Stage-Dependent Effect of Deferoxamine on Growth of *Plasmodium falciparum* In Vitro

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Deferoxamine (DF) has antimalarial activity that can be demonstrated in vitro and in vivo. This study is designed to examine the speed of onset and stage dependency of growth inhibition by DF and to determine whether its antimalarial activity is cytostatic or cytotoxic. Growth inhibition was assessed by suppression of hypoxanthine incorporation and differences in morphologic appearance between treated and control parasites. Using synchronized in vitro cultures of *Plasmodium falciparum*, growth inhibition by DF was detected within a single parasite cycle. Ring and nonpigmented trophozoite stages were sensitive to the inhibitory effect of DF but cytostatic antimalarial activity was suggested by evidence of parasite recovery in later cycles. However, profound growth inhibition, with no evidence of subsequent recovery, occurred when pigmented trophozoites and early schizonts were exposed to DF. At this stage in parasite development, the activity of DF was cytocidal and furthermore, the critical period of exposure may be as short as 6 hours. These observations suggest that iron chelators may have a role in the treatment of clinical malaria.

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NEW antimalarial drugs are urgently required in clinical practice. The spread of strains of *Plasmodium falciparum* that are resistant to commonly available antimalarials (chloroquine, sulfonamides, and antifolates) and the emergence of further strains that exhibit resistance to mefloquine and to quinine emphasize the deficits in current therapeutic regimens. Clinical observations suggesting that iron metabolism and malarial infection are closely interrelated have led to a number of studies designed to examine the effect of iron chelators on growth of *P. falciparum*. Deferoxamine (DF) inhibits parasite growth in vitro at concentrations ranging from 5 to 20 μmol/L. Similar inhibitory concentrations are found against the liver stage of *P. falciparum* in the human hepatocyte model. These concentrations of DF are attainable in patients. However, knowledge of the site or mechanism of action of DF in inhibiting growth of *P. falciparum* is essential before clinical trials can be designed that take into account dosage and duration of therapy. One approach is to study stage-dependent effects of DF since different stages in the parasite cycle are associated with different metabolic functions and may therefore exhibit different drug sensitivities. Such information is already available for several antimalarial compounds. Mature parasites are killed by drugs that inhibit protein and nucleic acid synthesis. Drugs that interfere with the food vacuole, eg, ammonium chloride, produce effects early in the parasite cycle. Drugs that have more than one mode of action may inhibit *P. falciparum* at different stages at different doses. Thus, while some studies have shown that trophozoite and schizont stages are sensitive to chloroquine, more recent work demonstrates that exposure to chloroquine at lower doses over longer periods of time inhibit ring stage parasites. Some antimalarials, eg, qinghaosu and tetracycline are equally effective at all stages of parasite development but have different rates of action. These are important considerations when assessing the suitability of a compound as an antimalarial and constructing drug protocols.

Preliminary studies of the effect of 24-hour pulses of DF on growth of asynchronous cultures of *P. falciparum* suggested that the later stages of parasite maturation are more sensitive than ring forms. In this study, we have assessed the inhibitory effect of short pulses of DF on in vitro growth of synchronized cultures of *P. falciparum*. In the first instance, we determined the rate of onset of action of DF by assessment of parasite morphology and measurement of hypoxanthine uptake within a single cycle. We then extended these observations to the second and third cycles after exposure to DF in the first cycle. The ability of the parasite to develop, reinvade red cells in the second cycle, and ultimately to recover normal metabolic function in the third cycle was used to determine the cytostatic or cytoidal activity of DF. These effects were related to the stage in the first parasite cycle in which DF exposure had occurred.

**MATERIALS AND METHODS**

*Parasites.* ITO4, an uncloned strain of *P. falciparum*, that originated from Brazil, was grown in blood group O red cells using a modification of the method of Trager and Jensen. An atmosphere consisting of 1% oxygen, 3% carbon dioxide, and 96% nitrogen was maintained throughout the experiments. Red cells were suspended at a hematocrit of 3% in RPMI 1640 (Northumbria Biologicals) that had been supplemented with 10% human serum, 2 g/L of glucose (British Drug Houses), 4 mmol/L of NaOH (British Drug Houses), 25 μg/mL of gentamicin (Sigma), 1.9 mmol/L of glutamine (Northumbria Biologicals), and 25 mmol/L of HEPES (Northumbria Biologicals) to form complete medium. For experimental purposes the red cells were further diluted to a final hematocrit of 1.5%. Synchronized parasite cultures were obtained using the following protocol. After sorbitol synchronization, the cultures were continued to the trophozoite/schizont stage and mature parasites recovered by gelatin flotation. These parasites were cultured for a further 4 hours before a second sorbitol lysis was performed. The parasites were diluted to a final parasitemia of 0.1% to 0.5%. This procedure ensured that only young ring forms remained.

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Submitted January 22, 1990; accepted May 23, 1990.

Supported in part by the Medical Research Fund and Nuffield Department of Medicine, University of Oxford and The Wellcome Trust.

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0006-4971/90/7606-0023$3.00/0

BLOOD, Vol 76, No 6 (September 15), 1990: pp 1250-1255
Parasite growth. Two methods were used to assess the growth of *P. falciparum*. In the first, 0.2 µCi of tritiated hypoxanthine (Amersham) per 100 µL of red cell suspension was added to the test culture. Subsequently, 200-µL aliquots of culture were dispensed into 96-well flat-bottomed microculture plates. At appropriate points in each experiment, cells from each well were harvested onto glass fiber filters and dried before placing in a bag with 10 mL of liquid scintillant. Parasite growth, in terms of beta emissions from each well, was measured in a flat bed liquid scintillation counter (Pharmacia-Wallac). No tritiated hypoxanthine uptake occurred in uninfected red cells. In the second method, 200-µL aliquots of each test culture were washed in RPMI 1640 and spun for 5 seconds at 10,000 x g before thin films were made from the red cell pellet. Films were made in duplicate, fixed in methanol, and stained with Giemsa (BDH) for light microscopy examination. This procedure allowed morphologic assessment of parasite growth that was necessary both to determine the effect of DF and to monitor the progress of the experiment when pulsing with DF at various intervals.

DF. Solutions of DF, at different concentrations, were prepared in complete medium before addition to the parasite suspension.

Dose response curve. Five-milliliter volumes of culture that contained newly synchronized ring parasites were incubated with tritiated hypoxanthine and DF at various concentrations in 50-mL flasks. Two hundred-microliter aliquots were removed in triplicate for growth assessment after 36 to 42 hours. This experiment was repeated on 10 separate occasions.

Effect of cation-DF complex on parasite growth. Solutions (10 mM) of copper sulfate, zinc sulfate, sodium sulfate, ferric citrate, and DF were prepared in distilled water. Chelates of each metal, in which the DF was fully bound, were obtained by mixing equimolar volumes of each salt with DF. Serial dilutions were prepared in complete medium before addition to parasite suspension that contained newly synchronized ring parasites and tritiated hypoxanthine. Each test culture was dispensed in quadruplicate into 96-well microculture plates and parasite growth assessed 38 hours later when schizonts were seen in red cells that had not been exposed to a salt, DF, or chelate.

Rate of inhibition of growth of *P falciparum* by DF. Volumes (5 mL) of culture that contained newly synchronized ring parasites were incubated with tritiated hypoxanthine, with or without the addition of 100 µmol/L of DF, in 50-mL flasks. Aliquots (200 µL) were removed in triplicate at 6, 10, 23, 29, 33, and 48 hours for assessment of parasite growth.

Stage specificity of the antimalarial action of DF. Tritiated hypoxanthine was added to a red cell suspension that contained newly synchronized ring parasites. Immediately the test culture was dispensed in 200-µL aliquots into a 96-well microculture plate. Parasites in six replicate wells were exposed to 100 µmol/L of DF at various intervals of 7 to 8 hours over a 42 hour period. In each case the effect of DF was quenched at the end of the 7- to 8-hour interval by the addition of an equimolar concentration of ferric citrate. At 42 hours, cells were sampled to assess parasite growth. Preliminary experiments showed that 100 µmol/L of ferric citrate or sodium citrate alone had no effect on parasite growth as judged either by morphologic assessment or tritiated hypoxanthine uptake.

Effect of DF on parasite multiplication and cell invasion. Newly synchronized cultures of ring parasites were dispensed in 3-mL volumes into separate 50-mL culture flasks. Different cultures were exposed to 6-hour or 12-hour pulses of DF at different stages during the first parasite cycle. At the end of each 6-hour interval, all cultures were washed twice in RPMI 1640 and the pellet resuspended in complete medium. At 42 hours, when cells from cultures that had not been exposed at any time to DF contained segmenting schizonts, tritiated hypoxanthine was added to the incubation medium of all the cultures. Cells were sampled for growth assessment 20, 44, and 51 hours later, ie, during the second cycle of the experiment, and again 68 and 94 hours later, ie, during the third cycle. By 94 hours, synchrony of the parasite cultures had clearly been lost on morphologic examination of red cells. Three similar experiments were performed in which cultures were exposed to various pulses of DF during the first cycle and cells were sampled at various stages during the second and third cycles.

RESULTS

Dose response. Exposure of *P. falciparum* in vitro to DF results in growth inhibition. Figure 1 represents the mean dose-response curve obtained from 10 separate experiments. Overall the ID$_{50}$ was found to lie between 10 to 20 µmol/L of DF.

Effect of cation-DF complexes on parasite growth. The antimalarial activity of DF is likely to be due to its property as an iron chelator, but it may act by chelating other metals. To examine this possibility, equimolar amounts of different metals were added to DF to form cation-DF complexes before addition to cultures of *P. falciparum*. Figure 2 shows that the inhibitory action of DF on the growth of *P. falciparum* was abolished when DF was in the form of ferric iron-DF chelate, whereas complexing of copper or zinc to DF, that have lower binding affinity, had no effect on the inhibitory action of DF. Thus, ferric iron inhibited the antimalarial effect of DF while copper or zinc did not suppress its antimalarial activity. Spectroscopy was used to demonstrate that ferric iron will displace zinc or copper from DF to form ferrioxamine (detected by spectroscopy at a 430-nm wavelength) within 60 seconds of addition of iron to solutions of either copper or zinc chelates. Therefore, DF appears to exert its antimalarial action by chelation of iron rather than copper or zinc.

Speed of onset of the antimalarial action of DF. The speed of onset of the antimalarial action of DF was studied by adding a single dose of 100 µmol/L of DF to the
incubation medium at the beginning of the experiment when parasites were at the ring stage of development and assessing parasite growth later in the same cycle. Figure 3 shows that DF had no effect on the rate of hypoxanthine incorporation in control and treated parasites during the first 10 hours of incubation. Subsequently, DF inhibited the rapid increase in hypoxanthine incorporation found in the control cultures. Concurrent sampling of cells for morphologic examination showed no detectable differences between treated and control parasites during the early stages of the experiment. Even at 23 hours no clear difference was discernible between the two groups. However, by 33 hours treated parasites had abnormally vacuolated cytoplasm and no evidence of nuclear division while untreated parasites had developed into easily recognizable pigmented trophozoites and early schizonts. These results show that the inhibitory effect of DF is detectable within a single parasite cycle. Similarly, at 25 μmol/L of DF, parasites were clearly differentiated from controls after 30 hours (data not shown).

Stage-specificity of the antimalarial action of DF. The sensitivities of different stages of parasite development to the inhibitory action of DF were examined by adding DF to the incubation medium at different times during the parasite cycle and assessing parasite growth later in the same cycle. Figure 4 shows the hypoxanthine incorporation by parasites harvested towards the end of the first cycle. Exposure to DF in 7- to 8-hour pulses during the first 23 hours of the experiment, when parasites were between ring and nonpigmented trophozoite stages, had no inhibitory effect on parasite growth. On the other hand, exposure to DF after 23 hours when parasites had matured into pigmented trophozoites and schizonts, resulted in more than 50% (P < .001) suppression of hypoxanthine incorporation. Morphologic examination of cells taken from these cultures showed increased numbers of abnormal, degenerate trophozoites. In all the remaining cultures morphologically normal parasites were seen. Therefore, sensitivity to the inhibitory action of DF, as judged by growth inhibition, is only seen if exposure to DF occurs late in the parasite cycle. These results were confirmed on two further occasions using different pulses of DF at concentrations ranging from 25 μmol/L to 100 μmol/L.

Effect of DF on parasite multiplication and cell reinvansion. The previous experiment clearly demonstrates that the inhibitory effect of DF is related to parasite development within a single cycle, but gives no indication of the potential for growth during subsequent cycles once the parasite is no longer exposed to DF. In order to study this question, parasites were exposed to DF at different stages during the
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first cycle of the experiment. Parasite growth was then assessed during the second and third cycles. Figure 5 shows the timing of the 6- or 12-hour pulses that were used during the first cycle in relation to the stage of parasite development in one representative experiment. At 42 hours, schizonts predominate in each culture. However, in the culture that had not been exposed to DF and in cultures where exposure to DF had been confined to the ring and trophozoite stages of the parasite cycle, young ring parasites could also be identified.

Parasite growth was then assessed over the following two cycles and the growth of treated cultures compared with that of untreated cultures. Figure 6A shows the growth inhibition, measured as hypoxanthine incorporation, that was seen when parasites were harvested during the second (44 hours) and third (68 hours) cycles following 6-hour pulses of DF. DF administered during the early ring and nonpigmented trophozoite stages of parasite development (6 to 12 hours) resulted in minimal growth inhibition. Morphologic examination of cells that were sampled from these cultures showed parasites that were indistinguishable from untreated parasites.

As parasite development progressed and exposure to DF coincided with the pigmented trophozoite stage of the first cycle, i.e., 12 to 18 hours, 18 to 24 hours, hypoxanthine incorporation was suppressed by 22% and 39%, respectively, when compared with untreated cultures although marked morphologic changes were not yet seen. However, by the end of the third cycle, growth inhibition in these cultures had decreased to 4% and 25%, respectively, and morphologic examination of parasites showed a mixed population of vacuolated, abnormal trophozoites and morphologically normal ring parasites. This reduction in growth inhibition can therefore be considered to represent a cytostatic rather than cytocidal action of DF. In contrast, exposure of *P. falciparum* to DF during the development of schizonts, i.e., 24 to 30 hours, resulted in apparent arrest of parasite development with only abnormal trophozoites and parasite remnants present on morphologic examination of cells. Growth inhibition in both the next two cycles was as great as in treated control cultures that had been exposed to DF between 0 to 30 hours. Under these circumstances, the effect of DF on growth of *P. falciparum* appears to be cytocidal. Figure 6B shows the growth inhibition that was found in later cycles when 12-hour pulses of DF were administered during the first cycle of parasite development. As before, exposure to DF during early stages of parasite development resulted in growth inhibition during the second cycle that had reduced by the third cycle whereas, treatment with DF during schizont development produced profound growth inhibition in both cycles. A similar pattern of growth inhibition of *P. falciparum* was obtained in three other experiments, when parasites were exposed to DF and growth assessed in the later cycles.

**DISCUSSION**

The results of this study demonstrate that the inhibitory action of DF on the growth of *P. falciparum* in vitro is stage dependent. Furthermore, exposure to DF during a critical period of schizont development is followed by objective evidence of a cytocidal effect measured as hypoxanthine incorporation and morphologic assessment in the second and third cycles of parasite development. At other times in the parasite cycle, exposure to DF results in growth inhibition but there is evidence of recovery in subsequent cycles. Under these circumstances the inhibitory effect of DF appears to be cytostatic.

Incorporation of tritiated hypoxanthine is linearly related to the growth of *P. falciparum* in vitro for up to 66 hours provided certain criteria are observed, namely initial parasitemia less than 0.4% and erythrocyte concentration less than 1.5% hematocrit. Thus, hypoxanthine incorporation is a...
Data and standard errors are taken from a single experiment.

During the third cycle at 44 hours (open square) by parasites that have been exposed to DF when compared with untreated controls. The horizontal axis shows the times of exposure to 100 μmol/L of DF during the first cycle. Each culture was sampled in quadruplicate. Data and standard errors are taken from a single experiment.

Fig 6. Growth inhibition of *P. falciparum* following exposure to (A) 6-hour pulses of DF at 6-hour intervals and (B) 12-hour pulses of DF at 8-hour intervals, during the first cycle. The vertical axis shows growth inhibition measured as the relative %hypoxanthine uptake during the second cycle at 44 hours (open square) by parasites that had been exposed to DF when compared with untreated controls. The horizontal axis shows the times of exposure to 100 μmol/L of DF during the first cycle. Each culture was sampled in quadruplicate. Data and standard errors are taken from a single experiment.

Useful method of assessing the effect of potential antimalarial compounds on the growth of large numbers of parasites. However, information about stage of parasite development or red cell reinvasion can only be obtained from morphologic examination of parasitized red cells. In these experiments, we used both metabolic and morphologic techniques to assess parasite growth in synchronous cultures in order to describe both the rate of onset and stage dependency of the inhibitory action of DF and to determine whether or not the antimalarial action of DF is cytostatic or cytocidal.

In agreement with previous work, we find that DF inhibits parasite growth at concentrations that exceed 20 μmol/L. In addition, our study shows that the inhibitory effect of DF on growth of *P. falciparum* in vitro occurs within a single parasite cycle. Such a rapid onset of antimalarial activity argues in favor of a direct toxic effect on parasite growth and against gradual depletion of iron stores that are available to the parasite. Furthermore, as previously reported by Raven-rost-Suarez et al., addition of iron (DF affinity constant, 10^{11}) to the culture system abolishes the antimalarial activity of DF. We have extended these investigations to include other salts that have been suggested as alternative targets for DF chelation. Addition of either copper (affinity constant, 10^{5}) or zinc (affinity constant, 10^{6}) to DF does not suppress its antimalarial activity. Possibly the copper or zinc is displaced from DF by the iron that is essential for parasite growth. These findings support the hypothesis that the antimalarial activity of DF is due to its property as a chelator and in addition, suggest that it is the chelation of iron that is of primary importance.

By using cultures of *P. falciparum* that have been manipulated in such a way that the age between the oldest and youngest parasite is no more than 4 hours, we can demonstrate that the inhibitory effect of DF on parasite growth shows marked stage specificity. Young parasites at ring and nonpigmented trophozoite stages exhibit little or no sensitivity to DF. On the other hand, exposure to DF during the development of pigmented trophozoites and early schizogony results in profound growth inhibition that is apparent even when parasites are assessed within the same cycle of treatment. We then examined the effect of DF on parasite multiplication and red cell reinvasion in an attempt to determine whether the growth inhibition of *P. falciparum* induced by DF is cytostatic or cytocidal. Since there is no recognized method of determining cytostatic or cytocidal activity, we elected to serially measure hypoxanthine uptake by parasites in the second and third cycles having added hypoxanthine at the end of the first cycle. Any increase in hypoxanthine incorporation between the second and third cycles was taken as an index of parasite recovery from a cytostatic effect. This information was then related to the morphologic appearances of the cultures. Over several experiments a trend becomes apparent. Parasites that are exposed to DF early in their cycle show minimal growth inhibition and any inhibitory effect that is obtained is followed by recovery. Under these circumstances, the antimalarial effect of DF is cytostatic. By contrast, following exposure to DF late in the parasite cycle there is no evidence of recovery. At this stage, the antimalarial effect of DF is clearly cytocidal. Our results indicate that the critical period may be as short as 6 hours. By producing a more tightly synchronous culture, it may be possible to more accurately define the limits of this period of cytocidal activity. Nevertheless, the cytocidal effect of DF appears to coincide with the period of maximal DNA synthesis by the parasite. Lederman et al. have demonstrated that in vitro DF inhibits DNA synthesis by human T and B lymphocytes. They suggest ribonucleotide reductase as the likely target for inhibition by DF. This is particularly interesting since iron that is bound to an active site by tyrosyl residues, as in ribonucleotide reductase, is directly available to chelators. Alternatively, DF may act by chelating the low molecular weight iron pool in the parasite thereby interfering with the supply of iron to a number of different parasite metabolites. Thus, the inhibitory effect of DF on growth of *P. falciparum* may be via several different mechanisms. Elucidation requires experiments that are designed to
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measure the size of the low molecular iron pool and the iron requirements of the parasite together with measurement of nucleotide incorporation into parasitic DNA with and without the presence of DF.

Four studies have examined the effect of DF in malaria infections in vivo.\textsuperscript{22-24} Models that have been used include Plasmodium vinckei in mice,\textsuperscript{23} Plasmodium berghei in rats,\textsuperscript{22} and Plasmodium falciparum in Aotus monkeys.\textsuperscript{24} DF was administered as either a continuous infusion or intermittently. In each study, suppression of malaria infection was achieved although no cures of established malaria were effected. Failure to cure the established infection may have been due to inadequate dosage since in vivo DF has a short plasma half-life that is not apparent in in vitro experiments. In addition, in experiments using the rat model there was difficulty in ensuring adequate drug delivery. The only study that documents use of DF to treat P. falciparum infection in vivo is that in Aotus monkeys. Unfortunately, only three animals were infected of which one died of complications that related to implantation of the DF pump and there was no follow-up of animals whose malaria was suppressed. This work demonstrates that DF may act as a rapidly-acting malarial drug and therefore should be considered for testing in clinical trials. However, although DF has been used in humans to treat iron overload for over 10 years with few side effects, its toxicity in patients without iron loading remains to be established.\textsuperscript{25}

ACKNOWLEDGMENT

We thank Prof Sir David Weatherall, C.I. Newbold, K. Marsh, and W.G. Wood for encouragement and helpful criticism during this study.

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Stage-dependent effect of deferoxamine on growth of Plasmodium falciparum in vitro

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