Immunotherapy of Cancer with Nonliving BCG and Fractions Derived from Mycobacteria: Role of Cord Factor (Trehalose-6,6′-Dimycololate) in Tumor Regression

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Delipidated and deproteinized cell walls from Mycobacterium tuberculosis H37Ra suspended in 1.25% mineral oil emulsion cured established tumors in the skin and metastases in draining lymph nodes of guinea pigs (strain 2) after intratumoral administration in 33% of the cases examined. This was increased to 83% when a mixture of cord factor and the delipidated cell walls was used. A similarly high percentage of cures was obtained after administration of lyophilized, killed BCG alone or with addition of cord factor in 1% mineral oil emulsion. Lyophilized, killed BCG dispersed in a solution of tocopherol acetate in peanut oil or in peanut oil alone showed a limited tumor-regressive activity (36%). However, a mixture of BCG and cord factor suspended in the above media cured 80 and 69% of the treated animals, respectively.

Guinea pigs with a transplanted hepatocellular carcinoma (Line 10) in their skin and metastases in their regional lymph nodes can be cured by the intralesional injection of living BCG (36). Killed BCG or BCG cell walls suspended in saline are not therapeutic in this system. Cures comparable to those produced by living BCG, however, have been produced by the intralesional injection of BCG cell wall fragments attached to mineral oil which was emulsified in an aqueous solution of saline containing Tween 80 (35). One of the components of the mycobacterial cell wall is cord factor (CF; trehalose-6,6′-dimycololate; [17, 24]), a compound which possesses some of the biological properties of living BCG. Both agents produce granulomas in lungs of mice after an intravenous injection (4). After administration into footpads of mice, both agents induce in the draining lymph nodes granuloma formation, hyperplasia in the paracortical region, and accumulation of macrophages, changes which are apparently responsible for increased antibody formation to an unrelated antigen, which is subsequently injected into the same site (5, 8). CF suppresses the development of urethane-induced tumors in the lungs of mice to an extent similar to that produced by living BCG. This suppression was attributed to the granulomatous reaction induced in the lungs of mice by both agents (6). It has also been reported that CF prevents the growth of Ehrlich ascites cells in the peritoneal cavities of mice as do living or dead BCG cells (33).

It was conceivable that CF (which simulates some of the biological activities of living BCG) plays a role in regression of tumors after intrallesional injections of living BCG or mycobacterial cell walls. The purpose of this work was to test this assumption and to attempt to cure established tumors with nonliving BCG.

MATERIALS AND METHODS

Animals. Male guinea pigs of the Sewall-Wright inbred strain 2 weighing 400 to 500 g were supplied by the Animal Production Section, National Institutes of Health and Frederick Cancer Research Center and Animal Farm (Fort Detrick, Frederick, Md.). They were fed Wayne guinea pig chow ad libitum and were given kale twice weekly. In skin tests with the preparation of mycobacterial fractions, guinea pigs of a local strain were used.

Preparation of mycobacterial fractions. CF was prepared as described previously (4) and purified by thin-layer chromatography; its identity was checked by optical rotation, infrared and mass spectra, and the analysis of its components: mycolic acids and trehalose (R. Tubiana and E. Lederer, in preparation). The cell walls of Mycobacterium tuberculosis strain H37Ra were prepared and freed of extraneous, noncovalently linked lipid, protein, and glycolipid by exhaustive extractions with organic solvents, as well as by enzymatic digestion (2, 25). After lipid extraction and digestion with proteolytic enzymes, the cell
walls were treated with lysozyme, and the supernatant of the digest was fractionated on a Sephadex G-50 column by a procedure similar to that described by A. Adam et al. (1). Two eluates were recovered; the first showed adjuvant activity and was used in these experiments. A detailed description of its chemical structure and adjuvant properties is in preparation.

BCG cells. Lyophilized heat-killed (at 70 C for 20 min) BCG bacilli supplied by the Pasteur Institute were used in therapy experiments.

Preparation of emulsions. The mycobacterial fractions and lyophilized BCG cells were incorporated into oil-in-water emulsions, containing 1 to 1.25% mineral oil. Fractions were weighed and placed in glass tubes, mineral oil (Drakeol 6-VF, Pennsylvania Refining Co., Butler, Pa.) was added, and the mixture was ground to a smooth paste with the aid of a Teflon pestle for 2 min. The paste was emulsified in 1-ml portions of saline containing 0.2 to 0.25% Tween 80. Usually, 4-1 ml portions were used, recombined, and reground to obtain emulsion ready for use. To prepare mixtures of CF with either delipidated and deproteinized cell wall or cell wall lysozyme digest or BCG cells, the CF was first dissolved in oil, and the other cell wall fractions or BCG bacilli were added and ground prior to emulsification.

Tumor line. The guinea pig model used to test the antitumor activity was developed at the National Cancer Institute (Bethesda, Md. [27]). An hepatocarcinoma designated Line 10 was induced with diethylnitrosamine in strain 2 guinea pigs and was subsequently adapted to the ascites form (27). Methods of tumor passage and inoculation have been described by Zbar et al. (37). After intradermal injection of 10^6 Line 10 cells, tumors grew progressively, metastasized to the draining lymph nodes within 7 days, and killed the recipient in 2 to 3 months (37).

Treatment of animals. To test the mycobacterial fractions and BCG cells, 10^6 tumor cells suspended in 0.1 ml of Medium 199 (Microbiological Associates, Bethesda, Md.) were injected intradermally into the plucked flank of a guinea pig. Seven days later, when tumors were 6 to 10 mm in diameter, 0.4 ml of the fractions and BCG in emulsion was injected into tumors. Animals were observed for at least 3 months with weekly measurement of intradermal tumors and regional lymph nodes.

RESULTS

Tumor-regressive activity of mycobacterial fractions. To test whether CF played a role in the tumor-regressive ability of mycobacterial cell walls, it was necessary to delipidate the latter and to see whether the cell wall fraction displayed tumor-regressive activity after delipidation. BCG cell walls were, in their chemical structure and biological activity, very similar to cell walls of other mycobacteria, as are CF from different strains of mycobacteria. In our experiments, we used cell walls from Mycobacterium tuberculosis strain H37Ra and CF from a human strain of M. tuberculosis Peurois. Besides the two fractions, another one derived from the H37Ra cell wall was also tested by lysozyme treatment (Table 1). It was observed that the emulsion of oil in Tween-saline after administration into the tumors retarded their development as compared with a group of untreated guinea pigs which were inoculated with 10^6 tumor cells (not shown in Table 1). In one out of 28 guinea pigs, regression of the tumor was observed locally and in the draining lymph nodes. This might have been a spontaneous regression or an effect caused by the oil emulsion. CF alone retarded the development of the tumors, but had no tumor-regressive activity. The delipidated and deproteinized cell wall of H37Ra cured four out of 12 animals locally in two experiments, and no metastases in the nodes were observed. The difference be-

<table>
<thead>
<tr>
<th>Material injected into tumor</th>
<th>Tumors regressed/tumors tested (in different experiments)</th>
<th>Total no. of cures/no. of animals treated</th>
<th>Percent of cures</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control emulsion</td>
<td>1/6, 0/5, 0/6, 0/6, 0/5</td>
<td>1/28</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>CF (150 μg)</td>
<td>0/6, 0/6</td>
<td>0/12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Delipidated and deproteinized cell walls (H37Ra; 150 μg)</td>
<td>2/6, 2/6</td>
<td>4/12</td>
<td>33.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Delipidated and deproteinized cell walls (H37Ra) + CF (150 μg + 150 μg)</td>
<td>5/6, 6/6, 4/6</td>
<td>15/18</td>
<td>83.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lysozyme digest of delipidated and deproteinized H37Ra cell walls (150 μg)</td>
<td>2/6, 3/6</td>
<td>5/12</td>
<td>41.6</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Versus the control emulsion group.
* Versus the group treated with delipidated and deproteinized cell walls. Concentration of mineral oil in the emulsions was 1.25%.
between these groups and the control emulsion groups was significant \((P < 0.05)\). It is noteworthy that the lymph nodes in these guinea pigs enlarged soon after injection of the cell wall fraction, and, at the time when the tumor was already locally healed, the enlarged nodes decreased in size and slowly became unpalpable. A clear-cut increase of complete cures was attained when CF was added to the delipidated and deproteinized cell walls. In three experiments, the healing of the tumors was already evident at the end of the first week, after administration of the mixture. Also in these cases, the draining nodes were enlarged even after the tumors were locally healed, but they slowly decreased to normal size. The results of the three experiments are statistically significant compared with groups treated with delipidated and deproteinized cell walls only \((P < 0.01)\).

The lysozyme cell wall digest, a product derived from the delipidated and deproteinized cell walls of H37Ra, was also able to cure the tumors to a degree similar to the delipidated and deproteinized cell walls. However, mixtures of the lysozyme cell wall fraction and CF, injected into the tumor, gave the following conflicting results.

Guinea pigs in which there was complete regression of the tumors locally and in the lymph nodes were rechallenged with \(10^4\) tumor cells on the flank opposite the original tumor site. Out of 18 guinea pigs 17 rejected the challenge, indicating that the cured guinea pigs acquired immunity to Line 10 tumor cells.

**Tumor-regressive ability of nonliving BCG.** Heat-killed BCG cells \((10^4\) to \(10^7\)) were unable to cause a cellular response in the lungs of mice after an intravenous injection or any significant change in the draining lymph nodes after administration into their footpads, unless the bacilli were attached to oil and emulsified in an aqueous solution of saline to which Tween 80 was added as an emulsifier. Similarly, a saline suspension of dead bacilli introduced into the skin of guinea pigs caused a slight and transient reaction (unpublished data; 7). It was assumed that BCG cells attached to oil should be able to cause regression of established tumors at least to the same degree as living BCG. It was also assumed that, since the tumor-regressive ability depends on the inflammatory cellular reaction of the host, the stronger this reaction the better the therapy result will be. Addition of CF to BCG cells would intensify this reaction and increase the effectiveness of the BCG cells. Before testing these assumptions, skin reactions in guinea pigs were observed after intradermal administrations of emulsion, CF alone, BCG cells plus cord factor, and BCG cells alone. All preparations induced an inflammatory reaction in the form of nodules with an erythema and induration. However, there were marked differences in their intensity. The weakest reaction was induced by emulsion and the strongest was induced by the mixtures of CF and BCG cells. In the latter case, necrosis was always evident in the center of the nodule. A slight necrotic center was also present at the site of injection of CF; after 10 days, no signs of reaction were present at the site of administration of emulsion. The reactions were observed for 20 days; at that time, induration at the site of injection of BCG and BCG plus CF was still evident. Figure 1 illustrates these reactions 18 days after administration of the preparations. The results of the immunotherapy tests are presented in Table 2. They clearly indicate that BCG cells attached to oil and emulsified in saline-Tween 80 cause regression of established tumors very effectively. Addition of CF seemed to improve the efficacy of BCG cells. More experiments on larger numbers of animals are needed to answer

![Fig. 1](http://iai.asm.org/)

**Fig. 1.** Inflammatory skin reaction in a guinea pig 18 days after intradermal administration of emulsions of 75 \(\mu\)g of nonliving, lyophilized BCG, 75 \(\mu\)g of BCG plus 25 \(\mu\)g of CF, 25 \(\mu\)g of CF and emulsion (from right to left). Emulsions contained 1% Drakeol emulsified in 0.2% Tween 80 in saline. Volume of injection was 0.1 ml.
this question definitely. However, there was a difference between the local inflammatory reactions after administration of BCG cells and the mixture of BCG plus CF. In the latter case, the reaction was much stronger. As soon as 4 days after injection of the mixture, the area of the tumor and beyond was covered with an almost black crust of necrotic tissue; the large nodule which appeared at the site of the tumor after administration of the mixture shrunk very rapidly; the crust of necrotic tissue was rejected and usually local cures are evident at the end of the second week after injection. At that time the regional node was enlarged, but then it decreased in size and slowly became impalpable. The process of tumor rejection in the BCG group was slower.

**Therapy of tumors with BCG cells suspended in solutions of tocopherol in peanut oil.** In the years that followed the introduction of complete Freund adjuvant, attempts were made to develop an adjuvant from vegetable oils (7, 11-13, 32). Only one such preparation (containing Adjuvant 65, composed of peanut oil; Arlcel A [mannide monooleate]; and aluminum monostearate), gave comparable results to those received with Freund adjuvant (15). At first, we attempted to substitute the mineral oil with a solution of tocopherol in peanut oil, and to use it as a vehicle for the BCG cells and CF. Dead, lyophilized BCG cells alone and BCG plus CF were dispersed by sonic treatment for 30 s in a MSE sonic oscillator in peanut oil containing 5% d,l-α-tocopherol acetate, which was taken out from ampoules of vitamin E for intramuscular injections in humans (“Duphar” Weesp, The Netherlands). Before testing these preparations in immunotherapy experiments, they were injected into the skin of guinea pigs to observe the inflammatory reaction the preparations provoked. Very intense inflammation was present at all sites except the site injected with vitamin E alone; the strongest was present at the site injected with the mixture of BCG plus CF. Erythema, induration, and tissue necrosis were present, especially at the sites of administration of CF and BCG plus CF. Signs of inflammation at the site of injection of vitamin E disappeared after 2 weeks. At the sites of administration of BCG plus CF and BCG alone, induration was still present after 3 weeks. At that time, only slight erythema at the site of CF injection was evident.

The results of the immunotherapy experiment are presented in Table 3. It was surprising that BCG cells did not show any tumor regressive activity in the first experiment. There were local healings, but metastases in the nodes appeared, grew, and killed the animals. However, in a second experiment, four out of six animals were cured. CF in one experiment cured two out of four animals but in another none out of five were cured. The mixtures of CF plus BCG were very effective; 80% of the animals were cured.

After establishing that administrations into the skin of guinea pigs of CF, BCG plus CF, and BCG alone in peanut oil caused a similar inflammatory reaction to the one caused by the medium of the oil with addition of tocopherol, therapy experiments were carried out using the

**Table 2. Regression of intradermal tumors and lymph node metastases in guinea pigs after intraleional administration of dead, lyophilized BCG cells**

<table>
<thead>
<tr>
<th>Material injected into tumor</th>
<th>Tumors regressed/tumors tested (in different experiments)</th>
<th>Total no. of cures/no. of animals treated</th>
<th>Percent of cures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control emulsion*</td>
<td>1/4, 0/4, 0/6</td>
<td>1/14</td>
<td>7</td>
</tr>
<tr>
<td>Lyophilized BCG (150 μg)</td>
<td>4/6, 4/5, 5/6</td>
<td>13/17</td>
<td>76</td>
</tr>
<tr>
<td>Lyophilized BCG + cord factor (150 μg + 50 μg)</td>
<td>4/5, 6/6, 5/6, 6/6, 4/6</td>
<td>20/23</td>
<td>87</td>
</tr>
</tbody>
</table>

* Concentration of mineral oil in the emulsion was 1%.
* Result after 2 months of observation.

**Table 3. Regression of intradermal tumors and lymph node metastases in guinea pigs after intraleional administration of dead, lyophilized BCG cells suspended in vitamin E**

<table>
<thead>
<tr>
<th>Material injected into tumor</th>
<th>Tumors regressed/tumors tested (in different experiments)</th>
<th>Total no. of cures/no. of animals treated</th>
<th>Percent of cures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E BCG (150 μg)</td>
<td>0/3, 0/4, 0/6</td>
<td>0/13</td>
<td>0</td>
</tr>
<tr>
<td>BCG + CF (150 μg each)</td>
<td>0/5, 4/6</td>
<td>4/11</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>6/6, 4/5, 2/4</td>
<td>12/15</td>
<td>80*</td>
</tr>
<tr>
<td>CF (150 μg)</td>
<td>2/4, 0/5</td>
<td>2/9</td>
<td>22</td>
</tr>
</tbody>
</table>

*The fractions were suspended in 5% of d,l-α-tocopherolacete in peanut oil.
* P = 0.05.
oil alone as the medium (Table 4). The results were similar to those presented in Table 3.

**Effect of the cellular (inflammatory) reaction induced by living BCG on tumor development.** It has been assumed that stimulation of the immune response by mycobacterial adjuvants (and among them, by living BCG) to unrelated antigens was due to the cellular inflammatory reaction of the host at the sites of the lodgment of the adjuvants and multiplication (in the case of living BCG). This may take place in the skin, lymph nodes, reticuloendothelial system, lungs, and peritoneal cavity (4–6, 8, 33). To test this assumption, \(1.45 \times 10^4\) living BCG cells were injected intradermally into four guinea pigs. The pathological change at the injection site began as usual with a very mild reaction which developed progressively into a granulomatous nodular lesion. Seven days after administration of BCG, \(10^6\) Line 10 tumor cells were injected into the indurated tissue at the site of injection and on the opposite site into normal skin. It soon became clear that the growth of the tumor in the granulomatous lesion was strongly inhibited, whereas on the opposite site it developed quite normally (Fig. 2). In two out of the four, the tumors regressed locally at the sites of the nodules. Similar results were obtained after treating the nodules with nonliving BCG alone or BCG plus CF in 1% mineral oil emulsions.

**DISCUSSION**

It is clear from the present study that trehalose-6,6'-dimycolate (CF), a component of the mycobacterial cell wall, takes part in the tumor-regressive activity of living BCG or of cell walls. The inflammatory reaction evoked by CF in an emulsion containing 1.25% of mineral oil was not enough to cause regression of the tumor. CF

<table>
<thead>
<tr>
<th>Material injected into tumor</th>
<th>Tumors regressed/treated animals (in different experiments)</th>
<th>Total no. of cures/no. of animals treated</th>
<th>Percent of cures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut oil</td>
<td>0/6, 0/6</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>BCG (150 (\mu)g)</td>
<td>1/4</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>CF (150 (\mu)g)</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>BCG + CF (150 (\mu)g each)</td>
<td>3/5, 3/5, 5/6*</td>
<td>11/16</td>
<td>69</td>
</tr>
</tbody>
</table>

* Result after 2 months of observation.

**FIG. 2.** Skin tumors in four guinea pigs at the site of injection of BCG (left) and at the opposite site (right) 26 days after administration of \(10^6\) Line 10 tumor cells.
had to be added to the delipidated cell wall to increase the tumor-regressive ability of the latter. An enhancement of antitumor activity of delipidated BCG cell walls by addition of mycobacterial lipid designated P3 has recently been reported (21). It has been observed during this study that the lipid- and protein-free cell wall of H37Ra and its lysozymal digest (injected intra-dermally into guinea pigs in amounts of 100 μg) caused delayed hypersensitivity to 2 μg of purified protein derivative (Connaught Medical Research Laboratories, Toronto, Ontario). CF had no such effect, and it seemed that to cause tumor regression, cellular components of a hypersensitivity reaction had to be present at the site of tumor. However, since CF alone in the peanut oil solution of tocopheryl acetate (or in peanut oil alone) showed tumor-regressive activity in some cases, it may be that effector cells appear in every chronic inflammatory reaction, but the outcome of antitumor activity may depend on their quantity and predominance of certain cells (probably lymphocytes T and B types or a third type, besides macrophages). This problem is under investigation.

The lysozymal cell wall digest of H37Ra cell walls showed tumor-regressive activity similar to that of the delipidated and deproteinized cell walls. However, mixtures with CF injected into tumors gave conflicting results. In one experiment, a high percentage of cures was achieved; in two others, no cures were achieved at all. In the latter case, the impression was left that there was an enhancement of the tumor growth. The observation that BCG lyophilized cells alone in peanut oil solution of tocopheryl acetate or peanut oil alone showed limited tumor-regressive activity, but addition of CF increased this activity, indicates once more the importance of CF. On the other hand, the fact that BCG in mineral oil emulsion was very effective in tumor regression points to the importance of the medium in which the agents are introduced into the host. Apparently, the cellular composition and intensity of the ensuing inflammation depends on it. The immunotherapy results with nonliving BCG cells alone or with CF in the 1% mineral oil emulsions were remarkable; 76 and 87% of cures are good results, compared with about 60% achieved in the same experimental model with living BCG (34). Also remarkable are the results obtained with the mixtures of BCG plus CF in peanut oil solution of tocopheryl acetate or peanut oil alone.

Living BCG have been used in man and have been effective in some cases against melanoma (22, 23, 26), as well as against advanced Hodgkin’s disease (30). It has also proved effective in lengthening remissions in acute leukemia (20). However, living BCG can cause disseminated disease in an immunosuppressed host (10, 14, 18, 19, 29). Although local reactions at the site of injections may be severe, they generally disappear (16, 31). Seventeen deaths from generalized infection with BCG have been reported also during large scale vaccination (10, 18, 29).

It seems that these adverse effects of using living BCG can be avoided by using lyophilized, killed BCG alone, with CF in 1% emulsions of mineral oil or with BCG and CF in peanut oil as used in this work. The high percentage of cures and the reproducibility of the results obtained in the present work justifies the assumption that the above preparations could also be more effective than living BCG in immunotherapy of cancer in humans. There are different living strains of BCG in use for vaccination; all of them are probably effective in vaccinating against tuberculosis, but not all of them are effective in immunotherapy of experimental cancer (Zbar, personal communication). The same situation exists in therapy (with living BCG) of cancer in man.

It is noteworthy that the content of CF may be different in different strains of BCG (3). This may explain the different efficacy of the strains in immunotherapy experiments. This effectiveness is apparently dependent on the cellular reaction induced in the host at the site of lodgment of the bacteria as has been shown in this work. The therapy results received in this work, and the evidence that a suitable inflammatory cellular reaction of the host is necessary for tumor destruction, call not only for application in humans, but also for further investigations, such as the mechanism and nature of tumor regression and the very important practical question of controlling tumors in sites other than the skin.

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LITERATURE CITED


