Enhancement of Tumor Growth following Immunization with Bacillus Calmette-Guérin Cell Walls

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SUMMARY

The effect of preimmunization with Bacillus Calmette-Guérin cell walls (BCGcw) on tumor growth was studied with the use of transplantable Morris Hepatoma 3924a in inbred ACI rats. There was an increase in tumor incidence, an increase in the i.m. tumor growth rate, and an increase in the average i.m. tumor mass in animals that were pretrained with BCGcw. The BCGcw-induced enhancement in tumor growth was dependent upon the amount of tumor present at the time of BCGcw immunization. No enhancement occurred in animals immunized with BCGcw 12 days after the inoculation of tumor cells.

INTRODUCTION

BCG, BCGcw, and other agents are potent stimulators of the immune response and most recently have been used in immunotherapy. These agents are given locally into tumor masses, and they are also used as systemic adjuvant therapy in the hope that they may in some way selectively stimulate the cellular immune response and thereby facilitate an antitumor response. BCG can increase the resistance to spontaneous, induced, and transplanted tumors. On occasion, use of immunostimulants has been associated with an enhancement of tumor growth. We undertook studies to determine whether an admixture of BCGcw and "soluble tumor antigen" could be an effective immunogen for the induction of tumor resistance. These studies showed that preimmunization with BCGcw alone or admixed with soluble tumor antigen was associated with an enhancement of tumor growth. Further studies were conducted to delineate this experimental phenomenon. In this paper, we report the effects of BCGcw preimmunization upon the i.m. tumor growth of weakly antigenic transplantable Morris Hepatoma 3924a.

MATERIALS AND METHODS

Animals. ACI inbred male rats (13 weeks old; 250 g) were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. They were housed in groups of 4 in stainless steel boxes with stainless steel grids and were supplied sawdust bedding, fresh water, and Purina laboratory rat chow ad libitum.

Tumor Line. The 3924a tumor is a poorly differentiated hepatocellular carcinoma induced after the dietary administration of N-2-fluorenyldiacetamide (14). It was received from Dr. H. Morris, Howard University, Washington, D. C., in the 299th transplant generation, and Transplant Generations 310 to 320 were used in this study.

Preparation of Tumor Cells. Tumor cell preparations were made by mild trypsinization of small chunks of viable tumor obtained from i.m. tumor as previously described (20). Cell viability was assessed with 0.1% trypan blue, as previously described, and cell preparations contained 80 to 90% viable tumor cells (20).

Tumor Extracts. To 1.0 g of whole minced tumor tissue were added 1.5 ml of 0.005 M PO4, pH 7.4, and 3 M KCI, and this mixture was mechanically homogenized. The homogenate was then incubated at 4° with stirring for 24 hr. If a single cell preparation was used, then 10° cells were exposed to 10 ml of 3 M KCI for 24 hr at 4°. After 24 hr, the 3 M KCl-extracted tumor tissue was spun at 35,000 x g at 20° for 40 min. The supernatant was decanted and dialyzed against 100 volumes of 0.15 M phosphate-buffered saline pH 7.4, at 4° with several changes being made over 48 hr. Protein was quantitated by the method of Lowry et al. (11).

Preparation of BCGcw. The mycobacterial cell walls were obtained from Dr. Edgar Ribi, Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Mont., and were prepared in a Drakeol Tween 80 0.9% NaCl solution as previously described (13). The dosage of BCGcw used was either 75 or 150 μg in a volume of 0.1 ml.

RESULTS

The initial observation that prior immunization with BCGcw was associated with an enhancement of tumor growth was first made from an experiment intended to determine whether animals could be immunized with solu-
able tumor antigens prepared by extraction with 3 M KCl. In this experiment, we chose to immunize animals with an admixture of tumor extract and BCGcw (75 μg). Such an immunizing inoculum was to promote the induction of a cellular antitumor immune response, and immunoprotection should have resulted. There were 4 experimental groups: BCGcw plus extract, extract alone, BCGcw alone, and a control group. Tumor challenge was performed 3 weeks following immunization. The results are summarized in Table 1.

Tumor growth, i.m., occurred in one-third of the control animals and i.m. tumors grew in one-third of the animals immunized with 3 M KCl 3924a tumor extracts. Five out of 6 of the animals immunized with only BCGcw grew tumors, whereas all of the 6 animals immunized with BCGcw and 3 M KCl 3924a tumor extracts grew tumors.

In the 2nd experiment, we inoculated BCGcw (150 μg) either 32 days before tumor cell inoculation, on the day of tumor cell inoculation, or 12 days after tumor inoculation. The results of this experiment are summarized in Table 2. The tumor cell inoculum grew in only 4 of 6 of the control animals. Tumor growth occurred in all of the animals in groups immunized with BCGcw, either 32 days before, or the day of, tumor cell inoculation. BCGcw immunization 12 days after tumor cell challenge did not appear to affect tumor incidence. In the animals immunized with BCGcw 32 days prior to tumor cell challenge, the average i.m. tumors on Day 55 (the day of sacrifice) were statistically significantly larger (p < 0.01), compared with those of the control group (Table 2). There was an increased tumor incidence in animals inoculated with BCGcw the same day as tumor cell inoculation, compared with the control group. There was a suggestion that there was an enhancement of tumor growth in the BCGcw-immunized animals, although the differences in average tumor mass were not statistically significant. The tumor incidence in the animals inoculated with BCGcw 12 days after tumor cell inoculation was approximately the same as that of the controls, but, again, slightly larger tumors were present in the BCGcw-immunized animals, although this difference was not statistically significant.

Three additional experiments were performed to determine what effect prior immunization with BCGcw had upon various challenge inocula of tumor cells. In these experiments, we chose to vary the time of BCGcw immunization in relationship to when the tumor would become palpable by varying the dose of the challenge inoculum of tumor cells. All animals were immunized with BCGcw 2 weeks prior to tumor cell challenge. These experiments are summarized in Table 3.

In Experiment A, we studied 2 dosage levels, 1 x 106 and 1 x 105. Tumor growth occurred in all animals inoculated with 1 x 106 tumor cells but, on the day of sacrifice (Day 51), tumors that were statistically significantly larger were present in animals that had been immunized with BCGcw. As was observed previously with 1 x 105 tumor cells, immunization with BCGcw was associated with an increased tumor incidence, and statistically significantly larger tumors were also present in the BCGcw-immunized animals.

In Experiment B, we observed that all of the animals inoculated with 1 x 106 tumor cells grew tumors, but when the animals were sacrificed on Day 38, the average tumor mass in the BCGcw-immunized animals was significantly larger than the average tumor mass in the unimmunized control animals. In the group of animals inoculated with 1 x 105 tumor cells, we again observed that the BCGcw-immunized animals had a higher incidence of tumors, compared with the control group. The average tumor mass on the day of sacrifice was again statistically significantly larger in the BCGcw-immunized animals, compared with the control group. Among animals inoculated with 1 x 105 tumor cells i.m. tumors did not grow in any immunized with BCGcw, but i.m. tumors grew in 3 out of 7 of the control animals. This protective effect of BCGcw immunization was a reverse of the enhancement we had previously observed.

The 3rd experiment was performed to further determine whether enhancement would occur following preimmunization with BCGcw and to determine whether the immunoprotective effect noted at low challenge dosage (Table 3, Experiment B) could be duplicated. The results are summarized in Table 3, Experiment C. In Experiment C, we repeated Experiment B, but added an additional dose titration of 1 x 106 tumor cells. In this experiment we again observed that there was marked enhancement of tumor growth at the dosage levels of 1 x 106 and 1 x 105. In the animals inoculated with 1 x 105 tumor cells, average i.m. tumors were significantly larger than the tumors in the control animals. Inoculation with 1 x 106 tumor cells resulted in approximately the same tumor incidence in both the BCGcw-immunized and control animals, but a statistically significant enhancement of the tumor growth was noted in the

### Table 1

<table>
<thead>
<tr>
<th>Tumor challenge given (1 x 106 cells)</th>
<th>No. with tumor/total no. of animals</th>
<th>Av. tumor mass on Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution controls</td>
<td>2/6</td>
<td>48 ± 9.5</td>
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<tr>
<td>3 M KCl-3924a tumor extract</td>
<td>2/6</td>
<td>78 ± 33.8</td>
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<td>BCGcw-3 M KCl-3924a tumor extract</td>
<td>6/6</td>
<td>39 ± 9.4</td>
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<td>BCGcw-3 M KCl-3924a tumor extract</td>
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<td>47 ± 9.2</td>
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<th>Av. tumor mass on Day 50</th>
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<tr>
<td>0.9% NaCl solution controls</td>
<td>4/6</td>
<td>35 ± 15.1</td>
</tr>
<tr>
<td>BCGcw-32 days before tumor</td>
<td>6/6</td>
<td>76 ± 10.1</td>
</tr>
<tr>
<td>BCGcw-day of tumor</td>
<td>6/6</td>
<td>52.8 ± 10.8</td>
</tr>
<tr>
<td>BCGcw-12 days after tumor</td>
<td>3/6</td>
<td>53 ± 29.7</td>
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* The dose of BCGcw injected intrafootpad was 150 μg/animal.
* The differences between the control and experimental groups were significant to p < 0.01.

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immunized animals. No tumor growth was observed in any of the animals inoculated with either $1 \times 10^4$ or $1 \times 10^5$ tumor cells.

**DISCUSSION**

Systemic immunization with BCG has a variable effect upon tumor development and subsequent growth. Studies have been performed that evaluated the effect that BCG immunization had on the induction of tumors. Old *et al.* (16) demonstrated a transient protection of mice both against chemically induced tumor and against spontaneous mammary tumors and leukemias. That prior immunization with BCG can delay tumor incidence has been shown for $^{60}$Sr induced osteogenic tumors in CBA mice (15), dimethylbenzanthracene-induced mammary tumors in rats (17), urethane-induced lung adenomas (3), and polyoma virus-induced tumors (9).

There have been instances in which prior immunization with BCG has been associated with an enhancement of tumor growth. This was somewhat inadvertently observed when BCG was given to animals that had palpable tumors (17). Piessens *et al.* observed, in a dimethylbenzanthracene mammary tumor model system in rats, that BCG immunization after the 1st palpable tumor significantly increased the appearance of the 2nd palpable tumor. Lavrin *et al.* (8) noted a similar time relationship, in that BCG immunization prior to or shortly after carcinogen administration inhibited tumor induction, but a marked enhancement of tumor growth occurred when BCG was given at the time that the animals began to develop palpable tumors.

A few investigators have examined the effect that immunization with immunostimulants has upon the growth of a quantitative tumor cell inoculum. Faraci *et al.* (6) showed that, when mice were immunized with BCG prior to the i.m. challenge with $1 \times 10^6$ S-91 melanoma cells, there was a 65% reduction in melanoma incidence (6). They observed no protection to challenge with a mammary tumor ($1 \times 10^5$) or to a methylcholanthrene fibrosarcoma ($1 \times 10^3$). That this beneficial effect of BCG immunization is dependent upon the dose of BCG inoculated was noted by Chee and Bodurtha (5). They found, upon using the B-16 mouse melanoma tumor, that mice preimmunized with a high dose (0.5 mg) of BCG had an accelerated growth rate, whereas, with a lower dose (0.005 mg) of the BCG, tumors grew at a reduced rate.

The variable effect of BCG immunization may be related to the tumor burden present. A study by Bansal and Sjogren (2) showed that administration of BCG 2 weeks prior to or at the time of isografting of a polyoma tumor resulted in inhibition of tumor growth, but when BCG was inoculated when the tumor was palpable, there was an enhancement of the tumor growth.

The various routes of BCG administration have variable effects on tumor growth. This was noted by Pimm and Baldwin (18), in that enhancement of intrapleural tumor growth occurred when BCG was inoculated i.v. or s.c., but no enhancement was observed when BCG was inoculated i.p.

Other immunostimulatory agents, such as methanol extraction residue of BCG (7), and Freund’s adjuvant (1, 12) have also been associated with enhancement of tumor growth. Methanol extraction residue of BCG, administered 14 and 3 days before challenge with syngeneic Moloney virus-induced mammary tumors, was associated with an increase in the incidence of the tumors compared with that of the 0.9% NaCl solution-treated controls (7). A study by Meltzer *et al.* (12) showed that prior immunization with complete Freund’s adjuvant delayed tumor rejection in ani-
mals and also effectively abrogated the induction of tumor immunity. A similar observation was made in hamsters treated with complete Freund’s adjuvant prior to challenge with adenovirus tumor cells (1).

Although these studies differ markedly, they have some similarities. (a) Enhancement following immunization does not occur in all tumor systems and, therefore, a particular host system must be present. In our model system, we have not yet shown that enhancement of tumor growth occurs with other tumor lines in inbred rats, but further studies concerning this are planned in the near future. (b) The dose, route, time of administration of BCG, and other immunostimulatory agents are critical, as enhancement is not consistently observed in all situations. In our own experiments, the greatest enhancement was noted when animals were immunized with BCGcw approximately 2 weeks prior to tumor challenge with 1 x 10^6 or 1 x 10^7 tumor cells, which would grow into palpable tumors by 3 and 6 weeks, respectively. That the BCG-induced enhancement was dependent upon the time following tumor injection was shown in Experiment 2, in which BCG administered 12 days following the inoculation of 1 x 10^5 tumor cells did not increase tumor incidence, and there was no significant increase in the tumor mass. Systematic experiments to evaluate what effect altering the dose of BCGcw had upon tumor challenge inoculum were not conducted. (c) The enhancement observed by others was in most cases a systemic phenomenon. Experiments are currently underway to determine whether this is true in our experimental model.

The mechanism(s) of tumor enhancement following immunostimulation are unclear, but some proposed explanations include the following: (a) Antigenic competition can exist between BCG and the tumor. Some tumor lines have antigenic determinants that cross-react with antiserum to BCG, and some animals immunized with BCG may form antibodies that cross-react with antigenic determinants present on tumor cells (4). (b) The induction of serum-blocking factors may occur in BCG-immunized animals, especially if there is an antigenic similarity between BCG and the tumor. Bansal and Sjogren (2), using in vitro studies, documented that when BCG was given at the time a tumor was palpable, there was no increase in the level of cell-mediated immunity, but there was an increase in the blocking activity of the serum. A similar phenomenon was noted in a patient who developed serum-blocking activity following immunotherapy with BCG (10). (c) Immunostimulation by BCG and other related agents causes a marked alteration in the morphological structure and functional activity of lymphoid organs. Such an alteration, especially in the local relative balance of B- and T-cells, could result in an inappropriate immunological response to a tumor cell inoculum, with the end result being an enhancement of tumor growth.

If immunotherapy with BCG and other agents is performed following tumor resection and curative chemo- and irradiation therapy, one must be cognizant of the fact that, on occasion, such immunostimulation may be a form of functional immunosuppression, with the end result being an increase in the growth rate of any residual tumor and the subsequent clinical deterioration of the patient.

REFERENCES

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