Intratumor Chemoimmunotherapy with Mitomycin C and Components from Mycobacteria in Regression of Line 10 Tumors in Guinea Pigs

Charles A. McLaughlin, John L. Cantrell, Edgar Ribi, and Eugene P. Goldberg

ABSTRACT

Intratumor chemotherapy with the use of mitomycin C and/or immunotherapy caused regression of line 10 carcinomas in strain 2 guinea pigs and resulted in development of tumor-specific immunity. The immunotherapeutic preparation consisted of oil-in-water emulsions containing Mycobacterium cell walls or residues of cell walls termed cell wall skeletons. The latter preparations were combined with trehalose dimycolate, which was isolated by microparticulate chromatography from whole cells of mycobacteria. Reducing mitomycin C to a single intratumor injection of 50 µg produced little necrotizing effect and a mean tumor regression rate of 17%. Intratumor immunotherapy 1 day after treatment with 50 µg of mitomycin C resulted in regression of 90% of the treated tumors as compared to mean regression rates of 30 to 50% for immunotherapy alone. In addition, chemoimmunotherapy was more effective than either chemotherapy or immunotherapy alone in producing regression of relatively large, as well as smaller, tumors.

INTRODUCTION

Intratumor, but not systemic, injections of mitomycin C or other chemotherapeutic agents into established line 10 carcinomas have not resulted in tumor regression and development of tumor immunity (2). Intratumor injections of immunopotentiating agents, such as viable BCG(1) (23, 24), cell walls from BCG (25, 26), and deproteinized-delipidated cell walls combined with trehalose dimycolate (11, 16), also produced regression of line 10 tumors. Chemotherapy combined with immunotherapy was more efficacious than either regimen alone in the treatment of neoplastic disease in humans (7, 13, 22) and animals (3, 8, 12). We are not aware, however, of any reported study wherein solid-tumor intratumor chemotherapy was combined with intratumor immunotherapy. This combined intratumor approach seemed promising in view of the effectiveness of each of the modalities alone. The purposes of this study were to determine whether intratumor treatment of tumors by chemoimmunotherapy was more effective than either treatment alone and to determine whether the combined therapy could be modulated to reduce the necrotizing effect of mitomycin C inoculated into the tumor.

1 The abbreviations used are: BCG, Mycobacterium bovis strain of Bacillus Calmette-Guerin; CWS, cell wall skeleton that is a deproteinized, delipidated cell wall residue; P3, α-α-trehalose, 6,6'-dimycolate purified by microparticulate chromatography.

Received October 20, 1977; accepted January 30, 1978.

MAY 1978 1311

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nonsporulated bacteria.

Incorporation of Radioactive Thymidine by Line 10 Cells. Line 10 cells grown in the peritoneal cavity were harvested, washed, and adjusted to 10⁶ cells/ml of Eagle's minimum essential medium. The cells (1.0-ml aliquots) were incubated with mitomycin C at 37°C for 1 hr in tightly sealed, screw cap plastic tubes. The cells were washed 3 times by centrifugation through 10 ml of Eagle's medium at 4°C. The washed cells were suspended in 20 ml of Eagle's medium, and 0.1-ml aliquots (5 x 10⁴ cells) were incubated with [³H]thymidine (13 μCi/ml) in flat-bottomed Linbro trays for 1 hr at 37°C. The cells were collected by use of a multiple automated sample harvester (Microbiological Associates, Bethesda, Md.) on glass fiber filters and washed with 0.15 M NaCl followed by 5% (w/v) trichloroacetic acid. The filters were air dried and were placed in 5 ml of PPO:toluene, and the amount of radioactive decay was determined by liquid scintillation counting with the use of the Beckman LS-150 counter (Beckman Instruments, Fullerton, Calif.).

Statistical Analyses. The probabilities that mean rates of tumor regression in treated groups differed from those of control groups or other treated groups were estimated by Student's t test. Probability values (p) were obtained from the tables of Finney (5) based on the Fisher calculation of exact probability in a 2 x 2 contingency table (19). The acceptable level of significance was set at p < 0.05 in a 2-tailed test. In addition, we applied Wilcoxon's signed-rank test (20) (see Table 3) to ascertain whether a particular treatment regimen produced regression rates that tended to be significantly different from another set of tumor regression rates.

RESULTS

Successful intratumor treatment of 6-day-old, line 10 tumors depended upon the dosage of mitomycin C (see Table 1). Maximal percentages of tumor regression (approximately 80%) were obtained with single injections of either 250 or 500 μg. The efficacy was reduced to 50 and 17% for 100- and 50-μg doses, respectively. Because intratumor injections of 500 μg caused appreciable necrosis of normal dermis (Fig. 1A), lower levels of mitomycin C were combined with immunotherapy to determine whether maximal percentages of tumor regression could be obtained with minimal necrosis. Indeed, this was the case when animals received 50 μg of mitomycin C 6 days after transplantation in addition to CWS plus trehalose dimycolate administered on Day 7 (Fig. 1C; Table 1). Combined treatment with mitomycin C and CWS plus trehalose dimycolate (300, 100, or 50 μg of CWS) was more effective (p < 0.005) than was mitomycin alone (50 μg). However, immunotherapy contributed significantly to the efficacy of chemoinmunotherapy. In fact, only when animals received 50 μg of mitomycin C in addition to 300 μg of CWS plus trehalose dimycolate was chemoinmunotherapy more effective than either chemotherapy or immunotherapy alone (p < 0.01).

Except for the 1 instance in which 300 μg of CWS plus trehalose dimycolate produced regression of only 3 of 10 treated tumors, all of the treatment regimens, alone or when combined, were beneficial when compared with the diluent-treated control groups (p < 0.025; Table 1). Variation between experiments in antitumor activity of CWS and trehalose dimycolate previously observed (E. Ribi, unpublished observations) may explain the low activity in this 1 instance. As shown in Table 1, 100 or 50 μg of CWS plus trehalose dimycolate produced tumor regression in 50% of the treated animals. Thus, immunotherapy alone produced regression rates varying from 30 to 50% in the experiments described herein.

To ascertain whether a more dramatic difference between either immunotherapy or chemotherapy alone versus chemoinmunotherapy could be demonstrated, we treated tumors at various stages of development (Table 2). Three reasonable statements can be made to summarize the results seen in Table 2. First, most (12 of 17) of the treated groups benefited from the treatment regimens when compared with diluent-treated control groups (p < 0.025). No beneficial effect was observed in those groups receiving immunotherapy alone on Days 12, 14, or 15 or in the group receiving immunotherapy on Day 14 and chemotherapy on Day 15. Secondly, the later such treatment was initiated, the less effective was each treatment regimen in producing tumor regression. For example, tumor regression rates varied from 10 to 100% for treatments administered 6 to 12 days after transplantation versus 0 to 29% if administered on Days 14 and 15. Third, a significant difference (p < 0.05) exists between immunotherapy and chemoinmunotherapy (Groups 7 versus 8 and 9 versus 10 of Table 2), but no significant difference exists with chemotherapy versus chemoinmunotherapy (p > 0.05). In most instances (6 of 7 groups), however, chemoinmunotherapy was as effective as chemotherapy versus chemoinmunotherapy (p > 0.05). In most instances (6 of 7 groups), however, chemoinmunotherapy was as effective as chemotherapy.
Strain 2 guinea pigs received an intradermal transplant of $10^6$ line 10 cells on Day 0. On the days indicated following transplant, each animal received, as indicated, 500 µg of mitomycin C, 300 µg of BCG cell wall, and/or 300 µg of BCG combined with 50 µg of trehalose dimycolate (P3). The volumes of emulsion used to treat 6- to 7- and 11- to 15-day-old tumors were 0.4 and 0.6 ml, respectively. Control animals were treated by single or multiple intratumor injections of oil-in-phosphate-buffered saline emulsions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 11</th>
<th>Day 12</th>
<th>Day 14</th>
<th>Day 15</th>
<th>Tumor regression$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mitomycin</td>
<td>78 (9)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>Cell wall</td>
<td>33 (9)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>Mitomycin</td>
<td>100 (9)</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
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<td>70 (20)</td>
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<tr>
<td>5</td>
<td>Cell wall</td>
<td>33 (9)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mitomycin</td>
<td>78 (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CWS + P3</td>
<td>10 (10)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>Mitomycin</td>
<td>90 (10)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>CWS + P3</td>
<td>78 (9)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>CWS + P3</td>
<td>0 (20)</td>
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</tr>
<tr>
<td>11</td>
<td>CWS + P3</td>
<td>50 (10)</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>Mitomycin</td>
<td>67 (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Mitomycin</td>
<td>29 (17)</td>
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</tr>
<tr>
<td>14</td>
<td>CWS + P3</td>
<td>20 (10)</td>
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</tr>
<tr>
<td>15</td>
<td>CWS + P3</td>
<td>0 (9)</td>
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<td></td>
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</tr>
<tr>
<td>16</td>
<td>Mitomycin</td>
<td>0 (10)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>17</td>
<td>CWS + P3</td>
<td>0 (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Oil-Tween phosphate-buffered saline-treated controls</td>
<td>0 (30)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ Percentages of tumor-bearing animals in which tumors regressed following treatment. Data from 3 separate experiments are pooled. $^b$ Numbers in parentheses, number of animals in each group.

As or more effective than either chemo- or immunotherapy alone.

To determine whether this trend was statistically significant, we applied Wilcoxon's signed-rank test (20). As shown in Table 3, we ranked 12 pairs of values from Tables 1 and 2. These values were the differences obtained by subtracting percentages of tumor regression obtained after treatment with chemotherapy or immunotherapy alone (which ever produced the higher rate of regression) from those obtained by treatment with chemoimmunotherapy. The negative rank sum was -3, and the positive rank sum was +75. The null hypothesis can be rejected with 12 pairs of values if the negative rank sum is less than 7. Therefore, we concluded that the trend is significant; i.e., chemoimmunotherapy tends to be more efficacious than is either treatment alone.

As described in “Materials and Methods,” each guinea pig that rejected the treated, primary tumor was challenged with a second transplant of line 10 cells. Without exception, all these animals rejected this challenge, which indicated that successful chemo-, immuno-, or chemoimmunotherapy resulted in tumor immunity. These same animals, however, failed to reject $10^6$ L2 C leukemic cells transplanted intradermally (data not shown). The leukemic cells grew at the site of implantation; metastasized; produced leukemia, splenomegaly, and lymphadenopathy; and killed the animals about 3 to 4 weeks after inoculation. This indicated that the tumor immunity was relatively specific for the line 10 tumor, although we cannot exclude the possibility that the immunity might have been shared with some tumor(s) other than the line 10 tumor.

Although Segerling et al. (18) do not discuss their data regarding the direct effect of mitomycin on line 10 cells, they do present data that suggest that there might be some
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cell death as measured by trypan blue uptake following incubation of line 10 cells with mitomycin (20 μg/ml) for 17 hr at 37°. We found that exposure of line 10 cells for 1 hr at 37° to increasing concentrations of mitomycin caused decreased uptake of [³H]thymidine (Table 4). The direct effect(s) of mitomycin on line 10 cells was most apparent at 50 and 100 μg of mitomycin per ml, where thymidine uptake was reduced to 54 and 29%, respectively, of untreated control values.

DISCUSSION

We have extended the studies of Meyer et al. (11), Ribi et al. (16, 25, 26), and Bast et al. (2) demonstrating that intratumor treatment with either microbial or chemotherapeutic agents produced regression of line 10 tumors in strain 2 guinea pigs. For several reasons, the intratumor administration of such agents has had limited application in treatment of spontaneous tumors in humans or other mammals. One reason is that tumors located at sites anatomically accessible for injection are amenable to more conventional treatment, such as surgical removal or radiotherapy. In addition, intratumor injections of immunopotentiating microbial components or chemotherapeutic agents can produce adverse reactions such as necrosis and ulceration at the sites of injection. Nevertheless, localization of antitumor substances at the site of neoplastic growth has potential advantages over systemic administration. For example, in the line 10 carcinoma system, intratumor administration of chemotherapeutic agents (2) or immunopotentiating microbial agents (23) was effective in producing tumor regression, whereas systemic administration of these substances was not effective. Conceivably, adverse effects of systemic administration of chemotherapeutic or immunopotentiating agents could be minimized by confining these materials to the site of the neoplasm. These considerations are not to be construed as a position taken against use of systemic chemo-, immuno-, or chemoimmunotherapy. Obviously, many important advantages, realized and potential, exist for systemic treatment of neoplastic disease.

One difficulty encountered in localizing antitumor agents by intratumor injection is that of rapid dissemination or active removal of substances from the injection site. Takeda et al. (21) found that bleomycin in an oil emulsion was more efficacious than it was in aqueous solution for producing tumor regression, and it was retained longer at the site of injection. Another approach to drug localization is the use of antitumor antibody to which are coupled cytotoxic substances such as metabolic inhibitors (4, 10, 15). The use of insoluble polymer matrices with added chemotherapeutic drugs (9, 17) for prolonged release of the drug at the site of neoplastic growth is still another possible means of locally confining antitumor drugs.

Thus, intratumor injection is perhaps a primitive approach to specific localization of antitumor agents. However, intratumor delivery of both a chemotherapeutic agent, such as mitomycin C, and an immunopotentiating prepara-

tion, such as CWS plus trehalose dimycolate, seemed a promising way to complement antitumor therapy. Data presented herein (Tables 1 and 2) indicate that the combined intratumor treatment with components of BCG in oil-in-water emulsions and mitomycin C is more effective than is either treatment alone. Tissue necrosis caused by mitomycin C was not observed when the dose was reduced to 50 μg (Fig. 1A). This small dose in combination with immunotherapy significantly increased the percentage of tumor regression as compared with either treatment regimen alone. Thus, tissue necrosis, a major disadvantage of intratumor chemotherapy, could be minimized or eliminated, and maximal tumor regressive potency could be retained by combining low-dose chemotherapy with immunotherapy (Fig. 1C; Table 1). Reducing the amount of the chemotherapeutic agent might also minimize any immuno-suppressive effects that such drugs may have. An additional positive aspect of chemoimmunotherapy was that relatively high percentages (78 to 90%) of large (11- to 14-day-old) tumors were successfully treated (Table 2).

In these studies, we did not evaluate the mechanism of enhanced antitumor activity of chemoimmunotherapy. One simple hypothesis is that mitomycin C has direct cytotoxic effects on line 10 cells (Table 4), with resultant reduction of tumor burden. Conceivably, immunological responses then would be more effective against the smaller numbers of tumor cells. In addition, the death of tumor cells might be accompanied by processing of cellular debris, including tumor antigens, by the host immune system. Development of tumor-specific immunity could ensue. The addition of immunotherapy to chemotherapy also might stimulate non-specific, antitumor immunological responses to tumor cells damaged by mitomycin C as well as to tumor cells unaffected by mitomycin. Conceivably, the antimetabolic effects of mitomycin might render line 10 cells more susceptible to cytotoxicity either by antibody plus complement-mediated lysis as shown by Segerling et al. (18) or by cell-mediated lysis. We hope that other studies now in progress will shed some light on the immunological mechanisms producing the additive, beneficial effect of combined chemoimmunotherapy in this model system.

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**Table 4**

<table>
<thead>
<tr>
<th>Mitomycin (μg/ml)</th>
<th>Uptake of [³H]thymidine</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10,621 ± 1,234*</td>
<td>100b</td>
</tr>
<tr>
<td>5</td>
<td>12,043 ± 1,302</td>
<td>113</td>
</tr>
<tr>
<td>25</td>
<td>8,274 ± 640</td>
<td>78</td>
</tr>
<tr>
<td>50</td>
<td>5,725 ± 211</td>
<td>54</td>
</tr>
<tr>
<td>100</td>
<td>3,105 ± 146</td>
<td>29</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

b Mean of 4 determinations. These mean values have been corrected for background radioactivity by subtraction of 410 cpm, which was the mean value for line 10 cells incubated at 4°, and were harvested immediately after addition of [³H]thymidine.

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We hope that other studies now in progress will shed some light on the immunological mechanisms producing the additive, beneficial effect of combined chemoimmunotherapy in this model system.
ACKNOWLEDGMENTS

We are grateful to Dr. M. Hanna and Dr. W. Payne for providing us with the strain 2 guinea pigs reared at the Litton Bionetics Animal Farm of the Frederick Cancer Research Center under support from the National Cancer Institute, Bethesda, Md. We warmly appreciate the superlative technical assistance of D. Goode, R. List, J. Kyle, L. Fadness, J. Simmons, W. Bickle, and S. Hargrave. We thank Dr. R. D. Angus of the United States Department of Agriculture, Ames, Iowa, for his generous provision of M. bovis strain AN5 whole cells. We are thankful for the expert advice of Dr. H. Goldberg, Department of Statistics, North Carolina State University, Raleigh, N. C., in the statistical analysis of our data.

REFERENCES


Fig. 1. Effect of mitomycin C on normal guinea pig skin and effect of chemoimmunotherapy on regression of line 10 tumors. A. sites of intradermal treatment of normal guinea pig with 50 µg (arrow) and 500 µg (black ulcer to right of arrow) mitomycin C 2 days after treatment. B. 8 representative line 10 tumors 28 days after cell transplantation. The tumors were treated with oil:Tween 80:phosphate-buffered saline 6 days after transplantation. C. 8 representative tumors photographed after treatment with 50 µg mitomycin C and 300 µg CWS plus 50 µg trehalose dimycolate. Mitomycin C and CWS with trehalose dimycolate were injected 6 and 7 days after tumor cell transplantation, respectively. The tumors or healing lesions were photographed 28 days after tumor cell transplantation. The tumor in C7 did not regress. None of the tumors in B regressed.
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