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Humoral and Cell-Mediated Immunity to the *Plasmodium falciparum* Ring-Infected Erythrocyte Surface Antigen in an Adult Population Exposed to Highly Endemic Malaria

H.-P. BECK,[1]* I. FELGER,[1] B. GENTON,[1] N. ALEXANDER,[2] F. AL-YAMAN,[1] R. F. ANDERS,[3] AND M. ALPERS[2]

Papua New Guinea Institute of Medical Research, Madang,[1] and Goroka,[2] Papua New Guinea, and The Walter and Eliza Hall Institute, Post Office Royal Melbourne Hospital, Victoria 3050, Australia[3]

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A parasitological and immunological survey was carried out in an area in Papua New Guinea highly endemic for malaria. Two hundred fourteen adult individuals were selected for studies to assess their immune responses against the malaria vaccine candidate ring-infected erythrocyte surface antigen (RESA). Total immunoglobulin G (IgG) antibodies directed against RESA as well as specific IgG1, IgG2, and IgG3 antibodies were determined. Humoral responses directed against RESA were frequent in all IgG subclasses. Only IgG3 responses were found to be age dependent. Total anti-RESA IgG antibodies were not correlated with protection against malaria as measured by parasite prevalence, parasite density, or health center attendance. In contrast, cytophilic antibodies (IgG1 and IgG3) were associated with reduced *Plasmodium falciparum* prevalence and reduced health center attendance. T-cell proliferation in general was low and very infrequent. No correlation between humoral and cellular immune responses could be found. Parasite density, parasite prevalence, and health center visits tended to be reduced in individuals with good humoral and cell-mediated immune responses.

The ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* is considered an important candidate for possible inclusion in a subunit vaccine against malaria. RESA is present in dense bodies of merozoites but after invasion is found associated with the membrane skeleton of the infected erythrocyte (1, 12). Humoral immunity to RESA increases with age, and the majority of adults living in areas of endemic malaria have high antibody titers against this antigen. Although many studies have been conducted to evaluate the immunity against RESA, little conclusive evidence exists that immunity against RESA is protective. Several investigators (21, 24, 35, 37) found trends of a negative correlation between anti-RESA antibodies and parasite prevalence or density, although Chougnet et al. (9) in Madagascar, where malaria recently had reappeared, could not find such a correlation. Riley et al. (26) in The Gambia found no difference in antibodies against the infected erythrocyte membrane in symptomatic versus asymptomatic malaria cases, but antibodies against peptides of the 3′ region of RESA were significantly associated with protection against clinical malaria.

Antibodies to RESA inhibit merozoite invasion in vitro (36), and passive immunization of *Aotus* monkeys with human anti-RESA immunoglobulin provided a degree of protection against *P. falciparum* challenge (6). Recombinant fragments of RESA used to immunize *Aotus* monkeys gave some protection against overwhelming parasitemia (10). However, in two later monkey trials, there was no protection (11, 25), although there was a negative correlation between antibody titer and peak parasitemia.

To further dissect the immune response against RESA, we conducted a cross-sectional study within an adult immune population of the Wosera area in Papua New Guinea. This study was performed as part of the Malaria Vaccine Epidemiology and Evaluation Project (2). By choosing an immune population, we hoped to be able to identify specific immunological factors which may be important for immunity against malaria. All individuals were tested for cellular and humoral immune responses. Since total immunoglobulin G (IgG) may not be the most suitable measurement for immunity (8), we decided to analyze the humoral response with respect to IgG isotypes. The objective was to test whether individuals with any strong immune response against RESA had lower *P. falciparum* densities, lower prevalence, or showed lower malaria-related morbidity as judged by their health center visits than those without such responses.

MATERIALS AND METHODS

Study area and population. The Wosera area of the Maprik District in the East Sepik Province, Papua New Guinea, has a population of about 25,000. The area is highly endemic for malaria. Transmission is perennial, but the distribution of rainfall is seasonal, with 65% falling from October to April. Ten villages are being monitored demographically and epidemiologically. For this study, we recruited 214 adults from two villages (Kunjingini 1 and 2), consisting of 125 females and 89 males, aged 12 to 72 years (mean age, 33.5 years).

Ethical clearance for this study was obtained from the PNG Medical Research Advisory Committee.

Information and blood collection. The assessment of humoral and cellular immune responses was performed in July 1992 during one of the cross-sectional surveys. For this purpose, 30 ml of blood was taken by venipuncture into tubes containing EDTA. Blood samples were processed not later than 6 h after collection. Demography data, history of sickness for the previous week, axillary temperature, and spleen size based on the Hackett grading system (28) were recorded. One-half milliliter of blood was taken for parasitological and hematological assessments. Samples were tested for ovalocytosis and glucose-6-phosphate-dehydrogenase (G-6-PD) deficiency.

For the 2 years preceding this study, all inpatients and outpatients were recorded at the rural health center in Kunjingini 1, which represents the only health facility in this area. Malaria morbidity was assessed by enumerating the number of health center visits with a diagnosis of presumptive malaria.

Parasitological and hematological testing. Blood films for microscopy were stained with 4% Giemsa stain, and 100 fields of a thick film were examined under

* Corresponding author. Present address: Institut für Zellbiologie, Universitat Witten-Herdecke, Stockumerstr. 10, D-58448 Witten, Germany. Phone: 49-2302-669 143. Fax: 49-2302-669 220.

TABLE 1. Antibody response and data regarding T-cell proliferation against RESA

Antibody (dilution)	Total participants					Men				Women			
	No. tested	No. positive	% Positive	OD		No. tested	No. positive	% Positive	Mean OD	No. tested	No. positive	% Positive	Mean OD
				Mean	Max[a]								
Total IgG (1:500)	214	185	86	0.675	1.328	89	79	89	0.714	125	106	85	0.647
IgG1 (1:100)	212	174	82	0.724	2.985	89*	79[b]	89	0.798*	123	95*	76	0.670*
IgG2 (1:100)	214	136	64	0.864	3.134	89	57	64	0.883	125	79	63	0.850
IgG3 (1:100)	214	208	97	0.733	2.937	89	86	97	0.818*	125	122	98	0.672*
SI	195	50	26	2.64[c]	8.35	84	25	30	2.47[c]	111	25	23	2.81[c]

[a] Max, maximum.
[b] Asterisk indicates that values are significantly different between men and women ($P < 0.05$).
[c] Means calculated for positive responders only.

oil immersion before being declared negative. Densities were recorded as the number of parasites per 200 leukocytes. Taking 8,000 leukocytes per μl as average, densities were converted to the number of parasites per μl of blood (29). Hemoglobin was determined immediately with a Hemocue photometer on the basis of a modified azidemethemoglobin reaction. Packed cell volume was determined with microcapillary tubes and a hematocrit centrifuge. Values were read on a manual hematocrit meter.

Antigens. The RESA peptide (recombinant RESA) used in T-cell proliferation and in enzyme-linked immunosorbent assay (ELISA) studies was a recombinant protein expressed in *Escherichia coli* and contains several B- and T-cell epitopes (19, 28, 33), which show no diversity between different strains and field isolates (22). rRESA was provided by Saramane Pty. Ltd. The antigen, designated Ag1505H, has a molecular mass of approximately 89 kDa and corresponds to the C terminus (approximately 70%) of the RESA polypeptide. The antigen has a short N-terminal extension which includes 6 histidine residues to allow purification by nickel-chelate chromatography. RESA was diluted in sterile phosphate-buffered saline (PBS) according to the assay requirements.

Proliferation assays. Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque; Pharmacia) of 20 ml of blood, and proliferation assays were performed as described elsewhere (28). Mononuclear cells (2×10^5) were seeded in each well in triplicate, and rRESA was added to a final concentration of 10 μg/ml and incubated in complete medium. Positive controls were stimulated in triplicate with concanavalin A (5 μg/ml), and eight wells containing medium only were used as negative controls. After day 6, half of the medium (100 μl) was removed and replenished with 100 μl of complete medium containing 1 μCi of [^{3}H]thymidine (Amersham TRK565). After approximately 16 h of incubation, the cells were harvested automatically onto filter mats and counted in an LKB betaplate liquid scintillation counting system. Proliferative responses were calculated as geometric mean counts per minute of triplicate samples. The stimulation index (SI) was calculated as SI = geometric mean test counts per minute/geometric mean control counts per minute. An SI of >1 was considered positive if the *P* value was <0.05 by the Mann-Whitney rank sum test, comparing three test wells with eight control wells. We are aware that this method will ignore weak positives and will detect only definitely stimulated samples.

Detection of RESA-specific antibodies. Total IgG antibodies and antibodies of IgG1, IgG2, and IgG3 isotypes were detected by standard ELISA techniques. Fifty microliters of an rRESA solution (5 μg/ml) per well was used to coat 96-well flat-bottom microtiter plates (Dynatech) overnight. The plates were blocked for 2 h with 5% milk powder–0.5% Tween 20 in PBS. Plasma was diluted 1:500 for the total IgG ELISA and 1:100 for IgG subclass ELISAs in blocking solution and incubated for 2 h. Goat anti-human IgG labelled with horseradish peroxidase (Kirkegaard & Perry Laboratories [KPL]) was used as the second antibody to detect total IgG. Mouse monoclonal antibodies against human IgG1 Fc, human IgG2 Fab, and human IgG3 hinge fragments were used to measure immunoglobulin subclasses (Serotec MCA514, MCA515, and MCA516). As the third antibody, rabbit anti-mouse IgG Fab fragment labelled with horseradish peroxidase was used (Serotec STAR43). The enzymatic reaction was revealed 2 h later by use of the ABTS system (1:1; KPL); optical densities (ODs) were determined photometrically at 405 nm. Each sample was tested in duplicate against antigen-coated and uncoated wells. Each plate contained serial dilutions of standard plasma (40 immune serum samples pooled) in duplicate. Assays were accepted when the standard curves were comparable in slope and level for the particular assay. Samples were considered positive when their mean OD with antigen minus mean OD with no antigen (blank) was greater than the mean of 15 Australian nonimmune serum samples plus 2 standard deviations. To test if individuals totally negative for IgG antibodies to RESA failed to recognize the RESA molecule, we determined their IgM concentrations. Briefly, plasma was depleted of IgG antibodies by use of Gullsorb (Gull Laboratories Inc.) as described in the supplier's instructions, and consequently, ELISAs were performed with a serum dilution of 1:100 as described above with mouse horseradish peroxidase-conjugated anti-IgM antibodies (KPL).

RESULTS

Malaria indices, hemoglobin characteristics, and morbidity. The percentages of prevalence of *P. falciparum*, *Plasmodium vivax*, and *Plasmodium malariae* in the adult populations of Kunjingini 1 and 2 in the survey were 20, 8, and 11%, respectively. The geometric mean *P. falciparum* density for the adult subsample was 225 parasites per μl of blood. The highest density found was 3,000 parasites per μl in an apparently healthy male. We found 33 individuals (16%) with enlarged spleens, of whom 4 (1.9%) were grade 1, 14 (6.6%) were grade 2, 14 were grade 3, and 1 (0.5%) was grade 4. In three individuals, spleen grades were not determined. Mean hemoglobin values were 113 g/liter, and G-6-PD deficiency was found in six individuals (five males and one female). Samples defined as partially deficient were found in 24 individuals (13 males and 11 females). No ovalocytosis was detected.

The health center survey revealed that 61 individuals visited the health center with a presumptive diagnosis of malaria at least once during the previous 2 years (mean number of visits for the 61 individuals, 2.0; range, 1 to 8).

All indices described above were randomly distributed throughout the whole age range.

Cellular and humoral responses. Antibody responses were tested in 214 individuals, and 195 individuals were tested for a proliferative response against RESA. Nineteen of 214 individuals were excluded from the proliferation assays because of technical problems. The results obtained are summarized in Table 1. Fifty (26%) of these individuals had a proliferative response after RESA stimulation, with no significant difference between men and women in the percentage of responders (28% versus 20%; $H = 1.48$, $P = 0.225$). In general, proliferation was low, with a mean SI for responders of 2.64. The maximum SI was 8.35 in an apparently healthy female. There was no correlation between percentage of responders and age, although the majority of responders were found in the age group of 20 to 40 years.

Anti-RESA antibodies (total IgG at 1:500) were detected in 185 of 214 individuals (cutoff OD point for seropositivity [cutoff OD], 0.163). Of the 29 IgG-negative individuals, 20 (69%) had detectable IgM titers (mean OD, 0.369; highest OD, 1.335), although they were still lower than in IgG-positive individuals (mean OD, 0.938 as determined with eight random samples). Nine of the IgG-negative individuals were also IgM negative (cutoff OD, 0.098). Six of these nine individuals were also negative when the three IgG isotypes were tested and must be considered true nonresponders.

The majority of individuals had significant levels of anti-RESA antibodies in each of the IgG subclasses. Antibodies of subclass IgG1 were detected in 174 individuals (81%; cutoff

TABLE 2. *P. falciparum* prevalence in different antibody responder groups against RESA

Anti-RESA antibody	Prevalence of parasite in:					
	Nonresponders		Low responders		High responders	
	No. positive/ total no.[a]	%	No. positive/ total no.	%	No. positive/ total no.	%
Total IgG	7/29	24	15/80	19	20/105	19
IgG1	9/39	23	24/121	20	8/54	15
IgG2	15/79	19	13/65	20	14/70	20
IgG3	2/6	33	29/128	23	11/80	14
IgG1 + IgG3	NA[b]	NA	30/126	24*[c]	11/88	13*

[a] Number of individuals positive for *P. falciparum*/total number of individuals tested.
[b] NA, not applicable (i.e., no natural cutoff point was established).
[c] Asterisk indicates that values are significantly different between low and high responders ($P = 0.040$).

OD, 0.203), antibodies of subclass IgG2 were detected in 136 individuals (64%; cutoff OD, 0.264), and antibodies of subclass IgG3 were detected in 208 individuals (97%; cutoff OD, 0.042). The antibody titers of the different subclasses were all positively correlated with each other ($P < 0.001$).

We categorized an individual as a low responder when the antibody titer was lower than the mean titer of the appropriate antibody and as a high responder if the titer was equal or higher than the mean. Sixty-five percent of individuals who showed a proliferative response were high antibody responders (total IgG) against RESA compared with 35% high antibody responders in individuals who had no proliferative response ($\chi^2 = 4.7$; $P = 0.03$).

Total IgG antibodies against RESA increased significantly with age over the whole age range ($r = 0.17$; $P = 0.011$), but this was due entirely to an increase in IgG3 only ($r = 0.20$; $P < 0.001$). In contrast, cellular responses against RESA decreased with age, although not significantly ($r = -0.13$; $P = 0.062$).

Individuals with enlarged spleens showed significantly higher antibody than individuals with normal spleens (mean OD, 0.902 versus 0.740; H = 6.74; $P = 0.009$). This difference was significant for all tested IgG subclass titers (IgG1, H = 4.21 and $P = 0.04$; IgG2, H = 4.57 and $P = 0.03$; IgG3, H = 4.47 and $P = 0.03$). The opposite was observed for T-cell proliferation; i.e., individuals with normal spleens showed a tendency to have higher proliferative responses than individuals with enlarged spleens (mean SI, 2.7 versus 1.87; H = 2.1; $P = 0.147$).

Gender had no effect on T-cell proliferation or overall IgG titers, but IgG1 and IgG3 titers were found to be significantly elevated in males (Table 1). G-6-PD deficiency had no effect on the immune response.

Neither parasite densities nor prevalence was correlated with T-cell proliferation (for parasite densities, $r = -0.04$, $P = 0.575$; for prevalence, H = 2.36, $P = 0.125$). Parasite prevalence was similar in low and high responders for total IgG and IgG2, but there was a reduction, although not significant, in *P. falciparum* prevalence in IgG1 and IgG3 high responders (Table 2). When the data for the cytophilic isotypes, IgG1 and IgG3, were pooled and individuals were categorized again as high or low responders, parasite prevalence was reduced significantly in high responders compared with that in low responders ($P = 0.040$) (Table 2), but parasite densities remained similar.

We tested whether the frequencies or mean numbers of health center visits differed between high responders and low responders. Indeed, high responders in any antibody class tended to visit the health center less often with a presumptive diagnosis of malaria. This tendency was observed with all antibody classes but reached significance only with IgG1 high responders (Table 3).

No combination of immune responses (e.g., high antibody titers and proliferation) was significantly associated with any of the parasitological or epidemiological parameters tested (data not shown). In general, individuals negative in any one response (total IgG or SI) had a higher parasite prevalence (19%) than all-positive responders (i.e., both SI positive and OD positive; 16%). The same was found with the health center visits (28% versus 19%), but none of the associations reached significance.

DISCUSSION

Isotypic differences of the humoral response may be important in immunity to malaria, and we analyzed our study with respect to the immunoglobulin isotypes of antibodies against RESA. Bouharoun-Tayoun and Druilhe (8) showed that individuals protected against clinical malaria had high concentrations of cytophilic antibodies and that sera with high concentrations of noncytophilic antibodies competed detrimentally with immune sera in antibody-dependent cell inhibition (ADCI) assays. Wåhlgren et al. (34) monitored Swedish malaria patients with recrudescent parasites and found increasing levels of IgG3 against *P. falciparum* blood-stage antigens after relapses. Also, compared with nonimmune sera, immune sera from Liberians contained elevated levels of IgG3. A similar observation was made in studies with mice (17). In contrast, Dubois et al. (16) found in Madagascar, where malaria recently reappeared, IgG1 levels against the central RESA repeat sig-

TABLE 3. Number and frequency of health center visits in different responder groups against RESA

Anti-RESA antibody	Nonresponders			Low responders			High responders		
	No. of HC visitors/ total no.[a]	% HC visitors	Mean no. of visits[b]	No. of HC visitors/ total no.	% HC visitors	Mean no. of visits	No. of HC visitors/ total no.	% HC visitors	Mean no. of visits
Total IgG	11/29	38	1.8	27/80	33	2.1	23/105	22	1.7
IgG1	13/39	34	1.7	34/121	28	2.2*[c]	14/54	27	1.3*[c]
IgG2	24/79	31	1.9	20/55	30	2.3	17/70	24	1.5
IgG3	2/6	33	2.5	37/128	29	1.9	22/80	28	1.8
IgG1 + IgG3	NA[d]	NA		36/126	29	2.1	25/88	29	1.6

[a] Number of health center (HC) visitors/total number of individuals tested from this group.
[b] Mean calculated for visitors only.
[c] Asterisk indicates that values were significantly different between low and high responders ($P = 0.018$).
[d] NA, not applicable.

nificantly higher in nonprotected individuals and found generally low IgG3 levels. Our data presented here provide evidence that cytophilic, opsonizing antibodies may play a pivotal role in humoral immunity in areas with stable, highly endemic malaria. We demonstrate here that parasite prevalence was reduced in individuals who had elevated levels of IgG1 and IgG3 isotype antibodies against the recombinant RESA molecule. Although visits of adults to the health center seem to be more erratic and perhaps more a question of convenience than of morbidity, in this context the observation that high IgG1 and IgG3 responders visited the health center fewer times than individuals with lower IgG1 and IgG3 antibody titers is important.

An increase of total anti-RESA antibody with age was rather surprising in an already immune adult population, although earlier findings demonstrated that anti-RESA antibodies correlate with exposure (23). It is interesting that in our study this increase was due entirely to an increase in antibodies of the IgG3 subclass. This and the high prevalence of detected IgG3 antibodies indicate that long-term exposure to RESA stimulates B cells to switch to IgG3 expression. The switch to IgG3 has been associated recently with interferon expression (30), and Al-Yaman and colleagues found a tendency of increased interferon expression in children with reduced parasite prevalence and reduced morbidity (3). The apparent discrepancy between 97% IgG3-positive and only 86% total IgG-positive individuals may be explained by the fact that our cutoff point for IgG3, derived from nonimmune Australian sera, was extremely low. All individuals negative for total IgG showed low IgG3 titers but were still positive above the cutoff point (except six individuals who were also IgG3 negative). Antibody titers of these individuals would have escaped detection at serum dilutions of 1:500.

The relatively high number of nonresponders or low responders in all subclasses against RESA in our population cannot be explained conclusively, but similar observations have been made in other areas where malaria is endemic with intense transmission (33). Two speculative explanations have been discussed: (i) that immune responses against RESA may be genetically restricted and (ii) that the consumption of antibodies by circulating parasites reduces the amount of detectable antibodies (23). Immune responses to RESA may be HLA restricted, although Troye-Blomberg et al. (32) failed to find any association with HLA class II variants. Whether a reduced humoral responsiveness is due to a failure of appropriate antigen processing or presentation remains open. The lack of anti-RESA antibodies is certainly not due to a lack of malaria exposure in this population. Studies involving these individuals showed that other malaria antigens are recognized universally (i.e., MSA2) or at a high frequency (i.e., CSP) (4, 5).

The finding of a generally low and infrequent proliferative response of T cells in the peripheral blood is interesting and also confirms earlier findings of Rzepczyk et al. (28), who found only 25% responders against a fairly similar RESA preparation (Ag1505). General immunosuppression due to malaria has been discussed frequently, and recently, nonresponsiveness of peripheral T cells against CSP peptides has been reported for individuals living in areas with high perennial transmission (15). Another speculative explanation is that infected individuals may sequester their reactive T cells in their spleens, thus withdrawing them from peripheral circulation (18). In our population, only 3 (9%) of 33 individuals with enlarged spleens showed proliferative responses, although 26 of 33 had no microscopically detectable parasitemia. In areas where malaria is endemic such as Wosera, the exposure to malaria is constant, and as a result, the immune system is stimulated permanently and the spleen increases in size as a result of rapidly proliferating T cells (and B cells) within it. Although the difference between responders and nonresponders failed to achieve a statistically significant level, we noted a higher prevalence of splenic enlargement in individuals lacking a proliferative response (17% versus 9%). Suppressed responses against other malarial antigens such as SPf66 and MSA2 have also been found in this population (4, 5). Increased levels of all isotypes of anti-RESA antibodies in individuals with enlarged spleens could support the hypothesis of T-cell homing because direct contact between T cells and resting B cells could enhance a T-cell-dependent B-cell activation (13, 20). Also, Desowitz showed in a Papua New Guinean population a high prevalence of IgE antibodies, which would imply that individuals are infected chronically with malaria (14).

With respect to morbidity, we found a nonsignificant reduction of parasite prevalence and health center visits due to malaria in the group with good humoral and cell-mediated immune responses against RESA. The infrequent and generally low cellular immune responses will require further investigation to test to what extent differential stimulation of T-helper cells occurs. In particular, the importance of differential stimulation of either T_H1 or T_H2 cells needs to be investigated to understand the underlying mechanism of isotype switching in malaria which stimulates the production of cytophilic immunoglobulin.

The lack of any association between T-cell proliferation or total IgG antibodies against RESA with parasite prevalence or density agrees with findings of Björkman et al. (7), who found no correlation between antibody titers to RESA and parasite densities in immune adults. The finding that the total amount of cytophilic, opsonizing antibodies was associated with reduced *P. falciparum* prevalence is interesting and could explain the lack of conclusive results in studies aimed to detect total IgG only. On the other hand, RESA is only one potential target for the immune response, and lack of immunity against a particular antigen should not necessarily compromise the degree of protection provided by overall immunity attained by adults living in areas where malaria is endemic. Our findings would clearly point in the direction of emphasizing the further delineation of the humoral immune response. Also, for the development of RESA as part of a subunit vaccine, it may be important to study the effect of adjuvants on the modulation of immunoglobulin isotype expression (31).

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