Inability of Anti-Asialo-GM₁ and 2-Chloroadenosine to Abrogate Maleic Anhydride-Divinyl Ether-induced Resistance against Experimental Murine Lung Carcinoma Metastases


Departments of Immunology Research [R. M. S., E. W. A., M. G. A.] and Infectious Disease Research [J. C. T., D. C. D.], Lilly Research Laboratories, Indianapolis, Indiana 46225

ABSTRACT

Both macrophages and natural killer cells have been implicated in the antimitostatic activity of maleic anhydride-divinyl ether (MVE-5). In the present study, we attempted to utilize anti-asialo-GM₁ antibody and 2-chloroadenosine, agents that kill natural killer (NK) cells and macrophages, respectively, to determine the relative contribution of each effector cell type to the overall host defense. These agents were tested in the M109 lung metastasis model in syngeneic BALB/c mice, and the cytotoxic activity of both peritoneal macrophages and splenic NK cells were followed. The most profound antitumor effect was observed when MVE-5 was given before rather than after i.v. tumor inoculation. Treatment i.p. with MVE-5 at 20 mg/kg produced >98% inhibition of subsequent lung metastases when given 2 days prior to tumor. Anti-asialo-GM₁ antibody (25 mg/kg, i.p.) and 2-chloroadenosine (50 mg/kg, i.p.) were administered concurrently with MVE-5. Although each agent exhibited greater selectivity for its respective target, the early (Day 2) inhibitory response was nonspecific. By Day 5 after MVE-5 treatment, 2-chloroadenosine only inhibited macrophage tumoricidal activity, and conversely, anti-asialo-GM₁ antibody only inhibited NK reactivity. Despite the ability of these agents to increase survival of metastases in control animals, they only slightly abrogated the antimitostatic activity of MVE-5. Our data suggest that caution should be exercised in using these agents to discriminate macrophage and NK responses.

INTRODUCTION

Recently, more attention has been paid to mechanisms of natural resistance, such as NK cells or activated (tumoridal) macrophages, in tumor surveillance. Since both effector cell types frequently coexist in the same population and since they share many of the same activation stimuli, it is difficult to assess the relative contribution of each in the overall host defense (1). Gray and coworkers (2) showed that the detection of either rapidly cytolytic macrophages or NK cells in "activated" peritoneal exudates from Bacillus Calmette-Guerin or Corynebacterium parum depends on the method of analysis and the target cell type. When the appropriate target cells (such as sarcoma M2) were used and the results of cell-fractionation experiments were evaluated in terms of recovery of total lytic units, the simultaneous presence of both cytotoxic macrophages and NK cells in peritoneal exudates could be readily demonstrated. The relative cytotoxic activity of either macrophages or NK cells was determined by the use of target cells with differential susceptibility.

The ability to discriminate between in vivo antitumor activi-

ties due to NK cells or activated macrophages appears to be more difficult. Treatment of mice with β-estradiol has been reported to cause a selective inhibition of NK cells and a resultant increase in the incidence of experimental and spontaneous pulmonary metastases of the syngeneic UV-2237 fibrosarcoma and K-1735 and B16 melanoma tumors (3). Numerous other investigators have utilized antibody against asialo-GM₁, a glycolipid surface marker on NK cells (4, 5), to indicate the involvement of NK cells in tumor resistance in vivo (6–9). Anti-asialo-GM₁ antibody has been shown to abrogate the antimitostatic activity induced by poly(I:C) (6), heparin (7), and prostaglandin I₂ (7) and increase the tumorigenicity of B-16 melanoma cells in an artificial metastasis model (9). Keller and coworkers (10) have observed that systemic inoculation of anti-asialo-GM₁ antibody selectively eliminated NK activity, leaving macrophage-type tumoricidal reactivity intact. In contrast, Saijo and coworkers (9) noted that the cytotoxic activity of macrophages activated by Nocardia rubra cell wall skeleton was decreased by in vivo treatment with anti-asialo-GM₁. Similarly, Akagawa and Tokunaga (11) noted that the cytotoxicity of peptide-induced peritoneal macrophages incubated with lymphokines or LPS was reduced by treatment with anti-asialo-GM₁ antibody plus complement.

2-Chloroadenosine has been shown to exhibit a selective lethal effect on mouse peritoneal macrophages (12, 13). These authors showed that splenic and thymic lymphocytes and polymorphonuclear cells were not killed, even by a long-term exposure to 2-chloroadenosine (13). In the present study, we sought to determine the effects of anti-asialo-GM₁ antibody and 2-chloroadenosine on the antimitostatic activity of MVE-5. In addition, we determined the effects of these agents on the enhanced cytotoxic activity of NK cells and macrophages induced by MVE-5.

MATERIALS AND METHODS

Mice. Male BALB/c mice, 6 to 8 wk old, were obtained from Harlan Industries, Inc., Madison, WI, and Charles Rivers Breeding Laboratories, Portage, MI. They were fed autoclaved chow and acidified-autoclaved water ad libitum.

Drugs. MVE-5 was supplied by Dr. D. S. Breslow (Hercules, Inc., Wilmington, DE). The compound was dissolved in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (GIBCO Laboratories, Grand Island, NY) at room temperature with constant stirring. Adenosine and 2-chloroadenosine were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit anti-asialo-GM₁ antibody was purchased from Wako Chemicals USA, Inc., Dallas, TX.

Induction of Experimental Lung Metastases. The M109 lung carcinoma, which arose spontaneously in a BALB/c mouse in 1964, was received from Dr. Joseph Mayo, Head, Mammalian Genetics, DCT, National Cancer Institute, Frederick Cancer Research Center, Frederick, MD. The M109 tumor has been maintained in our laboratory in BALB/c mice by s.c. inoculations of 2 × 10⁶ cells. A single cell suspension was prepared by trypsinizing nonneoplastic tumor sections for 1 h at room temperature in 0.25% trypsin (GIBCO Laboratories, Indianapolis, Indiana 46225).
DISCRIMINATION BETWEEN MACROPHAGE AND NK CELL

Grand Island, NY). After exposure to calf serum, tumor cells were washed twice and resuspended in serum-free Hanks' balanced salt solution without calcium and magnesium (M. A. Bioproducts, Walkersville, MD). The number of single viable cells was determined with a hemocytometer. To produce artificial pulmonary metastases, 3 to 7 x 10⁶ cells from four mice in each group were collected at various times after drug treatment by peritoneal lavage with calcium- and magnesium-free Hanks' balanced salt solution. The cells were washed and resuspended in RPMI 1640 medium (M. A. Bioproducts) prior to counting in a hemacytometer.

Preparation of Effector Cells. Noninduced BALB/c mouse peritoneal macrophages were prepared as previously described (14). Peritoneal cells from four mice in each group were collected at various times after drug treatment by peritoneal lavage with calcium- and magnesium-free Hanks' balanced salt solution. The cells were washed and resuspended in RPMI 1640 medium (M. A. Bioproducts) prior to counting in a hemacytometer.

For preparation of NK cells, spleens from four mice were pooled and minced through No. 60 stainless steel mesh cloth (Small Parts, Inc., IL). Cell suspensions were washed 3 times in RPMI 1640 medium. Cell pellets were pulse treated with distilled water to lyse RBC, washed in medium, and then incubated in nylon wool columns at 37°C for 20 min. The nylon wool nonadherent spleen cell populations were eluted from the column with prewarmed medium, washed, counted, and adjusted to the desired concentrations.

Labeling Target Cells. The murine mastocytoma cell line P815 was used as a target in the macrophage tumoricidal assay. Two x 10⁷ viable cells were resuspended in 2 ml of RPMI-FCS containing 200 μCi of Na₂⁵¹CrO₄ (New England Nuclear, Boston, MA; NEZ030S at 1 mCi/ml). The mixture was then incubated at 37°C in a CO₂ incubator for 90 min with occasional shaking. After incubation, the cells were pelleted by centrifugation and washed 3 times with 30-ml volumes of medium. The cells were then incubated an additional 1 h at 37°C to eliminate the rapid phase of Cr spontaneous release. Cr-labeled P815 cells were finally suspended at 2 x 10⁶/ml in RPMI-FCS.

The YAC-1 cell line, a Moloney virus-induced T-cell lymphoma of A/J origin, was purchased from the American Type Culture Collection (Rockville, MD) and used as a target cell for NK assays. YAC-1 cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, penicillin (ISO units/ml), and streptomycin (150 μg/ml). The YAC-1 cells obtained from suspension culture were washed twice with RPMI-1640 medium. Five million cells were resuspended in 1 ml of RPMI-1640 medium containing 100 μCi of Na₂⁵¹CrO₄. The mixture was then incubated at 37°C for 60 min with occasional shaking. After incubation, the cells were washed 3 times with RPMI-1640 medium containing 5% fetal calf serum to remove excess Na₂⁵¹CrO₄.

Macrophage Tumoricidal Assay. The ⁵¹Cr release tumoricidal assay used in these studies has previously been described (15). Briefly, noninduced peritoneal macrophages were resuspended at 1 x 10⁶ cells/ml in RPMI-FCS. Two hundred μl aliquots of this cell suspension were plated in wells on 96-well flat-bottomed tissue culture plates (Costar). The plates were incubated for 90 min at 37°C to allow the cells to adhere. After the adherence step, the wells were subjected to 2 cycles of vigorous washing to remove nonadherent cells. One hundred μl of RPMI-FCS and 100 μl containing 2 x 10⁴ ⁵¹Cr-labeled P815 cells were added to each well. All reactions were performed in triplicate. Plates were incubated at 37°C in 5% CO₂ in air. After an 18-h incubation, 100 μl of each culture supernatant were removed and analyzed for ⁵¹Cr release. Macrophage cytotoxicity to target cells was determined by the formula

\[
\text{Experimental release} - \text{spontaneous release} \times 100
\]

where experimental release = amount of isotope released from wells containing P815 targets and drug-treated macrophages; spontaneous release = amount released from wells containing P815 targets and non-drug-treated macrophages; and maximal release = amount obtained by freezing and thawing the target cells.

NK Cell Assay. The NK cell assay used in these studies has previously been described (16). Briefly, 5000 ⁵¹Cr-labeled YAC-1 target cells in 0.1 ml were added to wells on U-bottomed microtiter plates (NUNC; Grand Island Biological Co.). The nylon wool-nonadherent splenic effector cells were added in 3-fold serial dilutions into appropriate wells. Each experimental point was set up in triplicate. The plates were incubated at 34°C (humidified 5% CO₂ in air) for 16 to 18 h. After incubation, the plates were centrifuged for 2 min at 200 × g, and 100 μl of each supernatant were harvested and analyzed for ⁵¹Cr release. The percentage of ⁵¹Cr released was calculated as follows.

\[
\% \text{ of } ⁵¹\text{Cr release} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100
\]

where spontaneous release was determined by adding 100 μl of medium in place of effector cells to the target cells, and maximum release was determined by measuring total uptake of ⁵¹Cr by target cells.

RESULTS

Effect of 2-Chloroadenosine and Anti-Asialo-GM₁ Antibody on Macrophage Tumoricidal Activity. We tested whether simultaneous treatment with MVE-5 and either 2-chloroadenosine or anti-asialo-GM₁ antibody would inhibit the development of tumoricidal macrophages in vivo. As shown in Table 1, significant macrophage-mediated cytotoxicity against P815 cells was observed 2 days after MVE-5 treatment, peaked around 5 days, and had declined to base-line values by Day 9. At Day 2, 2-chloroadenosine and anti-asialo-GM₁ inhibited the MVE-5-induced response by 100% and 88%, respectively. Moreover, only macrophages from mice treated with 2-chloroadenosine did not respond to an additional activation stimulus (LPS) in vitro. At Day 5, when macrophage activation peaked, treatment with 2-chloroadenosine abrogated cytotoxicity by 69%. In contrast to macrophages taken 2 days after treatment, these cells could still respond to LPS in vitro. Anti-asialo-GM₁ antibody, unlike 2-chloroadenosine, did not affect macrophage function at Day 5.

### Table 1 Effects of anti-asialo-GM₁ antibody and 2-chloroadenosine on MVE-5-induced macrophage tumoricidal activity

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>Day of observation</th>
<th>% of tumoricidal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without LPS</td>
<td>With LPS*</td>
</tr>
<tr>
<td>PBS (0.2 ml)</td>
<td>2</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>PBS (0.2 ml) plus 2-CA (50 mg/kg)</td>
<td>2</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>PBS (0.2 ml) plus anti-ASGM₁ (25 mg/kg)</td>
<td>2</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg)</td>
<td>2</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg) plus 2-CA (50 mg/kg)</td>
<td>2</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg) plus anti-ASGM₁ (25 mg/kg)</td>
<td>2</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>PBS (0.2 ml)</td>
<td>5</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>PBS (0.2 ml) plus 2-CA (50 mg/kg)</td>
<td>5</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>PBS (0.2 ml) plus anti-ASGM₁ (25 mg/kg)</td>
<td>5</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg)</td>
<td>5</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg) plus 2-CA (50 mg/kg)</td>
<td>5</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>MVE-5 (20 mg/kg) plus anti-ASGM₁ (25 mg/kg)</td>
<td>5</td>
<td>41 ± 1</td>
</tr>
</tbody>
</table>

* Drugs were administered i.p. on Day 0.

* To test inducible cell function, LPS was added in vitro at 10 μg/ml.

* PBS, phosphate-buffered saline (Dulbecco's); 2-CA, 2-chloroadenosine; ND, not determined due to macrophage depletion in vivo by 2-chloroadenosine; anti-ASGM₁, anti-asialo-GM₁.

* Mean ± SE of triplicate determinations.
Effect of 2-Chloroadenosine and Anti-Asialo-GM\textsubscript{1} Antibody on NK Cell Activity. As shown in Table 2, NK cell cytotoxicity for YAC-1 targets peaked 2 days after i.p. MVE-5 treatment, was still evident on Day 5, and had declined to base-line values by Day 9. 2-Chloroadenosine at 50 mg/kg decreased the yield of nylon-wool-nonadherent cells per spleen by 50\% at Day 2. On Day 2, both 2-chloroadenosine and anti-asialo-GM\textsubscript{1} antibody inhibited >97\% of MVE-5-induced cytotoxicity (effector:target ratio, 30:1). In contrast, only anti-asialo-GM\textsubscript{1} antibodies significantly inhibited cytotctoxic activity at Day 5 (80\% inhibition at 30:1 ratio; \(P < 0.01\)). Anti-asialo-GM\textsubscript{1} antibody inhibited not only MVE-5-induced activity, but spontaneous NK activity against YAC-1 cells for up to 9 days.

Effects of MVE-5, 2-Chloroadenosine, and Anti-Asialo-GM\textsubscript{1} Antibody on Experimental M109 Metastases. Earlier studies have shown that treatment with pyran (MVE) initiated 5 days before to 1 day after i.v. tumor inoculation can significantly retard lung metastasis development (17, 18). We sought to determine the effect of delayed treatment. The results in Table 3 show that treatment delayed to Day 4 after tumor inoculation did not significantly retard formation of lung metastases, whereas treatment at earlier times was highly inhibitory (\(P < 0.05\)). These results indicate that the antimetastatic activity of MVE-5 is observed only in those animals placed on therapy soon after i.v. tumor cell challenge. This “window” of protective activity suggested that we could probe the mechanism of MVE-induced resistance with agents that compromise the tumoricidal activities of NK cells and macrophages during the first few days after tumor inoculation.

Treatment with either 2-chloroadenosine or anti-asialo-GM\textsubscript{1} 2 days prior to i.v. tumor inoculation promoted the formation of lung metastases 3.34-fold and 3.88-fold, respectively (\(P < 0.01\)) (Table 4). In sharp contrast, these same two treatments could only slightly increase the number of metastases in MVE-5-treated animals. Adenosine, a compound that does not significantly alter macrophage tumoricidal function or viability \textit{in vitro} (data not shown), did not increase the incidence of experimental metastases in control or MVE-5-treated mice.

DISCUSSION

A series of synthetic MVE polyanions with molecular weights ranging from 12,500 to 52,600 have been reported to activate macrophages (17–21) and augment NK activity (20, 21). Since both cell types have been implicated in MVE-induced tumor surveillance (17, 18, 21–23), the present study was undertaken to determine the relative protective activity of each of these two cell types in an experimental lung metastasis model (M109) in syngeneic BALB/c mice. The selective macrophage inhibitor, 2-chloroadenosine (12, 13), and the selective NK cell inhibitor, anti-asialo-GM\textsubscript{1} antibody (4, 5), were tested for their ability to (a) abrogate MVE-5-induced antimetastatic activity, and (b) inhibit the enhanced killing capacity of MVE-5-treated macrophages and NK cells.

Several investigators have utilized asialo-GM\textsubscript{1} to define the effector cell types involved in tumor resistance (6–10, 24, 25). Kawase and coworkers depleted NK cell activity \textit{in vivo} with rabbit anti-asialo-GM\textsubscript{1} serum and studied its effect on the growth of NK-sensitive and NK-resistant tumor cell variants (6). They showed that the inhibitory effect on NK activity was selective, as treatment with anti-asialo-GM\textsubscript{1} did not affect the development of other cytotoxic cells, including cytotctoxic macrophages, following injection of poly(I:C) and cytotoxic T-cells in response to allogeneic cells. Treatment of DBA/2 mice and BALB/c-nu/nu mice with anti-asialo-GM\textsubscript{1} serum led to a marked increase in tumorigenicity of NK-sensitive, but not NK-resistant tumor cell variants. Moreover, poly(I:C) treatment of mice boosted NK activity and markedly depressed the growth of an NK-sensitive subline of the murine lymphoma L5178Y. This protective activity was abrogated by anti-asialo-GM\textsubscript{1} serum. Gorelik and coworkers studied the antimetastatic effects of heparin and prostacyclin in normal mice and in mice with
depressed or activated NK cell activity (7). Both anticoagulants inhibited the formation of lung metastases after inoculation of the F1 or F10 sublines of B16 melanoma. Inhibition of NK cell activity by treatment with anti-asialo-GM₁ serum was associated with abrogation of the antitumoral effects of prostacyclin or heparin. Barlozzari and coworkers investigated the role of NK cells in the control of the metastatic spread of MADB106 mammary adenocarcinoma cells (8). They similarly showed that treatment with anti-asialo-GM₁ serum selectively impaired NK function without detectable effects on T-cell-mediated immunity or on the cytotoxic activity of alveolar macrophages. This treatment caused an increased incidence of experimental pulmonary metastases following injection of MADB106 tumor cells. We observed that treatment with either anti-asialo-GM₁ or 2-chloroadenosine 2 days prior to i.v. tumor inoculation promoted the formation of lung metastases 3.34-fold and 3.88-fold, respectively.

The presence of asialo-GM₁ on the surface of different cell types has raised the question of its value as a specific marker for NK cells (11, 26–28). Akagawa and Tokunaga presented evidence that the appearance of asialo-GM₁ on the macrophage surface is associated with induction of tumoricidal activity (11). They also observed the appearance of asialo-GM₁ on the cell surface of M1 myeloid leukemia cells during lymphokine-induced differentiation into macrophages (26). Arndt and coworkers studied the cellular distribution of asialo-GM₁ in the rat (27). They observed that this antigen is not only expressed on lymphocytes of the T-lineage and on macrophages, but also on eosinophilic cells. Wiltrout and coworkers studied the reactivity of anti-asialo-GM₁ with tumoral and nontumoral mouse macrophages (28). They noted that the use of eliciting agents, particularly thioglycollate, or eliciting agents in conjunction with activating agents can cause peritoneal macrophages to become reactive with anti-asialo-GM₁ serum. Saijo and coworkers observed that the tumoricidal activity of macrophages treated with Nocardia rubra cell wall skeleton was decreased by in vivo treatment with anti-asialo-GM₁ antibody (9). We similarly observed that antibody treatment impaired the early (Day 2) response of MVE-5-treated macrophages. Other investigators have noted that treatment with anti-asialo-GM₁ antibody did not diminish tumoricidal activity of macrophages induced by C. parvum (10) or OK-432 (25). It is not known whether the discrepancy between these results is due to differences in activating agents used, assay procedures, or the time after treatment when activity was assessed. Despite the ability of anti-asialo-GM₁ treatment to increase the tumorigenicity of M109 carcinoma cells (Table 4) and its ability to markedly inhibit both spontaneous and MVE-5-induced NK cell function over 5 days (Table 2), it only slightly increased the number of metastases in MVE-5-treated animals (Table 4).

2-Chloroadenosine has previously been demonstrated to be a useful cytotoxic agent for inhibiting the accessory functions of macrophages in vitro (12). This compound did not, however, affect viability of lymphocytes, polymorphonuclear cells, and a number of established tumor cell lines. In the present study, we noted that the tumoricidal activity of both natural killer cells and macrophages was totally inhibited at 2 days after treatment with 2-chloroadenosine at 50 mg/kg. By Day 5, only macrophage-mediated cytotoxicity was inhibited. Despite the ability of 2-chloroadenosine treatment to increase the tumorigenicity of M109 carcinoma cells (Table 4) and its capacity to markedly inhibit MVE-5-induced macrophage function over 5 days (Table 1), it only slightly increased the number of metastases in MVE-5-treated animals (Table 4).

Finally, our data show that the inhibition of hematogenous tumor metastasis by MVE-5 closely corresponded with the kinetics of macrophage and NK cell activation. Attempts to abrogate the antitumor activity of MVE-5 with agents which inhibit macrophage and NK cell function were unsuccessful. Further studies are needed to determine whether macrophage and NK cell functions were similarly impaired in the microenvironment of the tumor. The ability of 2-chloroadenosine and anti-asialo-GM₁ antibody to enhance the number of lung lesions developing in normal mice (Table 4) suggests that host defense mechanisms in the lung were compromised. The lack of total specificity of anti-asialo-GM₁ antibody and 2-chloroadenosine for macrophages and NK cells, respectively, suggests that caution be exercised in interpreting results with these inhibitors.

REFERENCES

DISCRIMINATION BETWEEN MACROPHAGE AND NK CELL


Inability of Anti-Asialo-GM₁ and 2-Chloroadenosine to Abrogate Maleic Anhydride-Divinyl Ether-induced Resistance against Experimental Murine Lung Carcinoma Metastases


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/11/5624