Serum Factors Inhibitory for *in vitro* Development of *Plasmodium falciparum* Blood-stage Parasites

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Sera from 29 individuals residing in a malaria-endemic region of Colombia were evaluated by an inhibition assay for their capacity to retard the growth of Plasmodium falciparum in vitro. *The inhibitory activity was found to be independent of antibody activity. Furthermore, the degree of inhibition of parasite development was variable, depending on the parasite isolate used for the assay and the season of malaria transmission. We selected sera with high inhibitory activity and carried out partial analytical characterization by anion exchange fast protein liquid chromatography (FPLC) to identify the chemical nature of the inhibitory factor(s). The results suggested that the* in vitro *inhibitory activity might result from the additive effect of different molecules. It appears that these molecules could be non-specifically induced by stimulation of the immune system, they seem to play a role in the immunity to malaria.*

Key words: malaria - *Plasmodium falciparum* - immunity - crisis factor - inhibition

It is known that both cell-mediated and humoral immune responses to malaria contribute to acquired immunity in man (Long 1993). Previous studies have demonstrated the presence of soluble non-dialyzable non-antibody factor(s) in sera from both experimentally infected animals, and human subjects previously exposed to *Plasmodium* infections (Jensen et al. 1982, Chulay et al. 1981). Such factors, although poorly characterized, seem to be correlated with immunity to malaria. Parasites exposed to these sera undergo variable degrees of degeneration first described as crisis forms of *P. brasilianum* parasites in immune *Cebus* monkeys (Taliaferro & Taliaferro 1944). In humans, crisis activity was originally detected in sera of malaria-immune subjects living in Sudan (Jensen et al. 1982). These authors referred to crisis form factor (CFF) the serum factor(s) responsible for the retardation of intracellular parasite development. The presence of CFF appeared to correlate with the seasonal transmission of malaria in Sudan (Vande Waa et al. 1984). The inhibitory activity of such sera however was not observed in studies carried out in areas of Indonesia (Jensen et al. 1984) and The Gambia (Marsh et al. 1987) with similar malaria transmission patterns.

In previous studies, we have demonstrated its presence in sera of individuals residing in endemic areas of Colombia (Herrera et al. 1987). It does not correlate with levels of parasitemia and different isolates of *P. falciparum* may vary in their *in vitro* sensitivity to this factor(s) (Perlaza et al. 1990). Moreover its presence does not show any correlation with ethnic differences (Herrera et al. 1990). In the present study, we explored the existence of seasonal variations in CFF activity in Colombia previously reported in Sudan (Vande Waa et al. 1984). In addition we partially characterized the CFF using sera from immune individuals. Here, we present evidence which suggests that the CFF activity may be due to a molecular complex, most likely of proteinous nature.

MATERIALS AND METHODS

EVALUATION OF CRISIS FORM FACTOR (CFF) ACTIVITY

EXPERIMENTAL SUBJECTS AND COLLECTION OF BLOOD SAMPLES

A total of twenty-nine individuals between 15 and 50 years of age, were followed for a period of one year. Study subjects resided in Docordó (Chocó), a malaria-endemic village in the rain forest

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of the Colombian Pacific Coast where malaria transmission is unstable. All individuals consented to medical examination and a blood sample was taken every 4 months during the one year period to assess the presence of malaria infection and serum crisis activity.

Blood (5 ml) was collected in a citrate phosphate dextrose (CPD) anticoagulant solution. Plasma were separated by centrifugation at 4500 x *g* for 15 min and in order to remove any possible antimalarial drug present in these samples, dialysis was carried out against RPMI 1640 medium diluted 1:100 in double-destilled water suplemented with 25mM Hepes and 0.21% sodium bicarbonate for 24 h at 4°C. Dialysis membranes of a 12-14 kD cutoff were used. After dialysis, plasma were centrifugated at 4500 x g for 15 min to remove insoluble material and kept frozen in aliquots at -70 °C until used.

IN VITRO PARASITE INHIBITION ASSAYS

Two *P. falciparum*, isolates FCB_1 from Colombia and T_4 from Thailand, were maintained in continuous *in vitro* culture according to the method described by Trager and Jensen (1976), to test the *in vitro* inhibition capacity of the test plasma, cultures were metabolically labelled with tritiated hypoxanthine $^3H(Hx)$ (Jensen et al, 1982). The mean $^3H(Hx)$ up-take value from 20 individual sera that supported the *in vitro* parasite growth was considered as reference values for normal parasite growth (100% growth). This value was similar to that produced by the pool of the same sera, which was used throughout the whole study. We consider 20% inhibition as the minimal degree of inhibition above which CFF activity may be found. The assay was also qualitatively evaluated by visual examination of thin blood films stained with Giemsa.

ANALYTICAL CHARACTERIZATION OF CFF

For isolation of CFF, we selected plasma from a *P. falciparum* patient infected with a high CFF activity and plasma from a healthy donor from a non-endemic area as control. Test samples were subjected to fast protein liquid chromatography (FPLC) using a Mono-Q (Anion- exchange) column in a gradient of sodium/potassium phosphate buffer pH 6.5; 0.16M (Buffer A) and 0.33M (Buffer B). Twenty individual fractions of plasma with CFF activity and 18 fractions from a healthy donor were collected. Pools of these fractions were tested on a parasite growth inhibition assay according to the method described above.

RESULTS

FOLLOW-UP OF PARASITE INHIBITORY ACTIVITY THROUGHOUT THE YEAR

None of the 29 individuals exhibited malaria symptoms or had detectable parasitemias at the time of blood collection. Furthermore none complained about malaria symptoms during the year of the study. All of them exhibited high antibody titers against both *P. falciparum* and *P. vivax* (data not shown).

We previously showed that the inhibitory activity of human sera was specific to certain parasite isolates (Perlaza et al. 1990). In the present study at least two *P. falciparum* isolates from different geographical regions of the world were tested. Fig. 1 shows a comparison of the inhibitory activity of the sera obtained in the months of May, October and March on the FCB_1 (Colombian) and the T_4 (Thai) isolates. Medians of inhibitory activity with 95% confidence intervals on the FCB_1 were 35% (15%-48%), 46% (22%-69%) and 26% (14%-46%), respectively, indicating that at least 30% of the study group maintained this inhibitory capacity throughout the entire year (Fig. 1A). The inhibition of the T_4 isolate depicts a more defined trend of seasonal variation; inhibitory activity was greatest with sera taken in May (72%) with a confidence interval between 63% and 87%, followed by sera obtained in October (45%) with a confidence interval between 26% and 71%. Clearly, no inhibition (5%) with confidence interval between 2% and 10% was observed with sera taken in March (Fig. 1B) (Campbell, 1974). The test plasma exhibited consistent CFF activity in five different experiments although the mean incorporation of $^3H(HX)$ varied approximately 20% through these experiments.

CFF CHARACTERIZATION FROM HUMAN PLASMA

Fig. 2 shows the inhibitory activity of the pools from both control and crisis plasma obtained after anion-exchange chromatography. The inhibitory activity was found in pool 3 containing fractions 6 to 10 of crisis plasma and non-inhibitory activity was found in fractions containing immunoglobulins (Pools 1-2). The apparent molecular weights that corresponded to these fractions was approximately

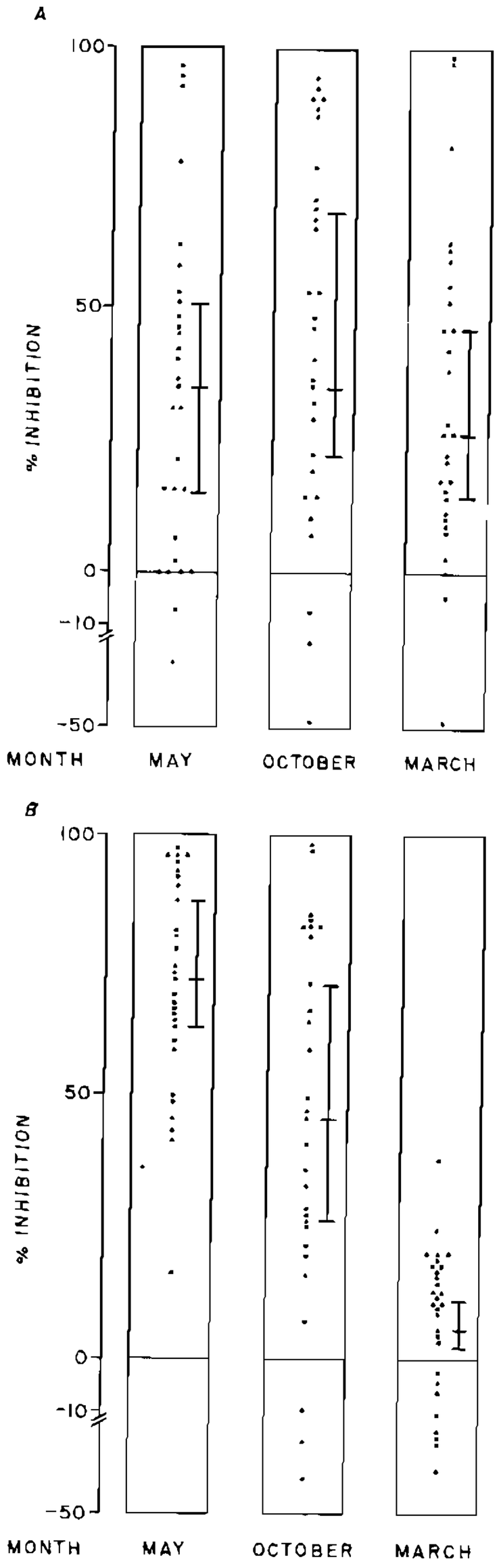

Fig. 1: follow up of *in vitro* inhibition of intra-erythrocytic *Plasmodium falciparum* development. Percent of inhibition of plasma samples collected during the months of May/89, October/89 and March/90 as measured by ^{3}H-Hx incorporation . (A) FCB_1 isolate is from Colombia and (B) T_4 isolate from Thailand. The figure indicate the median and 95% confidence intervals of the inhibitory activity for the study group. Each dot corresponds to the mean of triplicates or quadruplicates. Only values above 20% are considered significant.

100 kD as determined by gel electrophoresis (data not shown). Similar elution patterns were observed with control plasma, however, there was not inhibitory activity in any of the fractions obtained from control plasma.

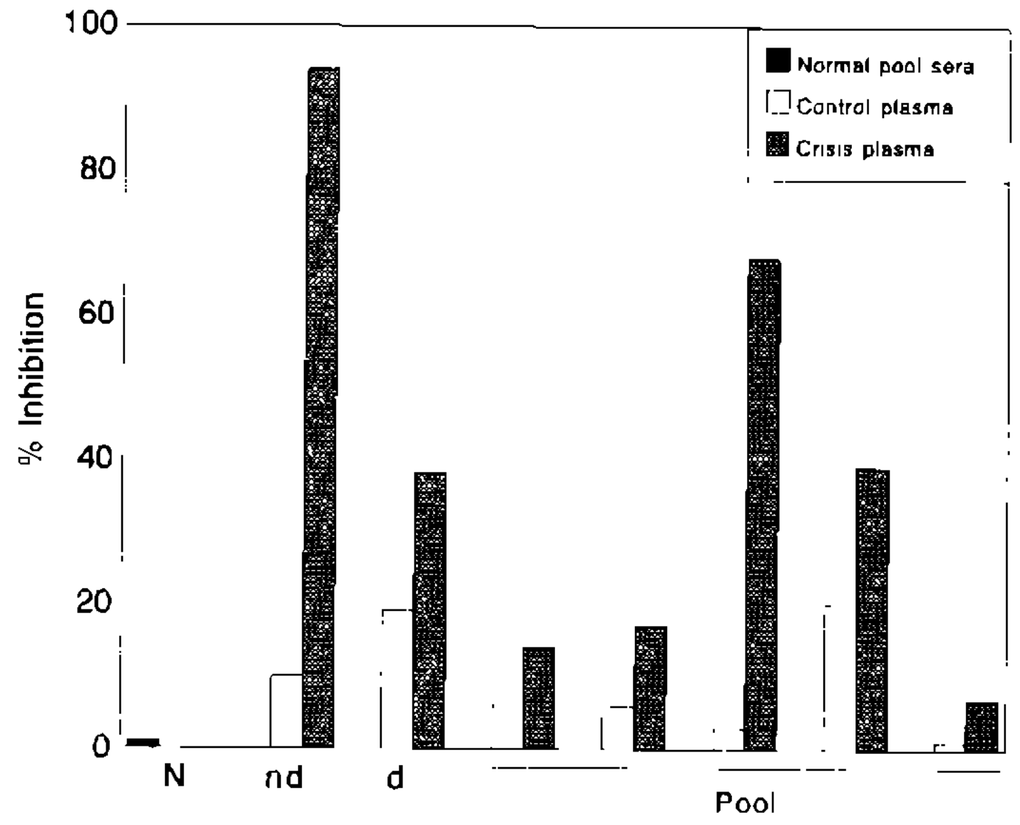

Fig 2: inhibition percent of plasma from healthy donor and plasma with crisis activity. The fractions were collected from Anion exchange column in five pools (1-5) and tested in an inhibition assay against FCB_1 isolate of *P. falciparum*. N: Normal pool sera used to support the *in vitro* parasite growth, d: dialyzed, nd: not dialyzed

DISCUSSION

Previous studies demonstrated inhibitory activity in sera from infected and non-infected individuals from malarious areas of Colombia (Herrera et al. 1987). This inhibitory activity did not correlate with levels of parasitemia nor with antibody titers, and surprisingly, different isolates of *P. falciparum* varied in their sensitivity to this inhibitory activity (Perlaza et al. 1990). To determine if the production of CFF was related to ethnic background, two independent communities (negroes and indians) living under the same malaria transmission in the region of Docordó, Colombia were previously studied (Herrera et al. 1990) and no difference was found between ethnic groups. This contrast with other studies performed in other regions like Indonesia (Jensen et al. 1984), The Gambia (Marsh et al. 1987) or Papua-New Guinea (Butcher et al. 1987) versus Sudan (Jensen et al. 1982).

In present study, the population from Docordó, Colombia was used as study subject. This village on Pacific Coast is in the middle of a tropical rain forest, and therefore is characterized by high rainfall levels throughout the whole year with unstable malaria transmission. In contrast with other malarious regions like Sudan where there are real dry seasons, the Colombian Pacific Coast has the lowest rainfall levels between December and April, about 30% of the maximal levels observed from May to Novem-

ber. Individuals were evaluated three times over a period of one year and inhibitory activity was always present in the population. The *in vitro* inhibitory effect of sera varied from one season to the other. Maximal CFF activity was observed in May, coinciding with the increase in rainfall indexes for the area, whereas CFF activity in October was intermediate and the lowest level was showed in sera samples obtained in March. These findings are in agreement with previous studies carried out by Vande Waa et al. (1984) who previously observed strong seasonal variation of CFF in Sudan, where it appears more prevalent during the rainy season. Additionally, our results show that the pattern of seasonal variation of the inhibition was different in the two isolates of *P. falciparum* studied. We favor the hypothesis that in addition to malaria other multifactorial stimulating events may occur during the higher rainfall season potentiating the prevalence of CFF activity in sera. The absence of clinical symptoms of malaria and parasites in the individuals suggests the influence of different stimuli in the CFF seasonal prevalence pattern. Some of the individual sera did not change seasonally. Instead, these maintained high levels of CFF activity throughout the study period.

The biochemical nature of CFF activity is poorly understood. Plasma selected for chemical analysis showed high inhibitory activity *in vitro* and it was found to be independent of antimalarial antibody activity tested by indirect fluorescent antibody test (IFAT) (data not shown). Analytical characterization by anion-exchange chromatography suggests the existence of a heterogeneous population of molecules with slightly different values of negative net charge.

According with the standards run in the anion exchange column, the immunoglobulin-containing fractions corresponded to pools 1 and 2. These pools did not show CFF activity (Fig. 2) confirming previous studies which showed that factors responsible for CFF are of non-antibody nature (Jensen et al. 1983, Perlaza et al. 1990).

On the other hand, studies have demonstrated that the cytokines TNF and IFN-γ mediate the gametocyte killing effects of serum taken during crisis of a malaria blood infection suggesting the existence of additional humoral or extra-cellular factors(s) in crisis serum that are necessary for the parasiticidal effects of TNF or IFN-γ (Naotunne et al. 1991). Similarly, the addition of recombinant cytokines to *P. falciparum in vitro* retarded the growth of the parasite and this effect was dependent of the concentration and the exposure time of the cytokines (Orago et al. 1993). Therefore, these findings suggests that the presence of oxygen radical intermediates from mononuclear cells (Ockenhouse et al. 1984), cytokines and other molecules of unknown function could present crisis activity.

The limited amount of plasma available from individuals from the endemic areas displaying crisis activity restricts the chemical analysis of these molecules. However, further analysis are in progress to better determine the chemical nature, origin and specificity of these malaria inhibitory molecules.

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