ABSTRACT
The objective of the present investigation was to compare the effects of three ornithine decarboxylase inhibitors on tumoricidal macrophage activity and antitumor activity in vivo. α-Difluoromethylornithine (DFMO), (2R,5S)-6-heptyne-2,5-diamine, and α-(fluoromethyl)dehydroornithine methyl ester (ΔMFMOme) were administered continuously in drinking water starting on Day 1 to B16F1 tumor-bearing mice. DFMO, (2R,5S)-6-heptyne-2,5-diamine, and ΔMFMOme reduced B16F1 tumor growth, measured on Day 18, up to 87, 79, and 95%, respectively. Similarly, all three ornithine decarboxylase inhibitors reduced B16F1 putrescine and spermidine levels. ΔMFMOme was substantially more effective both as an antitumor agent and in reducing polyamines. Both DFMO and ΔMFMOme augmented macrophage tumoricidal activity directed against B16F1 target cells. MAP had no effect on macrophage tumoricidal activity. Lipopolysaccharide-stimulated macrophages from ΔMFMOme-treated mice also exhibited an increase in interleukin and tumor necrosis factor levels. Furthermore, treatment with a known macrophage activator, γ-interferon, enhanced the antitumor activity of ΔMFMOme. ΔMFMOme did not alter natural killer cell activity; however, cytolytic T-lymphocyte induction was reduced by 40 to 50%. These results demonstrate that, in addition to their established antitumor activity, ornithine decarboxylase inhibitors may also potentiate specific tumoricidal effector cell generation in vivo.

INTRODUCTION
Naturally occurring host resistance has been established to be important in preventing tumor growth and metastasis. A variety of both specific and nonspecific host immune response mechanisms have been shown to be involved in antitumor immunity. CTLs are distinguished from other tumoricidal effector cells by their specific reactivity toward antigenically distinct tumor cells following sensitization (1). In contrast, NK cells and macrophages exhibit nonspecific cytolytic reactivity toward tumor cells without prior sensitization (2). Macrophages have been shown to be particularly important in tumor regression and are closely associated with a decreased incidence of metastasis (3–5).

The naturally occurring polyamines putrescine, spermidine, and spermine appear to be essential for cell growth and differentiation (6–8). ODC is the first enzymatic step in the polyamine biosynthesis pathway (9). Polyamine depletion induced by ODC inhibitors will result in inhibition of tumoral growth in vivo (10–13). Furthermore, polyamine biosynthesis is required for maintenance of circulating leukocyte levels (14). Nonetheless, relatively little is known regarding the requirement for polyamines in the function of various leukocyte subpopulations. In the present study, we have compared the effect of three different ODC inhibitors, DFMO (15), MAP (16), and ΔMFMOme (Ref. 17; Fig. 1), with respect to their antitumor activity, inhibition of polyamine biosynthesis, and macrophage tumoricidal function. Our results demonstrate that the most potent antitumor activity correlated with the highest detectable levels of macrophage tumoricidal activity.

MATERIALS AND METHODS
Mice. Male C57BL/6 mice, with an initial body weight of approximately 20 g, were obtained from the Charles River Breeding Laboratories, Wilmington, MA. They were housed in plastic cages, under standard laboratory conditions, with free access to food and water.

Reagents. DFMO, MAP, and ΔMFMOme were synthesized in the laboratories of Merrell Dow Research Institute. Recombinant IFN-γ was purchased from Amgen Biologicals (Thousand Oaks, CA). [3H]-dThd (specific activity, 25 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Na2[35]CrO4 (specific activity, 0.25 Ci/mg) was purchased from ICN Radiochemicals (Irvine, CA). Con A, LPS, and phytohemagglutinin were purchased from Sigma Chemical (St. Louis, MO). Recombinant TNF-B was obtained from Phillips Petroleum (Bartlesville, OK).

Target Cells. YAC-1 and P815 cells were maintained in RPMI-1640 supplemented with 10% FCS. B16F1 tumor cells were maintained in vivo by s.c. serial transplantation.

Drug Treatment. DFMO, MAP, and ΔMFMOme were administered p.o. via the drinking water as a 0.05 to 2% solution. Fluid intake was monitored daily, and average daily drug intake was calculated.

In Vivo Tumor Growth. Rapidly dividing 14-day-old tumors were removed and trypsinized. The resulting cell suspension was passed through sterile gauze, viability was determined (trypan blue), and 104 B16 tumor cells were injected s.c. into the intrascapular region. Tumors became palpable within 7 to 10 days. Mice were killed 18 days following tumor injection. The tumor tissue was weighed, and a portion was saved for polyamine analysis.

Polyamine Analysis. Intracellular polyamine levels were determined by reverse-phase HPLC as previously described (18). Briefly, a Waters gradient high-pressure liquid chromatographic system, equipped with two Model 510 pumps, a Model 680 automated gradient controller, and a Model 710B WISP autoinjector, was used for polyamine analysis. The fluorescence detector was a LDD/Milton Roy Fluoro-Monitor III, a 370-nm excitation filter, and a 418-nm emission filter. The separation was achieved on a Resolve 5 C18μBondapak column (Waters Associates) equipped with a Brownlee RP-18 guard column. Data were collected on a HP 1000 computer using CALS software.

The final conditions for HPLC analysis were a flow of 1.0 ml/min and 25-μl injection volume. Mobile Solvent A was acetonitrile/0.002 M sodium phosphate buffer, pH 7.0 (20/80). Mobile Solvent B was acetonitrile/0.002 M sodium phosphate buffer, pH 7.0 (80/20). The gradient was run from 0 to 100% mobile Solvent B over 25 min using a convex curve (No. 4), followed by 13 min isocratic at 100% Solvent B before returning to initial conditions.

The peaks of interest were identified and quantitated using polyamine standards (Sigma) at known concentrations. The elution order of the polyamine derivatives was determined to be putrescine, spermidine, and then spermine. Polyamine levels were detectable to levels of 1 pmol injected onto the column.
Macrophase-mediated Cytotoxicity. Peritoneal macrophase tumoricidal activity was assessed as previously described (19). Briefly, PEC were collected on Day 18 following tumor injection. The total number of viable cells was determined by trypan blue exclusion, and differential cell counts were made on Diff-Quik (Harleco, Gibbstown, NJ)-stained cell smears prepared by cytocentrifugation (Cytospin; Shandon Southern, Camberley, England). The PEC were added to flat-bottomed 96-well tissue culture cluster plates (No. 25020; Corning Glass Works, Corning, NY) for 24 h. Nonadherent cells were removed by repeated washings, and the adherent cells were labeled with 1.0 μCi/ml of tritiated thymidine for 24 h. Labeled tumor cells were then harvested by exposure to 0.5% trypsin (GIBCO), washed, and added in quadruplicate (10^4/well) to the plates containing the adherent macrophages. Following 72-h incubation at 37°C, 0.1 ml of supernatant fluid was removed from each well, and the released [3H]Thd was measured in a Beckman LS7800 beta counter. Supernatant fluids from target cells incubated alone were assayed for spontaneous release, and maximum release was determined by adding 1% sodium dodecyl sulfate. Cytotoxicity, expressed as the percentage of specific lysis, was calculated as follows.

\[
\% \text{ of specific lysis} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}} \times 100
\]

Natural Killer Cell Assay. Splenic NK cell activity was assessed as previously described (19). Briefly, spleens were aseptically removed, cleaned of extraneous tissue, teased apart in complete RPMI-1640, and filtered through sterile gauze to obtain single cell suspensions. The spleen cells were washed, viability was determined by trypan blue exclusion, and these cells were added (0.25 to 1.0 x 10^6/0.1 ml) to 96-well round-bottomed (Costar No. 3799) tissue culture plates. L929 cells were labeled with 100 μCi of ¹¹⁵I at 37°C for 1 h. Labeled target cells were then centrifuged at 50 x g for 3 min and incubated at 37°C for 4 h. Following this incubation period, the plates were centrifuged at 300 x g for 5 min, the supernatant fluids were harvested with a supernatant collection system (Skatron, Sterling, VA), and the released ¹¹⁵I was measured in a Beckman Model 5500 gamma counter. Spontaneous release, maximum release, and the percentage of specific lysis were determined as described above.

Cytolytic T-Lymphocyte Assay. Splenic CTL activity was assessed as previously described (19). Briefly, leukocytes (10^6/ml) were stimulated with Con A (2.5 μg/ml) for 48 h. The effector cells were treated with α-methyl mannosidase (50 mmol), washed, and assayed for cytotoxicity against ¹¹⁵I-labeled P815 targets in the presence of phytohemagglutinin (10 μg/ml) for 4 h.

Interleukin 1 Assay. Peritoneal macrophages (10^5/ml) were stimulated with LPS (100 μg/ml) for 24 h. Supernatant fluids were harvested and assayed for IL-1 activity in the thymocyte costimulator assay as described by Mizel et al. (20).

Tumor Necrosis Factor Assay. TNF activity obtained from LPS-stimulated macrophage cultures, prepared as described above, was assayed with modification according to the method of Satoh et al. (21). L929 cells were labeled with ¹³¹I for 1 h, washed, and plated in 96-well flat-bottomed tissue culture plates in RPMI-1640 with 5% FCS. Following a 24-h incubation, 2-fold serial dilutions of test supernatant fluid were added, and the plates were incubated for an additional 18 h. Supernatant fluids were harvested, and the released ¹³¹I was measured in a Beckman Model 5500 gamma counter. Supernatant fluids from cells incubated in medium alone were assayed for spontaneous release, and maximum release was determined by adding 1% sodium dodecyl sulfate. The dilution producing 50% ¹³¹I release was used as an endpoint of TNF activity, and units/ml were calculated based upon a laboratory reference standard (recombinant TNF-B).

Statistical Analysis. Statistical significance was determined by the two-tailed Student t test.

RESULTS

Comparative Antitumor Activity of DFMO, MAP, and ∆MFMOme against B16F1 Melanoma in Vivo. The effect of DFMO (0.5 to 2.0%), MAP (0.1 to 0.5%), and ∆MFMOme (0.1 to 0.5%), administered in drinking water, on the growth of B16F1 melanoma cells in mice is presented in Table 1. All three ODC inhibitors significantly (P < 0.001) reduced tumor growth, as judged by tumor weights, measured on Day 18. ∆MFMOme (0.5%) was the most effective, inhibiting tumor growth by 95%. Daily fluid intake was nearly constant for all drug-treated mice. However, a reduction in fluid intake was observed for the group receiving 0.5% MAP (Table 1).

Comparative Effect of DFMO, MAP, and ∆MFMOme on B16F1 Polyamine Levels. All three ODC inhibitors significantly reduced polyamine levels, in a dose-dependent manner, in B16F1 tumor cells in vivo (Table 2). DFMO (2%), MAP (0.5%), and ∆MFMOme (0.5%) reduced putrescine levels 96%, 84%, and 97%, respectively. Similarly, spermidine levels were reduced by 83%, 64%, and 84% following treatment with DFMO (2%), MAP (0.5%), and ∆MFMOme (0.5%). None of the inhibitors tested reduced tumor cell spermine levels.

Comparative Effect of DFMO, MAP, and ∆MFMOme on Macrophage Tumoricidal Activity. Peritoneal macrophage tumoricidal activity was determined, on Day 18 following tumor cell injection, against freshly isolated B16F1 tumor cell targets. DFMO (2.0%) and ∆MFMOme (0.5%) augmented macrophage tumoricidal activity 2- to 3-fold (Fig. 2). ∆MFMOme was the most effective, increasing cytolytic activity to 43% compared with 7% in the vehicle-treated control group at an E:T of 50:1. The effect of ∆MFMOme on macrophage tumoricidal activity was dose dependent (Fig. 2). MAP had no effect on macrophage tumoricidal activity (Fig. 2).

Macrophage function was also assessed in B16F1 tumor-bearing mice following a shorter duration of ∆MFMOme treatment. Similar to Day 18, mice treated with ∆MFMOme (0.1%) for 7 days exhibited a greater than 2-fold increase in macrophage tumoricidal activity directed against B16F1 target cells (Table 3). Furthermore, macrophages taken from ∆MFMOme-treated mice also displayed an increase in detectable IL-1 and TNF levels 24 h following LPS stimulation.

Effect of ∆MFMOme on Natural Killer Cell and Cytolytic T-Cell Activity. The effect of ∆MFMOme on splenic NK cell and...
high drug intake and tumor cell polyamine levels and did not potentiate macrophage tumoricidal activity.

**Table 3 Effect of AMFMOme on macrophage tumoricidal activity and IL-1 and TNF production**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Tumor size (% of initial)</th>
<th>IL-1* (units/ml)</th>
<th>TNF* (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.0 ± 1.0</td>
<td>21 ± 0.1</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>AMFMOme</td>
<td>40.0 ± 2.0^</td>
<td>41 ± 1.9</td>
<td>6.7 ± 0.4^</td>
</tr>
</tbody>
</table>

^ AMFMOme was administered as 0.1% in drinking water. Animals were killed on Day 7.  
\* Macrophage cytolytic activity was determined against [3H]dThd-labeled B16F1 tumor cells (E:T, 5:1).  
\* IL-1 activity was determined in the thymocyte costimulator assay.  
\* TNF activity was determined as cytotoxicity measured against L929 fibroblasts.  
\* Mean ± SE (n = 3).  
\* P < 0.001.

**DISCUSSION**

The results of this study demonstrate that, in addition to their inhibitory effects on tumor cell growth, ODC inhibitors such as DFMO and AMFMOme can also potentiate specific immune cell function. Both DFMO and AMFMOme augmented macrophage tumoricidal activity following 18 days of p.o. administration in B16F1 tumor-bearing mice. AMFMOme was the most effective compound in preventing tumor growth, reducing polyamine levels, and augmenting tumoricidal macrophage function. When corrected for total drug intake, AMFMOme was 2 to 3 times more potent than DFMO in inhibiting B16F1 growth in vivo. Similarly, when compared with MAP for the same total drug intake (e.g., 0.5 g/kg), AMFMOme was 2 to 3 times more effective in reducing tumor cell polyamine levels and did not potentiate macrophage tumoricidal activity.

Mamont et al. (23) have shown that, upon intracellular levels were reduced by 53%. Spermine levels were not reduced in either population.

**Effect of Combination Treatment with AMFMOme and IFN-γ on B16F1 Tumor Growth in Vivo**

We have recently demonstrated that IFN-γ, a macrophage activating agent, can enhance the antitumor activity of DFMO (22). Therefore, we assessed whether IFN-γ would enhance the antitumor activity of AMFMOme. As shown in Fig. 4, IFN-γ significantly (P < 0.001) increased the antitumor activity of AMFMOme administered at 0.05, 0.1, and 0.2% in drinking water. Enhancement was not observed at the highest dose of AMFMOme (0.5%) tested.

**Fig. 2.** The effect of 2% DFMO (•), 0.5% MAP (△), and 0.5% AMFMOme (□) on macrophage tumoricidal activity. B16F1 tumor-bearing mice were treated with DFMO, MAP, or AMFMOme for 18 days, starting on Day 1 following tumor injection. Peritoneal macrophage tumoricidal activity was assessed against [3H]dThd-labeled B16F1 target cells. Points and columns, mean; bars, SE. ○, control.
ODC Inhibitor and Immune Function

Fig. 4. The effect of combination treatment with ∆MFMOme and IFN-γ on B16F1 tumor growth in vivo. B16F1 tumor cells (10⁶) were injected s.c. at the intrascapular region. ∆MFMOme was administered starting on Day 1 in drinking water at the indicated doses. IFN-γ (2000 units) (W) or phosphate-buffered saline (✓) was administered s.c. every other day starting on Day 2 through Day 16. Mice were killed on Day 18, and the tumor tissue was removed and weighed. Columns, mean; bars, SE; n = 10.

Table 4 Effect of ∆MFMOme on peritoneal macrophage and splenic lymphocyte polyamine levels in vivo

<table>
<thead>
<tr>
<th>Cell type</th>
<th>∆MFMOmea (0.5%)</th>
<th>Putrescine (pmol/10⁶ cells)b</th>
<th>Spermidine (pmol/10⁶ cells)b</th>
<th>Spermine (pmol/10⁶ cells)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>+</td>
<td>49.4 ± 5.0</td>
<td>151.1 ± 16.1</td>
<td>220.8 ± 25.8</td>
</tr>
<tr>
<td>Splenic leukocyte</td>
<td>+</td>
<td>12.9 ± 13.1</td>
<td>135.4 ± 14.4</td>
<td>202.2 ± 19.9</td>
</tr>
</tbody>
</table>

Note: * See Table 1.  
  † See Table 2.  
  ‡ Mean ± SE (n = 5).  
  § P < 0.001.

hydrolysis, ∆MFMOme is converted to ∆MFMO. The affinity of both ∆MFMO and MAP for ODC is very similar (Kᵢ = 3 μmol), and better than that observed for DFMO (Kᵢ = 40 μmol) (14–16). The superior antitumor and polyamine-depleting ability of ∆MFMOme could reflect a capacity to more effectively penetrate B16F1 cells. In this regard, it has been previously shown that the absorption of ∆MFMOme in HTC cells was better than that of ∆MFMO (23). Furthermore, Bacchi et al. (24) have shown that ∆MFMOme accumulated in Trypanosoma brucei brucei 6 to 8 times faster than did ∆MFMO or DFMO.

In contrast to our results presented herein on B16F1, Pera et al. (25) have shown that MAP was more effective than DFMO or ∆MFMOme in inhibiting the growth of L1210 tumor cells in culture. More recently, Claverie and Mamont (26) similarly demonstrated that, when given via the drinking water, MAP was more effective than DFMO or ∆MFMOme against L1210 leukemia in vivo. However, they also showed that ∆MFMOme was more effective than MAP against Lewis lung carcinoma in vivo. Therefore, it seems clear that individual tumors will respond differently to various ODC inhibitors. Furthermore, based on the results presented herein, the immune sensitivity of a particular tumor may also be important regarding its susceptibility to ODC inhibitors. Therefore, both polyamine depletion and immunosensitivity should be considered when evaluating ODC inhibitors as antitumor agents.

Studies with ODC inhibitors indicate that polyamine biosynthesis is required for lymphocyte proliferation (27, 28). More recently, our laboratory (29, 30), and others (31, 32), have shown that polyamine biosynthesis is critical for the function of those lymphocyte subpopulations requiring clonal expansion for their full immunological potential. In this regard, we have recently established that MAP is a potent inhibitor of allogene-induced CTL induction in vitro and in vivo (28, 33). Similarly, as shown herein, ∆MFMOme treatment in vivo reduced subsequent polyclonal CTL generation in vitro. MAP has also been shown to reduce immunoglobulin production in vitro and in vivo (32, 34). In contrast, as shown herein with ∆MFMOme and previously with DFMO (19, 35), splenic polyamine depletion did not impair NK cell function. We have previously shown that DFMO could, however, augment macrophage yield and tumoricidal activity in B16F1 tumor-bearing mice treated with the immunomodulators, tuftsin (36), IFN-γ (22), or Corynebacterium parvum (35). Taken together with our current results on ∆MFMOme, at least some ODC inhibitors can potentiate macrophage function. ∆MFMOme treatment increased not only macrophage tumoricidal activity, but also the detectable levels of two macrophage products, IL-1 and TNF. Whether or not this increase accounts for any of the ∆MFMOme-mediated macrophage tumoricidal activity is unknown. In contrast to our results presented here, Kierszenbaum et al. (37) have shown that the binding and ingestion of the protozoan Trypanosoma cruzi, as well as latex beads, by macrophages were impaired in vitro by DFMO, MAP, and ∆MFMOme. Ehrke et al. (38) demonstrated that DFMO administration in vivo had little effect on Fc-dependent macrophage phagocytosis. We are currently assessing the in vitro effects of these compounds on macrophage tumoricidal activity and monokine production.

Inhibitors of ODC have generally not shown efficacy as single antitumor agents in humans (39). However, ODC inhibitors in combination with immunopotentiating agents have been shown to be more effective than either agent alone (35, 36, 40). In this regard, we have recently shown that IFN-γ, a known macrophage activator, enhanced the antitumor activity of DFMO.
against B16F1 tumor growth in mice (22). Similarly, as shown herein, ΔMFMOme-mediated antitumor activity was also enhanced by IFN-γ. Therefore, our current results further favor the use of ODC inhibitors in combination with immunomodulators in the treatment of human neoplasia.

ACKNOWLEDGMENTS

We would like to thank Dr. Nellikunja Prakash for reviewing this manuscript, John Zwolshen and Lisa Schmidt for technical assistance, and Cyrena Tyson and Norma Reddington for secretarial assistance.

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Effects of Three Irreversible Inhibitors of Ornithine Decarboxylase on Macrophage-mediated Tumoricidal Activity and Antitumor Activity in B16F1 Tumor-bearing Mice

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