Immunotherapy of Experimental Cancer by Intralesional Injection of Emulsified Nonliving Mycobacteria: Comparison of *Mycobacterium bovis* (BCG), *Mycobacterium phlei*, and *Mycobacterium smegmatis*

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Mycobacterium bovis (BCG), *Mycobacterium phlei*, and *Mycobacterium smegmatis* were each tested in emulsified form for their potency to cause regression of transplants of a syngeneic murine fibrosarcoma and of a syngeneic guinea pig hepatoma. On a weight basis, *M. phlei* and *M. smegmatis* were as effective as BCG in causing tumor regression. *M. phlei* and *M. smegmatis* were comparable to BCG in provoking delayed cutaneous hypersensitivity reactions in guinea pigs sensitized to *M. phlei* or *M. smegmatis*. In BCG-sensitized guinea pigs, *M. phlei* and *M. smegmatis* provoked weaker delayed cutaneous hypersensitivity reactions than did BCG. Purified protein derivative of *M. tuberculosis* was more active in eliciting delayed cutaneous hypersensitivity in BCG-sensitized guinea pigs than in animals sensitized with *M. phlei* or *M. smegmatis*.

Administration of living *Mycobacterium bovis* (BCG) to cancer patients or to animals with experimental cancer has sometimes caused systemic mycobacterial infection and toxicity. To avoid toxicity due to systemic infection, preparations of nonliving mycobacteria or their components have been tested as substitutes for living BCG in the immunotherapy of cancer. Qualitative evidence has been presented indicating that certain saprophytic mycobacteria may be effective substitutes for BCG in the treatment of malignant disease (1, 11). We now present a quantitative comparison of (i) the antitumor activity of killed *M. bovis*, *Mycobacterium phlei*, and *Mycobacterium smegmatis* against the line 10 guinea pig hepatocarcinoma and the murine fibrosarcoma 1023 and (ii) the capacity of the mycobacteria to provoke delayed cutaneous hypersensitivity (DCH) in guinea pigs sensitized to the organisms.

MATERIALS AND METHODS

Mycobacteria and emulsions. Viable BCG (Phipps substrain, TMC 1029) at 2×10^7 colony-forming units per ml of Middlebrook 7H9 broth were purchased from Trudeau Mycobacterial Collection, Saranac Lake, N.Y., and were used for sensitization of guinea pigs.

Autoclave-killed, lyophilized whole cells of mycobacteria were used for immunotherapy studies. *M. bovis* strain BCG was purchased from ITR Biomedical Research, University of Illinois at the Medical Center, Chicago. *M. phlei* strain 110 and *M. smegmatis* strain NIH TMC 1515 were obtained from Rocky Mountain Laboratory, Hamilton, Mont. Squalene ($C_{30}H_{50}$,

2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) was obtained from Eastman Kodak Co., Rochester, N.Y. Vitamin E (*dl*- α -tocopherol; T 3251) was obtained from Sigma Chemical Co., St. Louis, Mo. The ultrasonic method for preparing injectable emulsions of mycobacteria for treatment of mice has been described previously (10). The final concentrations of emulsion components were as follows: mycobacterial cells, 6 to 0.22 mg/ml; oil (squalene containing 2% vitamin E), 10%; and Tween 80, about 0.2%. For treatment of guinea pigs, the ultrasonic method has been modified as follows. Mycobacterial cells (4.5 to 0.17 mg) were placed in a polypropylene plastic tube. Oil (squalene containing 2% vitamin E; 0.06 ml) was added to the tube, and the mixture was sonicated for 3 min. A 0.5-ml amount of 0.85% NaCl solution (without Tween 80) was added to the mixture, and ultrasonication was applied for about 8 min by submerging the bottom of the tube in the water bath of an ultrasonic cleaner (model CC-.25; Beckman Instruments, Inc., Fullerton, Calif.). A 0.44-ml amount of a 0.85% NaCl solution was added to the emulsion, and the ultrasonication was continued for another 5 min. NaCl solution (0.85%) was added (1.0 ml) to the emulsion. Because of the absence of Tween, the emulsion contained large oil droplets that aggregated and floated to the surface of the liquid. For use in immunotherapy, unstable emulsions were mixed well before injecting, and the syringe was filled each time with a dose sufficient for only one guinea pig. The final concentrations of emulsion components were as follows: mycobacterial cells, 2.25 to 0.085 mg/ml; and oil (squalene containing 2% vitamin E), 3%.

Animals. Male C3H/HeN mice, 20 to 25 g, were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Adult male or female guinea pigs, Hartley outbred strain or Sewall Wright strain 2, were

obtained from stock maintained at the Frederick Cancer Research Center, Frederick, Md.

Murine tumor and treatment. A methylcholanthrene-induced fibrosarcoma, designated 1023, was maintained by serial subcutaneous passage in syngeneic mice. Transplant generations 15 and 17 were used in these experiments. Tumor was removed from the donor animal, trimmed of connective or necrotic tissues, and then minced and rinsed in Eagle minimal essential medium containing 100 U of penicillin and 100 μ g of streptomycin per ml. The minced tumor was digested with 0.22% pronase and 0.005% deoxyribonuclease in Dulbecco phosphate-buffered saline without calcium, magnesium, or antibiotics. The tumor cells were washed twice in Eagle minimal essential medium, and their concentration was adjusted to 2×10^7 viable cells per ml. Each mouse received 10^6 tumor cells intradermally on its dorsal surface in a volume of 0.05 ml. Five days later, when the average tumor diameter was 5 mm, mice were given a single intratumoral injection of one of the mycobacterial preparations in a volume of 0.1 ml. Mice in control groups received emulsion alone. Tumor incidence was determined by weekly observations for 2 months. Treated mice that remained tumor-free were rechallenged with 10^6 1023 tumor cells intradermally on their ventral surface. Injection sites were observed for 21 days thereafter for growth of rechallenge inoculum.

Guinea pig tumor line and treatment. Tumor line 10, an ascitic variant, was derived from a hepatocarcinoma induced by diethylnitrosamine in a strain 2 guinea pig. Inoculation of 10^6 tumor cells intradermally resulted in progressive local tumor growth, development of microscopic metastasis in the draining axillary lymph node by 1 week, and death 2 to 3 months after tumor inoculation (2). Animals were treated by intralesional injection of emulsified mycobacterial cells in 0.4-ml volumes 10 or 14 days after tumor implantation when the average diameter of the dermal tumors was 10 or 11 mm, respectively. Tumor incidence was determined by weekly observations for 2 months. Treated animals, whose dermal tumor regressed completely and with no outward evidence of metastatic disease, were rechallenged with 10^6 line 10 tumor cells intradermally on the contralateral flank. Injection sites were observed for 30 days thereafter for growth of rechallenge inoculum.

Test for DCH. Guinea pigs that remained tumor-free were tested for DCH reactions with graded doses of mycobacteria and purified protein derivative (PPD; Connaught Laboratories, Toronto, Canada, or National Institute of Allergy and Infectious Diseases, Bethesda, Md.) suspended in 0.85% NaCl solution to the desired concentrations. Mycobacterial cells were suspended in 0.85% NaCl solution by mechanical grinding in a glass tube equipped with a Teflon pestle and were injected intradermally in 0.1-ml volumes. The diameters of the skin reactions were measured 24 h after challenge and used to calculate the average diameter of the DCH reaction to each challenge.

The skin reactions to these mycobacterial antigens were also tested in Hartley guinea pigs 21 days after the animals were sensitized by the intradermal injection of 5×10^6 colony-forming units of living BCG.

Statistical evaluation. Differences among groups

were evaluated statistically by a 2×2 contingency table of Fisher's exact test.

RESULTS

Graded doses of each mycobacterium were tested for antitumor activity. Mice (in groups of 12), each with an established 5-day-old tumor, received intratumoral injections of a given dose of emulsified BCG, *M. phlei*, or *M. smegmatis*. In two separate experiments (Table 1), we observed that there were no statistically significant differences in the antitumor activity among the mycobacteria tested. From these results we calculated (3) the immunotherapeutic dose which might cause regression in 50% of the treated animals (Table 1).

The efficacy of the three mycobacteria in causing tumor regression was also studied in the guinea pig line 10 hepatoma model. The emulsified mycobacterial preparations were inoculated intralesionally 10 or 14 days (Table 2; experiments 1 and 2, respectively) after tumor implantation. Control animals were not treated. In each of the two separate experiments, comparable numbers of animals were cured of the dermal tumors and the regional lymph node metastasis. From these results we calculated the 50% immunotherapeutic dose for each preparation (Table 2). Cured animals rejected a contralateral challenge of 10^6 line 10 tumor cells given two months after the initial implant of tumor cells.

In addition to determining the relative therapeutic efficacy of these mycobacteria, we tested their capacity to induce DCH. DCH reactions were measured 24 h after intradermal injection of PPD or mycobacteria suspended in saline. Animals from the first experiment treated with 0.9 mg of mycobacterial preparations were divided into groups of five, and graded doses of each bacterial suspension and of PPD were tested. Unsensitized (control) animals were tested in the same way (Fig. 1). The mycobacteria induced a mild inflammatory reaction in control animals at a dose of 0.05 mg (2 or 3 mm in diameter). No skin reactions were found in the normal animals challenged with PPD (up to 0.002 mg) or mycobacteria at a dose of 0.01 mg or less. Animals presensitized with any of the three mycobacteria responded comparably to *M. phlei* or *M. smegmatis* suspended in saline. BCG suspended in saline elicited a greater skin reaction in BCG-presensitized animals. Two micrograms of PPD elicited a strong DCH reaction only in guinea pigs presensitized with BCG.

The skin reaction to all three of these mycobacteria was also tested in each of five Hartley guinea pigs presensitized with living BCG (Fig.

TABLE 1. Tumor regression in mice induced by intralesional injection of emulsified, heat-killed mycobacteria

Expt	Material injected ^a	Dose (mg)	No. of tumor-free animals/no. of animals treated (60 days)	P	ID ₅₀ ^b (mg)	No. of immunized animals/no. of animals cured (21 days)
1	BCG	0.6	12/12	<0.001	0.12	12/12
		0.2	7/12	<0.005		4/7
		0.067	5/12	<0.05		4/5
		0.022	0/12			
	<i>M. phlei</i>	0.6	7/12	<0.005	0.15	6/7
		0.2	10/12	<0.001		8/10
		0.067	1/12	NS ^c		1/1
		0.022	2/12	NS		2/2
	<i>M. smegmatis</i>	0.6	8/12	<0.005	0.09	8/8
		0.2	8/12	<0.005		6/8
		0.067	6/12	<0.01		6/6
		0.022	4/12	<0.05		3/4
	Emulsion		0/12			
2	BCG	0.6	10/12	<0.001	0.12	10/10
		0.2	8/12	<0.005		8/8
		0.067	4/12	<0.05		4/4
		0.022	2/12	NS		2/2
	<i>M. phlei</i>	0.6	9/12	<0.001	0.17	9/9
		0.2	6/12	<0.01		6/6
		0.067	4/12	<0.05		4/4
		0.022	1/12	NS		1/1
	<i>M. smegmatis</i>	0.6	9/12	<0.001	0.16	9/9
		0.2	7/12	<0.005		7/7
		0.067	3/12	NS		3/3
		0.022	1/12	NS		1/1
	Emulsion		0/12			

^a Emulsified mycobacterial preparations (10% oil and 0.2% Tween) were injected into 5-day-old tumors (no. 1023).

^b ID₅₀, Immunotherapeutic dose which might cause regression in 50% of the treated animals.

^c NS, Not statistically significant.

2). Five normal animals served as controls. As observed previously, 0.05 mg of any of the mycobacteria studied produced mild inflammation in the normal animals. In immunized animals the intensity of the skin reaction produced by PPD (2 µg) or by each dose of BCG in saline was significantly greater than that found with an equivalent dose of *M. phlei* or *M. smegmatis*.

The experimental results presented in Fig. 1 showed that guinea pigs treated with emulsions containing *M. phlei* or *M. smegmatis* did not respond with DCH reaction to 2 µg of PPD. In the next experiment (Fig. 3) we tested whether higher doses of PPD were required to induce DCH reactions in guinea pigs treated with *M. phlei* or *M. smegmatis*. Animals from the second experiment (Table 2) treated with 0.9 mg of mycobacterial preparations were divided into groups of five, and graded doses of PPD (up to 0.25 mg) were tested. Animals in a control group consisting of normal animals were tested in the same way. No DCH reactions were found in the normal animals challenged with PPD. Animals

presensitized with *M. phlei* or *M. smegmatis* showed less reactivity to PPD than those presensitized with BCG. A dose of 0.25 mg of PPD was required to produce DCH reactions in animals treated with *M. phlei* or *M. smegmatis*, whereas 0.01 mg of PPD produced similar reactions in BCG-treated animals.

DISCUSSION

The first report on the antitumor activity of different species of mycobacteria demonstrated that emulsified cell walls of *M. kansasii* were as active as emulsified BCG cell walls, whereas those of *M. smegmatis* were not active (11). Later it was reported that emulsified cell walls of *M. smegmatis*, which had been initially incorrectly classified as *M. phlei*, brought about elimination of 6- to 7-day-old line 10 tumors (1). The contradiction between these two reports may derive from the difference in the strains of *M. smegmatis* used or from the difference in the mineral oil content of the emulsified preparations: in the first report, the oil concentration

TABLE 2. Tumor regression in guinea pigs induced by intralesional injection of emulsified, heat-killed mycobacteria

Expt	Material injected ^a	Dose (mg)	No. of tumor-free ^b animals/no. of animals treated (90 days)	P	ID ₅₀ ^c (mg)
1	BCG	0.9	7/7	<0.005	0.07
		0.3	7/7	<0.005	
		0.1	6/7	<0.005	
	<i>M. phlei</i>	0.9	7/7	<0.005	
		0.3	7/7	<0.005	
		0.1	5/7	<0.05	
	<i>M. smegmatis</i>	0.9	7/7	<0.005	
		0.3	7/7	<0.005	
		0.1	5/7	<0.05	
	No treatment		0/7		
2	BCG	0.9	7/7	<0.005	0.07
		0.3	7/7	<0.005	
		0.1	4/7	<0.05	
		0.03	2/7	NS ^d	
	<i>M. phlei</i>	0.9	7/7	<0.005	0.06
		0.3	7/7	<0.005	
		0.1	5/7	<0.05	
		0.03	2/7	NS	
	<i>M. smegmatis</i>	0.9	6/7	<0.005	0.09
		0.3	5/7	<0.05	
		0.1	5/7	<0.05	
		0.03	1/7	NS	
	No treatment		0/7		

^a Emulsified mycobacterial preparations (3% oil and no Tween) were injected into 10- (experiment 1) or 14- (experiment 2) day-old line 10 tumors.

^b Complete disappearance of the dermal tumor, no clinical evidence of metastatic disease, and rejection of contralateral challenge (10^6 tumor cells) 2 months after the inoculation of the tumor transplant.

^c ID₅₀, Immunotherapeutic dose which might cause regression in 50% of the treated animals.

^d NS, Not statistically significant.

was 0.72%; in the second report, it was 1.5%. We reported that oil concentration has a significant effect on the antitumor activity of emulsified mycobacterial components (6, 7).

We have reported recently that on a weight

FIG. 2. DCH reactions to saline suspensions of PPD (from Connaught Laboratories), BCG, *M. phlei*, and *M. smegmatis* in guinea pigs presensitized with living BCG bacilli (5×10^6 colony-forming units).

FIG. 1. DCH reactions to saline suspensions of PPD (from Connaught Laboratories), BCG, *M. phlei*, and *M. smegmatis* in guinea pigs presensitized with 0.9 mg of intralesionally administered emulsion of BCG (open bars), *M. phlei* (hatched bars), or *M. smegmatis* (shaded bars).

FIG. 3. DCH reactions to PPD (from *The National Institute of Allergy and Infectious Diseases*; 1 µg = 30 IU) in guinea pigs presensitized with 0.9 mg of intraleisionally administered emulsions of BCG (open bars), *M. phlei* (hatched bars), or *M. smegmatis* (shaded bars).

basis, emulsions of heat-killed BCG bacilli were at least as effective as those of BCG cell walls in bringing about tumor regression, and their immunogenicity was similar as judged by DCH (9). A preliminary experiment showed that emulsified, killed *M. smegmatis* bacilli caused regression of 14-day-old line 10 tumors (10). Therefore, in the studies reported here, we compared emulsions of killed cells of BCG with those of *M. phlei* and *M. smegmatis*. We have also substituted squalene for mineral oil as a carrier of the bacilli, since we had found that squalene was an effective substitute for mineral oil in preparing emulsified mycobacterial components for the treatment of transplanted murine (8) and guinea pig tumors (9a). Squalene has some advantages over mineral oil as a vehicle for mycobacterial components in the immunotherapy of cancer: (i) it is normally present in various tissues of mammals and in some plants, (ii) its structure is known, (iii) it can be synthesized in vitro, and (iv) it contains only one component, unlike mineral oil which contains thousands of compounds. The emulsified mycobacterial preparations used for treatment of guinea pigs did not contain Tween 80, since BCG cell walls emulsified in squalene containing 0.2% or more Tween had

reduced antitumor activity in guinea pigs (9a). However, in mice the presence of 0.2% Tween did not reduce the tumor regression capacity of mycobacterial components emulsified in squalene (8).

The experiments in mice and guinea pigs reported here demonstrated that on a weight basis there was no statistically significant difference in the antitumor activity of emulsions of the three species of mycobacteria tested. BCG was the most efficient inducer of DCH against itself. Guinea pigs presensitized with living BCG or with killed BCG exhibited DCH reactions to *M. phlei* and *M. smegmatis*, but the reactions were significantly weaker than that produced by BCG. Animals cured by intralesional injection of emulsified *M. phlei* or *M. smegmatis* showed much less DCH reaction to PPD than those cured by BCG. Similar weak responses to PPD in animals cured with *M. phlei* cell wall skeletons plus P3 (a mycobacterial glycolipid known also as trehalose 6,6'-dimycolate or cord factor) have been reported previously (1).

These results suggest that saprophytic mycobacteria might be useful for intralesional cancer immunotherapy. The saprophytic mycobacteria differ from BCG in that they grow faster and in simpler media (1). The faster growth rate of the saprophytes as compared to BCG might make them more suitable than *M. bovis* as starting material in attempts to isolate and characterize the antitumor principle of mycobacteria. Recently, it has been reported that the emulsified combination of *M. smegmatis* cell wall skeletons and P3 was useful in the treatment of melanoma patients (4, 5).

ACKNOWLEDGMENTS

We thank E. Ribi for his generous supply of *M. phlei* and *M. smegmatis*. We also thank Kevin Rice for typing this manuscript.

The research of E. Y. was aided in part by grant 7820 from the Cancer Research Institute, Inc., New York, N.Y.

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