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Impairment of Macrophage Function by Inhibitors of Ornithine Decarboxylase Activity

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The effects of irreversible inhibition of ornithine decarboxylase on the capacity of murine macrophages to take up a protozoan organism (*Trypanosoma cruzi*) or inert particles were investigated. Incubation of macrophage cultures with four different ornithine decarboxylase inhibitors, namely, DL-α-difluoromethylornithine (DFMO, 0.5 to 20 mM), Δ-methyl-acetylenic putrescine (1 to 5 mM), monofluoromethyldehydroornithine ethyl ester (1 to 5 mM), and monofluoromethyldehydroornithine methyl ester (1 to 5 mM), before the addition of the parasites significantly reduced the percentage of macrophages with parasites, indicating that some of the host cells were no longer capable of binding or ingesting the parasite. The average number of trypanosomes per 100 macrophages was also diminished, denoting a lesser phagocytic capacity as a consequence of the treatments. These effects were reversible within 2 h after removal of excess DFMO. No alteration in parasite-macrophage interaction was seen when the trypanosomes were treated with DFMO. That the effects of DFMO on the macrophages probably resulted from a reduction in polyamine levels caused by inhibition of ornithine decarboxylase was indicated by the fact that these effects were not seen when the macrophages were incubated with DFMO in the presence of putrescine, the product of ornithine decarboxylation by ornithine decarboxylase. DFMO treatment of macrophages also inhibited the capacity of these cells to ingest killed parasites or latex beads and thus appeared to generally affect phagocytosis. An effect of DFMO on the susceptibility of macrophages to penetration by the parasites seemed less likely because no significant alteration in cell-parasite association occurred when myoblasts—which, not being phagocytic, can be infected only by membrane penetration—were treated with DFMO. Taken together, these results emphasize a role of ornithine decarboxylase activity and polyamine biosynthesis in macrophage function.

In the course of our studies on the role of polyamines and polyamine biosynthesis in the infectivity of *Trypanosoma cruzi*, the unicellular parasite causing Chagas' disease, we observed that α-difluoromethylornithine, a specific, irreversible inhibitor of ornithine decarboxylase (ODC) (1–3, 5, 8–10), significantly reduced the level of parasite-macrophage interaction. Interestingly, this effect was seen when DL-α-difluoromethylornithine (DFMO) was present during parasite-macrophage interaction but not when the trypanosomes were pretreated with DFMO, pointing to the macrophage as the cell affected by DFMO. Because of our limited knowledge of the role of polyamines in macrophage function (8), we decided to explore this possibility. In this paper we show that the inhibition of ODC activity in macrophages by either DFMO or other specific ODC inhibitors curtails their capacity to take up live or dead *T. cruzi* or inert particles.

MATERIALS AND METHODS

Animals. The 6- to 9-week-old inbred CBA/J mice used to obtain resident peritoneal macrophages were purchased from Jackson Laboratory (Bar Harbor, Maine). The 4-week-old Crl-CD-1(ICR)BR Swiss mice used to maintain and produce blood forms of *T. cruzi* were obtained from Charles River Laboratory (Portage, Mich.).

Mouse peritoneal macrophages. The methods for the purification of resident mouse peritoneal macrophages (referred to herein as macrophages) and for setting up cultures of these cells on the 3-mm-diameter glass wells cut on Teflon-coated microscope slides (Cel-Line, Newfield, N.J.) were described previously (11, 12). The macrophage and monocyte monolayers consisted of >99% cells with the typical morphology of these cells and positive staining for nonspecific esterase activity.

Rat heart myoblasts. Confluent monolayers of rat heart myoblasts, referred to herein as myoblasts (CRL 1446; American Type Culture Collection, Bethesda, Md.), were prepared on the gelatin-coated glass wells of Teflon-coated microscope slides (see above) with Dulbecco modified minimal essential medium containing 100 U of penicillin and 100 μg of streptomycin per ml (DMEM) and supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum.

Parasites. Blood was drawn from ether-anesthetized mice which had been infected 2 weeks previously with 2×10^5 Tulahuen strain *T. cruzi* intraperitoneally. The trypomastigotes were purified by centrifugation over Isolymph (Gallard Schlesinger, Carle Place, N.Y.) (4) followed by chromatography through a DEAE-cellulose column (6). The eluted trypomastigotes were concentrated by centrifugation (800 × *g*, 15 min, 20°C) and suspended at 3×10^6 to 5×10^6 organisms per ml in the desired medium (see Results). The parasite suspensions used in this study consisted of 100% organisms in the trypomastigote form, whose viability was >99% in terms of motility. When dead parasites were needed, trypomastigotes were suspended in a 0.01% solution of glutaraldehyde in phosphate-buffered saline (pH 7.2) and incubated at room temperature for 5 min. These parasites

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TABLE 1. Reduction of the capacity of mouse macrophages to associate with untreated blood forms of *T. cruzi* after pretreatment with DFMO[a]

DFMO concn (mM)	% of cells associated with parasites (% change)[b]	No. of parasites/100 cells (% change)[b]
0	68.7 ± 3.1	145.4 ± 12.3
0.1	68.4 ± 1.6 (−0.4)	153.4 ± 15.0 (5.5)
0.5	59.9 ± 5.6 (−12.8)	105.8 ± 8.7 (−27.2)
1.0	55.5 ± 2.6 (−19.2)	102.7 ± 2.1 (−29.4)
5.0	52.9 ± 3.4 (−22.9)	89.2 ± 5.0 (−38.7)
10.0	49.1 ± 4.1 (−28.5)	81.1 ± 5.6 (−44.2)

[a] Macrophage monolayers were incubated with the indicated concentration of DFMO for 18 h and washed with medium before being exposed to untreated parasites for 1 h. All differences between experimental values obtained with concentrations of DFMO of ≥0.5 mM and the corresponding control value were statistically significant ($P < 0.05$).

[b] In this and subsequent tables, % change = [(experimental − control)/control] × 100.

were then washed by centrifugation and incubated in 0.1 M lysine in phosphate-buffered saline at room temperature for 5 min. After the killed organisms were washed twice with DMEM, they were suspended in DMEM and counted microscopically with a hemacytometer. The concentration was then adjusted to 5×10^6 and 1×10^7 organisms per ml in the same medium to be used in experiments with macrophages and myoblasts, respectively.

Reagents. DFMO, Δ-methyl-acetylenic putrescine (MAP), monofluoromethyldehydroornithine ethyl ester (MFMOee) and monofluoromethyldehydroornithine methyl ester (MFMOme), irreversible inhibitors of ODC (2, 7), were synthesized at the Merrell Dow Research Institute, Cincinnati, Ohio. Solutions of these reagents were prepared in DMEM. The tested concentrations are described under Results.

Treatment of macrophages or *T. cruzi* with ODC inhibitors. Monolayers of macrophages were incubated with medium alone or containing various concentrations of the ODC inhibitor at 37°C for 18 h. The cells were then washed with DMEM and cocultured with untreated *T. cruzi* as described below. In some experiments, macrophage cultures were incubated with DFMO as described above in the presence of 0.2 mM putrescine, all other conditions remaining the same. Trypomastigote suspensions were incubated with medium alone or containing the appropriate ODC inhibitor at 37°C for 4 h. After the parasites were washed by centrifugation (800 × *g*, 10 min) with DMEM, they were counted microscopically, and their concentration was adjusted to 3×10^6 to 5×10^6 organisms per ml.

Host cell association with *T. cruzi*. The conditions to measure host cell-*T. cruzi* association have been described elsewhere (11–12). Briefly, monolayers of macrophages or myoblasts were incubated with the parasites at 37°C for 1 or 2 h in a 5% CO_2-in-air incubator. The initial parasite/cell ratio was 3:1 to 5:1 in experiments with macrophages and 10:1 when myoblasts were used. After the free parasites were removed by washing three times with DMEM, the monolayers were fixed with absolute methanol and stained with Giemsa. Not less than 200 cells were screened in each culture; the number of screened cells, the number of parasites associated with these cells, and the number of cells associated with one or more parasites were recorded. Each assay was set up in triplicate, and the results are expressed in terms of the mean percentage of cells associated with parasites ± 1 standard deviation and the average number of parasites per 100 cells ± 1 standard deviation.

Macrophage uptake of latex beads and killed *T. cruzi*. The methods for measuring the uptake of latex beads and killed parasites by macrophages were described elsewhere (12).

Presentation of results and statistics. Each set of results presented in the tables of this paper is typically representative of two to four repeat experiments. Differences between means were considered to be significant if P was <0.05 as determined by the Mann-Whitney U test.

RESULTS

Effects of ODC inhibitors on macrophage interaction with *T. cruzi*. Presented in Table 1 is a representative set of results showing that incubation of macrophage monolayers with concentrations of DFMO ranging from ≥0.5 to 10 mM caused a marked reduction in their capacity to interact with untreated trypomastigotes. Similar results were obtained in repeat experiments in which 20 mM DFMO was used (data not shown). In all cases, the inhibition was evidenced by significant reductions in both the percentage of infected cells and the average number of organisms per 100 cells with respect to the control values (mock-treated macrophages). Concentrations of DFMO up to 20 mM did not affect the viability (trypan blue-excluding capacity) of the macrophages under our experimental conditions (incubation at 37°C for 18 h). Pretreatment of macrophages with MAP, MFMOee, or MFMOme also decreased significantly the extent of cell interaction with untreated trypomastigotes (Table 2) and had no detectable toxic effect on the macrophages at the tested concentrations.

When the parasites were incubated with concentrations of DFMO ranging from 5 to 100 mM for 3 or 4 h and then cocultured with either untreated macrophages or rat heart myoblasts, their mutual association was essentially the same as that of organisms which had been mock treated with medium alone (data not shown). Pretreatment of parasites with MAP, MFMOee, or MFMOme for 4 h also failed to affect their level of association with macrophages (data not shown). We chose not to extend parasite incubation with ODC inhibitors beyond 4 h, because with washing times and

TABLE 2. Pretreatment of mouse macrophages with MAP, MFMOee, or MFMOme reduces their capacity to associate with untreated blood forms of *T. cruzi*[a]

Inhibitor and concn (mM)	% of cells associated with parasites (% change)	No. of parasites/100 macrophages (% change)
MAP		
0	79.6 ± 3.1	198.6 ± 3.1
1.0	54.4 ± 1.9 (−31.7)	96.8 ± 10.2 (−51.3)
5.0	53.6 ± 4.1 (−32.7)	83.4 ± 5.3 (−58.0)
MFMOee		
0	49.4 ± 2.3	77.3 ± 4.8
1.0	39.0 ± 3.1 (−21.0)	53.1 ± 3.1 (−31.3)
5.0	40.2 ± 6.4 (−18.6)	55.0 ± 12.0 (−28.8)
MFMOme		
0	51.0 ± 1.2	64.4 ± 4.3
1.0	45.7 ± 2.6[b] (−10.3)	65.7 ± 4.8[b] (2.0)
5.0	33.8 ± 4.2 (−33.7)	48.6 ± 7.2 (−24.5)

[a] Macrophage monolayers were incubated with the indicated concentration of inhibitor for 18 h and washed with medium before being exposed to untreated parasites for 1 h. The experiments with each inhibitor were carried out separately.

[b] Except for these values, all differences between experimental values and the corresponding control value were statistically significant ($P < 0.05$).

TABLE 3. Inhibitory effect of DFMO on macrophage association with *T. cruzi* is prevented by putrescine[a]

Treatment	% of macrophages associated with parasites (% change)	No. of parasites/ 100 macrophages (% change)
Medium	28.7 ± 1.6	32.3 ± 1.3
5 mM DFMO	19.6 ± 1.5[b] (−31.7)	21.9 ± 2.9[b] (−32.2)
5 mM DFMO–0.2 mM putrescine	30.2 ± 3.3 (−5.2)	37.1 ± 3.3 (14.9)
0.2 mM putrescine	30.4 ± 2.4 (5.9)	36.1 ± 3.8 (11.8)

[a] Macrophage monolayers were incubated with the indicated concentration of DFMO for 18 h in the presence or absence of putrescine and washed with medium before being exposed to untreated parasites for 1 h.
[b] The difference between this experimental value and the corresponding control value was statistically significant ($P < 0.05$).

the time of coculture with the host cells, trypomastigote transformation into other forms would have occurred (Wirth and Kierszenbaum, unpublished observations), making it difficult to interpret the results.

Effects of the presence of putrescine during ODC treatment of macrophages. If the decreased macrophage-*T. cruzi* interaction caused by macrophage treatment with DFMO were indeed due to ODC inhibition, the addition of putrescine—the product of ornithine decarboxylation—would be expected to overcome it. A typically representative set of results is presented in Table 3, showing that this was indeed the case. Putrescine alone had no significant effect on macrophage interaction with the parasites.

Effects of DFMO pretreatment of macrophages on the uptake of latex beads and killed *T. cruzi*. Experiments were set up with DFMO-treated macrophages in which latex particles or killed parasites were used to establish whether there was an impairment in phagocytic capacity. DFMO-treated macrophages presented a significantly reduced capacity to take up either particle relative to mock-treated macrophages (Table 4).

Effects of DFMO pretreatment of myoblasts on their capacity to associate with untreated *T. cruzi*. Since the methods used in this study do not distinguish between macrophage internalization of parasites via phagocytosis or active membrane penetration by the trypomastigotes, we conducted similar experiments with myoblasts, which are not phagocytic and can only be infected by *T. cruzi* via membrane penetration. The results indicated that DFMO treatment of myoblasts had no detectable consequence on their susceptibility to infection (Table 5).

TABLE 4. Pretreatment of macrophages with DFMO reduces their capacity to take up latex particles or killed *T. cruzi*[a]

Particle and DFMO (mM)	% of macrophages associated with particles (% change)	No. of particles/ 100 macrophages (% change)
Latex		
0	43.5 ± 2.9	97.0 ± 8.6
20	32.7 ± 3.2 (−24.8)	65.1 ± 7.6 (−32.9)
Killed *T. cruzi*		
0	27.0 ± 0.5	54.5 ± 4.4
20	20.8 ± 3.8 (−23.0)	37.1 ± 6.6 (−31.9)

[a] Macrophage monolayers were incubated with medium alone or containing the indicated concentration of DFMO for 18 h and washed with medium before being incubated with latex or killed *T. cruzi* for 1 h. All differences between experimental values and the corresponding control value were statistically significant ($P < 0.05$).

TABLE 5. Pretreatment of myoblasts with DFMO does not affect their capacity to associate with untreated blood forms of *T. cruzi*[a]

DFMO concn (mM)	% of myoblasts associated with parasites (% change)	No. of parasites/ 100 myoblasts (% change)
0	12.3 ± 3.1	15.6 ± 2.9
10	10.9 ± 2.1 (−11.4)	17.0 ± 2.4 (9.0)
20	12.9 ± 3.3 (4.9)	17.2 ± 5.1 (10.3)

[a] Myoblast monolayers were incubated with the indicated concentration of DFMO for 18 h and washed with medium before being exposed to untreated parasites for 2 h. None of the differences between experimental and control values was statistically significant.

Reversibility of the DFMO effect on macrophages. Incubation of DFMO-treated macrophages in fresh medium for various periods of time before exposure to untreated trypanosomes resulted in a partial but significant recovery from the inhibitory effect after 30 min and total recovery after 2 h (Table 6), implying that DFMO-blocked ODC was replaced by ODC via turnover.

DISCUSSION

These results demonstrated the capacity of specific inhibitors of ODC to inhibit internalization of the protozoan *T. cruzi* by macrophages, probably by impairing phagocytosis. Parasite treatment with concentrations of DFMO as high as 100 mM had no detectable consequence on its capacity to interact with either macrophages (phagocytosis plus membrane penetration) or myoblasts (penetration only). Thus, it is unlikely that ODC inhibitor incorporated by the macrophages could have reduced interaction with the parasite by acting on the latter during coculture. Loss of macrophage viability due to the tested treatments also seemed to be an unlikely explanation, because none of the tested inhibitors caused significant cell death under the conditions described herein. Furthermore, the DFMO effect was totally reversible.

The fact that four different drugs, DFMO, MAP, MFMOee, and MFMOme, which share the ability to irreversibly inhibit ODC, reduced the proportion of macrophages engaging the parasite (Tables 1 and 2) strongly implicated ODC activity in the process of binding or internalization (or both) of *T. cruzi*. Ornithine decarboxylation catalyzed by ODC produces putrescine, which in turn is metabolically converted into spermidine and spermine. Inhibition of macrophage ODC may have resulted in insuffi-

TABLE 6. Inhibitory effect of DFMO on macrophage association with *T. cruzi* is reversible[a]

Treatment	Time after treatment (min)	% of macrophages associated with parasites (% change)	No. of parasites/ 100 macrophages (% change)
Medium	0	43.7 ± 0.5	63.1 ± 2.2
DFMO	0	29.2 ± 4.7[b] (−33.2)	40.4 ± 8.9[b] (−36.0)
DFMO	15	31.6 ± 1.5[b] (−27.7)	43.7 ± 2.5[b] (−30.7)
DFMO	30	36.8 ± 1.5[b] (−15.8)	54.4 ± 2.5[b] (−13.8)
DFMO	60	32.5 ± 1.7[b] (−25.6)	51.3 ± 7.5[b] (−18.7)
DFMO	120	42.4 ± 4.7 (−3.0)	60.9 ± 4.1 (−3.5)

[a] Macrophage monolayers were incubated with the 10 mM DFMO for 18 hr, washed with medium, and further incubated with fresh medium for the indicated periods of time before being exposed to untreated parasites for 1 hr.
[b] The difference between this value and the corresponding control value was statistically significant ($P < 0.05$).

cient levels of one or more of these polyamines and in this way affected macrophage function. This notion found support in the finding that exogenous putrescine prevented the inhibition when it was present during macrophage pretreatment with DFMO.

Since DFMO treatment of macrophages also inhibited their capacity to take up latex beads or killed parasites (Table 4), the overall phagocytic capacity of the macrophages appeared to be curtailed. The latter observation also suggested that DFMO might affect the phagocytic capacity of the cells rather than reduce their susceptibility to parasite invasion. This notion was supported by the observation that the susceptibility of myoblasts (which are not phagocytic cells) to invasion by *T. cruzi* was not altered by DFMO (Table 5).

The gradual reversibility of the DFMO effect (Table 6) indicated that some macrophages could recover faster than others. Our results did not clarify whether this was due to the use of populations of cells at different differentiation stages. Alternatively, even at the highest tested concentrations the amount of inhibitor actually entering the macrophages by passive diffusion (3) may have represented a fraction of the exogenous concentration insufficient to inhibit all of the intracellular ODC. Unfortunately, we could not test this possibility by further increasing the concentrations of DFMO, MAP, MFMOee, or MFMOme because levels of these drugs greater than those described in Results were found to decrease macrophage viability (data not shown). On the other hand, some macrophages may have had greater stores of polyamines than others at the time of their treatment and may have relied on these to carry out their function during incubation with the parasites. The latter possibility finds support in the fact that exogenous putrescine, which would not prevent the binding of DFMO to ODC, did prevent the inhibitory effects of DFMO.

Although it was tempting to test whether ODC would have altered the course of *T. cruzi* infection in mice, we refrained because cells other than macrophages utilize ODC for polyamine synthesis and the results would have been inconclusive. In any case, our results indicate that inhibition of ODC affects the phagocytic capacity of macrophages and emphasize a requirement for at least putrescine in this function. We cannot at present specify the precise polyamine-dependent reaction(s) supporting effective phagocytosis.

It is noteworthy that polyamine biosynthesis is an important requirement for *T. cruzi* invasion of mammalian host cells and subsequent intracellular multiplication (4a). However, unlike macrophages, the parasite appears to utilize for this purpose arginine decarboxylase, an enzyme not found in mammalian cells.

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