Polyamine levels and the activity of their biosynthetic enzymes in human erythrocytes infected with the malarial parasite, *Plasmodium falciparum*

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Human erythrocytes contain only trace amounts of polyamines and lack active polyamine biosynthetic enzymes. A remarkable increase in polyamine content, and in the activity of ornithine and *S*-adenosyl-L-methionine decarboxylases, is noted in synchronous cultures of the malarial parasite, *Plasmodium falciparum*. Polyamine biosynthesis reached peak values during the early trophozoite stage, whereas nucleic acid and protein synthesis occurred later in mature trophozoites. DL- α -Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, did not interfere with merozoite invasion and with ring-form development, but prevented the transformation of trophozoites to schizonts. Concomitantly, the synthesis of proteins and nucleic acids was significantly inhibited. These inhibitory effects could be readily reversed by the diamine putrescine. Macromolecular synthesis and schizogony were normal when 5–10 mM-DL- α -difluoromethylornithine and 0.1 mM-putrescine were added to the cultures simultaneously.

The World Health Organization has reported that more than 600 million individuals all over the world are affected by tropical diseases. Among these, malaria is the most common, being a potential threat to more than two billion people in Asia, Africa and Central America (Lepes, 1981). *Plasmodium falciparum* causes the majority of cases and is responsible for the fatal disease in man. In the host, *P. falciparum* multiplies rapidly in the erythrocyte, producing up to 32 merozoites in 48 h.

Despite the high incidence of malaria in the various tropical and subtropical regions, no dramatic progress has been made in therapy and only a few new drugs have been introduced over the past three decades. In order to develop new therapeutic approaches, the physiology of the parasites and the host–parasite relationship should be better understood. It would be most desirable to detect quantitative differences in enzymes (or their products) and to utilize these differences for chemotherapy (Cohen, 1979).

The naturally occurring polyamines have been implicated in many growth processes (Cohen,

1971; Bachrach, 1973; Tabor & Tabor, 1976) and in cell differentiation (Heby, 1981). In general, polyamine levels are high and the activity of the biosynthetic enzymes, ornithine decarboxylase (ODC, EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase (SAMDC, EC 4.1.1.50) increases in rapidly growing systems. McCann *et al.* (1981) have reported that DFMO, an irreversible inhibitor of ODC, interferes with the growth *in vitro* of *P. falciparum* and that this inhibition is reversed by exogenous putrescine. It is the purpose of the present paper to extend these findings and to describe the changes in polyamine levels and in the activity of ODC and SAMDC throughout the asexual cycle of *P. falciparum* cultured in human erythrocytes. The role of DFMO as an inhibitor of parasite growth will also be discussed. A preliminary account of this work has been given previously (Assaraf *et al.*, 1983).

Experimental

Parasite culture

Plasmodium falciparum (FCR-3) was cultured by the method of Trager & Jensen (1976), with some modifications: the parasites were grown in 75 cm² disposable plastic flasks in an atmosphere of N₂/O₂/CO₂ (18:1:1). Suspensions enriched in tropho-

Abbreviations used: ODC, ornithine decarboxylase; SAMDS, *S*-adenosyl-L-methionine decarboxylase; DFMO, DL- α -difluoromethylornithine; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

zoites and schizonts were obtained by the gelatin-sedimentation technique of Jensen (1978) from cultures containing 10–20% parasitized erythrocytes. The upper layer containing 60–85% trophozoites and schizonts was washed, mixed with non-parasitized erythrocytes to yield a 3% final cell suspension and returned to the culture. After 9–15 h the erythrocytes were washed and resuspended in 5% sorbitol in distilled water for 15 min as described by Lambros & Vanderberg (1979). The resulting suspension, containing only ring forms, was incubated for an additional 30 h, after which the same process was repeated. The parasites still grew synchronously 5 days later.

Monitoring multiplication and growth of plasmodia

Parasitized erythrocytes were adjusted to 1% in a suspension of 5% non-infected erythrocytes. Aliquots (100 μ l) of this suspension were dispensed into 96-well microculture trays and incubated at 37°C in a candle jar (Trager & Jensen, 1976). Thereafter, growth media were changed once or twice a day for 4–6 days, and drugs were added to the media as indicated. Parasitaemia and stage distribution were estimated daily from Giemsa-stained smears by counting 5000 erythrocytes and 500 infected erythrocytes, respectively. Incorporation of radioactive hypoxanthine or isoleucine was used to monitor nucleic acid and protein synthesis by the method of Golenser *et al.* (1981) as follows. [3 H]Hypoxanthine (1 μ Ci, sp. radioactivity 10 Ci/mmol) or [3 H]isoleucine (1 μ Ci, sp. radioactivity 110 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.) was added in 25 μ l quantities of medium to the cultures and incubated at 37°C for 8 h. Cells were then collected on Whatman no. 3 paper, precipitated with 10% (w/v) trichloroacetic acid, washed, and the radioactivity counted (Golenser *et al.*, 1981). When necessary, parasitized erythrocytes were washed three times with RPMI medium (Trager & Jensen, 1976) and portions (0.5–1.0 ml) of packed erythrocytes were kept at –70°C until polyamine content was assayed and enzyme activity measured.

Polyamine assay

Polyamines were extracted from parasites with 3% (w/v) HClO₄. Their dansyl derivatives, prepared as described by Seiler (1970), were separated and identified by t.l.c. on 300 μ m-thick silica-gel plates, by using first ethyl acetate/cyclohexane (2:3, v/v) and then benzene/cyclohexane/methanol (85:15:2, by vol.) as solvents. Once identified, the dansyl derivatives were scraped off the plates and assayed at 365 nm excitation and 505 nm emission in a Turner model III Fluorometer (Turner Associates, Palo Alto, CA, U.S.A.). The

fluorescence corresponding to the scraped spots being compared with that of known standards.

Enzyme activity

Cells were suspended in ODC buffer containing 10 mM-sodium phosphate buffer, pH 7.2, 0.1 mM-EDTA, 3 mM-dithiothreitol (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 40 μ M-pyridoxal 5-phosphate, frozen and thawed three times at –70°C and 37°C respectively and centrifuged at 8000 g at 4°C. The ODC activity of the supernatant was determined by measuring the release of 14 CO₂ from [1- 14 C]ornithine (Bachrach, 1975). Reaction mixtures consisted of 200 μ l of cell extract and 0.2 μ Ci of [14 C]ornithine (sp. radioactivity 51.3 mCi/mmol; New England Nuclear) in 50 μ l of ODC buffer. Incubations were carried out in 17 mm $\times$ 150 mm glass tubes equipped with a rubber stopper supporting a polyethylene centre well (Kontes Glass Co., Vineland, NJ, U.S.A.) that contained 0.2 ml of Hyamine hydroxide (Packard Co., Zurich, Switzerland). After the tubes had been shaken at 37°C for 60 min, reactions were terminated by injecting 0.2 ml quantities of 3% HClO₄ through the rubber stopper. The mixtures were agitated for an additional 20 min at 37°C to ensure complete absorption of 14 CO₂. The centre wells were then removed and their radioactivity counted as previously described (Bachrach, 1975). SAMDC activity was similarly assayed, with *S*-[carboxy- 14 C]adenosylmethionine (sp. radioactivity 62 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) as substrate. The reaction mixture also contained 1.2 mM-putrescine (Sigma).

Results

Polyamine content of parasite-infected erythrocytes

To study the relationship between cellular polyamine levels and the growth of *P. falciparum*, human erythrocytes were infected with parasites and analysed for polyamines at various times. Fig. 1 shows that a fraction rich in trophozoites and schizonts contained much more polyamines than did the normal erythrocytes. The differences were most pronounced when putrescine and spermidine were compared (a 95-fold and a 51-fold increase respectively). Spermine levels were also elevated in parasitized cells. Different preparations with various parasitaemias, ranging from 1 to 22%, were analysed, and rendered similar results. The polyamine content in all samples was proportional to the parasitaemia (Y. G. Assaraf, unpublished work).

Activities of polyamine-biosynthetic decarboxylases in parasite-infected erythrocytes

ODC, which catalyses the conversion of ornith-

Fig. 1. Accumulation of polyamines in various stages of *P. falciparum*

Measurements are per 10^6 parasitized erythrocytes (PE). Ring forms and trophozoites were nearly 100% pure; the schizont preparation contained about 40% trophozoites. ▨, Putrescine; □, spermidine; ▩, spermine.

Table 1. Activity of polyamine-biosynthetic decarboxylases in *P. falciparum*-infected erythrocytes during the different stages of growth

	Activity (pmol of $^{14}\text{CO}_2$ released by 10^9 parasitized erythrocytes after incubation at 37°C for 1 h)	
Normal erythrocytes	0	0
Ring-form-infected erythrocytes	49.4	90.2
Trophozoite-infected erythrocytes	168.3	409.0
Schizont-infected erythrocytes	223.5	110.9

ine into putrescine, is known to be the rate-limiting enzyme in the polyamine biosynthetic pathway. Another polyamine-biosynthetic enzyme, SAMDC, is involved in the formation of spermidine from putrescine and that of spermine from spermidine. There was no measurable activity of ODC or SAMDC in fresh normal erythrocytes or after storage for 30 days. On the other hand, parasitized erythrocytes exhibited considerable activity of both enzymes. The increase was most obvious in trophozoites and schizonts (Table 1).

Effect of DFMO

In many systems cell division can be blocked by inhibiting polyamine biosynthesis. DFMO is an irreversible inhibitor of ODC that inhibits the growth of various parasites including *P. falciparum* (McCann *et al.*, 1981). We were able to confirm and extend previous findings (McCann *et al.*, 1981) and showed that DFMO (5–10 mM), completely

Fig. 2. Growth of *P. falciparum* in the presence of various concentrations of DFMO and putrescine

●, Control; □, 0.2 mM-DFMO; △, 1 mM-DFMO; ■, 5 mM-DFMO; ▲, 10 mM-DFMO; ○, 5 mM-DFMO + 0.1 mM-putrescine.

Fig. 3. Distribution of different stages of plasmodium treated with DFMO and putrescine
For DFMO concentrations, see the legend to Fig. 2.

blocked the development of the parasites (Fig. 2). DFMO did not affect the invasion of merozoites into erythrocytes and did not inhibit the early development to the ring form (Fig. 3a), but

Fig. 4. Stage distribution (a) and the effect of DFMO on the synthesis of nucleic acids (b) and proteins (c)
(a) Distribution: $\square$, rings; ▨ , trophozoites; ▩ , schizonts. (b and c) For DFMO concentrations, see the legend to Fig. 2.

prevented the transformation of trophozoites to schizonts (Figs. 3b and 3c).

Polyamine biosynthesis and accumulation occurred during the early trophozoite stage, whereas macromolecular synthesis reached peak values later, in mature trophozoites. The deleterious effect of DFMO on the development of the

parasite was accompanied by inhibition of the synthesis of nucleic acids and proteins. Fig. 4 illustrates the effect of DFMO on the incorporation of [3 H]isoleucine into cellular proteins and of [3 H]hypoxanthine into nucleic acids, as related to the developmental stages of the parasite. Putrescine readily reversed the inhibitory effect of DFMO, and normal development was achieved in the presence of a 0.1 mM concentration of this diamine (Figs. 3 and 4). The synthesis of proteins and nucleic acids also returned to the normal range when 0.1 mM-putrescine was added to DFMO-treated cells (Fig. 4). These findings are in line with the preliminary results reported by McCann *et al.* (1981).

Discussion

A rational approach to chemotherapy of parasitic diseases has been urged for some time. Thus Cohen (1979, 1982), advocated the search for differences in the structure and function of enzymes in normal and infected cells. Obviously, the appearance of a unique enzyme in the infected system could be exploited for chemotherapy. Human erythrocytes lack nuclei and are devoid of ornithine and S-adenosyl-L-methionine decarboxylase activity (Assaraf *et al.*, 1983). On the other hand, the activities of ODC and SAMDC were significantly elevated in erythrocytes infected with *P. falciparum*. This was followed by a dramatic increase in cellular polyamine levels. Thus the polyamine-biosynthetic pathway can be exploited to control the growth of malaria parasites.

It is not surprising that the activity of the decarboxylases and the concentration of polyamines were maximal during the early trophozoite stage. The major macromolecular synthesis occurs during this stage (Geary *et al.*, 1983) and resembles the late- G_1 phase in the eukaryotic-cell cycle. The activity of ODC is usually maximal during this phase (Tabor & Tabor, 1976) as it was also in the synchronous trophozoites.

Our experiments demonstrated that DFMO blocked the development of the parasites in the infected cells, thus confirming previous studies (McCann *et al.*, 1981). This inhibition could be reversed by micromolar concentrations of putrescine, the product of ODC activity. Parasites that are blocked in their development might serve as live immunogens incapable of inducing the disease.

Our study confirms the importance of polyamines in the growth of *P. falciparum* and suggests that drugs which inhibit polyamine biosynthesis also interfere with the development of the parasites.

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