Antibody-Independent Inhibition of Plasmodium falciparum In Vitro Cultures

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The sera of 100 Colombian individuals of African origin living in a malaria-endemic area of the Pacific coast were studied with regard to their capacity to inhibit Plasmodium falciparum cultures in vitro. Antimalarial antibody levels determined by indirect immunofluorescence were higher in the group of infected individuals than in the noninfected individuals, and inhibitory activity assessed by the inhibition of parasite incorporation of H-hypoxanthine in vitro was present in the sera of both the infected and noninfected patients. We believe that the noninfected patients had high inhibitory capacities for the P. falciparum FCB-1 isolate. When the inhibitory effects of some of the sera were tested by using four parasite isolates from different regions of the world, striking differences among them were found.

Serum transfer experiments have clearly demonstrated the importance of humoral factors in the control of malaria infections (5, 15). Furthermore, it is known that naturally acquired antibodies play a significant role in neutralizing the extracellular parasites (6, 17). Antibodies can also prevent the binding of infected erythrocytes to endothelial cells and may operate by inhibiting the sequestration of Plasmodium falciparum in the deep vascular system of the host (21).

It has been observed, both in vivo and in vitro, that immune sera of humans and experimental animals may contain, in addition to antibodies, soluble mediators capable of producing inhibition and damage of intraerythrocytic parasites (9, 18). The resulting damaged intracellular parasites have been called crisis forms (19) and can be produced by the presence of reactive oxygen radicals (1) or by crisis form factor (CFF) (9). Although the chemical nature of CFF has not yet been elucidated, it is known to be a nondialyzable factor which is different from antibodies, tumor necrosis factor, and alpha or gamma interferon (4, 12).

CFF seems to play an important role in acquired immunity to malaria. It was found in the sera of individuals from endemic areas of Sudan, sera which were functionally immune to malaria in the absence of high antibody titers. In contrast, CFF was not found in individuals from Indonesia, where acquired immunity to malaria was less effective and seemed to depend mainly on the presence of anti-merozoite antibodies in the population (11). Marsh et al. (14) studied a Gambian rural population with apparently the same malaria epidemiological characteristics as the population studied previously by Jensen et al. (9) in Sudan. In this study, no differences were found in the parasite growth when exposed to sera of nonimmune Europeans or immune Gambians. Butler et al. (3) studied individuals from three regions of Papua New Guinea with different malaria transmission rates. They found inhibitory activity in 55% of those individuals; however, the inhibition capacity of the sera was lost after dialysis, suggesting the occurrence of a dietary toxin with antimalarial properties.

In order to know if this inhibitory activity was present in individuals from malaria-endemic regions of Colombia and to define its possible protective role, the present study of a population of African origin living on the Colombian Pacific coast was performed.

MATERIALS AND METHODS

Experimental subjects. One hundred individuals of African origin, 15 to 50 years old, living in Buenaventura (a malaria-endemic region of the Colombian Pacific coast that has unstable transmission) were studied. All of the individuals submitted to medical examinations, blood counts, and thick smears; also, clinical histories were recorded.

Of the 100 subjects, 50 consulted the National Malaria Eradication Service in Buenaventura because of malaria symptoms. Each of these 50 subjects was diagnosed as positive for P. falciparum by thick smear. The other 50 subjects were healthy individuals who had been logging for several years in a paper factory in a forest where both P. falciparum and Plasmodium vivax are transmitted. This group was closely followed in the medical service of the paper company during the 3 months prior to the study. They did not present any malarial episode or symptoms related to the disease during this period and were, therefore, considered to be immune individuals. No individuals of this group had previously taken prophylactic antimalarial agents.

In addition, 20 healthy mestizos from the city of Cali, a malaria-free region, who had never experienced malaria and who had negative serology for plasmodia, were included in this study and considered the normal control group.

Sera. Ten milliliters of blood was taken from each subject by venipuncture; the blood was then allowed to clot for 1 h at room temperature, and sera were separated by centrifugation. Aliquots were kept frozen at -20°C until used for antibody detection and the inhibition assay.

Antimalarial antibodies. Antimalarial antibody levels were measured in each individual using the indirect immunofluorescence technique described by O'Neil and Johnson (16). Asexual blood forms of the FCB-1 Colombian isolate of P. falciparum were used as an antigen source.

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Parasitemia. Parasitemia was determined on Giemsa-stained thick smears and quantified on the basis of leukocyte counts.

Parasite cultures. Four different *P. falciparum* isolates (FCB-1 from Colombia, T-4 from Thailand, FCR-3 from Gambia, and FUP Palo Alto from Uganda) were maintained in continuous in vitro culture, according to the method of Trager and Jensen (20), and used for the inhibition assays.

Inhibition assays. Prior to performance of the inhibition assays, the parasites were highly synchronized to ring stages by a combination of the gelatin flotation method (8) and lysis of the mature stages with 5% sorbitol (13).

Parasites were cultured in 96-well flat-bottomed microculture plates in complete RPMI 1640 medium without hypoxanthine and containing 10% of the test serum.

In order to remove chloroquine or other antimalarial drugs which might be in the sera and to equilibrate with the parasite culture medium, all test sera were previously dialyzed for 24 h against RPMI 1640 medium diluted 1:100 in double-distilled water supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) and 0.21% sodium bicarbonate (dialysis medium). Each single serum was dialyzed against 1,000-fold its own volume.

Microcultures were set up in quadruplicate in volumes of 100 μl with 5% hematocrit, 3% of ring-infected erythrocytes, and medium supplemented with 10% of either test sera or normal pooled sera.

After 24 h of culture, the supernatants were removed and a fresh medium containing the same sera was added. At this time, microcultures were labeled with 3H-hypoxanthine (Du Pont, NEN Research Products) dissolved in RPMI 1640 medium at a concentration of 1 μCi per well of culture. Sixteen hours later, microcultures were harvested onto glass fiber filters (Cambridge Technology Inc.) with a cell collector (Mini-Mash, Microbiological Associates), and the incorporation of the 3H-hypoxanthine into parasite nucleic acids was measured by using a liquid scintillation system (LS 3801; Beckman Instruments, Inc.). The assay was also evaluated qualitatively by visual examination of thin films stained with Giemsa stain.

We selected as normal parasite growth the range of values produced by 10 serum specimens previously selected because of their good capacity to support the in vitro parasite growth. The mean of these individual values was similar to the growth produced by the pool of the same sera and was considered to be 100% growth. The same pool of normal sera was used throughout the whole study.

According to the behavior of the normal sera from both the pool and the normal control group, we arbitrarily labeled activity as normal (0 to 20% inhibition), moderate (21 to 50%), and severe (51 to 100%) (Fig. 1).

Serum fractions. Sera that showed inhibitory activity higher than 50% were precipitated with 35% (vol/vol) (NH₄)₂SO₄ for 8 h at 4°C, and both fractions, supernatants and precipitates, were dialyzed for 36 h at 4°C. For the first 24 h, each sample was dialyzed against 1,000 times its volume with 0.02 M phosphate buffer (K₂HPO₄, pH 8.0, and then against RPMI 1640 culture medium at the same ratio. Dialysates were changed at 24-h intervals.

The precipitates were diluted to 5% in complete RPMI 1640 medium supplemented with 5% of the normal pool sera and tested for inhibitory activity as described above. On the other hand, the supernatants were additionally passed through a chromatography column containing Sepharose CL-4B conjugated with *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals), according to standard protocols (7), in order to absorb immunoglobulin G antibodies that may have still been present in the supernatants. After this step, all supernatants were examined using a radioimmunoassay method (2) to determine the absence of residual immunoglobulins G and M and by indirect immunofluorescence to determine plasmid antibodies. Supernatants were then tested for inhibitory activity at a concentration of 10% (vol/vol) in complete culture medium upon addition of 5% normal pool sera.

Susceptibility of different isolates of *P. falciparum* to inhibitory activity. To test their susceptibility to inhibitory activity, the four parasite isolates were cultured, synchronized, and studied under the same conditions as for the inhibition assay with the FCB-1 isolate. A group of sera were selected and tested at the same time against the different isolates.

Each experiment was repeated at least twice, and the experimental variation was less than 10%, indicating that the inhibitory activity was conserved during storage of the supernatants at −70°C for several months.
Statistical analysis. Arithmetic means were obtained in order to establish the mean growth of the parasites. Inhibition is expressed in proportions as follows: percent inhibition = [1 – cpm (test serum)/cpm (control)] × 100. By using Student’s t test and 95% confidence intervals, we established whether there was a statistically significant difference between the growth of parasites exposed in vitro either to the test sera or to the control pool.

RESULTS

All malarious patients presented typical symptoms such as fever, chills, headaches, and myalgias. None of these individuals presented splenomegaly or symptoms of cerebral malaria. Parasitemias ranged from 0.2 × 10⁹ to 150 × 10⁹ parasitized erythrocytes per μL and were treated with a combination of chloroquine and primaquine. The patient with the highest parasitemia was hospitalized because of the severity of his symptoms and the risk of developing a complicated disease. Individuals from the control group were all negative for antimalarial antibodies by indirect immunofluorescence, whereas 80% of the individuals from the immune group presented positive reactions, indicating their previous contact with malaria and possible immunity. Of the infected individuals, 94% presented positive titers. Antibody levels were low in the immune group and moderate in the infected group, with geometric mean titers of 1:126 and 1:1,047, respectively.

No correlation was found between indirect immunofluorescence titers and inhibitory activity in either the currently infected group (Pearson correlation coefficient, −0.10; P = 0.70) or the immune group (r = −0.20; P = 0.88) (Fig. 2).

The control group showed an inhibition mean of 2.72% with a confidence interval between −2.16 and 7.60%, which was similar to the behavior presented by the individual sera of the normal pool (data not shown). The infected group showed a mean of 14.84% with a confidence interval between 6.56 and 23.12%, whereas the immune group showed a mean of 29.03% with a confidence interval between 20.13 and 37.93%. All groups presented several individuals on the negative side of inhibition (Fig. 1, dots below the zero line), meaning that these sera could stimulate the parasite growth as well as the normal sera.

There was no statistically significant difference in the percentage of inhibition between the infected and the control groups. We found, however, a significant difference between the control group and the immune set of sera. The sera of 36% of the individuals from the infected group and 58% of the immune group were inhibitory. Of the sera from infected patients and immune subjects, 12 and 28%, respectively, produced inhibitions above 50%.

Since there was no relationship between antibody titers and inhibitions, the nonantibody fractions of the sera were tested. All sera with high inhibitory activity were fractionated both by salt precipitation and affinity chromatography. Inhibitory activity was found exclusively in the soluble fraction. Not one of the immunoglobulin G precipitates produced significant inhibition of the culture (Table 1).

We did not find any correlation among the symptoms referred to by the patients, the number of previous malaria
episodes, and the parasitemia and their inhibitory capacity in vitro ($r = 0.046, P = 0.80$). Interestingly, one of the individuals (011) who showed the strongest inhibition in vitro carried at the same time the highest parasitemia ($152 \times 10^3$ parasites per μl) and an antimalarial antibody titer of 1:640.

This suggested that the inhibitory activity observed in vitro, but apparently ineffective in vivo, could be strain specific. Therefore, we tested the susceptibility of several isolates of *P. falciparum* to the inhibitory activity of serum supernatants obtained from some of the individuals we were investigating. There were some sera (004 and 013) which were inhibitory for all isolates and others (014, 035, and 036) which inhibited only one or two isolates. Some sera (001, 008, 010, 012, and 021) were inhibitory for all isolates except one (Table 2). Thus, there appears to be isolate variation in the susceptibility to inhibition by a given serum or variability in the effects of the different sera on a single isolate.

The percentage of inhibition of these sera was again calculated according to the growth induced in each parasite strain by the pool of normal sera.

All four strains of parasites were typed by using monoclonal antibodies (kindly provided by Jana S. McBride from the University of Edinburgh, Edinburgh, Scotland) in order to see whether there was any correlation between this different susceptibility to the CFF and antigenic diversity. It was impossible, however, to determine any pattern of correlation between serotyping and inhibition.

**DISCUSSION**

We have found that sera from individuals of African origin living in a *P. falciparum-* and *P. vivax-*endemic area of the Colombian Pacific coast do have inhibitory activity for parasite growth in vitro. The high prevalence (47%) of this inhibitory activity in individuals of the region and its nondialyzable nature are similar to the CFF activity described by Jensen et al. (10) in Sudan. However, we found that the inhibitory factor occurred in both infected and immune individuals. There was no apparent correlation among the inhibitory activity, frequency of previous malaria episodes, clinical symptoms, and parasitemia.

The inhibitory activity of these sera was present in the antibody-free fraction of the sera. Titers of specific anti-*P. falciparum* blood stage antibodies did not correlate with the presence of this factor.

Specific antimalarial antibodies in the infected individuals reached titers fourfold higher than those of the immune group, yet there was no significant difference in inhibitory activity. Furthermore, there was no correlation between

<table>
<thead>
<tr>
<th>Serum source</th>
<th>% Inhibition ± SD in:</th>
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<tbody>
<tr>
<td></td>
<td>Unfractionated serum</td>
</tr>
<tr>
<td>Control b</td>
<td>12 ± 3.00</td>
</tr>
<tr>
<td>011</td>
<td>97 ± 6.30</td>
</tr>
<tr>
<td>016</td>
<td>72 ± 5.70</td>
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<tr>
<td>022</td>
<td>71 ± 2.55</td>
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<tr>
<td>033</td>
<td>89 ± 2.00</td>
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a Percentage of in vitro inhibition for the FCB-1 isolate displayed by some of the most inhibitory sera before and after immunoglobulin precipitation with ammonium sulfate.

b Normal donor without malaria experience.

| Table 2. Susceptibility of isolates from different geographical origins to CFF
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Normal growth [μm] (x 6)</th>
<th>% Inhibition ± SD</th>
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<tbody>
<tr>
<td>001</td>
<td>FC-1</td>
<td>143.1 ± 0.1</td>
<td>000</td>
</tr>
<tr>
<td>004</td>
<td>FCB-3</td>
<td>86.4 ± 0.0</td>
<td>000</td>
</tr>
<tr>
<td>008</td>
<td>FC-2</td>
<td>78.6 ± 0.1</td>
<td>000</td>
</tr>
<tr>
<td>010</td>
<td>FCB-1</td>
<td>73.5 ± 0.0</td>
<td>000</td>
</tr>
<tr>
<td>012</td>
<td>FCB-2</td>
<td>66.5 ± 0.0</td>
<td>000</td>
</tr>
<tr>
<td>021</td>
<td>FCB-3</td>
<td>58.5 ± 0.0</td>
<td>000</td>
</tr>
<tr>
<td>025</td>
<td>FCB-4</td>
<td>55.5 ± 0.0</td>
<td>000</td>
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<tr>
<td>035</td>
<td>FCB-5</td>
<td>43.5 ± 0.0</td>
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</tr>
<tr>
<td>038</td>
<td>FCB-6</td>
<td>40.5 ± 0.0</td>
<td>000</td>
</tr>
</tbody>
</table>

- Normal growth: *O* = 50.0% inhibition; — = 0.0% inhibition.

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**TABLE 1.** Intraerythrocytic growth inhibition by serum fractions

<table>
<thead>
<tr>
<th>Serum source</th>
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a Percentage of in vitro inhibition for the FCB-1 isolate displayed by some of the most inhibitory sera before and after immunoglobulin precipitation with ammonium sulfate.

b Normal donor without malaria experience.
inhibitory levels and parasitemia. Interestingly, the sera of some individuals which induced strong inhibition of the in vitro cultures had at the same time high levels of parasitemia. This indicates that the sera had inhibitory activity against the FCB-1 isolate used for the in vitro test but apparently no inhibitory activity against the parasites infecting the patients themselves, thus suggesting variability in the parasite susceptibility to inhibition.

It was found that some sera presented wide variations in their capacity to inhibit in vitro isolates from different geographical origins. It was also observed that sera which were noninhibitory against the FCB-1 isolate could retard the growth of other isolates and vice versa. Some sera were inhibitory for all four isolates, whereas others inhibited only one or two of them. These results indicate a high degree of polymorphism in both the inhibitory effect and the parasite susceptibility to it.

Immune individuals who had been followed for more than 3 months and who had not had any malarial episode presented inhibitory activity more frequently.

The difference in the prevalence of the factor in both groups was not significant. If the inhibitory factor was specifically induced by malarial parasites, the fact that these individuals did not complain of malaria or have high titers of antimalarial antibodies would suggest that their infections may have been aborted before they produced any symptoms which would induce parasitemias and increase the levels of antibodies. Alternatively, it is possible that the immune system could be stimulated by other microorganisms to induce the production of cytokines, which might stimulate mononuclear cells to produce the inhibitory factor. This could explain why the group of immune individuals maintained such high levels of CFF in the absence of circulating parasites. In this case, the infection may occur only when the individuals are in contact with parasites which are not inhibited by the factor being produced. The polymorphism of inhibition displayed by the different isolates renders this possible.

So far, almost all studies on CFF have been conducted using only one isolate of P. falciparum. It is possible that by using several isolates of the parasite, the presence of inhibition might be detected in a number of other countries of high malaria endemicity.

In summary, our results indicate that sera of infected or immune individuals contain an inhibitory factor for parasite growth in vitro. The high prevalence and nonantibody nature of this factor implies that it may be similar to CFF described previously by Jensen et al. (10). Studies are being conducted to determine the chemical nature of CFF. In our case, the presence of the factor did not appear to correlate well with clinical immunity. A new finding was the diversity of inhibitory activity on the growth of different parasite isolates.

ACKNOWLEDGMENTS

We gratefully acknowledge the skilled assistance of René Gerrens and Frank Gagliardi in culturing P. falciparum for the inhibition assays. We are also grateful to Ruth S. Nussenzweig for discussions of our findings and to Gordon Langsley for reviewing the manuscript.

This investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, from the Instituto Colombiano para el Fomento de la Educación Superior, and from the Fondo Colombiano de Investigaciones Científicas-COLCIENCIAS.

LITERATURE CITED


