Increased Tumor Immunity in Mice Inoculated with Muconomycin A-treated B16 Melanoma Cells

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ABSTRACT

B16 melanoma cells were treated in vitro with muconomycin A, a long-lasting inhibitor of protein and glycoprotein synthesis, to reduce cellular sialic acid. Two i.p. inoculations of 10⁷ muconomycin-treated cells into female C57BL/6 mice, followed by challenge with homologous live cells, resulted in a significant decrease in tumor incidence when compared to the results of inoculation with untreated cells (p < 0.01). Inoculation of mice with cells treated with neuraminidase resulted in little or no decrease in tumor incidence. Effective immunity was dependent on the number of cells injected and was found only with the i.p. route of inoculation into female mice.

INTRODUCTION

Several studies have demonstrated that drug-treated cells may be used to increase immunogenicity against the parent tumor cells and that this increase may be due to the appearance of new antigens (7, 20, 22, 23, 30, 31). (For a review, see Ref. 24). The specific removal of sialic acid from the surface of tumor cells with VCN and the increased immunity resulting from their injection has aroused interest in both experimental and clinical settings. Results of experimental studies in mice from different laboratories have been contradictory, however, and both inhibition and enhancement of tumor growth have been reported. For example, if L1210 cells were treated with VCN and then injected into mice, strong tumor immunity resulted (2-5, 17, 27, 29). Methylcholanthrene-induced tumors and the B16 mouse melanoma partially or completely regressed if mice bearing these tumors were given injections of homologous VCN-treated tumor cells (26). Other reports, in contrast, noted enhanced tumor growth in mice following inoculation with VCN-treated cells in both the EARK leukemia systems (14) and B16 melanoma (15). VCN-treated cells have also been used for active immunotherapy in clinical trials on patients with acute myelogenous leukemia (2-4, 17). Although VCN removes about one-half of the sialic acid on the surface of the cell, sialoglycoproteins regenerate rapidly (18, 19). We thought that if this regeneration could be prevented by additional treatment with agents that prevent glycoprotein synthesis, the period over which VCN-treated cells could act as effective immunogens might be prolonged.

In work reported in previous papers (8, 9), we tested in vitro the ability of 2 inhibitors of protein synthesis, emetine and muconomycin A, to prevent restoration of sialic acid to cells that had been treated with VCN. B16 melanoma cells treated with 10⁻⁴ M emetine and then washed repeatedly (to reproduce in vivo conditions) recovered their ability to incorporate [³H]leucine in 12 hr. After similar treatment with muconomycin, incorporation of [³H]leucine remained inhibited for at least 30 hr. During this time the treated cells were able to exclude trypan blue dye and to retain nearly normal concentration levels of ⁸⁶Rb⁺ indicating that they had remained intact.

Treatment with VCN removed 35 to 45% of the sialic acid from the surface of B16 melanoma cells (9). Measurement of glycoprotein synthesis in cells treated with both VCN and muconomycin, as determined by sialic acid analysis, showed that muconomycin prevented restoration of sialic acid after VCN treatment. Moreover, even without prior VCN treatment, muconomycin caused sialic acid deficiency in cells after 24 hr, presumably because new glycoproteins were not being made as old molecules were lost from the cell. Hence muconomycin appeared to be useful both in producing and in maintaining sialic acid deficiency in intact tumor cells over a long period. To extend our earlier work to in vivo studies, we tested the immunogenicity of cells treated with various combinations of VCN, emetine, and muconomycin.

Here we present a short report describing the effectiveness of muconomycin-treated cells in inducing immunity when injected into mice that were then challenged with homologous live, untreated tumor cells. We chose the mouse melanoma because only weak tumor immunity has been found in this system and because contradictory results on the effects of inoculating VCN-treated B16 cells have been reported (15, 26). Both humoral and cellular immune mechanisms have been shown to be increased by immunization with VCN-treated cells (2-4, 11, 17, 27, 29), although enhancement of tumor growth may occur, even in the presence of cytotoxic lymphocytes (15). We have, therefore, along with others (5, 6, 14, 29), used the ability of the host to reject a live tumor transplant as the index of tumor immunity.

MATERIALS AND METHODS

Tumors, Mice, and Cell Cultures. The B16 melanoma and C57BL/6J mice (6 to 10 weeks old) were purchased from The Jackson Laboratory, Bar Harbor, Maine. B16 cells, cultured from the tumor, and syngeneic fibroblasts...
derived from skin explants, were grown in 100- x 20-mm culture dishes (Falcon Plastics, Oxnard, Calif.), in Dulbecco’s MEM (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum (Reheis Chemical Co., Phoenix, Ariz.) and penicillin and streptomycin. No evidence of contamination of cultures by Mycoplasma was found in periodic measurements of the ratio of incorporation of radiolabeled uracil to uridine (28). Because B16 cells appeared to become increasingly immunogenic after prolonged growth in culture (data not shown), cells used for all experiments reported here were set up freshly every 3 months from stocks frozen in liquid nitrogen. These stocks were originally prepared from a minced B16 tumor, grown for a short period in culture. Comparable cells from these stocks had previously been used to measure surface sialic acid after treatment with VCN and muconomycin. Because the reduction in sialic acid content 24 hr after treatment with these agents was very reproducible (9), we did not think it was necessary to repeat the sialic acid determinations on cells used for immunization.

For either immunization or challenge, cells were removed from culture dishes with 0.02% EDTA in calcium-magnesium-free phosphate-buffered saline and washed twice in Dulbecco’s MEM without serum.

Inhibitors of Protein and DNA Synthesis. An aqueous stock solution of emetine (Sigma Chemical Co., St. Louis, Mo.) was stored at −20°. Muconomycin, a gift of Dr. R. K. Johnson, NIH (Bethesda, Md.), was dissolved in dimethylsulfoxide as a 0.01 M stock solution and kept at 4°. Powdered mitomycin C (a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute) was mixed with NaCl in a ratio of 1:24 to increase solubility, and the solution (mitomycin at 500 µg/ml) was incubated at 37° overnight in the dark and then stored at 4°.

Treatment of Cells with VCN, Emetine, Muconomycin A, and Mitomycin C. In a previous study, we had found that treatment of tumor cells with inhibitors of protein synthesis did not prevent cell proliferation indefinitely; after 10⁸ M emetine, virtually all of the cells, and after 10⁻⁸ M muconomycin, about 20% of the cells, recovered the ability to grow if they were thoroughly washed (9). For experiments involving inoculation of cells into mice, we wanted to be certain that treated tumor cells did not proliferate and thereby complicate our interpretation of the results of tests for induction of immunity. Cells that were treated with any agent (VCN, emetine, or muconomycin), therefore, were also treated with mitomycin. [For a similar procedure, see the paper of Rios and Simmons (26).] Measurement of [¹⁴C]leucine incorporation into trichloroacetic acid-precipitable material showed that the concentration of mitomycin used did not affect protein synthesis for at least 24 hr although by 48 hr there was a 50% decrease (data not shown; Ref. 8).

Cells were removed from culture dishes and, after 2 samples were counted in a hemocytometer, the cells were treated with drugs. Six different drug treatments were used. Cells, both with and without prior treatment with VCN, were treated with mitomycin alone, with mitomycin and emetine, or with mitomycin and muconomycin. When cells were treated first with VCN, 9 ml of serum-free Dulbecco’s MEM containing 10⁶ cells were mixed with 1 ml of VCN (500 units; 1 unit of activity is equivalent to the release of 1 µg of N-acetylmuramic acid from a glycoprotein substrate at 37° in 15 min at pH 5.1 (Grand Island Biological Co.). Cells not treated with VCN were incubated in Dulbecco’s MEM alone. After 1 hr at 37°, cells were washed twice in serum-free Dulbecco’s MEM and then treated further with mitomycin and inhibitors of protein synthesis, as follows: 5 ml of the stock solution of mitomycin (500 µg/ml in 0.9% NaCl solution) were mixed with an equal volume of serum-free Dulbecco’s MEM containing 10⁴ cells and either emetine (10⁻⁵ M), muconomycin (10⁻⁸ M), or nothing else. The cell suspensions were incubated for 1 hr at 37°, washed twice, and injected into mice.

Injection of Mice. All cells were freshly prepared for each inoculation. Mice were given 2 injections of drug-treated cells, 2 weeks apart, i.p. or s.c., in the left thigh, followed by a dorsal s.c. challenge 10 days later with 2 × 10⁸ homologous live cells. (This inoculum is about 20 times the number of B16 cells needed to induce tumors in 50% of mice.) A control group that had received no immunizing inoculations was also challenged with live cells. All injections were made in a 0.5 ml volume and all suspensions were either pooled or diluted to 0.5 ml to reach the required cell concentration. Mice were examined twice weekly for the presence and size of tumors. Comparisons of results of different treatments were made by χ² analysis.

RESULTS

In the text and tables, cells treated with mitomycin alone are called “untreated.” When cells were also treated with VCN, emetine, or muconomycin, only these 3 drugs are mentioned and reference to mitomycin is omitted.

Immunogenicity of 10⁷ Cells Treated with VCN, Emetine, or Muconomycin A. Groups of female mice received 2 i.p. injections of B16 cells, untreated or treated with VCN, emetine, or muconomycin, alone or in combination followed by challenge with homologous live tumor cells. In unimmunized mice tumors began to appear within 1 week of challenge and mice began to die by about 30 days. By 40 days 20 to 90% of these mice were dead, depending upon the experiment. Tumor-bearing mice that died before 40 days were scored as tumor positive. In immunized mice, tumors sometimes developed later but were maximally present by 21 days after challenge. On several occasions during the first 14 to 21 days after challenge, we noted the appearance and subsequent disappearance of small tumors (<2 mm diameter) at the challenge site in mice immunized with muconomycin-treated cells; these mice were scored as tumor free. Tumors never regressed in mice immunized with untreated cells or with cells treated with VCN or emetine, either alone or in combination. Compared to unimmunized mice death of immunized tumor-bearing mice was not delayed. Hence we chose to terminate the experiments at 40 days after challenge.

Forty days after challenge with live tumor cells (Table 1), 34% of female mice given injections of cells treated with muconomycin alone showed tumors, as did 28% given injections of cells treated with both VCN and muconomycin. Both of these tumor incidences are significantly less than the 74% seen in mice given injections of untreated cells.
In mice given injections of cells treated with VCN alone, 46% showed tumors, an incidence less than in mice given injections of untreated cells, and greater than in the group receiving muconomycin-treated cells, but not significantly different from either group. Cells treated with emetine gave no protection. Thus, of all the drugs tested, muconomycin was most effective in inducing tumor immunity.

Male mice, unlike females, showed no immunity 40 days after challenge with live tumor cells (Table 2), even if they had been given injections of muconomycin-treated cells. In 2 separate experiments the tumor incidence in unimmunized male and female mice was found to be the same, but the initial growth rate was slower for females. In one experiment, the mean tumor diameter in a group of 8 female mice 14 days after challenge was 0.45 ± 0.10 cm (S.E.), while that of 8 males was 1.02 ± 0.13 cm.

Table 1
Immunogenicity of 10^7 B16 melanoma cells treated with VCN, emetine, or muconomycin A in female mice

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Summary 1</th>
<th>% with tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (not inoculated)</td>
<td>7/10</td>
<td>20/20</td>
<td>8/8</td>
<td>35/38</td>
</tr>
<tr>
<td>&quot;Untreated&quot;</td>
<td>7/10</td>
<td>7/10</td>
<td>4/8</td>
<td>18/28</td>
</tr>
<tr>
<td>VCN</td>
<td>5/10</td>
<td>4/10</td>
<td>4/8</td>
<td>13/28</td>
</tr>
<tr>
<td>Emetine</td>
<td>7/10</td>
<td>8/10</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/20</td>
</tr>
<tr>
<td>VCN and emetine</td>
<td>2/10</td>
<td>9/10</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/20</td>
</tr>
<tr>
<td>Muconomycin</td>
<td>7/20</td>
<td>5/20</td>
<td>4/8</td>
<td>16/48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>4/10</td>
<td>3/20</td>
<td>4/8</td>
<td>11/38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, not tested.
<sup>b</sup> Significant at p < 0.01 when compared to untreated group.

Effect of Number of Cells Inoculated and Route of Immunization on the Immune Response in Female Mice.

Mice were given injections of 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> B16 cells treated with VCN and muconomycin or with muconomycin alone. Table 3 shows that only mice given 10<sup>7</sup> cells were significantly protected (p < 0.01).

To see the effects of further increasing the number of cells used for immunization and to determine the most effective route of immunization, we immunized mice i.p. or s.c. with 3 x 10<sup>7</sup> cells that were untreated or treated with VCN, muconomycin, or both. Forty days after challenge, virtually all mice immunized i.p. were tumor free, while mice immunized s.c. were not (Table 4).

As a test of tumor specific immunity, C57BL mice were given 2 i.p. injections of 3 x 10<sup>7</sup> syngeneic fibroblasts treated with muconomycin and challenged with live B16 cells. Ten days after challenge with B16 cells 7 of 8 mice given injections of syngeneic fibroblasts had tumors, indicating that syngeneic fibroblasts and B16 cells do not share tumor rejection antigens.

Table 2
Immunogenicity of 10<sup>7</sup> B16 melanoma cells treated with VCN or muconomycin A in male mice

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Summary 1</th>
<th>% with tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (not inoculated)</td>
<td>8/8</td>
<td>8/8</td>
<td>16/16</td>
<td>100</td>
</tr>
<tr>
<td>&quot;Untreated&quot;</td>
<td>8/8</td>
<td>7/8</td>
<td>15/16</td>
<td>93</td>
</tr>
<tr>
<td>VCN</td>
<td>8/8</td>
<td>7/8</td>
<td>15/16</td>
<td>93</td>
</tr>
<tr>
<td>Muconomycin</td>
<td>7/7</td>
<td>7/8</td>
<td>14/15</td>
<td>83</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>6/8</td>
<td>8/8</td>
<td>14/16</td>
<td>87</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, not tested.
<sup>b</sup> Significant at p < 0.01 when compared to inoculum of 10<sup>5</sup> cells.

Table 3
Effect of the number of B16 cells inoculated on the immune response in female mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>No. of mice with tumor/no. of mice in group at 32 days after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (not inoculated)</td>
<td>6/9</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt; cells treated with</td>
<td></td>
</tr>
<tr>
<td>Muconomycin</td>
<td>7/10</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>8/10</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt; cells treated with</td>
<td></td>
</tr>
<tr>
<td>Muconomycin</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>7/10</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt; cells treated with</td>
<td></td>
</tr>
<tr>
<td>Muconomycin</td>
<td>2/10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>2/10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, not tested.
<sup>b</sup> Significant at p < 0.01 when compared to inoculum of 10<sup>5</sup> cells.

Table 4
Immunogenicity of 3 x 10<sup>7</sup> B16 melanoma cells in female mice

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Route of inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (not inoculated)</td>
<td>i.p.</td>
</tr>
<tr>
<td>&quot;Untreated&quot;</td>
<td>10/10</td>
</tr>
<tr>
<td>VCN</td>
<td>0/10</td>
</tr>
<tr>
<td>Muconomycin</td>
<td>1/20</td>
</tr>
<tr>
<td>VCN and muconomycin</td>
<td>1/10</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, not tested.
DISCUSSION

We have shown that immunization of female C57BL mice with B16 melanoma cells treated with muconomycin, with or without VCN, resulted in significant immunity when compared to immunization with untreated cells (p < 0.01). Cells treated with neuraminidase alone, however, were not significantly different from untreated cells in their ability to immunize (Table 1).

In contrast, injection of cells into male mice gave no immunity, even if cells were treated with muconomycin (Table 2). Thus an important difference exists between female and male mice in their ability to respond to immunization with B16 melanoma cells. Proctor et al. (25) have reported that B16 cells grow more slowly in female mice than in males but that, after oophorectomy, the growth rate of tumors in females is similar to that in both normal and castrated males. The authors conclude that there is (a) a clear association between hormone function of intact ovaries and a slower growth rate in vivo of the B16 melanoma and (b) no apparent effect of androgen depletion in males.

Our data support the results reported by Proctor et al. (25). Possibly, the increased immunity seen in female mice may reflect a synergism between female endocrine factors and the immune system. As a result, because the tumor grows more slowly in female mice, the immune system may effectively prevent the growth of the challenge inoculum, whereas tumor growth may outstrip the immune response in male mice. Similar sex differences have been reported in studies on allograft rejection in mice (13, 16).

The increased immunity produced by treatment of tumor cells with muconomycin was apparent only with a specific number of cells in the inoculum, given i.p. Thus, i.p. injection of 10^6 muconomycin-treated cells resulted in significant tumor immunity, but 10^6 cells were without effect. Similarly, i.p. injection of cells treated with VCN alone or s.c. injection of muconomycin-treated cells had little or no effect. However, if as many as 3 x 10^6 cells were injected i.p., even without prior treatment with muconomycin, strong immunity was induced. These results are similar to those of Bystryn et al. (10), who found that inoculations of large numbers of irradiated B16 cells into female mice produced immunity, and, more generally, with results reviewed by Prager and Baechtel (24).

Our data showing little or no increase in immunity with VCN-treated cells differ from those of others, perhaps because of variations in experimental design and tumor cell lines. Rios and Simmons (26), using female C57BL mice, found that s.c. injection of 10^6 cells treated with VCN resulted in partial regression of tumors. Different results are described by Froese et al. (15), who found that mice given 2 s.c. injections of 3 x 10^4 irradiated and VCN-treated B16 cells, and then challenged with live homologous cells, showed enhanced tumor growth.

Successful immunotherapy with VCN-treated L1210 cells has been associated with a marked increase in the concentration of serum immunoglobulins (2-4, 11, 17, 27), as well as with increased cell-mediated cytotoxicity (2-4, 15, 17). We do not know whether muconomycin-treated cells stimulate an increase in immune mechanisms or whether the increased immunogenicity of muconomycin-treated cells is related to their prolonged decrease in sialic acid. We suggest, however, 2 possible immunologically mediated mechanisms to explain our results.

First, muconomycin may have caused a decrease in turnover of glycoproteins on the surface of the tumor cells used for immunization. We have, in fact, previously shown that prolonged inhibition of protein and glycoprotein synthesis results in a net loss of cell surface sialoglycoproteins (8, 9). This decrease may then have resulted in exposure of antigenic sites on the cell surface because of loss (without replacement) of proteins and glycoproteins from the cell surface.

A second possibility, but one for which we have no data, is that treatment of cells with muconomycin may have reduced the amount of soluble antigen shed from the cell surface. The reduction in shedding, in turn, may have prevented the formation of blocking factors (1) and permitted the expression of cell-mediated (rather than humoral) immunity (20, 21). TA3-St tumor cells, for example, are known to shed small amounts of soluble antigen and these cells, unlike their shedding counterparts, Ta-3Ha, cannot grow in allogeneic recipients (12), perhaps because blocking factors were not produced.

In assessing the results of reports from different laboratories, it is important to note whether interpretations are based on similar criteria. In interpreting our data, for example, we compared the results of inoculation of muconomycin-treated or VCN-treated cells with untreated cells (i.e., cells treated with mitomycin alone), primarily to see whether treatment with muconomycin was more effective than treatment with VCN. We could, instead, have compared the results of inoculation of treated cells with controls that received no inoculation at all, except for challenge with live tumor cells (see Table 1). The tumor incidence in mice inoculated with VCN-treated cells (46%) could then be interpreted as significantly different from the incidence (92%) in mice not immunized. The results of muconomycin treatment would then appear to be even more striking.

Our results indicate that treatment of tumor cells with muconomycin increases their immunogenicity. If muconomycin-treated cells are to be used for studies on tumor immunity, optimum dose and treatment schedules will probably have to be developed for each tumor system.

REFERENCES

C. E. Brinckerhoff and M. Lubin


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