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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 $GLURP_{94–489}$ (R0) and IgG3 against $GLURP_{705–1178}$ (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.

Individuals repeatedly exposed to *Plasmodium falciparum* malaria infections gradually develop clinical immunity. Results from studies performed in vivo suggest that one of the mechanisms underlying clinical immunity to malaria is the containment of parasite multiplication by antibodies (12, 13, 15). It is reasonable to assume that if protective effects are mediated by antibodies, there is a relationship between the level, isotype, or function of the antibodies and the clinical outcome. Studies of the role of subclass responses in naturally developing clinical immunity to defined malaria antigens and fragments thereof are therefore important. Bouharoun-Tayoun and Druilhe found profound differences in the distribution of immunoglobulin (Ig) subclasses between clinically protected and nonprotected individuals, with cytophilic isotypes (IgG1 and IgG3) being dominant in protected individuals (5). This observation was later confirmed by Aribot (1), who found that the level of parasite-specific IgG3, but not total IgG, was inversely proportional to susceptibility to clinical malaria. In a study of severe malaria, Sarthou et al. demonstrated that only levels of *P. falciparum*-specific IgG3 were positively correlated with survival (16).

The 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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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 in July 1991. Only clinical data 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.

Enzyme-linked immunosorbent assay. Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.5, 0.5, and 0.1 μg of recombinant $GLURP_{94–489}$, $GLURP_{489–705}$, or $GLURP_{705–1178}$, respectively, per ml (21), diluted in 0.05 M bicarbonate (pH 9.6), blocked for 2 h with 2.5% (wt/vol) powdered-milk-containing phosphate-buffered saline (PBS), and reacted for 2 h with sera diluted 1/200 in 1.25% (wt/vol) powdered-milk-containing PBS–0.05% (vol/vol) Tween 20 (PBST) for 2 h. The secondary antibody was a peroxidase-conjugated anti-human IgG (no. P-214; Copenhagen Dako, Denmark) diluted 1/1,000 in 1.25% (wt/vol) powdered-milk-containing PBST. After 1 h of incubation, bound secondary antibody was quantitated by coloring with *o*-phenylenediamine and H_2O_2 in citrate buffer (Sigma, St. Louis, Mo.) for 30 min. The optical density (OD) at 492 nm was determined in a plate reader (Titertek Multiskan MCC 1340). The plates were washed extensively with PBST between each incubation step. Results are expressed in arbitrary units (AU) calculated as the ratio of the OD value of the test sample divided by the mean OD value + 3 standard deviations (SD) of six control sera. These control sera were selected from among 100 serum samples from French blood donors never exposed to malaria so as to reflect the mean of the entire range of antibody reactivity of the 100 sera. A ratio higher than 1 is considered positive.

For IgG subclass detection, which was performed at a later date, only 157 samples were abundant enough to allow a full isotype analysis. Monoclonal mouse anti-human IgG1 to IgG4 subclasses (clones NL16 [Boehringer], HP6002 [Sigma], Zg4, and RJ4 [both from Immunotech]) and IgM (clone MB11 [Sigma]) were diluted 1:2,000, 1:10,000, 1:10,000, 1:1,000, and 1:30,000, respectively in 1.25% (wt/vol) powdered-milk-containing PBST and incubated for 1 h at room temperature. Rabbit anti-mouse IgG conjugated to peroxidase diluted in 1.25% (wt/vol) powdered-milk-containing PBST was added to the wells and incubated for 1 h. The plates were washed extensively with PBST between each incubation step. Bound secondary antibody was revealed as described above. The above dilutions of each isotype-specific monoclonal antibody (MAb) had been deter-

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

Age group (yr) (*n*)[a]	Geometric mean of IgG responses (AU)[b] to:		
	R0	R1	R2
1–5 (36)	0.49	0.28	0.43
6–10 (32)	1.06	0.48	1.04
11–15 (24)	1.60	0.50	1.83
16–20 (19)	1.85	0.63	1.79
>20 (103)	2.37	0.72	2.12

[a] The number of persons in each age group is indicated in parentheses.
[b] Responses were transformed into AU by dividing the OD value of the test sample by the mean OD value + 3SD of six control sera from French blood donors never exposed to malaria.

mined previously as those discriminating between human Ig sub-classes, i.e. yelding no cross-reactions between sub-classes (5). However, it was later found that due to 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 Kruskas-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 can be controlled. Each isotype was included as main explanatory variable in the models, and their effects were tested collectively and individually as continuous responses (log-transformed AU values) or dichotomic variables (lower or equal and above the median value) by likelihood ratio statistics. Using the relative risk associated with each age group estimated by models including or models not including immune responses, the fraction of the clinical immunity acquired in each age group attributable to GLURP-specific isotype responses was calculated. All the immune responses were initially tested collectively, and those which did not reach significance were successively removed from the model according to a descending stepwise strategy.

RESULTS

Relationship between age and total IgG response to GLURP. The levels of IgG responses to $GLURP_{94–489}$ (R0), $GLURP_{489–705}$ (R1), and $GLURP_{705–1178}$ (R2) are given in Table 1. For R0 and R2, the mean levels increased rapidly with age and hence with exposure. Antibody responses to both antigens were strongly associated with age (Kruskall-Wallis test, $P < 10^{-6}$). For R1, the antibody levels and seropositivity rates were low in all age groups. Although there was a slight increase with age, this association was not statistically significant.

Relationship between age and levels of isotype responses to R0 and R2. Since the levels of antibody responses to R1 were very low, the subclass study was extended to include only the response to the R0 and R2 regions in the 157 blood samples which were available for this part of the analysis. GLURP-specific IgG responses in all age groups consisted mainly of IgG1, IgG2, and IgG3, whereas IgG4 was rarely detected. The geometric mean of R0-specific and R2-specific isotype responses is given for each age group in Table 2. Although the mean levels of IgG2, IgG3, IgG4, and IgM responses increased with age, this association was significant only for R0-specific IgG3 (Kruskall-Wallis test; $P < 0.05$). A nonsignificant negative relationship was observed between the levels of R0- and R2-specific IgG1 and age: the responses were high in the younger age groups and tended to decrease in the older age groups. Thus, the most abundant GLURP-specific IgG subclass in the very young age group was IgG1 whereas the most abundant subclass in the adult age group was IgG3 (Table 2).

R0-specific IgG2 and IgG3 and R2-specific IgG3 levels are associated with protection. Due to the relatively small numbers of adults who experienced a malaria attack, the cohort was stratified into three age groups defined as follows: group 1, 0 to 5 years of age ($n = 10$); group 2, 6 to 10 years of age ($n = 22$); group 3, ≥11 years of age ($n = 125$). Taking into account the number of days spent in the village (3,619, 7,093, and 39,944 person-days in groups 1 to 3, respectively), the mean numbers of malaria attacks observed over a period of 1 year were 2.72, 0.98, and 0.13 in the three groups, respectively. The risk of malaria attacks in children younger than 5 years was referred to as a baseline level of risk. Poisson regression models were built to take into account the effect of 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 [$CI_{95\%}$] = 32 to 79) in group 2 and 96% ($CI_{95\%}$ = 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 ($CI_{95\%}$ = 1.2 to 4.8), 2.3-fold ($CI_{95\%}$ = 1.2 to 4.3), and 1.85-fold ($CI_{95\%}$ = 0.97 to 3.6), respectively. Taking into account the effect of these three immune responses, the residual age protective effect was 47% ($CI_{95\%}$ = 0 to 72) in group 2 and 95% ($CI_{95\%}$ = 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 ($CI_{95\%}$ = 1.4 to 5.3) reduction in the risk of malaria attacks.

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

Age group (yr) (*n*)[a]	Geometric mean of IgG subclasses and IgM responses (AU)[b]									
	IgG1		IgG2		IgG3		IgG4		IgM	
	RO	R2	R0	R2	R0	R2	R0	R2	R0	R2
1–5 (10)	5.47	8.17	0.96	1.52	3.37	2.55	1.16	1.06	1.59	1.59
6–10 (22)	3.77	5.48	1.13	1.84	2.96	3.15	1.21	1.14	2.33	2.52
11–15 (21)	3.04	3.78	1.34	1.70	5.55	3.40	1.13	1.14	2.55	2.94
16–20 (15)	3.36	4.20	1.84	2.17	5.48	4.30	1.03	1.30	3.67	3.26
>20 (89)	3.30	4.17	1.81	1.85	5.30	3.78	1.45	1.23	2.92	2.74

[a] The number of persons in each age group is indicated in parentheses.
[b] Responses are transformed into AU as described in Table 1, footnote *b*.

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 ($P < 10^{-6}$). 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.

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C. Oeuvray and M. Theisen contributed equally to this work.

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