Tumor Regression and Enhancement Resulting from Immunotherapy with *Bacillus Calmette-Guérin* and Neuraminidase

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SUMMARY

The effect of *Bacillus Calmette-Guérin* (BCG) and/or neuraminidase (VCN) on tumor growth was studied in four murine fibrosarcomas. In the first tumor system, the intratumor injection of BCG resulted in increased tumor growth of both injected and uninjected tumors. In the second tumor system, the intratumor injection of BCG and VCN resulted in earlier growth of both injected and distant uninjected tumors. In the third tumor system, immunoprophylaxis with BCG made mice more resistant to subsequent challenge with tumor cells, and the intratumor injection of BCG, VCN, or BCG and VCN resulted in complete regression of established tumors. In the fourth tumor system, BCG, injected into the tumor or into a distant site, one-half this number of tumor cells, caused increased tumor growth.

INTRODUCTION

BCG immunotherapy has generated widespread interest since publication of the reports of Mathé *et al.* (25) and Morton *et al.* (30) demonstrating its effectiveness in certain clinical situations. In the laboratory, the intratumor injection of BCG or VCN has been shown to cause the regression of established cancers in selected experimental tumor models (10, 37, 38, 42). BCG given after operation can protect animals from spontaneous metastases (3, 39). There have been few reports of enhanced tumor growth in solid tumors following immunotherapy with either BCG or VCN (4, 5, 32, 33).

Our laboratory has carried out a number of experiments designed to establish the optimum conditions under which the injection of BCG and/or VCN causes regression of both primary and distant metastatic tumors. The 4 experiments reported herein demonstrate that the effect of BCG and VCN immunotherapy can vary from tumor regression to tumor enhancement. This information is important for the design of future controlled clinical trials of immunotherapy.

1 Supported by grants from the Veterans Administration and the California Institute of Cancer Research.

2 The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; VCN, *Vibrio cholerae* neuraminidase; AVMLR, analysis of variance of a multiple linear regression.

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MATERIALS AND METHODS

**Mice.** C3H/HeCr, C3Hf/HeCr, and C57BL/6Cr adult female mice, age 8 to 12 weeks, were obtained from Simonsen Laboratory, Gilroy, Calif. The animals were housed in covered plastic cages, 10 or fewer per cage, and were provided standard laboratory diet and water *ad libitum*.

**Tumors.** The characteristics of the 4 fibrosarcomas are listed in Table I. The immunogenicity data of BP, MCA-1, and MCA-2 were determined by the method of Prehn and Main (36). The immunogenic characteristics of MC-43 have been reported by Haywood and McKhann (19).

**BCG.** Mycobacterium bovis, Tice strain, was obtained from the Research Foundation, Chicago, Ill., in ampuls containing 3.45 X 10^8 viable organisms.

**VCN.** VCN was obtained from General Biochemicals, Chagrin Falls, Ohio. Each ampul contained 500 units of enzyme per ml (1 unit of activity is equivalent to the release of 1 µg of N-acetylneuraminic acid in 15 min from a glycoprotein substrate at 37°C at pH 5.5).

**Preparation of Tumor Cell Suspensions.** Single tumor cell suspensions were prepared by a modification of the method of Hammond *et al.* (17). Tumor tissue minced of necrotic and fibrous tissue was finely minced and placed in a trypsinizing flask. Eagle’s minimum essential medium containing 0.25% Pronase was added (10 ml/g of tissue) and the mixture was agitated at room temperature for 15 min, filtered through cheesecloth, and washed 3 times by centrifugation in Eagle’s minimum essential medium. The cells were then resuspended and counted in a hemocytometer by the trypsin blue dye-exclusion technique.

**Experimental Methods.** In each tumor system, the number of cells required for s.c. tumor growth in 90% of inoculated mice was determined by titration. Experimental mice were then inoculated with this number of tumor cells in the left flank (primary tumor). Metastases were simulated by inoculating at a distant site, the right flank, one-half this number of tumor cells. BCG and/or VCN was injected into the primary tumor or into a site separate from either the primary or simulated metastatic tumor. The details vary with each experiment and are described below.

All injections of BCG, VCN, or BCG and VCN were given in a volume of 0.1 ml. Since previous experiments in our laboratory have shown that there was no difference in tumor growth when 0.1 ml 0.9% NaCl solution was injected into a
Varied Effects of Immunotherapy with BCG and VCN

The 4 tumors used in these experiments were carcinogen induced and were carried in syngeneic mice. The immunogenicity data were determined by the method of Prehn and Main (36). The immunogenic characteristics of MC-43 are reported elsewhere (19).

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Inducing agent</th>
<th>Mouse strain carriers</th>
<th>Transplant generation</th>
<th>Inoculum (no. of cells)</th>
<th>% take in control mice</th>
<th>% take in immunized mice</th>
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<tbody>
<tr>
<td>BP</td>
<td>BP</td>
<td>C3Hf/HeCr</td>
<td>3</td>
<td>10^6</td>
<td>90</td>
<td>0</td>
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<tr>
<td>MCA-2</td>
<td>MCA</td>
<td>C57BL/6JCr</td>
<td>17</td>
<td>10^4</td>
<td>80</td>
<td>0</td>
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<tr>
<td>MCA-2</td>
<td>MCA</td>
<td>C3Hf/HeCr</td>
<td>4</td>
<td>10^4</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>MC-43</td>
<td>MCA</td>
<td>C3Hf/HeCr</td>
<td>6</td>
<td>10^4</td>
<td>95</td>
<td>20</td>
</tr>
</tbody>
</table>

* Agents were: BP, benzpyrene; and MCA, methylicholanthrene.

Tumor, control mice received no injections. Tumors were measured at least weekly, and the tumor volume was calculated by the formula $V = 0.4ab^2$, where $a$ is the major and $b$ is the minor diameter of the tumor parallel to the surface of the mouse (2).

In the 1st tumor system, $1 \times 10^6$ BP tumor cells were inoculated into the left flank and one-half that number, into the right flank of C3H mice. Fifteen days after tumor inoculation, at which time the tumors were palpable and measured more than 7 mm in diameter, either BCG ($3.45 \times 10^5$ organisms) or VCN (50 units) was injected into the tumor in the left flank.

In the 2nd tumor system, $1 \times 10^3$ MCA-1 tumor cells were inoculated into the left flank, and one-half that number, into the right flank of C57BL/6 mice. BCG ($3.45 \times 10^7$ organisms) and VCN (50 units) were injected on Day 7 into the s.c. tissue at the site of tumor inoculation in the left flank. At this time, tumors were not detectable by palpation.

In the 3rd tumor system, $1 \times 10^6$ MCA-2 tumor cells were inoculated into the left flank of C3H mice. Immunotherapy was given 2 weeks after tumor inoculation, at which time tumors were palpable and measured more than 7 mm in diameter. BCG, VCN, or both BCG and VCN were injected into the tumors to determine whether there was a local antitumor effect, and into the s.c. tissue of the right flank to determine whether there was a systemic antitumor effect. In an additional group, BCG was injected s.c. into the opposite flank 2 weeks prior to tumor inoculation.

In the 4th tumor system, $1 \times 10^4$ MC-43 cells were inoculated into the left flank, and $5 \times 10^3$ cells were inoculated into the right flank of C3H mice. Treatment consisted of various doses of BCG alone, BCG with VCN, and VCN alone, given both before (Day 9) and after (Day 23) the primary tumors were clinically detectable (Table 3). Injections were given s.c. into the site of primary tumor inoculation, into the primary tumor itself, or s.c. into the left axilla.

Statistical Analysis. Statistical analyses were applied to several parameters in these studies.

1. The mode of growth of each system was assumed to be semilogarithmic (1st-order exponential) and was verified to be such by subjecting the logarithms of tumor volume versus time growth curves to linear regression analysis and to a test for linearity of regression (9). This semilog treatment of the data was found to form a consistently acceptable working hypothesis ($p < 0.01$).

2. The probability of correct prediction of group membership (control versus therapy) from the logs of tumor volume and times of observation was calculated by an AVMLR (9). The significance of the $F$ value thus obtained was taken as the probability that the groups were the same. A $p$ value of 0.05 or less was considered to indicate a statistical difference.

3. Growth rates were calculated over the curve's 1st-order exponential range. A linear regression program was utilized to construct the best fit for a plot of log tumor volume versus time, and a $t$ test of the slopes thus derived indicated the significance of any apparent differences (9).

4. Elapsed time data were evaluated by Halperin's extension of the Wilcoxon-Mann-Whitney nonparametric significance test (16).

5. Differences in tumor incidence and regression ratios between treated and control mice were analyzed by Fisher's exact test for $\chi^2$, since theoretical frequencies were small.

6. Differences between the mean volumes of 2 groups were analyzed by Student's $t$ test.

RESULTS

BP. In the 1st tumor system, the intratumor injection of BCG resulted in greater average tumor volumes on both sides (Chart 1). Analysis of tumor growth by AVMLR showed a statistically significant difference on the untreated right side ($p < 0.05$) and a difference approaching significance ($p \sim 0.1$) on the left side. The intratumor injection of VCN had no significant effect on tumor growth on either side.

MCA-1. In the 2nd tumor system, the intratumor injection of BCG and VCN resulted in earlier growth of both the primary tumor on the left side and the metastatic tumor on the right side (Chart 2). The time between inoculation of tumor cells and detectable tumor growth averaged 27 days in the control group and 14 days in the treated group. The delay in tumor growth was significant ($p < 0.01$).

On the left side, 7 of 10 mice in each group eventually developed tumors (Chart 3). On the right side, 6 of 10 mice...
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Chart 1. In the BP tumor system, BCG or VCN was injected into the primary tumor on the left side 15 days after tumor inoculation. The intratumor injection of BCG resulted in increased growth of both the primary tumor on the left side, and the metastatic tumor on the right side. The difference was statistically significant (p < 0.05) on the left side.

Chart 2. In the MCA-1 tumor system, BCG and VCN were injected into the primary tumor site in the left side 7 days after tumor inoculation. Treated mice had earlier tumor growth in both the primary tumor in the left side and the metastatic tumor in the right side.

treated with BCG and VCN and 3 of 10 control mice eventually developed tumors. Analysis by AVMLR showed a statistically significant increase in tumor growth (p < 0.01) on both sides in mice receiving BCG and VCN. However, the rates of tumor growth were not significantly different by the t test of slopes of regression. Furthermore, once tumor growth became apparent, there was no difference in the time to death in treated and control mice.

MCA-2. In the 3rd tumor system, 8 of 9 control mice developed tumors (Chart 4). BCG immunoprophylaxis resulted in a decreased tumor incidence, 5 of 10 mice (p = 0.15). However, tumor growth curves were the same in both the control group and the group that received immunoprophylaxis with BCG. The results of the intratumor and systemic injections of BCG, VCN, and BCG and VCN are summarized in Table 2 and shown in Charts 5 to 10. Tumor growth in 19 of

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>No. with regression/ no. in group</th>
<th>pα</th>
</tr>
</thead>
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<tr>
<td>BCG</td>
<td>Intratumor</td>
<td>9/9</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>BCG</td>
<td>Systemic</td>
<td>1/8</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>VCN</td>
<td>Intratumor</td>
<td>3/9</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>VCN</td>
<td>Systemic</td>
<td>3/8</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>BCG-VCN</td>
<td>Intratumor</td>
<td>8/9</td>
<td>0.002</td>
</tr>
<tr>
<td>BCG-VCN</td>
<td>Systemic</td>
<td>5/9</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Calculated by Fisher's exact test, relative to control group (0/8 regressions).
52 treated mice did not differ significantly from that observed in control mice. In 29 mice, tumors regressed completely and the mice were "cured." Slower tumor growth was observed in 3 mice that received a systemic injection of VCN (Chart 8) and in 1 mouse that received a systemic injection of BCG and VCN (Chart 10). In these 4 mice, the time from tumor inoculation to detectable tumor growth was no different from that of controls, but survival times were increased ($p = 0.01$).

**MC-43.** In the 4th tumor system, a significant increase in tumor growth was observed in the primary tumor in 5 of 13 groups and in the metastatic tumor in 4 of 13 groups (Table 3). Enhancement was observed when BCG, both alone and with VCN, was injected into the tumor site on Day 9 or into the tumor on Day 23. BCG injected into the s.c. tissue of the axilla caused enhanced growth of tumors in both flanks (Group 1). Although the intratumor injection of VCN (Group N) caused an increase in tumor growth, the difference approached but did not reach significance ($p \sim 0.10$).

However, 6 of 27 primary tumors in Groups D, F, and G completely regressed when injections were given into the tumor site prior to detectable tumor growth. In these mice, tumors grew to an average volume of $241 \pm 40$ cu mm before regression occurred. The question arose as to whether this represented true tumor regression or merely resolution of BCG granulomas in mice in which no tumors would have grown. To resolve this question, BCG was injected into 20 mice, 19 of which developed BCG granulomas reaching an average volume
DISCUSSION

The mechanism of BCG-induced tumor regression is complex. Four processes may be involved: (a) a local non-tumor-specific, delayed hypersensitivity type immunological reaction, (b) a systemic tumor-specific immune response, (c) a generalized nonspecific stimulation of the entire reticuloendothelial system augmenting both of the above, and (d) a local nonimmunological direct antitumor effect.

The intratumor injection of BCG initiates a delayed hypersensitivity reaction within the tumor. Direct contact between BCG and tumor cells is important (4, 6, 42). Lymphocytes and macrophages sensitized to BCG kill tumor cells nonspecifically during their immune attack on BCG (12, 22, 24).

A systemic tumor-specific immunity also develops after the intratumor injection of BCG (6). Patients with malignant melanoma who have been treated with BCG, injected into a tumor nodule, develop increased levels of antimalanoma antibodies (30).

BCG is a potent nonspecific stimulator of the reticuloendothelial system and may enhance both the local nonspecific delayed hypersensitivity reaction and the systemic tumorspecific immune response. BCG can stimulate the formation of antibodies against unrelated bacterial and viral antigens, accelerate the rejection of skin allografts and tumor transplants, and delay the appearance of primary induced tumors when given shortly before or after tumor induction (32).

BCG itself has no direct nonimmune antitumor effect. BCG does not inhibit the growth of rat tumors in tissue culture (4). Murine tumor cells mixed in vitro with BCG are not killed and can grow in immunodepressed mice but not in control mice (6). The intratumor injection of BCG does not usually cause tumor regression in tuberculinegative patients (28).

Both the intratumor injection of VCN (37) and immunization with BCG- and VCN-treated tumor cells (38) can cause regression of established methylcholanthrene induced murine fibrosarcomas. VCN is thought to make tumor cells more immunogenic by removing sialic acid residues from the cell surface, thereby unmasking tumor antigens.

In the experiments with the MCA-2 tumor system reported here, the intratumor injection of BCG resulted in tumor regression in all 9 mice. The systemic injection of BCG or VCN, and the intratumor injection of VCN alone, did not result in significant tumor regression.

VCN treatment resulted in delayed tumor growth in 3 of 8 mice when given systemically and produced complete tumor regression in 3 of 9 mice when injected into the tumor. When added to BCG in a systemic injection, VCN caused complete tumor regression in 5 of 9 mice, compared with only 1 of 8 mice when BCG alone was given systemically.

These results suggest that VCN, when administered systemically, may have some other immunological or direct antitumor effect. In the doses used in our study, it is unlikely that a systemic injection of VCN could result in a concentration of VCN at the tumor-cell surface sufficient to strip off sialic acid residues and unmask tumor antigens. In the experiments reported by Simmons and Rios (37, 38), who used the same dose of VCN, multiple intratumor injections were necessary to cause tumor regression. A single intratumor injection of VCN or a systemic injection of VCN was not effective.

Enhancement is defined in these experiments as tumor growth that was significantly in excess of that observed in control animals. Both immunological and nonimmunological mechanisms probably induce enhancement by altering a variety of factors that tend to limit or retard tumor growth rather than by affecting the genetic information within the tumor cell (34). It has been demonstrated that BCG enhances tumor growth in certain situations (4, 5, 32, 33). Three processes may be involved: (a) antigenic competition-immunoparalysis, (b) serum "blocking factors" or antibodies, and/or (c) local nonimmunological factors.

Antigenic competition between BCG and the tumor specific antigens may occur, diverting the immune response from the tumor-specific antigens to BCG. Multiplication of BCG organisms at the site of inoculation or in the visceral organs could provide a steady source of antigen, resulting in a state of immunoparalysis instead of hypersensitivity. However, the data from our experiment did not support this concept. When BCG alone was injected into the MC-43 tumor on either Day 9 or Day 23, enhancement was noted only when the intermediate dose of BCG was used, and was not noted with the higher dose. In tumors with antigenic similarity to BCG (8), immunoparalysis might occur more readily.

Serum blocking factors may abrogate the effects of circulating sensitized lymphocytes (20). The intratumor injection of BCG has been associated with the development of blocking factors and accelerated tumor growth (23). Since we did not
Table 3
Tumor enhancement and regression (MC-43)

In the MC-43 tumor system, BCG alone in varying doses, BCG with VCN, and VCN alone was injected into the primary tumor site on Day 9 prior to clinical detection of the tumor or into the tumor on Day 23. Enhancement was observed in both the primary and metastatic tumor, as noted by the \( p \) values. Regression of the primary tumor, as defined in the text, also occurred in a limited number of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>BCG dose</th>
<th>VCN 50 units</th>
<th>Site</th>
<th>Day</th>
<th>Primary tumor</th>
<th>&quot;Metastatic&quot; tumor</th>
<th>Primary tumor</th>
<th>Metastatic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>9</td>
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<td>TPD</td>
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<td>&lt;0.01</td>
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<td>E</td>
<td>9</td>
<td>9</td>
<td>X</td>
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<td>&lt;0.01</td>
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<td>F</td>
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<td>TPD</td>
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<td>&lt;0.01</td>
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</tbody>
</table>

\( a \), 6.90 \times 10^6 \text{ organisms}; \( b \), 3.45 \times 10^6 \text{ organisms}; \( c \), 6.90 \times 10^6 \text{ organisms}.

Omitted \( p \) values indicate no apparent effect, \( p > 0.10 \) by AVMLR.

\( d \) \( p > 0.05 \) by Fisher's exact test.

\( p \) values indicate no apparent effect, \( p > 0.10 \) by AVMLR.

\( TPD \), primary tumor site prior to clinical detection; \( TAD \), primary tumor site (tumor >7 mm in size); \( Ax \), axilla.

Study antitumor immune response in vitro, discussion of the possible role of blocking factors in these experiments is not appropriate here.

Local nonimmunological factors may also cause enhancement. Topical application with inflammatory agents such as turpentine and oxazolone may enhance tumor growth (18). Edema produced within the tumor by these agents may provide more favorable conditions for tumor growth. The enhancement observed in our experiments following the intratumor injection of BCG or BCG and VCN may have been caused by similar nonimmunological processes. However, 3 observations make this unlikely. First, we have not observed enhancement following the intratumor injection of 0.9% NaCl solution. Second, there was no relationship between enhancement of the injected tumor and the dose of BCG in the MC-43 tumor system. Third, local nonimmunological factors could not explain the enhancement of the metastatic tumor on the contralateral side from the injections in the BP, MCA-1, and MC-43 tumor systems.

The intratumor injection of VCN alone was not associated with a statistically significant increase in tumor growth in these experiments. However, in the MC-43 tumor system, the intratumor injection of VCN caused increased growth of the uninjected metastatic tumor that approached statistical significance (\( p < 0.10 \)).

Other manipulations of the immune system that have enhanced tumor growth include the following: nonspecific stimulation with MER, the methyl-extractable residue of BCG (41) and complete Freund's adjuvant (1, 26), passive transfer of immune serum (7, 11, 21, 27, 31), active immunization with viable tumor cells (14), and infection with mammary tumor virus (2). The i.v. injections of tumor cells mixed with lymphocytes from normal, tumor-bearing, or nonspecifically sensitized mice have increased the incidence of pulmonary metastases, compared with that caused by the i.v. injection of tumor cells alone (13). Clumping of tumor cells caused by the lymphocytes is thought to account for this phenomenon.

Prehn (35) has postulated that the immune system has a dual function, i.e., stimulation of tumor growth early in the course of disease secondary to a weak immune response to weak tumor-specific antigens and inhibition of tumor growth at other times.

Elucidation of the underlying mechanisms is important for the understanding and prevention of tumor enhancement. Unfortunately, no uniform pattern emerges from our experiments that would allow one to predict the conditions under which enhancement or regression might be expected to occur following immunotherapy. Varied effects were noted in each tumor system we studied. Immunotherapy may produce regression, no effect, or enhancement, depending upon the strength and quantity of tumor-associated antigens on the cell surface or in the circulation and the immunological response at that particular time to these antigens.

BCG immunoprophylaxis and immunotherapy are being used with increasing frequency in treating patients with cancer. Results appear promising in some patients (15, 29). Complications arising from BCG immunotherapy can be significant and are dependent upon dose and route of administration (40). Awareness of these complications and the possibility of enhancement of tumor growth is necessary for design of controlled clinical trials with BCG and other immunological adjuvants.
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