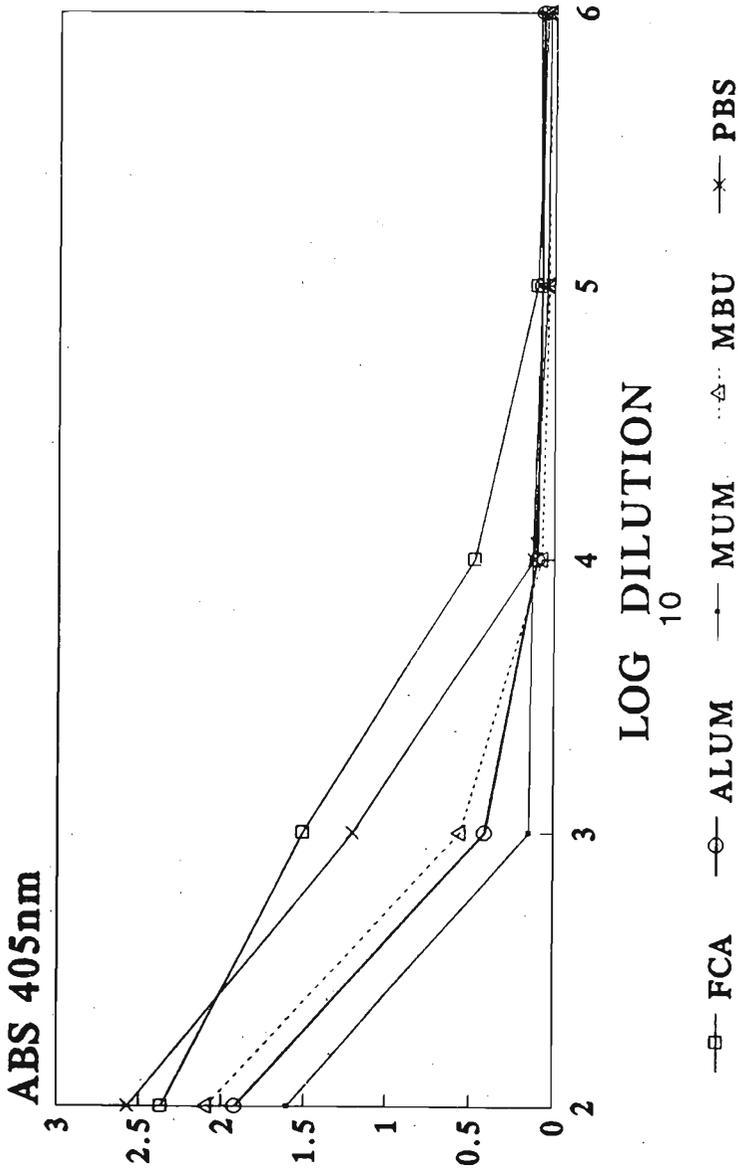**Figure 4:** Comparison of adjuvants in the anti-P102 response:bleed 3.

Figure 5: Comparison of adjuvants in the anti-P103 response:bleed 3.

when sera were tested against peptides conjugated to an unrelated carrier protein, keyhole limpet haemocyanin, (KLH), through glutaraldehyde.

Highest titres of anti-peptide antibodies were obtained with Freunds adjuvant when the sera obtained after three injections with P102-BSA (Figure 4) and P103-BSA (Figure 5) were tested by ELISA. While antibody titres of 10^{-4}-10^{-5} were observed with Freunds adjuvant, titres of 10^{-3}-10^{-4} were seen when alum was used as an adjuvant and 10^{-3} after immunization without adjuvant. MBU and MUM did not significantly increase antibody production when compared to injections given without adjuvant.

Three injections were generally necessary to obtain significant antibody production with P102-BSA and P103-BSA when Freunds adjuvant was not used since the antibody responses after two injections in such cases were weak (Figures 6 & 7). However, appreciable antibody response was detected with P103 after two injections when mice were immunized with P103-BSA in PBS only (Figure 7). A comparative analysis of the pre-immune sera and sera obtained after each injection of P102-BSA with alum as adjuvant clearly showed that three injections were needed for significant antibody production (Figure 8). Similar results were obtained with the corresponding anti-P103 sera.

DISCUSSION

The results show that immunization with Freunds complete adjuvant followed by Freunds incomplete adjuvant for subsequent injections gave the best result with peptide-BSA conjugates for anti-peptide antibody production. However, after three injections, the antibody response to injections of antigen without adjuvant and with alum as adjuvant were sufficiently strong to yield antibody titres of 10^{-3} - 10^{-4}. Similar results were obtained with two additional peptides conjugated to BSA (unpublished observations). Thus immunization without adjuvant or with alum as an adjuvant for peptide-carrier conjugates may be sufficient for many purposes and has the advantage that it avoids the use of an adjuvant that is toxic and produces locally severe lesions.

MBU and MUM did not show significant advantage as adjuvants in the present study. This may be because MBU and MUM, as is the case with MDP [5] are cleared rapidly from blood. Hence delivery in an aqueous medium [17] may be less effective than when MDP or its derivatives are given as an emulsion with mineral oil and antigen. [6] The covalent linkage of MBU and MUM to the peptide-carrier conjugate may be an alternative way of enhancing adjuvant action since this has been shown to be effective with MDP. [18]
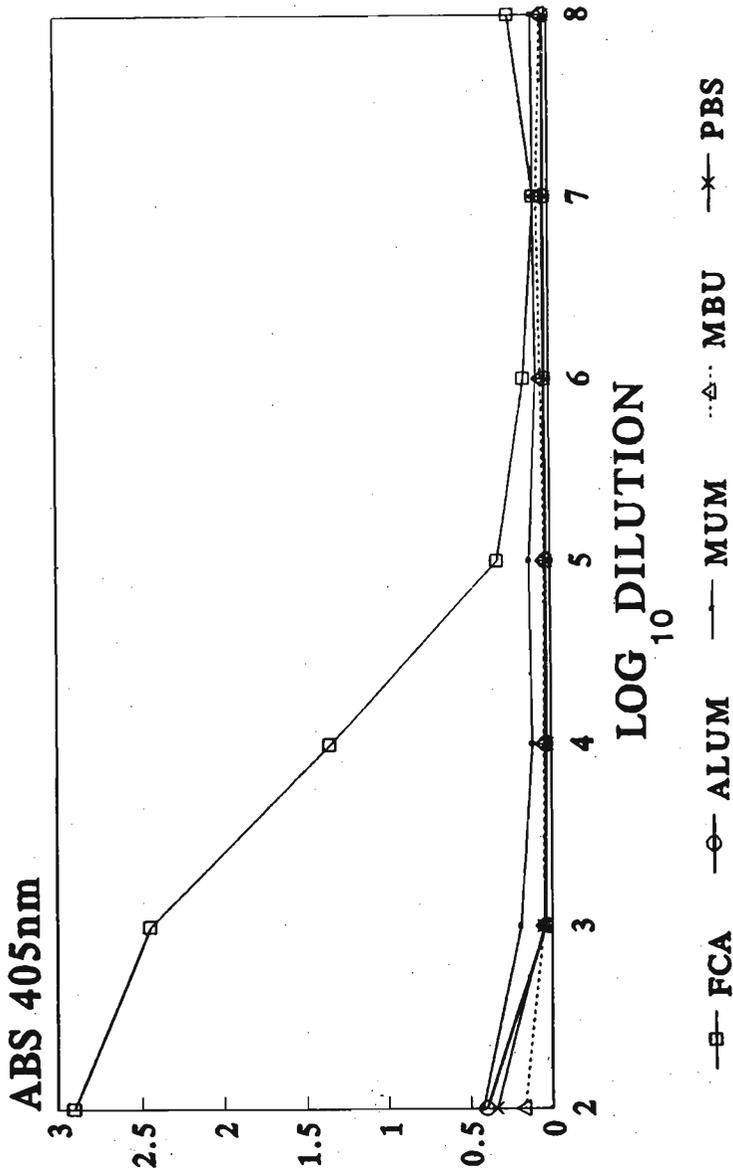
ABS 405nm
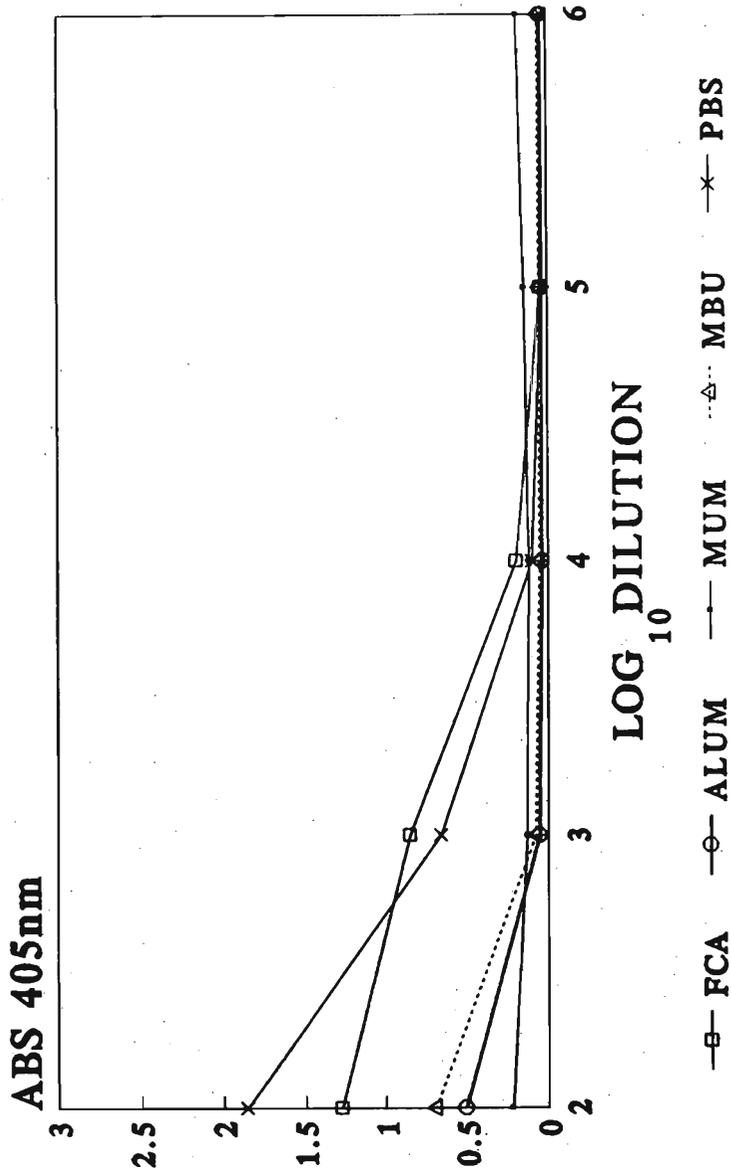
Figure 6: Comparison of adjuvants in the anti-P102 response:bleed 2.

─□─ FCA   ─◇─ ALUM   ── MUM   ···△··· MBU   ─✳─ PBS

Figure 7: Comparison of adjuvants in the anti-P103 response:bleed 2.

ABS 405nm
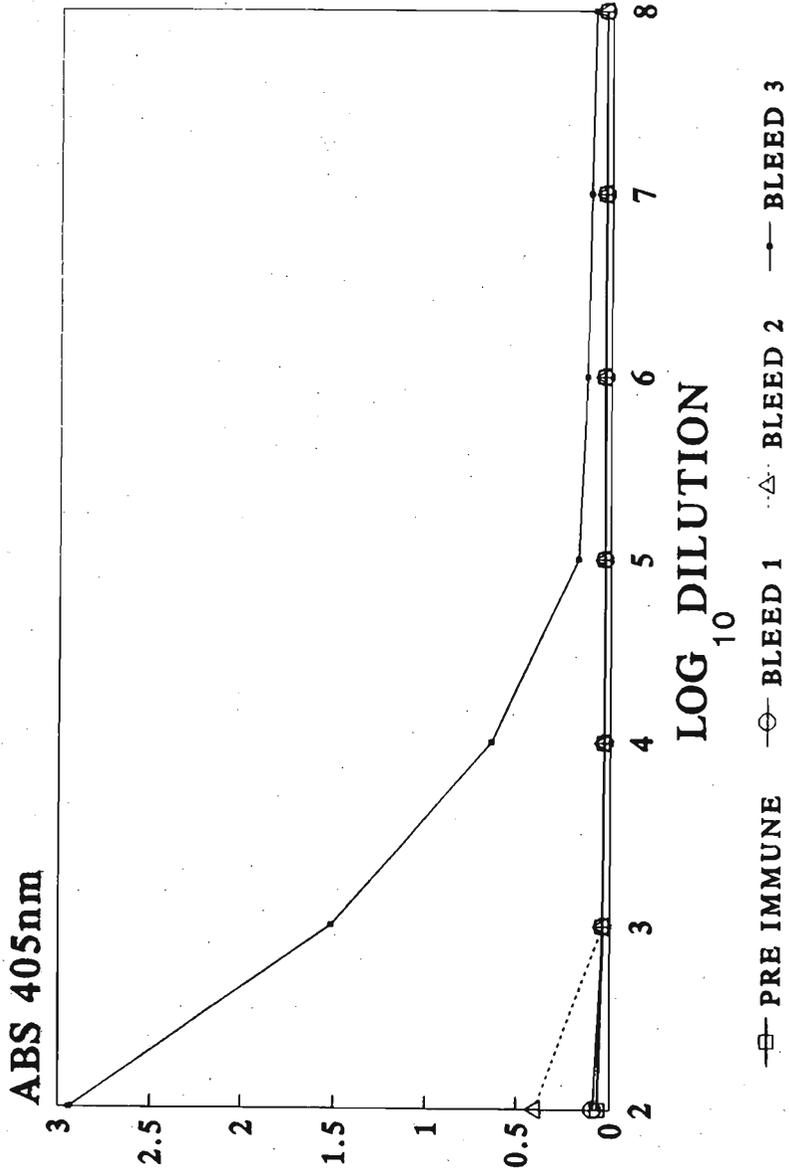
Figure 8: Antibody response to P102 with alum.

PRE IMMUNE   BLEED 1   BLEED 2   BLEED 3

LOG DILUTION

Immunofluorescence studies with acetone/methanol fixed asexual blood stages of *P.falciparum* showed that anti-P102 but not anti-P103 sera reacted with the merozoite surface (unpublished observations). This indicates that the peptide sequence and its location within GYMSSA may be important in eliciting antibodies reactive with native GYMSSA.

In general, the antibody responses to P102 and P103 induced by the different immunization schedules were similar except that P103 induced a greater antibody response when injected in PBS than in alum. The reason for this discrepancy is not clear, but it may be related to the presence of a T-cell activating determinant in P103[19] but not P102. Cellular as well as humoral immunity has been shown to act against asexual blood stage malaria parasites in man.[20] No attempt was made to measure T cell responsivenesss in the present investigation. Immunization with alum is generally considered to induce poor T cell immunity.[7] However, significant T-cell responsiveness was observed in mice immunized in alum with a herpes simplex virus glycoprotein derived peptide conjugated to ovalbumin.[21] Different immunoglobulin isotypes that are reported to be selectively induced by different adjuvants,[5] and which may have different effector functions in immunity, were also not measured in the present investigation. Immunization with peptide vaccines derived from the sporozoite[9] and asexual blood stage[10] *P. falciparum* proteins, in alum as an adjuvant, produced immunofluorescence and ELISA antibody titres of 1/20-1/400 in human sera. The poor antibody response to the sporozoite protein was attributed to previous exposure to the tetanus toxoid carrier inhibiting the antibody response,[9] a process which may be overcome with further antigen injections.[22] Antibody titres observed in our study are comparable to the serum antibody levels against a 185-200 kDa merozoite surface protein that completely protect owl monkeys against *P. falciparum* malaria (immunofluorescence titres of 10^{-3}-10^{-4}).[23] Higher ELISA antibody titres (10^{-5}) against sporozoite antigens have been produced in mice using alum absorbed liposomes containing lipid A and peptide-carrier.[24] The toxic effects of lipid A and liposomes however need to be further investigated before human use.[5] Our results show that useful antibody levels against peptides can be produced in mice, and potentially therefore in humans, after three injections of peptide-carrier conjugates in PBS or aluminium hydroxide, an adjuvant commonly used in man and domestic animals.

References

1. Anderer F.A. (1963). Preparation and properties of an artificial antigen immunologically related to tobacco mosaic virus. *Biochimica Biophysica Acta* **71**: 245-248.

2. Arnon R. (1986). Peptides as immunogens: prospects for a synthetic vaccine. *Current topics in Microbiology and Immunology* **130**: 1-12.

3. Lerne R.A. (1982). Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* **299**: 592-596.

4. Ramasamy R. (1991). Synthetic peptides in malaria research. *Peptide Research* **4**: 210-219.

5. Warren H.S., Vogel F.R. & Chedid L.A. (1986). Current status of immunological adjuvants. *Annual Reviews of Biochemistry* **4**: 369-388.

6. Ellousz F., Adam A., Ciorbarn R. & Lederer E. (1974). Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochemical Biophysical Research Communications* **59**: 1317-1325.

7. Nicklas W. (1992). Aluminium salts. *Research in Immunology* **143**: 489-494.

8. Scolnick E.M., McLean A.A., West D.J., McAleer W.J., Miller W.J. & Buynak, E.B. (1984). Clinical evaluation in healthy adults of a hepatitis B vaccine made by recombinant DNA. *Journal of the American Medical Association* **251**: 2812-2815.

9. Herrington D.A., Clyde D.F., Losonsky G., Cortesia M., Murphy J.R., Davis J., Bagar S., Felix A.M., Heimer E.P., Gillessen D., Nardin E., Nussensweig R.S., Nussensweig V. & Levine M.M. (1987). Safety and immunogenicity of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature* **328**: 257-259.

10. Pataroyo M.E., Amador R., Clavijo P., Moreno A., Guzman F., Romero P., Tascon R., Franco A., Murillo L.A., Ponton G. & Trujillo G. (1988). A synthetic vaccine protects humans against challenge with asexual stages of *Plasmodium falciparum* malaria. *Nature* **332**: 158-161.

11. Ramasamy R. (1987). Studies on glycoproteins in the human malaria parasite *Plasmodium falciparum*. Identification of a myristilated 45kDa merozoite membrane glycoprotein. *Immunology and Cell Biology* **65**: 419-424.

12. Smythe J.A., Coppel R.L., Brown G.V., Ramasamy R., Kemp D.J. & Anders R.F. (1988). Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences, USA* **85**: 5195-5199.

13. Ramasamy R. & Geysen H.M. (1990). Novel cross-reactive epitopes on asexual blood stage antigens of *Plasmodium falciparum*. *Parasite Immunology* **12**: 457-471.

14. Ramasamy R., Jones G. & Lord R. (1990). Characterization of an inhibitory monoclonal antibody defined epitope on a malaria vaccine candidate antigen. *Immunology Letters* **23**: 305-310.

15. Houghten R.A. (1985). General method for the rapid solid phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proceedings of the National Academy of Sciences, USA* **82**: 5131-5135.

16. Lee A.C.J., Powell J.E., Tregear G.W., Niall H.D. & Stevens V.C. (1980). A method for preparing -hCG COOH peptide carrier conjugates of predictable composition. *Molecular Immunology* **17**: 749-756.

17. Clough E.R., Audibert F.M., Barnwell J.W., Schlesinger D.H., Arnon R. & Chedid L.A. (1985). Biologically active antibodies elicited by a synthetic circumsporozoite peptide of *Plasmodium knowlesi* administered in saline with a muramyl dipeptide derivative. *Infection and Immunity*. **48**: 839-842.

18. Audibert F., Jolivet M., Chedid L., Arnon R. & Sela M. (1982). Successful immunization with a totally synthetic diphtheria vaccine. *Proceedings of the National Academy of Sciences, USA* **79**: 5042-5046.

19. Rzepczyck C.M., Ramasamy R., Mutch D.A., Ho P.C.L., Battistutta D., Anderson K.L., Parkinson D., Doran T.J. & Honeyman M. (1989). Analysis of human T cell response to two *Plasmodium falciparum* merozoite surface antigens. *European Journal of Immunology* **19**: 1797-1802.

20. Ramasamy, R. and Ramasamy, M.S. (1989). Progress towards a molecular vaccine against malaria. *Indian Journal of Malariology* **26**: 127-148.

21. Geerligs H.J., Weijer W.J., Welling G.W. & Welling-Wester S. (1989). The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus type 1 glycoprotein D. *Journal of Immunological Methods* **124**: 95-102.

22. Etlinger H.M. & Altenburger W. (1991). Overcoming inhibition of antibody responses to a malaria recombinant vaccinia virus caused by prior exposure to wild type virus. *Vaccine* **9**: 470-472.

23. Siddiqui W.A., Tam L.Q., Kramer K.J., Hui G.S.N., Case S.E., Yamaga K.M., Chang S.P., Chan E.B.T. & Kan S.C. (1987). Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences, USA* **84**: 3014-3018.

24. Richards R.L. Hayre M.D., Hockmeyer W.T. & Alving C.R. (1988). Liposomes, lipid A and aluminium hydroxide enhance the immune response to a synthetic malaria sporozoite antigen. *Infection and Immunity* **56**: 682-686.