The age-specific prevalence of *Plasmodium falciparum* in migrants to Irian Jaya is not attributable to agglutinating antibody repertoire

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The age-specific prevalence of *Plasmodium falciparum* in migrants to Irian Jaya is not attributable to agglutinating antibody repertoire

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Abstract

Previous observations have shown that individuals migrating from a malaria free area to a malaria endemic region in North Eastern Irian Jaya quickly acquire anti-parasite immunity, in an age-dependent manner. Sera from migrants and long-term residents in this area were examined for their ability to agglutinate a range of *Plasmodium falciparum* isolates and to disrupt erythrocyte rosettes. Antibody responses to merozoite surface protein 2 (MSP2) and ring-infected erythrocyte surface antigen (RESA) were also determined. The range of isolates agglutinated by sera from the migrants approached that seen in long-term residents. No difference was found between migrant adults and children in the range of agglutinating antibody, size of agglutinates, nor disruption of rosettes. Anti-MSP2 and anti-RESA antibodies were the only factors examined which showed a correlation with age. We conclude that although antibody to parasite neoantigens expressed on the surface of infected erythrocytes may play a role in the acquisition of immunity, the humoral response to other *P. falciparum* antigens is more likely to account for the age-dependent prevalence of parasitaemia observed. © 1997 Elsevier Science B.V.

Keywords: Malaria; *Plasmodium falciparum* acquired immunity; Irian Jaya

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1. Introduction

Antibodies directed against neo-antigens expressed upon the surface of trophozoite-infected erythrocytes have been associated with protection from *Plasmodium falciparum* malaria (Marsh et al., 1989). In vitro these antibodies are capable of agglutinating parasite infected cells (Marsh et al., 1986) and disrupting erythrocyte rosettes (Udomsangpetch et al., 1989). Agglutination assays using field isolates have shown a wide diversity in the surface antigens of infecting parasites (Forsyth et al., 1989; Southwell et al., 1989; Reeder et al., 1994) and it is believed that an important component of natural immunity is the building of a broad repertoire of antibody specificities following exposure to many variant antigenic types.

A valuable insight into acquired protection from malaria has been afforded by studies in Indonesia, following cohorts of people migrating from a malaria-free area in Java to a malaria endemic area of North Eastern Irian Jaya (Baird et al., 1991a, 1993). The first of these studies (Baird et al., 1991a), examined the migrant and indigenous inhabitants of a single village and discovered that migrants acquired good anti-parasite protection after 1–2 years of malaria exposure. The acquisition of immunity was further shown to be age dependent, as parasite prevalence decreased with age. The trend of age dependent prevalence of *P. falciparum* was reinforced in a second study (Baird et al., 1993), expanding the study group to six villages occupied by Javanese migrants for between 3 weeks and 72 months. Here, age dependency was not apparent during the first year, but became obvious after 1–2 years of exposure.

These observations left open the question of the mechanism of acquired immunity and the reasons for its age dependency. If protection was indeed due to infection with a number of waves of parasites of different variant antigenic types and exposure of the different age groups to infection was similar, why would parasite prevalence differ with age? Although ‘intrinsic immune factors’ have been suggested as an explanation (Baird, 1995), we felt it worthwhile to examine differences in the repertoire of agglutinating antibodies. Age related differences in the repertoire could in theory arise if adults demonstrate a greater capacity to generate responses to cross reacting epitopes or even an increased propensity for generating or tolerating antigenic variants in vivo (Roberts et al., 1993), thus allowing development of a wider range of agglutinating specificities.

An alternative explanation for the age-prevalence may lie in differences in response to antigens other than the erythrocyte surface antigens. Studies in neighbouring Papua New Guinea have shown humoral response to the merozoite surface protein 2 (MSP2) and the ring-infected erythrocyte surface antigen (RESA) to be both age/exposure dependent and related to the incidence of clinical malaria (Al-Yaman et al., 1995a).

In this study we examined the capacity of serum from migrant adults and children and long-term resident adults to agglutinate a panel of culture established *P. falciparum* isolates and disrupt erythrocyte rosettes, another response previously associated with age related acquisition of immunity (Rogerson et al., 1996) and
protection from severe disease (Carlson et al., 1990). We also determined antibody response to MSP2 and RESA.

2. Materials and methods

2.1. Study group

Serum samples were obtained during a survey in the village Arso PIR I, North Eastern Irian Jaya, in November 1989, 45 months after its occupation by Javanese migrants. A detailed description of this region and study group has been given elsewhere (Baird et al., 1991a,b, 1993). In the prior two surveys of migrants in Arso PIR I, after 35 and 38 months of residence, \textit{P. falciparum} prevalence had been 41/33\% in age group 6–15 years and 22/24\% in the age group over 15 years, respectively (Baird et al., 1993). At the time of sampling the prevalence in migrant children was 30\%, in migrant adults, 24\% and in adult long-term Irian Jayan residents, 10\%.

For the current analysis, 30 sera were obtained from three sub-groups in the study population, ten from indigenous Irian Jayan adults (IA) aged 20–30 years, ten from adult migrants from Java (JA) aged 20–30 years and ten from children migrants from Java (JC) aged 6–12 years. The serum samples were stored at $-70^\circ\text{C}$, then thawed, heat inactivated at 56°C for 1 h, absorbed on group AB erythrocytes for 1 h at 37°C and then held at $-20^\circ\text{C}$ prior to performing assays. The sera were coded in a random order and tested blind.

2.2. Parasites

Sixteen cryopreserved isolates of \textit{P. falciparum} were utilised to assess the breadth of the agglutinating repertoire of the test sera. These comprised seven laboratory lines (FAF6, E8B, Holmes, FC7, BE8, HCS3 and BB12), eight culture established isolates from Irian Jaya (2300, Arso1, Arso2, Arso3, Arso6, G73, CM87 and G132) and one isolate from Papua New Guinea (PNG44). Isolates were sequentially thawed and cultured in vitro. Cultures were performed at 37°C in RPMI 1640 media (Trace Biosciences, Australia) buffered with 25 mM HEPES and supplemented with 25 mM sodium bicarbonate, 2 mM L-glutamine, 25 \(\mu\)g/ml gentamicin, 0.5 ng/ml hypoxanthine and 10\% human blood group AB serum, in an atmosphere of 5\% CO₂, 1\% O₂ and 94\% N₂. Parasites were cultured for variable times until a minimum parasitaemia of 1\% had been achieved and most parasites were at the late trophozoite stage.

2.3. Parasite-infected cell agglutination

The method was essentially as previously described (Reeder et al., 1994). Briefly, after culturing parasites to late trophozoite stage, the infected erythrocytes were washed three times in human tonicity phosphate buffered saline (PBS). Cells were
then distributed in 50 μl aliquots, micro-centrifuged and resuspended in 25 μl volumes of PBS containing ethidium bromide (1 μg/ml). Heparin at 1000 U/ml was also added to agglutination reactions to disrupt rosettes (Brown et al., 1996). Patient serum for assay was then added (2.5 μl) and the tubes were incubated on a rotating wheel at 37°C for 1 h. All 30 sera were tested on each culture suspension at the same time. Two 10 μl aliquots of each sample were examined microscopically under ultraviolet light and a semi-quantitative assessment of agglutination was recorded using the arbitrary scale described in Table 1. A negative result was recorded if no more than three agglutinates of 3–5 cells were seen in either of the aliquots examined.

2.4. Rosette disruption

Assays of ability of the sera to disrupt erythrocyte rosettes were performed using the rosetting P. falciparum line BB12, as described previously (Rogerson et al., 1994, 1996). Briefly, a 25 μl aliquot of cultured BB12 (1–5% trophozoites, 30–70% of trophozoites forming rosettes) was incubated at 37°C for 30 min with 25 μl sera in PBS at a final dilution of 1 in 10. The cells were gently resuspended and 15 μl mixed with 5 μl ethidium bromide on a glass slide. Three hundred trophozoite-infected cells were counted by fluorescence microscopy and the number forming rosettes scored. Each assay was performed in duplicate and expressed as a percentage of the rosetting rate in the original culture.

2.5. Antibodies to MSP2 and RESA

ELISA assays were performed by standard protocols using serially diluted patient sera on microtitre plates coated with either MSP2 type 3D7 or RESA (1505H) at 2 μg/ml. Sheep anti-human IgG (Silentis, Australia) conjugated to horseradish peroxidase was used as the secondary antibody. Results were reported as the mean OD450 of duplicate samples at a serum dilution of 1:10000.

3. Results

3.1. Parasite-infected cell agglutination

Agglutination assays were performed on each of the 30 patient sera against the panel of 16 P. falciparum cultures. The scores are reported in Table 1. In order to assess the comparative breadth of agglutinating antibody repertoire a score was assigned to each serum based on the number of the 16 isolates tested which they agglutinated. The spread of the results is illustrated in Fig. 1A. The mean (standard deviation) numbers of isolates agglutinated by each group of sera were: Irian Jayan adults 6.8 (± 4.1), Javanese adults 3.9 (± 1.3) and Javanese children 4.9 (± 1.7). Analysis of the agglutination scores by t-test demonstrated no significant difference (P > 0.1) between the sera from Javanese adults and children, nor between Irian
Table 1
The results of agglutination assays against cultured *P. falciparum* isolates, rosette disruption, anti-MSP2 and anti-RESA antibody levels of sera from indigenous adults and migrant adults and children

<table>
<thead>
<tr>
<th>Serum Code</th>
<th>ELISAa</th>
<th>Rosette disruption (%)</th>
<th>Agglutination assaysb</th>
</tr>
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<tr>
<td></td>
<td>MSP-2</td>
<td>RESA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FAF6</td>
<td>E9B</td>
<td>FC7</td>
</tr>
<tr>
<td></td>
<td>BE8</td>
<td>HCS3</td>
<td>BB12</td>
</tr>
<tr>
<td></td>
<td>PNG44</td>
<td>2300</td>
<td>Aros1</td>
</tr>
<tr>
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<td>Aros2</td>
<td>Aros3</td>
<td>Aros6</td>
</tr>
<tr>
<td></td>
<td>G73</td>
<td>CM87</td>
<td>GJ32</td>
</tr>
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<td>1.5</td>
<td>1.1</td>
<td>47.5</td>
</tr>
<tr>
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<td>0.7</td>
<td>84.4</td>
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<td>0.4</td>
<td>0.3</td>
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</tr>
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<td>0.6</td>
<td>50.1</td>
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<td>50.3</td>
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<td>1.5</td>
<td>14.8</td>
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<tr>
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<tr>
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<tr>
<td>JC 10</td>
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<td>1.2</td>
<td>30.1</td>
</tr>
</tbody>
</table>

IA, Irian Jayan adult; JA, Javanese adult; JC, Javanese child.

*a* OD$_{405}$ at serum dilution 1:10 000

*b* Agglutinates of: - , 3–5 cells; + , >5–20 cells; ++ , >20–100 cells; +++ , >100 cells.
Fig. 1. (A) Number of positive agglutination reactions (out of 16 isolates tested); (B) rosette disruption (BB12); (C) Anti-MSP-2 antibody (OD450 at 1:10 000); and (D) anti-RESA antibody (OD450 at 1:10 000) in sera from indigenous adults and migrant adults and children.
Jayan adults and Javanese children. Irian Jayan adults in general agglutinated more isolates than did Javanese adults \((P < 0.05)\). Regression analysis showed no correlation \((r < 0.3)\) between the numbers of isolates agglutinated and either rosette disruption, anti-MSP2 or anti-RESA antibody.

As an arbitrary measure of the strength of agglutination, the mean (sd) agglutination score was calculated in each group. Irian Jayan adults \((\bar{x} = 2.1 \pm 0.3)\) had a larger agglutination size than Javanese adults \((\bar{x} = 1.7 \pm 0.4, P < 0.01)\) and Javanese children \((\bar{x} = 1.8 \pm 0.4, P < 0.05)\), but no difference existed between the two migrant groups \((P > 0.1)\).

3.2. Rosette disruption

The ability of sera to disrupt erythrocyte rosettes varied widely within each group (Table 1 and Fig. 1B) with mean (S.D.) values of 37.6 ± 27.9\% for Irian Jayan adults, 17 ± 21.2\% for Javanese adults and 32.8 ± 31.7\% for Javanese children. Analysis of these figures revealed no significant differences between any of the three groups \((P > 0.05)\). No correlation was observed between rosette disruption, number of agglutinations or anti-RESA or anti-MSP2 antibody \((r < 0.2)\).

3.3. Antibodies to MSP2 and RESA

The comparative levels of antibody to MSP2 (3D7) and RESA (1505H) in the sera are reported in Table 1, as OD\(_{450}\) values at a serum dilution of 1:10 000. The distribution of antibody levels in the three groups is illustrated in Fig. 1C and D. The level of antibody to MSP2 in migrant children \((\bar{x} = 0.4 \pm 0.4)\) was significantly lower than that seen in migrant adults \((\bar{x} = 1.2 \pm 0.7, P = 0.01)\) and Irian Jayan adults \((\bar{x} = 1.1 \pm 0.6, P = 0.02)\). There was no significant difference in anti-MSP2 antibody levels in the two adult groups \((P > 0.1)\). Antibody levels to RESA in Javanese children (mean 0.5 ± 0.4) appeared lower than in Irian Jayan \((\bar{x} = 0.9 \pm 0.7)\) or migrant adults \((\bar{x} = 1.1 \pm 0.8)\), but not significantly so \((P = 0.08)\). There was no significant difference in anti-RESA antibody levels in the two adult groups \((P > 0.1)\). In a similar manner to the previous studies (Baird et al., 1991a), those sera with an anti-RESA OD\(_{450}\) in excess of 0.3 at a serum dilution of 1:10 000 were categorised as strong positives. In both Irian Jayan adults and Javanese adults 70\% of the sera tested were strongly positive for anti-RESA. Only 40\% of Javanese children were in this category.

4. Discussion

Sera from 30 volunteers resident in a malaria hyperendemic region of North Eastern Irian Jaya for 45 months were examined and characteristics of their antibody response to parasite neo-antigens on the surface of *P. falciparum* infected erythrocytes determined. This was done in order to assess whether differences in the repertoire of these antibodies could contribute to the phenomenon of the age
specific prevalence of *P. falciparum* infection previously observed amongst recent migrants to this region (Baird et al., 1991a, 1993) and still present in the population studied.

Irian Jayan adults, with a lifetime of malaria exposure, were shown to have a broad range of agglutinating specificities. This supports the argument that immunity is acquired as agglutinating antibody repertoire increases over time, with exposure to a range of independent strain-specific antigenic types (Gupta and Day, 1994; Gupta et al., 1994). The breadth of agglutinating specificities seen in migrants was surprisingly extensive considering the relatively short time of exposure. The time taken for acquisition of a range of agglutinating specificities is, however, highly variable and dependent upon the region and pattern of exposure, as can be seen from the results of past studies in which children from The Gambia possessed agglutinating antibodies to a range of isolates (Aguiar et al., 1992), whereas serum from a group of Papua New Guinean children rarely recognised parasites other than the current infecting strain (Reeder et al., 1994). In the present study, no difference was apparent in the range of agglutinating antibody present in the sera of migrant adults and children, suggesting a similar level of exposure to malaria and a similar degree of response to the variant antigen.

Whilst first exposure to a particular variant antigenic type might confer some degree of anti-disease immunity, it has been suggested that the boosting of antibody by re-exposure to parasites of the same type may be required to eliminate parasites (Gupta and Day, 1994). Accordingly, we examined the magnitude of the agglutination reactions and found that Irian Jayan adults had higher mean agglutination scores than the migrant group.

The ability of sera to disrupt erythrocyte rosettes has been previously associated with exposure to malaria (Rogerson et al., 1996) but its relationship to immunity is unclear. Studies from Africa have correlated rosette disruption with protection from severe disease (Carlson et al., 1990), but this may be region specific, as similar studies from Papua New Guinea have not (Al-Yaman et al., 1995c). All sera in the present study were examined for their ability to disrupt rosettes, to assess whether this might contribute to differences in acquisition of immunity. No significant difference was found between the level of disruption by sera from either adults or children in the migrant group nor, interestingly, Irian Jayan adults. This contrasts with studies in Papua New Guinea (Rogerson et al., 1996) which found rosette disrupting ability to be infrequent in young children from a malaria endemic area yet common in adults. One must be cautious, however, of making comparisons of groups from different regions, as the testing utilises a single parasite strain (BB12) that may be better recognised by one group than another.

A number of previous studies, including extensive surveys in Papua New Guinea, have shown anti-MSP2 and anti-RESA antibody prevalence and concentration increases with age in a malaria endemic area (Al-Yaman et al., 1995a), even through adulthood (Al-Yaman et al., 1995b; Beck et al., 1995). This increase in antibody is strongly correlated with a reduction in malaria episodes (Al-Yaman et al., 1994). As the participants in these studies had lifelong exposure to malaria the apparent age-dependency of antibody levels could not be separated from the length
of exposure. However, in the current study, in which adults and children have been resident in a malaria endemic area for the same length of time and the malarialometric and agglutination profile data suggest similar levels of exposure to infection, it becomes possible to look for inherent age related differences in humoral response to these antigens.

The trend in anti-RESA antibody levels in the current study reflects the findings of the original survey in the Arso region (Baird et al., 1991a) where Javanese migrant adults had similar anti-RESA antibody levels to Irian Jayan adults, but the migrant children had lower levels than either adult group. More striking here was the highly significant difference in anti-MSP2 (3D7) antibody levels in adults and children in the migrant group. The indication of an age-dependent response to MSP2 (3D7) is particularly interesting in light of its prominence as a malaria vaccine candidate. However, one must be cautious in interpreting a direct causative relationship between antibody levels to these particular antigens and the increase in prevalence, as they may be only indicators of more important immune effector mechanisms.

Previous studies in the Arso region of Irian Jaya have shown that both adult and children migrants from a malaria free to a malaria endemic region quickly acquire anti-parasite immunity, yet the prevalence of parasites is age dependent (Baird et al., 1991a, 1993). The broad agglutinating repertoire and large mean agglutination score of the long-term residents of the area and the lower incidence of parasitaemia and symptomatic disease in this group (Baird et al., 1991a) conforms well to the model of agglutinating-antibody related immunity discussed. The lack of any difference in either the breadth of agglutinating specificities or the mean agglutinate size between migrant adults and children also supports the basic model of acquisition of strain-specific antibodies related to exposure, rather than suggesting any intrinsic difference in the manner in which adults and children respond to the variant antigens. The agglutinating-antibody response cannot therefore explain the age-specific prevalence of \textit{P. falciparum} observed in the migrant population. The finding that antibody to MSP2 and RESA was lowest in migrant children suggests that humoral response to \textit{P. falciparum} antigens other than those expressed on the erythrocyte surface is more likely to account for the age-dependent element of immunity observed.

\textbf{Acknowledgements}

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