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Isotypic Analysis of *Plasmodium falciparum*-Specific Antibodies and Their Relation to Protection in Madagascar

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We previously reported that antibodies to the central repeat (DDEHVEEPTVA) of Pf155/RESA, a major *Plasmodium falciparum* antigen, were negatively related to antimalarial protection. We measured levels of isotype antibodies to this epitope and to *P. falciparum* in 76 Madagascan subjects. Immunoglobulin G (IgG) and isotype antibodies specific for *P. falciparum* had similar levels in individuals who were considered protected and those who were not (as determined during a longitudinal follow-up). The levels of IgG1 antibodies to $(DDEHVEEPTVA)_3$ were higher in nonprotected subjects. The levels of total IgG and of other isotype antibodies had a similar tendency to be higher in nonprotected than in protected individuals.

In most tropical areas, *Plasmodium falciparum* malaria remains a major public health problem. Clinical manifestations are modulated by the host's immune response, the effector mechanisms of which are still poorly understood. Several malarial antigens potentially eliciting a protective response are characterized. One such antigen is Pf155/RESA. Antibodies (Abs) to Pf155/RESA inhibit merozoite invasion (16), and their levels increase with age in individuals in areas where malaria is endemic in parallel with protection (4, 10). Pf155/RESA includes three dominant B-cell epitopes included in repeated amino acid sequences: EENV and EENVEHDA at the 3′ terminus and DDEH VEEPTVA at the center of the molecule (5, 11).

We reported previously the unexpected result that the level of Abs to the Pf155/RESA 11-mer repeat was inversely related to clinical protection in Madagascar (1). In the same population, we have now measured the level of each isotype of Ab to this antigen and to *P. falciparum*. Serum samples from 76 adults were collected in January 1988 in Manarintsoa. The 37 individuals with a negative blood smear were monitored for 20 weeks; subjects who never developed a malaria attack were considered protected against clinical malaria (although many presented with asymptomatic parasitemia). Subjects who developed at least one malaria attack were considered nonprotected. Details of the epidemiological study design were reported previously (1).

Percoll-concentrated in vitro-cultured parasites (Palo Alto strain) were sonicated and stored at −80°C. Antibodies to the 11-mer repeat of Pf155/RESA were searched for by using the synthetic peptide $(DDEHVEEPTVA)_3$ reproducing this sequence and conjugated to bovine serum albumin (BSA) with glutaraldehyde (6).

Immunoglobulin G (IgG) Abs were detected by using goat anti-human IgG (Fc-specific) alkaline phosphatase-labelled Abs (Sigma, Saint Louis, Mo.). The monoclonal Abs used to measure isotype Abs were purchased from Caltag Laboratories (San Francisco, Calif.). These monoclonal Abs were HP 6069 (anti-IgG1), HP 6014 (anti-IgG2), HP 6047 (anti-IgG3), and HP 6023 (anti-IgG4). IgG isotypes were revealed by goat anti-mouse IgG human-absorbed alkaline phosphatase-conjugated Ab (Caltag Laboratories). From checkerboard titration (13), it was determined that the optimal concentrations of coating antigen were 2 μg/ml for *P. falciparum* and 5 μg/ml for $(DDEHVEEPTVA)_3$. The optimal dilutions of test sera were 1/200 (IgG) and 1/50 (IgG isotype). Monoclonal Abs were used at 1 μg/ml (IgG1), 0.5 μg/ml (IgG3), or 0.25 μg/ml (IgG2 and IgG4). Labelled Abs were used at dilutions of 1/1,000 (IgG2), 1/2,000 (IgG, IgG1, and IgG4), or 1/4,000 (IgG3).

Microtiter plates were incubated overnight at 4°C with the antigen in carbonate buffer (50 μl per well) and then blocked for 3 h with phosphate-buffered saline (PBS) containing 2% BSA. Duplicate wells were subsequently incubated with 50 μl each of (i) test serum in PBS–0.5% Tween 20 (overnight at 4°C), (ii) isotype-specific mouse monoclonal Ab (overnight at 4°C) (for isotype measurement), and (iii) enzyme-labelled anti-mouse (for isotype measurement) or anti-human (for IgG measurement) IgG Ab (for 2 h at 25°C). Then, *p*-nitrophenylphosphate (Sigma) was added and the A_{405}s were determined. Results were expressed in arbitrary units (AU) as the reactivity of a positive reference pool (in AU) × A_{405} (test serum)/A_{405} (positive reference pool). The reactivity of the reference pool was defined as 100 AU for whole IgGs to *P. falciparum* and to $(DDEHVEEPTVA)_3$. According to normal subclass distribution (18), the content of the reference pool was defined as follows: IgG1, 66 AU; IgG2, 23 AU; IgG3, 7 AU; and IgG4, 4 AU. Positivity was determined from the mean reactivities of a blood bank serum battery + 3 standard deviations. For *P. falciparum*, the thresholds of positivity were 1.3 AU (IgG), 3.4 AU (IgG1), 2.2 AU (IgG2), 0.3 AU (IgG3), and 0.7 AU (IgG4). For $(DDEHVEEPTVA)_3$, the thresholds were 45.5 AU (IgG), 25.2 AU (IgG1), 12.9 AU (IgG2), 1.2 AU (IgG3), and 3.1 AU (IgG4). Values were log transformed to normalize their distribution, and the geometrical means were calculated. Ab titers were compared by means of the two-tailed Student *t* test. Linear correlation coefficients were calculated between Ab reactivities ($P < 0.05$).

Total IgG was highly correlated with the sum of the four IgG subclasses, both for Abs to *P. falciparum* and for those to $(DDEHVEEPTVA)_3$ ($r = +0.71$ and $+0.43$, respectively; $P < 0.0001$ for both). For *P. falciparum*, the mean reactivities were 13.1 AU for IgG, 10.9 AU for IgG1, 3.7 AU for

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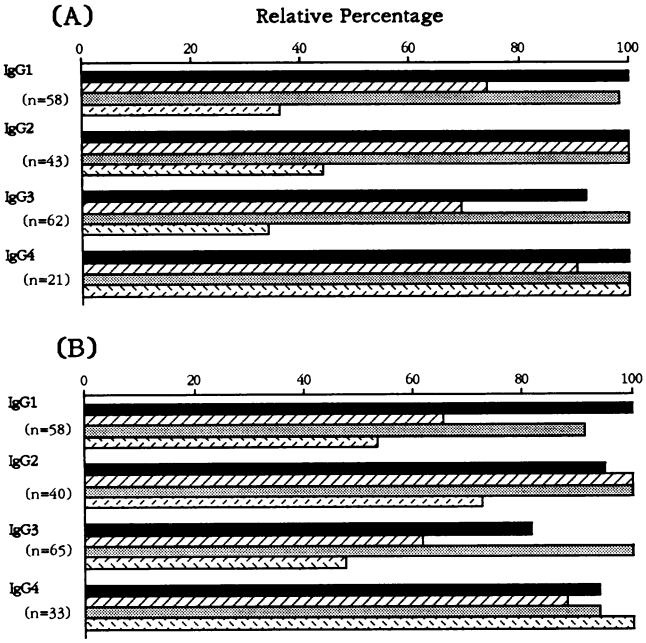

FIG. 1. Relative percentages of serum samples coexpressing antibodies of different IgG isotypes when a given isotype is expressed. Values were determined by enzyme-linked immunosorbent assays using a *P. falciparum* schizont-enriched antigen (A) and the synthetic peptide $(DDEHVEEPTVA)_3$ from Pf155/RESA (B). n, number of serum samples containing a given isotype antibody; ■, IgG1; ▨, IgG2; ▩, IgG3; □, IgG4.

IgG2, 0.9 AU for IgG3, and 0.6 AU for IgG4. For $(DDEHVEEPTVA)_3$, the means were 52.9 AU for IgG, 53.2 AU for IgG1, 15.0 AU for IgG2, 2.5 AU for IgG3, and 2.4 AU for IgG4. The reference pool included samples selected for high reactivity to *P. falciparum*, while reactivity to $(DDEHVEEPTVA)_3$ was not taken into account. However, the reactivities to both antigens were defined as 100 AU; this accounts for the relatively high values for Abs to $(DDEHVEEPTVA)_3$. The relative proportions of the IgG isotypes reacting with *P. falciparum* or $(DDEHVEEPTVA)_3$ in individual samples are shown in Fig. 1. For *P. falciparum*, IgG1 and IgG3 were coexpressed in more than 90% of cases. Among IgG1- or IgG3-containing samples, IgG2 and IgG4 were absent in one-third and two-thirds, respectively. In IgG2-containing samples, IgG1 and IgG3 Abs were present, while IgG4 Abs were absent in one-half of the samples. In more than 90% of the IgG4-containing samples, all three other isotypes were coexpressed. Similar reactivity patterns occurred with $(DDEHVEEPTVA)_3$.

Among the 37 noninfected subjects subsequently monitored, 26 were considered protected and 11 were considered nonprotected. The levels of IgG or isotype Abs specific for *P. falciparum* were intercorrelated (all $r > +0.43$; all $P < 0.01$) and were similar in protected and nonprotected individuals (Table 1). All isotype Abs to $(DDEHVEEPTVA)_3$ were intercorrelated (all $r > +0.34$; all $P < 0.05$). IgG1 and IgG3 levels were correlated to total IgG levels (both $r > +0.52$; both $P < 0.05$). The levels of IgG1 Abs to $(DDEHVEEPTVA)_3$ were higher in nonprotected ($P = 0.01$) than in protected subjects. IgG and other isotype Abs specific for $(DDEHVEEPTVA)_3$ exhibited a similar, but not significant, tendency to be higher in nonprotected than in protected individuals (Table 1).

In these inhabitants of Madagascar, IgG1 and IgG3 isotypes were the most frequent and, when present, were coexpressed in almost all serum samples. IgG2-containing samples also contained IgG1 and IgG3 Abs, while most serum samples containing IgG4 Abs also contained the three other isotypes. This was true for Abs to *P. falciparum* antigen and to $(DDEHVEEPTVA)_3$ from Pf155/RESA. Such a relation, previously described for *P. falciparum* Abs (16), demonstrates that natural antigenic stimulations first trigger B cells to produce IgG1 or IgG3 Abs. Subsequent stimulations will trigger B cells to produce IgG2 and lastly IgG4, suggesting that isotype expression reflects sequential activation of specific B cells by repeated antigenic stimulations.

This study also demonstrates the lack of relation between protection and Abs to total *P. falciparum* extract (16). Experimental data demonstrated that Abs to Pf155/RESA or to the 3′ terminus repeats inhibited merozoite invasion (2, 17). Epidemiological studies demonstrated an association between these Abs and age of patients or rate of malaria transmission (4, 6, 10). Moreover, the titers of Abs to Pf155/RESA or to the 3′ repeats were inversely related to parasite density and to the presence of clinical symptoms

TABLE 1. Titer of antibodies to *P. falciparum* and to the 11-mer amino acid sequence of Pf155/RESA, $(DDEHVEEPTVA)_3$, in 37 individuals (Madagascar, 1988)

Antibody type and class	Titer (95% confidence interval) for individuals[a]		P^b
	Not protected (n = 26)	Protected (n = 11)	
Anti-*P. falciparum*			
IgG	6.84 (3.16–14.88)	7.68 (3.06–19.25)	0.87
IgG1	8.44 (4.89–14.55)	7.67 (4.45–13.22)	0.83
IgG2	3.25 (1.80–5.87)	3.91 (1.98–7.72)	0.72
IgG3	0.65 (0.40–1.05)	0.60 (0.36–1.01)	0.84
IgG4	0.47 (0.25–0.89)	0.34 (0.15–0.78)	0.59
Anti-$(DDEHVEEPTVA)_3$			
IgG	45.33 (37.18–55.28)	39.68 (33.02–47.69)	0.43
IgG1	70.70 (41.31–120.76)	29.89 (20.42–43.64)	0.01
IgG2	15.57 (8.86–27.32)	6.64 (2.93–15.06)	0.10
IgG3	2.04 (1.58–2.62)	1.54 (1.26–1.86)	0.09
IgG4	3.04 (1.70–5.45)	1.27 (0.46–3.50)	0.13

[a] Values are geometric means. Individuals were considered either protected or not protected against clinical *P. falciparum* malaria (see text).
[b] Value in Student's t test for protected versus nonprotected individuals.

(12, 14–16). In contrast, no such relation was observed with the 11-mer central repeat Abs (6, 7, 14). We reported the opposite relation, i.e., that anti-$(DDEHVEEPTVA)_3$ IgG Ab level is inversely related to protection (1). In the present study, this negative relation was observed between protection and IgG1 Abs to $(DDEHVEEPTVA)_3$.

The role of Abs in protection against malaria is still unclear, and the effect of each isotype is controversial. Among serum samples from *P. falciparum*-primed donors, those with high *P. falciparum* Ab titers contained IgG-specific Abs of all four isotypes, while most low-titerd samples contained primarily IgG3 and IgG1 (16). In another study, nonprotected individuals presented with either an overall low response (6 subjects of 10) or an isotype imbalance with overexpression of IgG2 and a low level of IgG3 Abs (4 of 10) compared with African immune adults (3). In our population, among Abs specific for $(DDEHVEEPTVA)_3$, IgG1 isotype levels were inversely related to protection. Nevertheless, a genuine specific isotype imbalance remains questionable, as other isotypes showed a similar tendency. In *Schistosoma mansoni* infection, blocking Abs inhibit the eosinophil-mediated killing of schistosomula by other Abs cross-reacting with the same target antigen. These blocking Abs are isotype specific (to IgG4 and to a lesser extent to IgG2) (8). Abs inhibiting the activity of other Abs may also be present during *P. falciparum* malaria. Given the very peculiar epidemiological status of this area of Madagascar (*P. falciparum* was reintroduced a few years before our study after having disappeared for more than 2 decades) (9), most individuals were building up effector mechanisms against malaria. The isotype balance may differ from that of populations in stable malaria areas.

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