HUMORAL IMMUNE RESPONSES TO THE *PLASMODIUM FALCIPARUM* ANTIGEN Pf155 RESA IN ADULTS WITH DIFFERENTIAL CLINICAL CONDITIONS FROM AN INDIAN ZONE WHERE MALARIA IS ENDEMIC

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Abstract. Ring infected erythrocyte surface antigen of *Plasmodium falciparum* (Pf155/RESA) has been considered as a vaccine candidate. However, the relative immunogenicity of this antigen has not been studied in Indian populations. Pf155/RESA was investigated for its immunogenecity by studying humoral immune responses against Pf155/RESA and Pf155/RESA derived peptides (P1, P2 representing immunodominant epitopes from the 3' and P3 from the 5' repeat regions) by erythrocyte membrane immunofluorescence (EMIF) assay and by enzyme linked immunosorbent assay (ELISA) in *P. falciparum* primed donors living in hyperendemic malarious areas (Orissa State, India) where *P. falciparum* infections are highly prevalent. Subjects of different clinical status namely acute (A), clinically immune (CI) and acute with history of repeated *P. falciparum* infections (R) were included in the study. All the donors were seropositive against the crude antigen. There was considerable variation in the responses among the donors. While humoral responses in the plasmas against the P2 peptide $(EENV)_4$ were significantly higher in magnitude and in frequency in the CI donors than in the A donors, no positive response was seen in the R donors. The responses to the peptides P1 $(EENVEHDA)_2$ and P3 $(DDEHVEEPTVA)_2$ were poor both in the A and in the CI groups. Whereas, most of the R donors were seropositive against the P3. The present results indicate that Pf155/RESA contains B cell epitopes which were recognized differently by the immune system of individuals living in malaria-hyperendemic areas of India who have been primed by natural infection. Our studies also suggest that in order to investigate the possible functional role of a given antigen, study of immune responses against the antigen in donors of different clinical status may be useful.

INTRODUCTION

In endemic areas, clinical manifestations of malaria are mainly seen in infants and young children. Whereas, adults develop a functional but non-sterilizing immunity. Both antibody dependent and antibody-independent immune responses confer the protection. The various mechanisms behind this protection are poorly understood. However, the protective capacity of any immunogen depends on the presence of immunologically active and conserved structures (epitopes) which can mediate protective responses (Astagneau *et al*, 1991). It is of high importance to identify such epitopes in some known vaccine candidate antigens which are recognized by subjects from malaria-endemic areas. Dissection of the humoral response against various immuno-dominant sites from a conserved antigen, in subjects of differential clinical conditions living in endemic areas, as compared with the malaria unexposed (never suffered from clinical malaria) individuals might lead to identification of epitopes of likely significance of clinical immunity.

Plasmodium falciparum is responsible for high mortality and morbidity in malaria endemic areas (Walgate, 1991). About two million cases of malaria (35% due to *P. falciparum*) are reported each year in India (Sharma, 1987; Walgate, 1991). The current status of malaria indicates that almost one-third of the cases of *P. falciparum* infection and one-half of all malaria deaths in the country as a whole occur only in the state of Orissa. In some malaria endemic areas of Orissa, slide positivity rates upto 38% were found (Yadav *et al*, 1990; 1991).

Several malaria vaccine candidate antigens have been characterized at the molecular level. One such antigen is Pf155/RESA ("ring infected erythrocyte

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surface antigen"), which is deposited in the erythrocyte membrane during or shortly after merozoite invasion (Coppel *et al*, 1984; Perlmann *et al*, 1984). There are several reports indicating that Pf155/RESA contains certain conserved (Perlmann *et al*, 1987) regions which consist of protective epitopes (Berzins *et al*, 1991; Wahlin *et al*, 1984). However, there is lack of information about B-cell recognition sites in Pf155/RESA in Indian areas where malaria is endemic.

Here, we have studied the humoral responses to three synthetic peptides, P1 $(EENVEHDA)_2$, P2 $(EENV)_4$, (3' terminal repeat region), and P3 $(DDEHVEEPTVA)_2$ (the center of the 5' terminal) of Pf155/RESA (Favaloro *et al*, 1986) in subjects of differential clinical conditions living in malaria-hyperendemic area of India.

MATERIALS AND METHODS

Parasites

Seven *P. falciparum* isolates used in these experiments were collected from *P. falciparum* infected individuals (adults) living in malaria-endemic area of India, where malaria transmission is high and perennial. Parasites were cultured *in vitro* in blood group O^+ erythrocytes in RPMI 1640 medium (Centron Research Laboratories, Bombay, India) containing 10% normal human sera (AB^+) by the method described by Trager and Jensen (Trager and Jensen, 1976). Three field isolates namely FJB, D1, D2, D9 (sensitive to chloroquine) and D4 (resistant to chloroquine) were obtained from the adjoining areas of Jabalpur, Madya Pradesh State. Two isolates (sensitive to chloroquine) were obtained from the State of Uttar Pradesh. One isolate R1 (resistant to chloroquine) was obtained from Rohini, a residential area in Delhi, where *P. falciparum* transmission is low and seasonal.

Study subjects

Heparinized venous blood sample (2 to 3 ml) was collected from adults (age 18-45 years, male and female in equal numbers) who have been living in villages of Orissa State, India, where malaria is highly endemic with a stable situation. In this area, *P. falciparum* accounts for nearly 80% of total malaria cases. Chloroquine resistant falciparum malaria have also been reported from these areas. The total population of the study area is around 2,000. Plasma samples were collected from thirty-four clinically immune individuals (36 ± 12: mean age ± SD); afebrile at the time of blood collection and did not suffer from clinical malaria during the past one to two years (CI group), twenty-five acute individuals (*P. falciparum* infection confirmed by blood smear) (32 ± 9: mean age ± SD) (A group) and ten from acute patients with the history of repeated episodes of malaria (19 ± 4: mean age ± SD) (R group). They were not under anti-malarial therapy during the time of plasma collection. Active malaria surveillance in the study area is very well established by Malaria Research Center, Field Station, Rourkela. Thus, these repeated infections were confirmed as malaria from the clinical history and also through blood smear examination by the trained paramedical staff. Plasma from ten healthy volunteers (laboratory staff) who had not experienced clinical malaria were used as control (C group).

Indirect immunofluorescence

Antibodies to intraerythrocytic parasites were assayed using monolayers of air-dried but unfixed *P. falciparum* infected erythrocytes from *in vitro* cultures of all seven isolates. The dilution of the plasma, used in this assay was 1:1,000. Antibodies against Pf155/RESA antigens were determined by a modified erythrocyte membrane immunofluorescence (EMIF) as described elsewhere (Perlmann *et al*, 1984). Briefly, in multi-test slides, monolayers of early stage infected erythrocytes (1 to 2% parasitemia) from *in vitro* cultures (synchronized) of different isolates were fixed with 1% glutaraldehyde (GDA) and air dried. Immunofluorescence was performed by sequential incubation of various dilutions of human sera, biotinylated goat antibodies to human Ig and fluorescence conjugated (FITC) avidin (Sigma Chemicals, St Louis, USA). To visualize parasitized erythrocytes, the monolayers were counterstained with ethidium bromide (Sigma chemicals, St Louis, USA). Slides were scored with a 100 × oil immersion lens in incident ultraviolet light in a Nikon (Japan) microscope equipped for simultaneous observation of fluorescence staining (green) and nuclear staining (orange) (excitation filter 510-560 nm, barrier filter 590 and interference filter 580). The ultraviolet source was a HBO 100 mercury lamp.

Antigen and peptide preparations

The parasite antigen (Ei) was a pooled sonicates of trophozoite/schizont-enriched fractions obtained by Percoll gradient centrifugation of the *in vitro* cultures of *P. falciparum* (D2 isolate). Supernatants from sonicate of normal erythrocytes (RBC ghosts) were used as control antigens (Eo) (Kabilan *et al*, 1987).

Synthetic peptides

The synthetic peptides, P1 and P2 from the 3'-repeat region and P3 from the 5'-repeat region were synthesized by the solid phase procedure (Manzar and Rao, 1991). The amino acid sequence in single letter code of P1 (EENVEHDAEENVEHDA), P2 (EENVEENVEENVEENV), and P3 is (DDEHV-EEPTVADDEHVEEPTVA). The peptide polymers $(EENVEHDA)_2$ $(EENV)_4$ and $(DDEHVEEPTVA)_2$ were prepared using 0.25% glutaraldehyde as described (Manzar and Rao, 1991). Peptide purity was checked by HPLC and amino acid analysis composition. Peptides were conjugated to BSA (bovine serum albumin, ratio 1:3) using 1% glutaraldehyde.

ELISA

Antibody reactivity against the Pf155/RESA peptides, Ei and Eo in plasma were tested by ELISA. Briefly, 96 well plastic plates (Tarson, India) were coated with BSA conjugated Pf155/RESA peptides (100ng/well), Ei, Eo (1 μg/well and BSA alone and incubated at 4°C overnight. After blocking with 1% BSA, plates were incubated with serial dilutions (1: 500 and 1:1,000) of the plasmas overnight at 4°C. For determination of anti Pf155/RESA peptide antibodies in plasma samples, a dilution of 1:500 was found optimal and used. Bound antibodies were detected by horse radish peroxidase-conjugated rabbit antibodies specific for human IgG (Dakopatt, Denmark), with o-phenylenediamine/H_2O_2 as substrate (Sigma chemicals, St Louis, USA). The reactions were stopped with 8 N H_2SO_4 and the absorbance was measured at 490 nm by microplate reader (model EL-302, Biotek, USA). Donors were considered as positive if the OD (optical density) value was greater than the mean OD + 2 SD obtained from the antibody responses in control donors.

In vitro merozoite invasion inhibition assay

The biological activity of the anti-malarial antibodies was tested in *in vitro* merozoite reinvasion inhibition assay as described previously (Perlmann *et al*, 1987; Wahlin *et al*, 1984). In brief, *P. falciparum* cultures (D2 isolates) containing late trophozoite and schizont (1% parasitemia) in quadruplicate were incubated for 20 hours in microtiter plates with or without (control) diluted plasmas (1:100 dilution) which had high titer antibody against the peptide P2 as elicited in ELISA. Thereafter, the cultures were washed and monolayers were prepared and fixed with 1% glutaraldehyde. Parasites were stained with acridine orange and the infected erythrocytes were counted using a fluorescence microscope (Wahlin *et al*, 1984). Five thousand erythrocytes were screened per well and the parasitemia was counted and average was determined. Invasion inhibition was calculated using the formula; 100 × (percentage of parasitemia in control – percentage of parasitemia in test)/(percentage of parasitemia in control).

Statistical analysis

Differences between antibody responses of acute and immune individuals were analyzed by using student's *t*-test. Correlation analysis was done by simple linear regression analysis.

RESULTS

Plasma samples used in this studies were collected from permanent residents of the villages of Orissa State, India, and thus were likely to have been exposed to similar levels of infection and also similar strain of *P. falciparum* over the years. All plasma except that from control donors were positive for *P. falciparum* antibodies by conventional immunofluorescence at a titer of ≥ 1,000 on unfixed air-dried monolayers of infected erythrocytes.

Sixty-nine plasma samples from *P. falciparum* primed donors (34 (CI), 25 (A) and 10 (R) (For details refer to material and methods) were titrated in the EMIF assay, on monolayers of ring stage infected erythrocytes isolated from seven *P. falciparum* infected donors belonging to different geographical areas. The results shown in Table 1 were obtained by analyzing 21 different plasma samples. Plasma samples showed similar pattern of immunofluorescence staining in different isolates. However, there was variation in the titers of the plasma.

Sixty-eight percent (40/59) of the plasma contained anti-Pf155/RESA antibodies at titers ranging

Table 1

Titers determined by EMIF of twenty-one human plasma samples on seven *P. falciparum* isolates.

Donors	RI	P30	A6	D1	D2	D4	D9
CI1	50	1,250	250	50	250	250	1,250
CI2	50	50	250	1,250	250	50	1,250
CI3	50	250	50	250	250	50	250
CI4	250	1,250	1,250	1,250	250	250	1,250
CI5	250	1,250	1,250	1,250	250	250	1,250
C10	250	1,250	1,250	1,250	250	50	1,250
CI9	-	-	-	-	-	-	-
A1	1,250	1,250	250	250	1,250	1,250	1,250
A2	50	50	250	250	250	50	250
A3	50	1,250	1,250	1,250	250	50	250
A4	1,250	50	250	250	250	50	250
A6	50	50	50	50	50	50	50
A5	-	-	-	-	-	-	-
R1	-	-	-	-	-	-	-
R2	-	-	-	-	-	-	-
R3	-	-	-	-	-	-	-
R4	-	-	-	-	-	-	-
R5	-	-	-	-	-	-	-
C1	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-
C3	-	-	-	-	-	-	-

Reactivity of twenty-one human plasma with RESA of seven different field *Plasmodium falciparum* isolates from various geographical regions in India by Erythrocyte Membrane Immunofluorescence (EMIF). CI = Clinically immune, A = Acute, R = Acute with history of repeated attacks of malaria, C = Healthy controls.

from 1/50 to 1/1,250 as determined by EMIF assay. Positivity by EMIF was in higher percentage and in end titers (Fig 2) among the plasma from clinically immune donors (29/34, 85%) in comparison with that of acute donors (11/25, 44%) (Chi sq = 7.8 p = 0.005). The difference in EMIF titer between plasma of acute and clinically immune was signifi-cant ($p < 0.04$). None of the plasma collected from ten adults who were acutely ill and complained of re-peated attacks of *P. falciparum* infection at different intervals gave a positive staining by EMIF assay. Control sera did not show positive reactivity to Pf155/RESA of any isolates (Table 1).

Anti-malarial antibody profiles in donors of different clinical conditions

The "clinically immune" and acute individuals were all adults and there was no difference in the age of these two groups. Whereas, the repeated infection individuals were teenagers and the difference in age was significant when compared with the CI and A group ($p < 0.001$ for both). Antibody binding to Ei, Eo and three Pf155/RESA derived peptides was determined in plasma samples of CI and A groups by ELISA (Fig 1a, b). There was considerable variation in the magnitude of the antibody responses to the malaria antigens. Table 2 shows some representative results of antibody responses among different clinical groups. All plasma from malaria exposed donors contained antibodies against Ei at varying levels (Table 2). The differences between the responses in the malaria exposed individuals and the control donors were highly significant ($p < 0.001$ for all the groups). Whereas, differences in concentration of anti-Ei antibodies among the groups were not significantly different. Few plasmas from A and CI groups contained antibodies against Eo above the levels obtained from the plasmas of the control donors

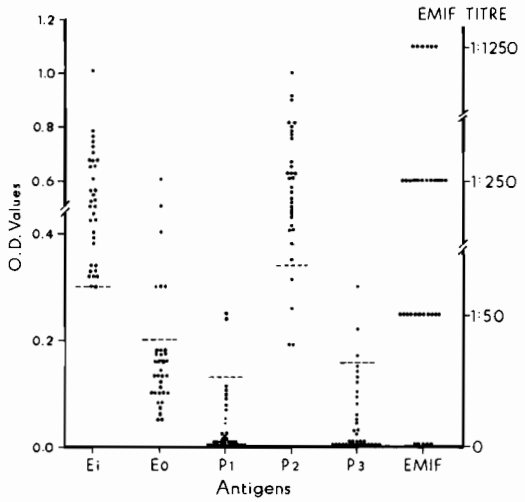

Figure-1(a)

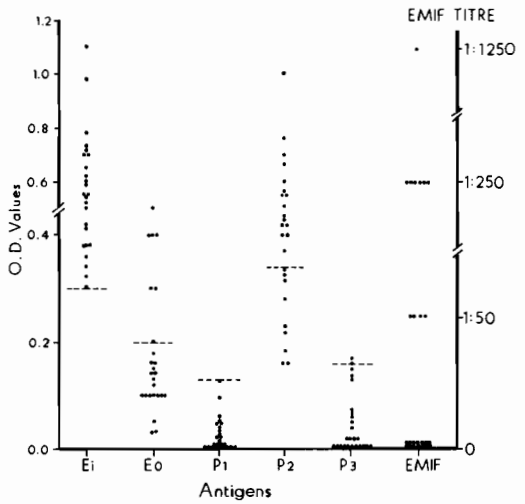

Figure-1(b)

Fig 1a, b–Scatter diagram of binding of antibodies in plasma from malaria exposed donors ((a) clinically immune and (b) acute), to three sequences of RESA (P1 - EENVEHDAEE NV-EHDA; P2 = EENVEENVEENVEENV; P3 = DDEHVEEPTVADDEHVEEPTVA) by ELISA and expressed as OD (optical density) after correction for background values. ------ = mean OD ± 2 SD from the plasma samples of control donors. Antibody responses determined by EMIF using monolayers of D2 isolates are expressed in titer values.

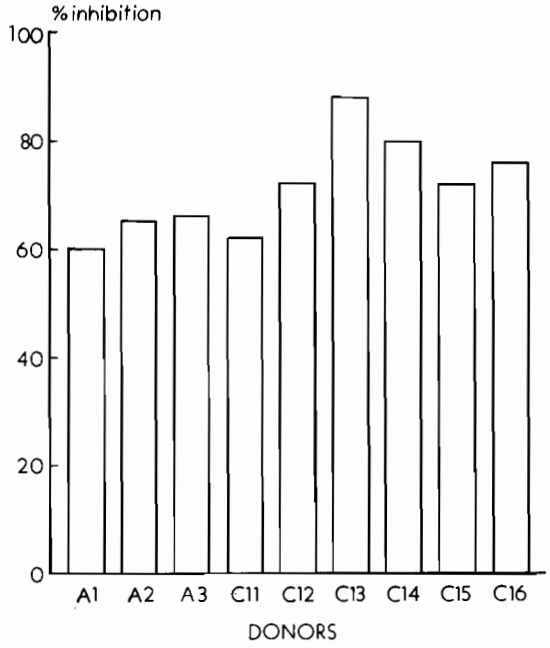

Fig 2–Merozoite invasion inhibition by human plasmas (1:100 dilution). A = Acute, CI = Clinically immune. Data were expressed as percentage inhibition as described in Materials and Methods.

(Table 2). Positive antibody responses to Eo was detected at higher frequency among R group (Table 2). However, the antibody responses in all groups to Ei was significantly greater than the response to Eo ($p \leq 0.001$ for A and CI; $p \leq 0.01$ for R donors).

Investigation of antibody profiles (Fig 1a, b) by peptide ELISA of individual donor revealed that responses to peptides were heterogeneous. Antibodies from most of the donors from A and CI groups reacted to the peptides with different degrees of binding. Stronger responses at a higher frequency was found only with peptide P2 in both the groups (Fig 1a, b). However, high concentration of anti-P2 antibodies was seen in plasma from the CI group than from the A group ($p < 0.01$) (Table 2). The other two peptides were recognized less frequently by the plasma from both groups (Fig 2, Table 2). For the R donors, the most frequent antibody responses with significant difference were against P3 peptide (5/10, $p < 0.01$ for A and CI; Table 2) and there was no positive responses against P2 and P1 ($p < 0.0001$ for A and CI).

Table 2

Anti-malarial antibody responses in the plasma of *P. falciparum* primed donors.

Donors	Ei	Eo	P1	P2	P3	EMIF
CI1	1.2	0.1	0.05	0.9	0.06	250
CI2	1.1	0.3*	0.01	1.01	0.01	1,250
CI3	0.9	0.1	0.02	0.91	0.01	250
CI4	0.8	0.07	0.01	0.91	0.02	1,250
CI5	0.7	0.5*	0.05	0.81	0.1	1,250
CI6	1.0	0.05	0.1	0.6	0.04	250
CI7	0.5	0.11	0.0	0.35	0.04	0
CI8	0.6	0.16	0.0	0.2	0.05	0
A1	1.3	0.1	0.01	1	0.01	1,250
A2	0.6	0.4*	0.07	0.7	0.06	250
A3	0.4	0.16	0.12	0.44	0.02	0
A4	0.5	0.15	0.0	0.28	0.06	50
A5	0.4	0.3*	0.05	0.19	0.02	0
R1	0.5	0.2*	0.04	0.1	0.27#	0
R2	0.7	0.3*	0.08	0.04	0.31#	0
R3	0.8	0.6*	0.02	0.12	0.16	0
R4	0.6	0.5*	0.04	0.05	0.3#	0
R5	0.25	0.16	0.01	0.01	0.2#	0
C1	0.15	0.1	0.05	0.1	0.06	0
C2	0.25	0.12	0.06	0.15	0.13	0
C3	0.14	0.14	0.02	0.08	0.08	0

Anti-malarial antibody responses in sera from unexposed [controls (C)] and exposed subjects of different clinical conditions (CI = clinically immune, A = acute, R = acute with history of repeated malaria episodes) were determined by ELISA in response to Ei (crude malaria antigen), Eo (normal red blood cell lysate), Pf155/RESA peptides P1, P2, and P3 (sequence as given in legend for Fig 1). * = Positive against Eo; # = positive against P3. Antibody responses determined by EMIF are expressed in titer values. Monolayers from D2 isolates were used for EMIF assay.

Significant difference in concentration of anti-Ei antibodies and anti-P2 antibodies was seen in all groups ($p < 0.05$, $p < 0.01$ $p < 0.005$ for A, CI and RI respectively). The antibody response to P2 peptide was significantly greater than the response to Eo ($p < 0.001$ for CI and A). Whereas, antibody response in RI donors against Eo was higher than anti-P2 response ($p < 0.001$).

Correlation analysis between EMIF and sero-reactivities against P2 and Ei revealed a positive correlation only with anti-P2 antibody titers and not with anti-Ei antibody titers ($r = 0.7$, $p < 0.05$, $r = 0.6$, $p < 0.05$ (EMIF and anti-P2); $r = 0.13$ $p < 0.01$, $r = 0.16$ $p < 0.01$ (EMIF and anti-Ei) for CI and A donors respectively

***In vitro* merozoite reinvasion inhibition assay**

To test the biological activity of anti-RESA antibody activity of the plasma, *in vitro* merozoite reinvasion inhibition assay was set up with nine plasma (3 from A and six from CI groups) which had positive anti-EENV (P2) responses. The plasma showed varying degrees of inhibition (40 to 88%) (Fig 2). There was a statistically significant positive correlation ($r = 0.7$; $p < 0.02$) only between anti-EENV antibodies and the merozoite reinvasion inhibition and not with anti-Ei antibodies ($r = 0.08$; $p > 0.2$ for both CI and A groups).

DISCUSSION

Both antibody dependent and antibody independent responses to immunodominant epitopes contribute to protective immunity to malaria. The effector response is both epitope specific and epitope dependent

(Astagneau *et al*, 1991). The design of a malaria vaccine will require identification of immunodominant epitopes in conserved protective antigens and also investigation of the role of such epitopes. The blood stage vaccine candidate antigen Pf155/RESA has been reported to have B cell and T cell recognition domains (Kabilan *et al*, 1987; 1988). In the present investigation, we studied anti- Pf155/RESA activity of Indian *P. falciparum* isolates by EMIF assay. We also studied B cell responses to three synthetic peptides representing the repeat sequence of the C-terminal region of Pf155/RESA in malaria exposed adults of differential clinical background living in malaria hyperendemic area of India.

Repeated exposure to malaria infections result in production of anti-malaria (Ei) antibodies. Humoral reponse to Eo was seen in individuals exposed to malaria, but not in the control donors. This confirms our previous finding (Kabilan *et al*, 1987; 1988) that individuals exposed to malaria become sensitized to normal blood cell components also. However, the high level of autoantibodies (Eo) seen in R group may be due to polyclonal activation of B- cells and/ or induction of specific B cell responses to the extensively destroyed red cell components (Rosenberg *et al*, 1972) due to repeated episodes of malaria.

Although, there may be other Plasmodial cross reacting antigens on the surface of infected erythrocytes (Sulzer *et al*, 1988), Pf155/RESA is the major antigen which is detected by EMIF (Perlmann *et al*, 1987). In Orissa State, *Plasmodium malariae* and *Plasmodium vivax* infections are also prevalent (Yadav *et al*, 1990). However, *P. malariae* contributes only 1% of the total Plasmodial infections. Thus, it is very unlikely that the typical erythrocyte surface staining seen in the EMIF assay with *P. falciparum* isolates in our studies is due to cross reacting antibodies. Different plasma showed different titers with different isolates. If epitopes are conserved then one would expect very similar titers. Whether, end point titer variations of plasma seen with EMIF assay in different isolates may reflect the differences in Pf155/RESA expression in different isolates rather than antigenic diversity remain to be investigated. However, failure of reactivity to possible diverse epitopes would not be distinguished by EMIF technique which was used in our study. This could be detected by specific anti-Pf155/RESA monoclonal antibodies. Demonstration of polymorphism in field isolates is also possible at DNA level.

Significant age differences between highly susceptible (R) and CI group indicate that the development of protective immunity is age dependent. However, the A group in our studies comprises of adults considered to be poorly immune to malaria. These individuals (A) had high anti-Ei antibody titers in comparison with RESA epitope titers. In general, the seropositivity percentage against the peptide P2 was higher in the CI donors than the A donors, suggesting that the CI individuals who had high levels of anti-Pf155/RESA antibodies and no recent history of malaria could be considered relatively immune to malaria.

Acute malaria might induce specific alterations in immune reactivity such as suppression of some T-cell reactions to specific stimuli, induction of tolerance in neonates etc (Goonewardene *et al*, 1990; Pombo *et al*, 1988). The low humoral responses against Pf155/RESA seen in the A and in the R donors may suggest the involvement of anti-Pf155/RESA antibodies in the neutralization of parasitemia and low or no protection against *P. falciparum* infections in subjects with low antibody responses against the epitopes of Pf155/RESA. The inhibitory activity of the antibodies against merozoites seen in our studies also suggest that probably the antibodies against the P2 peptide has an active role in clearing the parasitemia. Although, there are several reports on the inhibitory capacity of anti-RESA antibodies (Perlmann *et al*, 1987; Wahlin *et al*, 1984), affinity purified antibodies on P2 peptide from large number of plasma need to be tested to confirm this finding, since there may be other antibodies which are capable of inhibition of merozoite invasion (Coleman and Jensen, 1984). Further detailed studies are needed to investigate the influence of different humoral responses to different specific epitopes in mediation of protective immunity.

Variation in seropositivity against malaria antigens seen among different groups may be due to genetic restriction of immune response (Good *et al*, 1987) and/or difference in recognition by individuals of epitopes (Björkman *et al*, 1991, Wahlgren *et al*, 1991; Perlmann *et al*, 1989). However, recent studies elsewhere failed to detect associations between MHC (Major Histocompatibility Complex) antigens and immune response to Pf155/RESA antigens in *P. falciparum* primed donors (Troye-Blomberg *et al*, 1991).

Effector responses to different conserved immunodominant epitopes may vary in different subjects depending on the immune status even they are

derived from the same antigen. The present study shows that the C' terminal region of Pf155/RESA contains B cell epitopes which are recognized differently by plasmas from individuals of differential clinical status of malaria. These subjects are living in areas of India where malaria is a serious hazard. Differences in anti-Pf155/RESA antibody responses among this study groups may suggest that studies on epitopes specific immune responses of protective antigens in subjects of differential clinical conditions primed under natural malaria infections has to be taken into consideration in designing a malaria vaccine.

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