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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ABSTRACT

The objective of the present investigation was to compare the effects of three ornithine decarboxylase inhibitors on tumoral macrophage and antitumor activities in vivo. α-Difluoromethylornithine (DFMO), (2R,5R)-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,5R)-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 enhanced 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. CTLs2 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 tumor 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). Na235CrO4 (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 105 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 C18-Bondapak 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.

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2 The abbreviations used are: CTL, cytolytic T-lymphocyte; DFMO, α-difluoromethylornithine; MAP, (2R,5R)-6-heptyne-2,5-diamine; ΔMFMOME, α-(fluoromethyl)dehydroornithine methyl ester; NK, natural killer; ODC, ornithine decarboxylase; IL-1, interleukin 1; TNF, tumor-necrosis factor; IFN-γ, γ-interferon; E:T, effectortarget ratio; LPS, lipopolysaccharide; dThd, thymidine; Con A, concanavalin A; FCS, fetal calf serum; HPLC, high-pressure liquid chromatography; PEC, peritoneal exudate cells.
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. 3596; Costar, Cambridge, MA) for 24 h. Nonadherent cells were removed by repeated washings.

B16 melanoma cells were used as tumor cell targets for macrophase-mediated cytotoxicity. Fresh B16 tumor cell preparations were allowed to adhere to 100-mm plastic Petri dishes (No. 25020; Corning Glass Works, Corning, NY) for 24 h. Nonadherent cells were then harvested by exposure to 0.5% trypsin (GIBCO), washed, and added in quadruplicate (10^5/well) with the effector cells treated with Con A (2.5 mg/ml) for 48 h. The effect of AMFMOme on macrophage tumoricidal activity was determined, on Day 18 following tumor injection, against freshly isolated B16F1 tumor cell targets.

Natural Killer Cell Assay. Splenic NK cell activity was assessed as previously described (19). Briefly, spleens were aseptically removed, cleaned of extraneous tissue, and teased apart in complete RPMI-1640, 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 plate tissue culture cluster plates (No. 3599) for 24 h. Following 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.

% of specific lysis = \( \frac{cpm_{\text{experimental}} - cpm_{\text{spontaneous}}}{cpm_{\text{maximum}} - cpm_{\text{spontaneous}}} \times 100 \)

RESULTS

Comparative Antitumor Activity of DFMO, MAP, and AMFMOme against B16F1 Melanoma in Vivo. The effect of DFMO (0.5 to 2.0%), MAP (0.1 to 0.5%), and AMFMOme (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. AMFMOme (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 AMFMOme 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 AMFMOme (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 AMFMOme (0.5%). None of the inhibitors tested reduced tumor cell spermine levels.

Effect of DFMOme on Natural Killer Cell and Cytolytic T-Cell Activity. The effect of DFMOme on splenic NK cell and
Mice were killed on Day 18, and the tumor tissue was removed and weighed. *Mean±SE (n = 10).

**Table 2 Effect of ornithine decarboxylase inhibitors on B16F1 polyamine levels in vivo**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage (% in drinking water)</th>
<th>Putrescine (nmol/g)</th>
<th>Spermidine (nmol/g)</th>
<th>Spermine (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>15.2 ± 6.7</td>
<td>533 ± 170</td>
<td>360 ± 67</td>
</tr>
<tr>
<td>DFMO</td>
<td>0.5</td>
<td>8.7 ± 3.6</td>
<td>320 ± 64</td>
<td>746 ± 102</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.1 ± 0.2*</td>
<td>90 ± 13</td>
<td>560 ± 53</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.6 ± 0.1^</td>
<td>88 ± 7</td>
<td>396 ± 54</td>
</tr>
<tr>
<td>MAP</td>
<td>0.1</td>
<td>21.1 ± 7.3</td>
<td>501 ± 103</td>
<td>783 ± 80</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>13.2 ± 7.7</td>
<td>297 ± 80</td>
<td>809 ± 40</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.5 ± 1.4</td>
<td>167 ± 58</td>
<td>557 ± 93</td>
</tr>
<tr>
<td>ΔMFMOme</td>
<td>0.1</td>
<td>2.4 ± 0.5</td>
<td>157 ± 8</td>
<td>733 ± 46</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.4 ± 0.1^</td>
<td>88 ± 5</td>
<td>482 ± 22</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5 ± 0.1^</td>
<td>83 ± 16</td>
<td>429 ± 19</td>
</tr>
</tbody>
</table>

*See Table 1.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumoricidal* (% of specific lysis)</th>
<th>IL-1* (units/ml)</th>
<th>TNF* (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.0 ± 0.7^</td>
<td>21 ± 1.1^</td>
<td>4.0 ± 0.3^</td>
</tr>
<tr>
<td>ΔMFMOme</td>
<td>40.3 ± 2.0^</td>
<td>41 ± 1.9^</td>
<td>6.7 ± 0.4^</td>
</tr>
</tbody>
</table>

*ΔMFMOme was administered as 0.1% in drinking water. Animals were killed on Day 7.

**DISCUSSION**

The results of this study demonstrate that, in addition to their inhibitory effects on tumor cell growth, ODC inhibitors such as DFMO and ΔMFMOme can also potentiate specific immune cell function. Both DFMO and ΔMFMOme augmented macrophage tumoricidal activity following 18 days of p.o. administration in B16F1 tumor-bearing mice. ΔMFMOme was the most effective compound in preventing tumor growth, reducing polyamine levels, and augmenting tumoricidal macrophage function. When corrected for total drug intake, ΔMFMOme was 3 to 4 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), ΔMFMOme was 2 to 3 times more effective in preventing tumor growth. MAP was also the least effective in reducing tumor cell polyamine levels and did not potentiate macrophage tumoricidal activity.

Mamont et al. (23) have shown that, upon intracellular...
ODC INHIBITOR AND IMMUNE FUNCTION

Fig. 4. The effect of combination treatment with \(\Delta MFMOme\) and IFN-\(\gamma\) on B16F1 tumor growth in vivo. B16F1 tumor cells (10^5) were injected s.c. at the intrascapular region. \(\Delta MFMOme\) was administered starting on Day 1 in drinking water at the indicated doses. IFN-\(\gamma\) (2000 units) (B) or phosphate-buffered saline (C) 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 \(\Delta MFMOme\) on peritoneal macrophage and splenic lymphocyte polyamine levels in vivo

<table>
<thead>
<tr>
<th>Cell type</th>
<th>(\Delta MFMOme) (0.5%)</th>
<th>Putrescine (pmol/10^6 cells)</th>
<th>Spermidine (pmol/10^6 cells)</th>
<th>Spermine (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>+</td>
<td>49.4 ± 5.0f</td>
<td>151.1 ± 16.1</td>
<td>220.8 ± 25.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12.9 ± 13.1f</td>
<td>135.4 ± 14.4</td>
<td>202.2 ± 19.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>11.2 ± 1.2</td>
<td>175.5 ± 20.2</td>
<td>126.6 ± 13.5</td>
</tr>
<tr>
<td>Spleenic leukocytes</td>
<td>+</td>
<td>5.1 ± 0.5f</td>
<td>83.2 ± 7.9f</td>
<td>163.9 ± 15.4</td>
</tr>
</tbody>
</table>

* See Table 1.

* See Table 2.

* Mean ± SE (n = 5).

* *P < 0.001.

In contrast to our results presented herein on B16F1, Pera et al. (25) have shown that MAP was more effective than DFMO or \(\Delta MFMOme\) in inhibiting the growth of L1210 tumor cells in culture. Moreover, Claverie and Mamont (26) similarly demonstrated that, when given via the drinking water, MAP was more effective than DFMO or \(\Delta MFMOme\) against L1210 leukemia in vivo. However, they also showed that \(\Delta MFMOme\) 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 alloantigen-induced CTL induction in vitro and in vivo (28, 33). Similarly, as shown herein, \(\Delta MFMOme\) 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 \(\Delta MFMOme\) 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-\(\gamma\) (22), or Corynebacterium parvum (35). Taken together with our current results on \(\Delta MFMOme\), at least some ODC inhibitors can potentiate macrophage function. \(\Delta MFMOme\) 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 \(\Delta MFMOme\)-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 \(\Delta MFMOme\). 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-\(\gamma\), 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.

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