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Effect of the H37Ra Strain of *M. tuberculosis* and of a Mycobacterial RNA Fraction on Tumor Growth[1] (38434)

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BCG has been found useful in the prophylaxis and treatment of a variety of experimental tumors as well as in the treatment of certain malignancies in man (1–6). In addition, at least two nonviable mycobacterial fractions (MER, a methanol extracted residue, and cell walls of tubercle bacilli) have been shown to increase immunological resistance to tumors (3–5). Immunological defenses are important in both tuberculosis and malignant disease. Several reviews have been published concerning the mechanism of resistance to tuberculosis after vaccination (7–8). The acquired cellular immunity to tuberculosis, whether induced by vaccination or by actual infection consists of an increased capability of the reticuloendothelial system (RES) to prevent intracellular multiplication and both lymphatic and hematogenous spread.

In 1955, Youmans, Millman and Youmans (9) first demonstrated that a subcellular particulate fraction isolated from *Mycobacterium tuberculosis* strain H37Ra could produce an immunizing activity against tuberculosis in mice. This work was continued (10, 11) with the successful isolation and characterization of an active immunizing moiety rich in RNA. This fraction was demonstrated to be free of tuberculin in that it neither induced a tuberculin hypersensitivity in guinea pigs or mice nor was it able to provoke a reaction of delayed hypersensitivity in animals already rendered sensitive to tuberculin (12, 13).

This report will provide evidence that the H37Ra strain *Mycobacterium tuberculosis* as well as a Mycobacterial RNA containing fraction are effective inhibitors of tumor growth.

Materials and Methods. Mice. Approximately 10-week old syngeneic BALB/c × C3H males or, in other experiments, C3H females were supplied by the animal colony of the Institute for Cancer Research (ICR). All mice were of comparable weight and in good health. Mice were fed mouse pellets and water *ad lib* and were housed six per cage on sheet metal flooring with wood shavings for bedding.

Tumor. We used three chemically induced transplantable fibrosarcomas: (a) T-1967, induced by a paraffin ("Paraplast," A. H. Thomas, Philadelphia, PA) pellet containing 5% methylcholanthrene that was implanted subcutaneously in a BALB/c female, (b) T-1979, induced in a C3H female with a 20% nitroquinoline oxide, paraffin pellet, and (c) T-2060, induced with a 5% methylcholanthrene paraffin pellet in a C3H female. In order to avoid selection against tumor specific transplantation antigens the tumors were routinely passaged prior to use by subcutaneous transplantation in syngeneic females that had been immunosuppressed by thymectomy followed by 400 *r* whole body X-irradiation. The tumors were studied in their fifth to tenth transplant generations. Tumor cells were dispersed enzymatically (pronase 0.25%, DNAse 0.01%), washed three times and made up to a concentration of 10^6/0.1 ml in medium 199 (Grand Island Biological Co., Grand Island, NY).

Immunizing mycobacteria and fractions. The H37Ra strain of tubercle bacilli was cultured and harvested as 2 week old pellicle growths on modified Proskauer and Beck medium (14). A suspension was standardized by Hopkins tube determination to contain 0.5 mg/ml (wet wt).

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BCG was a lyophilized preparation obtained from Tice Institute, University of Illinois, Chicago. The culture was reconstituted with distilled water (provided by Tice) and then diluted with buffer (provided by Tice) to contain 5×10^6 cells/0.05 ml. An RNA fraction was prepared from a ribosomal fraction of the H37Ra strain as described in detail elsewhere (10, 11). Briefly it was purified from one of several fractions obtained by differential centrifugation of ruptured viable H37Ra cells by sodium dodecyl sulfate treatment and alcohol precipitation. The RNA fraction, used in the experiments described here, produced a sharp 260 nm absorption peak after being pelleted through a cesium chloride gradient of density 1.4 g cm^{-3}. This material, when separated on a cesium sulfate gradient (1.55 g cm^{-3}) containing 0.2% Sarkosyl, could be shown to contain RNA (degraded by NaOH) but no DNA. For immunization, the RNA fraction was neither centrifuged through cesium salts nor treated in any way beyond lyophilization and storage in the cold. On the day of immunization the lyophilized fraction (5.0 mg RNA equivalent (15) was dissolved in 2.5 ml of medium 199. 1.0 ml of this was emulsified with 3.0 ml of incomplete Freund's adjuvant made by mixing 1 part of Aquaphor (Duke Laboratories, Inc., South Norwalk, CN) with two parts of heavy mineral oil (E. R. Squibb & Sons, NY). All preparations were kept cold (ice bath) at all stages of preparation and during immunization.

Results. Table I outlines our protocol. In a representative experiment utilizing T-1967 in BALB/c × C3H mice, the size of the tumors at 7, 14 and 21 days resulting from the challenge innocula are shown in Table II. It is clear that both viable H37Ra microorganisms and the RNA fraction derived from this strain inhibited the growth of tumor cells. A statistical analysis utilizing an extension of 2 × 2 multisample comparison of proportions showed that the differences from the control of the H37Ra and RNA groups were highly significant ($P < 0.001$). The tumor size of the group that received BCG represents a composite of hypersensitivity to BCG and tumor growth. Thus, the BCG mice had tumors of considerable size at 7 days. We have biopsied a number of tumors of a comparable BCG group in a separate experiment and found nests of tumor cells contained in a marked inflammatory granulomatous reaction. Because in the BCG group measured tumor size was in part due to an inflammatory response induced by the challenge with BCG microganisms the results as relates to tumor cell growth are not strictly comparable as between BCG and the other groups. We have repeated the experiment outlined in Table I, using the same tumor as well as T-1979 and T-2060 (in C3H mice). Different batches of H37Ra, BCG and RNA fraction were used. The results were similar to those shown in Table II in all cases.

Discussion. The mechanism by which the H37Ra strain of *M. tuberculosis* and a RNA fraction derived from this strain prevents the growth of neoplastic cells is, at the present time, uncertain. In a preliminary experiment we found that mixtures of tumor cells and H37Ra (or RNA fraction) produced tumors in immunosuppressed syngeneic mice (thymectomy plus X-irradiation) just as well as comparable suspensions of tumor cells lacking mycobacteria. However, when these same suspensions were introduced into normal mice, there was marked inhibition in those groups given tumor cells mixed with mycobacterial preparation (as opposed to the control group). Bartlett *et al.* (4) reported similar results in comparing the effect of BCG on tumor growth in intact as against immunosuppressed mice. Thus the mycobacterial effect does not appear to be simply direct toxicity, but to involve a manipulation of the host response.

Zbar *et al.* (1, 2) and Bartlett *et al.* (4) have implied that the mechanism of action of BCG is immunological and perhaps related to tuberculin hypersensitivity. These investigators suggested that there was an alteration of the host's cellular response enabling "altered cells" to respond not only to the challenge of tubercle bacilli but also to the accompanying tumor cells. In other experiments, it has been shown that the administration of BCG prior to tumor induction by certain chemicals or viruses can either delay tumor appearance or reduce the total tumor burden. Although some evidence exists that intralesional BCG treatment causes a rise in antitumor antibody, the role of antibody remains uncertain and evidence exists that its effect may be detrimental (16). The role of cellular immunity is emphasized by several studies in cancer patients that correlated better prognosis with responsiveness to one or more of a battery of standard delayed hypersensitivity skin tests (17–19). It is difficult to directly apply this mechanism to the results obtained with the ribosomal RNA frac-

TABLE I. Experimental Protocol.[a]

Prechallenge					
Group	Mice	Treatment	Amount injected per mouse	Volume injected	Route
1	18	living H37Ra	8×10^6	0.4 ml	ip
2	18	RNA fraction	50 μg	0.1 ml	ip
3	18	Medium 199	Control	0.4 ml	ip
4	18	BCG (Tice)	5×10^6	0.05 ml	ic

Challenge Group			
1	2	3	4
H37Ra (4×10^6 viable particles/0.1 ml)	RNA Fraction (50μg/0.1 ml)	Medium 199 (Control)	BCG (5×10^6 viable particles/0.1 ml)

[a] Five weeks after the prechallenge procedure, the four groups of normal syngeneic mice were challenged with tumor cell suspension (10^6/0.1 ml) mixed with equal volumes of the respective materials listed above. The challenge dose was 0.2 ml given subcutaneously (dorsally). All injections were randomly spaced by cage and the entire procedure lasted no longer than 1 hr.

tion since this material neither induces nor provokes tuberculin hypersensitivity (12, 13). However, delayed hypersensitivity to some protein in the ribosomal fraction cannot be ruled out.

Our results with BCG were disappointing when compared with those reported by Bartlett (4). However, the BCG preparations that we used were lyophilized and are known to contain a large percentage of nonviable bacilli; in contrast, Bartlett *et al.*, in their experiments, used a different strain of BCG (Phipps) and one which was not lyophilized. Our success with the H37Ra strain of *M. tuberculosis* was striking and compares favorably with results of Bartlett *et al.* with BCG. This cannot be accounted for simply on the basis of viability since we have had good results with the lyophilized nonliving ribosomal RNA fraction. Indeed it is possible that when the conditions for maximal stabilization of the ribosomal RNA fraction are known and an optimal dosage schedule has been worked out we will find the activity of this nonliving preparation considerably improved. BCG, currently being studied on human neoplasia, has the capability of replicating and causing clinical infection in patients whose immunological responses have been depressed by disease and therapy. From a clinical viewpoint, nonviable mycobacterial material with anti-tumor activity equal to or better than BCG (or H37Ra) is highly desirable. The favorable response with the nonliving ribosomal RNA fraction is encouraging. Methods are available for further purification of the RNA fraction which, hopefully, may allow characterization of the active moiety.

Summary. Data are presented to show that both an H37Ra strain of *Mycobacterium tuberculosis* and a ribosomal RNA fraction from this strain are effective inhibitors of tumor growth in mice. Mice were pretreated with tubercle bacilli or RNA fraction and challenged 5 weeks later

TABLE II. Comparison of Various Treatments for Effect on Tumor Growth.

	7 days			14 days			21 days		
	Tumor free	<5mm	≥5mm	Tumor free	<5mm	≥5mm	Tumor free	<5mm	≥5mm
RNA	17	0	0	11	4	2	5	5	7
H37Ra	18	0	0	17	1	0	17	0	1
BCG	0	2	16	0	2	16	1	0	17
Media	13	3	1	0	2	15	0	3	14

with mixtures of tumor cells and the respective pretreatment agents. Comparative data with BCG are presented. Inhibition did not appear to be due to any direct toxic effect of tubercle bacilli or ribosomal RNA fraction on tumor cells. The necessity, for an inhibitory effect, of tuberculin hypersensitivity was deemed unlikely because the ribosomal RNA fraction neither induces nor provokes tuberculin hypersensitivity. The inhibition realized with mycobacterial extract avoids the risk to the tumor-bearer of viable organisms.

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