Effect of Corynebacterium liquefaciens on a C3Hf Mouse Squamous Cell Carcinoma

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SUMMARY

The antitumor effect of anaerobic Corynebacterium liquefaciens was compared with that of specific immunization. Experimental tumors were fourth or fifth generation isotransplants of a NR-SI squamous cell carcinoma that arose spontaneously in a C3Hf/He female mouse. Specific immunization failed to exhibit an antitumor effect, whereas a single administration of the bacterium markedly inhibited the growth of the tumor. This growth inhibition was most effective when C. liquefaciens was administered 2 to 4 days before transplantation of tumor cells, but marked inhibition was also observed when this agent was administered after transplantation. The inhibitory effect was independent of dose within a range of 0.1 to 2.0 mg/mouse; a single dose of less than 0.05 mg/mouse did not exhibit antitumor effect. Multiple administrations of large doses, if given with short treatment intervals, were no more effective than one small dose. Multiple doses given at 14-day intervals resulted in marked growth retardation. The dose of cells that produced 50% tumor takes in C. liquefaciens-treated animals was not significantly different from that in nontreated animals, indicating that this bacterium exhibited no lethal effect on the tumor cells studied.

INTRODUCTION

Anaerobic corynebacteria have been reported to inhibit the growth of various animal tumors (3, 6, 8, 13, 15, 20, 26, 30, 37). These bacteria are potent stimulators of the reticuloendothelial system (9, 21) and may increase macrophage activity (9, 16, 17, 32, 36). Cpa (16, 19, 31, 39) and CG (2, 20-23, 25) inhibit tumor growth and, in some animal tumor systems, have produced marked regression of growing tumors. In many studies, chemically induced tumors have been used (3, 6, 8, 13, 23, 30, 32). This communication will describe the effects of CL and specific immunotherapy on tumor growth and tumor take rate, with the use of a squamous cell carcinoma that arose spontaneously in 1 of our SPF female mice (34, 35). CL used in this study recently became available in Japan and has the same immunological properties as CP (1).

MATERIALS AND METHODS

Animal-Tumor System

Animals were 8- to 12-week-old C3Hf/He mice derived from our SPF mouse colonies. They were kept in a SPF animal facility and provided with sterilized Purina pellets and chlorinated water ad libitum. Fourth or 5th generation isotransplants of a squamous cell carcinoma that arose spontaneously in a C3Hf/He female mouse and was designated as NR-SI were used throughout. To obtain 4th- or 5th-generation tumors, 1st to 3rd-generation isotransplants were stored in a liquid nitrogen refrigerator and 3rd- or 4th-generation tumors were grown in flank tissue of several female mice to provide sufficient tumors for experimental use.

Tumor Cell Suspension

Animals bearing 3rd- or 4th-generation tumors were sacrificed. Intact tumor tissue was removed and minced finely by scissors. The mince was trypsinized in Ca²⁺- and Mg²⁺-free Dulbecco’s solution containing 0.2% trypsin (1:250; Difco Laboratories, Detroit, Mich.) at 37°C for 40 min. This suspension was removed into test tubes and settled for 15 min in crushed ice. The supernatant was passed through a Swinney filter, and the filtrates were centrifuged for 5 min at 1500 rpm. The sediment was diluted with Hanks’ medium containing 5% fetal calf serum. Viable tumor cells were counted by means of the trypsin blue exclusion test on a hemocytometer. Ten μl of this single cell suspension were transplanted s.c. into the right hind legs of experimental animals.

Immunization

Specific Immunization. Recipients were immunized with 3 weekly injections each of 5 x 10⁶ LR tumor cells, which had been irradiated with 10,000 rads of X-rays (33). For the 1st injection, LR cells were mixed with the same volume of Freund’s complete adjuvant (Difco) and then injected into inguinal and axillary fossae s.c., whereas the 2nd and 3rd injections were made i.p. without mixing. Viable tumor cells were transplanted 7 days after the last injection of LR cells.

Nonspecific Immunization. Heat-killed and phenol-extracted CL was kindly supplied by Kowa Co., Ltd., Tokyo, Japan. Twenty mg of this bacterium were suspended in a 1-
ml solution for i.p. injection. CL that was originally isolated from human bone marrow and identified according to Pre-vot's classification (27) was found to be an immunopotentiator (7, 10-12, 29) and to possess the same immunological properties as CP (1).

Experimental Assay Methods

TG or TGD Time. The growth of tumor was measured by TG time, i.e., the time required for a tumor to reach 500 cu mm after transplantation. Three diameters of each tumor, a, b, and c, were measured by a caliper at least every other day. Tumor volume was calculated by the formula \( \pi abc/6 \) and was plotted as a function of time on a semilogarithmic graph. Based on this TG curve, TG time was obtained graphically in each tumor. A difference between TG time of a treated and a nontreated tumor was defined as TGD time. Six to 8 animals were used in an experimental group.

TD\(_{50}\) Assay. The cytocidal effect caused by immunotherapy was assayed by the TD\(_{50}\) method. A single-cell suspension containing a known number of viable tumor cells was serially diluted 2- to 3-fold with Hanks' medium into 6 to 8 titration doses. Animals were randomly arranged in groups before transplantation; 4 to 6 recipients served for each dilution, and a total of 40 to 50 mice were used for an assay. The sites of transplantation were palpated for possible TG once a week for 60 days. If a tumor grew to more than 10 mm in average diameter, it was scored as a take. If an animal died without a palpable tumor before 60 days, it was excluded from the assay. If an animal died with a tumor smaller than 10 mm in diameter, tumor take was confirmed by autopsy. TD\(_{50}\) values were calculated by the logit analysis method on the basis of the tumor take frequency in 60 days.

Statistical Analysis

Results were evaluated statistically by Student's t test. A difference between groups was considered significant if the \( p \) value was 0.05 or less.

RESULTS

Specific Immunization

Animals were actively immunized with 3 injections each of \( 5 \times 10^8 \) LR tumor cells, and 7 days later they received a transplant of viable tumor cells. This immunization prolonged TG time slightly from 17.8 to 20.1 days, i.e., TGD time was 2.3 days.

The killing effect of this immunization on tumor cells was also studied. The TD\(_{50}\) in specifically immunized animals was 2.9 (1.8 to 4.7) \( \times 10^4 \), whereas in nontreated mice it was 9.9 (5.1 to 19) \( \times 10^2 \). This increment in TD\(_{50}\) in immunized animals was, however, not statistically significant.

Nonspecific Immunization

Preliminary Study. Two mg of CL were administered i.p. 7 days before or 7 days after transplantation or on the day of transplantation. TG was markedly inhibited by these treatments (Chart 1). Growth retardation was most pronounced when the bacterium was administered on the day of transplantation or 7 days after transplantation.

Effect of the Interval between Transplantation and Administration. CL (2.0 mg) was injected i.p. at various times, varying from 14 days before transplantation to the day of transplantation, and the effect was measured in terms of TGD time. The results are summarized in Chart 2, together with TGD data from the preliminary study. Growth inhibition was most prominent when CL was administered 3 days before transplantation, but, as noted above, inhibition was
also apparent when the bacterium was administered after tumor transplantation. The effectiveness of pretransplantation treatment decreased as the interval between administration of CL and tumor transplantation increased beyond 3 days, and it was negligible by 14 days (Chart 2).

**Effect of Dose of CL.** Single doses of CL varying from 0.02 to 2.0 mg were injected 3 days before transplantation (when the effect was most prominent in the above experiment), and TGD times were determined. As illustrated in Chart 3, less than 0.05 mg of CL had no effect on tumor growth, although doses of 0.1 mg or more were highly effective. TGD time after 0.1 mg was 6.5 days and was not significantly different from that after 2.0 mg, i.e., 8.2 days (Chart 3), suggesting that above a certain threshold the effect of CL is independent of dose.

**Effect of Multiple Administrations.** The finding that the effect of CL disappeared by 2 weeks after administration and that there was a threshold dose suggested the necessity of studying the effect of multiple injections of this bacterium.

Three multidose treatment regimens were studied. In the 1st regimen, a large dose of 2 mg was followed by 6 doses of either 1 or 0.2 mg (Table 1). In the 2nd regimen (Table 2), animals were given 6 equal doses of 0.02 mg (slightly less than the threshold dose) or 6 equal doses of 0.1 mg (slightly more than the threshold dose). In the 3rd regimen (Table 3), CL was given as an initial dose of 0.1 mg 14 days before tumor transplantation and then as a series of 6 doses given 2 doses/week. In this 2nd course of treatment, 2 different doses were used, 0.1 mg (above threshold dose) or 0.02 mg (less than threshold).

The 1st regimen retarded tumor growth, but the effect was no greater for multiple doses than if only a single dose of 2 mg was given (Table 1).

In the 2nd regimen, multiple doses, each of 0.02 or 0.1 mg, were administered. TGD time after 6 doses of 0.1 mg was 6.6 days and was equivalent to that from a single dose of the same total amount. Also, 6 doses of 0.02 mg (each of which is smaller than the threshold dose), if given repeatedly, inhibited tumor growth as effectively as a higher dose regimen (Table 2).

The 3rd regimen comprised 2 treatment courses separated by 14 days. This interval was chosen to allow the antitumor effect induced by the 1st course of treatment to dissipate (Chart 2). The 1st treatment was a single administration of 0.1 mg given 14 days before tumor transplantation. The 2nd course of treatment was a series of 6 injections, each of 0.1 or 0.02 mg, beginning on the day of transplantation and repeated twice weekly for 2.5 weeks. These protracted treatments with multiple small doses inhibited tumor growth significantly but less effectively than the single course treatment (Table 3), indicating that animals were capable of responding to a 2nd treatment for at least 2 weeks.

**Effect on Tumor Cell Transplantation.** A final study was made to investigate the effect of administration of CL on the survival of transplanted tumor cells. Animals were given 2.0 mg of CL, and 2 days later various numbers of tumor cells were transplanted to estimate the TD50. CL did not reduce significantly the transplantability of the tumor cells: the TD50 values for CL-treated animals and nontreated controls were significantly the transplantability of the tumor cells: the TD50 values for CL-treated animals and nontreated controls were

![Effect of Corynebacterium on a Squamous Cell Carcinoma](chart3.png)

**DISCUSSION**

Specific immunization failed to inhibit TG and did not raise the TD50, indicating that the NR-Si tumor cells were not detectably immunogenic. However, administration of CL markedly inhibited the growth of this tumor. These results imply that different mechanisms may be involved in specific and nonspecific immunization. Recent experimental evidence suggests that some corynebacteria and mycobacteria stimulate the reticuloendothelial system nonspecifically and exhibit antitumor activity (9, 21, 28). This antitumor effect is thought to be macrophage mediated (2, 9, 16, 17, 24, 32, 36). Tuttle and North (32) reported that 175 μg of CP injected into the footpad stimulated the activity of macrophages 2 days after administration. The stimulation reached its maximum 6 days later and disappeared within 15 days of injection. This report is consistent with our finding that pretransplantation treatment with CL inhibited TG, but that this effect was lost by 14 days.

An important finding in the present study was that CL inhibited TG without any lethal damage to tumor cells, as evidenced by no change in transplantability. Similar findings were observed by Boyle and Ormerod (4) and by Scott (30). The mechanisms involved in this cytostatic effect of CL are not clear, but studies by Janik and Steel (14) suggest that it results from reduced growth fraction or increased cell loss factor, or both. Our recent results clearly indicate prolongation of the generation time and decrease of the growth fraction (unpublished data).

Although we have found no change in the TD50 after administration of CL, Milas et al. (20) found a marked lethal effect on methylcholanthrene-induced fibrosarcoma in animals treated with CG, some established tumors showing
complete regression. However, the tumor used by Milas et al. was chemically induced and possessed strong immunogenicity, whereas the NR-SI tumors used in this study arose spontaneously and exhibited no detectable immunogenicity. The antitumor activity of Corynebacterium and other nonspecific immunostimulants may depend upon the immunogenicity of animal tumors, but even in the tumors in this study CL exhibited a prominent effect. This contrasts with the results of Milas et al. (20, 23), who found that CG inhibited the growth of a strongly immunogenic fibrosarcoma but had little effect on a weakly immunogenic spontaneous mammary carcinoma.

Multiple small doses of CL were more effective than a single dose, but above a certain total dose (given as single or multiple injections) no additional antitumor activity was elicited by further injections. Similar observations have been made by Scott (30) and Milas et al. (18), although Woodruff et al. (38) reported that a 2nd large dose enhanced the antitumor activity of CP. Also, Wolmark and Fisher (36) showed that a 2nd injection of CP increased the number of bone marrow macrophages. However, even if large doses of corynebacteria were given, the antitumor activity decreased by 14 days. Hence, from the viewpoint of possible clinical use of CL, the size of each dose and the spacing of multiple doses must be considered.

ACKNOWLEDGMENTS

We thank Dr. H. R. Withers for assistance in preparing this paper.

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### Table 1
**Effect of multiple doses of CL on TG time**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose/fraction (mg)</th>
<th>No. of fractions</th>
<th>Total dose (mg)</th>
<th>Time of administration</th>
<th>TG time (days)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.9 ± 0.9*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2.0</td>
<td>1</td>
<td>2.0</td>
<td>0</td>
<td>13.6 ± 1.1</td>
<td>1-2: p &lt; 0.001</td>
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<tr>
<td>3</td>
<td>8</td>
<td>2.0</td>
<td>1</td>
<td>8.0</td>
<td>0</td>
<td>15.4 ± 1.2</td>
<td>2-3: NS*</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>2.0</td>
<td>1</td>
<td>3.2</td>
<td>0</td>
<td>13.2 ± 1.6</td>
<td>2-4: NS</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* On the day of tumor cell transplantation, 2.0 mg of CL were administered i.p., and this was followed by twice a week administration for 3 weeks.
* NS; not significant.

### Table 2
**Effect of multiple doses slightly less or slightly more than the threshold dose of CL**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose/fraction (mg)</th>
<th>No. of fractions</th>
<th>Total dose (mg)</th>
<th>Time of administration</th>
<th>TG time (days)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14.9 ± 1.5*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.02</td>
<td>6</td>
<td>0.12</td>
<td>0-+17</td>
<td>20.2 ± 2.1</td>
<td>1-2: p &lt; 0.001</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.1</td>
<td>6</td>
<td>0.6</td>
<td>0-+17</td>
<td>21.5 ± 1.4</td>
<td>1-3: p &lt; 0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* On the day of tumor cell transplantation, 2.0 mg of CL were administered i.p., and this was followed by twice a week administration for 3 weeks.

### Table 3
**Effect on TGD of protracting treatment time**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose/fraction (mg)</th>
<th>No. of fractions</th>
<th>Total dose (mg)</th>
<th>Time of administration</th>
<th>TG time (days)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>−14</td>
<td>14.9 ± 1.5*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.1</td>
<td>1</td>
<td>0.1</td>
<td>−14</td>
<td>14.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.02</td>
<td>6</td>
<td>0.22</td>
<td>0-+17</td>
<td>16.8 ± 2.1</td>
<td>2-3: NS</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.1</td>
<td>1</td>
<td>0.7</td>
<td>−14</td>
<td>18.9 ± 1.5</td>
<td>2-4: p &lt; 0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* A single administration of 0.1 mg of CL was given 14 days before tumor cell transplantation. The 2nd course of treatment, i.e., twice a week administration for 3 weeks, was initiated on the day of transplantation.
* NS, not significant.
REFERENCES


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