The effect of purified aminoaldehydes produced by polyamine oxidation on the development *in vitro* of *Plasmodium falciparum* in normal and glucose-6-phosphate-dehydrogenase-deficient erythrocytes

D. M. L. MORGAN,* U. BACHRACH,† Y. G. ASSARAF,†† E. HARARI‡§ and J. GOLENSER†

*Section of Vascular Biology, Medical Research Council, Clinical Research Centre, Harrow, Middx., U.K. and †Department of Molecular Biology and ‡Department of Parasitology–The Kuvim Centre for the Study of Infectious and Tropical Diseases, The Hebrew University–Hadassah Medical School, Jerusalem, Israel

Purified aminoaldehydes produced by polyamine oxidation were toxic to the malarial parasite, *Plasmodium falciparum*, cultured in human erythrocytes. There was a profound effect on young ring forms, and, during maturation, parasites became more sensitive to the aldehydes. Oxidation of the aldehydes abolished the lethal effect. The plasmodia within glucose-6-phosphate-dehydrogenase (G6PD)-deficient erythrocytes were more sensitive to mono- and di-aldehydes than were parasites in normal erythrocytes. G6PD-deficient erythrocytes were also more sensitive to pretreatment with the dialdehyde produced by the oxidation of spermine. Pretreatment prevented further invasion by the parasites.

INTRODUCTION

The naturally occurring polyamines spermidine $[\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2]$ and spermine $[\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2]$ are oxidized by bovine serum amine oxidase (EC 1.4.3.6) to produce respectively an aminomonoaldehyde [*N'*-(4-aminobutyl)-aminopropionaldehyde] and a dialdehyde [*NN'*-bis-(3-propionaldehyde)-1,4-diaminobutane] as well as NH_3 and H_2O_2 (Tabor *et al.*, 1964; Scheme 1). The same polyamines may also be cleaved by tissue polyamine oxidases (such as the rat liver or human placental enzymes) to yield 3-aminopropionaldehyde, spermidine or putrescine and H_2O_2 (Höltta, 1977; Morgan, 1985*a,b*; Scheme 2).

The cytotoxic effects of the products (aminoaldehydes, NH_3 , H_2O_2) formed by the action of bovine serum on spermine or spermidine have long been known (Bachrach,

1970, 1981). An interesting development has been the finding that the products of oxidation can kill intra-erythrocytic parasites without lysing the host cell (Morgan & Christensen, 1983; Morgan *et al.*, 1981; Ferrante *et al.*, 1983; Rzepczyk *et al.*, 1984). To examine this phenomenon in more detail, and in particular to determine the effectiveness of the products of the human enzyme, the aminoaldehydes formed by both types of polyamine oxidases were prepared, isolated, and applied to synchronous cultures of the malarial parasite, *Plasmodium falciparum*. Thus the effects of the aminoaldehydes could be examined in the absence of H_2O_2 .

Malaria parasites grown in G6PD-deficient erythrocytes, rather than in normal erythrocytes, are usually more sensitive to physiological changes induced by naturally occurring factors. These include isouramil, derived from fava beans (*Vicia faba*) (Golenser *et al.*, 1983), and malaria crisis-form factor(s) (J. Golenser,

Scheme 1. Oxidation of spermine and spermidine by bovine serum amine oxidase

Scheme 2. Cleavage of spermine and spermidine by polyamine oxidase

Abbreviations used: G6PD, glucose-6-phosphate dehydrogenase; PBS, phosphate-buffered saline (PBS 'A'; Dulbecco & Vogt, 1954).
§ Deceased.

unpublished work). Therefore we have used the G6PD-deficient parasitized erythrocytes as sensitive target cells to explore further the effect of the aminoaldehydes.

Two experimental approaches were examined: the first was pretreatment of the erythrocytes before infection and the second was to treat parasitized erythrocytes.

EXPERIMENTAL

Chemicals

Spermine tetrahydrochloride, spermidine trihydrochloride, alcohol dehydrogenase (1.3 units/mg, from horse liver) and Dowex-50 ion-exchange resin (H^+ form; 2% cross-linked, 200–400 mesh) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; 3-aminopropan-1-ol was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; [3H]hypoxanthine (10 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.

Aldehydes

The monoaldehydes and dialdehydes of spermidine and spermine were prepared by incubating the polyamine (1 mM) and bovine serum amine oxidase (100 mg; specific activity 25 units/mg) (Tabor *et al.*, 1954; Bachrach, 1974) in 20 ml of phosphate buffer (0.1 M- Na_2HPO_4 adjusted to pH 7.2 with 0.1 M- NaH_2PO_4) for 3 h at 37 °C. The reaction was terminated by addition of trichloroacetic acid (50%, w/v; 2 ml) and the precipitated protein was sedimented by centrifugation. The supernatant fluid was applied to a column (3 cm $\times$ 6 cm) of Dowex-50 (H^+ form) ion-exchange resin and the aminoaldehydes were eluted with a linear gradient of 0–3 M-HCl (300 ml; flow rate 0.5 ml/min). Fractions (5 ml) containing aldehyde [determined by the method of Bachrach & Reches (1966)] were pooled and concentrated to a small volume in a centrifugal evaporator at 37 °C. Solutions of the aldehydes in 3 M-HCl were stored at 4 °C for not more than 48 h and were neutralized with NaOH immediately before use. No difference in aldehyde content or biological activity was observed between freshly prepared aminoaldehydes and those stored as described. Kimes & Morris (1971) have shown that aqueous solutions of these aminoaldehydes are stable for at least 1 year at –60 °C and for over 2 h at pH 3.5 and 37 °C. At pH 7.0, the half-lives of spermine dialdehyde and spermidine monoaldehyde were 42 min and 37 min respectively.

In the absence of a suitable preparation of the human enzyme, 3-aminopropionaldehyde [the aldehyde formed by the action of human polyamine oxidase on spermine and spermidine (Morgan, 1985b)] was prepared by the action of alcohol dehydrogenase (10 mg) on 3-aminopropan-1-ol (100 μ l) in 20 ml of glycine buffer (0.1 M-glycine adjusted to pH 8.5 with 10 M-NaOH) containing NAD (5 mg).

Blood

Blood collected in acid/citrate/dextrose was obtained from normal individuals or from healthy males with G6PD-deficiency (Mediterranean type) and kept at 4 °C. Experiments were carried out within 24 h of obtaining the blood samples.

Parasite

Plasmodium falciparum (FCR-3) was cultured by the candle-jar method of Trager & Jensen (1976). Synchroni-

zation of cultures was accomplished by using a combination of gelatin sedimentation (Jensen, 1978) and sequential treatments with 5% (w/v) sorbitol (Lambros & Vandenbergh, 1979). Suspensions enriched in trophozoites and schizonts were obtained by the gelatin-sedimentation technique from cultures containing 10–20% parasitized erythrocytes. The upper layer, consisting of 70–90% trophozoites and schizonts, was used in experiments requiring a minimal amount of non-infected erythrocytes in the inoculum, as described by Miller *et al.* (1984).

Monitoring development of plasmodia

Synchronous cultures (at 5% haematocrit with an initial parasitaemia of 5–18%) were dispensed into 96-well micro-culture trays. Thereafter, growth medium was changed for media containing the various compounds. Parasitaemias, stage distribution and incorporation of [3H]hypoxanthine (1 μ Ci/well) were performed as described elsewhere (Assaraf *et al.*, 1984) and the cells were harvested with a cell harvester (Jensen *et al.*, 1982).

In some experiments, erythrocytes were pretreated with the aminoaldehydes before the addition of the parasites. Erythrocyte suspensions (20%) were incubated with the various compounds in growth medium at 37 °C for 60–90 min with gentle agitation. Thereafter the cells were washed three times with medium. A fraction with parasitized erythrocytes enriched with trophozoites and schizonts (the inoculum) was added to a 5% -erythrocyte suspension (the recipient cell population) so that the initial parasitaemia was 2% of the cell suspension and contamination by the inoculum cells was less than 1% of the recipient cell population. The cell suspensions were dispensed into 96-well micro-culture trays as described above.

RESULTS

The effect of three different purified amino aldehydes, aminopropionaldehyde, *N'*-(4-aminobutyl)-3-aminopropionaldehyde and *NN'*-bis-(3-propionaldehyde)-1,4-diaminobutane was examined on normal and G6PD-deficient erythrocytes, parasitized with young or mature stages of *P. falciparum*. The parasitized erythrocytes were incubated with the aldehydes for 105 min, washed and incubated with radioactive hypoxanthine for an additional 2 h. Thereafter parasitaemia and radioactivity were monitored.

Fig. 1 shows the effect of the various aldehydes on the incorporation of hypoxanthine by parasites in normal erythrocytes. The various aldehydes had a marked, similar, effect on both young and mature stages of the parasite. The effect was more pronounced in the mature plasmodia. This finding was confirmed by microscopic examination of the development of the parasites after the treatment. Ring forms also were treated as mentioned above, and parasitaemias and stage distribution were determined 13 h later (Fig. 2).

Similar experiments were performed, comparing normal and G6PD-deficient erythrocytes, in which erythrocytes parasitized with trophozoites were treated with the aldehydes (Fig. 3). Table 1 depicts the ID_{50} (dose causing 50% inhibition) of the various compounds in different experiments. It is clear from Table 1 that the effect of the aldehydes was more pronounced in the mature stages: in young stages, about 3–5-fold higher aldehyde concentrations were needed to obtain the same

Fig. 1. Effect of purified aminoaldehydes on the incorporation of [^3H]hypoxanthine by young and mature stages of *P. falciparum*.

Synchronous cultures of 99% ring form (a) or over 95% parasites at the trophozoite stage (b) were treated with various concentrations of aminopropionaldehyde (○) *N*-(4-aminobutyl)-3-aminopropionaldehyde (□) or *NN'*-bis-(3-propionaldehyde)-1,4-diaminobutane (△). After incubation for 105 min, cultures were washed twice with growth medium and pulse-labelled with [^3H]hypoxanthine (1 μCi /well, mature and young parasites for 2 and 12 h respectively). Results are expressed as the ratio of the mean incorporations in the presence or absence of aminoaldehydes.

effect. Moreover, parasites cultured in G6PD-deficient erythrocytes were more sensitive than those grown in normal erythrocytes.

Treatment of parasitized erythrocytes with the oxidation products of the aldehydes, namely putrescine, β -alanine and spermic acid, had no effect on parasite growth, even at concentrations of 3 mM; spermine, spermidine and putrescine were also without effect (results not shown).

Pretreatment of erythrocytes with the dialdehyde before the addition of parasites revealed another difference between the G6PD-deficient and normal erythrocytes: the pretreatment exerted an irreversible change in the erythrocyte, preventing further invasion by

Fig. 2. Effect of aminoaldehydes on parasite maturation.

Experimental conditions were exactly those described in the legend to Fig. 1, except that, 13 h after treatment of cultures (at the ring-form stage) with the aminoaldehydes, triplicate thin blood films were prepared and stained with Giemsa stain. The percentage of ring forms (■) or trophozoites (□) was determined by counting 500 parasitized erythrocytes. (a) Zero time; (b) after 13 h.

merozoites (Table 2). The other two aldehydes had no effect.

DISCUSSION

Polyamine oxidation leads to the formation of products toxic to different parasites: *Trypanosoma lewisi* and *T. musculi* (Ferrante *et al.*, 1984), *Babesia microti* and *B. rodhaini*, *P. yoelii* (Morgan & Christensen, 1983) and *P. falciparum* (Ferrante *et al.*, 1983; Rzepczyk *et al.*, 1984). Aldehydes, H_2O_2 , free radicals and NH_3 are all products of polyamine oxidation. The role of the aldehydes in plasmodium destruction was shown indirectly by the addition of catalase and the free-radical scavenger superoxide dismutase, which did not decrease the toxicity (Rzepczyk *et al.*, 1984). These workers also suggested that NH_3 should not be considered as an anti-plasmodial agent, since the addition of this compound had only a marginal effect.

The results presented here show that the aminoaldehydes used, which are the products formed by enzymic oxidation of spermine or spermidine by either bovine or human polyamine oxidase, are indeed toxic to malaria parasites within the erythrocyte in the absence of H_2O_2 or NH_3 . A similar effect of purified aminodialdehydes on bacteria has been reported (Bachrach & Rosenkranz, 1969). There was a significant effect on young ring forms, and maturation of the parasite increased the sensitivity to all aldehydes. Further oxidation of the aminoaldehydes to the corresponding acids prevented the lethal effect. It cannot be decided at this time whether the effect is directed against the parasite or mediated through some changes in the erythrocyte, i.e. in membrane components.

Table 1. Comparison of ID₅₀ values for parasite growth in normal and G6PD-deficient erythrocytes after treatment with the different aminoaldehydes

Experimental conditions are those described in legend to Fig. 2. Results are means for two to three different experiments. Concentrations of aldehydes are expressed as μM -aldehyde; since oxidation of spermine produces a dialdehyde, the molar concentration of the active species will be half that given here.

Erythrocytes ... Parasites ...	ID ₅₀ (μM)		
	Normal		G6PD-deficient
	Ring form	Trophozoite	Trophozoite
Aminopropionaldehyde	851	331	295
Spermidine monoaldehyde	692	223	135
Spermine dialdehyde	1259	244	158

Fig. 3. Effect of aminoaldehydes on the incorporation of [³H]hypoxanthine by parasites in normal and G6PD-deficient erythrocytes

Parasites were cultured synchronously in normal (a) or G6PD-deficient (b) erythrocytes and treated with the various aminoaldehydes at the trophozoite stage. For details, see the legend to Fig. 1.

Table 2. Invasion of normal and G6PD-deficient erythrocytes by parasites after pretreatment with the dialdehyde resulting from spermine oxidation

Normal and G6PD-deficient erythrocytes (4×10^8 cells/ml) were incubated in growth medium containing the aminoaldehyde for 2 h at 37 °C with gentle agitation. Thereafter cells were washed twice with normal growth medium and an inoculum of enriched parasitized erythrocytes was added and cultures, in triplicate, were incubated for an additional 18 h.

[Dialdehyde] (mM)	Percentage parasitaemia 18 h after infection	
	Normal	G6PD-deficient
3	4.2	0.5
0.6	5.9	5.3
0 (PBS control)	4.8	5.9

G6PD-deficient parasitized erythrocytes are extremely sensitive target cells for examining immunological and physiological mechanisms affecting the parasites (Allison & Eugui, 1983). Plasmodia within G6PD-deficient erythrocytes were more sensitive to all the aldehydes than were plasmodia in normal cells, as shown by inhibition of tritiated-hypoxanthine incorporation and reduced parasitaemias. G6PD-deficient erythrocytes were also more sensitive to pretreatment with the dialdehyde produced by the oxidation of spermine. The pretreatment exerted a non-reversible change in the erythrocyte, preventing further invasion by malaria merozoites.

It is not surprising that the dialdehyde rather than the monoaldehydes was the more efficient in interfering with invasion. Dialdehydes have two reactive groups and may cross-link proteins in the membrane of the erythrocyte, thus preventing the deformation needed for successful penetration (Pasvol & Wilson, 1982).

The overall results indicate that there are (at least) two separate mechanisms by which aminoaldehydes may affect the fate of the parasite: the first is interference with invasion and the second is by affecting intracellular development. As suggested by Morgan & Christensen

(1983), this may give a clue to general processes of non-specific resistance against malaria.

It has long been suggested that G6PD deficiency confers some degree of protection against infection with malaria parasites (Allison, 1960; Motulsky, 1960). Our experiments with the aminoaldehyde products of polyamine oxidation provide another indication of the advantage of G6PD-deficient individuals in this respect.

This study was supported by research contract no. NO1-A1 22668-HIH-NIAID Epidemiology and Control of Arthropod Borne Diseases. D.M.L.M. acknowledges support from the Royal Society-Israel Academy Program.

REFERENCES

- Allison, A. C. (1960) *Nature* (London) **185**, 531–532
- Allison, A. C. & Eugui, E. M. (1983) *Annu. Rev. Immunol.* **1**, 361–392
- Assaraf, Y. G., Golenser, J., Spira, D. T. & Bachrach, U. (1984) *Biochem. J.* **222**, 815–819
- Bachrach, U. (1970) *Annu. Rev. Microbiol.* **24**, 109–134
- Bachrach, U. (1974) in *Methods of Enzymatic Analysis*, vol. 4. (Bergmeyer, H. U., ed.), pp. 1744–1748, Verlag-Chemie, Weinheim
- Bachrach, U. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 156–168, Dekker, New York
- Bachrach, U. & Reches, B. (1966) *Anal. Biochem.* **17**, 38–48
- Bachrach, U. & Rosenkranz, H. S. (1969) *Biochem. Pharmacol.* **18**, 243–246
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Ferrante, A., Rzepczyk, C. & Allison, A. C. (1983) *Trans. R. Soc. Trop. Med. Hyg.* **77**, 789–791
- Ferrante, A., Rzepczyk, C. & Saul, A. (1984) *J. Immunol.* **133**, 2157–2162
- Golenser, J., Miller, J., Spira, D. T., Navok, T. & Chevion, M. (1983) *Blood* **61**, 507–510
- Hölttä, E. (1977) *Biochemistry* **16**, 91–100
- Jensen, J. B. (1978) *Am. J. Trop. Med. Hyg.* **27**, 1274–1276
- Jensen, J. B., Boland, M. T. & Hayes, M. (1982) *Exp. Parasitol.* **54**, 416–424
- Kimes, B. W. & Morris, D. R. (1971) *Biochim. Biophys. Acta* **228**, 223–234
- Lambros, C. & Vandenberg, J. (1979) *J. Parasitol.* **65**, 418–420
- Miller, J., Golenser, J., Spira, D. T. & Kosower, N. S. (1984) *Exp. Parasitol.* **57**, 239–247
- Morgan, D. M. L. (1985a) *Biochem. Soc. Trans.* **13**, 322–326
- Morgan, D. M. L. (1985b) *Biochem. Soc. Trans.* **13**, 351–352
- Morgan, D. M. L. & Christensen, J. R. (1983) *Adv. Polyamine Res.* **4**, 169–174
- Morgan, D. M. L., Christensen, J. R. & Allison, A. C. (1981) *Biochem. Soc. Trans.* **9**, 563–564
- Motulsky, A. G. (1960) *Hum. Biol.* **32**, 28–63
- Pasvol, M. B. & Wilson, R. J. M. (1982) *Br. Med. Bull.* **38**, 133–140
- Rzepczyk, C. M., Saul, A. J. & Ferrante, A. (1984) *Infect. Immun.* **43**, 238–244
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954) *J. Biol. Chem.* **208**, 645–661
- Tabor, C. W., Tabor, H. & Bachrach, U. (1964) *J. Biol. Chem.* **239**, 2194–2203
- Trager, W. & Jensen, J. B. (1976) *Science* **193**, 673–675

Received 13 December 1985/3 February 1986; accepted 20 February 1986