Cytophilic Immunoglobulin Responses to Plasmodium falciparum Glutamate-Rich Protein Are Correlated with Protection Against Clinical Malaria in Dielmo, Senegal

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The goal of this study was to analyze antibody responses to Plasmodium falciparum glutamate-rich protein (GLURP) using clinical data and plasma samples obtained from villagers of Dielmo, Senegal. This molecule was chosen because it is targeted by human antibodies which induce parasite growth inhibition in antibody-dependent cellular inhibition (ADCI) assays. The results showed a strong correlation between protection against malaria attacks and levels of immunoglobulin G2 (IgG2) and IgG3 against GLURP489–705 (R0) and IgG3 against GLURP705–1178 (R2) when corrected for the confounding effect of age-related exposure to malaria. Thus, GLURP may play a role in the induction of protective immunity against P. falciparum malaria.

Materials and Methods

Study area and population. The village of Dielmo (13°45′ N, 16°25′ W) is located in an area of Senegal where malaria is holoendemic. The number of infective bites per person during the first year of follow-up was estimated at 101.2, 19.9, and 8.9 for P. falciparum, P. malariae, and P. ovale, respectively. The entire population of the village was involved in a prospective study initiated in May 1990 (22).

Clinical surveillance and blood sampling. All villagers were under active daily surveillance by medical staff present 24 h a day, 7 days a week, to identify and define all episodes of morbidity (14, 22). A malaria attack was defined by an episode of fever associated with a parasite density above the age-dependent pyrogenic threshold described for this village (14, 21). The existence of a pyrogenic threshold allowed the use of parasite density to distinguish malaria attacks from other causes of fever. The plasma used in the present study was collected from 214 of the 247 villagers covering all age groups. All the samples used were collected from January to December 1991 from nonpregnant villagers were used in the present analysis. Informed consent was obtained individually from all participants or their parents. This protocol was approved by the Conseil de Perfectionnement de l’Institut Pasteur de Dakar, which is headed by the Senegalese Minister of Health.

GluTamate-rich protein (GLURP) of P. falciparum is synthesized during all stages of the parasite in the vertebrate host, including on the surface of newly released merozoites (2). Immunoepidemiological studies have demonstrated a high prevalence of antibodies against recombinant GLURP fragments in adults from Liberia (20) and have shown that GLURP-specific IgG was associated with low parasite densities (10, 11) and the absence of disease (8) in West African children.

Motivated by these results and our recent findings that highly affinity-purified human IgG antibodies to GLURP were able to promote a strong monocyte-dependent inhibition of P. falciparum growth in vitro (19), we have investigated the distribution of isotypes to nonrepetitive and repetitive regions of GLURP in plasma from 214 villagers in Dielmo, Senegal, and its correlation to clinical protection.

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mined previously as those discriminating between human Ig sub-classes, i.e. yielding no cross-reactions between sub-classes (5). However, it was later found that the differences in affinity of each MAb, the resulting OD values did not faithfully reflect the actual amounts of each isotype in a reference serum. Therefore, we used a reference serum in which the accurate content of each subclass (non-malaria specific) had been determined. Thereafter, to correct for differences in MAb affinities, the observed OD values were transformed into corrected OD values by means of correcting factors, calculated as described below by using a standard serum pool from six French blood donors. This serum pool was diluted 20,000-fold, and the OD values obtained for each IgG subclass and IgM were first divided by the OD value obtained for IgG1 and then divided by the ratio of the concentration of the corresponding IgG subclass and IgG1. These calculations led to correcting factors of 1, 2.7, 0.67, 0.22, and 1.5 for IgG1 to IgG4 and IgM, respectively. The malarial antigen-specific OD values obtained in enzyme-linked immunosorbent assays were corrected using the above factors and expressed in arbitrary units (AU) corresponding to their ratios to the mean ± 3SD of OD obtained with six French controls tested concurrently in the same plate.

Statistical analysis. The Kruskal-Wallis test was used for the comparisons of antibody responses between different age groups. The relationship between the pattern of isotype distribution and the risk of malaria attack from 6 months before to 6 months after the blood sampling (i.e., over a 1-year period) was tested using a Poisson regression model where the effect of covariates such as age, hemoglobin AS phenotype, gender, *P. falciparum* infection, and transmission prior to testing the effect of the immune responses. Only the covariates with a significant effect, i.e., age and *P. falciparum* infection, remained in the baseline model.

Without taking into account the effect of the immune responses, the estimated protective effect of age was 62% (95% confidence interval [CI95%] = 32 to 79) in group 2 and 96% (CI95% = 93 to 98) in group 3 compared to the reference group (group 1).

A series of Poisson models was fitted to test and estimate to what extent the protective effect could be attributed to the level of each isotype. In an initial model, in which the immune responses were dichotomized according to their median values, R0 IgG2 and R2 IgG3 were significantly associated with clinical immunity and R0 IgG3 was almost significantly associated with protection. For each of these three immune responses, AU values over the median were associated with a reduced risk of 2.4-fold (CI95% = 1.2 to 4.8), 2.3-fold (CI95% = 1.2 to 4.3), and 1.85-fold (CI95% = 0.97 to 3.6), respectively. Taking into account the effect of these three immune responses, the residual age protective effect was 47% (CI95% = 0 to 72) in group 2 and 95% (CI95% = 89 to 97) in group 3 compared to the reference group. This indicates that R0-specific IgG2 and IgG3 and R2-specific IgG3 responses collectively could account for 25% [(62 – 47)/62 = 25%] of the protective effect in group 2 and 2% in group 3. In a second model where the immune responses were considered continuous variables, only the IgG3 response to R2 was significantly associated with clinical immunity: a 10-fold increase in AU was associated with a 2.7-fold (CI95% = 1.4 to 5.3) reduction in the risk of malaria attacks.

### Table 1. IgG levels in plasma from 214 villagers of Dielmo to recombinant GLURP

<table>
<thead>
<tr>
<th>Age group (yr) (n)</th>
<th>Geometric mean of IgG responses (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R0</td>
</tr>
<tr>
<td>1–5 (36)</td>
<td>0.49</td>
</tr>
<tr>
<td>6–10 (32)</td>
<td>1.85</td>
</tr>
</tbody>
</table>

* The number of persons in each age group is indicated in parentheses.

### Table 2. IgG subclass and IgM levels in plasma from 157 villagers of Dielmo to recombinant GLURP

<table>
<thead>
<tr>
<th>Age group (yr) (n)</th>
<th>Geometric mean of IgG subclasses and IgM responses (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>R0</td>
<td>R2</td>
</tr>
<tr>
<td>1–5 (10)</td>
<td>5.47</td>
</tr>
<tr>
<td>6–10 (22)</td>
<td>3.77</td>
</tr>
<tr>
<td>11–15 (21)</td>
<td>3.04</td>
</tr>
<tr>
<td>16–20 (89)</td>
<td>3.36</td>
</tr>
<tr>
<td>&gt;20 (89)</td>
<td>3.30</td>
</tr>
</tbody>
</table>

* The number of persons in each age group is indicated in parentheses.

* Responses are transformed into AU as described in Table 1, footnote b.

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Taking into account the effect of this immune response, the residual age protective effect was 54% (CI 95% = 15 to 75) in group 2 and 95% (CI 95% = 90 to 98) in group 3 compared to the reference group. The second model suggests that R2-specific IgG3 alone could account for 13% [(62 − 54)/62 = 13%] of the protective effect in group 2 and 1% in group 3.

There was no significant interaction between the effects of the immune responses and the effect of age in either model. The reduction of risk associated with these immune responses was similar in each age group.

**DISCUSSION**

The 220-kDa GLURP of *P. falciparum* has been located in all the developmental stages of the parasite in humans, including on the surface of newly released merozoites (2). The results of immunoepidemiological studies show that high levels of anti-GLURP antibodies correlate with a low grade of parasitemia (3) and the absence of disease (8). In this study, we found that plasma samples from the villagers of Dielmo frequently contained antibodies to two of the three recombinant GLURPs representing the N-terminal nonrepetitive region (R0) and the C-terminal repeat region R2. Both antibody responses were highly correlated with age (P < 10⁻⁵). In contrast, the acquisition of antibodies to the central repeat region, R1, was age independent and far less frequent. These findings confirm and extend earlier studies performed with sera from individuals in Liberia (20).

Since the GLURP R0 and R2 regions are targets of human antibodies which, in cooperation with monocytes, mediate the inhibition of *P. falciparum* growth in vitro (19), it was of interest to determine if any of the IgG subclasses were associated with clinical protection. Because levels and prevalences of R0- and R2-specific IgG responses increase with age, the data were analyzed in Poisson regression models taking into account the effect of age. The Poisson regression models consistently identified R2-specific IgG3 as a strong predictor of protection, irrespective of age, and this immune response alone could account for 13 and 1% of the protective effect in groups 2 and 3, respectively. The decrease in the attributable fraction between the two age groups suggests that other factors play a role in the maintenance of clinical protection in older children and adults. Using the same type of Poisson analysis, a previous study also points to IgG3 as a major component of clinical immunity to malaria in the villagers of Dielmo (1). In this study, it was found that IgG3 against a whole-parasite extract accounted for 35% of the protective effect in 3- to 6-year-old children. Considering the large number of proteins that might play a role in the acquisition of protection to malaria, it is highly surprising that the immune response to part of a single protein, GLURP, accounts for 13% of the IgG3-mediated protective immunity in young children. Furthermore, a second Poisson regression model suggested that R0-specific immune responses also contribute to protection, since the combined levels of R2-specific IgG3 and R0-specific IgG2 and IgG3 could account for 25% of the protective effect in the 6- to 10-year-old children. Similar results have been obtained in a study using data and plasma samples from a cohort of children living in coastal Ghana (7). For Ghanaian children, it was found that levels of R0-specific IgG1 and R2-specific IgG3 are significantly correlated with clinical protection from *P. falciparum* malaria after exclusion of the confounding effect of age. Although a linkage between the anti-GLURP responses and other immunological effector mechanisms cannot be excluded, we believe that these data collectively suggest that cytophilic antibodies against both the R0 and the R2 regions of GLURP contribute to the development of clinical immunity in West African children.

The significant involvement of antibodies in protection against the asexual blood stage of *P. falciparum* has been well documented by experiments carried out by Cohen and McGregor (12, 13) and by Sabchaeron et al. (15). Antibodies, however, may not act alone; rather, they seem to control parasitemia in cooperation with monocytes, as suggested by in vitro findings (4). Our observation that cytophilic antibodies against GLURP, a target for antibody-dependent cellular inhibition (ADCI) active antibodies, predominate in West African children who are protected from clinical disease is consistent with the hypothesis that protective antibodies act mainly in collaboration with monocytes to control parasite multiplication in vivo, and may indicate that cooperation between cytophilic antibodies and cells bearing Fcγ receptors, like monocytes, is essential for the control of circulating parasites in vivo. These results also suggest that among the IgG subclasses, IgG3 may play a major role in the protection of young children in Dielmo. In Kenyan adults, IgG1 seems to play a more important role than IgG3, since plasma samples with high levels of IgG1 antibodies and a higher IgG1/IgG3 ratio were associated with the highest ADCI activity (17).

The association between clinical protection and the possession of high levels of R0-specific IgG2 in older children may be related to the observation that weak binding of IgG2 to FcγRII receptors does occur (18, 23) and that IgG2 purified from a myeloma cell line could trigger the production of tumor necrosis factor alpha from human blood monocytes (9). Tumor necrosis factor alpha is one of the soluble factors which was shown to mediate parasite killing in ADCI (6). Alternatively, it may be speculated that GLURP-specific IgG2 acts to control parasite multiplication in a monocyte-independent manner. It is highly likely that such mechanisms are effective in clinically immune individuals, but it should be mentioned that affinity-purified GLURP-specific human IgG preparations have so far failed to display inhibition of parasite growth in vitro in the absence of monocytes.

We have recently identified two B-cell epitopes P3 (amino acid residues 216 to 229) and S3 (residues 407 to 434) in the GLURP R0 region as targets for ADCI-effective human antibodies (M. Theisen, S. Soe, S. G. Jessing, L. M. Okkels, S. Danielsen, C. Oeuvray, P. Druilhe, and S. Jepsen, submitted for publication). More detailed epidemiological studies using peptide antigens representing these epitopes would be of interest so that we can investigate the correlation between single epitope-specific subclass responses and protection against malaria.

In conclusion, a significant association between levels of cytophilic R0- and R2-specific subclass antibodies and clinical protection against malaria is found in the young children of Dielmo, suggesting that GLURP B-cell epitopes may play a role in the induction of protective immunity against *P. falciparum* malaria.

**ACKNOWLEDGMENTS**

C. Oeuvray and M. Theisen contributed equally to this work. We thank all the team and the inhabitants of the village of Dielmo.

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**REFERENCES**

lin isotype response to *Plasmodium falciparum* blood stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, West Africa).


