IMMUNE RESPONSE AGAINST THE NON-REPEAT REGION (293-310) OF THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM VIVAX*

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Abstract. Immunization by peptides based on the repeat sequences of *Plasmodium falciparum* or *P. vivax* antigen(s) have shown inconsistent results during clinical trials in humans. This could be attributed to the lack of T-cell help or antigenic polymorphism. Thus, attention has been focused towards the more conserved non-repeat regions. The present study was undertaken to map the antigenic determinant in the vicinity of region II (outside the repeat) of CS protein of *P. vivax*. The immunogenicity of the peptide was studied alone and after linking with polytuftsin (PT), using alum and Freund's adjuvant, in inbred strains of mice with different genetic backgrounds. The humoral response and antigen induced T-cell proliferation assays clearly demonstrated the immunomodulatory activity of PT. Comparable results were observed with antigen(s) administered either in alum or Freund's adjuvant. The induction of IgG2a and IgG2b antibody isotypes by both, peptide as well as the conjugate, may indicate that the T-helper response involved is of Th1 type. Further the immunofluorescence studies have shown that antibodies recognized the air dried sporozoites of *P. cynomolgi*. The results thus show that the above sequence has overlapping B and T-cell determinants and that alum can be substituted for Freund's adjuvant in generating an effective immune response.

INTRODUCTION

Plasmodium vivax malaria is a major cause of morbidity (Bruce-Chwatte, 1985), particularly in the developing world. The circumsporozoite is a membrane protein and is present on the surface of sporozoites. It consists of the central repeat region which is known to be immunodominant as most of the monoclonals and the endemic sera primarily recognize the repeat region. Unlike the *P. falciparum* CS protein, this repeat region has shown phenotypic heterogeneity or polymorphism among different isolates from different geographical areas (Rossenberg *et al*, 1989). Sequences designated as regions I and II, flanking the repeats, are homologous among several malaria species (Arnot *et al*, 1985; McCutchan *et al*, 1985).

Clinical trials in humans using recombinant and a synthetic vaccine derived from the repeat region of the CS protein of *P. vivax* failed to show any correlation between antibody levels and protection (Herrington *et al*, 1991). This has been attributed due to (a) lack of T-cell help (b) presence of antigenic polymorphism at the parasite level (c) genetic variability of the immune response at the host level.

Even though there is antigenic variation, we believe the parasite still preserves certain conserved sequences for its survival within the host. Hence we focused our attention on a sequence in the vicinity of region II, an area shown to contain a T-cell epitope in all the CS proteins (Nardin *et al*, 1991). The sequence between the residues 293-310 of the CS protein has shown to be mainly hydrophilic, highly antigenic and contains two helical turns between the residues 295-307 using computer aided algorithmic prediction (Hopp and Wood, 1981).

Since small peptides are known to be poor immunogens and are usually MHC restricted, coupling with polytuftsin was used to overcome this problem. The selection of the carrier was based on our earlier observations that it induces class II molecules on the APCs and also increases the secretion of IL-1 as a costimulatory signal for T-cell growth and differentiation (Pawan *et al*, 1994; Dhawan *et al*, 1995).

Immunoadjuvants are known to modulate the final outcome of the immune responses since they influence the balance between the humoral and cell mediated response. To be effective in a vaccine, a

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permissible adjuvant should not only be able to elicit good primary response but also memory at both T and B-cell level. In the present study, we have studied the immune response to the carboxy-terminal peptide of CS protein (*P. vivax*) conjugated to polytuftsin, in mice with different genetic background using alum as an adjuvant.

MATERIALS AND METHODS

Mice

Groups of 4-6 mice, of 8-12 weeks of age, weighing between 10-20 g were used. B10.3R (H-2^{i3}), DBA/2J (H-2^{d}), C57BL/10J (H-2^{b}), CBA/J (H-2^{k}), RIII (H-2^{r}) and FVB/J (H-2^{q}) were procured from the breeding facilities of National Institute of Immunology, New Delhi.

Peptide

The C-terminal peptide of the CS protein of *P. vivax*, 293-310 (PNAKSVKEYLDKVRATVG), was synthesized by solid phase technic using the HoBt/Dcc coupling procedure of our reported protocol (Manzar *et al*, 1991). After the usual purification and physiochemical characterization, the peptide was found to be > 90% pure. Polytuftsin (PT, kindly gifted by BB Ivanov, NIH, USA) is a polymer of tuftsin, containing 35-40 repeats of the sequence RTKP with an average MW of 15-20 kDa determined by gel filtration technic.

Coupling of CS peptide with PT

The CS peptide was coupled to PT using glutaraldehyde method of our reported protocol (Pawan *et al*, 1994). In brief, 0.7 mg of peptide and 0.7 mg of PT were taken in the molar ratio of 10:1 (peptide:carrier). At the end of coupling, an average of 6-8 peptide moieties were found to be linked per molecule of PT as determined by amino acid analysis.

Immunization

Aluminum hydroxide adsorbed antigen was prepared using the adjuvant alhydrogel (2%, Superfos Biosector, Denmark) at 1:1 ratio (v/v, Ag: adjuvant) at 4°C for 24 hours on an end to end shaker. The FCA formulation was prepared by mixing equal volumes of antigen and adjuvant.

Mice were immunized subcutaneously at the base of the tail with 50 µg of the antigen (peptide alone or conjugate) in the appropriate adjuvant formulation (alum or FCA). 25 µg of the respective antigens were given in IFA or alum as boosters on days 21 and 32. Mice were bled from the retro-orbital plexus on days 27 and 37. The sera tested by ELISA for the presence of anti-peptide antibodies.

Enzyme linked immunosorbent assay (ELISA)

The CS peptide conjugated to BSA at a molar ratio of 1:12 (BSA:Peptide), using the glutaraldehyde method, was used as a capture antigen in ELISA.

Total IgG

100 µl of peptide-BSA conjugate (50ng/well) was used to coat 96 well plates (Nunc, maxisorb). After washing with PBS-Tween-20 (0.05%) and blocking with skimmed milk powder (4%), two fold dilutions of the mice sera (100µl/well) in PBS containing 0.1% skimmed milk powder were added and the plate was incubated at 37°C for 2 hours. Bound antibodies were detected using goat anti mouse IgG-HRPO conjugate (Reagent bank, NII, New Delhi) with OPD as chromogen and H_2O_2 as substrate. After stopping the reaction, absorbance was read at 490 nm. Pre-immune sera and wells coated with sham coupled BSA were used as controls. All samples were taken in triplicates and the mean absorbance was calculated (Mean test - Mean control).

IgG subclasses

IgG subtyping was done using the same protocol as described above for the "total IgG" except that the first antibody was incubated at a single dilution of 1:25 with the peptide-BSA coated wells. After usual washing with PBS-T, second antibody (goat anti mouse IgG-specific for each class, Sigma subtyping kit) was incubated with the above complex for 90 minutes at 37°C at a dilution of 1:1,000 for IgG1, IgG2a, IgG3 and 1:2,000 for IgG2b. After washing again with PBS-T, third antibody

(rabbit anti-goat IgG-HRPO conjugate, 1:5,000 dilution) was incubated with the above Ag-Ab complex at 37°C for 90 minutes. After adding the substrate and stopping the reaction, the absorbance was read at 490 nm and the mean absorbance was calculated as described above.

Antigen induced T-cell proliferation

The assay protocol consisted of the following groups of mice immunized with

a. Peptide alone and

b. Peptide-PT conjugate

The spleen cells were stimulated *in vitro* with the respective homologous or heterologous antigen. In brief, mice were injected in the foot pad with 50 µl of FCA emulsion containing 50 µg of the antigen. The booster immunization with the homologous antigen (25 µg) was given in IFA on day 8. Animals were sacrificed on day 12 and the spleen cell suspension were prepared under aseptic conditions. The majority of the B-cells were removed by panning with goat anti-mouse Ig and 2×10^5 cells/well were plated in 96 well plate in RPMI-1640 containing glutamine, penicillin and streptomycin supplemented with 2% heat inactivated FCS. The cells were stimulated with the peptide alone or the conjugate at different concentrations in a final volume of 200 µl. PHA (5 µg/ml) was used as a positive control. On day 4, the cultures were pulsed with 0.5 µci ^{3}H-thymidine (Sp. Activity 6.2 Ci/mmole, BARC, Trombay, Bombay) per well. After 18 hours, the cells were harvested on glass fiber filters and incorporation of ^{3}H-thymidine was measured in a liquid scintillation counter. The data is presented as the mean cpm of triplicate cultures minus the cpm without antigen.

Immunofluorescence assay

Immunofluorescence assay was carried out using the standard technic (Nardin *et al*, 1982). Briefly, *P cynomolgi* B (Berok strain) sporozoites were aliquoted onto teflon coated IFA slides and air dried at room temperature and stored at -20°C. At the time of use, the slides were taken out and washed three times in PBS (pH 7.2) for 10 minutes each. Lyophilized serum samples resuspended in PBS were applied in to the wells at a dilution of 1:10, incubated at room temperature for 2 hours and washed thrice in PBS. Goat anti-mouse IgG labeled with FITC was added in to the antigen coated wells at a dilution of 1:40 in PBS (15 µl/well) and slides were incubated in humid chamber for one hour at room temperature. Slides were washed again in PBS, mounted in glycerol and observed under the epifluorescence microscope.

Statistical analysis

The data for the humoral response was analyzed using Student's *t*-test and paired *t*-test whereas in case of antigen induced T-cell proliferation, Wilcoxan's signed rank test was used.

RESULTS

Antibody response

Total IgG: The peptide-PT conjugate showed significantly ($p < 0.01$) higher antibody levels than the CS peptide alone in all six strains, irrespective of the nature of the adjuvant. This is reflected both in primary and secondary responses. Further, the conjugate showed significantly higher ($p < 0.05$) secondary response, than the primary response. Though there is a rise in antibody levels (albeit marginal) in the groups immunized with the Freund's adjuvant, it was statistically insignificant ($p > 0.05$) as compared to the group immunized in alum except in mice bearing the haplotype H-2^k ($p < 0.05$). Mice bearing the haplotype H-$2^{k\&q}$ showed the maximal antibody levels in Freund's adjuvant and alum respectively, among all the strains (Fig 1a-c).

An interesting observation made during the study was that no detectable antibodies were found against polytuftsin itself.

IgG subclass: The conjugate as well as the peptide alone predominantly showed IgG2a and IgG2b isotype response, in all the strains, irrespective of the nature of the adjuvant. Mice bearing the haplotype H-$2^{k,r\&q}$ showed marginally higher levels of IgG2a as compared to IgG2b isotype while mice bearing the haplotypes H-$2^{b,d\&i3}$ showed marginally higher levels of IgG2b, irrespective of the nature of the antigen or the adjuvant (Fig 2a-f).

Antigen induced T-cell proliferation

A dose dependent T-cell stimulation was observed in all strains of mice and the optimum dose

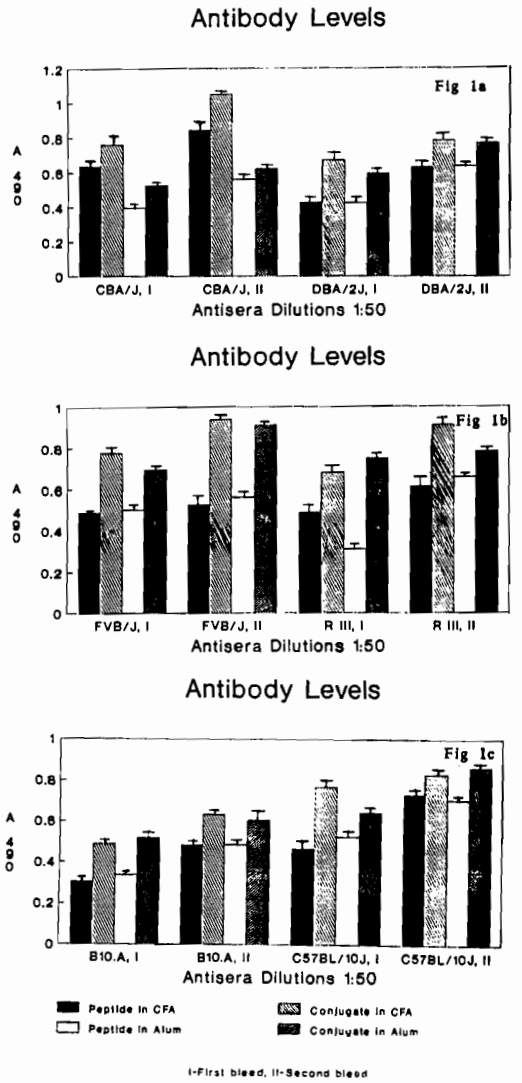
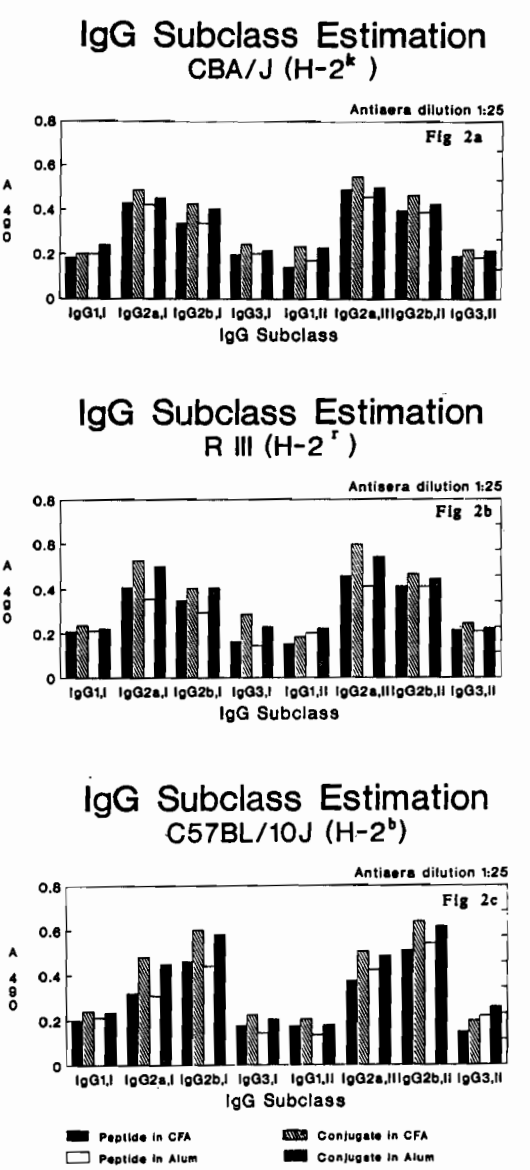


Fig 1a-c–Total IgG levels in various strains of mice (four mice per group) against CS peptide (alone and linked to polytuftsin), immunized in alum and Freund's adjuvant. Absorbance value are the average of the four mice, from the bleeds obtained on days 27(I) and 37 (II).



was found to be 20 μg/ml. This was highly significant ($p < 0.001$). Though mice bearing the haplotype H-$2^{k\&q}$ showed higher stimulation, the difference among all the haplotypes was statistically insignificant. When the cultures from the group primed with the peptide were pulsed with the same, much less proliferation was observed. However, when the CS peptide was used to pulse the cultures from

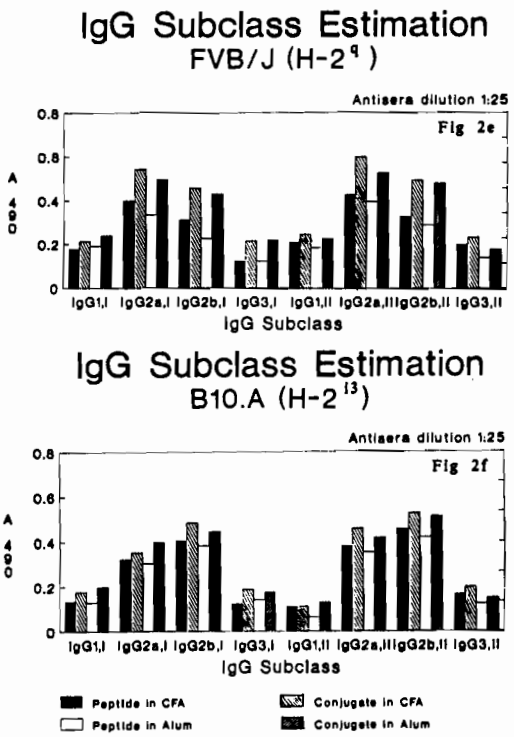


Fig 2a-f–IgG isotype responses to the CS peptide and CS peptide-polytuftsin conjugate, immunized in alum and Freund's adjuvant, in various strains of mice. The sera was collected on days 27(I) and 37(II).

the group primed with the conjugate, an increased stimulation (although marginal) was observed as compared to the previous group but this difference was significant ($p < 0.05$) in mice bearing the haplotypes H-$2^{k\&r}$ (Table 1a-c).

Indirect immunofluorescence

The IFA studies reveal that antibodies raised against the peptide showed a stronger fluorescence as compared to the conjugate, both in alum and Freund's adjuvant. However, the sera from the group immunized with peptide in alum showed higher reactivity as compared to sera from the group immunized in Freund's adjuvant. Sera from the mice bearing the haplotype H-2^k showed the maximal reactivity (Table 2).

DISCUSSION

The use of synthetic peptides as an alternative approach for vaccine design requires the identification of protective B-cell determinant(s) along with appropriate T-helper or cytotoxic epitope(s) in a given antigen. To improve the immunogenicity of peptide based vaccines, these are often linked to macromolecules like BSA or TT or DT or KLH. The inclusion of such carriers with vaccine formulations have shown limited applicability or suitability in view of their cross reactivity, epitopic suppression and tolerance (Schultze *et al*, 1985; Sakata and Atassi, 1981; Herzenberg *et al*, 1980). This has led to the mapping of pathogen derived T-cell determinants for producing memory response as well as long lasting immunity. This approach, though showed encouraging results for various immunogens, the influence of immunoadjuvants or potentiators to further improve vaccine efficacy by

Table 1a

Antigen stimulated T-cell proliferation Mice primed with conjugate.

Mice (Strain)	Cultures pulsed with conjugate (1μg/ml) Δ cpm ± SE	(10μg/ml) Δ cpm ± SE	(20μg/ml) Δ cpm ± SE
C57BL/10J	14,088 ± 1,365	26,431 ± 1,648	31,558 ± 2,304
DBA/2J	12,345 ± 1,102	24,678 ± 1,407	30,342 ± 2,132
R III	16,356 ± 1,309	29,456 ± 1,993	34,652 ± 1,849
CBA/J	11,453 ± 908	23,567 ± 1,704	29,876 ± 1,643
FVB/J	12,454 ± 1,108	28,476 ± 2,089	33,465 ± 2,617
B10.A	10,987 ± 907	23,536 ± 1,843	29,866 ± 1,984

Table 1b

Antigen stimulated T-cell proliferation mice primed with peptide.

Mice (Strain)	Cultures pulsed with peptide (1μg/ml) Δ cpm ± SE	(10μg/ml) Δ cpm ± SE	(20μg/ml) Δ cpm ± SE
C57BL/10J	1,408 ± 134	2,643 ± 268	3,455 ± 335
DBA/2J	2,834 ± 284	3,467 ± 245	5,034 ± 515
R III	2,635 ± 394	3,945 ± 312	4,465 ± 493
CBA/J	1,145 ± 245	3,356 ± 382	4,787 ± 502
FVB/J	1,543 ± 312	3,543 ± 414	4,365 ± 432
B10.A	1,233 ± 362	3,243 ± 203	4,153 ± 382

Table 1c

Antigen stimulated T-cell proliferation mice primed with conjugate.

Mice (Strain)	Cultures pulsed with peptide (1μg/ml) Δ cpm ± SE	(10μg/ml) Δ cpm ± SE	(20μg/ml) Δ cpm ± SE
C57BL/10J	3,408 ± 363	5,643 ± 498	7,155 ± 654
DBA/2J	2,834 ± 284	4,467 ± 453	6,034 ± 535
R III	4,635 ± 494	6,945 ± 612	8,465 ± 693
CBA/J	3,145 ± 345	5,356 ± 476	7,779 ± 654
FVB/J	4,345 ± 312	4,637 ± 414	5,376 ± 432
B10.A	3,956 ± 382	4,356 ± 453	4,976 ± 482

Lymphocyte stimulation as measured by tritiated thymidine incorporation. Mice were immunized in foot pad with the CS peptide alone or the CS peptide-polytuftsin conjugate on days 0 (50 μg) and 8 (25 μg). Spleen cells collected on day 12 were stimulated *in vitro* with the homologous and heterologous antigen. The results are expressed as mean +SE unstimulated cells CPM 500-600.

stimulating the appropriate arm of the immune response, has actively been studied in the recent years. This approach has paved the way for developing superior immunogens by linking B and T-cell, determinants with inbuilt adjuvanticity (Ritu and Rao, 1992; Sinigaglia *et al*, 1988), efficient and controlled delivery through MAP (Morein *et al*, 1984), liposomes (Allison and Gregoriadis 1974), ISCOMs (Morein *et al*, 1984), cytokines (Good *et al*, 1988; Gokulan and Rao, 1997). Thus appropriate selection of a carrier or the adjuvant or both can influence the final outcome of the immune response to a given peptide antigen.

In the present study, we focused our attention on a sequence outside the repeat region of the CS protein of *P. vivax*, so as to delineate the B or T-cell determinants. Immunogenicity of the peptide and the conjugate was studied in inbred strains of mice with different genetic background, in two adjuvant formulations, *ie* CFA/IFA *vs* alum. The pathways of antigen processing and presentation has shown to be more or less similar in murine and humans, we therefore used different inbred strains of mice to ascertain the immunogen applicability on a wider out-bred population. Since Freund's adjuvant has a number of disadvantages like causing acute or chronic inflammatory reactions at the injection site, so its adjuvanticity was compared with alum, a permissible adjuvant in humans, to check whether alum could replace it. The peptide alone and the conjugate were also separately tested for their po-

Table 2

Indirect immunofluorescence assay.

Haplotype	PA	CA	PC	CC
k	3+	0	2+	1-2+
r	2+	0	0	0
b	1+	1+	1-2+	1+
d	2+	1+	1-2+	1+
q	1+	1+	0	0
i3	1+	1+	1+	1+

PA- Peptide in alum, CA- Conjugate in alum
PC- Peptide in CFA/IFA, CC- Conjugate in CFA/IFA

Indirect immunofluorescence assay (IFA). The antisera was assayed for reactivity with *P. cynamolgi* B sporozoites. Fluorescence was graded from 0 to 3+, with 0 equal to weak fluorescence and 3+ equal to strong fluorescence. Pre-immune sera was taken as negative control.

tential to induce T-cell proliferation in different strains of mice. Maximal proliferative response was observed when conjugate was used to pulse the cultures of spleen cells from the group immunized with the conjugate (Table 1a). Since there is not much statistical difference among different haplotypes in T-cell proliferative response, it emphasizes the importance of polytuftsin in modulating the immune response to overcome the MHC restriction. This is further supported by the fact that polytuftsin has been shown to induce the release of lymphocyte growth factors thereby enhancing the further expansion of T-cell clones (Gokulan and Rao, 1997). The possible reason for the CS peptide showing marginal T-cell proliferation (Table 1b) could be that this peptide sequence (293-310) shares a few amino acids with a known murine T- cell epitope (308-320, Rodrigues *et al*, 1991) and also with a corresponding T-cell epitope (300-319) in higher animals like chimpanzee (Nardin *et al*, 1991). This clearly suggest that a T-cell epitope lies within the residues 308-319, which needs further verification. An increase in T-cell proliferations (albeit marginal) was observed with spleen cells pulsed with the peptide, though these were primed with the conjugate (Table 1c). This highlights the importance of the role played by polytuftsin to stimulate a few clones which can recognize the peptide upon subsequent *in vitro* stimulation. This observation has considerable relevance in natural circumstances, especially in an endemic area where the individuals are constantly being exposed to malarial sporozoite infection.

The results of "total IgG estimation" have clearly shown that polytuftsin provides the necessary T-cell help, as the immunogenicity of the conjugate was found to be superior over the peptide irrespective of the genetic background of the animal (Fig 1a-c). Peptide as well as the conjugate showed secondary booster response with both the adjuvants in all the haplotypes, thereby indicating that this sequence has an inbuilt T-cell epitope. However, the inclusion of polytuftsin undoubtedly showed an additive effect in enhancing the immunogenicity of the peptide or conjugate in either of the adjuvants. The results of the study clearly demonstrated that Freund's adjuvant can be substituted with alum (Jaroslav *et al*, 1994; Barbieri *et al*, 1992). Whether this is true for all the peptides or depends on the nature of the sequence, needs further verification.

Protection against infectious disease frequently depends on the stimulation of an appropriate isotype of antibody (Kaminiski *et al*, 1986). The induction of IgG2a and IgG2b isotypes by the peptide as well as the conjugate, in either of the adjuvants (Fig 2a-f), indicate that the T-helper response seen is predominantly of Th1 type. Induction of similar antibody isotype following vaccination with different adjuvants, has also been reported elsewhere (Robinson *et al*, 1994). In the murine system IgG2a is under the control of IFN-γ (Snapper *et al*, 1988). Lymphocyte derived cytokines such as IL-2 and IFN-γ specifically act on T and B-cells and induce IgG2a and IgG2b isotypes. Since the conjugate showed comparatively higher levels of these isotypes, this effect could be visualized as a direct effect of polytuftsin thereby activating the macrophages (Dhawan *et al*, 1995), eventually leads to the expansion of IFN-γ producing Th1 cells. IgG1 isotype has been found to have a lower protective capacity than IgG2a or IgG2b isotypes (Kenney *et al*, 1989). It has also been reported that the IgG2a isotypes promotes phagocytosis, activates the complement system and mediates antibody dependent cellular cytotoxicity (Ey *et al*, 1979; Burton, 1985). Animals depleted of complement were also efficiently protected with IgG2b antibodies (Pelkonen and Pluschke, 1989).

Since *P. cynamolgi* B CS protein has sequence homology with *P. vivax* (293-310) to a great extent

near region II, we have used these sporozoites for the indirect immunofluorescence assay. Since the air dried sporozoites showed fluorescence with the antibody raised with all the four preparations, this sequence must be present on the surface which is accessible to the immune system.

Thus in the present study, a CS peptide (PNAK-SVKEYLDKVRATVG, 293-310) of *P. vivax* generated IgG2a and IgG2b protective isotype antibodies and immunization with alum generated levels of antibodies comparable with those of Freund's adjuvant. Further the inclusion of polytuftsin has shown further enhancement of the immune response against the CS sequence.

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