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Antagonism by polyamines of the curative effects of α-difluoromethylornithine in *Trypanosoma brucei brucei* infections

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Polyamines are aliphatic polycations thought to be important in cell division and nucleic acid and protein synthesis [1, 2]. The commonly occurring polyamines are putrescine (1,4-diaminobutane), spermidine (*N*-[4-aminobutyl]-1,4-diaminobutane), and spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-diaminobutane). All three are usually found in eukaryotic cells, but only putrescine and spermidine in prokaryotes [3, 4]. The cellular content of these amines, especially putrescine, increases in dividing cells and decreases in non-proliferating populations [5]. Synthesis of putrescine in most eukaryotic cells is from ornithine, catalyzed by ornithine decarboxylase (ODC; EC 4.1.1.17), the main rate-limiting enzyme of polyamine biosynthesis. Activity of this enzyme is closely regulated by transcriptional, translational, and post-translational mechanisms [6–8].

The pathogenicity of some hemoflagellate protozoa and the lack of effective non-toxic drugs have fostered in-depth study of their metabolism as an avenue for novel approaches to chemotherapy. In this regard, polyamine metabolism has been investigated in a number of recent studies [9–13]. One important hemoflagellate—*Trypanosoma brucei brucei*, an agent of African sleeping sickness in cattle—is susceptible to attack by a specific, enzyme-activated ornithine decarboxylase inhibitor, DL-α-difluormethylornithine (α-DFMO). α-DFMO, which binds irreversibly to ODC, cures *T. b. brucei* infected mice when administered in drinking water [14]. It was also demonstrated that the drug inhibits ODC activity in bloodstream and culture forms of *T. b. brucei*.

In previous work we have reported the ability of polyamines to reverse *in vivo* the trypanocidal activity of the anti-protozoal babesicides, amicarbalide and imidocarb [15, 16] and the known anti-tumor agents, the bleomycins [17]. Though the mechanisms of these reversals are unknown, amicarbalide and imidocarb have a noticeable structural resemblance to the natural polyamines [16] while the bleomycins contain amines as part of their active site [17]. In a continuing effort to study the antagonism of certain drugs by polyamines, we expanded this study to the highly specific inhibitor of polyamine synthesis, α-DFMO. Polyamines have been shown previously to reverse the effects of α-methylornithine and α-DFMO on the replication of mammalian cells in culture [18, 19]. We describe here the effects of *in vivo* polyamine administration on the progress of *T. b. brucei* infection in α-DFMO-treated mice.

Materials and methods

Organism. The EATRO 110 isolate of *T. b. brucei* has been rodent-passaged in this laboratory for several years and its management has been detailed [10, 14, 16]. The organism produces a rapidly fatal (3–6 days) parasitemia in rats and mice.

Materials. α-DFMO (RMI 71,782) was synthesized by the Merrell Research Center, Cincinnati, OH. Polyamines and diamines were obtained from the Sigma Chemical Co., St. Louis, MO.

Inoculum and drug administration. Experiments were essentially as described previously [14, 16, 20]. Batches of fifty Swiss–Webster mice (20–25 g, mixed males and females) were inoculated with 5×10^5 trypanosomes from a 72-hr rat infection; 24 hr after inoculation, animals were randomly separated into groups of five mice and treatment was begun. α-DFMO was administered as a 1% or 2% solution in drinking water; animals were treated with the drug solution for 3 days. The total dose consumed per mouse was estimated at 150 or 300 mg based on intake of 5 ml of water per day of a 1% or 2% solution. Polyamines were administered as a single i.p. dose (in aqueous) daily for 3 days concurrent with α-DFMO treatment. Control groups receiving polyamines alone were also included.

Results are expressed as average survival time (days) beyond death of control animals. Cages were checked daily at approximately the same time of day. Blood for trypan-

Table 1. Effects of α-DFMO and polyamines on *T. b. brucei* infections in mice*

Polyamines	mg/kg	Average survival in days	
		1% α-DFMO	2% α-DFMO
None		>30.0 (10)	>30.0 (15)
Spermine·4 HCl	50	2.0 (2)	7.7 (6)
Spermidine·3 HCl	100		14.1 (6)
Putrescine·2 HCl	300	10.2 (2)	
Putrescine·2 HCl	500	12.2 (2)	13.4 (6)
Cadaverine·2 HCl	500		>30.0 (3)
1,3-Diaminopropane·2 HCl	500		>30.0 (3)

* α-DFMO was administered in the drinking water for 3 days, beginning 24 hr post-infection. Polyamines were given to groups of five animals once daily by i.p. injection (volume 0.2 ml) concurrently with α-DFMO administration. Polyamines given alone to animals did not alter their survival after infection as compared to untreated animals. Results are expressed as average survival in days beyond detah of controls. The number of experiments is given in parentheses. Other details are given in Materials and Methods.

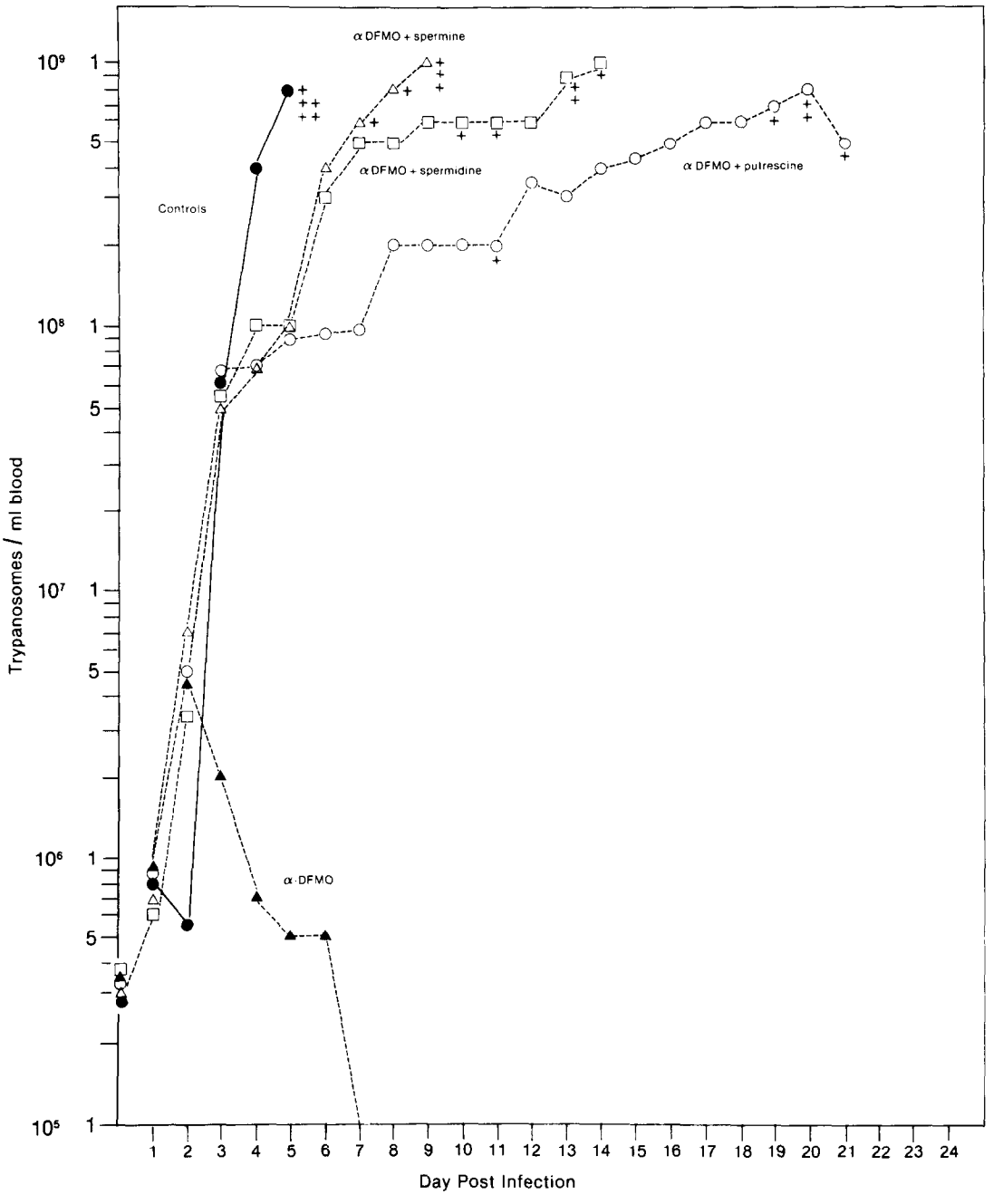

Fig. 1. Effect of 2% α-DFMO and polyamines on parasite levels in blood. Results of a single experiment. Mice (five in each group) were infected on day 0 with 5×10^5 trypanosomes and treatment was begun 24 hr post-infection. There were no parasites in α-DFMO-treated animals after day 7. A (+) sign indicates day of death of individual animals. Polyamines and α-DFMO were administered as indicated in Table 1. Key: (●) controls, (▲) α-DFMO alone, (△) α-DFMO + spermine (50 mg/kg), (□) α-DFMO + spermidine (100 mg/kg), and (○) α-DFMO + putrescine (500 mg/kg).

osome counts was taken from tail vein punctures using B-D Unopettes (Becton-Dickinson No. 5855). Cells were counted with a Neubauer improved hemocytometer.

Results

One or two percent α-DFMO, administered in the drinking water for 3 days, cured *T. b. brucei* infected mice (Table 1). Animals were scored as "cured" upon survival of more than 30 days beyond controls, with no parasites detected in peripheral blood smears. The polyamines putrescine, spermidine, and spermine, administered once daily concurrently with α-DFMO, reversed the action of the drug and caused the death of animals so treated (Table 1). Polyamines, administered alone to infected animals, did

not affect the usual lethal course of infection and the animals died at the same time as controls.

The diamines 1,3-diaminopropane and cadaverine (1,5-diaminopentane) are not normally present in eukaryotic cells but, if so, occur in much lower concentration than putrescine, spermidine, and spermine that are synthesized in a common pathway [1]. Therefore, to test whether the reversal of α-DFMO action was attributable simply to the multiple amine groups of any di- or polyamines, we coadministered these two putrescine homologs to infected α-DFMO-treated animals. Cadaverine and 1,3-diaminopropane did not interfere with cure by α-DFMO (Table 1).

As a check on progress of the infection in animals receiving polyamines together with α-DFMO, parasitemia levels were monitored throughout an experiment by taking daily cell counts (Fig. 1). Some initial parasite cell division also occurred; however, with 2% α-DFMO treatment, infections became undetectable by 7 days post-inoculation. Animals receiving α-DFMO together with polyamines had parasitemias increasing at a rate nearly comparable to controls. This was followed by a somewhat protracted but intense infection and death of the animals at parasitemias again comparable to that of the control untreated animals. This was particularly evident in animals given spermine with α-DFMO (Fig. 1). The reason for the somewhat different life-spans of animals treated with different polyamines is not known but pertinent variables might include differences in uptake of polyamines by the parasites and in rates of their catabolism and excretion by the host. Alhonen-Hongisto *et al.* [21] have found recently that transport of putrescine and spermidine into Ehrlich ascites-carcinoma cells is greatly enhanced by treatment with α-DFMO.

Discussion

These results demonstrate the efficacy of α-DFMO in inhibiting polyamine metabolism *in vivo* in African trypanosomes and, moreover, that this target in the parasites is clearly an extremely sensitive one. As noted previously, the compound was a potent inhibitor of ODC activity and of putrescine formation in both bloodstream and culture trypomastigotes of *T. b. brucei* [14]. Other studies involving standard cationic trypanocides and babesicides, e.g. diamidines and quinapyramine (Antrycide), also strongly suggest that polyamines are potential targets since the curative action of these compounds against *T. b. brucei* is reversible by spermidine or spermine although not by putrescine [15, 16]. Similar effects were obtained in studies with the antitumor bleomycins in which spermidine and spermine annulled cures by commercial Blenoxane (a mixture of five bleomycins) as well as the purified A_2 and B_2 congeners [17]. The results with the cationic trypanocides and the bleomycins differed from this presently described reversal of α-DFMO action in that putrescine as well as spermidine and spermine reversed α-DFMO cures. A fundamental difference in the action of α-DFMO and the other trypanocides thereupon emerges for, unlike α-DFMO, neither the cationic trypanocides nor the bleomycins seem to directly inhibit polyamine biosynthesis [16; C. Bacchi and J. Garofalo, unpublished observations], and may act by displacing polyamines *in situ* in the cell.

Since α-DFMO is a potent blocking agent of polyamine biosynthesis, its potential chemotherapeutic use includes disorders characterized by rapid cell proliferation, e.g. certain solid tumors, leukemias, and psoriasis [22–24]. Use of the drug against parasitic protozoa, therefore, seems a practical possibility, especially since, as in the present study, the division time of many parasites is less than 8 hr. Thus, it was also recently found that α-DFMO was highly effective against the protozoan parasite *Eimeria tenella*, an agent of coccidiosis in poultry and against division *in vitro* of the malaria parasite *Plasmodium falciparum* [25].

Intensification of studies on the synthesis and function of polyamines in protozoan parasites with a view towards developing novel chemotherapeutic regimens thus seems justified. Since α-DFMO, the cationic trypanocides, babesicides, and bleomycins apparently act on different aspects of trypanosomatid polyamine metabolism, examination of their activity in combination therapy is a logical expression of this idea: combination of drugs having interconnected target sites may prove synergistic and thereby allow reduction in dosage of agents such as cationic trypanocides which by themselves have poor therapeutic indices.

In this work we have demonstrated that a specific antagonist of polyamine metabolism, α-DFMO, which cures a virulent *T. b. brucei* infection in mice can be annulled by coadministration of the polyamine precursor putrescine, or the polyamines spermidine or spermine. These effects were demonstrable in infected α-DFMO-treated animals by persistence of the infection and death of the animals with parasitemias similar to that of untreated controls. Although the functions of polyamines in trypanosomes are not clear, this study points to polyamine metabolism as a sensitive and specific target for polyamine antagonists in these parasites.

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Interaction of histamine with specific membrane receptors on gastric mucosal cells*

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Histamine stimulates the secretion of acid and the formation of cyclic AMP in gastric mucosa of several species [1, 2], and it increases both cyclic AMP and [^{14}C]aminopyrine uptake in isolated gastric cells from guinea pig, dog and rat [3–10]. It also stimulates adenylate cyclase activity in guinea pig gastric mucosal membranes [11]. Direct studies of histamine with biologically active H_2-receptors in gastric cells have been hindered by the lack of suitable isolated cell system. To investigate further the biological significance of histamine–receptor interaction *in vitro*, it is desirable to study simultaneously the biological response of an intact cell system to histamine under conditions wherein direct binding to the receptors on intact cells could be determined.

In the present work we used our recently developed technique for isolating gastric mucosal cells from guinea pig stomach to study the kinetics, stoichiometry, and specificity with which [^{3}H]histamine interacts with H_2-receptors on mucosal cells.

Dispersed gastric cells were prepared as previously described [12]. The cells were suspended in standard solution containing Hanks' buffer (GIBCO) plus 15 mM Hepes [4-(2-hydropyethyl)-1-piperazine-ethanesulfonic acid], pH 7.2 [12]. Binding of histamine (7–10 Ci/mmole, New England Nuclear Corp., Boston, MA) was determined by our centrifugation technique as previously described [5]. Gastric cells (2–4 × 10^6 cells) were incubated in 0.5 ml standard solution containing 0.1 to 0.25 μCi [^{3}H]histamine. The cells were washed twice with 20 vol. of iced standard solution, suspended in 1% (v/v) Triton X-100, and dispersed in Aquasol. To determine specific binding of [^{3}H]histamine, 1 mM histamine was added to parallel incubations. The cell-associated radioactivity in these incubations was subtracted from the total observed binding to obtain specific binding. Binding of [^{3}H]histamine was a linear function of cell concentration from 1 to 15 million cells/ml. Cellular cyclic AMP and uptake of [^{14}C]aminopyrine (18 mCi/mmole, New England Nuclear) were determined as described in detail elsewhere [3, 5].

Binding of [^{3}H]histamine to gastric cells at 37° was moderately rapid ($T_{\frac{1}{2}}$, 14 min) and reached steady state by 40–50 min of incubation (Fig. 1A). Adding unlabeled histamine (1 mM) to the incubation solution reduced tracer binding by 85 per cent, indicating that binding of [^{3}H]histamine occurred to a finite number of receptors which could be saturated at a high concentration of histamine. Reducing the incubation temperature from 37 to 4° decreased [^{3}H]histamine binding by 94 per cent (Fig. 1A), as seen in other histamine-receptor binding studies [13]. To examine the reversibility of the binding process, cells were incubated with [^{3}H]histamine at 37° for 60 min, washed to remove free radioactivity, and resuspended in fresh standard solution containing no radioactivity. The loss of bound [^{3}H]histamine followed a first-order process with a dissociation rate coefficient of 0.04 min^{-1} (Fig. 1B and insert). Adding 1 mM histamine to the cells did not change the rate of dissociation, suggesting the absence of

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