Eradication by Active Specific Immunotherapy of Established Tumor Transplants and Microscopic Lymph Node Metastases

Ellyahu Yarkoni,1 Michael P. Ashley, Berton Zbar,2 Tohru Sugimoto, and Herbert J. Rapp3

ABSTRACT

Guinea pigs, each with an established syngeneic dermal line 10 tumor and microscopic lymph node metastases, were immunized by injection of a mixture of irradiated line 10 tumor cells and an oil-in-water emulsion containing heat-killed cells of Mycobacterium bovis strain Bacillus Calmette-Guérin. Squalane or squalene-in-water emulsions, prepared by ultrasonication and containing mg doses of mycobacterial cells, were effective adjuvants. Immunization eradicated established dermal tumors (about 10 mm in diameter) and prevented growth of microscopic lymph node metastases. Untreated animals, animals treated by irradiation, or animals treated by intraderal administration of Bacillus Calmette-Guérin cells attached to oil droplets alone or with irradiated tumor cells alone, all died with progressive tumor growth.

INTRODUCTION

A principal objective of experimental tumor immunologists has been to identify factors essential for the success of active specific immunotherapy (1–11, 15, 16). Animal models have been developed in which immunization retarded or eliminated the growth of established tumors or microscopic metastases (2, 4, 7, 8, 16). Successful immunization generally required injection of a mixture of live or irradiated tumor cells with a bacterial immunostimulant (3, 4, 7, 8). Therapy was dependent both on the dose of tumor cells and on that of adjuvant (1, 3–5). Histopathological studies (6) and studies of in vitro tumor cell cytotoxicity of cell populations from immunized animals (10) have been performed in an effort to identify factors that control active specific immunization. Knowledge of components of effective vaccines permitted Peters and Hanna (11) to identify the importance of tumor cell viability in cryopreserved, immunotherapeutically active vaccines. Analysis of vaccine efficacy as a function of time after tumor implantation permitted Mills et al. (10) to demonstrate that loss of vaccine potency correlated with inability of the vaccine to evoke a cytolytic response in regional lymph nodes. Neither histopathological nor in vitro studies of tumor cell cytotoxicity have provided clear guides in the design of vaccines with ability to enable the host to reject larger numbers of tumor cells than do already existing vaccines. In this report, we describe a nontumorigenic vaccine which enabled immunized guinea pigs to eradicate a tumor burden consisting of established dermal tumors and microscopic lymph node metastases. When injected in admixture with irradiated tumor cells, SQE or SQA-in-water emulsions, prepared by ultrasonication and containing mg doses of BCG organisms, were potent immunostimulants.

MATERIALS AND METHODS

BCG and Emulsions. BCG KC (Tice strain) was purchased from ITR Biomedical Research Center (University of Illinois at the Medical Center, Chicago, III.), and was used for immunotherapy studies. In one experiment, we used BCG CW Lot 286 purchased from the Rocky Mountain Laboratory, Hamilton, Mont. SOA and SQA were obtained from Eastman Kodak Co., Rochester, N. Y. Vitamin E was obtained from Sigma Chemical Co., St. Louis, Mo. SQE-in-water emulsions containing BCG KC or BCG CW were prepared as described (13). The final concentrations of emulsion components were as follows: BCG CW or BCG KC, 5 mg/ml; SQA, 3%; and Tween 80, about 0.2%. Emulsions containing SQE-vitamin E and BCG KC were prepared as described previously (14). The final concentrations of emulsion components were as follows: BCG KC, 1 to 5 mg/ml; SQE (SQA containing 2% vitamin E), 3%. Vitamin E was added to SQE to minimize oxidation. Because of the absence of Tween, emulsions made with SQE were unstable; oil droplets in these emulsions aggregated and floated to the surface of the liquid. For use in immunotherapy, emulsions containing SQE were mixed well before admixture with L10 cells and again before injection.

Animals. Adult male or female Sewall Wright strain 2 guinea pigs were obtained from stock maintained at the Frederick Cancer Research Center, Frederick, Md.

Tumor Line. L10, an ascitic variant, was derived from a hepatocarcinoma induced by diethylnitrosamine in a male strain 2 guinea pig. Inoculation of 105 L10 cells i.d. led to the formation of a dermal tumor nodule; by 1 week after i.d. injection, tumor cells were present in the SDA lymph nodes; guinea pigs died 2 to 3 months later with widespread lymph node metastases (12).

Irradiation. L10 cells (about 30 × 106/ml) were irradiated with a dose of 10,000 rads delivered by X-irradiation or by γ-irradiation (60Co or 137Cs). The percentage of L10 cells that excluded 0.1% trypan blue after irradiation was 95 to 98%. Two to 3 hr after admixture with KCE, the viability of the L10 cells was about 90%.

Vaccine Preparation. Vaccines containing BCG KCE (or BCG CWE) plus L10 cells were prepared not more than 0.5 hr before use. Suspensions of L10X were centrifuged at 140 × g, and the pellet was resuspended in emulsion. The injection volume for a guinea pig was 1.2 ml.

Animal Model for Active Immunotherapy. One million L10 cells were injected i.d. on the left flank about 2.5 cm posterior to the SDA lymph node on Day 0. Animals were immunized on Day 7; at this time, the abbreviations used are: SQE, squalene (C30H50; 2,6,10,15,19,23-hexamethylerotenocane); SQE, squalene (C30H50; 2,6,10,15,19,23-hexamethylerotenocane); SQA, squalane (C30H50; 2,6,10,15,19,23-hexamethylerotenocane); BCG, Bacillus Calmette-Guérin strain of Mycobacterium bovis; BCG KC, autoclave-killed, lyophilized, whole Bacillus Calmette-Guérin; BCG CW, Bacillus Calmette-Guérin cell walls; L10, tumor line 10; i.d., intraderal; SDA, superficial distal axillary; KCE, autoclave-killed, whole Bacillus Calmette-Guérin cell walls in emulsified form; BCG CWE, Bacillus Calmette-Guérin cell walls in emulsified form; L10X, irradiated tumor line 10 cells.

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Received September 28, 1981; accepted March 18, 1982.

CANCER RESEARCH VOL. 42

2544
ERADICATION OF DERMAL TUMORS AND LYMPH NODE METASTASES AFTER VACCINATION WITH IRRADIATED TUMOR CELLS AND BCG KCE: EFFICACY OF CONTRALATERAL VACCINATION

**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>BCG KCE total dose (mg)</th>
<th>L10X total dose (x 10^6)</th>
<th>Injection locations</th>
<th>No. of tumor-free animals/no. tested (90 days)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
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<td>100 A, B, C</td>
<td>6/10^6</td>
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<td></td>
<td>7/10^6</td>
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<td>2 Control</td>
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<td></td>
<td>13/18^6</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>3 100 C</td>
<td></td>
<td></td>
<td>10/18^6</td>
<td>&lt;0.001</td>
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</table>

^a SQA and SQE-Ve droplets served as carriers of BCG KC. ^b Vaccine was divided equally among the locations. ^c There is no statistically significant difference between test groups.

**Table 2**

<table>
<thead>
<tr>
<th>BCG KCE total dose (mg)</th>
<th>L10X total dose (x 10^6)</th>
<th>Injection locations^a</th>
<th>Type of oil</th>
<th>No. of tumor-free animals/no. tested (90 days)</th>
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<td>SQA</td>
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<td>&lt;0.001</td>
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<tr>
<td>3 100 A, B, C</td>
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<td></td>
<td>SQE-Ve</td>
<td>10/10</td>
<td>&lt;0.001</td>
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</tbody>
</table>

^a Vaccine was divided equally among the locations.

**Table 3**

<table>
<thead>
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<th>Experiment</th>
<th>BCG KCE total dose (mg)</th>
<th>L10X total dose (x 10^6)</th>
<th>No. of tumor-free animals/no. tested (90 days)</th>
<th>p</th>
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<td>NS^b</td>
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<td>100</td>
<td></td>
<td>9/10</td>
<td>&lt;0.001</td>
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</table>

^a SQE-Ve droplets served as carriers of BCG KC. Vaccine was divided equally among Locations A, B, and C. ^b NS, not significant.
were required to cause a statistically significant cure rate (Table 3, Experiment 2).

We compared the immunotherapeutic effectiveness of the i.d. and s.c. routes of vaccine administration. The results presented in Table 4 show that i.d. inoculation of the vaccine caused complete regression of the tumor in 9 of 10 animals, whereas s.c. administration was less effective (only 3 of 10 animals were cured).

We compared the efficacy of BCG CWE as an adjuvant with that of BCG KCE and found that BCG CWE was as effective as BCG KCE (Table 5).

**DISCUSSION**

Vaccines containing L10X plus SQA or SQE-in-water emulsions containing BCG cells or CW were highly effective in elimination of remote, relatively large (10-mm-diameter) dermal tumors and microscopic lymph node metastases. To our knowledge, this is the first report of complete regression of syngeneic established, metastasizing solid tumors by active specific immunotherapy with a nontumorigenic vaccine. We demonstrated that a vaccine containing L10X admixed with mineral oil-in-water emulsions containing BCG CW eradicated lymph node metastases remaining after surgical excision of 7-day-old dermal tumors (1). Treatment was antigenically specific (3, 16).

This vaccine also eradicated L10 cells remaining in lymph nodes after limited surgery for Stage II disease as well as i.v. injected L10 cells (9) but was not effective in guinea pigs with both established dermal tumors and microscopic lymph node metastases. The exact explanation for the difference in efficacy of the vaccine described in the present report and the vaccine described in Ref. 1 is unknown, but since both vaccines contained the same dose of L10X, the difference appears to reside in adjuvant formulation. In the previous study (1), emulsions were prepared by grinding BCG CW in mineral oil; dose-response studies indicated that mg doses of BCG CW were supraoptimal. In this report, emulsions were prepared by ultrasonication of BCG cells or CW in SQA or SQE; dose-response studies indicated that best results were achieved with mg doses of BCG KC. Difference in efficacy of the 2 vaccines may reflect differences in the carrier oil (mineral oil versus SQA or SQE-vitamin E), method of emulsification (grinding versus ultrasonication), or concentration of oil (10% versus 3%).

We found that the i.d. administration of the vaccine was immunotherapeutically more effective than was the s.c. route. Bartlett et al. (2) did not observe this difference using a model of immunization and challenge on the same day. The discrepancy might be explained by differences in tumor burden in the 2 reports. Bartlett’s vaccine (L10X plus viable BCG) was immunotherapeutically effective when given (s.c. or i.d.) immediately after i.d. challenge of 105 L10 cells.

Features of the improved vaccine were the use of BCG KC and SQE-vitamin E. BCG KC are stable, easily standardized, and inexpensive; emulsions of BCG KC can be prepared in the absence of detergent (Tween 80). SQE is a metabolizable oil, a natural constituent of some mammalian tissues.

### REFERENCES

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