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Epidemiological Correlation between Levels of Antibodies Promoting Merozoite Phagocytosis of *Plasmodium falciparum* and Malaria-Immune Status

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A phagocytosis assay was used to measure the prevalence of cytophilic antibodies directed against *Plasmodium falciparum* merozoites in three groups of subjects from Africa: (i) preimmune (individuals aged 2 to 15 years), (ii) immune (adults), and (iii) postimmune (African adults residing out of the endemic area). Results show that levels of antibodies promoting merozoite phagocytosis (APMP) increase slowly and gradually with age. The production of high levels of APMP requires about 15 years of continuous exposure to malaria and concurs with the ability of exposed individuals to control a high parasitemia and its pathological consequences, such as spleen enlargement. In the absence of antigenic restimulation for more than 1 year (postimmune subjects), APMP titers decrease abruptly. No correlation was found between APMP levels and levels of antimalarial antibodies detected by fluorescence and precipitation assays. Low levels of APMP in subjects susceptible to clinical manifestations of the disease and high levels in subjects in a state of premunition suggest that the results of the merozoite phagocytosis assay more closely reflect clinical immunity than do other markers of antimalarial humoral immunity.

The role of merozoite antigens has been emphasized in naturally occurring and artificially raised protective immunity to malaria (for a review, see reference 6).

We have previously reported that the uptake of *Plasmodium falciparum* merozoites by human blood monocytes was enhanced in immune African adults (9) and that this effect was mediated by high levels of antimerozoite cytophilic immunoglobulin G (IgG) (7). In contrast with the findings of other studies, intraerythrocytic parasites were seldom the target of peripheral blood phagocytes in the conditions of our assay.

These high levels of antimerozoite cytophilic antibodies contrasted with the low levels found in sera from subjects recovering from a primary attack. The latter subjects are highly sensitized to malarial antigens, as shown by high indirect fluorescent assay (IFA) titers to blood stages, but are not protected and present clinical symptoms after reinfection. This difference between the results of the IFA test and of the phagocytosis assay was thought possibly to reflect the relevance to clinical immunity of the antibodies measured by the phagocytosis assay.

To further investigate the possible relationship between levels of antibodies promoting merozoite phagocytosis (APMP) and clinical immune status, we designed a complementary study with three groups of malaria-sensitized patients presenting different levels of immunity.

MATERIALS AND METHODS

Merozoite phagocytosis assay. The methods for cultivation of the *P. falciparum* FCR3/FMG strain, merozoite isolation, preparation of blood monocytes from healthy subjects, and the phagocytic assay have been described previously (7). Briefly, mononuclear cells from healthy individuals were isolated on a Ficoll-Hypaque gradient (Pharmacia), and monocytes were separated from lymphocytes by adherence to 13-mm-diameter cover slips in multiwell culture plates (10^6 cells per well). To the monocyte monolayers in 0.2 ml of RPMI medium was added 0.2 ml of either test or control sera. Cells were incubated for 1 h at 37°C in a 5% CO_2 atmosphere and washed three times in cold Hanks medium. *P. falciparum* merozoites were collected in supernatants from 4-h cultures of Percoll-enriched schizont preparations of strain FCR3. Merozoites ($n = 10^6$) in 0.5 ml of RPMI medium were added to each monocyte well. After incubation for 45 min at 37°C, cover slips were washed twice, fixed in methanol, and stained with Giemsa stain, and the percentage of phagocytosis was determined microscopically. The phagocytosis rate was evaluated by counting the number of monocytes that had ingested one or more *P. falciparum* merozoites per 500 monocytes in each test performed with each serum in duplicate. Because two monocyte preparations were required for the sera from the African village and one was required for the other group of sera, we expressed results as the ratio of phagocytosis rates obtained with each test serum over the pooled phagocytosis rates from the three normal control sera used in each experiment.

Other antibody assays. IFA tests were done by using as antigen acetone-fixed thin blood films of FCR3/FMG-infected erythrocytes. The second serum was a 1/200 dilution of fluorescein-conjugated goat anti-human IgG, IgA, and IgM (Pasteur Institute).

Countercurrent immunoelectrophoresis was done on cellulose acetate membranes as previously described (3). Results are expressed as the number of precipitin bands obtained with each serum.

Sera. (i) African village sera. Fifty sera were selected at random for the various age groups from sera collected from 364 individuals aged 2 to 75 years in the village of Donsé, 50 km north of Ouagadougou in Burkina Faso. In this savanna area, the transmission of malaria is seasonal and occurs after the summer rainfalls. As in most tropical African areas,

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cerebral malaria is infrequent in people older than 5 years, and other clinical manifestations are rare and abortive in adults. Sera collected in December, at the end of the transmission season, were divided into portions, frozen in the field, and kept at −70°C until used.

Spleen rates were estimated by palpation of standing subjects, and therefore only major spleen enlargements were recorded. Parasites were searched for and scored by microscopic examination of 100 fields of Giemsa-stained thick blood films. The density per cubic millimeter was estimated as compared with the number of leukocytes on the basis of 8,000 leukocytes per mm^3.

(ii) Postimmune African sera. We collected in Paris 20 sera of African adults from Mali, Senegal, Burkina Faso, and the Ivory Coast, selected on the basis of a clear epidemiological background: rural subjects, who were born and resided in a malaria hyper- or holoendemic area until they moved to France as adults and who had not returned to the endemic area. Eleven had arrived in Paris less than 10 months before, and nine had arrived between 16 and 36 months before. The latter were called postimmune since we observed (unpublished data), as others have (10), that on reinfection during holidays spent in their home country they could present patent clinical symptoms, high parasitemia, and occasionally cerebral manifestations.

RESULTS

In subjects from the African village, variations in levels of APMP were observed within each age group, especially in the older subjects (Fig. 1A). In the two youngest groups, APMP activity was low (range, 6 to 16%; arithmetic means, 10.6 and 10.5%) and only slightly above that of normal control sera (mean values, 4.5 ± 1.5 and 6.0 ± 1). The exposure and susceptibility to malaria of these subjects was demonstrated by a high prevalence of positive blood smears (Fig. 2A) and high levels of antimalarial antibodies detected by IFA and countercurrent immunoelectrophoresis (Fig. 1B and C).

Levels of APMP increase slightly during adolescence, with 9 of the 11 subjects aged 10 to 15 years presenting titers higher than the average of those less than 10 years old (mean phagocytosis rate, 14.7%; range, 7.3 to 23.6%). High levels were found mainly in adults. Significantly increased titers were present in all but one of the individuals aged 20 to 75 years (mean, 23.8%; range, 9 to 35.3%). A phagocytic ratio higher than 3 was recorded in 96% of the adults but in only about half of the adolescents and in none of the children younger than 10 years. No correlation was found between results of the phagocytosis assay and results of the IFA test and countercurrent immunoelectrophoresis ($r = 0.18$ and $r = 0.28$, respectively).

Figure 2 allows a comparison of the above parameters with spleen rates and blood stage prevalences in the total population studied in Donsé. The prevalence of individuals harboring parasites was high in young children and increased up to the age of 13, and thereafter a moderate decrease occurred progressively with age. Parasites were detected in 40% of the adults. However, when a discrimination is made between very low and higher parasitic loads, it appears that most adolescents and all adults harbor only small numbers of parasites and that high parasitic loads are encountered only in children. The pattern of age variation of spleen indexes is very similar, showing a steady decrease with age and the lowest prevalence in adults. The pattern of prevalence recorded in the 50 subjects whose sera were used in the phagocytosis assay is close to that observed in the total population (prevalence of high parasitic loads in the five age groups, in order of increasing age: 42, 20, 10, 0, and 0%; prevalence of enlarged spleen, respectively: 28, 30, 20, 0, and 0%). This slow pattern of acquisition of premunition is in agreement with several previous studies in similar areas (2, 4, 5, 13).

There is an inverse but close relationship between the increase of mean APMP levels per age group and the prevalence either of high parasitic loads or of spleen enlargement per age group ($r = 0.95$, $P < 0.01$; $r = 0.98$, $P < 0.01$, respectively) (Fig. 3).

In the African population studied in Paris, the levels of APMP have a clear bimodal distribution (Fig. 4). In individuals who arrived from the endemic area less than 1 year before blood sampling, results were essentially similar to those of the adults of the Burkina Faso village. In contrast, in subjects who remained free of reinfection for 16 to 36 months, APMP levels were low, similar on average to those of the youngest children of the African village or to those found in European subjects recovering from a primary infection (7, 9). The difference between APMP levels in the two groups studied is significant ($P < 0.01$). In contrast, no significant difference is found between IFA titers in the two

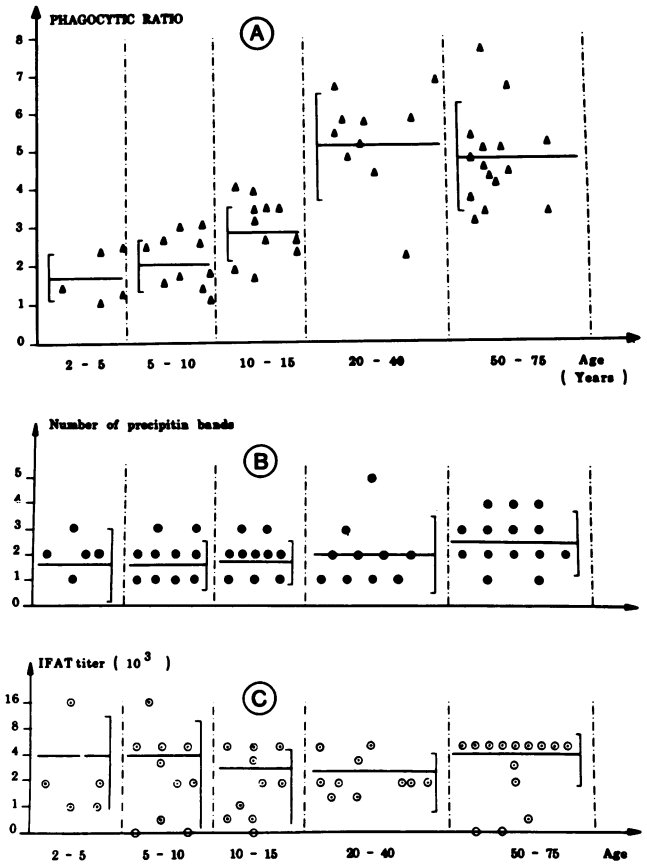

FIG. 1. (A) Merozoite phagocytosis rates in the village of Donsé. Results are expressed as the ratio of the phagocytosis rate recorded with each serum over the mean obtained with three normal sera used in each of two experiments (percent phagocytosis with normal sera, 4.5 ± 1.5 to 6.0 ± 1; serum values of patients ranged from 6.0 ± 1.0 to 35.3 ± 6.6%). Each value is the mean of duplicate tests. (B) IFA test (IFAT) titers for the same subjects. (C) Immunoelectrodiffusion assay for the same subjects.

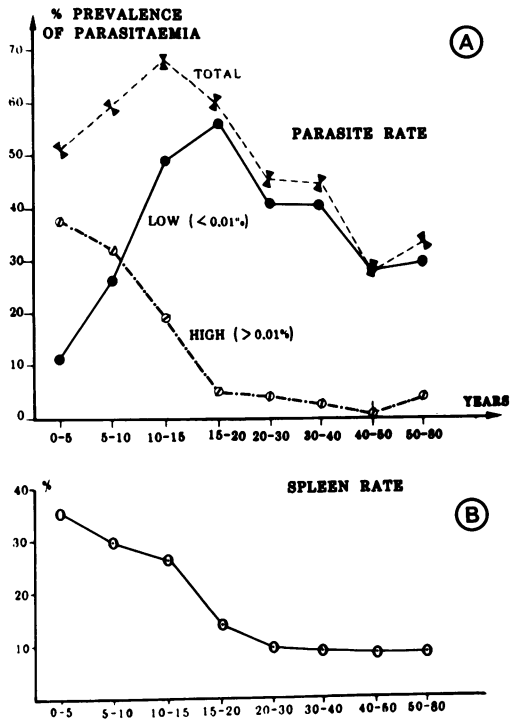

FIG. 2. (A) Prevalence of blood parasitemia. The figure shows the total prevalence of positive thick blood smears and the separate prevalences of parasitemia higher or lower than 1 parasite per 10,000 erythrocytes in the 364 subjects studied in the village of Donsé. (B) Prevalence of subjects with enlarged spleens in the same population (expressed as a percentage).

groups (arithmetic means of titers: 5,980 and 3,942, respectively; $P > 0.5$).

DISCUSSION

The design of an immunological assay reflecting effective immunity to malaria is an important goal, but it is difficult to achieve since protection can be clearly demonstrated only by the clinical response upon challenge. Since this experimental approach is not acceptable, we must rely on naturally occurring challenges in endemic areas to study the development and persistence of effective immunity to malaria.

We took advantage of two well-known epidemiological features of malarial immunity to investigate the protective component of the humoral response: (i) the fact that premunition is acquired very slowly over many years of continuous exposure to the parasite and untreated blood parasitemia lasting for weeks or months, and (ii) the fact that premunition does not last long in individuals without natural rechallenge. Therefore, the study of correlations between results of a given immunoassay in subjects in various epidemiological and clinical situations appears to be one means to try to define an assay reflecting acquired protection.

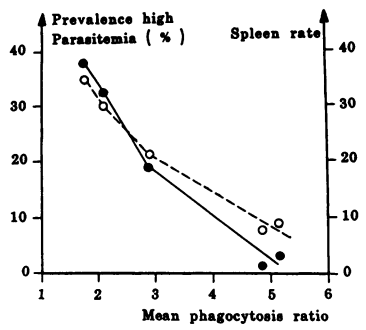

FIG. 3. Correlation between mean phagocytic ratios per age group and the prevalences of high parasitic loads (●) (>0.01%) and spleen enlargements (○) in the same age groups.

In the present study, we selected and compared three groups of patients: those in the process of acquiring protective immunity (African children), those who have reached this state (African adults), and those who are likely to have lost protection after residence away from the endemic area (postimmune African adults). We thought that, with a fourth group made up of European adults recovering from a primary attack, previously studied (7), the pattern obtained would help determine the significance of the assay used. Only one group was protected, whereas the three others were true controls; the latter three were sensitized to malarial antigens, but all were known to be susceptible to the pathogenesis induced by the parasite with high parasitic loads and concomitant clinical symptoms.

Cytophilic antibodies, involved in recognition and capture of free merozoites by armed monocytes, were found previously to have a restricted distribution among malaria-sensitized individuals (9). The present results confirm and extend these findings.

(i) High levels of APMP were recorded only in the adults from the African village and in African adults who had stayed in Paris for less than 1 year. Significantly lower titers of APMP were found in the other subjects, who were sensitized but not protected. Results show that the acquisition of high APMP levels is a slow and gradual process. Five years of exposure to the disease do not induce levels higher than those found in subjects recovering from their first attack (2, 7). Individual variations observed within each age group may reflect either different individual ability to respond to the antigen or different degrees of exposure to malaria or may simply depend on the delay between the last antigenic restimulation and the blood sampling.

(ii) Most interestingly, the gradual acquisition of APMP

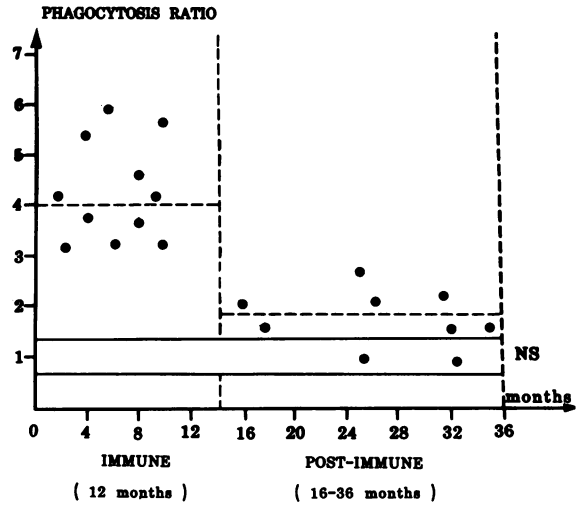

FIG. 4. Merozoite phagocytosis rates in African adults staying in Paris. Results are expressed as in Fig. 1. The number of months refers to the length of residence away from the endemic area. NS, Normal serum.

with age correlates positively with the ability of the individual to prevent development of a high parasitic load and its pathological consequences, such as spleen enlargement; that is, it correlates with markers of clinical immunity.

(iii) Despite their slow increase, with more than 15 years being necessary to reach high titers, APMP levels drop abruptly when African adults remain free of antigenic restimulation for more than 1 year. This result is reminiscent of the T-cell response pattern obtained with a similar group of patients. The specific proliferative response to merozoites of T-lymphocytes from immune African adults was high, whereas that of postimmune subjects was not significantly different from that of normal subjects (1).

(iv) As in previous studies (7) no correlation was found between the results of the phagocytosis assay and those of two serologic assays which measure total antiparasite antibodies. This suggests that antimerozoite cytophilic antibodies account for a small part of parasite-specific antibodies.

Therefore, the rate of antibody-dependent merozoite phagocytosis appears to be more closely related to the presumed protective status than are other antibody tests. Recently, antibodies to RESA, an antigen expressed on glutaraldehyde-treated infected erythrocytes, were also found to increase on average with exposure to malaria (12). However, anti-RESA antibody levels vary widely within each age group and, in contrast with APMP, low levels can be found in subjects with clinical immunity. From our results, it cannot be concluded that antibodies involved in recognition and capture of merozoites by monocytes are directly involved in the protection mechanisms. We show only a close epidemiological correlation between both phenomenons, which may correspond as well to the simultaneous appearance of other antibodies. However, the correlation we observed is also supported by in vitro studies demonstrating that immune IgG-monocyte cooperation has a far stronger inhibitory effect upon the parasite growth than does antibody alone (8).

In the present study, we made no attempt to characterize the antibody specificities involved in the phagocytosis process. We can only speculate that the antibodies measured should recognize accessible antigens, i.e., rhoptry or merozoite surface antigens, and we expect that the molecules involved are still present and antigenically active in the merozoites we used. However, the same sera were used to study antibody specificities in each group by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (P. Dubois, P. Druilhe, M. Jendoubi, and D. Ariat, Ann. Inst. Pasteur Immunol., in press). The most striking differences which appeared from that comparison of immune versus pre- and postimmune subjects concerned two polypeptides of 96 and 100 kilodaltons.

From an immunological point of view, the long delay required to reach some degree of protection against malaria has been difficult to explain (11), as large amounts of antigens are present for several weeks per year in infected individuals. This question also applies to the antibodies measured by the phagocytosis assay. It hardly seems possible that the target antigen(s) on merozoites is so poorly immunogenic that more than 15 years of continuous exposure are required to build up a strong immune response. Since no strain restriction of cytophilic antibodies recognizing merozoites was observed in previous (7) and more recent studies (F. Lunel et al., manuscript in preparation), the delay suggests mainly that the parasite is able to interfere in some way with the normal function of the immune system. However, such a hypothesis does not explain why this humoral response, once established, is short-lived in the absence of the parasites.

Whatever the reasons for the unusual kinetics of production of these antibodies, our epidemiological data suggest that antibodies directed to some undefined merozoite antigen might play a role in the defense against malaria blood stages. Therefore, this functional-immunity assay could be of use in studying the corresponding stimulating antigens and the conditions of appearance of a humoral immune response effective against malaria.

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