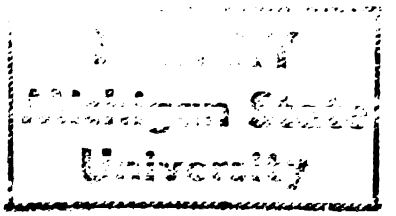


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James B. Jensen
Major professor

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# CHARACTERIZATION OF VARIOUS EFFECTS OF CRISIS FORM INDUCTION ON PLASMODIUM FALCIPARUM IN VITRO

BY

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ABSTRACT

CHARACTERIZATION OF VARIOUS EFFECTS OF CRISIS FORM INDUCTION ON PLASMODIUM FALCIPARUM IN VITRO

By

Joseph Michael Carlin

The various effects of crisis form induction on Plasmodium falciparum in vitro has been examined. Sudanese parasite isolates were analyzed for chloroquine and mefloquine sensitivity by two techniques differing in serum source. A 72% test failure rate was noted when parasites were analyzed in the patient's serum as compared to a 28.8% rate in nonimmune serum. In subsequent experiments, sera containing crisis form factor (CFF) were used to supplement standard test plates containing parasites of known drug sensitivities. These sera retarded parasite development in the presence or absence of drug. Inhibition of growth by drug and Sudanese serum combinations was additive.

A variety of known or suspected inducers of crisis form parasites; Sudanese CFF sera, TB patient sera, rabbit tumor necrosis sera, and human γ-interferon, were compared in vitro for cytotoxic effects on P. falciparum and mouse L-M cell cultures. Inhibition was determined by measurement of incorporation of radiolabeled nucleic acid precursors. When compared to normal serum, parasites cultivated in a 1:4 dilution of rabbit tumor necrosis sera, TB patient sera, or CFF sera were metabolically inhibited 73%, 75% and 95%,

respectively. Human γ-interferon had no direct effect on parasite growth. However, only rabbit tumor necrosis sera were inhibitory to L-M cells, inhibiting incorporation by 80% at a 1:1000 serum dilution. These findings suggest that tumor necrosis factor is apparently not responsible for induction of parasite crisis forms by the inhibitory human sera tested.

CFF was examined for stage- and time-dependent effects. The erythrocytic cycle was divided into 8 h intervals, and synchronized parasites of each interval were exposed to CFF for various time periods. At cell harvest, hypoxanthine and phenylalanine incorporation and glucose consumption were compared to values obtained in normal serum. The most profound derangement of metabolism occurred in parasites 0-8 h post-invasion. Inhibition decreased in tests started with progressively older parasites.[73] Cultivation in CFF serum for 8 h caused maximal inhibition of precursor incorporation; longer exposure did not increase inhibition. However, the effect on glucose consumption varied inversely to the duration of exposure, decreasing as parasites matured, showing little reduction as they entered schizogony.

This dissertation is dedicated to my wife, Dawn, for her constant encouragement, support, understanding, and love; and to our daughter, Lindsey, for the incredible joy she has added to our lives. I love you both dearly.

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# INTRODUCTION

## Incidence and control of malaria

Malaria remains the most lethal of the major parasitic diseases. The World Health Organization estimates a worldwide incidence of over 150 million cases per year.[46] In Africa alone, annual mortality in children is greater than one million.[42] Although eradication programs had reduced malaria incidence, the world is presently experiencing an alarming resurgence in this often fatal disease, due in part to the spread of drug-resistant strains of Plasmodium falciparum and insecticide-resistant mosquito vectors. Parasite drug resistance in humans has been demonstrated for the anti-folates, the quinolines and quinine.[52] In relevant mosquito vectors, insecticide resistance has been documented for the organochlorines, organophosphates, carbamates and pyrethroids.[23] Clearly, the need exists for alternative control measures, including the development of malaria vaccines. Research is currently in progress on the creation of effective vaccines against potentially susceptible stages in the parasite life cycle, including sporozoite, merozoite and gametocyte stages.[19,21,42,45,48]

It has been demonstrated that monoclonal antibody to the circumsporozoite protein in malaria sporozoites is able to specifically block attachment and entry of sporozoites into cultured human hepatoma cells.[29] Furthermore, polyclonal antibodies raised in rats against sporozoites of P.

falciparum exhibit strain cross-reactivity.[49] Limited success has also been achieved in blocking merozoite invasion into erythrocytes with antibodies produced in response to merozoite preparations.[19,20,21,65] Despite the progress that has been made in these areas, serious problems remain to be solved, including the completeness of protection provided by sporozoite immunization (successful invasion of one sporozoite can lead to fatal disease[42,45]), and the effect of antigenic variation[5,6] and diversity among strains and geographic isolates[39,43,65] on merozoite-induced immunity.

Roles of various components of immunity in malaria

Although much time and money has been invested in antimalarial vaccines, questions remain as to the relationship of vaccine-induced immunity to acquired immunity in man, a process generally accepted as being multifactorial, including components of both antibody-based and cell-mediated immunity.[54] It is clear that antibody plays several roles in effective immunity to malaria. Protection has been passively transferred to West African children by inoculation of purified IgG from immune adults.[22] Specific immune serum can agglutinate merozoite stages, inhibiting dispersal from mature schizonts and thus interfering with subsequent reinvasion.[27] Purified IgG specific to a 155,000 $M_r$ ring-stage-infected erythrocyte surface antigen strongly inhibits reinvasion in vitro.[66] Immune serum also enhances phagocytosis of parasite-infected erythrocytes by neutrophils,[4,11,12]

monocytes,[10,11,30,37,61,64] and eosinophils;[61] and antibody-dependent cellular cytotoxicity has been demonstrated in Plasmodium falciparum in vitro.[3]

Several lines of evidence suggest the importance of T-cell mediated immunity to malaria.[54] First, many manifestations of T-cell activation correlate with protective immunity, including delayed-type hypersensitivity to parasitized erythrocytes, T-cell production of lymphotoxin and macrophage activation.[31] Second, transfer of immunity has been accomplished with T lymphocytes from animals immune to malaria.[26,32,53] And, third; although T-cell or B-cell deprived mice cannot control normally non-lethal P. yoelii infections,[31,56] those B-cell deficient mice drug-rescued from infection resisted reinfection with the same parasite.[56] This indicates that some function of T-cells other than that of helper cells in antibody formation is important in malaria immunity[1], and that this function may interact nonspecifically with the malaria parasite, possibly leading to intraerythrocytic deterioration (crisis forms) and parasite death.

Crisis form induction

Crisis form malaria parasites were originally observed in P. brasilianum infection of Cebus capucinus, Ateles geoffroyi, and A. dariensis monkeys.[58] These crisis forms were characterized by reduction in average merozoite number per segmenter, retardation of development to maturity, and intraerythrocytic deterioration. Similar observations have been

described for P. knowlesi infections of immunized Macaca mulatta,[8] P. berghei infections of albino rats,[2] P. vinckei and P. chabaudi infections of mice previously innoculated with killed Propionibacterium acnes (formerly Corynebacterium parvum)[15] and P. vinckei and P. berghei yoelii infections of Mycobacterium bovis strain Bacille Calmette-Guérin (BCG) stimulated mice.[14] Although these deteriorating parasites have been observed in various animal models, they had not been described in human infections due to the sequestration and adherence of maturing stages of P. falciparum to venous capillary endothelium, and the paucity of these stages in peripheral blood.[40,44] It was not until after the in vitro technique for cultivation of falciparum malaria was developed[35,62] that crisis forms of human parasites were seen. The existence of crisis form factor (CFF), a non-antibody serum factor able to induce crisis form production in cultivated P. falciparum, has been demonstrated in the blood of Sudanese adults functionally immune to malaria,[34] and has been associated with histories of clinical immunity.[33] In general, parasite retardation and growth inhibition were greatest when parasites were cultivated with sera from individuals both from endemic regions and with no clinical histories of malaria. Moreover, greater parasite inhibition was observed in sera from holoendemic versus hyperendemic regions with no inhibition noted in sera from hypoendemic areas. Furthermore, in an extensive survey of individuals in a hyperendemic region experiencing seasonal

transmission of malaria, significant, nearly threefold, increases in CFF activity were found in wet-season sera as compared to dry-season sera.[63] Crisis form activity has also been observed in sera from patients diagnosed as suffering from cerebral malaria and meningitis.[7,60] Parasites grown in these sera and examined ultrastructurally exhibited loss of internal organelles and parasite membranes, and cytoplasmic vacuolation, all clear signs of parasite stress.

Relationship of CFF to mononuclear cell products

The focus of much research into the induction of crisis parasite formation has been on products of mononuclear cells. Rodents vaccinated with agents known to activate cells of the reticuloendothelial system, BCG and killed P. acnes, have enhanced resistance against malaria and other hemoprotozoan diseases.[14,15,18,47] Parasites observed inside the erythrocytes of these stimulated animals appear degenerate, much like the crisis forms described in the Plasmodium-Cebus model.[58] Similar studies in athymic nude mice have shown that prior injection of P. acnes, but not BCG, can protect mice from Plasmodium infections,[25] indicating that the effector phase of nonspecific immunity activated by P. acnes bypasses a T-cell requirement. Several in vitro parasite cultivation studies have been performed with sera obtained from animals previously vaccinated with BCG or P. acnes and subsequently inoculated with endotoxin.[28,59,67] Not only were these stimulated animal sera rich in macrophage secretory products, including tumor necrosis factor (TNF)[9] interleukin 1 (IL 1)[41] and type I interferon,[57] but they were

also quite toxic to P. falciparum and other Plasmodium spp., producing typical crisis forms in vitro. Further studies have shown that mice infected with Plasmodium spp. and then inoculated with endotoxin also have detectable TNF, IL 1, Type I interferon and antiparasitic activity,[17,59] demonstrating that Plasmodium infection is able to substitute for vaccination with BCG or P. acnes in this immunostimulatory treatment.

Another approach to crisis form induction research involves examining the production of reactive oxygen species by monocytic cells undergoing respiratory burst and the effect of these oxygen radicals on malaria parasites. In one report,[51] P. yoelii were cultivated adjacent to lymphokine-activated macrophages, separated by a 0.45 μm filter. When phorbol myristate acetate (PMA) or P. yoelii antigen were added to the macrophage cultures, $H_2O_2$ was elaborated, destroying the parasite cultures. When catalase was added to the cultured macrophages prior to $H_2O_2$ release, the antiparasitic activity was abrogated, indicating the toxic effect on malaria cultures was due to $H_2O_2$ production. Another study demonstrated that human γ-interferon-activated macrophages induced crisis form production when co-cultured with P. falciparum.[50] Parasite killing correlated with the magnitude of the oxidative response of the macrophages.

The effects of reactive oxygen species on malaria parasites were also examined in systems where the free radicals were generated with enzyme-substrate reactions. Parasites

cultivated with xanthine-xanthine oxidase or glucose-glucose oxidase combinations were killed by the resultant production of $H_2O_2$.[24,50,67] This effect was inhibited with catalase but not other reactive oxygen scavengers. In addition, inoculation of alloxan in P. vinckei infected mice caused hemolysis and parasite death in vivo, most likely due to the production of hydroxyl radicals.[16]

The relationship of CFF to reactive oxygen species appears remote. First, no leukocytes are present to generate oxidative intermediates in the cultivation system used to detect CFF activity.[35] Second, all serum tested is previously dialysed, and any free radicals present in the serum would be expected to be removed by this procedure. Third, in experiments to determine if CFF itself could generate reactive oxygen, a variety of oxygen scavengers, anti-oxidants and reducing agents, including catalase, superoxide dismutase, α-tocopherol and reduced glutathione have been included in assays for CFF, with no effect on inhibition (T. G. Geary, M. T. Boland, and J. B. Jensen, submitted for publication).

CFF may however, be related to various lymphokines and monokines elaborated by the immune system. Non-oxidative mechanisms of immune protection have been described for other parasites.[13,38] In addition, it has been demonstrated that lymphokine-activated macrophages isolated from a patient with chronic granulomatous disease, in which oxidative metabolism is impaired, were able to inhibit P. falciparum growth in a reactive oxygen-independent manner.[50]

Furthermore, crisis forms have been observed when parasites have been cultivated with PMA-stimulated neutrophils.[36] The addition of various oxygen scavengers to the cultures was ineffective in blocking the induction of crisis forms, indicating the possibility of non-oxidative inhibitory activity functioning in this system. It has been suggested that CFF may be TNF.[55] TNF has tumoricidal activity, and is produced in large quantities in animals vaccinated with BCG with subsequent lipopolysaccharide (LPS) injection.[9] Several reports have indicated that BCG-LPS serum is quite toxic to malaria parasites, inducing crisis forms <u>in vitro</u>.[28,59,67] It has not however, been clearly demonstrated that TNF is responsible for crisis form induction in these sera. Since the immuno-stimulatory treatment of the animals releases many macrophage secretory products into circulation, the possibility exists that both CFF and TNF are present in BCG-LPS sera. Despite the report that antibody raised to partially-purified TNF blocks the anti-parasitic activity of BCG-LPS serum,[28] CFF and TNF may be antigenically related molecules, and antisera generated against one may inhibit both activities. Indeed, one study has demonstrated the cross-reactivity of antibody generated against guinea pig lymphotoxin to guinea pig TNF and macrophage cytotoxic factor.[68] Thus the relationship of CFF to other lymphokines and monokines remains unclear.

Purpose of dissertation

The purpose of this dissertation is to further characterize the effects of CFF on parasite cultures and to begin to clarify the relationship of CFF to other inducers of malaria parasite crisis forms. Chapter 1 is concerned with the effect of CFF on *in vitro* field tests of drug resistance in isolates of *P. falciparum*. In addition, parasites cultivated with both CFF and the antimalarial drugs chloroquine and mefloquine are examined for serum-drug interactions. In Chapter 2, known or suspected inducers of crisis form parasites in cultivated *P. falciparum* are compared. Sera from Sudanese residents of malaria endemic areas, sera from American tuburculosis patients, human γ-interferon and rabbit sera containing tumor necrosis factor are assayed *in vitro* for cytotoxic activities against falciparum malaria and mouse L-M cell cultures. And finally, Chapter 3 contains an analysis of stage- and time-dependent effects of CFF on malaria parasite metabolism. Highly synchronous cultures were exposed to CFF for increasing lengths of time. At cell harvest, hypoxanthine and phenylalanine incorporation into nucleic acids and protein, respectively, and glucose consumption by parasites cultivated in CFF serum are compared to values obtained in normal human serum.

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CHAPTER ONE

# AFRICAN SERUM INTERFERENCE IN THE DETERMINATION OF CHLOROQUINE SENSITIVITY IN *PLASMODIUM FALCIPARUM*

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## ABSTRACT

Isolates of *Plasmodium falciparum* from villagers in central Sudan were tested for chloroquine and mefloquine sensitivity using the W.H.O. microtechnique test procedure and a modified 48-h *in vitro* test for drug resistance. No drug-resistant strains were noted. In the W.H.O. procedure, in which parasites were cultivated in the presence of the patient's plasma, 72% of the isolates failed to mature to the schizont stage, but when infected erythrocytes were washed free of the patient's plasma and cultivated in pooled nonimmune serum only 28.8% of the isolates failed to develop to the schizont stage. In subsequent experiments, sera from *P. falciparum*-infected patients or from noninfected "immune" adults were used to supplement standard *in vitro* test plates which contained parasites of known chloroquine sensitivities. Sera from malaria-infected patients, or from immune adults, retarded parasite development in the presence or absence of drug. The effect of these humoral factors and the antimalarial drugs was additive. The replacement of the patient's plasma with nonimmune serum in drug sensitivity tests performed with African isolates is recommended.

## INTRODUCTION

The spread of chloroquine-resistant *Plasmodium falciparum* presents a potentially severe problem to the nations of Africa. An increasing number of studies have described chloroquine-resistant malaria acquired in East Africa by nonimmune European and North American visitors,[1,2,4,15] although chloroquine resistance in indigenous Africans has not been well documented.[16,19,20,22] To monitor the spread of chloroquine-resistance in Africa, both the Rieckmann/World Health Organization (W.H.O.) microtechnique[24,26] for *in vitro* determination of chloroquine sensitivity (24-h test) and the 48-h test described by Nguyen-Dinh and Trager[18] have been developed as field tests requiring minimal laboratory equipment and which can be performed by most individuals after modest training. These standard *in vitro* assays differ primarily in the source of serum/plasma for culture, the former utilizing the patient's plasma whereas the latter employing sera pooled from nonimmune individuals. Since serum from Africa has been previously shown to retard parasite development *in vitro*,[11] it is possible that this inhibition could interfere with assays for drug sensitivity in Africans tested by the 24-h microtechnique. Reports are available suggesting that a high percentage of such tests are ineffective due to failure of controls to grow[20] (Walter Wernsdorfer, personal communication). Thus, in an attempt to characterize the effects of African serum on interpretation of drug sensitivity, parasite isolates from central

Sudan were assayed for chloroquine sensitivity by both procedures. In addition, the effects of African serum on chloroquine sensitivity of well characterized laboratory strains were examined.

# MATERIALS AND METHODS

## Field study area

Field studies were conducted in the Blue Nile Province, Sudan, a region hyperendemic for P. falciparum malaria, with peak transmission during the months of October and November, following annual rains. Villages in the area rely on local health clinics for malaria treatment where microscopic confirmation of infection is unavailable. Thus, nearly all febrile episodes are treated with chloroquine and those which fail to respond are considered drug resistant. Chloroquine is essentially the only antimalarial drug used in this area of Sudan.

Sudanese serum, frozen to -70°C, was transported on blue-ice gel to our laboratory in Michigan[13] to be tested with cultivated parasite strains of known chloroquine sensitivities. Sera from individuals living in Bahr El Ghazal Province in Southern Sudan where P. falciparum malaria is holoendemic and treatment with antimalarial drugs is limited were also examined.

## Collection of parasitized blood and sera

Febrile patients seen in local clinics were screened for malaria parasites with the aid of Giemsa-stained thick blood films and individuals with P. falciparum infections who denied a recent history of chloroquine treatment were asked to donate a blood sample for in vitro chloroquine sensitivity assay. Blood, collected in siliconized vacutainers with and without citrate-phosphate-dextrose-adenine

anti-coagulant (CPD-A)[3] was kept on wet ice 2 to 4 h during transport to the laboratory before being processed. Samples drawn with CPD-A were divided into two parts. One remained as whole blood, while the other was washed free of plasma with RPMI 1640 medium. Samples drawn without anti-coagulant were allowed to clot at 4°C overnight before centrifugation and separation of the serum from the cellular elements.

Drug sensitivity assays and parasite culture

Parasite isolates were tested for drug sensitivity by two techniques. The first, the 24-h test,[26] was used according to the W.H.O. protocol. Whole blood containing CPD-A was diluted 1:9 with RPMI 1640 and dispensed into 96-well microtiter plates prepared by W.H.O. with either chloroquine or mefloquine (W.H.O. plates). Results were obtained 24 h later by determining in Giemsa-stained thin blood films the number of parasites that matured to the schizont stage /200 asexual parasites. In the second assay, a modification of the 48-h test of Nguyen-Dinh and Trager,[18] infected erythrocytes were washed free of plasma with RPMI 1640 and suspended in fresh medium containing pooled $AB^+$ serum obtained in Michigan from American Red Cross. Parasitized erythrocytes were dispensed, 3 µl/well, into 96-well microtiter plates to which 200 µl of RPMI 1640 containing various concentrations of chloroquine was added, then cultivated in a candle jar for 48 h at 37°C. Sensitivity was determined by comparing the parasitemias in drug-containing wells with untreated control wells by examination

of stained thin films. Some assays were conducted by measuring incorporation of [$^3$H]hypoxanthine into parasite nucleic acid using scintillation spectrometry.[5,7,12] In these experiments, 1-2 μCi of [$^3$H]hypoxanthine were added to each well during the final 24 h of cultivation. The 96-well plates were subsequently harvested onto glass-fiber filter strips using a Bellco Microharvester cell harvester, and label incorporation was determined with a Beckman LS-7500 scintillation spectrometer. Each test well was compared to the control well and percent inhibition was calculated for each drug concentration using the formula:

$$\% \text{ inhibition} = \frac{\text{CPM of control well} - \text{CPM of test well}}{\text{CPM of control well}} \times 100$$

In some experiments, sera obtained from infected patients and from healthy Sudanese adults, both nonimmune and semi-immune, were examined for possible serum factor-drug interactions in cultivated laboratory strains of *P. falciparum* with well documented *in vivo* and *in vitro* sensitivities to chloroquine. All of these serum samples were heat-inactivated, dialysed 1:10$^6$ against RPMI 1640, and sterilized by filtration as previously described.[12]

Parasite strains, grown in type O$^+$ erythrocytes, were synchronized to the ring stage using a modification of the sorbitol method of Lambros and Vanderberg[17] described previously.[12] The synchronized ring stages, used in serum-drug interaction experiments, were mixed with undialyzed pooled nonimmune A$^+$ serum (PNS) and dialyzed Sudanese serum to give final concentrations of 5% PNS and 5% Sudanese serum in RPMI

1640 at 5% hematocrit. PNS was included to compensate for possible nutritional deficiencies in the Sudanese sera.[11] This concentration of PNS has been shown previously[6] to be the minimum serum requirement for optimal growth, and serum concentrations of up to 10% do not further increase the growth of parasites. This cell suspension was then added to W.H.O. plates.

Parasite strains employed in serum-drug interaction experiments included FCN-1, a chloroquine sensitive isolate,[18] FCR-$3_{TC}$[7,10] and FCR-1[7,9] which exhibit RI and RIII chloroquine resistance,[25] respectively. These strains have been maintained in continuous culture by the candle jar technique.[8]

Serum histories

Sera W-1260, W-1262, and W-1278 were obtained from 50-, 11- and 30-year-old males respectively in the village of Besselia, located in Bahr El Ghazal Province. No recent malaria histories were available for these individuals. Sera S-155 and S-157 were from 10- and 14-year-old females residing in Umm Shoka, Blue Nile Province. Both young women had patent P. falciparum infections when their sera were obtained.

## RESULTS

Sixty-six parasite isolates were tested in Sudan for chloroquine sensitivity using the modified 48-h test in which the patient's plasma was replaced in the cultures with PNS (Table 1). It is evident from Table 1 that the minimum inhibitory concentration (MIC), i.e. the drug concentration at which the parasitemia is $\leq$50% of that obtained in wells containing no drugs, was at most $5.62 \times 10^{-2}$ $\mu\underline{M}$. More than half of the isolates were sensitive to chloroquine at even lower concentrations. Since no parasites developed in $10^{-1}$ $\mu\underline{M}$ chloroquine, all isolates were considered to be chloroquine sensitive. Nineteen parasite isolates (28.8%) failed to grow in any wells, including those without chloroquine.

Using the 24-h microtechnique, 25 of the above isolates were tested for chloroquine and mefloquine resistance in W.H.O. plates (Table 2). As shown in Table 2, all parasite isolates were chloroquine and mefloquine sensitive. In contrast to the results of the 48-h test, however, 72% of the cultivated parasites failed to grow in any of the wells. Even in the drug-free wells, parasites were retarded in development, and appeared shrunken, pyknotic, and karyorrhexic.

All parasite isolates that developed to schizonts in control wells of the 24-h test underwent schizogony, producing new ring stages in control wells of the 48-h test. However, 61% of the isolates that grew in the 48-h test failed to develop in the 24-h test wells. For example, parasites

TABLE 1

Chloroquine minimum inhibitory concentrations (MIC)[a] obtained in the 48-h drug sensitivity assay.

| MIC | Isolates | |
|---|---|---|
| | | % of |
| (μM) | Number | Total No. |
| $1.00 \times 10^{-1}$ | 0 | 0.0 |
| $5.62 \times 10^{-2}$ | 29 | 43.9 |
| $3.16 \times 10^{-2}$ | 16 | 24.2 |
| $1.00 \times 10^{-2}$ | 2 | 3.0 |
| Control[b] | 19 | 28.8 |

[a]Concentration of the drug at which parasitemia $\leq$ 50% of that in drug-free control wells.

[b]Parasites failed to grow in control wells (Tables 1, 2).

TABLE 2

Chloroquine/mefloquine minimum inhibitory concentrations (MIC)[a] obtained using the 24-h drug sensitivity assay.

| Chloroquine | | | Mefloquine | | |
|---|---|---|---|---|---|
| MIC | Isolates | | MIC | Isolates | |
| (μM) | Number | % of Total no. | (μM) | Number | % of Total no. |
| $1.14 \times 10^{-1}$ | 0 | 0.0 | $8.00 \times 10^{-2}$ | 0 | 0.0 |
| $8.00 \times 10^{-2}$ | 0 | 0.0 | $4.00 \times 10^{-2}$ | 3 | 12.0 |
| $4.00 \times 10^{-2}$ | 3 | 12.0 | $2.00 \times 10^{-2}$ | 2 | 8.0 |
| $2.00 \times 10^{-2}$ | 4 | 16.0 | $1.00 \times 10^{-2}$ | 2 | 8.0 |
| Control[b] | 18 | 72.0 | Control | 18 | 72.0 |

[a]Concentration of the drug at which the number of schizonts/200 asexual parasites $\leq$ 50% of that in drug-free control wells.

[b]See Table 1.

obtained from patients S-155 and S-157 did not mature in the 24-h test, but increased in number in the 48-h test, with MIC values for chloroquine of $3.16 \times 10^{-2}$ and $5.62 \times 10^{-2}$ μM respectively.

The failure of so many of the Sudanese parasite isolates to develop in drug-free wells of the W.H.O. plates suggested strongly that: (a) the patients' plasmas were preventing parasite maturation *in vitro*; or (b) the test plates were in some way defective. To test these two possibilities, sera from some of the patients whose parasites were field-tested for chloroquine sensitivity were reexamined in the U.S.A. in a new lot of W.H.O. plates against parasite strains of known drug sensitivities. Three laboratory strains were tested by the 24-h microtechnique in W.H.O. plates using either dialyzed PNS or various dialyzed Sudanese sera. In tests utilizing sera from semi-immune or malaria-infected Sudanese, parasite structure was identical to that seen in field tests conducted in Sennar, making stage determination difficult and unreliable. Assays utilizing only PNS or serum from nonimmune Sudanese (data not shown) resulted in structurally normal parasites, indicating that the semi-immune serum itself, rather than the test plates, was the cause of test failure.

To examine further the role of African serum in drug sensitivity assays, quantification of parasite development was determined by measuring incorporation of [$^3$H]hypoxanthine into parasite nucleic acid (Figure 1). Parasites grown

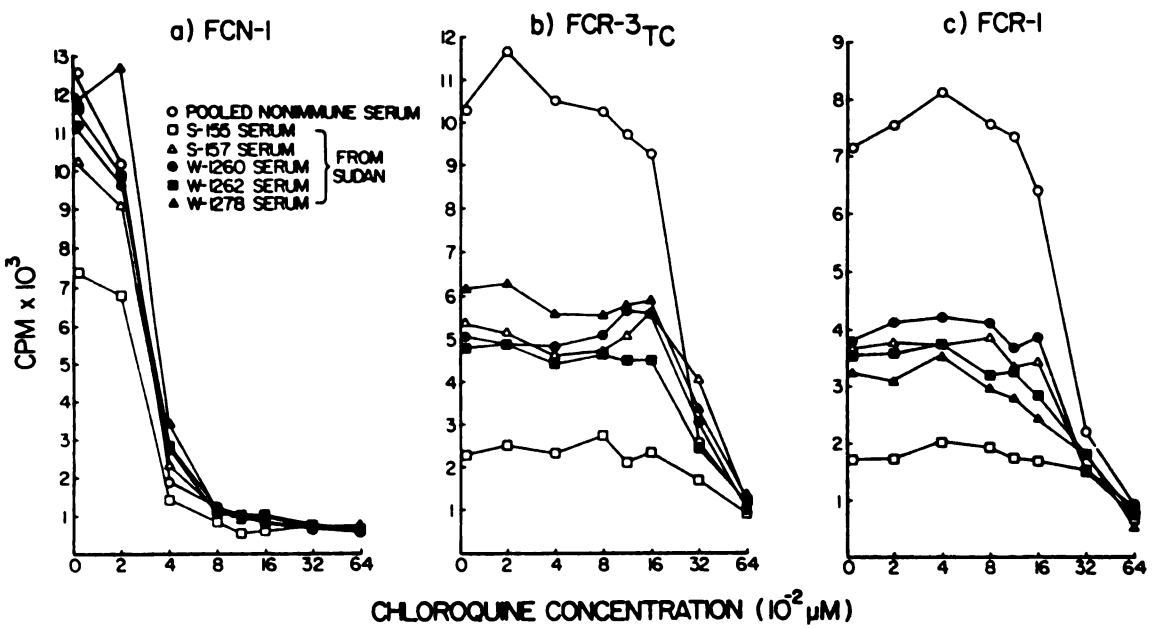


Figure 1. Results of the 24-h drug sensitivity tests with various sera, using parasite strains differing in clinical drug resistance. a. Counts per minute due to incorporation of [$^3$H]hypoxanthine by FCN-1, a drug-sensitive parasite, at various chloroquine concentrations. b,c. Data obtained with strains FCR-$3_{TC}$, an RI parasite, and FCR-1, an RIII parasite, respectively

in inhibitory Sudanese sera incorporated much less of the radiolabel, e.g. FCN-1 grown in the presence of serum S-155 without chloroquine incorporated less than 60% of the [$^3$H]hypoxanthine incorporated by parasites grown in PNS (Figure 1a). Differences were even greater with chloroquine resistant strains FCR-$3_{TC}$ and FCR-1. Parasites grown in S-155 serum incorporated less than 25% of the label than when grown in PNS (Figure 1b, c).

To determine if the inhibition altered parasite sensitivity to the drugs, data were expressed as percent inhibition in comparison to that noted for the homologous serum control value; chloroquine sensitivity remained unchanged in every strain (Figure 2). Although tests conducted in semi-immune Sudanese sera incorporated less label, parasites were still inhibited by the same concentration of drug as in PNS. Thus, the results illustrated in Figure 1 reflect additive effects of the sera and drugs, without synergistic interaction. This pattern also held true for experiments performed with mefloquine (data not shown). In addition, with PNS alone employed in sensitivity assays, no differences were found between the results of 48-h and 24-h tests, irrespective of whether [$^3$H]hypoxanthine incorporation or microscopic determination of parasitemia and parasite development was used as the basis for parasite quantification (Figure 3).

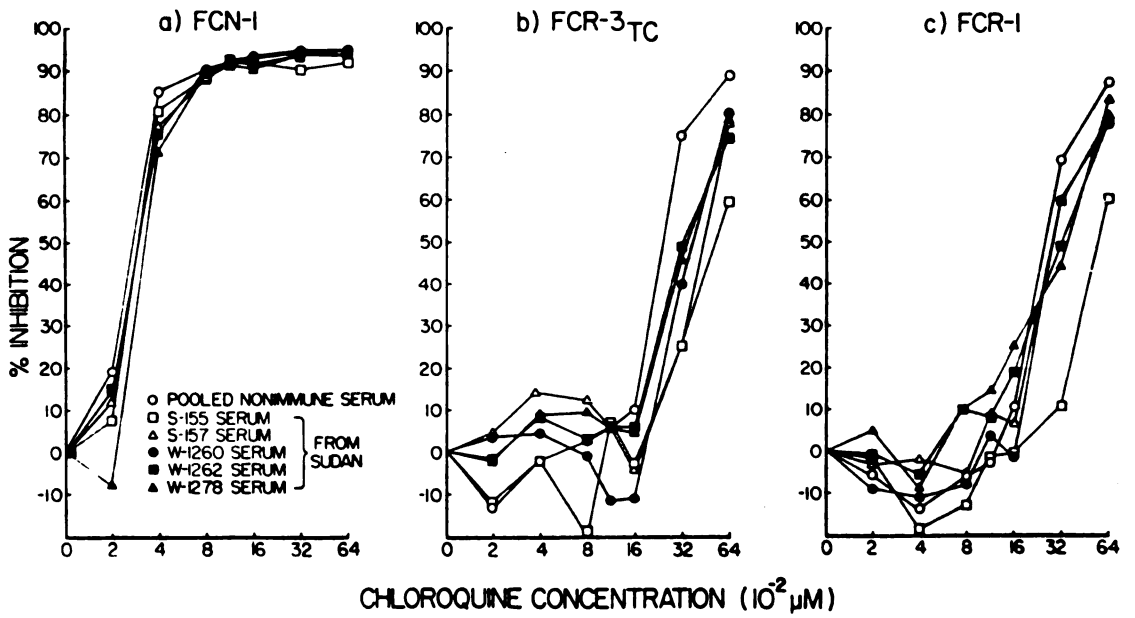


Figure 2. The data are plotted as % inhibition in comparison to the homologous serum control. a-c. Data obtained with strains FCN-1, FCR-$3_{TC}$ and FCR-1, respectively

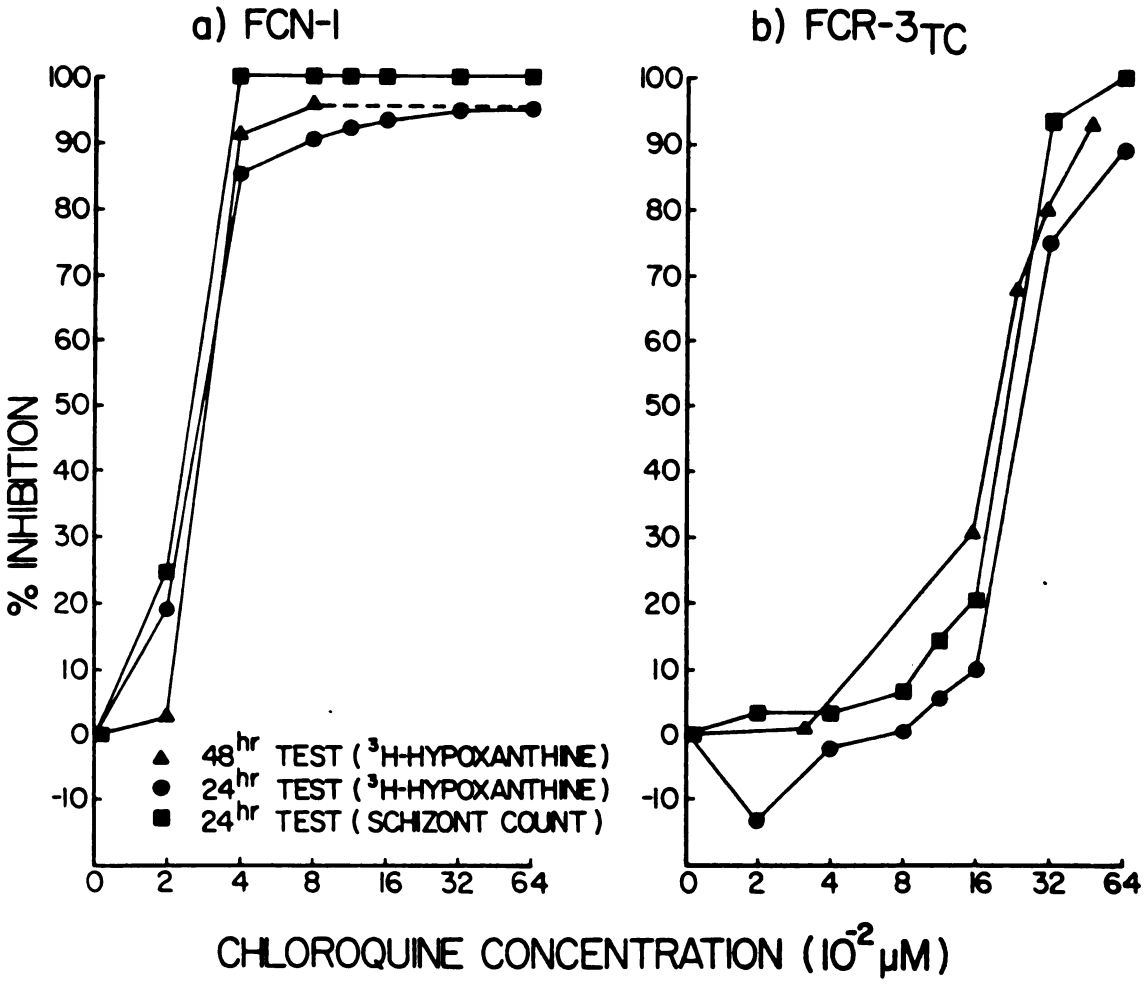


Figure 3. Comparison of results obtained with the 48-h test and 24-h test conducted in the presence of pooled nonimmune serum (PNS). The 48-h test was quantified by measuring incorporation of [$^3$H]hypoxanthine; the 24-h test results were determined by incorporation of the radiolabel and calculating the percentage inhibition of development to the schizont stage. a. Results of a comparison with FCN-1. b. Results obtained with FCR-$3_{TC}$

## DISCUSSION

Our experience using W.H.O. plates in Sudan indicates that some yet undefined components of the patient's plasma inhibit parasite development *in vitro* even in the absence of chloroquine. It might be argued that these failures were due to the presence of chloroquine in the patient's plasma, as suggested by Onori[20] who observed unexplainable failure of development in control wells. This may have been the case for a few of the samples tested in the field, since we did not test for the presence of chloroquine in the patients' urine. However, the cause of most test failure appears to be undialysable serum factors in the patients' serum, since we have demonstrated that dialysis quantitatively removes chloroquine.[12] Furthermore, the Sudanese sera were more inhibitory to chloroquine-resistant strains FCR-$3_{TC}$ and FCR-1 than to FCN-1, a chloroquine-sensitive strain. The fact that FCN-1 grew better in inhibitory serum than the others may be a strain specific phenomenon. The retardation of intraerythrocytic development seen in parasites cultivated with the patients' sera probably represents serum-induced crisis forms.[11,23]

The failures of the 24-h microtechnique in *in vitro* determination of chloroquine sensitivity were due to the presence of factors in the patients' plasma/serum and not to defects in the test plates themselves, since: (a) the plates sustained parasite growth when the patients' plasmas were replaced with PNS; and (b) supplementing the patient's serum with PNS still resulted in crisis forms.

It should be noted that the high failure rate in 24-h field tests might have been due in part to an age bias in selecting parasite and serum donors. Since large blood samples were needed to conduct this comparative study and serum analysis, only older patients, with presumably greater immunity to malaria, were chosen. Furthermore, most studies on chloroquine sensitivity in Africa report only successful tests, with no mention of the rate of test failures which range between 30 and 60% (Harrison Spencer; Phuc Nguyen-Dinh; Walter Wernsdorfer; et al., personal communication).

We conclude from our experience with the 48-h test that strains of P. falciparum isolated in central Sudan were chloroquine sensitive. These observations agree with previous reports.[7,16] Furthermore, since few of the malaria infections in the Blue Nile Province are confirmed microscopically and since the area is also endemic for typhoid fever, a febrile disease whose clinical manifestations are often confused with malaria, we believe that the chloroquine resistance reported by the clinicians in Blue Nile Province (M. Akood, personal communication) has yet to be demonstrated by in vitro testing of parasite isolates. However, with the spread of proved chloroquine resistance from Kenya and Tanzania, the situation in Sudan may soon change.

It is possible that chloroquine-resistant malaria in Africa is transmitted to nonimmune European and North Americans from semi-immune indigenous populations. Since sera from Sudanese residents can contain factor(s) that inhibit intracellular parasite development, one may hypothesize that

such factors may be masking the incidence of chloroquine-resistant malaria in Africans. Indeed, Spencer[22] and Schwartz et al.[21] have demonstrated malaria infections that by *in vitro* sensitivity assay proved to be chloroquine-resistant, but were subsequently cleared in the patients during the course of the standard W.H.O. *in vivo* test. Both investigators have speculated that immune responses may play a role in modifying the expression of drug resistance in *in vivo* and *in vitro* drug sensitivity tests. More experiments are required to test this hypothesis. It is of interest that in an Indonesian population we have recently studied, despite high titers of parasite-specific antibody, there existed no serum crisis forming activity; unlike the situation among Africans, more than 80% of the *P. falciparum* infections were chloroquine-resistant.[14]

Notwithstanding the extensive use of chloroquine in Africa, most infections are probably eliminated, or at least controlled, by immunity to the disease. Nevertheless, as control programs increase, population immunity will undoubtedly decrease, creating a situation where: (a) control of infections must rely more heavily on chemotherapy, and (b) drug resistant parasites already present in the indigenous population will no longer be controlled by immune responses. We postulate that these conditions favor the spread of drug-resistant parasites.

In summary, we have demonstrated that serum factors from Sudanese individuals interfere with determination of chloroquine sensitivity *in vitro*. After extensive dialysis

to remove chloroquine, the only antimalarial drug used in the field test area, the sera remained inhibitory to parasite development. If our findings are confirmed in other regions of Africa, *in vitro* drug sensitivity test results may be questionable due to antiparasitic factors present in semi-immune sera. Under such circumstances, it may be advantageous to replace the patient's plasma with PNS in *in vitro* field tests of drug sensitivity in Africa.

## ACKNOWLEDGMENTS

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CHAPTER TWO

# COMPARISON OF INDUCERS OF CRISIS FORMS IN *PLASMODIUM FALCIPARUM* IN VITRO

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## ABSTRACT

A variety of known or suspected inducers of crisis form parasites in cultivated Plasmodium falciparum were examined. Sera from Sudanese residents of malaria-endemic areas, sera from American tuberculosis patients, and rabbit sera containing tumor necrosis factor were assayed in vitro for cytotoxic activities against P. falciparum and mouse L-M cell cultures. Inhibition was determined by measurement of incorporation of radiolabeled nucleic acid precursors. When compared to normal serum, parasites grown in the presence of a 1:4 dilution of rabbit sera containing tumor necrosis factor, TB patient sera, or Sudanese sera were metabolically inhibited 73%, 75%, and 95% respectively. However, only the rabbit sera containing tumor necrosis factor were cytotoxic to L-M cells, inhibiting radiolabel incorporation by 80% at a 1:1000 serum dilution. These findings suggest that tumor necrosis factor is apparently not responsible for the induction of parasite crisis forms by the inhibitory human sera tested. In addition, human gamma-interferon had no effect on parasite growth.

## INTRODUCTION

The term "crisis form" was applied originally by Taliaferro and Taliaferro to describe abnormal parasites observed in *Plasmodium brasilianum* infections of *Cebus capucinus* monkeys.[20] These parasites were characterized by reduction in average merozoite number per segmenter, retardation of development to maturity, and intraerythrocytic deterioration. Since their initial study, several other investigators have reported induction of crisis forms of *Plasmodium* spp. by a variety of methods. Jensen *et al.* have demonstrated the existence of a human non-antibody serum factor that induces crisis forms in cultivated *Plasmodium falciparum*.[12] This crisis form factor (CFF), found in serum from Sudanese adults functionally immune to malaria, is both positively correlated with malaria endemicity[22] and associated with clinical immunity to malaria in Sudan,[11] and thus is probably part of acquired immunity to *P. falciparum*. The metabolic activity of parasites cultivated in the presence of sera containing high titers of CFF is inhibited by greater than 90% as determined by scintillation spectrometry of incorporation of [$^3$H]hypoxanthine into parasite nucleic acids.

Rodents vaccinated with agents known to activate macrophages, such as *Mycobacterium bovis* strain *Bacille Calmette Guérin* (BCG) and *Propionibacterium acnes* (formerly *Corynebacterium parvum*), have enhanced resistance against malaria and other hemoprotozoan diseases.[3,4] Parasites

examined from these animals appear morphologically similar to the crisis forms described by Taliaferro and Taliaferro[20]. Mechanisms postulated for intracellular parasite killing include secretion of non-specific effector molecules by activated macrophages into the serum,[6] and damage due to the production of free radical intermediates of the reduction of oxygen by effector cells which bind to the surface of parasitized erythrocytes.[1] Sera from animals vaccinated with BCG and subsequently inoculated with a bacterial lipopolysaccharide (LPS) contain tumor necrosis factor (TNF)[2], a monokine cytotoxic to mouse L cells. These TNF containing sera (BCG-LPS sera) are also inhibitory to Plasmodium spp. in vitro, and promote the formation of crisis form parasites.[8,21] In addition, investigations have shown that the generation of $O_2$-derived free radicals within in vitro and in vivo Plasmodium cultivation systems leads to parasite death.[5,24]

In the present study, we have examined various known or suspected inducers of crisis form malaria parasites in vitro (CFF sera from Sudan, BCG-LPS sera from rabbits; and sera from tuberculosis patients, human gamma-interferon [HuIFN-$\gamma$] respectively). In order to clarify the relationship between these agents, they were assayed for their ability to induce crisis form parasites in cultivated P. falciparum and tested for tumoricidal activity with mouse L-M cell cultures.

## MATERIALS AND METHODS

### Parasite and cell cultivation

*P. falciparum* strain $FCR_3TC$[14] was routinely cultivated by the candle-jar system,[15] using RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% pooled human $A^+$ sera (PHS), 25mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 0.2% sodium bicarbonate (RP-5). We have previously found minimal differences in parasite growth rates when normal human serum was used at 10% v/v concentration in RPMI 1640 medium.[9] The use of human sera pooled from 20 individuals allows for the reduction of required serum in culture medium to 5% v/v with parasite growth equal to nonpooled sera used at 10%.[7] Parasites to be employed in cytotoxicity assays were synchronized to a six hour age differential with a modification of the sorbitol technique.[16] Resultant ring stages were allowed to mature to the late schizont-stage before concentration by the gelatin flotation method[10] and subcultivation to 0.5% parasitemia in freshly washed, aged human $O^+$ erythrocytes devoid of viable leukocytes.

L-M cells (American Type Culture Collection No. CCL 1.2-derivative of NCTC clone 929) were used as target cells for tumoricidal activity. These cells were maintained in Medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 0.5% Bacto-peptone (Difco Laboratories, Detroit, MI) and 0.22% sodium bicarbonate (M-199BP) in an atmosphere of 5% $CO_2$, 95% air at 37°C.

Collection of human serum

The procedure for collection of sera from Sudan has been described previously.[13] Briefly, blood was drawn in siliconized vacutainers (Becton-Dickinson, Rutherford, NJ) from healthy adult volunteers residing in Blue Nile Province, Sudan, a region hyperendemic for P. falciparum.

Blood from tuberculosis patients was obtained through the Ingham Medical Center Chest Clinic, Lansing, MI. TQ, CJ, DW, and SG were classified as type III tuberculosis patients (positive for PPD skin tests, acid-fast bacillus stain, and chest X-ray); MS was a class II TB patient (positive for PPD skin test, with no other evidence of tuberculosis). All patients were undergoing antibiotic therapy at the time of blood drawing.

Production of BCG-LPS sera

BCG-LPS sera were prepared by the method of Matthews and Watkins.[19] Female New Zealand white rabbits (2-2.5 kg) were bled for control serum and subsequently inoculated with 4-16 x $10^7$ viable BCG organisms (University of Illinois at Chicago Medical Center, Chicago, IL). After two weeks, the rabbits were inoculated i.v. with 100 µg of lipopolysaccharide B from Escherichia coli 055:B5 (Difco Laboratories, Detroit, MI). Blood for BCG-LPS serum was collected two hours after LPS treatment. Control sera were also obtained from rabbits inoculated with LPS only, BCG only, or saline only.

Serum preparation

All blood samples were allowed to clot at 4°C overnight before centrifugation and separation of the serum from the cellular elements. Serum samples obtained in Sudan were immediately frozen and stored at -20°C until transported on blue-ice gel to our laboratory in Michigan, where they arrived still frozen. Other sera were kept at -20°C until use. All sera were heat-inactivated at 56°C for 30 min and dialysed $1:10^6$ against RPMI 1640 or Medium 199 in Spectrapor 12000-14000 MWCO tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) to remove all drugs and equilibrate the sera nutritionally for radiometric studies. Previous studies have shown that dialysis removes chloroquine[13] and antitubercular drugs from serum (T.G. Geary, East Lansing, MI, personal communication). After dialysis, sera were sterilized by filtration through 0.45 µm pore membranes (Schleicher & Schuell, Keene, NH).

Parasite inhibition assay

This assay was performed as described in detail elsewhere.[13] Synchronized schizont-stage parasites were dispensed into 96-well microtiter plates, 3 µl/well. Various concentrations of test sera in RPMI 1640 containing 40 µg/ml of gentamicin sulfate (Valley Biologics, Inc., State College, PA) and 5% PHS were assayed for total inhibition (merozoite invasion inhibition and growth retardation) by exposing triplicate wells to 200 µl of each serum concentration for 48 h, and for intraerythrocytic parasite growth

inhibition by allowing merozoite invasion to occur in normal serum before cultivating the parasite in test serum. The presence of 5% background PHS in all sera tested provides completely for the nutritional requirements of the parasites. Control wells were set up with PHS or matched individual pre-treatment rabbit sera at the same concentration as test sera. Metabolic inhibition was determined by scintillation spectrometry. Each microtiter well was pulsed with 1 µCi of [$^{3}$H]hypoxanthine (sp. activity = 10.0 Ci/mmol, New England Nuclear, Boston, MA) for the final 24 h of cultivation, before harvesting the parasites onto glass-fiber filter strips using a cell harvester (Bellco Glass, Inc., Vineland, NJ). Incorporation of radiolabel into parasite nucleic acid was measured with a Beckman LS 7500 scintillation spectrometer. Results obtained with parasites grown in non-inhibitory control sera usually ranged from 10,000-20,000 CPM. All values were corrected for background (incorporation of label by uninfected erythrocytes) and expressed as percent inhibition $\pm$ standard deviation. Percent inhibition was calculated with the following equation:

$$\% \text{ inhibition} = \frac{\text{CPM of control well} - \text{CPM of test well}}{\text{CPM of control well}} \times 100\%$$

Statistical comparisons were made using Student's $t$ test. Giemsa-stained thin films were prepared from identical wells for visual assessment of parasite inhibition.

In experiments assessing the effect of HuIFN-γ on parasite metabolism, HuIFN-γ or interferon diluent were serially diluted in RP-5 containing gentamicin sulfate. Concentrations of 10, $10^2$, $10^3$, and $10^4$ U/ml were added to ring-stage parasites and assayed for intraerythrocytic parasite inhibition as above. HuIFN-γ (Immunomodulators Laboratories, Staffort, TX) was a gift from Dr. Harold C. Miller, Michigan State University. It was obtained from culture supernatants of leukocytes stimulated with staphylococcal enterotoxin B and phytohemagglutinin, and was supplied lyophilized, $10^6$ U/vial, with diluent (0.05 M Tris and 0.5 M lysine, pH 7.5). Activity was $10^{6.5}$ U/ml in a Sindbis virus assay.[23]

L cell cytotoxicity assay

Cytotoxicity was determined by a modification of an assay described by Matthews and Watkins.[19] Microtiter plates were seeded with 75 µl of L-M cells at a concentration of $2.5 \times 10^5$ cells/ml M-199BP and incubated at 37°C in 5% $CO_2$ for 3 h. After cells had adhered to the plates, 75 µl of various concentrations of test sera in M-199BP containing 100 U penicillin and 100 µg streptomycin/ml (Gibco Laboratories, Grand Island, NY) were added to triplicate wells. Control wells were set up with PHS or matched individual pre-treatment rabbit sera at the same concentration as test sera. Plates were then incubated for either 24 or 72 h. During the final 24 h of cultivation, cells were pulsed with 0.5 µCi of [methyl-$^3$H]thymidine/well (sp. activity = 2.0 Ci/mmol, Research Products International Corp., Mount

Prospect, IL). At the end of cultivation, medium was replaced with distilled water, and the plates were refrigerated at 4°C overnight. Cells were then harvested and incorporated radiolabel was determined as in the parasite inhibition assay.

# RESULTS

## Toxicity of various sera to P. falciparum

BCG-LPS sera, sera from patients infected with TB, and CFF sera from Sudan were all found to be toxic to intraerythrocytic stages of P. falciparum in comparison to normal rabbit or human sera (Tables 1, 2 and 3, respectively), producing typical crisis form parasites in vitro. The antimalarial effects of the sera were assayed in two ways; by measuring total inhibitory activity (both merozoite invasion inhibition and growth retardation) and by measuring retardation of intraerythrocytic parasite growth. To determine total inhibition, parasites were cultivated in test sera 48 h, from schizont to schizont-stage, and pulsed with [$^{3}$H]hypoxanthine during the final 24 h of cultivation. Intracellular growth retardation was determined by measuring radiolabel incorporation by parasites grown in test sera for 36 h, from ring to schizont-stage, and pulsed during the final 24 h.

With BCG-LPS sera, total inhibition in a 48 h period was significant ($p<.001$) with averages of 73% inhibition at a 1:4 dilution and 47% at a 1:8 concentration (Table 1). Control sera (from BCG, LPS, or saline inoculated control rabbits) showed no toxic effects on malaria cultures. Similar to results obtained with BCG-LPS sera, TB patient sera significantly inhibited parasite cultures ($p<.01$), averaging 75% at a 1:4 serum dilution (Table 2). Serum concentrations

Table 1

Percent inhibition of *P. falciparum* [$^3$H]hypoxanthine incorporation by rabbit BCG-LPS serum.

| Rabbit Serum | Dilution | % Inhibition 36 h[a] | % Inhibition 48 h[b] |
|---|---|---|---|
| Rb-1 | 1:4 | 68.0 ± 7.8 (3)[c] | 94.7 ± 3.9 (6) |
| | 1:8 | 16.8 ± 1.6 (3) | 55.4 ± 12.3 (6) |
| Rb-2 | 1:4 | 36.1 ± 10.6 (3) | 79.1 ± 4.9 (6) |
| | 1:8 | 19.9 ± 6.8 (3) | 26.8 ± 9.6 (5) |
| Rb 3 | 1:4 | 86.8 ± 3.7 (5) | 98.8 ± 2.0 (6) |
| | 1:8 | 49.1 ± 30.9 (5) | 92.5 ± 3.4 (8) |
| Rb 4 | 1:4 | 25.2 ± 12.2 (7) | 61.9 ± 16.2 (8) |
| | 1:8 | 18.8 ± 10.1 (7) | 24.2 ± 7.2 (10) |
| Rb 5 | 1:4 | 20.7 ± 10.2 (8) | 31.2 ± 15.6 (6) |
| | 1:8 | 19.7 ± 1.5 (6) | 22.0 ± 16.2 (6) |
| Rb 6 | 1:4 | 58.6 ± 21.2 (10) | 76.0 ± 10.8 (7) |
| | 1:8 | 41.1 ± 23.2 (8) | 57.6 ± 19.6 (6) |
| Total[d] | 1:4 | 46.5 ± 27.0 (36) | 73.1 ± 24.4 (39) |
| | 1:8 | 29.2 ± 20.9 (32) | 47.0 ± 28.8 (41) |

[a]Intraerythrocytic parasite development retardation, rings to schizonts, exposed 36 h to serum.

[b]Total parasite retardation, schizont to schizont, exposed 48 h to serum.

[c]Percent inhibition ± standard deviation. Number of replicates in parentheses.

[d]Mean of six rabbits.

Table 2

Percent inhibition of *P. falciparum* [$^3$H]hypoxanthine incorporation by sera from humans with TB.

| Serum Donor | Dilution | % Inhibition 36 h[a] | % Inhibition 48 h[b] |
|---|---|---|---|
| TQ | 1:4 | 83.6 ± 2.7 (2)[c] | 93.8 ± 0.4 (2) |
| | 1:8 | 57.1 ± 2.1 (2) | 77.5 ± 1.7 (2) |
| | 1:16 | 36.9 ± 4.2 (2) | 57.6 ± 3.0 (2) |
| MS | 1:4 | 38.0 ± 1.0 (2) | 59.3 ± 21.9 (2) |
| | 1:8 | 14.4 ± 0.7 (2) | 18.0 ± 8.5 (2) |
| | 1:16 | 8.1 ± 1.4 (2) | 18.6 ± 3.3 (2) |
| CJ | 1:4 | 89.0[d] (1) | 96.5 (1) |
| | 1:8 | 79.8 (1) | 91.6 (1) |
| | 1:16 | 64.3 (1) | 81.7 (1) |
| SG | 1:4 | 47.5 (1) | 66.5 (1) |
| | 1:8 | 40.5 (1) | 53.6 (1) |
| | 1:16 | 36.2 (1) | 34.8 (1) |
| DW | 1:4 | 43.2 (1) | 53.8 (1) |
| | 1:8 | 25.2 (1) | 38.1 (1) |
| | 1:16 | 20.6 (1) | 35.6 (1) |

Table 2 (Continued)

| Serum Donor | Dilution | % Inhibition | |
|---|---|---|---|
| | | 36 h[a] | 48 h[b] |
| Total[e] | 1:4 | 60.5 ± 23.6 (7) | 74.7 ± 21.1 (7) |
| | 1:8 | 41.2 ± 24.8 (7) | 53.5 ± 30.1 (7) |
| | 1:16 | 30.2 ± 19.9 (7) | 43.3 ± 23.3 (7) |

[a]See Table 1.

[b]See Table 1.

[c]See Table 1.

[d]Only one well tested due to insufficient sera.

[e]Mean of five tested sera.

Table 3

Percent inhibition of P. falciparum [$^3$H]hypoxanthine incorporation by Sudanese sera with high CFF activity.

| Serum Donor | Dilution | % Inhibition 36 h[a] | % Inhibition 48 h[b] |
|---|---|---|---|
| 830 | 1:4 | 70.0 ± 1.4 (2)[c] | 89.1 ± 2.8 (2) |
| | 1:8 | 54.0 ± 4.4 (2) | 76.8 ± 4.0 (2) |
| S-81-15 | 1:4 | 88.1 ± 2.8 (2) | 98.5 ± 0.3 (2) |
| | 1:8 | 64.9 ± 1.8 (2) | 94.6 ± 1.1 (2) |
| S-81-55 | 1:4 | 72.0 ± 4.4 (2) | 91.4 ± 2.7 (2) |
| | 1:8 | 50.1 ± 1.6 (2) | 74.8 ± 1.3 (2) |
| S-81-132 | 1:4 | 83.5 ± 7.8 (2) | 97.9 ± 0.3 (2) |
| | 1:8 | 64.4 ± 2.8 (2) | 92.3 ± 0.1 (2) |
| S-81-139 | 1:4 | 79.4 ± 4.2 (2) | 96.2 ± 1.7 (2) |
| | 1:8 | 56.2 ± 3.1 (2) | 88.4 ± 2.1 (2) |
| Total[d] | 1:4 | 78.6 ± 7.9 (10) | 94.6 ± 4.1 (10) |
| | 1:8 | 57.9 ± 6.6 (10) | 85.8 ± 8.2 (10) |

[a]See Table 1.

[b]See Table 1.

[c]See Table 1.

[d]Mean of five Sudanese sera.

of 1:8 resulted in 54% parasite inhibition. Total inhibition due to cultivation in Sudanese sera was significant ($p<.001$) and averaged 95% at a 1:4 concentration and 85% at 1:8 (Table 3). These sera were selected for their high levels of CFF for purposes of comparison.

When parasites were cultivated 36 h, from ring to schizont-stages in the presence of various sera, hypoxanthine incorporation was still significantly reduced. At a 1:4 concentration, BCG-LPS sera produced an average of 46% inhibition ($p<.001$), TB sera inhibited by 60% ($p<.05$), and Sudanese CFF sera reduced incorporation by 79% ($p<.001$). Since no reinvasion took place during this part of the malaria cycle, all inhibition observed was due to serum effects on intracellular stages. Microscopic examination of inhibited cultures demonstrated crisis form parasites, with retarded development and shrunken cytoplasm when compared to control cultures.

Toxicity of various sera to L-M cells

The three groups of sera (BCG-LPS, TB, and Sudanese) were assayed for tumor cell cytotoxicity. Data from these experiments were pooled for Figure 1, a graphic representation of the effects of various sera during a 24 h period on thymidine incorporation by L-M cells. In contrast to results obtained with malaria parasites, only BCG-LPS sera were inhibitory to the target cells. BCG-LPS sera were cytolytic at serum concentrations greater than 1:32000. As serum concentration was decreased, a corresponding decrease in residual cell bodies was observed. In the case of TB and

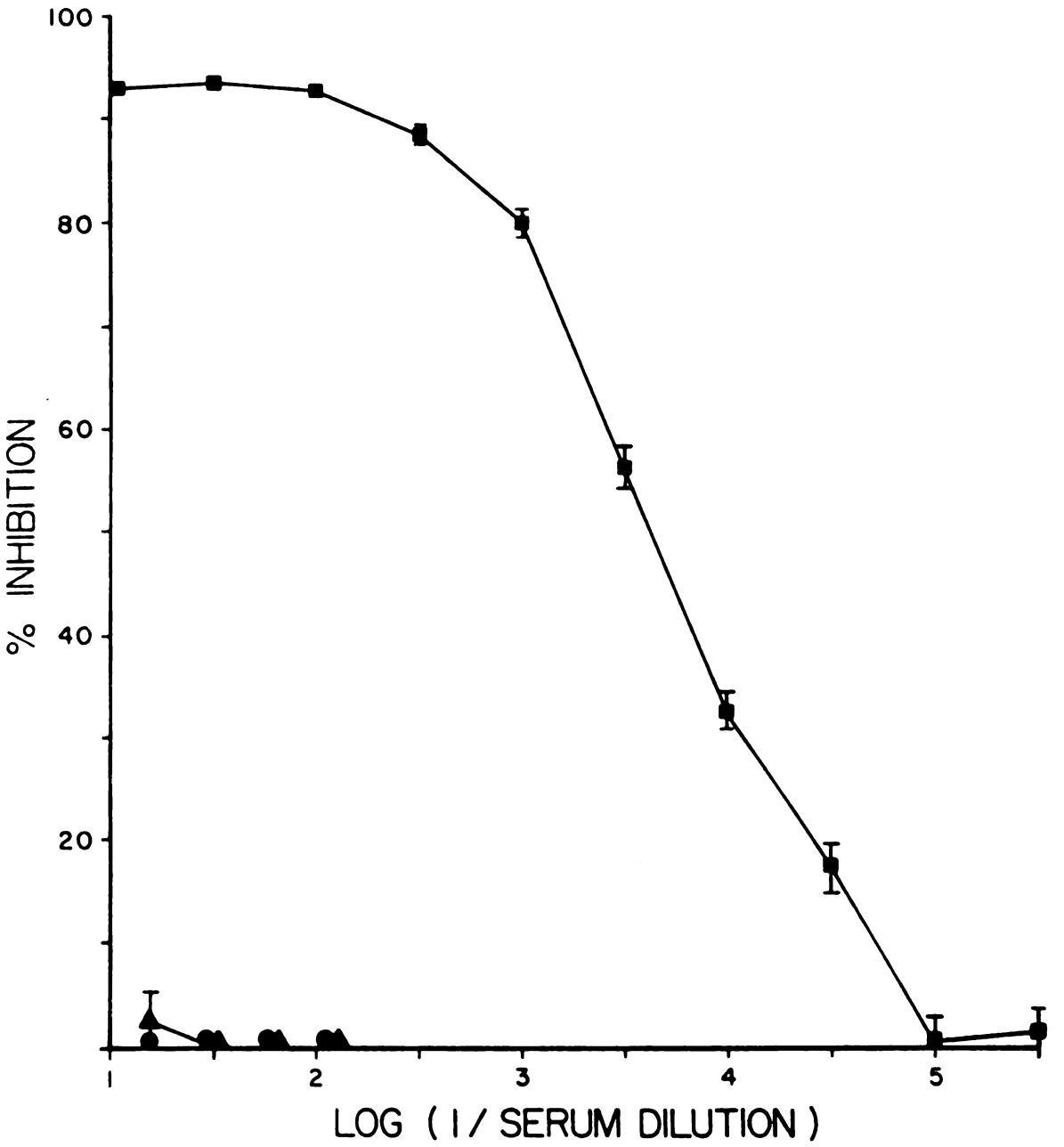


Figure 1. Effects of sera from a variety of sources on [$^{3}$H]thymidine incorporation by L-M cells during a 24-h period. Results are expressed as percent inhibition ± standard error of the mean. Symbols: BCG-LPS sera, ■; TB sera, ▲; Sudanese sera, ●

Sudanese CFF sera, no cell lysis was observed at any concentration. In fact, incorporation of radiolabel in the presence of these sera was slightly higher than that seen when cells were cultivated either with PHS or in the absence of serum. Results obtained when cells were grown for 72 h in the presence of various sera were similar to those from 24 h cultures (data not shown).

BCG-LPS serum IC50s

The concentration of BCG-LPS serum required for 50% inhibition of radiolabel incorporation in L-M cells was nearly 700 times less than that needed to produce the same effect in malaria parasites (Table 4). The IC50s for parasites ranged from 1:1.0 to 1:30.3, averaging 1:6.6; whereas, IC50s for tumor cells ran from 1:2449 to 1:6281, with a mean of 1:4414.

Effect of HuIFN-γ on P. falciparum

Previous studies had shown that human alpha-interferon had no effect on growth of P. falciparum.[11] It was observed that HuIFN-γ also had no effect on the incorporation of hypoxanthine into parasite nucleic acids. Parasites cultivated in various amounts of HuIFN-γ were not inhibited significantly when compared to parasites cultivated in the diluent control (data not shown).

Table 4

*P. falciparum* and L-M cell IC50s of BCG-LPS sera.

| Rabbit Serum | IC50 | |
|---|---|---|
| | Parasite[a] | Tumor cell[b] |
| Rb-1 | 1: 8.8 | 1:5408 |
| Rb-2 | 1: 5.9 | 1:2449 |
| Rb-3 | 1:30.3 | 1:5188 |
| Rb-4 | 1: 5.0 | 1:5248 |
| Rb-5 | 1: 1.0 | 1:3266 |
| Rb-6 | 1:10.7 | 1:6281 |
| Total[c] | 1: 6.6 | 1:4414 |

[a]Concentration at which parasite incorporation of [$^3$H]hypoxanthine is reduced by 50%.

[b]Concentration at which cell line incorporation of [$^3$H]thymidine is reduced by 50%.

[c]Mean of six rabbits.

# DISCUSSION

The present studies were conducted to clarify the relationship between various inducers of malaria parasite crisis forms. We found that HuIFN-γ had no effect on parasite incorporation of purine label. However, BCG-LPS sera, TB patient sera, and Sudanese CFF sera were all able to induce crisis forms in *in vitro* cultures of *P. falciparum*. This induction was observed in synchronous parasites cultivated from ring to schizont-stages, demonstrating that merozoite invasion was not required for the serum factors to have an effect on parasite development.

Although all three types of sera were active against *P. falciparum*, only TNF-containing rabbit BCG-LPS serum was cytotoxic to L-M cells. Since other investigators have demonstrated that TNF generated in rabbit,[19] human[18] and mouse[17] systems are all active at high dilutions against the standard L cell target, and since we have shown that rabbit BCG-LPS serum is about 700 times more active against L-M cells than malaria parasites, it appears likely that TB and Sudanese CFF sera contained no appreciable amounts of TNF, and that TNF was not responsible for crisis form induction in these human sera. In addition, the production of TNF usually requires both treatment with macrophage activating agents and subsequent stimulation with LPS. Männel *et al.* have shown that tumor necrotic activity peaks 2 hours after LPS treatment of BCG-infected mice, and is absent after 6 hours.[17] Alternatively, studies by Clark *et al.*[3,4] have

shown that mice injected with BCG or killed P. acnes alone are protected from lethal Plasmodium and Babesia infections. Since the TB and Sudanese sera used in our study were obtained without endotoxin treatment of the donors, and the anti-parasitic activity in their sera remains relatively stable over time, crisis form induction by these sera would appear more closely related to the rodent model described by Clark[3,4] than to those models requiring LPS injection.

One might argue that the antimalarial activity in Sudanese CFF sera is due to TB infection. However, the Sudanese serum donors did not have any clinical evidence of infections, and in previous studies we have noted that many of the inhibitory sera were from PPD skin test-negative donors (unpublished observations).

Thus, the relationship between TNF and CFF remains unclear. They may be entirely different molecular species both capable of inducing crisis forms of malaria parasites. Alternatively, BCG-LPS sera may contain both TNF and CFF. Despite the fact that Haidaris et al.[8] were able to block the cytotoxic activities of mouse BCG-LPS serum with antibody made to partially purified rabbit TNF, CFF and TNF may be antigenically related molecules, and antibody generated against one may inhibit the other. Indeed Zacharchuk et al.[25] have shown that antibody prepared against purified guinea pig lymphotoxin neutralizes not only lymphotoxin, but also guinea pig TNF as well as macrophage cytotoxic factor, indicating that these lymphokines and monokines have strong

antigenic relationships. Studies on the effect of highly purified TNF and CFF on malaria cultures are required to answer these questions.

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CHAPTER THREE

# STAGE- AND TIME-DEPENDENT EFFECTS OF CRISIS FORM FACTOR ON *PLASMODIUM FALCIPARUM IN VITRO*

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Submitted to the Journal of Parasitology

## ABSTRACT

To determine the stage- and time-dependent effects of crisis form factor (CFF) on Plasmodium falciparum metabolism in vitro, the parasite erythrocytic cycle was divided into sequential 8 h time intervals, and highly synchronous parasites were exposed to CFF for various lengths of time. Hypoxanthine and phenylalanine incorporation into parasite nucleic acids and proteins, respectively, and glucose consumption by the parasites were compared in cultures grown in CFF-containing or nonimmune sera. The most profound derangement of metabolism occurred in parasites 0-8 h post-invasion. Inhibition correspondingly decreased in tests started with progressively older parasites. Cultivation in CFF serum for 8 h caused maximal inhibition of purine and amino acid incorporation; longer periods of exposure did not increase inhibition In contrast, CFF's effect on glucose consumption varied inversely to the duration of exposure to CFF. As the parasites matured in the presence of CFF, inhibition of the rate of glucose utilization decreased, with little or no reduction in consumption observed as parasites entered schizogony. Of the three metabolic parameters studied, hypoxanthine was the most sensitive indicator of metabolic inhibition throughout the cycle.

## INTRODUCTION

"Crisis form" malaria parasites were originally described[22] as abnormal parasites characterized by their reduced average number of merozoites per segmenter, retarded development to maturity and intraerythrocytic deterioration. Although crisis form parasites had been observed in several rodent[1,5,6] and simian models,[3,22] it was not until after the *in vitro* technique for cultivation of *Plasmodium falciparum* was developed[24] that crisis forms of human parasites were seen. Jensen *et al.*[13] have demonstrated the existence of a human serum factor able to induce crisis forms in malaria parasites. This factor (CFF) has been found to be both associated with clinical immunity to malaria in Sudan,[15] and positively correlated with malaria endemicity;[25] thus, it is probably part of acquired immunity to *P. falciparum*. Furthermore, other investigators[2,23] have demonstrated intraerythrocytic parasite growth inhibition by human sera from malarious regions.

Despite the fact that crisis form parasites had been described as early as 1944,[22] little is known about how crisis form inducers affect cell metabolism. Jensen *et al.*[14] have shown that parasites cultivated in the presence of sera containing high titers of CFF are inhibited in incorporation of [$^3$H]hypoxanthine by greater than 90% during a 48 h period. However, since certain aspects of *Plasmodium* metabolism have demonstrable stage dependence,[7,11,19,20,21,27] the possibility exists that CFF may affect the *in vitro*

development of P. falciparum in a stage-specific manner. The experiments reported here demonstrate stage- and time-dependent effects of CFF, characterized by several metabolic criteria, on synchronous cultures of P. falciparum in vitro.

## MATERIALS AND METHODS

### Parasite cultivation

*Plasmodium falciparum* strain $FCR_{3TC}$[16] was routinely cultivated by the candle jar method[17] using RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 5% pooled human $A^+$ sera (PHS), 25 mM N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid (HEPES) and 0.2% sodium bicarbonate (RP-5). However, in metabolic inhibition assays, RPMI 1640 was replaced with the minimal medium described by Divo *et al.*[8] This substitution enhances parasite incorporation of radio-labeled phenylalanine, facilitating measurement of CFF inhibition of this process.

Parasites to be employed in cytotoxicity assays were synchronized to a 4 h age differential with the following procedure. Late stage schizont-infected cells were concentrated by the gelatin flotation method,[12] diluted to 25% parasitemia in freshly washed $O^+$ erythrocytes, suspended in RP-5 at a 2.5% hct, and incubated in a candle jar atmosphere at 37°C to allow merozoites to invade fresh cells. After 4 h, invasion was terminated by sorbitol lysis[18] of all remaining schizonts, resulting in young ring-staged parasites 0-4 h old. Parasitemia was then adjusted to 10% with additional red blood cells (RBC).

### Serum preparation

The procedure for collection of sera and the region of Sudan from which the sera were obtained has been described previously.[4] Both normal human sera (NHS) and sera

containing enough CFF to reduce parasite incorporation of hypoxanthine by 50% when used at 25% serum concentration in culture were each pooled from 20 individuals. Following heat-inactivation at 56°C for 30 min, the serum lipoproteins were removed by precipitation with 0.05% w/v dextran sulfate[26] (500,000 MW, Sigma Chemical Co., St. Louis, MO). Residual dextran sulfate was precipitated with 5% v/v saturated $BaCl_2$. The serum pools were then dialysed $1:10^6$ against minimal medium in Spectrapor 12,000-14,000 MWCO tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) to remove any remaining $BaCl_2$ and equilibrate the sera nutritionally for metabolic studies. After dialysis, sera were sterilized by filtration through 0.45 µm pore membranes (Schleicher & Schuell, Keene, NH).

Metabolic inhibition assays

Inhibition was determined by a modification of a technique described previously.[14] The CFF and NHS pools were diluted to 25% serum concentration in minimal medium supplemented with 5% undialysed PHS. The presence of 5% background PHS in each pool tested provided completely for the nutritional requirements of the parasites.[9] Synchronized parasites 0, 8, 16, 24, and 32 h post-invasion (p.i.) were dispensed into 96-well microculture plates, 1 µl packed cell volume/well, and exposed to CFF serum for various lengths of time (Figure 1). Since all experiments used parasites synchronized to a 4 h age differential, these times actually represent parasites 0-4, 8-12, 16-20, 24-28, and 32-36 h

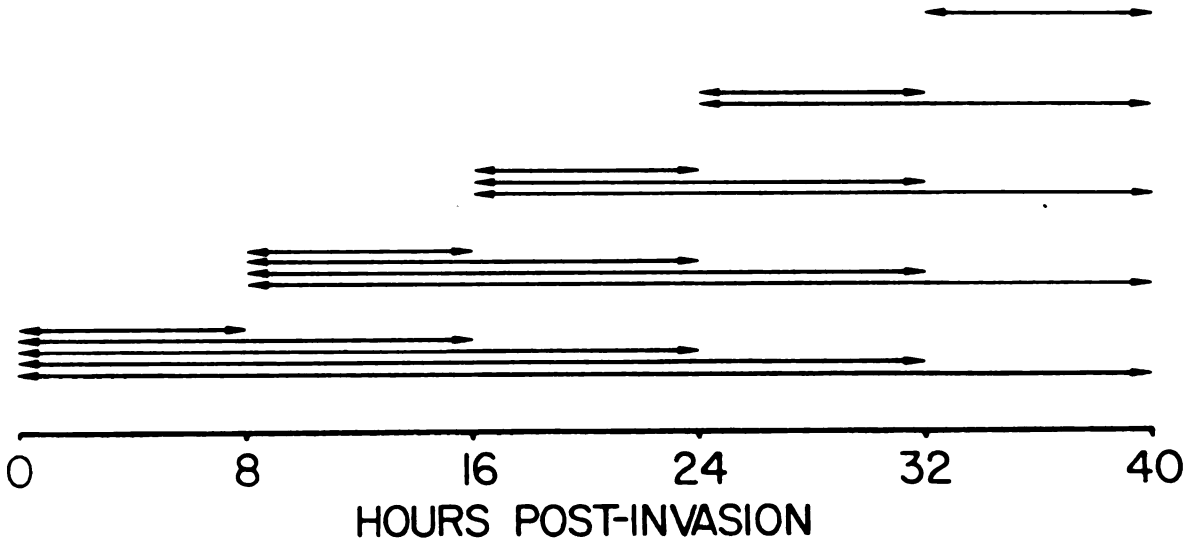


Figure 1. Periods of exposure of P. falciparum to CFF at various times post-invasion

old, but for convenience and clarity, the age spread shall not be given throughout the report. Metabolic inhibition was determined after exposure of triplicate wells to 200 µl of each serum pool and cultivating parasites of various ages for 8, 16, 24, 32 or 40 h.

Inhibition of purine and amino acid incorporation were determined by scintillation spectrometry. Each microculture well was pulsed with 2 µCi of [$^{3}$H]hypoxanthine (sp. activity = 10.0 Ci/mmol, New England Nuclear, Boston, MA) and 0.2 µCi of [U-$^{14}$C]phenylalanine (sp. activity = 450 mCi/mmol, ICN Radiochemicals, Irvine, CA) for the final 8 h of cultivation, before harvesting the parasites onto glass-fiber filter strips using a cell harvester (Bellco Glass, Inc., Vineland, NY). Parasite incorporation of radiolabel was measured with a Beckman LS 7500 scintillation spectrometer. All values were corrected for background incorporation of label by uninfected erythrocytes and expressed as percent inhibition $\pm$ standard error of the mean. Percent inhibition was calculated with the following equation:

$$\% \text{ inhibition} = \frac{\text{CPM of NHS well} - \text{CPM of CFF well}}{\text{CPM of NHS well}} \times 100\%$$

Inhibition of glucose consumption was determined by UV spectrophotometry. At the time of cell harvest, 100 µl aliquots of culture medium were removed from each well for measurement of glucose concentration using a glucose assay kit (Glucose No. 15-UV, Sigma Chemical Co., St. Louis, MO). The assay procedure is based on the quantitative conversion

of glucose to glucose-6-phosphate and then to 6-phosphogluconate by hexokinase and glucose-6-phosphate dehydrogenase, respectively. The latter reaction generates NADPH from NADP, of which the absorbance at 340 nm was measured with a Gilford Model 2000 spectrophotometer. Change in absorbance ($\Delta A$), proportional to parasite-specific glucose consumption, was determined by subtracting the absorbance of culture medium from parasite-infected erythrocyte wells from that of uninfected RBC control wells. All values were expressed as percent inhibition $\pm$ standard error of the mean. Percent inhibitino was calculated with the following equation:

$$\% \text{ inhibition} = \frac{\Delta A \text{ of NHS well} - \Delta A \text{ of CFF well}}{\Delta A \text{ of NHS well}} \times 100\%$$

Statistical comparisons were made using Student's $t$ test. Giemsa-stained thin films were prepared from identical wells for visual assessment of parasite inhibition.

# RESULTS

## Stage-dependency

The parasite erythrocytic cycle was divided into 5 sequential 8 h time periods. Synchronized parasites corresponding to each period were exposed to CFF serum, and the serum's effect on each of three parasite metabolic processes; hypoxanthine and phenylalanine incorporation into nucleic acids and proteins, respectively, and glucose consumption, was determined (Figure 2). The greatest inhibition in the measured metabolic parameters occurred during the first time period, the 0 h to 8 h p.i. ring-stage parasites. Glucose utilization, purine incorporation and amino acid incorporation were significantly inhibited 63.0% ($p<0.05$), 38.6% ($p<0.001$) and 21.9% ($p<0.05$), respectively. Percent inhibition progressively decreased in parasites exposed to CFF during later periods. In parasites cultivated from 8 h to 16 h p.i. in CFF, glucose consumption was reduced 38.8%, while hypoxanthine and phenylalanine incorporation were inhibited 28.9% ($p<0.01$) and 17.9% ($p<0.05$), respectively. During the third time period examined (16 h to 24 h p.i.), corresponding to the transition from ring to trophozoite-stage parasites, CFF serum continued to inhibit purine and amino acid incorporation by 26.8% ($p<0.01$) and 19.2% ($p<0.05$), respectively, but glucose utilization was no longer affected. Parasites exposed to CFF during the fourth or fifth time intervals (24 h to 32 h and 32 h to 40 h p.i., respectively) were not significantly inhibited in any of their observed metabolic processes.

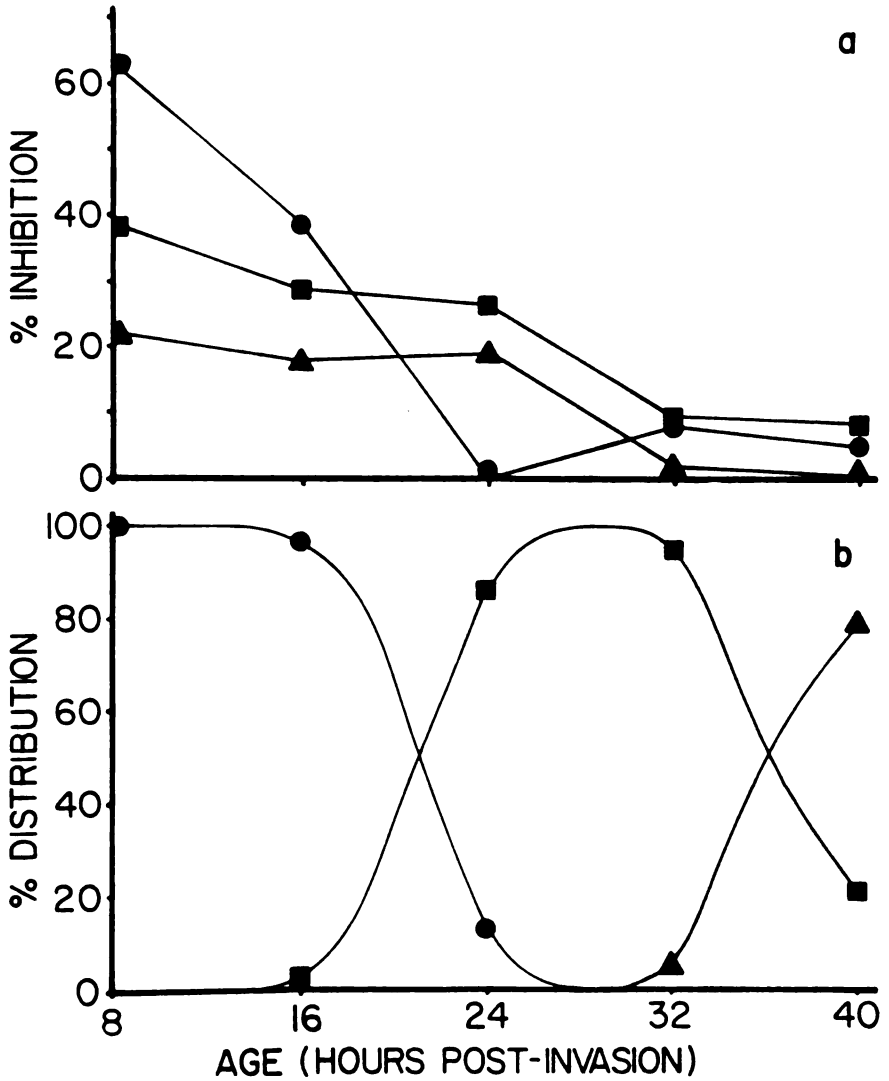


Figure 2. Effect of CFF on various stages of P. falciparum. a. Effect of 8 h exposure to CFF serum on three metabolic processes in parasites harvested at various times. Results are expressed as percent inhibition compared to control cultures. Symbols: hypoxanthine incorporation, ■; phenylalanine incorporation, ▲; glucose consumption, ●. b. Differential distribution of various stages expressed as percentage of total parasites present at cell harvest. Symbols: ring stages, ●; trophozoite stages, ■; schizont stages, ▲

Time-dependency

Data given above demonstrated that parasites exposed to CFF for 8 h at various times post-invasion were more sensitive as younger stages, but different metabolic processes were selectively affected. In experiments conducted to determine the effects of longer exposure to CFF, the various metabolic parameters exhibited either time-dependent or time-independent sensitivity to the action of CFF. Parasites of various ages were exposed to CFF for increasing duration and the degree of inhibition observed was compared in cultures initiated at the same parasite age. The sensitivity of incorporation of radiolabeled hypoxanthine to CFF (Table 1) was the greatest (significant at $p<0.001$) in cultures initiated with early ring stages (0 h p.i.). In those cultures, inhibition determined after continuous exposure to CFF for one, two, three, four, and five 8 h pulses was 38.6%, 41.5%, 52.6%, 48.4% and 47.1% respectively; thus remaining relatively constant, independent of the duration of CFF exposure. Parasites initially exposed to CFF 8 h and 16 h p.i. and maintained in CFF serum for longer duration were also significantly inhibited ($p<0.001$) in purine incorporation, with mean inhibitions of 31.2% and 30.9%, respectively. As seen before, the length of time during which the parasites were grown in the presence of CFF did not significantly affect the amount of inhibition seen. Parasite cultures initiated with 24 h trophozoite stages were still

TABLE 1

Inhibition of hypoxanthine incorporation in *P. falciparum* by CFF

| Cultivation | | Hypoxanthine Incorporation | | % Inhibition | |
|---|---|---|---|---|---|
| Start[a] | End[b] | Normal Serum | CFF Serum | | |
| 0 | 8 | 1272 ± 76[c] | 781 ± 26 | 38.6 | (6)[d] |
| | 16 | 2255 ± 165 | 1319 ± 28 | 41.5 | (5) |
| | 24 | 8424 ± 632 | 3997 ± 199 | 52.6 | (6) |
| | 32 | 23608 ± 1152 | 12172 ± 534 | 48.4 | (6) |
| | 40 | 66552 ± 1231 | 35219 ± 778 | 47.1 | (6) |
| 8 | 16 | 2391 ± 67 | 1701 ± 114 | 28.9 | (6) |
| | 24 | 8137 ± 714 | 5862 ± 508 | 28.0 | (5) |
| | 32 | 25592 ± 1479 | 16210 ± 669 | 36.7 | (6) |
| | 40 | 80154 ± 1178 | 55066 ± 2070 | 31.3 | (6) |
| 16 | 24 | 7551 ± 263 | 5528 ± 459 | 26.8 | (6) |
| | 32 | 22530 ± 1451 | 13536 ± 615 | 39.9 | (6) |
| | 40 | 77818 ± 957 | 57570 ± 2314 | 26.0 | (6) |
| 24 | 32 | 18345 ± 1003 | 16641 ± 937 | 9.3 | (6) |
| | 40 | 77913 ± 1737 | 66327 ± 3635 | 14.9 | (6) |
| 32 | 40 | 65368 ± 1216 | 59988 ± 3299 | 8.2 | (6) |

[a]Parasite age post-invasion (h) at initiation of assay

[b]Parasite age post-invasion (h) at termination of assay

[c]Incorporation (CPM/well) ± standard error of the mean

[d]Number of replicates in parentheses

sensitive to CFF ($p<0.05$), but much less so than with cultures started with younger parasites. Inhibition was 9.3% after one 8 h pulse and 14.9% after two 8 h pulses, averaging 12.1%. Hypoxanthine incorporation was not inhibited by CFF when parasites were exposed 32 h p.i.

Similar data were seen in inhibition assays using phenylalanine incorporation (Table 2); however, inhibition was less than that observed in assays measuring hypoxanthine incorporation.

Although the effect of CFF on both hypoxanthine and phenylalanine incorporation appeared independent of the number of time intervals during which the parasites were exposed to CFF, such was not the case for CFF's effect on glucose utilization by the parasite. Early ring stages cultivated for one and two 8 h pulses were significantly inhibited 63.0% ($p<0.05$) and 69.8% ($p<0.01$), respectively (Table 3). However, when these parasites were continuously exposed to CFF for three, four, and five 8 h periods, inhibition progressively decreased to 39.7% ($p<0.05$), 24.4% ($p<0.05$) and 11.1% ($p<0.01$), respectively. A similar pattern was observed in tests initiated with 8 h p.i. parasites. As the duration of exposure of parasites to CFF serum was increased, inhibition of glucose consumption decreased, from 38.8% after one 8 h pulse to 7.3% after four 8 h periods. Furthermore, no inhibition of glucose utilization was seen in cultures begun with parasites $\geq$ 16 h p.i.

TABLE 2

Inhibition of phenylalanine incorporation in *P. falciparum* by CFF

| Cultivation | | Phenylalanine Incorporation | | % Inhibition | |
|---|---|---|---|---|---|
| Start[a] | End[b] | Normal Serum | CFF Serum | | |
| 0 | 8 | 1849 ± 90[c] | 1444 ± 58 | 21.9 | (6)[d] |
| | 16 | 2912 ± 199 | 2219 ± 77 | 23.8 | (5) |
| | 24 | 5134 ± 262 | 3055 ± 141 | 40.5 | (6) |
| | 32 | 7748 ± 447 | 4945 ± 208 | 36.2 | (6) |
| | 40 | 13644 ± 235 | 9258 ± 434 | 32.2 | (6) |
| 8 | 16 | 3050 ± 105 | 2505 ± 92 | 17.9 | (6) |
| | 24 | 4732 ± 274 | 3702 ± 228 | 21.8 | (5) |
| | 32 | 7880 ± 514 | 6073 ± 237 | 22.9 | (6) |
| | 40 | 15709 ± 196 | 12472 ± 696 | 20.6 | (6) |
| 16 | 24 | 4829 ± 109 | 3900 ± 292 | 19.2 | (6) |
| | 32 | 7606 ± 471 | 5501 ± 258 | 27.7 | (6) |
| | 40 | 15331 ± 517 | 12419 ± 503 | 19.0 | (6) |
| 24 | 32 | 7137 ± 381 | 7013 ± 330 | 1.7 | (6) |
| | 40 | 15679 ± 448 | 14767 ± 604 | 5.8 | (6) |
| 32 | 40 | 15378 ± 249 | 15459 ± 737 | 0.0 | (6) |

[a]Parasite age post-invasion (h) at initiation of assay

[b]Parasite age post-invasion (h) at termination of assay

[c]Incorporation (CPM/well) ± standard error of the mean

[d]Number of replicates in parentheses

TABLE 3

Inhibition of glucose utilization

in P. falciparum by CFF

| Cultivation | | Glucose Utilization | | % Inhibition | |
|---|---|---|---|---|---|
| Start[a] | End[b] | Normal Serum | CFF Serum | | |
| 0 | 8 | 3.5 ± 0.6[c] | 1.3 ± 0.6 | 63.0 | (9)[d] |
| | 16 | 6.3 ± 1.0 | 1.9 ± 0.7 | 69.8 | (10) |
| | 24 | 11.2 ± 1.6 | 6.8 ± 0.9 | 39.7 | (7) |
| | 32 | 21.5 ± 1.5 | 16.2 ± 1.3 | 24.4 | (6) |
| | 40 | 38.7 ± 0.5 | 34.3 ± 0.8 | 11.1 | (4) |
| 8 | 16 | 4.1 ± 0.9 | 2.5 ± 0.9 | 38.8 | (10) |
| | 24 | 8.3 ± 1.1 | 6.3 ± 1.3 | 24.0 | (8) |
| | 32 | 20.0 ± 0.8 | 17.8 ± 1.9 | 11.3 | (6) |
| | 40 | 38.8 ± 1.2 | 36.0 ± 1.5 | 7.3 | (4) |
| 16 | 24 | 4.2 ± 0.8 | 4.3 ± 0.6 | 0.0 | (8) |
| | 32 | 15.9 ± 1.2 | 15.5 ± 0.8 | 2.6 | (6) |
| | 40 | 35.1 ± 1.1 | 34.1 ± 0.8 | 2.8 | (4) |
| 24 | 32 | 12.5 ± 1.4 | 11.6 ± 1.0 | 7.9 | (6) |
| | 40 | 32.2 ± 0.6 | 30.6 ± 1.4 | 5.2 | (4) |
| 32 | 40 | 19.1 ± 1.1 | 18.1 ± 1.7 | 4.9 | (4) |

[a]Parasite age post-invasion (h) at initiation of assay

[b]Parasite age post-invasion (h) at termination of assay

[c]Glucose utilized (nM/well) ± standard error of the mean

[d]Number of replicates in parentheses

# DISCUSSION

Since certain aspects of Plasmodium metabolism have demonstrable stage dependence,[7,11,19,20,21,27] the present studies were conducted to determine if CFF affected the in vitro development of P. falciparum in a stage- and time-dependent manner. A moderately inhibitory serum pool was used in these experiments to determine the effects of CFF on parasite metabolism since more inhibitory sera quickly kill the parasites, rendering longer exposure times to CFF meaningless. The erythrocytic cycle was divided into sequential 8 h time periods and highly synchronous parasites corresponding to each period were exposed to CFF serum for various lengths of time. The effects of CFF on hypoxanthine and phenylalanine incorporation into nucleic acids and proteins, respectively, and glucose consumption were then examined for stage- and time-dependency. In contrast to the activity reported for the antimalarial drug chloroquine,[27] in which ring stages were the least sensitive, it was found that early ring-staged parasites were the most sensitive to an 8 h exposure to CFF, regardless of the metabolic parameter examined. Assays begun with older parasites and cultivated for 8 h in CFF serum showed progressively less inhibition. Although inhibition of glucose utilization was not observed in tests initiated with parasites $\geq$ 16 h p.i., purine and amino acid incorporation remained inhibited in parasites first exposed to CFF at this age. However, in experiments begun with parasites $\geq$ 24 h p.i., CFF had little or no effect on any of the three measured metabolic parameters.

Both hypoxanthine and phenylalanine incorporation exhibited no time-dependent effects. Regardless of the number of 8 h periods that parasites were continuously exposed to CFF, percent inhibition in tests initiated with parasites of identical ages remained relatively constant. However, in contrast to the time-independent inhibition observed in the incorporation studies, CFF's effect on glucose consumption varied inversely to the duration of exposure to CFF. As the parasites matured in the presence of CFF, inhibition of glucose utilization decreased, with little or no reduction or consumption observed as parasites entered schizogony. It appears likely that the effect of CFF on glucose utilization is not persistent, and once the parasite is past the 16 h p.i. ring stage, it becomes refractory to CFF inhibition of this process. A possible explanation of this observation may be found in the studies of Sherman and Tanigoshi[21] who showed that the permeability of P. lophurae-infected erythrocytes was markedly enhanced for a glucose analog, and this enhancement was due to increases in both carrier-mediated and simple diffusion components of transport of the sugar. Furthermore, the size of the parasite positively correlated with this observed enhancement. The increased erythrocyte permeability observed in maturing parasites might then account for the decrease in CFF inhibition of glucose utilization seen in maturing parasites. If glucose transport was being inhibited by CFF, the increased glucose permeability of maturing

P. falciparum-infected erythrocytes could overcome this effect, allowing glucose utilization to proceed at normal rates. Further experimentation will be required to determine if such is the case.

It is important to note the implications these results have on determination of CFF titers in malaria-immune sera using cultured parasites. First, although our results have shown that hypoxanthine incorporation is a more sensitive indicator of CFF inhibition than phenylalanine incorporation, this observation does not lead to the conclusion that CFF affects nucleic acid more than protein synthesis. Hypoxanthine is an exogenously required medium constituent,[10] whereas phenylalanine is not,[8] thus protein synthesis may continue without incorporation of the radiolabeled amino acid, even though it is readily incorporated if available to the parasite. Second, despite the fact that CFF affected the three metabolic parameters differently, metabolism in young ring stages was always inhibited more than in other stages. Thus, the amount of inhibition any given immune serum may have on cultured P. falciparum will depend upon the degree of synchrony of the parasites, and their post-invasion age at the time of initial CFF exposure.

## ACKNOWLEDGMENTS

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## SUMMARY

The objective of this research was to further characterize the various effects of crisis form induction on Plasmodium falciparum. As a result of this effort, the following conclusions have been reached:

1) The presence of CFF in patient serum interferes with field tests of parasite drug sensitivity, resulting in a test failure rate > 70%.

2) Inhibition of parasite development due to CFF and the antimalarial drugs chloroquine and mefloquine are additive in effect.

3) Although human CFF and TB sera and rabbit BCG-LPS sera all induce crisis forms in cultivated P. falciparum, only BCG-LPS sera are cytotoxic to L-M cell cultures, indicating that TNF is most likely not responsible for crisis form induction in the human sera tested.

4) Human $\gamma$-interferon has no direct effect on parasite growth.

5) CFF exhibits stage-dependent inhibition as determined by various metabolic criteria. Hypoxanthine and phenylalanine incorporation into parasite nucleic acids and protein, respectively, and glucose consumption are most

inhibited in cultures initiated with parasites 0 h post-invasion. Inhibition correspondingly decreases in tests started with progressively older parasites.

6) Variation in length of exposure to CFF has little effect on inhibition of parasite incorporation of hypoxanthine and phenylalanine. However, in contrast to the time-independent inhibition observed in purine and amino acid incorporation, inhibition of glucose consumption varies inversely to the time of cultivation in the presence of CFF. As the parasites mature in the presence of CFF, inhibition of glucose utilization decreases, with little or no reduction of consumption observed as parasites enter schizogony.