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Effects of α-difluoromethylornithine on protein synthesis and synthesis of the variant-specific glycoprotein (VSG) in *Trypanosoma brucei brucei*

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Protein synthesis in *Trypanosoma brucei brucei* was rapidly inhibited during polyamine depletion by DL-α-difluoromethylornithine (DFMO) *in vitro* and *in vivo*. [^{3}H]Leucine incorporation was depressed 30–40% by 24 h and 80–90% by 48 h of DFMO treatment. Concomitantly there was an apparent decrease in the synthesis of the variant-specific glycoprotein (VSG) in DFMO-treated trypanosomes, as measured by decreased incorporation of [^{3}H]myristic acid into VSG. The discovery of decreased protein synthesis in *T. b. brucei* during DFMO treatment is noteworthy, because it was reported previously that protein synthesis was paradoxically stimulated 2–4-fold during DFMO treatment in these organisms. Decreased protein synthesis probably relates to the biochemical mechanism of action of DFMO on trypanosomes.

INTRODUCTION

Administration of α-difluoromethylornithine (DFMO, eflornithine hydrochloride) effects cures of African trypanosomiasis in both experimental murine infections (Bacchi *et al.*, 1980) and human sleeping sickness (Schechter & Sjoerdsma, 1986; McCann *et al.*, 1986). Since it is a catalytic irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978), DFMO rapidly depletes the intracellular polyamines putrescine and spermidine in *Trypanosoma brucei brucei* (trypansomes do not contain detectable spermine) and inhibits trypanosome DNA synthesis and proliferation (Bacchi *et al.*, 1983), as it does in a number of rapidly proliferating cells (Pegg & McCann, 1982).

One of the earliest effects ascribed to DFMO in mammalian cells is an inhibition of protein synthesis, as evidenced by a change in leucine incorporation as well as a perturbation in polyribosome formation (Rudkin *et al.*, 1984). These changes parallel the depletion of spermidine caused by DFMO and are readily prevented by the presence of putrescine or spermidine in addition to DFMO, i.e. by-passing the enzymic block of ornithine decarboxylase. The occurrence of a similar rapid decrease in protein synthesis has not been described in trypanosomes. In fact, it was reported previously (Bacchi *et al.*, 1983) that there is a paradoxical 2–4-fold stimulation of protein synthesis in trypansomes during DFMO treatment. A negative effect of DFMO on protein synthesis in trypanosomes would be interesting, not only because of the general depression of metabolic activity that could ensue, but also because of potential effects on the synthesis of the variant-specific glycoprotein (VSG) which completely coats the trypanosome's plasma membrane and protects it from immune attack (Vickerman, 1969).

The major immune response to trypanosomes is an antibody response directed towards the VSG (Vickerman, 1978; Boothroyd, 1985). Trypanosomes normally undergo antigenic variation by switching their VSG coat, thus avoiding the host immune response (Vickerman, 1978; Boothroyd, 1985). However, if synthesis of new VSG was inhibited either specifically or concurrent with a general decrease in protein synthesis, then the trypanosome could not evade the immune system and parasites would presumably be eliminated. We showed previously that the immune response to trypanosomes is a necessary component of DFMO-induced cures in rats and mice (Bitonti *et al.*, 1986; deGee *et al.*, 1983). The latter finding is reasonable, since DFMO is regarded as a cytostatic rather than a cytotoxic agent. Thus it is possible that, by limiting protein synthesis (VSG synthesis), DFMO allows the host's immune response to the trypanosome effectively to remove the parasites from the blood.

We now show that DFMO does, in fact, inhibit protein synthesis in *T. b. brucei* and also apparently inhibits the synthesis of the VSG coat. It is proposed that the inhibition of protein synthesis contributes to the therapeutic effect of DFMO in trypanosomiasis.

EXPERIMENTAL

Chemicals

[9,10-^{3}H]Myristic acid (22.4 Ci/mmol) and [3,4,5-^{3}H]leucine (141 Ci/mmol) were purchased from New England Nuclear; putrescine, spermidine and bovine serum albumin (BSA) were from Sigma. DFMO was synthesized as described previously (Metcalf *et al.*, 1978).

Organisms

Trypanosoma brucei brucei (EATRO 110) was harvested from the blood of 72 h-infected rats by using DEAE-cellulose chromatography (Lanham & Godfrey, 1970). Columns were equilibrated and eluted and trypanosomes were washed with 90 mM-Tris/HCl (pH 7.8) containing 50 mM-NaCl and 2% (w/v) glucose (TSG).

Abbreviations used: DFMO, DL-α-difluoromethylornithine; VSG, variant-specific glycoprotein; BSA, bovine serum albumin; PAGE, polyacrylamide-gel electrophoresis.

Culture of trypanosomes

Bloodstream-form trypomastigotes were cultivated essentially as described previously (Brun *et al.*, 1981). *T. b. brucei* was obtained from rats as described above by aseptic cardiac puncture. Trypanosomes were separated from blood cells by differential centrifugation, washed three times with culture medium consisting of Eagle's minimal essential medium supplemented with 30 mM-Hepes, pH 7.4, non-essential amino acids, 2 mM-glutamine, 20% (v/v) fetal bovine serum, 2% (w/v) glucose and penicillin/streptomycin, and then used to inoculate culture flasks containing a feeder layer of embryonic bovine tracheal cells (EBTr; ATCC CCL-44) in the above medium. Cultures of EBTr cells were inoculated with 1×10^5 trypanosomes/ml, and these multiplied to approx. 1×10^6 trypanosomes/ml in 24 h, except in the DFMO-treated cultures, in which cell division slowed and then stopped within 24 h. Each day some cultures were harvested for incubation with radioactive substrates, and other cultures were refed and diluted and kept for labelling experiments on succeeding days.

To obtain polyamine depletion *in vitro*, 100 μM-DFMO was added to trypanosome cultures. This concentration of DFMO has been shown to give maximal polyamine depletion in 24 h (Giffin *et al.*, 1986) and also represents a rough approximation of an average plasma concentration obtained in rats after overnight administration of DFMO in drinking water (Fairlamb *et al.*, 1987).

Biosynthetic labelling of VSG

Biosynthetic labelling of the membrane-form VSG followed closely the procedure described by Ferguson & Cross (1984). Trypanosomes were sedimented from culture medium by centrifugation at 600 *g* for 10 min or were purified from rat blood on DEAE-cellulose as described above. The cells were washed twice with TSG, once with RPMI 1640 medium containing 25 mM-Hepes (pH 7.4) and 1 mg of defatted BSA/ml, and counted with a haemocytometer. Trypanosomes (2×10^7) were then incubated at 37 °C in 0.5 ml of RPMI 1640 medium containing 50 μCi of [^{3}H]myristic acid complexed to defatted BSA and 1 mg of defatted BSA/ml. After 60 min the trypanosomes were washed twice with TSG and then immediately boiled in a solution of 2% (w/v) SDS, 100 mM-dithiothreitol, 10% (v/v) glycerol, 62.5 mM-Tris/HCl (pH 6.8) and 0.005% Bromophenol Blue in preparation for gel electrophoresis.

SDS/PAGE

Radioactively labelled proteins were analysed by SDS/PAGE by the method of Laemmli (1970) on 10%-acrylamide gels. After electrophoresis, proteins were stained with Coomassie Blue to be certain that similar amounts of protein were present in each trypanosome sample. Radioactive proteins were then observed by impregnation of the gels with EN^{3}HANCE (New England Nuclear) and exposure of dried gels to Kodak X-OMAT AR film at −70 °C.

[^{3}H]Leucine incorporation

In another set of experiments, 4×10^6 trypanosomes, which were isolated from EBTr cultures of separated from rat blood, were washed twice in leucine- and isoleucine-free RPMI 1640 medium (Selectamine Kit; Gibco) containing 20% fetal bovine serum, and then mixed in microtitre wells with 5 μCi of [^{3}H]leucine in a total volume of 200 μl/well. Trypanosomes were incubated for 60 min at 37 °C and the incorporation of [^{3}H]leucine into proteins was determined by using a multiple automatic cell harvester as described previously (Bitonti *et al.*, 1985).

In a previous study (Bacchi *et al.*, 1983), a simple phosphate-buffered salts/glucose/BSA solution was used for [^{3}H]leucine incorporation experiments with *T. brucei*. Comparison of this simple medium with leucine-free RPMI 1640 medium + fetal bovine serum showed that the latter complex medium yielded data more consistent with the specific incorporation of [^{3}H]leucine into protein. [^{3}H]Leucine incorporation conducted in the more complex medium showed more sensitive responses to known inhibitors of protein synthesis such as cycloheximide, puromycin and several specific inhibitors of peptidyltransferase (Bitonti *et al.*, 1985). Therefore the experiments presented herein were conducted in the more complex medium.

Table 1. [^{3}H]Leucine incorporation in *T. brucei* treated *in vivo* and *in vitro* with DFMO

T. b. brucei was grown in rats (*a*, *b*) or in tissue culture (*c*) as described in the Experimental section. In each case, the trypanosomes were washed twice in leucine-free RPMI 1640 medium and then incubated for 60 min in the presence of 5 μCi of [^{3}H]leucine at a cell density of 2×10^7/ml.

	Treatment time	[^{3}H]Leucine incorporation (c.p.m./4×10^6 trypanosomes) (±S.E.M.)	(% of control)
a	0 h	87524±3663	100
	24 h	54706±15724	63
	48 h	9798±1932	11
b	0 h	75280±10105	100
	24 h	52591±5524	70
	48 h	13862±293	18
c	24 h, control	52197±4266	100
	24 h, DFMO	17056±1025	33
	48 h, control	52815±600	100
	48 h, DFMO	17134±1335	32

RESULTS

Trypanosomes isolated from rats treated with 2% DFMO in drinking water were markedly deficient in their ability to incorporate [^{3}H]leucine into protein (Table 1, parts *a* and *b*). After exposure to DFMO for 1 day, [^{3}H]leucine incorporation was decreased by 30–40%, whereas 2 days' exposure to DFMO resulted in a decrease in incorporation of 80–90%. A similar decrease in protein synthesis was noted in trypanosomes which were exposed to 100 μM-DFMO for 24 h or 48 h *in vitro* (Table 1, part *c*). In the latter case, however, maximal inhibition was attained by 24 h and protein synthesis did not apparently decrease any further during 48 h of DFMO exposure. The decrease in protein synthesis at 48 h could not be reversed by adding putrescine (100 μM)

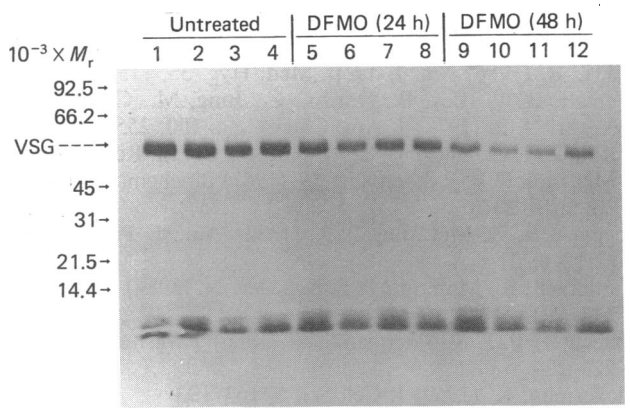

Fig. 1. Effects of DFMO treatment *in vivo* on [^{3}H]myristic acid incorporation into VSG

[^{3}H]Myristic acid incorporation was carried out as described in the Experimental section with trypanosomes obtained from untreated rats (lanes 1–4) or trypanosomes exposed to DFMO *in vivo* for either 24 h (lanes 5–8) or 48 h (lanes 9–12). Radioactive proteins were separated by SDS/PAGE. Protein markers and their M_r values were: phosphorylase *b*, 92500; BSA, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soya-bean trypsin inhibitor, 21500; lysozyme, 14400.

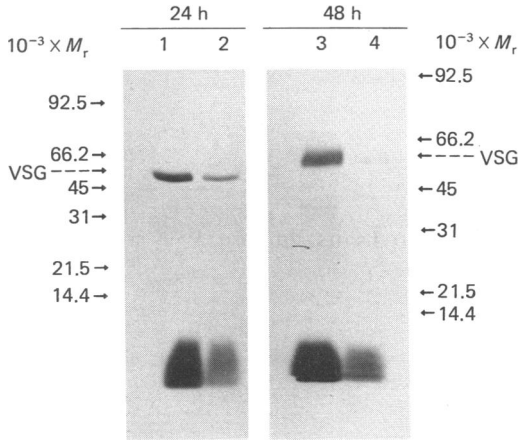

Fig. 2. Effects of DFMO treatment *in vitro* on [^{3}H]myristic acid incorporation into VSG

[^{3}H]Myristic acid incorporation was carried out as described in the Methods section with trypanosomes from EBTr-cell cultures containing no DFMO (lanes 1 and 3) or 100 μM-DFMO for 24 h (lane 2) or 48 h (lane 4). Radioactive proteins were separated by SDS/PAGE. Protein markers and their M_r values are the same as those described in the legend for Fig. 1.

or spermidine (100 μM) to incubations of DFMO-treated trypanosomes plus [^{3}H]leucine (results not shown).

The effects of DFMO on the synthesis of VSG were also examined under the same conditions as for general protein synthesis. By SDS/PAGE, the incorporation of [^{3}H]myristic acid into VSG was shown to be markedly inhibited in trypanosomes harvested from rats which had been treated for 24 h with DFMO (Fig. 1). Treatment with DFMO for 48 h resulted in a further decline of [^{3}H]myristic acid incorporation into VSG. Similar results were obtained if VSG synthesis in trypanosomes (bloodstream trypomastigotes) grown in culture was measured. DFMO (100 μM) exposure decreased VSG synthesis at both 24 h and 48 h (Fig. 2).

DISCUSSION

The data presented herein show that a consequence of exposure of trypanosomes to DFMO either *in vivo* or *in vitro* is a decrease in general protein synthesis as well as a decreased rate of synthesis of VSG. It was reported previously that DFMO treatment resulted in a paradoxical stimulation of protein synthesis at times when DNA and RNA syntheses were markedly depressed (Bacchi *et al.*, 1983). Our results here more closely parallel the finding in mammalian cells, in which inhibition of protein synthesis is an early consequence of DFMO treatment (Rudkin *et al.*, 1984).

The finding that we could not reverse the effects of DFMO on protein synthesis by addition of either 100 μM-putrescine or 100 μM-spermidine to DFMO-inhibited trypanosomes may suggest that, as in the study with HTC cells (Rudkin *et al.* 1984), there may be changes in the make-up of the polyribosomes in the trypanosome, owing to polyamine depletion, which are not rapidly reversible by polyamines. In fact, the previous study (Rudkin *et al.*, 1984) showed that a finite amount of time (60 min) was required for reversal by polyamines of the disruptive effects on polyribosomes caused by prior DFMO-induced polyamine depletion. It is possible that a DFMO-induced change in protein synthesis in *T. brucei* is more resistant to reversal by polyamines than are the similar changes in mammalian cells and did not occur during the 60 min incubations used herein. However, as has been shown previously, polyamines will reverse growth-inhibition and morphological effects of DFMO (Nathan *et al.*, 1981; Giffin *et al.*, 1986) when the trypanosomes are exposed simultaneously to the polyamines and DFMO either *in vivo* or *in vitro*. No-one has attempted previously to reverse acutely the effects of DFMO on trypanosomes.

We have also shown that incorporation of [^{3}H]myristic acid into VSG is inhibited by DFMO. This may indicate that synthesis of the glycoprotein coat is slowed, as might be expected when general protein synthesis is inhibited. Inhibition of VSG synthesis may be related to the overall effect of DFMO on the trypansome *in vivo*, inasmuch as it is known that the VSG is the major antigenic determinant of the trypanosome against which host antibodies respond (Vickerman, 1969, 1978; Boothroyd, 1985), and antibody responses are necessary for DFMO-induced cures of trypanosomiasis (Bitonti *et al.*, 1986; deGee *et al.*, 1983). Slowing of VSG synthesis would seemingly allow a better antibody response to be mounted against the trypanosome, thus rendering DFMO more effective in curing a parasitic infection.

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