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How specific is the immune response to malaria in adults living in endemic areas?

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It is documented that people living in malaria endemic areas acquire immunity against malaria after repeated infections. Studies involving passive transfer of IgG from immune adults to the nonimmune subjects have shown that circulating antibodies play an important role, and that immune adults possess protective antibodies, which susceptible malaria patients do not. Through a differential immunoscreen, we have identified several novel cDNA clones, which react exclusively and yet extensively with immune sera samples. Specific antisera raised against the immunoclones inhibit the growth of parasites in culture. The clones studied so far turn out to be novel conserved *Plasmodium* genes. In order to study the response of sera of adults from malaria endemic areas of India and Africa to these immunogens, we carried out ELISA assays using these immunopeptides, other *P. falciparum* specific antigens, peptides, antigens from other infections such as mycobacterial infections and other proteins such as BSA. Children from the same areas and normal healthy urban people showed very little activity to each of these categories. A large percentage of adults from endemic areas responded positively to all the malarial immunogens tested. However, the same persons also showed high response to other antigens and proteins as well. The implications of these results are reported in this paper.

Key words Circulating malaria antibodies – immunoclones – immunopeptides – malaria

Plasmodium falciparum, the causative agent of the lethal form of malaria, elicits a complex immune response. The parasite exhibits sophisticated mechanisms of immune-evasion and antigenic variation, and these may be the reasons why, even after a hundred years of research on malaria, we do not have an effective malaria vaccine. However, immunity to malaria does exist. It develops gradually, after many attacks and over many years, in adults living in highly endemic areas[1]. The successful passive transfer of this immunity by injecting antibodies from malaria immune persons to malaria patients has demonstrated that the transfer of antibodies alone can trigger protection[2–5]. These experiments worked across geographic borders, as IgG from malaria immune west Africans could cure east Africans[3,4] as well as Thai[5] malarial patients. However, the mechanisms of action, as well as the molecular specificities of such protective IgG, are not very clearly established.

Since passive transfer of IgG from immune adults can cure the patients, the IgG that triggers the curative process must be present differentially in the immune sera. Therefore, a differential immunoscreening of an erythrocytic stage specific cDNA expression library of *P. falciparum* was designed and performed in our laboratory, using malaria immune and acute patient

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sera[6]. This resulted in the identification of several novel cDNA clones, which reacted exclusively and yet extensively with immune sera samples[6]. These coded for a ribosomal protein P0[7], epitopes homologous to SEC65 protein of yeast[8], ion-channel protein of influenza virus[6], switching antigen of *Paramoecium,* and others[9]. With the availability of the genomic data base, the molecular properties of the genes are presented in Table 1. Antisera against most of these epitopes were found in about 50–60% of sera samples used, showing that it is a combination of such protective IgG species that may confer protection amongst immune adults. Most of these protein epitopes are conserved amongst the *Plasmodium* species. We have tested antibodies raised against five of such proteins, and four of these show inhibition of the growth of *P. falciparum* in culture. We have shown that IgG purified from antisera raised specifically to PfP0 inhibit the growth of *P. falciparum* in culture, as well as protected mice against challenge with the lethal 17XL variant of the rodent malarial parasite, *P. yoelii*[10–12]. We have demonstrated that PfP0 protein, in addition to its ribosomal role, is also present on the surface of merozoites and gametocytes, and constitutes the target for blocking invasion into red cells[11–13].

In order to study whether the B-cell antibody response to PfP0, Pf2 and Pf9 epitopes correlated with the age of the residents in regions other than Orissa, India, ELISA assays were carried out using sera samples from Kenya as well. To compare the results with a standard malaria immunogen, we cloned and expressed the apical membrane antigen (AMA1) of *P. falciparum.* Epidemiological data are presented here.

Material & Methods

Human sera samples : Human blood samples were collected from various endemic areas of Orissa and Kenya. Approximately 0.1 to 1.0 ml of blood was collected using heparanised capillaries or tubes and the plasma samples were separated and used at a dilution of 1 : 200. Appropriate ethical clearances were taken for such collection.

Synthetic peptides and AMA1 protein used for ELISA : Synthetic peptides were designed on PfP0 protein based on a hydrophilicity profile. Peptides N1 and N2 represent the amino terminal domain of PfP0, and C1 and C0 represent the carboxy-terminal domains of PfP0[11,14]. The idea for synthesis of C1 peptide was that, it represents the least conserved domain of P0 protein across various organisms[11]. Of the four peptides representing PfP0, C0 is hydrophobic and it undergoes into solution only in presence of ~20% dimethyl sulfoxide. C0 peptide is equivalent to the au-

Table 1. Properties of differential immunoclones

Clone name	Gene name	Southern blot	Predicted protein	Protective	Surface expression
Pf4	PF 11_0313	+	Ribosomal protein P0 (Conserved)	Yes	Yes
Pf2	PF 13_0027	+	Hypothetical 4 TM (Conserved)	Yes	Yes
Pf9	Not found	+	Homologue of *Paramecium* surface protein (Conserved)	Yes	Yes
Pf1	PF 10_0080	+	Put. Endonuclease	ND	ND
Pf3	PF 14_0570	+	(Conserved)	ND	ND
Pf10	PF 14_0737	ND	Hypothetical (Conserved) Viral ion channel Homologue (from cDNA sequence)	ND	ND

ND – Not done.

toimmune P-peptide domain[15,16], antibodies against which have been shown to be invasion blocking[14]. Pf2 and Pf9 peptides were also designed based on their hydrophilicity and immunogenic properties[6,9]. The sequences of all the peptide antigens used for ELISA are given below.

N1	–	DNVGSNQMASVRKSLR (16 residues)
N2	–	SVRKSLRGKATILMGKNT (18 residues)
C1	–	AKADEPKKEEAKKVE (15 residues)
C0	–	EEEEEEDGFMGFGMFD (16 residues)
Pf 2	–	KLMNIKFFEDKNIKLGKC (18 residues)
Pf 9	–	RTCLDAAWTTDKMCS (15 residues)

Another potential malaria vaccine candidate is apical merozoite antigen 1 (AMA1)[17]. A ~35 kDa expression protein from the extreme carboxy terminal domain of *P. falciparum* AMA1 was also used as an antigen for ELISA. This protein was expressed as HIS-tagged protein in pQE30 vector (Savithri *et al*—unpublished data).

ELISA : 96 well (Nunc, Maxisorp) plates were coated with 100 ng of peptide antigens and 200 ng of AMA1 protein in a total volume of 100 μl. Antigens were coated overnight at 4°C. The plates were then blocked with 5% milk in PBS for 1 hr at 37°C. Plates were washed with PBST (PBS containing 0.05% Tween-20) and probed with human sera at a dilution of 1 : 200 in PBS. The antibody binding was allowed to take place at 37°C for 2 h. The plates were washed extensively with PBST and then probed with secondary anti-human antibodies (Boehringer Mannheim) at a dilution of 1 : 2000 in PBS. The secondary antibody binding was carried out at 37°C for 1 h and the plates were then extensively washed with PBST. The reaction was developed using ABTS (Boehringer Mannheim) for 10 min and the absorbance was measured at 405 nm in an ELISA reader (EL808, Biotek Instruments). All the samples were screened in duplicates and the readings represent the geometric mean of the two values. Proper positive and negative controls were also included in each plate.

Results & Discussion

Although PfP0 protein has been expressed as a recombinant GST fusion protein, it was not possible to use this for the ELISA assay, as GST alone gave a very high reactivity with most human sera samples. Thus it was decided to use various synthetic peptides to assess the antibody reactivity of the samples. PfP0 was represented with four peptides; N1 and N2 representing the amino terminal domain, and C1 and C0 representing the carboxy-terminal domains. Pf 2 and Pf9 peptides were used for these two antigens. The carboxy-terminal 250 amino acid domain of AMA1 was used as a HIS-tag fusion protein.

Fig. 1 shows the ELISA assays with serum samples from Orissa, while Fig. 2 shows the results with serum samples from Kenya. It is clear that the response of adults is much higher as compared to that of children for each of the peptide antigens used. This is consistent with our differential screen, which had used immune versus susceptible sera for screening. These results also show that the reactivity of these peptide antigens with sera from immune adults is also observed in Kenya and Africa, and is not a peculiarity of Orissa in India. Thus, the reactivity of these samples showed that immune response is generated against these epitopes in Africa as well as in India. It was observed that the same immune serum samples reacted with the peptides, although the cut-off parameters predicted different frequencies of reactivities for different epitopes. It has been shown that none of these peptides are cross-reactive and that these are distinct antigens on *P. falciparum.* Irrelevant peptides such as transcription factors and RNase1 also showed similar patterns of activity, although the reactivity was much lower (Fig. 3). Such data suggest that the immune responsiveness is a state attained by the immune adults, and that there is a general polyclonal activation achieved against several parasite determinants in the immune adults. Hypergammaglobulinimia has been reported earlier for such persons[2]. However, the reactivity against AMA1 protein did not fit that pattern, and was indeed more prevalent amongst children as

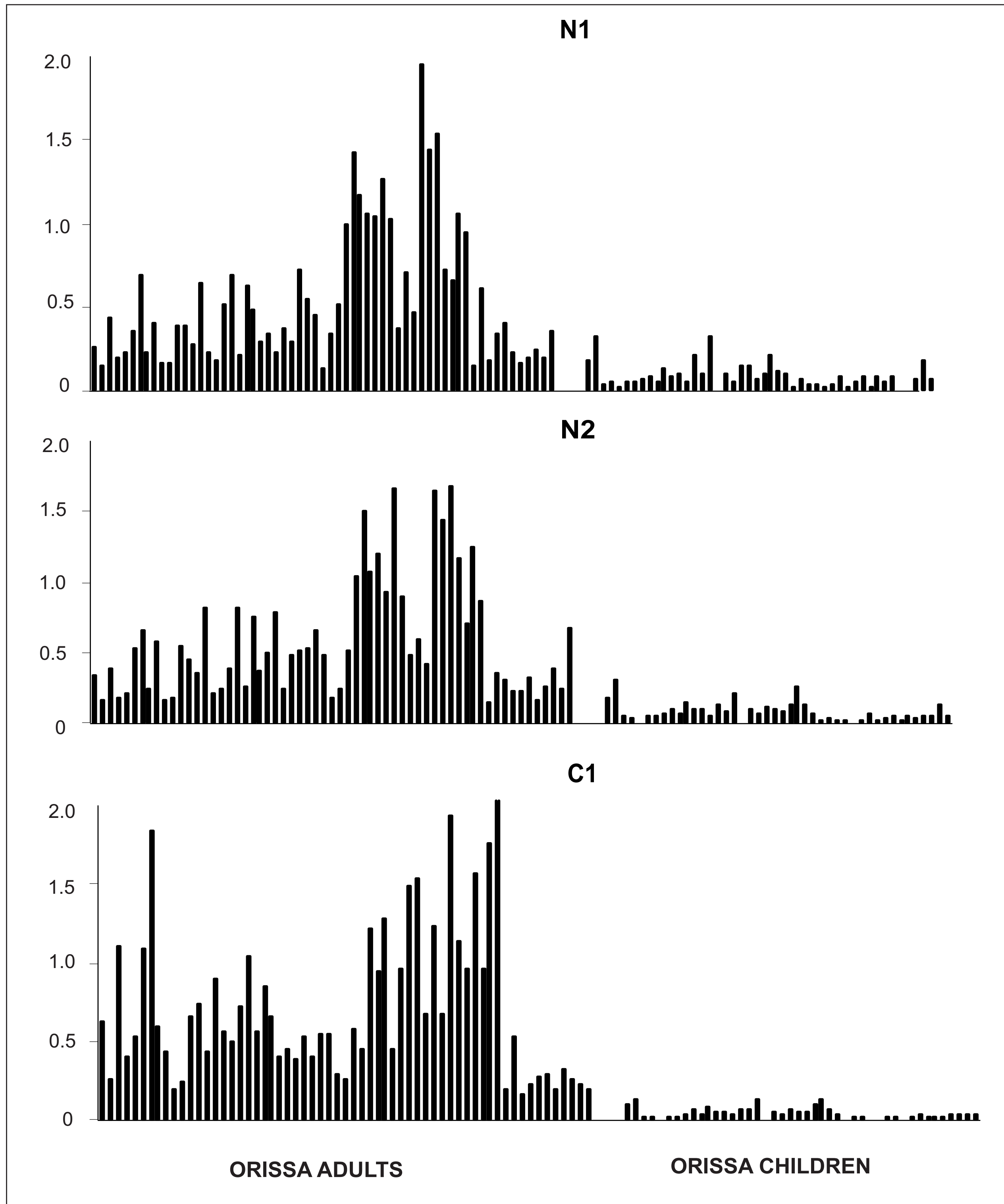

Fig. 1: ELISA analysis histogram of 60 and 40 sera samples from adults (>12 years) and children (< 12 years) respectively, residing in the Keonjhar district of Orissa for the three peptides N1, N2 and C1 of the PfP0 protein. Y-axis—— OD_{405} value

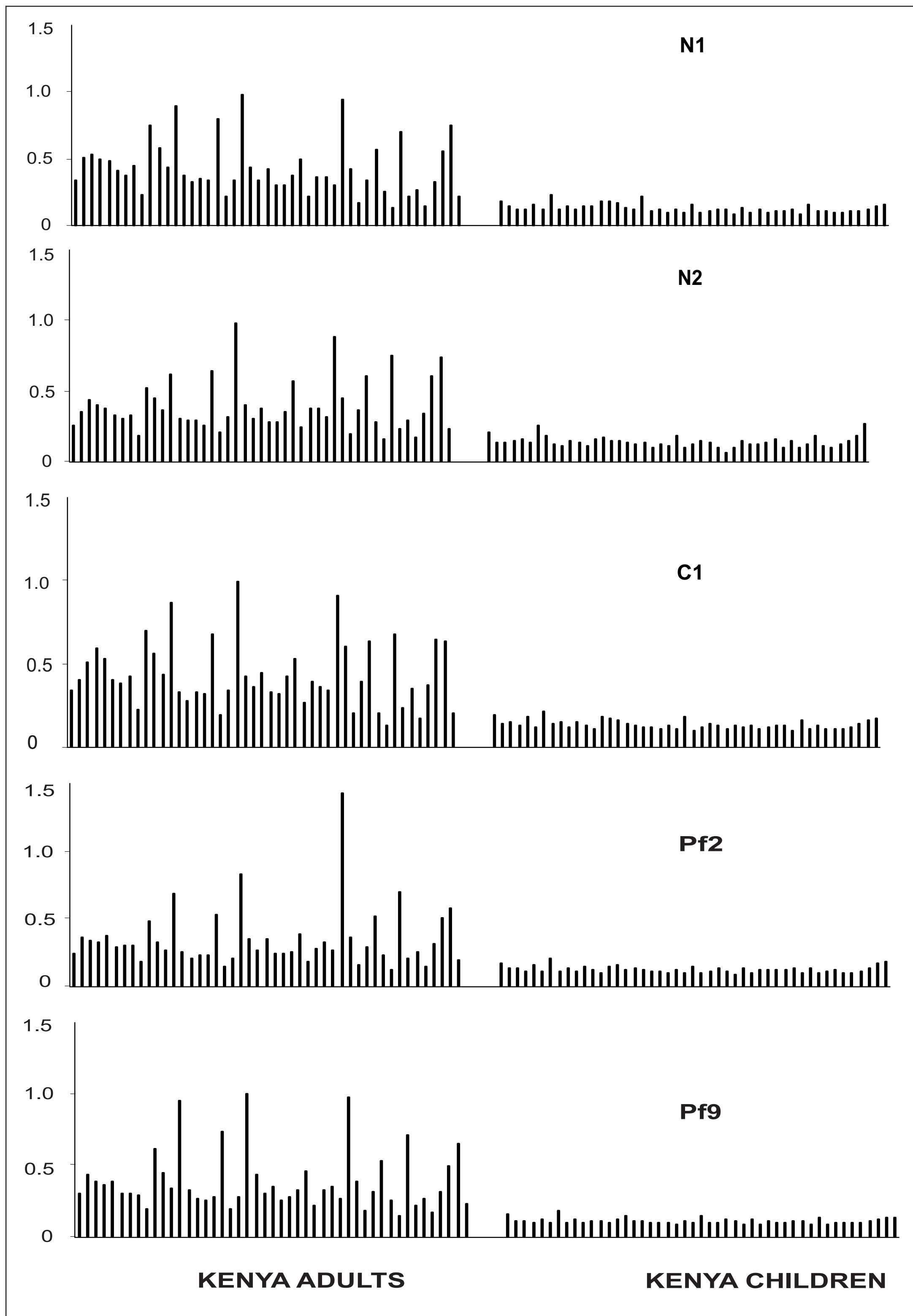

Fig. 2: ELISA analysis of 47 sera samples from adults (>12 years) as well as children (<12 years), residing in the Msambweni district of Kenya for the five peptides; N1, N2 and C1 of the PfP0 protein; Pf2 and Pf9 peptides. Y-axis— OD_{405} value

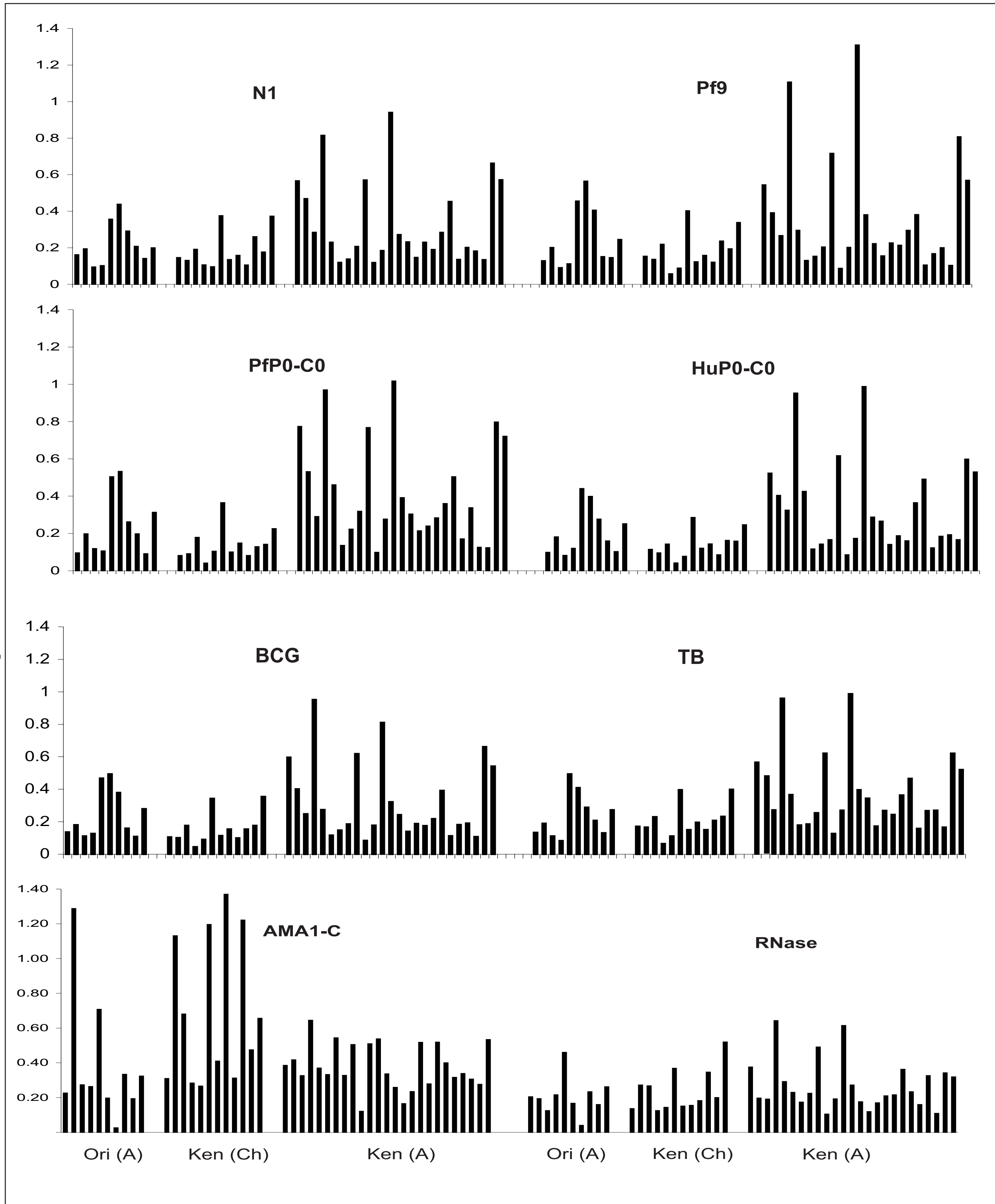

Fig. 3: ELISA analysis of sera samples from adults (>12 years) and children (<12 years) residing in the Keonjhar district of Orissa, India and the Msambweni district of Kenya for the peptides and antigens. Antigens used were : N1 and PfP0-C0 peptides of the PfP0 protein; HuP0-C0 : the equivalent C0 peptide of the human P0 protein; Pf9 peptide; BCG; TB : a surface antigen from *Mycobacterium tuberculosis*; RNase, and AMA1-C : AMA1-carboxyterminal domain protein. Y-axis— OD_{405} value

compared to adults from these malaria endemic areas (Fig. 3). Longitudinal study of natural immune responses to AMA1 has been carried out in several holoendemic regions of Africa, and different domains of AMA1 appear to react differently[17–19]. Invasion blocking antibodies to the amino-terminal domain of AMA1 has been reported[19], but the response to the carboxy-terminal domain is as yet unreported. From our results it appears as though the response to the carboxy-terminal domain is restricted in immune adults.

It is observed that parasites protect themselves from host immune responses by using proteins that mimic host cells. The ribosomal protein P0 itself is closer to the mammalian counterpart, as compared to other protozoan species[7]. Other malaria epitopes that are established to be crucial for hepatocyte and red cell invasion (TRAP and MSP1) are also related to host motifs (thrombospondin and epidermal growth factor (EGF-like folds). Chronic malaria is known to generate antibodies that cross-react with host red cells and other host-proteins. However, it is also documented that persons living in malaria endemic areas suffer less with autoimmune disorders than people in malaria free areas[20]. How can we explain this?

It is envisaged that the protective IgG present in the malaria immune adults can only limit the parasites, and not clear the parasitaemia completely. The protective immune response is hypothesised to be dependent on the presence of small sub-clinical levels of parasite[21,22]. Indeed, such naturally acquired immunity is lost if the subject moves out of endemic areas, and thus the exposure to pathogen is lost. The establishment of a host-parasite interaction such that the host is clinically normal, and the parasite is also transmitted, is an equilibrium situation normally favoured in evolution. This state arises when there is enough immune response to control the levels of parasitaemia. It is known that malarial parasite generates immune evasive 'smoke-screen epitopes' to keep the host immune response busy[23]. The immune response against such smoke-screen epitopes may not control the parasite levels effectively. An effective control occurs only when immune responses against crucial epitopes are generated. Since the crucial epitopes are essentially mimics of the host, we start seeing the auto-reactivity. However, the presence of the parasite appears to limit such immune response. Elegant models have been proposed indicating a feedback mechanism, such that if the parasite level falls below a certain limit, the 'effective immune response' gets turned off[21]. Even though molecules such as TNF and NO have been implicated in such a process[20], the actual mechanism(s) is (are) not yet understood. In order to use the naturally acquired immunity to malaria for the purpose of a vaccine, we need to understand these close interactions between parasites and host immune cells.

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