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Research Note

GROWTH INHIBITION OF CULTURED *PLASMODIUM FALCIPARUM* BY IMMUNE SERUM FROM TANZANIA.

THOMAS B. NYAMBO[1] AND HIROJI KANBARA

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Abstract: The inhibition titers of sera from adult residents in Dar es Salaam, Tanzania to intraerythrocytic growth of *Plasmodium falciparum* were not correlated to their antibody titers by immunofluorescence and enzyme immunoassay, and did not exhibit specificity to the strain with a certain MSA-1 gene. Another inhibitory factor(s) was found in a low molecular fraction with molecular weight less than 30,000 that was separated from a high molecular fraction by centrifugation on a membrane filter (centriprep-30, Amicon). This factor induced morphological changes of intraerythrocytic parasites and was thought to correspond to crisis form factor (CFF) by Jensen *et al.* (1982). The factor was shown to remain in the serum of an adult Tanzanian who had lived in a non-malarious area six months prior to the commencement of this study and to be contained in a hyperimmune serum from a Japanese patient even though on a low level.

INTRODUCTION

Various humoral factors in immune sera to inhibit malaria parasite growth were described by Mcgregor and Wilson (1988). Although intraerythrocytic growth inhibition has been considered to be associated mostly with antibodies, Jensen *et al.* (1982, 1983 & 1984) have shown that sera from Sudanese living in malaria endemic areas contained factors independent of antibodies which induced crisis forms of erythrocytic stages of *P. falciparum* and which were absent in Indonesian immune sera with high immunofluorescence antibody (IFA) titer. These results suggested the presence of two different and ethnogenetically biased humoral mechanism for elimination of asexual blood forms. In the present work we examined the inhibitory pattern of Tanzanian sera to cultured *P. falciparum* in relation to anti-malaria antibodies. Furthermore serum samples were devided into a high (HMF) and a low molecular fraction (LMF) by centrifugation on a cut off membrane filter for Mr 30,000 to specify the growth inhibitory factors.

MATERIALS AND METHODS

Serum samples and parasite strains.

Twenty five serum samples were collected from adult malaria patients in Muhimbili Hospital in Dar es Salaam, Tanzania where malaria is hyperendemic. The samples were collected in sterile tubes and preserved with 0.05% sodium azide prior to transportation. A serum sample T was obtained from a Dar es Salaam resident, a 39-year-old man who had lived in a nonendemic area, Japan for six months prior to this study and another sampe M was from a Japanese malaria patient, a 43-year-old man who had contracted the disease while on visit to Mozambique four months before and repeated recrudescence till the serum collection. Before use the Tanzanian serum samples were freed of sodium azide by Sephadex G-25 column chromatography, and were screened for chloroquine (CHQ) according to the modified Hanskins method (Hanskins MM II) as described by Mount *et al.* (1987). Control samples were obtained from voluntary persons in Japan. Part of the serum samples were diluted 2.5 fold with serum free RPMI 1640 media, devided into two fractions by centrifugation at 3,500 rpm for about 55 minutes using

Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852, Japan
[1]Present address, Department of Biochemistry, Faculty of Medicine, Muhimbili University College of Health Sciences, 65001 Dar es Salaam, Tanzania

centriprep-30 concentrator (Amicon, USA) at a cut-off of Mr 30,000. The filtered low molecular fraction (LMF) was kept alone and the remaining high molecular fraction (HMF) was adjusted to the original volume by adding serum free RPMI 1640 media and kept at 4°C till further use. Three *P. falciparum* strains FCR, K1 and Thai 836 were used in the inhibition assays. Briefly the strain FCR was used due to its easiness of culture and the latter two strains were because they had the distinct genotype of the precursor of the major merozoite surface antigen (MSA-1) (Jongwutiwes *et al.*, 1991). *P. falciparum* parasites were usually cultured in group O red blood cells adjusted to 5% hematocrit in a RPMI 1640 medium supplemented with 10% human serum as described by Tragar and Jensen (1976) at humidified conditions of 5% carbon dioxide, 5% oxygen, 90% nitrogen and then overlaid media were changed with fresh ones everyday.

Enzyme linked immunosorbent assay (ELISA).

FCR strain infected red blood cells with 13% parasitemia were processed for antigen preparation according to the method by Spencer *et al.* (1979). Protein estimate by the method of Lowry in the final supernatant which was the source of antigen was 3.2 mg/ml. The antigen was aliquoted and stored at -80°C till further use. Microplates (ELISA Plate, MS-3496, Sumitomo-Bakelite) were coated with 0.2 ml antigen diluted in 0.06 M carbonate buffer, pH 9.6 at 1 : 300 overnight at 4°C, washed 3 times with PBS, sealed and kept at 4°C till further use. Prior to use in the next step the microplates were blocked by Block Ace solution (Dainippon Pharmaceuticals) according to the manufacturer's instruction. This solution was also used as a diluent for the following steps. Test serum was diluted at 1 : 200. 0.2 ml of diluted serum was run in duplicate on the antigen coated microtiter plates, incubated at 37°C for two hours. The plates were washed three times in PBS-Tween and incubated with 0.2 ml of dilute (1 : 500) horseradish peroxidase-conjugated goat IgG fraction to human IgG (Organon Teknika Co.) for two hours at room temperature. The plates were read at 400 nm after 30 min incubation with 0.3 ml of substrate (o -Phenylenediamine) at room temperature. The ELISA titer was expressed as the ratio of OD value of each sample to that of control.

Immunofluorescence assay (IFA).

Dried thin layer blood slides of parasitised cells were treated with various dilutions of immune sera and incubated in moist chamber at 37°C for one hour. The slides were then washed 3 times with PBS and incubated with 100 times dilute fluorescein conjugated anti-human IgG (Fc fragment) (Organon Teknika Co.) in PBS at 37°C for half an hour. The slides were then 3 times washed with PBS and mounted with 5% glycerol in PBS for fluoroscopy. The IFA titer was expressed as the highest dilution that showed positive.

Growth inhibition assays.

Sorbitol synchronized parasites were prepared as described by Lambros and Vandergerg (1979). After an overnight culture the synchronous culture with more than 95% trophozoites was adjusted to 0.5 to 2.5% parasitemia depending on the experiment and to a hematocrit of 5% by freshly washed group O red blood cells. Assays containing final volume of 0.2 ml with ten times dilute immune serum or LMF or HMF (at final concentration of 10%) were prepared in flat bottomed 96 well culture plates. Where immune serum was used together with the normal serum, the latter was adjusted to give the filal concentration of 10%. In one experiment the immune sera were adjusted to final dilutions from 5 times to 160 times. Culture conditions were as described earlier and the overlying media were changed daily for fresh media. Giemsa stained blood films were examined under the light microscope to ascertain the level of parasitemia and possible morphological changes. The inhibition rate was expressed as the ratio of the difference of parasitemia between control (C) and sample (S) to control; (C-S/C)x100.

Table 1. IFA titer and growth inhibition rate after 72h incubation to three *P. falciparum* strains by four Tanzanian immune sera.

Sample No.	FCR		K1		Thai 836	
	IFA	Inhibition (%)	IFA	Inhibition (%)	IFA	Inhibition (%)
8	≧3,200	57	1,600	79	1,600	65
34	≧3,200	58	≧3,200	81	≧3,200	79
5	400	46	400	78	200	74
7	400	41	400	71	400	65

Table 2. CHQ levels and serum antibody titers to FCR strain of immune sera and their inhibition rates after 72h incubation.

Sample No.	CHQ (μM)	ELISA	IFA	Inhibition (%)
11	0	2.7	100	54
10	0	3.0	800	45
8	1.5	8.1	≧3,200	74
34	61.5	18.3	≧3,200	80
15	2.3	5.6	100	61
5	56.3	4.1	400	53
7	0	5.9	400	68
3	62.3	7.5	1,600	65
4	61.0	3.3	800	65
T[a]	0	4.1	400	73
M[b]	0	>18.3	≧3,200	57

[a] A serum from a Dar es Salaam resident living in Japan for 6 months.
[b] A serum from a Japanese patient with chronic infection for 4 months.

Results and Discussion

Using the preliminary ELISA 9 samples were selected from 25 for further examination. They had IFA titers ranging from 100 to more than 3,200 that well corresponded to ELISA titers from 2.7 to 18.3 (Table 2). Of these samples four were used in the growth inhibition assay of *P. falciparum* strain FCR, K1 and Thai 836 (Table 1).

The 72 hours inhibition pattern of the immune sera did not correspond well with their respective antibody titers (Table 1 and 2). Although the Thai 836 (MAD 20 type) is slightly less susceptible to the inhibition assay than the K1 type, the least susceptibility was seen in the FCR. This we attributed to the fact that FCR strain is better adapted to in vitro culture conditions than the other two strains. Eleven serum samples were analysed for a widely used anti-malaria drug CHQ and its metabolite desethylchloroquine and used in the inhibition assay of synchronous culture of *P. falciparum* FCR strain (Table 2). Levels of CHQ and its metabolites above 2.0 μM were considered significant. However, although serum samples 34, 5, 3 and 4 exhibited high drug levels close to the 75 μM considered to be the maximum therapeutic serum levels in the treatment of malaria (Staiger *et al.*, 1981) they did not influence the growth inhibition by the immune serum. We consider this to be due to the low CHQ concentration levels in the final culture volumes because the immune sera were 10 times dilute in the final inhibition assays. This could also have been augmented by possible CHQ resistance by the parasite strains used.

Table 3. Growth inhibition after 72h incubation to *P. falciparum* FCR strain by LMFs and HMFs of different immune sear.

Serum sample		Average inhibition (%)
CONTROL	LMF	10
	HMF	0
M	LMF	27
	HMF	37
T	LMF	57
	HMF	16
34	LMF	39
	HMF	33

The analysis of the inhibitory capacity of the LMFs and HMFs of the serum M, T and 34 on synchronously growing *P. falciparum* FCR strain revealed a stronger inhibition tendency of LMF than HMF except the Japanese serum M (Table 3), although the ELISA titer by the dilution method (Spencer *et al.*, 1979) for the Japanese serum M was two times higher than the serum sample 34 which was the highest value 10,240 in Tanzanian samples. Surprisingly the sample T from a Tanzanian who had lived in Japan for six months still maintained high inhibitory level of LMF despite his low antibody titer. This finding may partly explain reported findings by various groups that *P. falciparum* infection does not cause the severe complication of acquired immunodeficiency syndrome, AIDS (Wabwire-Mangen *et al.*, 1989, Lucas, 1990 and Simooya *et al.*, 1991).

Further analysis of the LMF and HMF inhibition using the serum T showed that the inhibition was dose -dependent in either case. Inhibition capacity of the

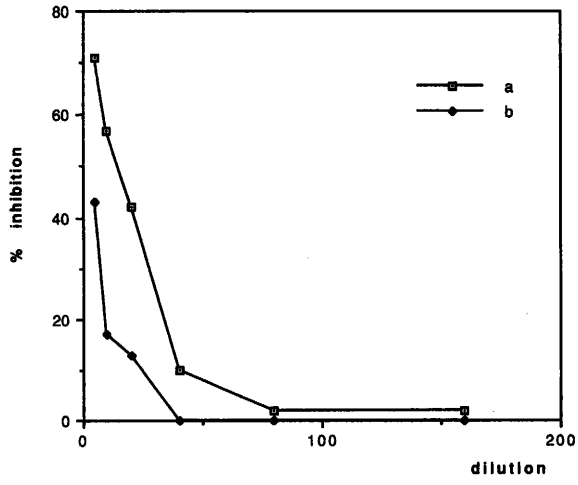
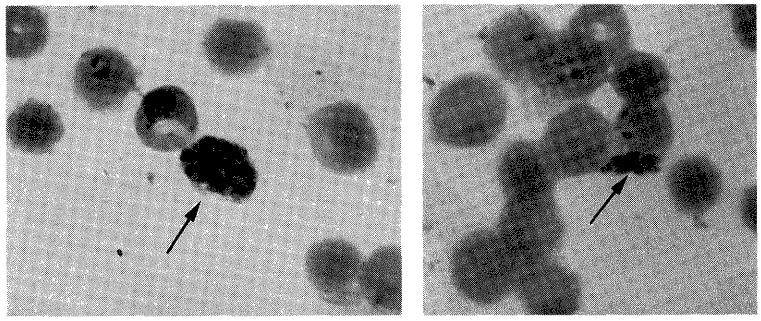


Figure 1 The effect of concentration on the inhibition capacity of LMF (a) and HMF (b) from the serum T on synchronously growing *P. falciparum* FCR strain in 48 hours.



a b

Figure 3 Giemsa stained thin blood films of synchronous culture after 72h culture with a nonimmune serum (a) and with a immune serum T(b). Note the number of produced merozoites (→).

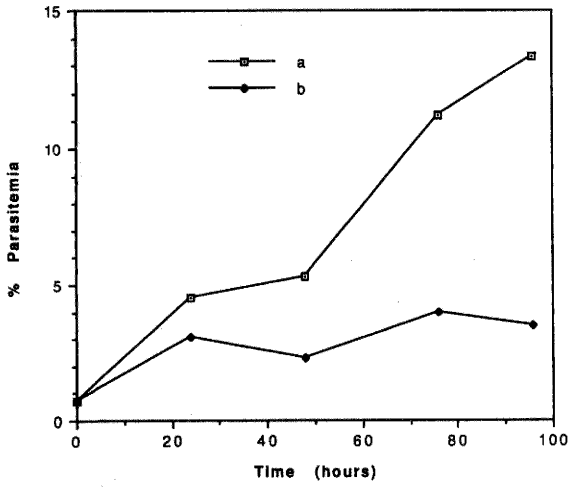


Figure 2 Growth of *P. falciparum*, FCR strain in the presence of LMF of T serum (b) and in the absence (a). At time points 0, 48, and 96h the predominant forms were trophozoites while at time points 24 and 76h ring forms predominated.

serum fractions was almost absent after 40 times dilution (Figure 1). To determine the life stage of the parasite that was affected by the immune sera, the growth pattern was monitored everyday. Synchronization was observed to start disappearing after 48 hours. Crisis forms were noted with typical characteristics of bulky ring appearance, poorly staining IRBC, denaturated schizonts and mature schizonts showing remarkably fewer merozoites (between 4 and 7) as described by Jensen *et al.* (1983) instead of Laverania characteristic of the number of merozoites between 12 and 16 (Figure 3). Inhibition was observed at all stages as characterised by low parasitemia and a negative gradient in the immune serum, implicating intracellular growth impairment (Figure 2).

We have shown here that the LMF with a molecular weight less than 30,000 was a more potent inhibitor than the HMF which contained the immunogloblins. Infected red blood cells cultured with the LMF, washed and incubated with fluorescein conjugated anti-human IgG could not show malaria antibodies, while with the HMF we could detect antibodies on the surface of the infected red blood cells (results not shown). We thus report presence in the Tanzanian immune sera, of an anti-malaria factor(s) that is capable of growth inhibition of intracellular parasites and formation of crisis forms, which support previous findings where it was refered to as crisis form factor (CFF) by Jensen *et al.* (1982), originally by Taliaferro and Taliaferro (1944). We could not induce crisis forms by the HMF of the immune serum. This mechanism of malaria defense is possibly a parallel mechanism to antibody defence and our findings suggest that they do occur together in an individual at varying degrees of potency. Further studies are necessary to define the nature of the factor.

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