Immunological Characteristics of Malaria Antibodies in Two Regions of Madagascar

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Antibodies directed against antigens of the asexual blood stages of Plasmodium falciparum were studied in the plasma of 29 individuals infected with P. falciparum and living in two areas of Madagascar. These plasma samples were investigated by four immunological methods: indirect fluorescence, immunoprecipitation of radiolabeled P. falciparum polypeptides, inhibition of the in vitro growth of P. falciparum, and double diffusion in a gelose plate. A multifactorial correspondence analysis of the results obtained for each sample revealed that the nature of several of the antibodies varied according to the age and place of residence of the subjects. In comparison with plasma samples from older individuals, specimens from young children had a higher immunofluorescence titer, immunoprecipitated several additional peptides (90, 110, and 118 kilodaltons), revealed more precipitation lines in the Ouchterlony plate technique, and did not inhibit the in vitro growth of P. falciparum to the same extent. Furthermore, as opposed to plasma samples from individuals living in the high central plateau, plasma samples from individuals living on the east coast of the island inhibited the penetration of erythrocytes by merozoites of one of the two studied P. falciparum strains and preferentially immunoprecipitated low-, rather than high-, molecular-weight peptides.

One of the main characteristics of malaria parasites is their antigenic complexity. Unlike sporozoites, the asexual erythrocytic stages express a great number of different antigens (13), several of which have been identified and whose respective roles in inducing an immune response to the parasites remain to be determined. To develop a vaccine against the asexual forms of Plasmodium falciparum, the selected antigen must be immunogenic and induce protection, but it must also be common to all P. falciparum strains.

In a previous study, we showed that immunoprecipitable antimalarial antibodies in the plasma of P. falciparum-infected individuals from Madagascar varied according to the region where they lived (3). This study is complemented by the present work, in which antimalarial antibodies were analyzed by four techniques: indirect immunofluorescence, immunoprecipitation of radiolabeled plasmodial polypeptides, inhibition of P. falciparum in vitro cultures, and double diffusion in a gelose plate. Data corresponding to each plasma sample were processed by multifactorial correspondence analysis (MCA).

MATERIALS AND METHODS

Subjects. Twenty-nine individuals who were 3 to 43 years old and had P. falciparum parasitemia (4) were bled by venipuncture into a VACUTAINER tube containing citrate and glucose before any treatment. Nineteen of the subjects were living on the east coast of Madagascar (Manakara and Foulpointe), a hyperendemic region for malaria with year-round transmission. Ten subjects were living in Ankazobe on the high central plateau, a mesoendemic area with a 6-month transmission season (J. A. Ramananirima, personal communication). The blood samples were centrifuged, and the plasma was separated and kept at 4°C for about 1 week and then was kept at −20°C.

Parasites. FCM22, a P. falciparum strain derived from a Madagascar isolate, was used throughout this study. In addition, FCM6, derived from a Thailand isolate, was used for the in vitro culture inhibition experiment. These two strains were maintained in continuous culture in vitro for about 1 year by the technique of Trager and Jensen (16).

Indirect immunofluorescence. The titration of fluorescent antibodies was performed with in vitro-cultured P. falciparum parasites by the thick blood film technique described by Sulzer et al. (15). The significance level was a 1:64 dilution.

Immunoprecipitation of L-[35S]methionine-labeled antigens. The plasma samples were centrifuged for 20 min at 19,000 × g, and then 10 μl of supernatant was incubated with 50 μl of parasite extract from a culture of FCM22 labeled with L-[35S]methionine (3). Immunoprecipitation of the labeled polypeptides was performed with Staphylococcus aureus by the method of Kessler (10). One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli and Favre (11) in a 10% polyacrylamide gel. The gel was fluorographed with En3Hance, dried, and exposed to Kodak X-Omat AR film.

Inhibition of in vitro cultures of P. falciparum. Two millionst of each plasma sample was dialyzed at 4°C against culture medium (RPMI 1640, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], 25 mM NaHCO3) to eliminate any chloroquine that might be present. The dialysis medium was renewed three times at 24-h intervals, enabling 97% of the chloroquine to be eliminated (8). After dialysis, the plasma samples were diluted in 9
volumes of a 2.5% parasitized-erythrocyte suspension in culture medium containing 5% normal human serum.

The inhibitory activity of the plasma was assessed by the technique described by Desjardins et al. (5) with strains FCM6 and FCM22 previously synchronized in gelatin (60 to 75% of schizonts) (7). Parasite growth was assessed by the uptake of [G-3H]hypoxanthine added after 20 h of culturing and was compared with that of a control culture with normal human plasma diluted under the same conditions. Six 16-mm-diameter wells were used for each plasma sample and each plasmoidal strain. Three of these were cultured for 44 h with test plasma and used to measure the overall inhibition of the in vitro culture. The remaining three wells were cultured with normal plasma for the first 20 h and then with test plasma for the next 24 h. These wells were used to assess the inhibition of intraerythrocytic maturation from the ring stage to the schizont stage. After 44 h of incubation, the contents of each culture well were processed through a cell harvester and collected on filter paper. The filter paper was dried and inserted into a liquid scintillation counter. The corresponding counts per minute were used to assess the parasite growth in each culture well. The mean counts-per-minute value of triplicate test wells was divided by the mean counts-per-minute value of control wells and used to assess the overall inhibition of the in vitro culture (wells cultivated for 44 h with test plasma) and the inhibition of intraerythrocytic maturation from the ring stage to the schizont stage (wells cultivated with test plasma from hour 20 to hour 44).

In both strains FCM6 and FCM22, the inhibition of the penetration of erythrocytes by merozoites was calculated from the following formula: inhibition of erythrocyte penetration = 1 - (percent overall maturation/percent maturation to schizonts).

Double diffusion in a gelose plate. The Ouchterlony plate technique was carried out by filling the eight outside wells of a gelose plate with threefold-concentrated test plasma samples. The central well was filled with a supernatant of an FCM22 culture concentrated 20-fold by dialysis. The precipitation pattern of each plasma sample was determined with this antigenic preparation either unheated or heated for 5 min at 100°C to keep only the heat-stable S antigens (19).

Statistical analysis. MCA (1, 6, 12), a multidimensional descriptive and explicative method of analysis available in the case of contingency tables in which the cell entry denotes the occurrence of a couplet, was used. The rows and columns of the table are called individuals and parameters, respectively. The 25 variables measured in individuals (see legend to Fig. 1) were as follows: (i) the origin (region of Manakara and Foulpointe or region of Ankazobe) and the age of the subjects (12 years and under or over 12 years); (ii) the rate of inhibition of in vitro P. falciparum cultures split into two modalities (absent [or under 30%]) and greater than 30%; and (iii) the response to serological tests split into three modalities for indirect immunofluorescence (negativity, positivity at a titer of ≤1:256, and positivity at a titer of 1:1,024) and two modalities (absence or presence) for the other tests.

Therefore, the 25 variables were split into 51 binary dummy modalities corresponding to significant classes of original variables to constitute a complete disjunctive datum table Z (n, 51). If Z' denotes the transposed matrix of Z, B = Z'Z represents the Burt contingency table associated with Z. Each subject could be represented as a point in a multidimensional space, of which one dimension was allocated for each variable included in the study. Within this space, subjects were scattered as a cloud of points, which was difficult to visualize except by projection on planes. It was possible by MCA to determine the few planes (the so-called factorial planes) on which the projection of these points would provide a satisfactory image of the subject's cloud structure while minimizing information losses. Similarly, the set of measured parameters could be represented as a cloud of points in a multidimensional space, of which one dimension was allocated for each studied subject. A satisfactory image of this parameter cloud could also be obtained on the factorial planes; the Benzécri \( \chi^2 \) metrics (1) made it possible by measuring distances both in the parameter space and in the subject space.

The methodology of MCA was based on the research of a reduced reference system generated by new axes (principal axes) obtained by a rotation of the original coordinate system into an orientation corresponding to the directions of maximum variance. The weighted variance of coordinates was the eigenvalue \( \lambda \) of each axis. The axes were deemed to be ordered by their eigenvalues. The two factorial axes \( F_1 \) and \( F_2 \) associated with the greatest eigenvalues were used to represent a projection of the set of modality points on a factorial plane \( (F_1, F_2) \). The units of those axes were arbitrary and, in those units, the standard error of the coordinates was equal to the square root of the eigenvalue. The points for which the coordinates were at more than 2.3 standard errors from the center were replaced within the limit of the graph. On the same factorial plane \( (F_1, F_2) \) was represented the projection of the subject points, identified by origin and age. The closeness between certain subjects and certain parameters indicated the tendency for those subjects to assume a definite immunogenic profile. Indeed, a given subject was close to the parameters to which he had the highest degree of association (Fig. 2) and vice versa.

Additional analyses were carried out by using the chi-square test with and without the Yates correction for continuity. Significance was considered to be \( P \leq 0.05 \).

**RESULTS**

**Indirect immunofluorescence.** Of the 29 plasma samples, 26 contained fluorescent antimalarial antibodies at a significant titer, most often at the 1:256 or 1:1,024 dilution.

**Immunoprecipitation of \( [G-3H] \)methionine-labeled antigens.** Five polypeptides (82, 56, 44, 18, and 16 kilodaltons) were recognized by nearly all the plasma samples. Thirteen others (ranging from 140 to 25 kilodaltons) were recognized by various plasma samples. Only these latter antigens were used in MCA.

**Inhibition of in vitro cultures of P. falciparum.** A plasma sample was considered as inhibitory when the uptake of \( [G-3H] \)hypoxanthine by the corresponding culture was re-

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**Table 1.** Number of plasma samples producing a significant (>30%) inhibition of in vitro cultures of *P. falciparum* FCM6 and FCM22

<table>
<thead>
<tr>
<th>Age (yr) of subject</th>
<th>No. of samples tested</th>
<th>No. (% of plasma samples that inhibited the following:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FCM22</td>
<td>Maturation Penetration</td>
</tr>
<tr>
<td>≤12</td>
<td>15</td>
<td>11 (73) 9 (60)</td>
</tr>
<tr>
<td>&gt;12</td>
<td>14</td>
<td>12 (86) 4 (29)</td>
</tr>
<tr>
<td></td>
<td>FCM6</td>
<td>Maturation Penetration</td>
</tr>
<tr>
<td>≤12</td>
<td>15</td>
<td>6 (40) 10 (67)</td>
</tr>
<tr>
<td>&gt;12</td>
<td>14</td>
<td>4 (29) 6 (43)</td>
</tr>
</tbody>
</table>
duced by more than 30% of that in the control. With strain FCM22, intraerythrocytic maturation was inhibited by 23 of 29 plasma samples; the penetration of erythrocytes was inhibited by 13 (Table 1). The median inhibitory rates were 41 and 33%, respectively. With strain FCM6, intraerythrocytic maturation was inhibited by 10 plasma samples (of these, only 1 was from the high central plateau area); the penetration of erythrocytes was inhibited by 16. The median inhibitory rates were 22 and 41%, respectively.

**Double diffusion in a gelose plate.** When unheated concentrated culture supernatant was used as antigen, three precipitation lines (A, B, and C) were distinguishable according to their increasing distance from the antigen well. They were detected by 21, 2, and 16 plasma samples, respectively. With previously heated antigen, one line (C') corresponding to precipitated S antigen was detected by 7 of the 16 plasma samples recognizing line C.

**Statistical analysis.** MCA revealed on factorial axis 1 (horizontal), which corresponds to the greatest eigenvalue \( \lambda_1 = 0.262 \), that the following two categories were opposed (Fig. 1). (i) The Manakara-Foulpointe region was associated with children aged 12 years and under; with a significant inhibition of the intraerythrocytic maturation of strain FCM6 and of the penetration of erythrocytes by merozoites of both strains; with a positive immunoprecipitation response for bands of 25, 27, 40, 47, and 90 kilodaltons and a negative one for bands of 46, 67, 96, 110, 118, and 140 kilodaltons; and with the absence of lines A and C when tested by double diffusion in a gelose plate. (ii) The Ankazobe region was associated with adults and children over 12 years of age; with the absence of inhibition of the penetration of erythrocytes by merozoites of strain FCM6; and with a positive immunoprecipitation response for bands of 96 and 110 kilodaltons and a negative one for bands of 25, 27, 40, 47, and 90 kilodaltons.

The following two categories were opposed on factorial
axis 2 (vertical; \( \lambda_2 = 0.131 \)). (i) Children aged 12 years and under were associated with an immunofluorescence titer of 1:1,024; with the absence of inhibition of the intra-erythrocytic maturation of both strains and of the penetration of erythrocytes by merozoites of strain FCM22; with a positive immunoprecipitation response for bands of 90, 110, and 118 kilodaltons and a negative one for bands of 67, 76, and 100 kilodaltons; and with the presence of lines A, B, C, and C' when tested by double diffusion in a gelose plate. (ii) Adults and children over 12 years were associated with an immunofluorescence titer \( \leq 1:256 \); with a significant inhibition of the intraerythrocytic maturation of strain FCM6 and of the penetration of erythrocytes by merozoites of strain FCM22; with a positive immunoprecipitation response for bands of 46, 67, 76, and 100 kilodaltons and a negative one for bands of 90, 110, and 118 kilodaltons; and with the absence of lines A, C, and C' when tested by double diffusion in a gelose plate.

Neither the regions on factorial axis 1 nor the ages of the two factorial axes could be distinguished by the other modalities because the projections on the \( F_1, F_2 \) plane were too near the center of gravity.

A total of 276 chi-square tests were computed to investigate the relationship between the 25 variables taken 2 by 2.

Of these 276 chi-square tests, 49 were significant at \( P \leq 0.05 \). No relationship was detected between the lines when tested by double diffusion in a gelose plate and any of the other variables. However, numerous relationships, positive or negative, were significant between different immunoprecipitation bands, bearing out MCA findings. In particular, bands of 25, 27, and 40 kilodaltons were positively related to each other and to 47- and 90-kilodalton bands. Inhibition of schizont maturation of either of the two strains was not related to any other variable. For both strains, inhibition of the penetration of erythrocytes by merozoites was positively related to the 25-kilodalton band and negatively related to the 96-kilodalton band. The presence of the latter band was also more frequent in adults (10 of 14; 67\%) than in children (5 of 15; 33\%). On the other hand, the presence of the 25-kilodalton band was more frequent in children (10 of 15; 67\%) than in adults (4 of 14; 29\%). In both cases, the difference was significant \( (\chi^2 = 4.2; P < 0.05) \).

DISCUSSION

Given the small numbers involved in MCA, only the relationships between complementary modalities were considered; i.e., for both the Manakara-Foulpointe and
Ankazobe regions, the significant inhibition of the penetration of erythrocytes by FCM6 merozoites and the presence of antibodies immunoprecipitating low-molecular-mass peptides (25, 27, 40, 47, and 90 kilodaltons) but not high-molecular-mass peptides (96 and 110 kilodaltons) at Manakara-Foulpointe could be opposed to opposite results at Ankazobe. In the factorial plane, axis 2 reflected the difference between the ages of the individuals. Plasma samples from children aged 12 years and under had an immunofluorescence titer of 1:1,024 and did not significantly inhibit penetration of erythrocytes by merozoites of strain FCM22. These plasma samples immunoprecipitated peptides with apparent molecular masses of 90, 110, and 118 kilodaltons and recognized lines A, C, and C' when tested by double diffusion in a gelose plate. These results could also be opposed to opposite results for adult plasma.

In this study, adolescents and adults can be supposed to have been exposed to P. falciparum infection for a longer time and consequently to have developed a higher level of protective immunity than children under 12 years old. For this reason, the higher titer of antibodies revealed by indirect immunofluorescence and the higher rate of precipitation of antigens, particularly S antigens, revealed by the Ouchterlony plate technique for the sera from children are surprising. However, these results could be related to a more recent infection at a higher grade or for a longer duration in children than in adults, in whom the intensity of antigenic stimulation is probably reduced by their immune status.

Protective immunity has been reported to be related to the ability of plasma to inhibit intraerythrocytic growth of the parasite (9, 14) and merozoite penetration into erythrocytes (2, 9, 18). However, the former relationship was found only with Thailand strain FCM6, and the latter relationship was found only with Madagascar strain FCM22, demonstrating the strain specificity of these inhibitory effects previously reported by others (9, 17). This specificity limits the application of this method in determining the level of premunition against malaria.

The combined use of different techniques demonstrated immunological differences in the plasma samples of individuals exposed to malarial infections in various areas of Madagascar. We previously reported that plasma samples from Manakara-Foulpointe preferentially immunoprecipitated low-molecular-weight peptides and that plasma samples from Ankazobe preferentially immunoprecipitated high-molecular-weight peptides (3). Another difference was the ability of plasma samples from Manakara-Foulpointe and not of those from Ankazobe to inhibit the penetration of erythrocytes by merozoites of strain FCM6. The differences in anti-P. falciparum antibodies between these two distinct regions of Madagascar might be related to a variable antigenic stimulation, which is reflected in the wide diversity of P. falciparum strains in different areas of the island. Although it is not possible to determine the role of these antigenic variations in antimalarial protective immunity, to be effective in all geographic regions a future vaccine must take these antigenic variations into account and act on a common antigen.

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LITERATURE CITED