Brief Communication: Immunotherapy of Cancer With Nonliving Mycobacteria and Cord Factor (Trehalose-6,6'-dimycolate) in Aqueous Medium

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ABSTRACT—Heat-killed BCG, cord factor (trehalose-6,6'-dimycolate), or killed BCG plus cord factor in aqueous medium, admixed with tumor cells and injected into the skin of guinea pigs, inhibited the growth of a hepatocellular carcinoma. Intradermal administration of killed BCG or Mycobacterium kansasii coated with cord factor, in the same medium, caused regression of established tumors in 48 and 45% of the treated animals, respectively.—J Natl Cancer Inst 57: 963-964, 1976.

Intratumor administration of lyophilized heat-killed BCG plus cord factor (trehalose-6,6'-dimycolate) in 1% mineral oil emulsion cured 87% of guinea pigs (23 animals) with transplanted carcinoma in their skins and metastases in the regional lymph nodes. A similarly high percentage of cures was obtained when mixtures of heat-killed BCG and cord factor dispersed in a solution of tocopherol acetate and peanut oil or in peanut oil alone were used (1). The efficacy of a vaccine containing nonliving BCG in mineral oil emulsion and living tumor cells was significantly increased when cord factor was added to the vaccination mixture (2). Here we show, although in a much smaller percentage of treated animals, that it is possible to obtain cures in the same experimental model with similar preparations of killed BCG and Mycobacterium kansasii in aqueous medium.

MATERIALS AND METHODS

Animals.—Inbred strain 2 guinea pigs (400-500 g) (Animal Production Section, Weizman Institute, Rehovoth, Israel) were fed guinea pig chow ad libitum.

Tumor line.—The guinea pig model used and the hepatocarcinoma designated line 10 were described in (3, 4). After intradermal injection of $10^6$ line 10 cells, tumors grew progressively, metastasized to the draining lymph nodes within 7 days, and killed the animals within 2-3 months.

Bacterial cells.—Lyophilized BCG cells were supplied by the Pasteur Institute, Paris, France. M. kansasii (strain P21) were grown in Sauton medium, killed with 2% phenol, washed with distilled water, and extracted three times with acetone. M. smegmatis was grown in medium described in (5), washed with distilled water, and lyophilized. Chromatographically pure cord factor (trehalose-6,6'-dimycolate) from the human virulent strain of M. tuberculosis Peurois was provided by Dr. E. Lederer.

Preparation of bacterial suspensions.—Cord factor was weighed, placed in a glass tube, and dissolved in ether. Weighed amounts of lyophilized bacterial cells were added to the ether solution, and after evaporation of the ether, the mycobacterial cells coated with cord factor were homogenized in saline containing 0.2% Tween 80. The bacterial suspensions were heated for 20 minutes at 70°C.

MER of BCG.—A suspension of 10 mg/ml was provided by Dr. M. Weinberg from the Department of Immunology, Hebrew University-Hadassah Medical School.

Living BCG.—BCG (strain Phipps) was grown in Dubos medium with Tween 80 (Difco Laboratories, Detroit, Mich.) for 7 days. The culture was distributed into tubes and kept viable at −70°C. The number of colony-forming units was $5.2 \times 10^7$/ml.

Treatment of animals.—To test the tumor regressive activity of the mycobacteria, $10^6$ tumor cells suspended in 0.1 ml medium 199 were injected intradermally into the plucked flanks of the guinea pigs. Seven days later, when the tumors were about 10 mm in diameter, 0.4 ml of the bacterial preparation was injected intratumorally. Animals were observed for at least 3 months, and the tumors and regional lymph nodes were measured weekly.

RESULTS AND DISCUSSION

Growth of L10 Tumor Cells at Sites of Administration of Cord Factor, Killed BCG, or Cord Factor Plus Killed BCG

Mixtures of $10^6$ L10 tumor cells with 500 µg cord factor, killed BCG, and killed BCG plus cord factor were injected intradermally into guinea pigs (table 1). All preparations inhibited the growth of the injected tumor cells. It has been reported that $6 \times 10^6$ heat-killed BCG organisms in aqueous suspensions were unable to inhibit the growth of $10^6$ L10 tumor cells in the same experimental model (7). That negative result was apparently due to the smaller amounts of BCG cells used.

<table>
<thead>
<tr>
<th>Material injected with $10^6$ L10 tumor cells</th>
<th>Growth suppression</th>
<th>Number of tumor rejections/No. of animals tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG (500 µg)</td>
<td>3/3, 2/4</td>
<td>3/5</td>
</tr>
<tr>
<td>Cord factor (500 µg)</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>BCG (600 µg) + cord factor (500 µg)</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Control</td>
<td>0/3</td>
<td>—</td>
</tr>
</tbody>
</table>

* Number of tumors suppressed/No. inoculated.
* Animals in which there were no signs of tumor growth after 2 months of observation were challenged intradermally with $2 \times 10^6$ L10 cells.

Tumor cells were mixed with 0.2% Tween 80 in saline.

**ABBREVIATION USED:** MER=methanol extraction residue.

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3 I thank Professor E. Lederer (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France) for the generous supply of cord factor and Mycobacterium kansasii.
Although the tumor-regressive activity of heat-killed BCG plus cord factor in aqueous medium is much weaker than that of the same components in 1% mineral oil emulsion (48% vs. 87%), such nonliving preparations can probably replace living BCG organisms for clinical applications without the adverse side effects encountered in tumor immunotherapy with the living organisms.

REFERENCES


(2) BEKIERKUNST A: Immunotherapy and vaccination against cancer with killed BCG and cord factor (trehalose-6,6'-dimycolate). Int J Cancer 16:442-447, 1975


Table 2.—Regression of intradermal tumors in guinea pigs after intralesional administration of mycobacteria and cord factor in aqueous medium

<table>
<thead>
<tr>
<th>Material injected into tumor</th>
<th>Number of regressed tumors/ No. of animals treated</th>
<th>Percent cured</th>
<th>Total No. of cures/No. of animals treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/5, 0/3, 0/4, 0/2</td>
<td>0</td>
<td>0/14</td>
</tr>
<tr>
<td>Heat-killed BCG (1 mg)</td>
<td>1/5, 0/5, 1/5</td>
<td>2</td>
<td>2/15</td>
</tr>
<tr>
<td>Cord factor (1 mg)</td>
<td>0/10, 2/5, 1/6</td>
<td>3</td>
<td>3/21</td>
</tr>
<tr>
<td>Heat-killed BCG (1 mg)+cord factor (1 mg)</td>
<td>4/5, 4/10, 3/5, 1/5</td>
<td>14</td>
<td>12/25 (0.05)</td>
</tr>
<tr>
<td>M. kansasii (1 mg)</td>
<td>1/5, 1/5, 3/5</td>
<td>33</td>
<td>5/15</td>
</tr>
<tr>
<td>M. kansasii (1 mg)+cord factor (1 mg)</td>
<td>2/5, 2/5, 2/5, 3/5</td>
<td>45</td>
<td>9/20</td>
</tr>
<tr>
<td>M. smegmatis (150 μg)+cord factor (1 mg)</td>
<td>0/5, 1/5</td>
<td>10</td>
<td>1/10</td>
</tr>
<tr>
<td>MER (1 mg)</td>
<td>0/6</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>M. smegmatis (150 μg)</td>
<td>2/4</td>
<td>50</td>
<td>2/4</td>
</tr>
<tr>
<td>M. smegmatis (150 μg)+cord factor (150 μg)</td>
<td>4/5, 3/5</td>
<td>NS</td>
<td>7/10</td>
</tr>
<tr>
<td>Living BCG</td>
<td>3/5</td>
<td>60</td>
<td>3/5</td>
</tr>
</tbody>
</table>

a Multiple results for one treatment group represent different experiments.

b Versus group treated with killed BCG alone.

c Not significant versus the group treated with M. kansasii alone.

d Bacteria were suspended in 1% mineral oil emulsion (1).

* 2.08x10^6 colony-forming units injected intratumorally.

Such amounts cause a weak and transient granulomatous reaction; however, a strong reaction is necessary to mount a specific immunologic response able to cope with the tumor cells (1, 2). Most animals in which the growth of the tumor cells was completely inhibited rejected a challenge with 2x10^6 L10 tumor cells (table 1).

Tumor Regressive Activity of Aqueous Preparations of Killed Mycobacteria and Cord Factor

Heat-killed BCG, M. kansasii, and M. smegmatis, coated with cord factor and homogenized in 0.2% Tween 80 in saline, were injected into 7-day-old established skin tumors (table 2). Killed BCG in aqueous suspensions and cord factor alone could occasionally cause regression of tumors. The percentage of cures increased significantly when cord factor was added to the bacilli. The same was true with M. kansasii, although the difference between the groups of M. kansasii alone and M. kansasii with cord factor was not statistically significant.

It is interesting that M. smegmatis plus cord factor in aqueous medium had an insignificant effect but that in smaller amounts the same components in 1% mineral oil emulsion had a clear-cut strong tumor regressive activity. This was also true for heat-killed BCG bacilli. Killed BCG (150 μg) with 50 μg cord factor, in a medium containing 1% mineral oil, was able to cure 87% of the animals (1).

No tumor regressive activity was shown by MER. MER seems to have an activity similar to that of killed BCG from which it differs in having the phosphatidyl-inositol-pentamannosides and a small amount of cord factor removed by the methanol extraction (8). Such a methanol-soluble fraction of M. butyricum may have some antitumor activity (9) probably due to cord factor. MER, admixed with the tumor cells and injected into the animals (10), like the heat-killed BCG in this report, restricted the subcutaneous growth of rat sarcomas and a hepatoma.