IgG1 and IgG2 antibody responses to *Plasmodium falciparum* exoantigens correlate inversely and positively, respectively, to the number of malaria attacks

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Abstract

In Manarintsoa, near Antananarivo, Madagascar, two groups of patients were defined in terms of malaria clinical immune status: Group MA+ consisted of 36 patients who suffered from between one to four malaria attacks (MA) during the 20-week study, and Group MA− who comprised of 48 persons who did not have any malaria attacks during this time. In group MA+, IgM and IgG antibody levels to *Plasmodium falciparum* exoantigens (E-Ag) were inversely related to the number of malaria attacks. The level of IgM antibodies were significantly higher in group MA+. In contrast, IgG, IgG1, IgG2, IgG3 and IgG4 antibodies to E-Ag were significantly higher in group MA−. The level of IgG1 antibodies was inversely correlated, and IgG2 antibodies were positively correlated to the number of malaria attacks.

Keywords: Plasmodium falciparum; Malaria attack; Subclass antibodies

1. Introduction

In malaria, clinical manifestations have been modulated by the host’s immune responses against the parasite, and the effector mechanisms remain poorly understood. The erythrocytic stages of *Plasmodium falciparum* have presented a particular challenge for vaccine development [1]. Thus, various malarial antigens potentially eliciting a protective response have been characterized. Exoantigens (E-Ags) of *P. falciparum* are soluble, partially stable to 100°C for 5 min and excreted in the culture fluid (CF) of the asexual erythrocytic stage of *P. falciparum*. In their native form, E-Ags have low isoelectric points and high relative masses [2]. In their denatured form, E-Ags have high relative masses of 100,000, 135,000 and 170,000 [3]. Immunization of *Saimiri* monkeys with E-Ags induced a partial protection against an homologous challenge and, to a lesser extent, against an heterologous infection [4]. E-Ags have been released at the time of schizogony and their function has not yet been determined.

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although antibodies to E-Ags have been more frequently found in adults than in children [3,5].

In malaria, two stages of protective immunity were proposed: a period of 'clinical immunity in children, and a period of 'antiparasite' immunity in adults of malaria endemic areas with stable transmission [6]. The first stage could present transient high and asymptomatic parasitaemia but non-malaria attacks. In the second stage adults developed a state of non-sterilizing immunity with low parasitaemia. Therefore, the relationships among parasite density, malaria attacks (MA), IgG, IgG1, IgG2, IgG3, IgG4, and IgM antibodies responses directed against *P. falciparum* E-Ag in a malaria endemic area were studied.

2. Materials and methods

2.1. Study area

The study was conducted in Manarintsoa, a village located 20 kilometers from Antananarivo in the Madagascar highlands plateau (1200–1300 m). Eradication of malaria in this area occurred in the 1950's but *P. falciparum* malaria returned in 1985 [7]. The climate is tropical and characterized as hot and rainy from November to April (average maximum temperature > 25°C and average minimum temperature > 15°C) and cool and dry from May to October (average minimum temperature < 15°C). Rainfall varies from 120 to 160 cm per year with only 10 to 14 cm from May to October. Malaria, reintroduced in this zone, was meso- to hypo-endemic with discontinuous transmission due to *Anopheles arabiensis* and *A. funestus* [8]. *P. falciparum* is by far the most common species but *P. ovale* and *P. vivax* are also present [7,9].

2.2. Investigated population

A total of 120 persons were enrolled in the study over the whole malaria transmission season. Clinical and parasitological data were recorded weekly for each person for 20 weeks (from January to May). In addition, blood specimens were collected every other week by venipuncture in heparin tubes. An episode of malaria attack was defined as a febrile illness (axillary temperature > 37.5°C) with an asexual *P. falciparum* parasitaemia (> 5000 parasites/mm³). When a MA was confirmed, a 3-day antimalarial curative treatment, quinine-resorcinol, quinidine-resorcinol and cinchonine-resorcinol bichlorohydrate (90 mg kg⁻¹) or a 4-amino-quinoline (25 mg kg⁻¹), was immediately administered by the physician. Only 84 individuals, from 9 to 62 years old (mean 27.7 ± 13.0), donating more than six samples (a total of 726) were included in the longitudinal study and tested in all immunological and parasitological assays.

2.3. Microscopic observation

Thick and thin films were made for microscopic examination after standard Giemsa staining. Parasite densities were calculated by *P. falciparum* counting obtained from the number of microorganisms observed per approximately 20,000 erythrocytes, assuming a constant erythrocyte count of 5 × 10⁶ per µl of blood. Only *P. falciparum* was counted.

2.4. Antibody assays

E-Ag was prepared by SP-Trisacryl chromatography from the culture fluid (CF) of in vitro-cultured *P. falciparum* of SGEI strain (Gambia, Africa) as previously described [5]. Antibodies to E-Ag were detected by ELISA [3]. Briefly, PBS supplemented with 0.05% Tween 20, and 5% skimmed dry milk was used for dilution of plasma and conjugate. Conjugates used were: (a) anti-human IgG heavy chain (from goat) labelled with peroxidase (1:750, Miles Yeda); anti-human IgG1 (1:1500, from sheep); anti-human IgG2 (1:1500, from sheep); anti-human IgG3 (1:1500, from sheep) and anti-human IgG4 (1:1500, from sheep); (b) anti-human IgM (1:1500, Miles Yeda) and the corresponding anti-sheep IgG (Fc-specific) labelled with peroxidase (1:1500, Miles Yeda, France). The chromogene-substrate was orthophenylendiamine 2HCl – H₂O₂. After 10 min, the reaction was stopped with 2N H₂SO₄ and the absorbance at 492 nm (A₄₉₂nm) of the contents of each well was measured using a multiscan photometer (Flow Laboratories). Results were expressed as the difference between A₄₉₂nm values obtained in coated and uncoated plates. As controls, 50 plasma
samples from healthy Europeans, provided by the Blood Transfusion Centre of Grenoble (France) were tested. The non-specific baseline readings were 0.49 for anti-E Ag of IgG class; 0.52 for anti-E Ag of IgG1 subclass; 0.53 for anti-E Ag of IgG2 subclass; 0.82 for anti-E Ag of IgG3 subclass; 0.81 for anti-E Ag of IgG4 subclass and 0.68 for anti-E-Ag of IgM class. These values were computed from averages of absorbance values plus twice the respective standard deviation.

2.5. Circulating S-antigen

Circulating S-Ag were detected by enzyme linked immunoelectrodiffusion Assay (ELIEDA) as previously described [5]. Briefly, after electrophoretic migration for 2 h at 110 V followed by washing and coating for 30 min in phosphate-buffered saline 0.05% Tween 20, (PBS-T) - 5% dry not-fat milk, a peroxidase-conjugated affinity-purified goat anti human heavy-chain IgG (1:750; Miles) was added to the cellulose membranes and incubated for 2 h. The strips were washed three times for 5 min in PBS-T and once in 20 mM Tris-(hydroxymethyl)amino-methane, 500 mM NaCl, pH 7.5 (TBS). Then, 10 ml of TBS supplemented with 4-chloro-1-naphthol (6 mg) and 30% H₂O₂ (6 μl) was added to the membranes, and the mixture was incubated for 30 min in a dark environment and washed twice for 5 min in distilled water.

3. Results

3.1. Group of patients

Two groups of patients were defined according to bioclinical and parasitological data: Group MA+ comprised 36 patients (9 to 52 years old) who suffered from one to four MA during the study, and Group MA− was composed of 48 persons (11 to 62 years old) who did not have any MA during the study. The neurological complications induced by P. falciparum were not observed in the study population. A significant partial correlation between number of MA and age was noted (Table 1) although a 52-year-old man suffered a MA.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>r' (n = 726)</th>
<th>r' (n = 726)</th>
<th>r' (n = 312)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>-0.26 (P &lt; 0.0001)</td>
<td>ND</td>
</tr>
<tr>
<td>P. falciparum μl⁻¹</td>
<td>1</td>
<td>0.18 (P &lt; 0.0001)</td>
<td>1</td>
</tr>
<tr>
<td>MA+</td>
<td>0.18 (P &lt; 0.0001)</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>IgG Ab to E-Ag</td>
<td>NS</td>
<td>NS</td>
<td>-0.28 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>IgG1 Ab to E-Ag</td>
<td>NS</td>
<td>-0.10 (P &lt; 0.0001)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG2 Ab to E-Ag</td>
<td>NS</td>
<td>0.10 (P &lt; 0.0001)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG3 Ab to E-Ag</td>
<td>NS</td>
<td>0.11 (P &lt; 0.05)</td>
<td>0.14 (P &lt; 0.01)</td>
</tr>
<tr>
<td>IgG4 Ab to E-Ag</td>
<td>NS</td>
<td>NS</td>
<td>-0.21 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>IgM Ab to E-Ag</td>
<td>NS</td>
<td>NS</td>
<td>ND</td>
</tr>
</tbody>
</table>

n: number of samples; NS: non-significant; ND: non-calculated.

MA: number of malaria attacks in both groups.

MA+: number of malaria attacks in Group MA+.

Log ((P. falciparum μl⁻¹) + 1).

Antibodies to E-Ag (Aθ2nm).

2.6. Statistical analysis

Aθ2nm of antibodies were compared by the Kolmogorov-Smirnov two-sample test. To correlate data and to take into account the influence of putative confounding factors, a multivariate method with partial correlation coefficients (r') was subsequently used. P values ≤ 0.05 were considered significant. 'Dbase' (Microsoft) and 'Statgraphics' (Statistical Graphics Corporation) software were used.
3.2. Prevalence percentage of the study parameters

The prevalence percentage \( P. falciparum \) among all blood samples was 25.3:21.0% for Group MA\(^-\), and 31.5% for Group MA\(^+\). All the persons during the 20-week study were parasitized at least once but persons of the Group MA\(^-\) remained asymptomatic.

In both groups, prevalences of IgG, IgG1, IgG2, IgG3, IgG4 and IgM antibodies to E-Ag were 22.2%, 20.6%, 26.3%, 12.3%, 10.8%, and 42.5%, respectively. In Group MA\(^-\), prevalences of IgG, IgG1, IgG2, IgG3, IgG4 and IgM antibodies to E-Ag were 33.6%, 30.7%, 32.9%, 18.2%, 15.7% and 36.0%, respectively. In Group MA\(^+\), prevalences of IgG, IgG1, IgG2, IgG3, IgG4 and IgM antibodies to E-Ag were 5.8%, 6.6%, 17.1%, 3.9%, 3.9% and 51.2%, respectively.

Among IgG subclasses, the greatest difference between both groups concerned IgG1 anti-EAg antibody prevalence and the lowest one was IgG4 prevalence. Moreover, in both groups, IgG2 anti-EAg antibody prevalence was the most frequent.

3.3. Parasite load, IgG, IgM, IgG1, IgG2, IgG3 and IgG4 anti-EAg antibody levels

The data concerning the different study parameters in terms of time are shown in Figs. 1 and 2. Fig. 1 shows the variation of the parasite load. Fig. 2 shows the average levels of IgG, IgM, IgG1, IgG2, IgG3 and IgG4 antibodies to E-Ag.

3.4. Kolgomorov-Smirnov two-sample test

Taking into account the 20-week study, the levels of IgG, IgG1, IgG2, IgG3 and IgG4 antibodies to E-Ag were significantly higher in group MA\(^-\) than in group MA\(^+\) \((P < 0.0001)\). In contrast, IgM antibodies to E-Ag were significantly higher in the MA\(^+\) group \((P < 0.002)\).

3.5. Multivariate analysis

This analysis was made after taking into account all the data. The significant partial correlations \((r')\) between the study variables are shown in Table 1. In Group MA\(^+\), IgM and IgG antibody levels to \( P. falciparum \) E-Ag were negatively related to the number of MA. In addition, IgG antibody levels to \( P. falciparum \) E-Ag were significantly higher \((P < 0.001)\) in Group MA\(^-\) than in Group MA\(^+\). The same trend was observed for IgG1, IgG2, IgG3 and IgG4 antibody isotypes to E-Ag but the difference was only significant for IgG1 antibodies which were inversely correlated to the number of MA (Table 1). No relationship was found among antibodies and parasite levels.

3.6. Individual variation

S-Ag, IgM, IgG, IgG1, IgG2, IgG3 and IgG4 level variations from two persons of the MA\(^-\) group are shown in Fig. 3. Individual immunological responses varied from one person to another, although they have similar levels of malaria clinical immunity during the period of study.

The analogous patterns between persons of group MA\(^-\) and MA\(^+\) differed more than between persons of the same group.
Fig. 2. Levels of antibodies (A492nm) to E-Ag obtained by ELISA (logarithmic scale) during the 20-week longitudinal study in a malaria endemic area of Madagascar: (A) IgG; (B) IgM; (C) IgG1; (D) IgG2; (E) IgG3, and (F) IgG4. The error bars represent the standard deviation. For Group MA+ and Group MA' see legend of Fig. 3.
4. Discussion

Acquired immunity to blood-stage *P. falciparum* is mainly stage specific. This requires acquired malaria immunity to be predominantly mediated by T-cell mechanisms and cytokines which are essential in both regulating antibody formation and in inducing antibody-independent immunity [10,11].

In endemic malaria areas with stable transmission, young children experience clinical symptoms from malaria while adults develop a state of functional but non-sterilizing immunity [12]. The exact timing of these events varies with the degree of transmission. In the central highland plateau of Madagascar after a period of eradication, malaria returned in 1985 [7]; the people examined were still in the process of building up effector mechanisms against malaria in 1988. Therefore, in this population, age cannot be used as a classical parameter which helps to classify persons as clinical protected or unprotected. However, we observed a significant partial correlation between number of MA and age indicating that clinical protection or antitoxic immunity [6] could take longer to develop in this malaria area than in, for example, Bobo-Dioulasso (Burkina Faso) area [5] where the population is normally protected under 20 years of age. For the inhabitants of Manarintsoa, the level of anti-malarial protection was poor [9,13]. In fact, both groups (MA+ and MA−) differed with regard to the level of parasites and the number of MA. Therefore, Group MA+ was not considered to be protected but it had weak immunity to malaria, and Group MA− was not considered fully protected because asymptomatic high parasitaemia were observed in this group. The MA− group had a higher level of clinical malaria protective immunity than the MA+ group.

The longer time required to acquire clinical protection [11] may be a good model for study since it indicates the different steps to naturally acquired immunity. To evaluate the role of E-Ag antibody responses, the population investigated was divided into two levels of malaria immunity, as indicated above. In the present study, the levels of IgG antibodies to E-Ag were lower than those obtained in another malaria mesoendemic area in Bobo Dioulasso [5]. E-Ag antibody responses may play a partial role in the naturally-acquired immunity against malaria.

In the group MA+, when the effects of confounding factors were eliminated by the multivariate analysis, IgG4 antibody responses to E-Ag were positively correlated to the number of MA. In addition, in the whole study population, the levels of IgG2 antibodies to E-Ag correlated positively with the number of MA. In *Schistosoma mansoni* infection, IgG4 and to a lesser extent IgG2 specific antibodies, inhibit the eosinophil-mediated killing of schistosomula by competition with other isotype antibodies reacting with the same target antigen [14]. Therefore, IgG2 and IgG4 antibodies to E-Ag might be involved negatively in building-up clinical malaria immunity. Our observations might also explain why only a partial protection against a *P. falciparum*
challenge was obtained in *Saimiri sciureus* with E-antigens [4].

In contrast, in the whole population, IgG1 antibodies to E-Ag values were negatively correlated to the number of MA. Human IgG1 and IgG3 mediated opsonization against *P. falciparum* infected erythrocytes [15]. The natural process of malaria protection may involve cytophilic properties of IgG1 since no correlation was found between IgG1 levels and the parasitaemia.

Nevertheless, the role of antibodies in the development of clinical immunity is still unclear, and the effect of each IgG subclass is controversial. It was reported that high-titred antibody samples from *P. falciparum*-primed donors contained all four IgG isotypes while the low titred had mainly IgG3 and IgG1 [16]. In another study, the levels of IgG1 antibodies to the central repeat (DDEHVEEPTVA)₃ of Pf155/RESA were higher in non-protected subjects [17]. In contrast, in a different study, IgG1 and IgG3 antibodies predominated in protected subjects [18]. In the same study, non-protected individuals had either an overall low response or a subclass imbalance in which IgG2 were over-expressed with relative low levels of IgG3 antibodies when compared to the African protected individuals. The multivariate analysis demonstrated an intermediate shape with IgG1 antibodies to E-Ag levels negatively related to the number of MA and IgG2 ones positively related to them. Taking into account antibody prevalences, a similar conclusion was found here. It is well known that natural antigenic stimulation first triggers B cells to IgG1 or IgG3 antibodies and a subsequent stimulation will encourage these cells to produce IgG2 and finally IgG4. In the present study, in the whole population IgG2 antibody to E-Ag isotype was the most frequent. This may be due to the nature of the E-Ag differing from the central repeat (DDEHVEEPTVA)₃ of Pf155/RESA [17].

In group MA⁺, evidence of down-regulation was observed concerning IgG, IgG1, IgG2, IgG3 and IgG4 antibodies to E-Ag. This may be associated to the higher parasite load in group MA⁺. Another explanation is that conserved parasite epitopes of *P. falciparum* may not be immunodominant and take many years to induce the adequate synthesis of protective antibodies [11]. In a malaria mesoendemic area, the levels of antibodies to E-Ag were higher in adults than in children [5]. Moreover, at least partial immunological identity was found among E-Ag from different stains [2]. Therefore, further studies are necessary to sequence conserved epitopes of E-Ag.

In conclusion, the complex relationships found in this area, where malaria has been reintroduced, show isotype imbalance could differ in terms of the nature of the investigated antigens, the levels of personal protection and the specific shape of the malaria transmission. IgG2 and IgG4 antibodies to E-Ag may have a negative effect on building-up clinical acquired immunity. Inversely, IgG1 antibodies to E-Ag may be involved positively in the phase of ‘clinical’ immunity against malaria.

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