Eradication by Immunization with Mycobacterial Vaccines and Tumor Cells of Microscopic Metastases Remaining after Surgery

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ABSTRACT

Guinea pigs, each with a growing tumor from hepatoma cells implanted in the skin, were treated either by surgical excision alone of the dermal tumor or by surgery followed by administration of emulsified Bacillus Calmette-Guérin (BCG) cell walls alone or mixed with hepatoma cells. Animals treated by surgery alone developed malignant disease characterized by progressive lethal growth of lymph node metastases. Injections of cell walls intradermally between the site of excision and the draining lymph node (regional injection) prevented the development of palpable metastasis in some animals; cell walls given intradermally on the side contralateral to the surgical site (remote injection) cured no animals. Similar treatments with preparations containing both cell walls and hepatoma cells prevented the development of malignant disease in a significant number of animals whether the injections were regional or remote. Treatment with mixtures consisting of living BCG and tumor cells was not as effective as treatment with cell walls mixed with tumor cells. A mixture consisting of modified BCG cell walls known as skeletons, trehalose dimycolate also known as cord factor or P3, and endotoxin was an effective substitute for BCG cell walls. There was no particular advantage in the use of the mixture containing BCG cell wall skeletons, trehalose dimycolate, and endotoxin instead of BCG cell walls. Neither preparation produced any gross systemic toxicity, and there was no statistical difference in their therapeutic efficacy. Under degraded cell wall emulsions were easier and less costly to prepare than was the three-component mixture. Animals treated by surgery followed by administration of BCG cell walls together with cells from another syngeneic hepatoma, immunologically distinct from the dermally implanted tumor, failed to eradicate microscopic lymph node metastases.

INTRODUCTION

Development of effective methods to eliminate tumor remaining after cancer surgery is a major goal of oncologists. Some progress has been made toward this goal. Postoperative chemotherapy has been effective in prolonging disease-free intervals in selected patients with osteogenic sarcoma (10) and carcinoma of the breast (6, 9), yet many patients with resectable neoplasms eventually succumb to progressive growth of tumors originating from occult metastasis present at the time of surgery. Immunotherapy, because it produces specific and systemic antitumor responses, is in theory capable of eliminating malignant disease remaining after surgery. Thus far, attempts to apply this method to the treatment of human cancer have not been conspicuously successful (18). Animal models may help to establish conditions for successful postoperative immunotherapy. Optimal analogs of human cancer would be animals with autochthonous carcinomas that frequently spread from the primary site to regional lymph nodes and/or other sites such as brain, bone, liver, and lung.

Transplantable syngeneic metastasizing tumors may be useful in selecting promising immunotherapies. These tumor models provide an opportunity to test the efficacy of a number of treatment regimens in a homogeneous population of animals within a relatively short time. Selected treatments may be further evaluated in animals with autochthonous tumors. We have studied the effects of immunotherapy on growth of a transplantable syngeneic hepatoma of inbred guinea pigs. After i.d.3 injection, this hepatoma regularly metastasizes to lymph nodes; usually, this hepatoma does not metastasize to visceral organs. We demonstrated that i.l. injection of living BCG (21) or CW (25) led to regression of dermal tumors and prevented growth of lymph node metastases. These observations provided the impetus and guidelines for a trial of this form of therapy in cattle with naturally occurring autochthonous tumors. Injection of CW i.l. caused regression of bovine ocular carcinomas in a majority of treated animals (13). The results of the bovine study were consistent with the findings in the guinea pig model. To provide an additional approach to the treatment of metastases, we searched for conditions required for successful postoperative immunotherapy. Here we report the immunotherapeutic eradication of microscopic metastasis remaining in regional lymph nodes after excision of a syngeneic guinea pig hepatoma established by i.d. injection of tumor cells. Three preparations were tested for immunotherapeutic potency. Living BCG was evaluated because it has been used extensively in clinical cancer immunotherapy. CW were chosen because experiments in animals indicated that this agent was at least as effective as and less toxic than living BCG (24). CWS-TDM-ET was selected because of data suggesting that this mixture has greater antitumor activity than do CW (12). This study may provide information on some of the conditions required for eradicating occult metastases remaining after surgical removal of primary tumors.

MATERIALS AND METHODS

Animals. Male Sewall-Wright strain 2 guinea pigs were obtained from the Laboratory Aids Branch, Division of Research

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1 The abbreviations used are: i.d., intradermal; i.l., intralesional; BCG, Bacillus Calmette-Guérin; CW, Bacillus Calmette-Guérin cell walls; CWS, Bacillus Calmette-Guérin cell wall skeleton; TDM, trehalose dimycolate; ET, endotoxin; CFU, colony-forming units.
Services, NIH, and from the Experimental Animal Breeding Facility of the National Cancer Institute, Frederick Cancer Research Center, Frederick, Md. Caged in groups of 6, the animals were fed NIH guinea pig ration and given water ad libitum. All animals were 3 to 4 months old and weighed 500 to 550 g.

**Tumor Line.** We used a transplantable syngeneic hepatocellular carcinoma designated line 10. This diethylnitrosamine-induced tumor has been converted to ascites form and is passed i.p. in weaning strain 2 guinea pigs. Inoculation of 10⁶ line 10 tumor cells i.d. in the flank leads to progressive i.d. tumor growth, metastasis of tumor cells to regional axillary lymph nodes, and death (22). In one experiment, we used a transplantable syngeneic hepatoma, designated line 1. This diethylnitrosamine-induced tumor has been converted to ascites form. Injection of 3 × 10⁶ line 1 tumor cells in the flank leads to temporary i.d. tumor growth (26). Previous studies demonstrated that line 10 and line 1 tumor cells each contain individually specific tumor rejection antigens (23).

**Mycobacteria.** BCG (Trudeau Mycobacterial Collection No. 1029) was obtained from the Trudeau Institute, Saranac Lake, N. Y. BCG was grown in Proskauer-Beck medium with Tween 80, fresh frozen, stored in aliquots at −90°, and thawed at 37° immediately before use.

**Preparation of Oil-in-Water Emulsions Containing CW.** CW were separated from other cell constituents by methods described in Ref. 1. CW (16.84 mg; Lot 217b) were placed in a 15-ml tissue grinder equipped with a Teflon pestle. A light mineral oil (0.270 ml) was added to the CW. The mixture was ground to a smooth paste. A 0.85% NaCl solution containing 0.2% Tween 80 (6.74 ml) was added to the paste, and grinding was continued until a well-dispersed oil-in-water emulsion was obtained. The emulsion was poured into a sterile container, and 2 ml Tween-0.85% NaCl solution diluent were added to the grinding tube. Grinding continued for 2 to 3 min. The 2 emulsions were combined and ground for an additional 2 to 3 min. This emulsion contained (per ml) 1.87 mg CW, 30 µl oil, and 2 µl Tween.

**Preparation of Oil-in-Water Emulsions Containing CWS, TDM, and ET.** The residue remaining after treatment of the undegraded cell walls of BCG with proteolytic enzymes and organic solvents has been referred to as CWS (1). TDM (also known as cord factor or P3), a family of compounds containing trehalose, each molecule of which is linked to 2 molecules of mycolic acid, was extracted from whole mycobacterial cells as described in Ref. 1. ET was extracted from polysaccharide-deficient *Salmonella typhimurium* by the phenol-water method (19). CWS (Lot 770323 WB), TDM (Lot D-17), and ET (Lot SMS 761202/405) were combined as described (12) so that the grinding tube contained 12 mg CWS, 6 mg TDM, and 12 mg ET. Mineral oil (0.160 ml) was added, and the mixture was ground to a smooth paste. Oil-in-water emulsions were prepared as detailed above except that the total amount of 0.85% NaCl solution containing Tween was 8 ml. The final emulsion contained (per ml) 3.75 mg bacterial products, 20 µl oil, and 2 µl Tween.

**Composition of Mixtures Used for Treatment.** CW were used alone at a concentration of 1.87 mg/ml of emulsion. Mixtures of CW and tumor cells were prepared by adding emulsified CW (1.87 mg/ml) to a pellet of tumor cells. The pellet contained 15 × 10⁶ cells for each ml of CW. CWS-TDM-ET was used after dilution of the stock emulsion with an equal volume of Tween-0.85% NaCl solution to a concentration of 1.87 mg/ml of emulsion. CWS and ET each accounted for 40% and TDM accounted for 20% of the 1.87 mg. Mixtures of CWS-TDM-ET and tumor cells were made by mixing a given volume of the stock emulsion with an equal volume of a suspension of live tumor cells containing 30 × 10⁶ cells/ml. Suspensions of living BCG (0.2 to 1 × 10⁶ CFU/ml) were mixed with an equal volume of diluent (Dulbecco’s phosphate-buffered saline containing 1% gelatin) and used alone at a concentration of 0.1 to 0.5 × 10⁶ CFU/ml. Mixtures of living BCG and tumor cells were made by mixing the organisms at a concentration of 0.2 to 1 × 10⁶ CFU/ml with an equal volume of tumor cells containing 30 × 10⁶ cells/ml. The following quality control measures were followed. Batches of tumor cells containing more than 10% trypan blue-stained cells were discarded. Each oil-in-water emulsion was examined microscopically to determine whether CW granules were incorporated into spherical oil droplets. Emulsions consisting of transparent or nonspherical oil droplets were discarded. Emulsions and tumor cells were mixed just before inoculation.

**Tumor Excision.** Tumors located i.d. were removed by local excision 7 days after injection of 10⁶ line 10 tumor cells. Previous studies indicated that at this time tumor cells were present in the superficial distal axillary lymph nodes (21).

**Immunotherapy.** Emulsions and mixtures were injected i.d. (0.4 ml, 0.1 ml in each of 4 injection sites) 2 days after excision of dermal tumors (see Chart 1 for location of injections).

**Measurements.** Animals were examined at weekly intervals after surgery to detect growth of tumors. Measurements were made of the size of regional lymph nodes in the axilla, because invariably this was the site of tumor growth in animals that were not cured. For 3 to 4 weeks after immunostimulant treatment, it was not possible to distinguish between lymphadenopathy resulting from immunostimulation and that resulting from tumor growth. Axillary adenopathy detected 4 weeks after immuno-stimulant treatment was a reliable indicator of tumor progression. Unsuccessfully treated animals died 60 to 90 days after tumor cell injection with widespread lymph node metastases (axillary, inguinal, and cervical lymph nodes) and anasarca. There was no evidence of metastases to internal organs. There were no recurrences of tumor at the site of excision. Date of death was recorded. Significance of results was determined with the Fisher exact test, the x² analysis, and the Mann-Whitney U test. Experimental animals were observed for a minimum of 90 days after injection of tumor cells; the usual observation period was 120 days.

**RESULTS**

The experimental design used for tests is illustrated in Chart 1. Tumor cells (10⁶ line 10) were injected i.d. on Day 0. Seven days after injection of tumor cells, i.d. tumors were excised. Immunotherapy was administered 2 days after surgery. The efficacy of treatment with adjuvant alone was evaluated since similar treatments have been widely used in the clinic for treatment of patients with cancer. The influence of the site of administration of adjuvant alone on the outcome of therapy was analyzed. Also tested was the efficacy of treatment with...
The minimum observation period was 90 days after injection of tumor cells. Animals in Group 1 did not receive immunotherapy. Composition of vaccine Location of vaccination No. of animals alive and tumor free/no. tested in following experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Bacterial immunostimulant Dose</th>
<th>Tumor antigen</th>
<th>Location of vaccination</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td></td>
<td></td>
<td>0/20^a</td>
<td>0/12</td>
<td>0/15</td>
<td>0/11</td>
<td>0/14</td>
<td>0/17</td>
<td>0/9</td>
<td>3/12</td>
<td>0/10</td>
<td>3/120 (3)^b</td>
</tr>
<tr>
<td>2</td>
<td>CW 750 µg</td>
<td>Ipsi.</td>
<td>3/15</td>
<td>1/15^d</td>
<td>1/15</td>
<td>1/15</td>
<td>1/15</td>
<td>1/15</td>
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<td>1/15</td>
<td>1/15</td>
</tr>
<tr>
<td>3</td>
<td>CW 750 µg +</td>
<td>Ipsi.</td>
<td>12/14</td>
<td>10/12</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>CW 750 µg −</td>
<td>Contra.</td>
<td>0/9</td>
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<tr>
<td>5</td>
<td>CW 750 µg + Contra.</td>
<td>10/15</td>
<td>8/9</td>
<td></td>
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</tr>
<tr>
<td>6</td>
<td>CWS-TDM-ET 750 µg</td>
<td>Ipsi.</td>
<td>6/15</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>CWS-TDM-ET 750 µg +</td>
<td>Ipsi.</td>
<td>13/14</td>
<td>12/12</td>
<td>12/14</td>
<td>6/6</td>
<td></td>
<td></td>
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<tr>
<td>8</td>
<td>CWS-TDM-ET 750 µg −</td>
<td>Contra.</td>
<td>0/9</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>9</td>
<td>CWS-TDM-ET 750 µg + Contra.</td>
<td>3/15</td>
<td>12/12</td>
<td>5/10</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>Living BCG 10^6 CFU</td>
<td>Ipsi.</td>
<td>0/15</td>
<td>4/13</td>
<td>2/10</td>
<td>4/9</td>
<td>6/10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>16/57 (28)</td>
</tr>
<tr>
<td>11</td>
<td>Living BCG 10^6 CFU +</td>
<td>Ipsi.</td>
<td>4/14</td>
<td>8/9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12/23 (52)</td>
</tr>
<tr>
<td>12</td>
<td>Living BCG 10^6 CFU −</td>
<td>Contra.</td>
<td>0/9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/19 (0)</td>
</tr>
<tr>
<td>13</td>
<td>Living BCG 10^6 CFU + Contra.</td>
<td>1/14</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>1/14 (7)</td>
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</tr>
</tbody>
</table>

^a Experiment 1: Group 2 versus Group 1, NS; Group 3 versus Group 1, p < 0.002; Group 4 versus Group 1, NS; Group 5 versus Group 1, p < 0.003; Group 3 versus Group 2, p < 0.002; Group 6 versus Group 1, p = 0.003; Group 7 versus Group 1, p < 0.002; Group 8 versus Group 1, NS; Group 9 versus Group 1, NS; Group 7 versus Group 6, p = 0.004. Experiment 2: Groups 3, 5, 7, 9 versus Group 1, p < 0.002. Experiment 3: Group 6 versus Group 1, p = 0.050; Group 7 versus Group 1, p < 0.002; Group 9 versus Group 1, p = 0.005; Group 6 versus Group 7, p = 0.002. Experiment 4: Group 6 versus Group 1, p = 0.035. Group 7 versus Group 1, p < 0.001; Group 6 versus Group 7, p = 0.026. Experiment 5: no significant difference in tumor incidence; median survival times of Groups 6 and 10 significantly different than median survival time of Group 1, p < 0.005. Experiment 6: Group 10 versus Group 1, p = 0.026; Group 11 versus Group 1, p = 0.032; Group 13 versus Group 1, NS. Experiment 7: Groups 10 and 12 versus Group 1, NS. Experiment 8: Group 10 versus Group 1, NS; Group 11 versus Group 1, p = 0.006; Group 10 versus Group 11, NS. Experiment 9: Group 10 versus Group 1, p = 0.005.

^b Numbers in parentheses, percentage of tumor-free animals.

^c Ipsi., ipsilateral or adjacent to the site of tumor excision; Contra., contralateral to the site of tumor excision; NS, not significant.

d Median survival times for animals in Experiment 5 were: Group 1, 73 days; Group 2, 82 days; Group 4, 75 days; Group 6, 95 days; Group 8, 76 days; Group 10, 89 days; and Group 12, 73 days.
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and tumor cells contralateral to the site of excision was also effective in preventing the growth of microscopic lymph node metastases.

**Experiment 5.** Regional and remote treatments with immunostimulants alone (CW, CWS-TDM-ET, and living BCG) were compared in this experiment (Chart 3). None of the immunostimulants injected regionally or remotely significantly reduced the incidence of metastases. The median survival times of guinea pigs treated by contralateral injection of immunostimulants alone were not significantly different from those of animals not receiving immunostimulant treatment (Table 1, Footnote d). Regional treatment with CWS-TDM-ET or living BCG significantly prolonged median survival times.

**Experiments 6 to 9.** These 4 experiments were performed to determine whether vaccines containing living BCG with or without tumor cells would be effective in preventing growth of microscopic lymph node metastases. The effects of regional treatment with BCG alone were variable. Regional treatment with BCG alone significantly inhibited tumor growth in Experiment 6 and 9 but not in Experiments 5, 7, and 8. In contrast to CW and to CWS-TDM-ET, regional treatment with vaccines containing both living BCG and tumor cells was not significantly more effective than regional treatment with living BCG alone (Experiments 6 to 8). Remote injection of living BCG and tumor cells was ineffective (Experiment 6) (Chart 4).

Results of Experiments 1 through 9 are combined and summarized in Tables 2 to 4. Conclusions reached from study of results of individual experiments on CW and on CWS-TDM-ET (Tables 2 and 3) are in agreement with the summarized data. The combined data indicate that treatment with regional CWS-TDM-ET and tumor cells was significantly more effective than was remote treatment. The efficacy of CW and CWS-TDM-ET

**Table 2**

<table>
<thead>
<tr>
<th>Composition of vaccine</th>
<th>Ipsilateral vaccination</th>
<th>Contralateral vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW</td>
<td>A. 4/30 (13)</td>
<td>B. 0/18 (0)</td>
</tr>
<tr>
<td>CW + tumor cells</td>
<td>C. 22/26 (84)</td>
<td>D. 18/24 (75)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of tumor-free animals.

\[ \chi^2: \text{Group A versus Group B, } 2.62 (p > 0.10 < 0.20); \text{ Group C versus Group D, } 0.72 (p > 0.3); \text{ Group A versus Group C, } 28.2 (p < 0.001); \text{ Group B versus Group D, } 23.76 (p < 0.001).\]

**Table 3**

<table>
<thead>
<tr>
<th>Composition of vaccine</th>
<th>Ipsilateral vaccination</th>
<th>Contralateral vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWS-TDM-ET</td>
<td>A. 18/55 (32)</td>
<td>B. 0/19 (0)</td>
</tr>
<tr>
<td>CWS-TDM-ET + tumor cells</td>
<td>C. 43/46 (93)</td>
<td>D. 20/37 (54)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of tumor-free animals.

\[ \chi^2: \text{Group A versus Group B, } 8.62 (p < 0.01); \text{ Group C versus Group D, } 17.18 (p < 0.001); \text{ Group A versus Group C, } 8.55 (p < 0.001); \text{ Group B versus Group D, } 16.03 (p < 0.001).\]
Immunotherapy of Lymph Node Metastases

was compared by analyzing data summarized in Tables 2 and 3. No significant difference in therapeutic efficacy was found between CW and CWS-TDM-ET when these agents were given together with tumor cells (Tables 2 and 3, Groups C and D). On the basis of the summarized data, animals treated with CWS-TDM-ET alone adjacent to the site of excision fared better than animals treated with CW alone adjacent to the site of excision (Tables 2 and 3, Group A) ($\chi^2 = 3.79; p < 0.05$). This conclusion was not supported by the results of individual experiments. Data on effects of treatment with living BCG are given in Table 4. The summary indicates that addition of tumor cells to living BCG improved the effect of BCG treatment, but this conclusion is not supported by the results of the individual experiments.

To test whether effective therapy required tumor cells in the vaccine antigenically similar to the microscopic lymph node metastases, animals were treated by immunization with CW alone, with CW admixed with cells of an antigenically dissimilar syngeneic tumor (line 1) and with CW admixed with line 10 tumor cells (Table 5). Since it was possible that injection of line 1 tumor cells and CW adjacent to the tumor site might retard growth of line 10 lymph node metastases, treatments were given ipsilaterally to some groups of animals; other groups of animals were treated contralaterally. The results were clearcut. Injection of CW and line 1 tumor cells adjacent to the site of excision cured a few animals. Addition of line 1 tumor cells to CW did not significantly improve efficacy of CW treatment. Injection of CW and line 10 tumor cells adjacent to the site of excision cured all treated animals. When CW alone or CW and line 1 tumor cells were injected contralaterally to the site of excision, no animals were protected. Injection of CW and line 10 tumor cells contralateral to the site of excision protected a significant number of animals.

Rarely, tumor growth was observed at the site of vaccination. Of 167 treated animals, tumor growth at the site of injection occurred in 4. All animals that had tumor growth at the site of vaccination failed to prevent the growth of lymph node metastases. Animals in Experiments 1 and 2 alive and tumor free 90 days after injection of tumor cells were challenged by i.d. injection of living line 10 tumor cells. Of 32 tested animals, 28 rejected the tumor challenge. Injection i.d. of vaccines containing CW or CWS-TDM-ET did not produce any gross systemic toxicity.

Recently, we tested whether tumor cells rendered nontuberculous by X-irradiation could effectively substitute for living tumor cells in vaccines. Animals were treated by i.d. injection of X-irradiated line 10 tumor cells (20,000 R; $30 \times 10^6$ cells) admixed with CW ($500 \mu g$). Treatments were given contralateral to the site of excision. Results are a summary of 4 experiments. Of 33 immunized animals, 20 were alive and tumor free at the end of the observation period; of 55 animals that were not immunized, 1 animal was tumor free. ($\chi^2 = 38.9; p < 0.001$)

DISCUSSION

The results of this investigation provide additional insight into the conditions required for immunotherapeutic eradication of residual malignant disease following surgery. Injection of immunostimulants alone remote from the surgical site was ineffective. This failure of postoperative adjuvant therapy in animals with defined residual metastatic cancer is analogous to the failure of BCG scarification treatments to improve significantly the course of patients with cancer (15, 16). Additionally, previous studies in this (17, 22) and several other animal models have emphasized the ineffectiveness of systemic adjuvant therapy in the treatment of experimental cancer (2, 5).

Regional postoperative adjuvant treatment with living or nonliving bacterial immunostimulants alone had a weak inhibitory effect on growth of microscopic lymph node metastases. These results may be analogous to those observed following treatment of patients with Stage I lung cancer by postoperative intrapleural injection of living BCG (14). Previous reports from this and other laboratories have emphasized the importance of close contact between bacterial immunostimulants and tumor for beneficial results. Injection of immunostimulants into the skin adjacent to the regional lymph node presumably assures that the agent will reach the site of metastasis.

Remote or regional postoperative treatment with nonliving bacterial immunostimulants (CW or CWS-TDM-ET) and tumor cells was effective treatment against microscopic metastases. The success of active immunization in this model stands in contrast to the failure of this modality of treatment to benefit patients with solid tumors (8, 15). In our studies, syngeneic living tumor cells were used as the source of antigen. Many human trials have used allogeneic tissue-cultured tumor lines as the source of antigen. These lines may not possess the relevant tumor rejection antigens. Additional factors that may account for the different result of active immunotherapy in humans and in the line 10 tumor model in guinea pigs include tumor immunogenicity, tumor burden, dose and form of tumor antigen, and dose and form of bacterial immunostimulants. It is

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Table 4

<table>
<thead>
<tr>
<th>Composition of vaccine</th>
<th>No. of animals alive and tumor free/no. of animals tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPSILATERAL VACCINATION</td>
<td></td>
</tr>
<tr>
<td>Living BCG</td>
<td>A. 16/57 (28)*</td>
</tr>
<tr>
<td>Living BCG + tumor cells</td>
<td>C. 12/23 (62)</td>
</tr>
<tr>
<td>CONTRALATERAL VACCINATION</td>
<td></td>
</tr>
<tr>
<td>Living BCG</td>
<td>B. 0/19 (0)*</td>
</tr>
<tr>
<td>Living BCG + tumor cells</td>
<td>D. 1/14 (7)</td>
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</tbody>
</table>

* Numbers in parentheses, percentage of tumor-free animals.

b X^2: Group A versus Group B, 6.71 (p < 0.01); Group C versus Group D, 7.65 (p < 0.01); Group A versus Group C, 4.26 (p < 0.05); Group B versus Group D, 1.4 (p > 0.30).

Table 5

<table>
<thead>
<tr>
<th>Composition of vaccine</th>
<th>No. of animals alive and tumor free/no. of animals tested</th>
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</thead>
<tbody>
<tr>
<td>IPSILATERAL VACCINATION</td>
<td></td>
</tr>
<tr>
<td>CW</td>
<td>A. 0/11*</td>
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<tr>
<td>CW + line 1 tumor</td>
<td>C. 2/12</td>
</tr>
<tr>
<td>CW + line 10 tumor</td>
<td>E. 12/12</td>
</tr>
<tr>
<td>CONTRALATERAL VACCINATION</td>
<td></td>
</tr>
<tr>
<td>CW</td>
<td>B. 0/12</td>
</tr>
<tr>
<td>CW + line 1 tumor</td>
<td>D. 0/12</td>
</tr>
<tr>
<td>CW + line 10 tumor</td>
<td>F. 9/12</td>
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</table>

* Results of Fisher exact test: A versus B, NS; C versus D, NS; E versus F, NS; A versus C, NS; A versus E, p < 0.005; B versus D, NS; B versus F, p < 0.001; C versus E, p < 0.002; D versus F, p < 0.001; NS, not significant.
known that the tumor used in our studies is immunogenic. Suitably immunized guinea pigs will reject an inoculum of living line 10 tumor cells (22). It is not known whether the human tumors possess similar tumor rejection antigens. The tumor burden in this animal model was approximately $10^6$ tumor cells located within a single axillary lymph node. Many patients treated by active immunization had tumor burden far in excess of that which can be effectively treated in this animal model. Dose-response tests have not yet been performed in the model described in this report, but previous investigations with this tumor indicate the critical importance of an adequate dose of tumor antigen to effect successful immunization (4). The nonliving bacterial immunostimulators that we used were highly effective adjuvants for the treatment of residual malignant disease, and they gave more reproducible results than did living BCG. These adjuvants have not yet been extensively used in human trials.

Remote or regional treatment with living BCG admixed with tumor cells was not more effective than regional treatment with living BCG alone. Several additional experiments (data not shown) were performed with living BCG mixed with tumor cells to prevent growth of microscopic metastases; in 2 of 3 experiments, vaccination failed to prevent growth of microscopic metastases. It is not clear why living BCG functioned poorly in this residual disease setting, since it is clear that vaccine containing living BCG can prevent growth of i.v. injected tumor cells (3, 11). It has been suggested that infection with mycobacteria may have both immunostimulatory and immunosuppressive activities (7, 24); under certain circumstances, these suppressive properties may interfere with active immunization.

CW vaccines contained 3 times more mineral oil than did CWS-TDM-ET vaccines. Since concentration of mineral oil influences the antitumor activity of mycobacterial emulsions (20), it is possible that CWS-TDM-ET would have been more effective than CW had the 2 preparations been tested at equivalent oil concentrations.

Injection of living autochthonous tumor cells with adjuvant into human beings with minimal residual malignant disease might be unacceptable because of the possibility of tumor growth at the vaccine site. Studies are under way to determine whether nonreplicating tumor cells can effectively substitute for living tumor cells. Preliminary data indicate that tumor cells rendered nontumorigenic by X-irradiation when admixed with CW function to eradicate microscopic lymph node metastases. Cure rates equivalent to those found with living tumor cells were obtained when 5 times more X-irradiated tumor cells were incorporated in the vaccine mixture. Irradiated tumor cells alone injected i.d. contralateral to the site of excision failed to cure any guinea pigs.

Experiments are currently being performed to define the stage(s) of disease amenable to treatment by systemic vaccination and to improve existing vaccines. Data indicate that both the time of vaccination and the quantity of tumor cells and adjuvant in vaccines have pronounced effects on treatment efficacy.

Our results permit some speculation about the mechanisms of immunological destruction of lymph node metastases. The failure of contralateral treatment with adjuvant alone to retard the growth of microscopic metastases suggests that a systemic host response to mycobacterial antigens is insufficient to destroy cancer metastases. The success of regional postoperative immunotherapy with adjuvant alone indicates that the local host response to mycobacterial antigens can lead to inhibition of growth of cancer metastases.

In our model, metastases were situated at one site of generation of immunological responses. Metastases within lymph nodes could serve as antigen for development of tumor rejection responses. After delivery of adjuvant to the lymph node containing metastases, immunization to antigen might occur leading to destruction of these metastases. Regional adjuvant treatment did lead to destruction of lymph node metastases in a few animals. Regional treatment with nonliving adjuvant and tumor cells led to destruction of metastases in more than 80% of animals. These observations suggest that the number of tumor cells within lymph nodes provided insufficient antigen for generation of rejection responses. Addition of tumor cells to the nonliving bacterial immunostimulant may have provided sufficient tumor antigen to effect immunological destruction of nodal metastases. The view that specific tumor immunity was required for destruction of lymph node metastases is supported by the following observations: (a) the ineffectiveness of treatment with adjuvant alone; (b) the marked improvement in results when animals were immunized with adjuvant and line 10 tumor cells; and (c) the ineffectiveness of treatment with adjuvant and line 1 tumor cells (a tumor containing tumor rejection antigens distinct from those of line 10 tumor cells).

Some work toward characterizing the factors necessary for optimal immunization against residual metastatic cancer has already been performed in this tumor system (3, 4, 11). Vaccines containing irradiated line 10 tumor cells and living BCG were effective in preventing the growth of a simultaneous contralateral i.d. challenge but were not effective in preventing the growth of microscopic axillary lymph node metastases. Vaccines containing irradiated line 10 tumor cells and living BCG were effective in preventing the growth of i.v. injected tumor cells. Animals that received a second injection of tumor cells 6 days after the first were capable of rejecting a larger challenge inoculum than those receiving only a single injection. It may be valuable to compare the potency of the vaccine and vaccination procedure described in this report with those described for prevention of growth of i.v. injected tumor cells and to test the ability of these vaccines to control growth of metastases in bone, brain, or liver.

An issue of some concern is the relevance of our findings to human cancer treatment. Given the major biological differences between a rodent-transplantable syngeneic tumor and autochthonous human cancers, it is possible that the findings reported have no relevance to human cancer immunotherapy. To put our findings in perspective, it will be necessary to test the efficacy of the methods described with other transplantable syngeneic tumors, autochthonous animal tumors, and guinea pigs with advanced malignant disease. These research programs are in progress.

The results presented indicate that a single systemic or regional treatment consisting of i.d. injection of nonliving mycobacterial components in oil-in-water emulsion in admixture with living tumor cells prevented growth of lymph node metastases remaining after local surgery. These results provide a guide to the treatment of animals with autochthonous tumors and occult microscopic metastases.
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Eradication by Immunization with Mycobacterial Vaccines and Tumor Cells of Microscopic Metastases Remaining after Surgery

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