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Immunotherapy of Guinea Pig Line 10 Hepatoma with Nonliving BCG Cells in Aqueous Medium

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Killed BCG cells suspended in 1.5% carboxymethylcellulose cured guinea pigs with established line 10 tumors in a high percentage of cases. The bacterial preparation of BCG in carboxymethylcellulose displayed a stronger tumor regressive activity and the process of healing was accelerated when endotoxin from a rough (Re) strain of *Salmonella typhimurium* was added to the BCG bacilli.

The tumor regressive activity of heat-killed BCG cells in the guinea pig model strongly depends on the medium in which the bacilli are suspended. It has been demonstrated that 1 mg of killed BCG in aqueous medium caused complete regression of the tumors in only 13% of animals compared to 76% of cures produced by 0.15 mg of BCG suspended in 1% mineral oil emulsion (3, 5). Plain peanut oil could be substituted for the mineral oil emulsion (5); alternatively, a 20% emulsion of peanut oil in saline containing 0.2% Tween as emulsifier was effective (in preparation). We now report that a high percentage of cures of tumor in the same guinea pig model can be achieved in an aqueous medium when the BCG bacilli are suspended in a solution of carboxymethylcellulose and that the process of healing can be accelerated by adding endotoxin (ET) to the mycobacterial preparation.

MATERIALS AND METHODS

Animals. Inbred Strain-2 guinea pigs (400 to 500 g) from the Production Section of the Weizmann Institute, Rehovot, Israel, were used.

Tumor line. The guinea pig model used and the hepatocarcinoma designated line 10 have been described (23). After intradermal injection of 10^6 line 10 cells, tumors grow progressively, metastasize to the draining lymph nodes within 7 days, and kill the animals within 2 to 2.5 months.

Preparation of bacterial suspensions. Weighed amounts of lyophilized killed BCG cells were placed in glass tubes and homogenized with a Teflon grinder in solutions of carboxymethylcellulose in saline, containing 0.1% Tween 80. Carboxymethylcellulose sodium salt (CMC), low viscosity, was purchased from BDH Chemicals, Poole, Dorset, England.

Treatment of the animals. To test the tumor regressive activity of the mycobacteria, 10^6 cells suspended in 0.1 ml of medium 199 were injected intradermally into the plucked flanks of the guinea pig. Seven days or more later, 0.5 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.

ET from a rough (Re mutant) strain of *Salmonella typhimurium* was kindly provided by E. Ribi. The product is obtained by first extracting lyophilized bacteria with chloroform-methanol (4:1, vol/vol) and then extracting the dried residue with phenol-water (1:1, vol/vol). The ET is recovered from the aqueous phase (E. Ribi et al., manuscript in preparation).

Preparations of BCG cells and ET. To a weighed amount of BCG in a glass tube, a methanol suspension of ET was added. The bacteria were homogenized with a Teflon grinder, and after homogenization the methanol was evaporated under nitrogen. The dried BCG cells associated with ET were subsequently homogenized in CMC solutions.

RESULTS

Tumor regressive activity of BCG in 0.5% CMC. Amounts of 1, 1.5, and 3 mg of BCG cells in 0.5% of CMC were injected into 7-, 9-, and 11-day-old tumors. The results are presented in Table 1.

One injection of killed BCG in the range of 1 to 3 mg in 0.5% solutions of CMC cured 60 to 83% of 7-day-old tumors. These percentages are significantly different from those obtained after injections of killed BCG in saline, and are comparable to those obtained with killed BCG attached to oil in mineral oil emulsions (76%). There is no doubt that the tumor regressive activity of the BCG cells depends on the size of the tumor and on the number of tumor cells that metastasized to the draining lymph nodes; and accordingly the therapeutic effect on large tumors was significantly less pronounced. However, four cures in 12 guinea pigs treated by two injections into 9-day-old tumors with an average size of 13.8 mm seemed to be a good result.

Tumor regressive activity of BCG in 1.5%

CMC. In preliminary experiments on mice injected intraperitoneally with different concentrations of CMC, it was observed that macrophages from such mice showed an increased acid phosphatase activity as compared with normal macrophages. The highest increase was observed in macrophages injected with 1.5% CMC; therefore, further therapy experiments were conducted with bacteria suspended in this concentration of CMC (Table 2). In two experiments, 9 out of 11 guinea pigs were cured (81%), and even one injection of 1 mg of BCG into large tumors (average size 13.9 ± 2.3) caused complete regression in two out of six animals.

Tumor regressive activity of BCG and ET in 1.5% CMC. ETs from gram-negative bacteria have been shown to have tumor-necrotizing properties (21, 22). In studying this activity of ET, Carswell et al. found that the serum of BCG-infected mice treated with ET contains a tumor-necrotizing substance which mimics the necrotic action of ET itself (6).

In an ongoing series of studies, Ribi and collaborators have shown that specific ETs from Re (heptoseless) mutants of *S. typhimurium* and *Salmonella minnesota* exhibit very impressive antitumor activity in the line 10 system when the ET is combined with cord factor preparations (P3) from any of a variety of mycobacteria, and even with much simpler synthetic analogs of these glycolipids (20, 24). As improved variants, combinations of P3, ET, and mycobacterial cell wall skeleton have also proved very effective (17). All of these agents have required incorporation of the components into oil and dispersion in Tween-saline. It was of interest therefore to see whether in an oil-free system BCG and ET would show a stronger tumor-regressive activity than BCG alone (Table 3). In all 10 guinea pigs injected with 1 mg of BCG and 1 mg of ET, the tumors regressed completely. Two injections of ET alone in CMC had no effect; ET in saline under the same conditions had a negligible effect. It may be added that, according to numerous reports from Ribi, ET alone in oil-saline emulsion is also ineffective (see ref. 20).

In this experiment healing of the skin tumors was notably accelerated, apparently because of contributions by the ET. Already in the middle of the second week after injection, in most of the treated animals only scars were evident at the site of the tumors. The process of healing in guinea pigs treated with BCG alone was much slower. Evidence of healing with BCG alone was usually apparent in the middle of the third week after injection. At 15 weeks, all of the cured animals rejected a second challenge with 10^6 tumor cells, with vigorous delayed hypersensitivity reactions at the challenge site.

TABLE 1. *Regression of intradermal tumors in guinea pigs after intralesional administration of BCG in 0.5% solutions of CMC*

Material injected (amount)	Average size of tumors ± SD[a] (mm)	Age of tumor (days)	Total no. of cures/no. of animals	% Cures
BCG (1 mg)	11 ± 0.6	7	3/5	60
BCG (1.5 mg)	9.8 ± 0.5	7	4/6	66
BCG (3 mg)	11.8 ± 1.8	7	5/6	83
BCG (1 mg in saline)	10 ± 1	7	0/5	0
Control—0.5% CMC	11 ± 0.5	7	0/5	0
BCG (3 mg)	14.2 ± 0.7	11	0/6	0
BCG (3 mg)	11.7 ± 1.2	9	2/5	40
BCG (1 mg[b])	14.7 ± 3.2	9	2/6	33
BCG (1 mg[b])	13 ± 1.3	9	2/6	33
Control—0.5% CMC	9.2 ± 1	7	0/5	0

[a] SD, Standard deviation.
[b] Two injections spaced at 4-day intervals.

DISCUSSION

It is clear from the above results that high percentages of cures in the guinea pig model can be achieved with BCG in CMC solutions and that the process of healing can be accelerated by adding ET to BCG. It is evident that CMC can substitute for the mineral oil, although the mode

TABLE 2. *Regression of intradermal tumors in guinea pigs after intralesional administration of BCG in 1.5% CMC*

Material injected (amount)	Average size of tumors ± SD[a] (mm)	Age of tumor (days)	Total no. of cures/no. of animals	% Cures
BCG (1 mg)	11 ± 0.6	7	4/5	80
BCG (1 mg)	11.3 ± 1.2	7	5/6	83
BCG (1 mg)	13.9 ± 2.3	10	2/6	33
1.5% CMC	14 ± 0.5	10	0/6	0

[a] SD, Standard deviation.

TABLE 3. *Tumor regressive activity of BCG and ET in 1.5% CMC*[a]

Material injected (amount)	Average size of tumors ± SD[b] (mm)	Age of tumors (days)	Total no. of cures/no. of animals	% Cures
BCG (1 mg) + ET (1 mg)	10 ± 0.5	7	10/10	100
ET (1 mg[c])	9.1 ± 0.5	7	0/5	0
ET (1 mg in saline[c])	9.4 ± 0.7	7	1/5	20
1.5% CMC	10 ± 0.5	7	0/5	0

[a] After 15 weeks, the cured animals were rechallenged with 10^6 line 10 cells. All rejected the challenge.
[b] SD, Standard deviation.
[c] Two injections spaced at intervals of 7 days.

of action of the two vehicles could be different. Mycobacterial cells to which a layer of mineral oil is attached cause an influx of macrophages and lymphocytes at the site of injection. Apparently owing to the mineral oil, they are carried not only to the peripheral parts of the draining lymph nodes but also to the paracortical areas, eliciting, besides the granulomatous response, proliferation of thymus-dependent lymphocytes (3, 18). The presence of the mineral oil also promotes the persistence of the bacilli at the site of their lodgement, hence the chronicity of the inflammatory granulomatous reactions. How CMC acts is less clear. However, there are reports in the literature on the antitumor activity of some polysaccharides, which may be relevant to the results described in this paper. One of these, lentinan (7), extracted from a kind of edible mushroom, is a β-(1-3) glucan. It was suggested to be a T-cell adjuvant (12).

A glucan with a similar structure derived from yeasts was reported by DiLuzio et al. to induce proliferation and activation of macrophages, to inhibit tumor growth, and to promote survival when given to rats either before or after tumor cell administration (13). However, according to a recent report, glucans from yeast have no significant antitumor activity in guinea pigs and mice (16). Immunotherapy experiments with killed BCG suspensions in solutions of glucan are underway in this laboratory.

In the experiments reported in this paper, CMC alone injected into the tumors had a minimal effect on the growth of the tumors. Their growth was sometimes retarded in comparison with tumor-bearing control animals, but a cure was never observed. How CMC affects the distribution of the BCG bacilli from the site of intratumoral injection to the draining lymph nodes, and how it affects macrophages and lymphocytes, are questions under investigation. As indicated above, macrophages from mice pretreated with solutions of CMC have shown an increased acid phosphatase activity, but the relevance of this change to antitumor activity has to be shown. Although ET by itself in CMC solution did not cause regression of the tumors, it strongly affected the tumor-regressive activity of the BCG cells. It not only increased the efficacy of the preparation, but also accelerated the process of healing. Apparently the immunopotentiating effects of ET described in the literature (1, 2, 8, 11, 15, 24-27) are relevant to this action.

Since CMC is an innocuous material used as a vehicle for drugs, the results that we describe may be applicable in the treatment of neoplasia in humans with mycobacterial components (e.g., killed BCG) with or without ET. In the experience of one of the authors (A.B.), the therapy of skin tumors in humans may be achieved with substantially smaller amounts of nonviable BCG than have been used in guinea pigs (9, 10). A recent report by McLaughlin et al. (19) describes a similar synergistic tumor-regressive activity between deproteinized (not delipidated) BCG cell walls and Re ET in oil-in-water emulsion. When the components either alone or in combination were tested in young mice for toxicity, lethal toxic reactions did not occur, but the authors nevertheless considered the transient effects that were seen (malaise, conjuctivitis, diarrhea, or ruffled hair), along with transient weight loss, as potentially hazardous. Intratumor immunotherapy with BCG cell walls and cord factor (in oil-in-water emulsions) has been studied (Richman et al. [25]) and found to have significant benefit in patients with melanoma and breast cancer, and no serious toxicity was noted. Additional studies with increased doses "will provide a data base for controlled studies with even more promising microbial fractions," e.g., Re ET. It seems worthwhile, therefore, to assess the toxic effects of our CMC preparations and to compare these with their counterparts in oil.

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