Potentiation of Natural Killer Cell Activity and Tumor Immunity by Diacetylpentrescine

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ABSTRACT

The objective of the present investigation was to evaluate the immunomodulating properties of tetramethylenedisuccinimide (N,N'-diacetylputrescine, DAP), a known inducer of cellular differentiation. We examined the effect of DAP administration in vivo on splenic and nonadherent peritoneal natural killer (NK) cell activity. A single i.p. injection of DAP (100 mg/kg) enhanced cytolytic activity directed against YAC-1 and MCA-38 tumor target cells 2- to 3-fold. Cytolytic activity peaked 3 days following DAP injection. DAP treatment increased the frequency of asialo-GM1-positive splenocytes to 15% compared with 5% for vehicle treated controls. Furthermore, cytolytic activity could be eliminated by treatment with anti-asialo-GM1 antibodies and complement. Lysis of NK-resistant P815 and EL4 tumor target cells was not observed in leukocytes from DAP-treated mice. DAP treatment of mice given injections i.p. of MCA-38 tumor cells increased survival time of the mice by 37%, curing 10% of the animals. DAP treatment of mice given injections intrasplenically of MCA-38 tumor cells reduced both the number and the size of the hepatic metastases. The antitumor effect of DAP in vivo could be eliminated by pretreating mice with anti-asialo-GM1 antibodies or utilizing NK cell deficient beige (bg/bg) mice. These results indicate that the observed anti-tumor activity of DAP is mediated, at least in part, by NK cells.

INTRODUCTION

There has been increasing evidence that host resistance mechanisms are important in preventing tumor growth and metastasis. Tumorocidal effector cells which exhibit natural cell-mediated cytotoxicity demonstrate nonspecific cytolytic reactivity toward tumor cells without prior sensitization (1). Natural cell-mediated cytotoxicity has been shown to be mediated primarily by NK cells. NK cells have been shown to be particularly important in the destruction of incipient tumors (1–3). An NK cell deficiency can result in higher susceptibility to neoplastic growth (2). Similarly, potentiation of NK cell activity was shown to enhance antitumor activity in vivo (3). Therefore, NK cell augmentation may be beneficial in the therapy of human malignant diseases. The naturally occurring polyamines putrescine, spermidine, and spermine are closely associated with lymphocyte differentiation and proliferation (4). A putrescine analogue, DAP, has been shown to be a potent inducer of cellular differentiation (5). In this report we have investigated the effect of DAP on murine NK cell activity. Our results indicate that DAP can potentiate NK cell activity and antitumor activity in vivo.

MATERIALS AND METHODS

Mice. Normal and beige (bg/bg) 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. Tetramethylenedisuccinimide (N,N'-diacetylputrescine) and Tilorone were synthesized in the laboratories of the Merrell Dow Research Institute. Rabbit anti-asialo-GM1 antibodies were purchased from Wako Chemical Co., Dallas, TX. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was purchased from Southern Biotechnology Associates, Birmingham, AL. Na251CrO4 (specific activity 0.25 Ci/mg) was purchased from ICN Radiochemicals (Irvine, CA). Mouse-absorbed guinea pig complement was purchased from Cedarlane, Hornby, Ontario, Canada.

Tumor Cells. The YAC-1 cells, a Moloney virus-induced lymphoma of A/SN origin, were generously provided by Dr. John R. Ortaldo (National Cancer Institute). The MCA-38 cells, a dimethylhydrazine-induced colon adenocarcinoma of C57BL/6 origin, were generously provided by Dr. Steven A. Rosenberg (National Cancer Institute). P815 mastocytoma cells (DBA/2 origin) and EL4 thymoma cells (C57BL/6 origin) were both obtained from the ATCC. YAC-1, EL4, and P815 tumor cells were maintained in vitro in RPMI 1640 (GIBCO) containing 10% fetal calf serum. MCA-38 tumor cells were propagated and maintained in vivo by serial transplantation of dissociated tumor cells into the interscapular region of C57BL/6 mice. MCA-38 tumor cells used in all studies were restricted to rapidly dividing tumors of less than 10 passages.

Natural Killer Cell Assay. Splenic and peritoneal cell cytolytic activity were determined as previously described in detail (6). Peritoneal cells were separated into plastic adherent and nonadherent populations prior to use as effector cells. Freshly isolated effector cells were directly assayed for cytolytic activity against 51Cr-labeled YAC-1, P815, EL4, and MCA-38 tumor cell targets. Target cells (106) were labeled with 100 μCi of 51Cr for 1 h at 37°C. Labeled cells were then washed (3 times) and added in triplicate (106/well) to 96-well microtiter plates containing different numbers of effector cells to give various effector:target ratios (100:1–12:1). The plates were incubated for 4 h at 37°C, supernatant fluid was harvested, and the released 51Cr was measured in a Beckman Gamma 5500 counter. Cytotoxicity, expressed as the percentage of specific lysis was calculated as:

\[
\text{cpm experimental} - \text{cpm spontaneous} \times 100 \\
\text{cpm maximum} - \text{cpm spontaneous}
\]

One lytic unit is defined as that number of effector cells required to produce 30% lysis in the 4-h assay period based upon regression analysis.

Antibody Treatment. Effector cells (106/100 μl) were treated, in vitro, with anti-asialo-GM1 antibodies (10 μg/ml) and/or complement (1/20 dilution) for 1 h at 37°C. These cells were then used as effectors in the 51Cr release assay as described above. For in vivo studies, anti-asialo-GM1 antibodies (1 mg/kg) were administered i.p. on days 1, 4, 7, and 10 after tumor inoculation.

In Vivo Tumor Studies. MCA-38 tumor cells (3 × 105) were injected i.p. or intrasplenically on day 0. Following i.p. injection mice were monitored until death or held until 60–120 days from the time of inoculation at which time they were considered cured. Hepatic metastases were induced by intrasplenic injection as described by Lafreniere and Rosenberg (7). Briefly, mice were anesthetized with pentobarbital, the spleen was exposed, and 1 ml of tumor cells was injected into the spleen. Following a 1-min period the spleen was removed, the incision was closed, and all animals were randomly allocated to their respective treatment groups. On day 14 the mice were given a tail vein injection of India ink (15%) and killed by cervical dislocation. Liver metastatic foci, visible as white spots on the surface of the liver, are then counted.
under low magnification. A single daily i.p. dose of DAP (100/kg), in phosphate-buffered saline, was given from day 1 through day 10. Vehicle (phosphate-buffered saline)-treated animals served as controls.

Surface Phenotyping. The percentage of asialo-GM$_1$-positive splenic and peritoneal leukocytes was determined by staining with anti-asialo-GM$_1$ antibodies and analysis by flow cytometry. Leukocytes (10$^6$) were incubated with anti-asialo-GM$_1$ antibodies (1.2 mg/ml) or control rabbit IgG (1.2 mg/ml) for 30 min at 4°C. These cells were then washed and stained with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (10 lg/ml) for 30 min at 4°C. The cells were washed and analyzed on a Coulter Epics C flow cytometer with an argon laser at 488 nm. The percentage of asialo-GM$_1$-positive population was determined by setting a positive/negative gate containing >98% of the control IgG-staining population in the negative population. A minimum of 10$^6$ cells was analyzed for each sample.

Statistical Significance. Statistical significance was determined by a two-tailed Student's $t$ test.

RESULTS

The effect of a single dose of DAP (100 mg/kg) on splenic cytolytic activity, directed against NK-sensitive YAC-1 target cells, is presented in Figs. 1 and 2. DAP increased NK cell activity levels 2-3 times, compared with vehicle-treated control mice. Peak NK cells activity was observed 3 days following DAP treatment (Fig. 1). The increase in NK cell activity was not limited to the splenic population, since DAP also increased NK cell activity in the nonadherent peritoneal exudate cell population (Fig. 2). Cytolytic activity was not observed in the adherent peritoneal exudate cell population (data not shown). Furthermore, the effect of DAP on NK cells was dose dependent (Table 1). The NK cell activity observed in DAP-treated mice was slightly lower than that observed with Tilorone (Table 1), an established IFN and NK cell inducer (8).

It has been well established that murine NK cells express the surface antigen asialo-GM$_1$ (9). As shown in Fig. 3, DAP (100 mg/kg) treatment increased the frequency of splenic asialo-GM$_1$-positive leukocytes. In the DAP-treated group, approximately 15% of the leukocytes were asialo-GM$_1$ positive compared with 5% in the vehicle-treated control group. Treating the splenic leukocyte population, isolated from DAP (100 mg/kg)-treated mice, with anti-asialo-GM$_1$ antibody and complement in vitro completely eliminated detectable NK cell activity (Fig. 4). Furthermore, DAP did not induce any cytolytic activity capable of killing NK-resistant P815 or EL4 target cells (data not shown). However, the increase in splenic cytolytic activity seen in DAP-treated mice was not limited to YAC-1 targets. DAP treatment also potentiated NK cell activity directed against a NK-sensitive murine colon adenocarcinoma (MCA-38) (Fig. 5).

**Table 1** DAP increases NK cell activity in a dose-dependent manner

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Splenic NK cell activity$^a$ (lytic units/10$^6$ effector cells)</th>
<th>Peritoneal NK cell activity$^a$ (lytic units/10$^6$ effector cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>DAP (1 mg/kg)</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>DAP (10 mg/kg)</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Tilorone (100 mg/kg)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* DAP (i.p.) and Tilorone (p.o.) were administered 72 and 18 h, respectively, prior to assessing NK cell activity.
* NK cell activity was assessed against $^{31}$Cr-labeled YAC-1 target cells.
* PEC, peritoneal exudate cells; NT, not tested.
DAP POTENTIATES NK CELL ACTIVITY

The observed stimulation of NK cell activity by DAP prompted evaluation of the antitumor activity of the compound. In initial studies, MCA-38 tumor cells were injected i.p. and the mice were monitored daily for survival. Daily doses of DAP (100 mg/kg) increased mean survival time, with 10% of the treated animals alive for at least 60 days and considered cured (Table 2). Treating mice with asialo-GMa antibodies eliminated the increase in survival time observed for the DAP-treated group. Furthermore, mice treated with asialo-GMa antibodies exhibited no detectable NK cell activity, compared with mice treated with control IgG antibodies (data not shown).

The antitumor effect of DAP against MCA-38 tumor cells was also examined in an experimentally induced liver metastatic model. Tumor cells were injected directly into the spleen and liver metastatic foci were counted 2 weeks later. Daily doses of DAP (100 mg/kg) reduced the number of metastatic foci by 50% (Table 3). It is also noteworthy that the remaining tumor foci were substantially smaller in the DAP group than those found in control mice. DAP was without effect in the liver metastatic tumor model when NK-deficient beige mice (bg/bg) were used (data not shown), further indicating NK cell involvement in the observed antitumor activity of DAP.

**DISCUSSION**

We have shown in the present investigation that DAP displayed both NK cell potentiating activity and antitumor activity in vivo. The antitumor activity in DAP-treated mice could be eliminated by treating with asialo-GMa antibodies or utilizing NK-deficient beige mice. These results suggest that DAP antitumor activity, at least in part, is NK cell mediated. DAP is one of a series of acetylated diamines (polymethylene bisacetamides) which have been shown to be potent inducers of human and murine tumor cell differentiation (5, 10). One of these analogues, hexamethylenebisacetamide, has been recently shown to produce regression of cutaneous metastases in patients with breast and colorectal carcinomas (11). We have shown here that DAP has antimetastatic activity against a murine colon adenocarcinoma. Our results suggest that bisacetamide-mediated antitumor activity may be, at least in part, immune mediated.

DAP can be converted intracellularly to putrescine, spermidine, spermine, and acetylpolyamine derivatives (12). Whether an alteration in intracellular polyamine pools accounts for DAP-immunomodulating activity is unknown. We have previously established that inhibition of polyamine biosynthesis, utilizing an ornithine decarboxylase inhibitor a-difluoromethylornithine, did not impair NK cell activity in vivo (6), nor NK cell potentiation by IFN and IFN inducers (13).

DAP has been shown to have other immunomodulating properties. DAP is known to reduce B-cell proliferation in vitro without effecting T-cell proliferation (14). However, relatively high concentrations (≥3 mM) are required, which are very similar to those concentrations necessary to demonstrate an effect on cellular differentiation for most cell lines in vitro (5, 10). Similarly, we have observed herein that relatively high concentrations (10–100 mg/kg) are required to achieve increases in NK cell activity in vivo. Whether or not the immunomodulating activity observed is a result of differentiation-inducing ability of DAP remains to be determined. However, we have shown an increase in the frequency of asialo-GMa-positive cells, which may reflect an increase in NK cell differentiation. Other well-established NK cell inducers, such as IFN,

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**Table 2 Effect of DAP on survival of mice given injections of a murine colon adenocarcinoma (MCA-38)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (days)</th>
<th>% of increase</th>
<th>Cures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>16.7 ± 0.4 (46)</td>
<td>0/46</td>
<td></td>
</tr>
<tr>
<td>DAP (100 mg/kg)</td>
<td>22.8 ± 1.8 (41)</td>
<td>37/46</td>
<td></td>
</tr>
<tr>
<td>DAP + asialo-GMa</td>
<td>15.1 ± 0.4 (10)</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

* PBS or DAP (100 mg/kg) was administered i.p. on days 1–10. Anti-asialo GMa antibodies were administrated i.p. on days 1, 4, 7, and 10. Data compiled from 5 separate experiments (mean ± SE; n value is given in parentheses).

**Table 3 Effect of DAP on MCA-38 experimental liver metastasis**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metastatic foci</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>93.1 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>DAP (100 mg/kg)</td>
<td>46.7 ± 5.8e</td>
<td>50</td>
</tr>
</tbody>
</table>

* See Table 1 (mean ± SE, n = 39).

**Fig. 4. Effect of anti-asialo-GMa, and complement (C') treatment on splenic NK cell activity. DAP was administrated on day 0. On day 3 splenic leukocytes were treated with anti-asialo-GMa antibodies (ab) and/or complement. These cells were then used as cytolytic effectors against [5Cr-labeled YAC-1 cells (effector:target ratio, 50:1). Data are from one representative experiment. Columns, mean of triplicate determinations; bars, SE. Treatment with control IgG antibodies (ab + C') had no effect on cytolytic activity.**

**Fig. 5. Effect of DAP on splenic NK cell activity directed against MCA-38 tumor cell targets. Phosphate-buffered saline (O) or DAP (100 mg/kg) were administrated on day 0. NK cell activity was determined against [5Cr-labeled MCA-38 tumor cells on day 3. Data are from one representative experiment. Points, mean of triplicate determinations; bars, SE.**
DAP POTENTIATES NK CELL ACTIVITY

are known to increase the differentiation of NK cell precursors into mature cytolytic cells (15).

It is also possible that the NK cell-potentiating activity of DAP may require accessory cell function. This could explain why peak NK cell potentiation occurs several days following DAP treatment. In preliminary experiments, we have observed that DAP can potentiate IL-2 production.3 IL-2 is a well-established potentiator of NK cell activity (16). Experiments are in progress to assess the relationship, if any, between DAP, IL-2 production, and NK cells.

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