Immunogenicity of Multiple Antigen Peptides Containing *Plasmodium vivax* CS Epitopes in Balb/c Mice

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Multiple antigen peptide systems (MAPs) allow the incorporation of various epitopes in to a single synthetic peptide immunogen. We have characterized the immune response of BALB/c mice to a series of MAPs assembled with different B and T cell epitopes derived from the Plasmodium vivax *circumsporozoite (CS) protein. A B-cell epitope from the central repeat domain and two T-cell epitopes from the amino and carboxyl flanking regions were used to assembled eight different MAPs. An additional universal T cell epitope (ptt-30) from tetanus toxin protein was included. Immunogenicity in terms of antibody responses and in vitro T lymphocyte proliferation was evaluated. MAPs containing B and T cell epitopes induced high titers of anti-peptides antibodies, which recognized the native protein on sporozoites as determined by IFAT. The antibody specificity was also determined by a competitive inhibition assay with different MAPs. A MAP containing the B cell epitope (p11) and the universal epitope ptt-30 together with another composed of p11 and the promiscuous T cell epitope (p25) proved to be the most immunogenic. The strong antibody response and specificity for the cognate protein indicates that further studies designed to assess the potential of these proteins as human malaria vaccine candidates are warranted.*

Key words: malaria - *Plasmodium .vivax* - vaccines - synthetic peptides

Repeated immunization with radiation-attenuated malaria sporozoites protects animals (Vanderberg et al. 1969) and humans (Clyde et al. 1975) against challenge with normal sporozoites. This immunization procedure induces a strong antibody response mainly directed against the central repeat domain of the CS proteins. T cell responses are induced by several epitopes located throughout the CS protein (Nardin & Nussenweig 1993). Passive transfer of monoclonal antibodies specific to the *Plasmodium berghei* CS protein have partially or totally protected mice against sporozoite challenge (Potocnjak et al. 1980, Egan et al. 1987) and monoclonal antibodies against the repeat regions of both *P. falciparum* and *P. vivax* were able to neutralize and abolish their infectivity *in vitro* (Hollingdale et al. 1982). In addition, complete protection was also obtained by passive transfer of murine CS-specific cytotoxic lymphocyte (CTL) clones (Kumar et al. 1988, Romero et al. 1989), whereas T-cell depletion of mice rendered the animals completely susceptible to sporozoite challenge (Weiss et al. 1988). Because of this evidence for the possibility to prevent malaria infection by sporozoite immunization, efforts have focused on the development of a sporozoite-based vaccine for humans. Several vaccine trials have been carried out in human volunteers using both recombinant and synthetic peptides based on an immunogenic B cell epitope located in the CS repeat domain. These trials however were shown to confer limited protection (Ballou et al. 1987, Herrington et al. 1987).

The recent development of the Multiple Antigen Peptide systems (MAPs) allows the incorporation of multiple B and T cell epitopes in a single immunogenic macromolecule (Tam 1988). MAPs containing multiple copies of the immunodominant B-cell epitope plus a T-helper cell (Th) epitope or the Th epitope alone, all from the *P. berghei* CS protein have recently shown the capacity to induce high titers of anti-sporozoite antibodies and to significantly protect immunized mice (Tam et al. 1990,

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Migliorini et al. 1993). Although the role played by these antibodies seems to be important, antibodies against other regions of the CS protein (Franke et al. 1992) as well as efficient helper and CTL epitopes could be required to induce functional protective responses *in vivo*. In the search for such protective human B and T-cell epitopes, the responses of immune individuals to the *P.vivax* CS protein have been studied (Herrera et al. 1992).

Herein we assessed the immunogenicity of a series of MAPs containing B and/or T-cell epitopes of the *P. vivax* CS protein. Antibody responses to each synthetic MAP and to the native protein as well as *in vitro* T cell proliferation were evaluated in BALB/c mice.

MATERIALS AND METHODS

SYNTHETIC PEPTIDES

Eight tetra-branched MAPs were synthesized on a core of three lysines separated by glycine residues as described elsewhere (Tam 1988). Each of the MAPs contained branches based on dimer or trimer of T and B cell epitopes (group B and T) or combinations of thereof (group B+T) that have been previously shown to correspond to epitopes recognized by human T cells (Herrera et al. 1992) or immune sera (Herrera et al. in preparation). Peptide 11 (p11) is located within the central B-cell domain and was included as a B-cell epitope in these MAPs. Two other sequences that map either the amino (p6) or the carboxyl (p25) flanking regions were included as T-cell epitopes. An additional T-cell epitope (ptt-30) derived from the tetanus toxin (Panina-Bordignon et al. 1989) was included in some of the MAPs (Table I). The same linear epitopes synthesized by the F-moc solid phase technique (Atherton et al. 1981) were used for the *in vitro* tests.

Mice immunization - Six-week-old BALB/c mice (3 mice/group) were immunized s.c. at the base of the tail with 50 µg/ml of each MAP diluted in PBS and emulsified in CFA on days 0 and 20. Mice were bled and sera were collected on day 10 after the second immunization and stored frozen at -70°C until use.

Antibody response - The humoral immune response to the MAPs was measured by an ELISA test. Flexible 96-well microtiter plates (Falcon-3911) were coated with different concentrations of each MAP diluted in PBS and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS

TABLE I

Composition of the Multiple Antigen Peptides (MAPs). Group B compose of MAP I and II containg a monoepitopic B cell epitope p11. Group T compose of MAP III - V are mono or diepitopic based on T cell epitopes p6, p25 and p-tt30. Group B+T, MAPs VI, VII and VIII assembled with combinations of the all B and T cell epitopes (p6, p11, p25 and ptt30)

Epitope group	MAP code	construct
B	I	p11 2x
	II	p11 3x
T	III	p25(1x)
	IV	ptt-30(1x)
	V	p25 (1x)-p6(1x)
B+T	VI	p11 (3x)-p25(1x)
	VII	p11 (3x)-p30(1x)
	VIII	p11 (3x)-p25(1x)-p6(1x)

for 1 hour at 25°C. After several washes, serial dilutions of test and control sera in PBS-1% BSA-0.05% Tween 20 were added and incubated once more for 1 hour at room temperature. Each serum was tested against all the MAPs. Plates were further incubated in the presence of horseradish peroxidase-conjugated goat antimouse IgG (Sigma, St. Louis MO) for 1 hour at 25°C. After washing, 100 µl of O-phenylenediamine (400 ug/1 ml citrate buffer pH 5.0) substrate containing 0.05% H_2O_2 were added and incubated for 1 h. Absorbance was read at 492 nm in a Titertek Multiskan reader (Flow Laboratories, McLean, VA). Specificity of the antibody response was evaluated by a competitive inhibition assay (Shi et al. 1993). The 50% binding point dilution was established with a pool of sera from mice immunized with MAP VII. The pool was diluted 1/10,000 and adsorbed with several concentrations (0.001, 0.1, 1, 10 and 20 µg/ml) of the related MAPs II, III, IV and witn an unrelated MAP made from *P. falciparum* MSP-1 epitopes. The ELISA test was completed as described above.

The presence of anti-sporozoite antibodies was determined by an indirect immunofluorescence assay (IFAT) as previously described (Ballou et al. 1987). Air-dried *P.vivax* sporozoites containing the classical repeat sequence of the CS protein (Arnot et al. 1985) were incubated with two-fold dilutions

of mouse sera and antibody titers were determined with an fluorescein isothiocyanate (FITC) conjugate anti-mouse IgG (Sigma, St. Louis MO). Titers are expressed as the reciprocal of the last serum dilution showing positive fluorescence.

Lymphocyte Proliferation Assay - Inguinal and periaortic lymph node cells (LN) were obtained prior to immunization or 20 days after the booster dose and an *in vitro* T cell proliferation test was performed as described elsewhere (Herrera et al. 1992).

RESULTS

ANTIBODY RESPONSES

MAPs I and II (group B) that contained only the B cell epitope (p11) assembled as a dimer or trimer were not immunogenic. Sera from animals immunized with these two MAPs did not recognize the homologous peptide nor any other MAP construct containing the same epitopes, however they were able to induce *in vitro* T-cell proliferative responses. Although MAPs III-V (group T) based on individual or combined T cell epitopes were not expected to induce antibody production, all three constructs were recognized by the sera of mice immunized with the corresponding molecules or with MAPs containing the same epitopes, except for MAP IV. Mice immunized with MAPs VI-VIII (group B+T) were the most immunogenic. All sera of this group reacted with their corresponding MAP as well as with the others containing the same epitopes. Surprisingly, sera of animals immunized with MAP VI composed of p11-p25 were able to recognized MAP VII containing of p11-p30 whereas they did not recognize other MAPs that contained these two epitopes, i.e., MAPs I, II, IV (Table II).

Antibody titers determined for all positive sera are shown in Table III. Titers ranged from 1/100 to 1/80,000. Sera from all animals immunized with MAPs included in groups B and B+T were able to recognize the homologous synthetic molecule yet only antisera to MAPs VI-VIII reacted with the native CS protein by IFAT. Sera from mice immunized with MAP VII showed the highest antibody titer by ELISA and the best recognition of the cognate antigen. The competition assays performed with this serum indicated that although this construct elicited mainly specific antibodies, the cross

TABLE II

Presence of antibodies in sera of BALB/c mice immunized with MAPs by ELISA technique. Serum were diluted 1/100 and tested in microtiter plates coated with the different MAPs. Samples were considered positive when absorbance was above the mean of pre-immunization OD_{492} plus 3 SD.[a] Negative sera were used to determine the cutoff value: 0.212

Epitope Group	Immunogen MAP	OD_{492} BALB/c mice immunized with MAPs							
		Coating MAPs							
		I p11 2x	II p11 3x	III p25	IV p30	V p25-6	VI p11-25	VII p11-30	VIII p11-25-6
B	I	0.080	0.128	0.103	0.155	0.082	0.125	0.180	0.107
	II	0.148	0.156	0.163	0.107	0.095	0.131	0.105	0.115
T	III	0.075	0.097	**0.541**	0.122	**0.256**	0.100	0.065	0.063
	IV	0.098	0.087	0.145	**>2.0**	0.080	0.094	0.039	0.078
	V	0.044	0.095	**>2.0**	0.120	**>2.0**	**>2.0**	0.039	**>2.0**
B+T	VI	0.106	0.065	**1.206**	0.156	0.149	**>2.0**	**0.500**	**0.946**
	VII	0.179	**1.742**	**1.168**	**>2.0**	0.191	**>2.0**	**>2.0**	**>2.0**
	VIII	0.061	0.131	**>2.0**	0.073	**>2.0**	**0.707**	**1.250**	**>2.0**
	Control[a]	0.041	0.167	0.082	0.131	0.094	0.134	0.167	0.153

TABLE III

Titer and specificity of IgG antibody by ELISA and IFAT test in mouse sera. Values are the reciprocal of the highest positive antibody dilution. The ELISA titers are expressed as the reciprocal of the last positive deletion $x10^{-3}$

Epitope group	Immunogen MAP	Antibody titer in immunized mice: Coating MAPs I	II	III	IV	V	VI	VII	VIII	IFAT titer
B	I	0	0	0	0	0	0	0	0	0
	II	0	0	0	0	0	0	0	0	0
T	III	0	0	0.5	0	0.1	0	0	0	0
	IV	0	0	0	40	0	0	0	0	0
	V	0	0	40	0	40	40	0	40	0
B+T	VI	0	0	10	0	0	40	0.5	5	80
	VII	0	20	10	40	0	40	80	40	1260
	VIII	0	0	40	0	40	1	10	40	10

reaction observed with other MAPs was absorbed with those molecules (Fig 1).

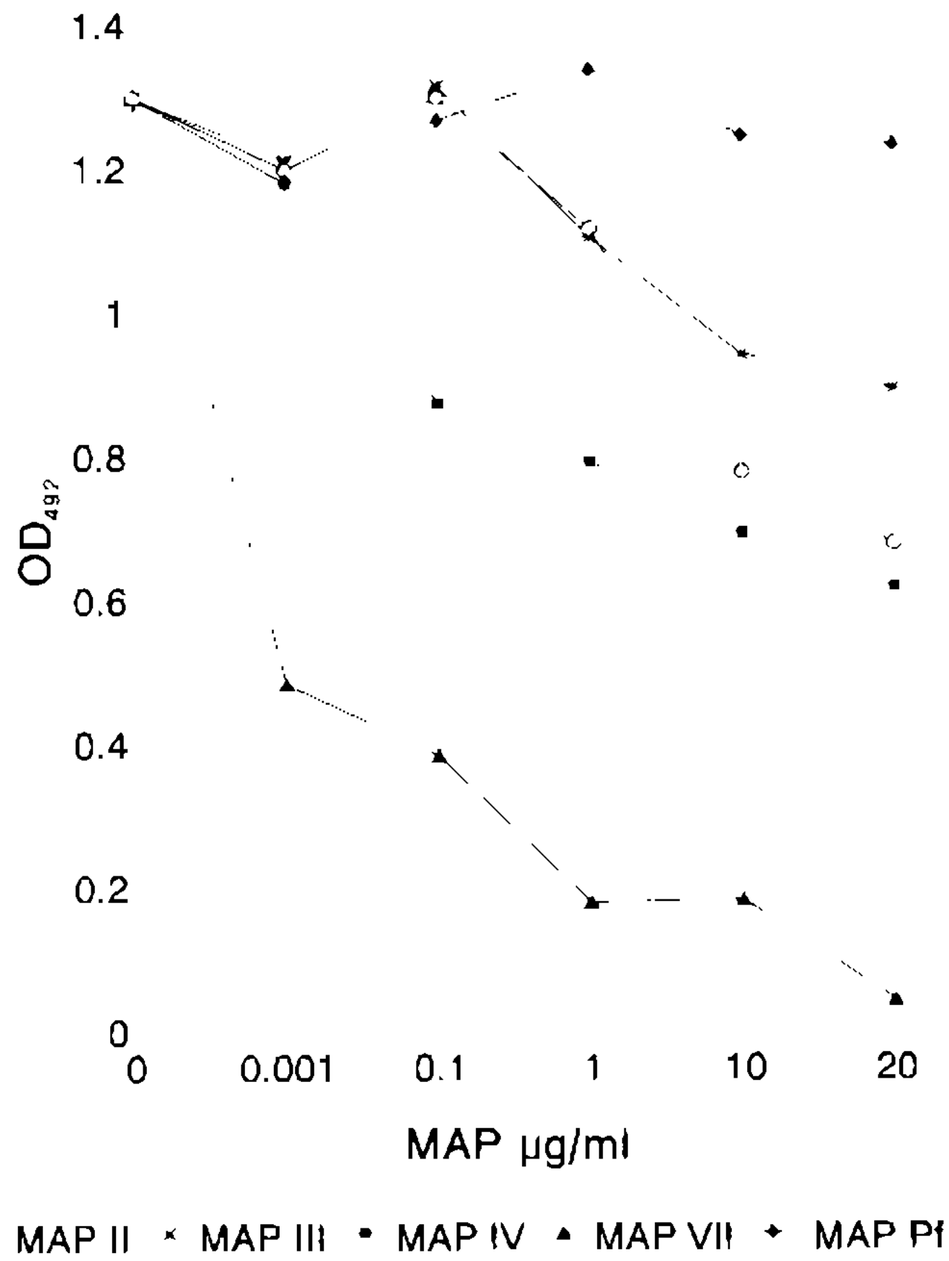

Fig. 1: competitive MAP inhibition assay. Sera of mice immunized with MAP VII .

T CELL PROLIFERATION

Table IV shows the *in vitro* proliferative T-cell responses induced by MAP constructs and the individual epitopes. All cultures stimulated with MAPs showed uniformly low responses and only MAPs VI-VIII induced moderately positive stimulation indexes. When individual peptides were used, only p11 and p25 induced proliferation of cells from mice immunized with MAP I and VI. All other animals presented negative *in vitro* proliferation responses.

DISCUSSION

The *P.vivax* CS epitopes used to assemble the MAPs studied in the present experiment were selected because of their documented reactivity with T lymphocytes (Herrera et al. 1992) and antibodies from malaria immune donors. The universal T cell epitope from the Tetanus toxin is routinely recognized by murine and human cells (Panina-Bordignon et al. 1989).

Our previous studies indicated that the T cell epitope contained in the p6 peptide induced strong *in vitro* T cell proliferative response although its recognition was rather restricted to a limited number of human donors (Herrera et al. 1992). In contrast, p25 was broadly recognized by a wide range of

TABLE IV

Stimulation indexes (SI) of lymph node cells from each BALB/c mouse after stimulation with 100, 10 and 1 µg/ml of each MAPs or 10 µg/ml of their corresponding individual peptide. Values above 3 SI are considered positive

Epitope group	Immunogen MAP	MAPS µg/ml			Individual Peptides 10 µg/ml			
		100	10	1	p6	p11	p25	p30
B	I	1.3	1.3	1.9	—	4.6	—	—
	II	0.5	0.2	1.8	—	1.9	—	—
T	III	0.5	0.9	0.6	—	—	0.5	—
	IV	1.1	2.2	2.4	—	—	—	1.6
	V	0.8	1.0	1.0	—	—	1.6	1.0
B+T	VI	2.4	2.7	6.4	—	9.8	17.4	—
	VII	4.9	1.3	1.6	—	2.1	—	1.8
	VIII	4.0	1.7	1.3	1.8	2.4	2.0	—

donors but its proliferative response was moderate. p11 was included in the present study as a B cell epitope since it is recognized by most sera from immune individuals from endemic areas that also react with the human CS protein. Apart of its B cell function, this peptide also behaves as T cell epitope (Herrera et al. 1992, Nardin et al. 1991).

Due to the limited knowledge regarding optimal MAPs structure, we decided to assemble molecules of different B and T-cell epitopes combination as well as control MAPs containing only B or T cell epitopes. As expected the constructs based on combinations of B and T cell epitopes were highly immunogenic. Most of them induced high antibody titers after only 2 immunization doses. All three MAPs (VI-VIII) were strongly recognized by sera of mice immunized with the homologous peptides, and these sera reacted with the same peptides assembled in different constructs. Interestingly, MAP VI based on the combination of p11 and p25 elicited antibodies that recognized p25 but apparently not p11 at least not when assembled as a monoepitopic construct (MAP I-II). MAP VII induced the strongest antibody response and these antibodies reacted with several different MAPs including some composed of unrelated epitopes. Although sera against all three MAPs (B+T) recognized the native protein by IFAT, serum against MAP VII produced the strongest reaction.

These results suggest that although MAP systems are capable of inducing high antibody responses, at least a fraction of these antibodies may be directed against non revelants sequences. P25 and ptt-30 do not share any sequence homology, however MAPs containing these two epitopes induce cross-reactive antibodies. In fact, the competition assay demonstrated that the other MAPs to which this serum was reactive were only capable of partially compete with the homologous MAP VII. The fact that neither the MAP constructs nor the individual epitopes were able to elicit a strong proliferative response *in vitro* does not reflect the *in vivo* situation as the vigorous B cell response against the multiepitopic MAPs as compared to the poor response to B cell monoepitopies can only be explained by a strong T helper reaction. As expected MAPs constructs based on either B or T cell epitopes alone were very poor immunogens. MAPs I-II based exclusively on the p11 B-cell epitope did not induce detectable antibodies, whereas MAPs III-V based on T-cell epitopes induced some anti-peptide antibodies.

In conclusion, we have confirmed the high immunogenicity of the MAP systems containing both B and T cell epitopes and have identified at least two combinations of B-T epitopes that may have the potential of inducing protective immune responses to *P.vivax* infection. Although, p25 produced a good helper response, the MAP VII containing ptt30 was the most immunogenic. This confirms the great potential of ptt30 as T helper epitope. Experiments designed to determine the protective efficacy of such MAPs in non-human primates are currently in progress.

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