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Stage-specific sensitivity of *Plasmodium falciparum* to antifolates*

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Abstract. Highly synchronous cultures of *Plasmodium falciparum* were exposed to therapeutic concentrations of sulfadoxine or pyrimethamine at different developmental stages to investigate the effect on subsequent growth. Morphological observations showed that schizont formation from uninuclear trophozoites was the only process inhibited by the drugs. Segmentation of mature schizonts, merozoite invasion and development of the ring stage remained unaffected. These results support earlier reports suggesting that DNA synthesis is most pronounced in 32–42 h old trophozoites. The possible relevance of our results to the metabolism of *P. falciparum* is discussed.

Introduction

Malaria is still the most important infectious disease of man. More than half the population of the world lives in malariaendemic areas. Increasing resistance both of mosquitos to insecticides and parasites to chemotherapeutics pose severe problems for malaria eradication. As practically all malaria cases in South East Asia are resistant to chloroquine, Fansidar has been the favoured drug for some time. Yet during the last five years, Fansidar resistance has spread rapidly, and not only in this area. Fansidar is composed of the sulfonamide sulfadoxine and the diaminopyrimidine pyrimethamine. Both block folic acid biosynthesis and thereby, theoretically, affect both DNA and amino acid metabolism.

This study was undertaken to investigate the dependence of the different developmental stages of *P. falciparum* on folic acid, thus allowing us to speculate about the biochemical processes in *P. falciparum* involving folic acid.

* Dedicated to Prof. Dr. W. Frank on the occasion of his sixtieth birthday

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Material and methods

Parasites. The K1 strain of *P. falciparum* was used throughout this study. The strain was obtained from Dr. Y. Yuthavong, Mahidol University, in 1983.

Cultivation. Parasites were maintained in Waymouth medium (Gibco) containing 10% (v/v) extensively dialyzed human serum and 4% (v/v) homologous human erythrocytes and were cultured according to the candlejar method (Trager and Jensen 1976). Waymouth medium was chosen because it does not contain para-aminobenzoic acid, an antagonist of sulfonamide.

Synchronization. Synchrony was achieved by treating infected cells three times with 5% (w/v) sorbitol at 37° C for 20 min (Lambros and Vanderberg 1979). The second treatment was 40 h after the first (Vial et al. 1982) and the third 44 h after the second. Parasites treated in this manner grew synchronously for 6–7days.

Drug treatment. For each drug, nine parallel cultures with a parasitemia of 2.0% highly synchronous very young rings were each established in 6-well culture plates (Falcon, culture volume 2 ml). Treatment (0–6, 6–12, 12–18, 18–24, 24–30, 30–36, 36–42, 42–48 h after start of culture) consisted of exposing one culture at a time to 20 μM sulfadoxine (Hoffmann-La Roche) or 10 nM pyrimethamine (same company) in Waymouth medium. After 6 h of drug exposure, the respective culture was washed twice with 5 ml of normal medium and returned to the incubator. Subsequently cultures were treated in the same way. All experiments were duplicated.

Morphological observations. Giemsa-stained thin smears were prepared from each culture every 6 h, when the medium was changed. The number of infected cells per 2,000 erythrocytes and the stage distribution and number of dead plasmodia per 500 parasites were determined by microscopic examination. The effect of each drug was expressed as a percentage of dead cells compared to the untreated control. All cultures were examined under a microscope over 48 h after the end of the experiment, to check for a second life cycle.

Results

Parasites grown in the drug-free medium exhibited a normal life cycle of 48 h (Fig. 1a). When ring stage parasites (aged 0–6 or 6–12 h) or young uninuclear trophozoites (18–24 h old) were treated, no effect was observed at all and the cultures continued to grow normally after removal of the respective drug (Figs. 1b, c). Polynuclear trophozoites (aged 30–36 h) were all killed by the treatment (Fig. 1d) yet cultures containing large numbers of segmented schizonts (aged 42 h) survived at least partially (Fig. 1e). The overall effect of both drugs was very similar (Fig. 2).

Dead parasites possess a faintly stained and vacuolated cytoplasm, only very small scattered points of deep blue nuclear staining and the same large mass of brown deposits of malarial pigment as typical for viable old trophozoites. These dead cells could be seen in the cultures for about 20 h, after which they disappeared, probably through lysis of their host erythrocytes.

Discussion

The sensitivity curve for antifolates (Fig. 2) confirms the results of Gritzmacher and Reese (1984) and Inselburg and Banyal (1984a) who found that DNA synthesis is most pronounced in 32–40 h old trophozoites. Our results also corroborate the reports of the inhibition of *P. falciparum* by

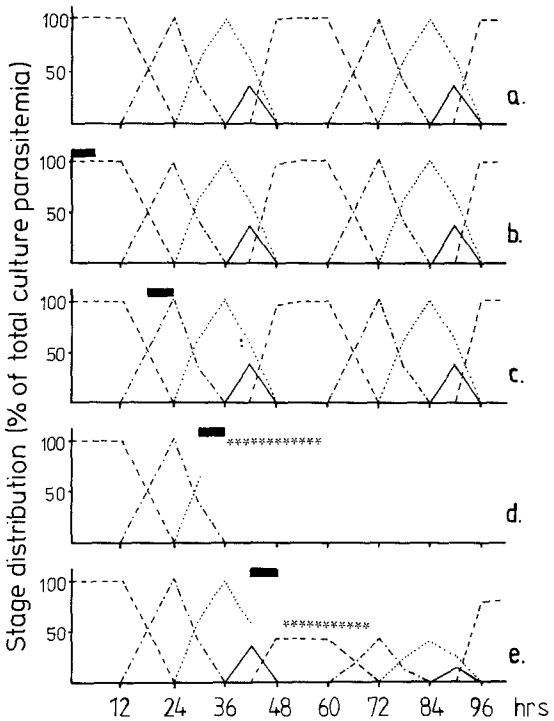

Fig. 1a–e. Effect of antifolates on different developmental stages of *Plasmodium falciparum*. *Black horizontal bars* indicate time of antifolate treatment. **a** control, **b–e** 4 of 9 experiments with antifolate treatment. – – – ring forms – · – · uninucleate trophozoites, · · · multinucleate trophozoites, —— mature (segmented) schizonts, ***** dead parasites. The number of trophozoites after 72 h in Fig. 1e is related to the preceeding number at the ring stage, as the dead part of the population disappeared

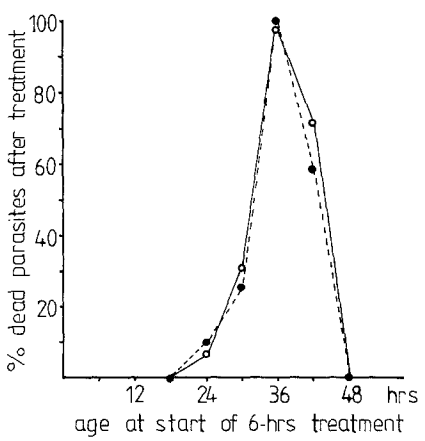

Fig. 2. Stage-specific effect of sulfadoxine (o–o–o) and pyrimethamine (●–●–●) on *Plasmodium falciparum*. All values are averages from two experiments

DNA synthesis inhibitors (Inselburg and Banyal 1984b; Assaraf et al. 1984). Moreover, we demonstrated that DNA synthesis is the only process in the development of *P. falciparum* which can be inhibited by antifolates. Growth of ring stages, which exhibit an extensive protein turnover, is not affected by antifolates (Fig. 1b). This is remarkable because it implies that the provision of methionine in *P. falciparum* could be folic acid independent, pro-

vided the parasite does not utilize erythrocytic folate, thereby overcoming the antifolate inhibition.

The surviving cells in Fig. 1e can be accounted for by the presence of mature schizonts. Thus, merozoite liberation and invasion also do not involve folic acid dependent processes, with the restrictions stated above.

Figure 1b also shows that antifolates do not exert a delayed effect on ring stage parasites. Therefore we conclude that rings barely, if at all, accumulate and store antifolates until they reach the trophozoite stage. This supports our own results on the uptake of antifolates by *P. falciparum* (Dieckmann and Jung 1986a, b).

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