In areas with intense and stable malaria transmission, acquired immunity develops gradually. Immune adults are able to control high parasitaemia and are protected from severe disease. The relative contributions of different immune mechanisms to protection against malaria infection and disease have still not been determined. However, the importance of humoral immune responses has been demonstrated by the protective effect of passively transferring serum from immune to non-immune malaria patients. A major issue in current vaccine development is the identification of antigens that might contribute to such a protective response.

Many asexual blood stage antigens have been identified, but assessment of their role in inducing protective immune responses is difficult. Several factors determine if a response is protective, including the quantity of antibodies produced, functional specificity and differential affinity of antibodies to malaria antigens. Nevertheless, many studies have demonstrated associations between malaria morbidity and/or infection and immune responses to specific malaria antigens, such as ring-infected erythrocyte surface antigen (RESA), merozoite surface protein 1 (MSP1), merozoite surface protein 2 (MSP2). Another common feature in all studies is that there is heterogeneity in immune responses to specific malaria antigens and the prevalence of responses to given antigens is never 100%. Since such variability in immune responses could lead to ineffectiveness of a vaccine, there is considerable interest in identifying factors that are responsible for the regulation of the amount of protective antibodies produced.

Assessment of different sources of variation in the antibody responses to specific malaria antigens in children in Papua New Guinea

HA Stirnadel, F Al-Yaman, B Genton, MP Alpers and TA Smith

Background A potential problem for malaria vaccine development and testing is between-host variation in antibody responses to specific malaria antigens. Previous work in adults in an area highly endemic for *Plasmodium falciparum* in Papua New Guinea found that genetic regulation partly explained heterogeneity in responsiveness. We have now assessed the relative contributions of environmental and genetic factors in total IgG responses to specific malaria antigens in children, and quantified temporal variation within individuals of total IgG responses.

Methods Total IgG responses against schizont extract, merozoite surface protein-1, merozoite surface protein-2, ring-infected erythrocyte surface antigen, and SPf66 were measured by ELISA. Variance component analysis was used to estimate the variation explained by genetic and environmental factors in these antibody responses. Intra- and inter-class correlations of antibody responses within relative pairs were estimated. We adjusted for age, *P. falciparum* density, sex and village differences either within or prior to the analysis.

Results For all malaria antigens, temporal variation in the total IgG response was the predominant source of variation. There was substantial familial aggregation of all IgG responses, but it remained unclear how much this clustering was attributable to genetic factors and how much to a common environment in the household. The remaining variance, which could not be explained by either of the above, was very small for most of the antigens.

Conclusions Temporal variation and clustering of immune responses to specific malaria antigens need to be taken into account when planning, conducting and interpreting immuno-epidemiological and vaccine studies.

Keywords IgG, malaria, variance component analysis, children, Papua New Guinea

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The importance of both environmental and genetic factors influencing the regulation of humoral immune responses in general has been known for a long time. In malaria research, the majority of studies have been concentrated on associations between MHC genes and immune responses to specific malaria antigens. A limited number of twin studies also demonstrated genetic regulation of immune responses to specific malaria antigens in Liberia, Madagascar, and The Gambia. In addition, family studies assessed the relative contribution of environmental in relation to genetic components in immune responses to specific malaria antigens in Papua New Guinea (PNG). Overall, most of these studies have concentrated on immune adults and on measures of immune responses at a single time point. Since children are the actual target group for vaccination and they suffer most from infection and disease, they should be included in such an analysis. Variability in the antibody acquisition may suffer most from infection and disease, they should be included in such an analysis. Variability in the antibody acquisition may be just as relevant as variation in the final levels attained. In addition, examining longitudinal data would be useful since temporal individual variation in immune responses to specific malaria antigens has been demonstrated in children, but was small in adults.

This study reports on the quantification of the relative contribution of environmental and genetic factors in the regulation of immune responses to specific malaria antigens, measured at different time points, in children. Variance component analysis was conducted in order to assess genetic and environmental components in the antibody responses and to estimate antibody variation over time within each individual. Estimates of variance components were then used to calculate heritabilities. In addition, inter- and intra-class correlations of antibody responses to specific malaria antigens within relative groups were assessed.

Materials and Methods
Study area and population

The data were collected and processed within the Malaria Vaccine Epidemiology and Evaluation Project (MVEEP) of the PNG Institute of Medical Research. This project is based in the Wosera area in the East Sepik Province of PNG, where malaria is highly endemic and transmitted throughout the year. Plasmodium falciparum, P. vivax, and P. malariae are the common parasite species in this area. The malaria epidemiology of the study area has been described earlier. For this study, we analysed data from three cross-sectional surveys (October–November 1992, March 1993 and July 1993). There were no clear seasonal patterns to malaria transmission during this period. Within these surveys, blood samples were collected from 236 children aged between 0.5 and 15 years old in two adjoining study villages, Kunjingini 1 and Apusit, comprising all available children whose parents gave consent. The house of residence was recorded for each child. In addition, P. falciparum densities were assessed by microscopy and recorded as number of parasites per 200 white blood cells. Further details of field work are given by Al-Yaman et al.14

Structured questionnaires were used in order to gather information on genetic relationships in the study population of the MVEEP. All present and willing adults were asked about their first- and second-degree relatives. Validation of familial relationships of adults was conducted using HLA class I and II information as stated previously. Additional interviews were performed in order to confirm the familial relationships between children and their parents in the study area. Therefore, all present parents were visited at their houses and asked again about the names and ages of their putative genetic children. This information was then cross-checked with data already available. Genetic relationships of only three children from two families did not match previous data. With additional questioning, they could be assigned to the appropriate families.

Malaria antigens

Schizont extract was prepared from the FUP (Falciparum-Uganda-Palo Alto) strain as described earlier. RESA was a recombinant protein expressed in Escherichia coli as described in Al-Yaman et al. Three different recombinant proteins of MSP2—two full length constructs FC27 and 3D7 and one MSP2 (3D7) with the repetitive part deleted d3D7—were used as described previously. The parasite-derived MSP1 molecule and two recombinant antigens of MSP1, 195A and Bvp42 were used, which were described earlier. SPf66 was a synthetic peptide as described previously.

Humoral responses

Indirect enzyme-linked immunosorbent assays (ELISA) were performed in order to measure antibodies against schizont extract, MSP1, MSP2, RESA and SPf66 (description by Al-Yaman et al.). Antibody units were assessed by comparison of optical densities measured at 405 nm with a standard curve derived from a twofold serial dilution of a standard high-titre serum pool. Detailed description of the prevalence and distributions of antibody responses has been given by Al-Yaman et al.

Statistical analysis

Prior to all analyses, normal score transformations were applied to all immune responses for each survey separately. Parasite density was log-transformed preceding all analysis. For descriptive purposes, the mean antibody responses were calculated for each individual over the three surveys, before assessing differences between sexes and villages and correlation with age, and parasite density. The effects of age, sex, village and parasite density were assessed by fitting an analysis of variance model to normalized data, allowing for repeated measurements on the same child with a random effect.

In order to quantify the relative contribution of genetic and environmental factors in antibody responses to specific malaria antigens, variance component analysis was performed using the FISHER program. Hence, the overall variation in antibody responses against each antigen was partitioned into genetic and non-genetic variance. The genetic variance is the sum of the additive genetic (AV) and dominance variance (DV). Whereas the AV is the variation deriving from additive effects of individual alleles at one or more loci, the DV is the variation due to dominance deviation from additivity at the same locus. Dominance variance contributes only to relatives who share both alleles IBD (identical by descent). The non-genetic variance consists of the house variance (HV), within child variance (WCV) and the remaining non-genetic variance (NGV). The HV describes the variation in the antibody responses which is explained by living in the same house, and
is implemented into the variance component model as described previously. The variation in the antibody responses against a specific antigen between surveys is quantified by WCV.

preceding variance component analysis data were corrected for age, village and parasite density using multiple regression for each trait separately. The measured antibody responses of each survey were treated as three separate traits, which were analysed at the same time.

To assess the goodness-of-fit, likelihood ratio tests (LRT) were performed between two models, as minus twice the difference of the log-Likelihood (lnL). The degrees of freedom (d.f.) for lysed at the same time.

The number of informative relative pairs, which have immune

The distributions of the antibody responses against different malaria antigens for each survey are summarized in Table 2. Antibody responses varied within and between surveys depending on the antigen. The median antibody responses against schizont extract, all MSP2 antigens and MSP1 increased from November 1992 to July 1993, but decreased for antibody responses against RESA and SPf66. The antibody responses against 195A and BVp42 were higher in November and July than in March. Overall, antibody responses differed significantly between the surveys, except for the antibody response against MSP2 (3D7) (Table 2).

The effect of age, sex, village and parasite densities on antibody responses against the specific malaria antigens (Table 3) was assessed, in order to determine which of the covariates needed to be included in subsequent analysis. All antibody responses increased significantly with age and infection level, independent of the specific malaria antigen. In addition, antibody responses against schizont extract, 195A and BVp42 were significantly higher in females than in males. The median antibody responses against all antigens were higher in Apusit, which reflects the higher parasite prevalence in this village. These differences between the two main study villages were significant for antibody responses against RESA, SPf66, MSP1 and 195A.

Table 1 Number of relative pairs used in variance component analysis

<table>
<thead>
<tr>
<th>Types of relatives</th>
<th>Kinship</th>
<th>No. of pairs</th>
<th>No. of pairs living in the same house</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full sibs</td>
<td>0.25</td>
<td>191</td>
<td>183</td>
</tr>
<tr>
<td>Half sibs</td>
<td>0.125</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Avuncular</td>
<td>0.125</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Cousins</td>
<td>0.0625</td>
<td>327</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL no. of pairs</td>
<td></td>
<td>539</td>
<td>197</td>
</tr>
</tbody>
</table>

The number of relative pairs refers to the numbers of pairwise combinations of children of the same family who had immunological data, and which could therefore be included in the analyses.

a In this analysis only relative pairs were used where each relative had responses measured for all three surveys. For the total number of relative pairs present see Table 5.

Table 2 Distribution of antibody responses against specific malaria antigens in three surveys

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median 25, 75 %tile</td>
<td>Median 25, 75 %tile</td>
<td>Median 25, 75 %tile</td>
</tr>
<tr>
<td>Schizont</td>
<td>21.7 5.5, 81.3</td>
<td>19.4 7.8, 78.5</td>
<td>34.8 9.5, 125.7</td>
</tr>
<tr>
<td>RESA</td>
<td>110.8 24.0, 277.7</td>
<td>53.0 28.3, 107.8</td>
<td>47.3 24.7, 111.9</td>
</tr>
<tr>
<td>MSP2 (FC27)</td>
<td>21.9 9.2, 188.3</td>
<td>37.4 7.6, 324.4</td>
<td>103.4 14.1, 452.3</td>
</tr>
<tr>
<td>MSP2 (3D7)</td>
<td>54.7 11.4, 311.5</td>
<td>63.3 10.2, 415.3</td>
<td>82.9 13.8, 543.6</td>
</tr>
<tr>
<td>MSP2 (d3D7)</td>
<td>28.4 11.6, 208.4</td>
<td>38.6 10.3, 345.7</td>
<td>93.0 15.1, 612.4</td>
</tr>
<tr>
<td>SPf66</td>
<td>121.8 45.3, 274.0</td>
<td>54.7 31.0, 91.7</td>
<td>ndb nd nd</td>
</tr>
<tr>
<td>MSP1</td>
<td>35.9 12.1, 399.3</td>
<td>76.2 11.3, 324.6</td>
<td>119.2 21.3, 461.8</td>
</tr>
<tr>
<td>195A</td>
<td>26.64 12.5, 193.6</td>
<td>17.1 11.1, 34.3</td>
<td>22.4 11.5, 208.8</td>
</tr>
<tr>
<td>BVp42</td>
<td>56.4 17.9, 271.9</td>
<td>30.6 15.7, 92.9</td>
<td>104.2 17.2, 459.7</td>
</tr>
</tbody>
</table>

a Kruskal-Wallis test (x² approximation).

b Not done.

*** P < 0.0001, ** P < 0.001.* P < 0.01.
The results of the variance component analysis, quantifying different sources of variation, are shown in Table 4. For each antibody response, two models are represented: model 1 without the house effect included and model 2 with the house effect included. Most importantly, the majority of the total phenotypic variance could be explained by the within-child variation of antibody responses between surveys in all models. If the house effect was ignored (model 1) then the remaining variation due to other non-genetic factors was very small, except for the antibody response against RESA. In addition, genetic variance, mainly additive genetic variance, was present for antibody responses against all malaria antigens. The heritability varied from 9.7 up to 48.2%, depending on the different antigens (Table 4).

However, when a house effect was included in the analysis (model 2), most or all of the genetic variance could be explained by living in the same house, except for the antibody response against SP66, where there was no house effect present. Consequently, heritability was reduced for the antibody response against schizont extract, MSP2 (d3D7), MSP1 and BVp42 or disappeared completely for RESA, MSP2 (FC27), MSP2 (3D7) and 195A (Table 4). As a special case for the antibody response against MSP2 (d3D7), genetic variation could be mainly explained by dominance variance and partly by house variation. This suggests that the genetic variation was highly confounded by the sharing of common environments by closely related relatives.

Comparing the fit of the two models showed that the model with the house effect included (model 2) fitted better for antibody responses against schizont extract, MSP2 (FC27), MSP2 (d3D7), MSP1 and BVp42 or disappeared completely for RESA, MSP2 (FC27), MSP2 (3D7) and 195A (Table 4). As a special case for the antibody response against MSP2 (d3D7), genetic variation could be mainly explained by dominance variance and partly by house variation. This suggests that the genetic variation was highly confounded by the sharing of common environments by closely related relatives.

In order to assess familial correlations of antibody responses in different relative pairs, intra-class correlation coefficients were estimated (Table 5). Antibody responses against all antigens were significantly correlated in full-sib pairs. The correlations in cousin pairs were positive for the antibody responses against all antigens, but were only significant for antibody responses against schizont extract, MSP2 (FC27), SP66 and MSP1. In half-sib and avuncular pairs the pattern shown was not uniform: there was strong negative correlation in half-sib pairs in immune responses against RESA, MSP2 (3D7) and MSP2 (d3D7). In addition, there was a much higher positive correlation in avuncular pairs than in full-sib pairs in antibody responses against MSP2 (FC27), MSP2 (d3D7), and 195A.

**Discussion**

The aim of the present analysis was to assess the relative contribution of several measurable sources of variation in total IgG responses against schizont extract and specific malaria antigens in children. We used variance component analysis, and were able to partition the variation of total IgG responses into four different sources, including between-survey variation, genetic, house variation and remaining non-genetic variation. These factors contributed by varying amounts to the observed heterogeneity in antibody responses, and the results of the analysis allow us to infer their relative importance in the context of immuno-epidemiological and vaccine studies.

The most important source of variation was related to surveying the children at different points in time, which explained at least half of the total variation for each antigen after age, sex and infection status adjustments. This was true even for MSP2 (d3D7), where there were no significant differences in the mean antibody responses among surveys. We cannot be sure of the underlying cause of the variation because only three surveys were conducted, and they were each several months apart. One reason is seasonal variation, related to fluctuations in transmission levels.49 This would concur with the results of Taylor...
Table 4: Results of variance component analysis

<table>
<thead>
<tr>
<th>Model</th>
<th>Model</th>
<th>AV</th>
<th>SE</th>
<th>DV</th>
<th>SE</th>
<th>HV</th>
<th>SE</th>
<th>NGV</th>
<th>SE</th>
<th>WCV</th>
<th>SE</th>
<th>LogL</th>
<th>(\chi^2)</th>
<th>AIC</th>
<th>H</th>
<th>SE</th>
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</thead>
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<td>Schizont</td>
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<td>0.164</td>
<td>0.011</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0004</td>
<td>0.000</td>
<td>0.364</td>
<td>0.009</td>
<td>-551.849</td>
<td>1119.70</td>
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<tr>
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<td>2</td>
<td>0.053</td>
<td>0.016</td>
<td>0.000</td>
<td>-</td>
<td>0.122</td>
<td>0.018</td>
<td>0.0001</td>
<td>0.000</td>
<td>0.374</td>
<td>0.010</td>
<td>-527.567</td>
<td>4856.50</td>
<td>1.073</td>
<td>0.097</td>
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<tr>
<td>RESA</td>
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<td>0.063</td>
<td>0.024</td>
<td>0.000</td>
<td>-</td>
<td>0.038</td>
<td>0.012</td>
<td>0.178</td>
<td>0.016</td>
<td>0.432</td>
<td>0.011</td>
<td>-1020.457</td>
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<td>0.012</td>
<td>0.178</td>
<td>0.016</td>
<td>0.432</td>
<td>0.011</td>
<td>-1019.257</td>
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<td>0.041</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>0.038</td>
<td>0.012</td>
<td>0.178</td>
<td>0.016</td>
<td>0.432</td>
<td>0.011</td>
<td>-1019.257</td>
<td>2.4</td>
<td>2056.51</td>
<td>0.000</td>
<td></td>
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<tr>
<td>MSP2 (3D7)</td>
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<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>0.012</td>
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<td>2056.51</td>
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<td>MSP2 (d3D7)</td>
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<td>0.006</td>
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<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0001</td>
<td>0.000</td>
<td>0.468</td>
<td>0.014</td>
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<td>-</td>
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<td>0.000</td>
<td>0.468</td>
<td>0.014</td>
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<td>1559.72</td>
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<td>-</td>
<td>-</td>
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<td>0.000</td>
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<td>0.014</td>
<td>-1276.95</td>
<td>59.24</td>
<td>2571.90</td>
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</tr>
</tbody>
</table>

a Additive genetic variance.
b Standard error.
c Dominance variance.
d House variance.
e Non-genetic variance.
f Within-child variance.
g Log likelihood.
h Akaike's information criterion.
i Broad-sense heritability.
et al., who showed that antibody responses to MSP1 and MSP2 vary by season in children in The Gambia. The variation is not due to differences in laboratory techniques since all three samples from each child were tested on the same ELISA plate. However, we would anticipate some variability even if bloods were drawn on consecutive days, or if the same samples were tested repeatedly. Whatever the explanation, our results indicate that immune responses measured at a single time point might not always be representative. This could be especially critical when assessing the protective effect of specific immune responses against infection and disease.

The second most important source of variation in responses against all antigens and schizont extract, before inclusion of the house effect, was genetic variation. This confirms and extends previous reports which have suggested genetic regulation of antibody responses to RESA, MSP2, MSP1, SPf66, and SP166. However, inclusion of a house effect explained most of the apparent genetic variation, in a manner similar to that found in a previous analysis of total IgG responses against RESA in adults in the same study population. It is worth noting that familial aggregation of antibody responses against these antigens, although not to the same degree. For antibody responses to schizont extract, SPf66, MSP1 and BVp42 there was very little non-genetic variation with or without the house effect included, indicating that we identified all major sources of variation in responses to these antigens. The non-genetic variation of responses to other antigens was comparatively high when the house effect was included, and always relatively high for RESA. These results indicate that other, non-specified, factors might have played a role in determining individual responses. Variation in exposure is probably important, and like other immuno-epidemiological studies in endemic areas we are not able to distinguish between effects of immunological memory and of continual antigenic challenge.

Taken together, the results of this analysis make it clear that temporal variation and familial aggregation, whether determined by house or genetic effects, were the most important sources of variation in total IgG responses to all antigens among the study children. A serious shortcoming of population-based analyses of heritability is that, as in our study, it is often not possible to separate clearly the environmental effects of shared houses from genetic effects. In general, the evaluation of effects of shared houses should be considered an important test of the robustness of estimates of heritability in human parasitic diseases.

Table 5 Correlations of antibody responses in relative groups of children

<table>
<thead>
<tr>
<th></th>
<th>Siblings</th>
<th>Half-sibs</th>
<th>Avuncular</th>
<th>Cousins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizont</td>
<td>0.33***</td>
<td>–0.12</td>
<td>0.15</td>
<td>0.15**</td>
</tr>
<tr>
<td>RESA</td>
<td>0.31***</td>
<td>–0.42*</td>
<td>–0.23</td>
<td>0.09</td>
</tr>
<tr>
<td>MSP2 (FC27)</td>
<td>0.39***</td>
<td>–0.09</td>
<td>0.61**</td>
<td>0.14**</td>
</tr>
<tr>
<td>MSP2 (3D7)</td>
<td>0.39***</td>
<td>–0.44*</td>
<td>0.19</td>
<td>0.07</td>
</tr>
<tr>
<td>MSP2 (d3D7)</td>
<td>0.33***</td>
<td>–0.55*</td>
<td>0.53*</td>
<td>0.001</td>
</tr>
<tr>
<td>SPf66</td>
<td>0.31***</td>
<td>–0.34</td>
<td>0.10</td>
<td>0.29***</td>
</tr>
<tr>
<td>MSP1</td>
<td>0.44***</td>
<td>0.17</td>
<td>0.08</td>
<td>0.17***</td>
</tr>
<tr>
<td>195A</td>
<td>0.24***</td>
<td>0.07</td>
<td>0.47*</td>
<td>0.09</td>
</tr>
<tr>
<td>BVp42</td>
<td>0.32***</td>
<td>–0.27</td>
<td>–0.11</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, *** P < 0.001.

In avuncular pairs for some of the antibody responses, which disagrees with any Mendelian genetic model. This phenomenon may be the result of low numbers of half-sib and avuncular pairs being unrepresentative of the population, and also because all members in these pairs came from only two families. In summary, the lack of a uniform pattern in the correlation coefficients, the low correlations in cousin pairs, and the high degree of aggregation of similar responses in the same houses, indicate that non-genetic factors within houses may be more important determinants of familial clustering than genetic factors.

The final source of variation was the remaining non-genetic variation. This was found in the responses to all antigens, although not to the same degree. For antibody responses to schizont extract, SPf66, MSP1 and BVp42 there was very little non-genetic variation with or without the house effect included, indicating that we identified all major sources of variation in responses to these antigens. The non-genetic variation of responses to other antigens was comparatively high when the house effect was included, and always relatively high for RESA. These results indicate that other, non-specified, factors might have played a role in determining individual responses. Variation in exposure is probably important, and like other immuno-epidemiological studies in endemic areas we are not able to distinguish between effects of immunological memory and of continual antigenic challenge.
studying immune responses, since ignoring such factors might lead to incorrect inferences or misinterpretation of results.

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