JOURNAL OF BACTERIOLOGY, Mar., 1966
Vol. 91, No. 3
Copyright © 1966 American Society for Microbiology
Printed in U.S.A.

Effective Nonliving Vaccine Against Experimental Tuberculosis in Mice

EDGAR RIBI, CARL LARSON, WILLIAM WICHT, ROBERT LIST, AND GRANVILLE GOODE

Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Hamilton, Montana, and Duncan Memorial Institute, University of Montana, Missoula, Montana

Received for publication 2 October 1965

ABSTRACT

RIBI, EDGAR (Rocky Mountain Laboratory, Hamilton, Mont.), CARL LARSON, WILLIAM WICHT, ROBERT LIST, AND GRANVILLE GOODE. Effective nonliving vaccine against experimental tuberculosis in mice. J. Bacteriol. **91**:975–983. 1966.—Antituberculosis vaccines were prepared in one of three manners: lyophilized BCG suspended in light mineral oil was disrupted in a Sorvall pressure cell and the "oil disruption product" was collected by centrifugation; BCG was disrupted in water, lyophilized, and worked into a paste with a small amount of oil (about 0.16 ml per 50 mg); BCG was disrupted in water, and the cell wall fraction was isolated, lyophilized, and prepared in an oil paste. These vaccines were suspended in Tween-saline to a concentration of 5 mg/ml and heated at 65 C for 30 min. In protection tests based on pulmonary infection with *Mycobacterium tuberculosis* H37Rv, the median number of virulent organisms in lung tissue of mice immunized with a few hundred micrograms of these three vaccines was 3 to 4 logs lower than in unvaccinated control mice. A similar dose of viable BCG standard vaccine reduced the lung count 1 to 2 logs below the controls. Protection afforded by nonviable, whole BCG, with or without oil, was of only borderline significance. Since oil-treated fractions containing cell walls produced effective immunity, while the oil-treated protoplasm or whole cells were not active, the protective antigen appeared to be an inner component of the cell wall, exposed when the cell was disrupted, and activated by oil. Extraction of oil from immunogenic disruption products resulted in loss of ability of the products to confer protection against the aerosol challenge, whereas high protection against the conventional challenge by intravenous infection with up to 1.4×10^8 cells of *M. tuberculosis* H37Rv was retained. Retreatment with oil of these nonimmunogenic products restored the immunogenicity if the oil was applied to dried products. The consistent finding that moisture interferes with the enhancement of the vaccine potency by oil suggested that such enhancement may not be the same as that ordinarily produced by water-in-oil emulsions.

It has been reported that mice immunized with a heat-stable, nonliving vaccine (BCG disrupted in light mineral oil) were as resistant to experimental tuberculosis as mice immunized with viable BCG (3, 6). This resistance could be demonstrated after aerosol or intravenous challenge of mice with virulent tubercle bacilli. It appeared that the vaccine potency could be correlated with the cell wall fraction of the disrupted BCG; however, the mineral oil prevented removal of protoplasmic material. It was not possible, therefore, to correlate the protective antigen definitely with a cell wall constituent, and the vaccine was designated "oil disruption product." Furthermore, since the immunogenicity of heat-killed whole BCG was not increased with the addition of mineral oil, it could be hypothesized that the protective antigen in the disrupted cells was an inner component of the cell wall or a protoplasmic constituent.

When it was found that BCG disrupted in water or in buffered sucrose solution was as poor a vaccine as nonviable whole cells, we considered that the oil might preserve the protective antigen during the disruption process. However, if the oil activated the immunogen rather than merely protecting it, cells disrupted in aqueous suspension should show increased immunogenicity after treatment with oil. This does, indeed, seem to be the case. Further data presented in this report

show that the oil cannot be completely removed from the disruption product without impairing the potency of the vaccine.

Materials and Methods

Preparation of vaccines. A strain of BCG obtained from the Pasteur Institute of Paris was maintained and stored on Sauton's potato medium. After one subculture in Sauton's liquid medium, it was grown as a pellicle in this medium for 10 days and was harvested by filtration onto sterile gauze supported by a stainless-steel screen (6). The moist cell mass was employed as starting material to prepare the various fractions used in this study.

Oil disruption product. If not otherwise stated, 20 g of the moist cell mass was suspended in 100 ml of light liquid petrolatum (Pharmaceutical Laboratory, Perry Point, Md.), and was disrupted in a Sorvall refrigerated pressure cell at a pressure of 40,000 to 45,000 psi at 5 to 10 C. The effluent was centrifuged at 20,000 × *g* for 1 hr, and the pellet was washed three times with oil by resuspension and centrifugation. The final product, designated "oil disruption product," was either resuspended in 0.85% sodium chloride containing 0.2% Tween 80 (Tween-saline) to a concentration of 5 mg (dry weight) per ml and heated for 30 min at 65 C, or was extracted with petroleum ether or acetone in a Soxhlet apparatus for 6 hr prior to suspension and heating in Tween-saline. Amounts of 50 mg of the extracted products also were washed three times with oil as described before or mixed with 4 or 8 drops of oil to form a paste prior to resuspension in Tween-saline and heat treatment.

Water disruption product, cell walls, and protoplasm. A 20-g amount of wet cell mass was suspended in distilled water to a volume of 100 ml and ruptured in the pressure cell. A portion of the effluent was dried immediately from the frozen state to yield the "water disruption product," and the remainder was centrifuged at 20,000 × *g* for 1 hr to provide a sediment of cell walls and a supernatant fluid containing the protoplasm. Both the cell wall and protoplasmic fractions were dried from the frozen state. The three dried products, the "water disruption product," the cell walls, and the protoplasmic fraction, were prepared in one or more of the following ways. They were suspended in Tween-saline, they were washed three times with oil and the oil-soaked sediment was resuspended in Tween-saline, or they were mixed with merely enough oil to form a paste with the aid of a Teflon grinder (about 0.16 ml of oil per 50 mg of powder was required) and the resulting product was resuspended in Tween-saline in the same tube with the same grinder. (Cell walls could not be readily resuspended to a concentration greater than 5 mg/ml of Tween-saline.) Each of the final suspensions was heated at 65 C for 30 min and then diluted with saline to the desired concentration for testing in mice. Amounts of 2 to 5 mg (dry weight) of each of the various heated fractions derived from oil or water disruption products were plated on Dubos Tween-albumin medium. No growth was observed after 30 days of incubation at 37 C.

Particulate fraction. This fraction was prepared according to the procedure of Youmans and Youmans (8). One-half of the final pellet containing the particulate fraction was suspended in 0.01 M phosphate buffer (*p*H 7.0) and stored at 4 C for 1 day before testing. The remainder of the pellet was suspended in oil and centrifuged. The particulate fraction did not sediment but formed a floating layer on the oil. Excess oil was removed with a pipette. About 100 mg of the moist particulate fraction was plated on Dubos albumin agar both prior to and after treatment with oil. By this means, averages of 90 and 60 viable BCG per mg were detected.

Protection tests. The methods used were based on airborne or massive intravenous challenge with virulent *Mycobacterium tuberculosis* H37Rv of mice vaccinated intravenously or intraperitoneally with fractions of BCG or with Rosenthal's living BCG standard vaccine. Both tests have been described elsewhere (6).

Results

Experiment I. A moist mass of BCG was divided into two portions, one-half suspended in oil and the remainder in distilled water. Both samples were disrupted in the pressure cell to yield an "oil disruption product" and a "water disruption product." The former was washed with oil in the usual manner; the latter was lyophilized. One-half of the lyophilized material was washed three times with oil, and the other half was held as a control. Tween-saline suspensions of these three preparations and a sample of the starting cells were heat-sterilized and injected intravenously into mice to test their ability to protect against challenge by aerosol containing *M. tuberculosis* H37Rv. Data presented in Table 1 show that the heat-sterilized whole cells and the water disruption product did not confer significant immunity. Treatment of the "water disruption product" with oil engendered immunizing properties in this previously inactive fraction, equal to that of the conventional "oil disruption product," and superior to that afforded by the viable BCG standard vaccine. These results indicate that disruption of the cells in oil is not essential for enhancement of immunogenicity; it is sufficient merely to add oil to a water disruption product.

Experiment II. A moist mass of BCG was prepared in the usual manner to obtain a conventional "oil disruption product." A sample of this product was extracted with petroleum ether for 6 hr in the Soxhlet apparatus. Table 2 shows protection afforded mice by the "oil disruption product" before and after extraction with petroleum ether. Extraction of the disrupted cells with petroleum ether did not alter significantly their immunogenicity, as shown by the ability of this

TABLE 1. *Protection of mice inoculated intravenously with heat-sterilized fractions from BCG or with living BCG, and challenged 4 weeks later with aerosol containing Mycobacterium tuberculosis H37Rv (experiment I)*

Vaccine	Immunizing dose (dry wt)	Results 30 days after challenge	
		No. with lung lesions/ no. of mice tested	Viable-cell coun t*
	μg		
Whole cells, heated	1,000	12/20	1.1 × 10^6
	250	19/20	9.0 × 10^5
"Oil disruption product"	1,000	0/19	1.0 × 10^2
	250	3/20	3.0 × 10^3
"Water disruption product," lyophilized	1,000	20/20	1.6 × 10^6
	250	20/20	9.5 × 10^5
"Water disruption product," lyophilized and washed with oil	500	0/20	6.1 × 10^2
	250	1/20	8.0 × 10^2
Viable BCG standard vaccine	1,500†	1/19	1.3 × 10^4
Controls	None	30/30	3.7 × 10^5

* Median number of viable *M. tuberculosis* H37Rv per 100 mg of lung tissue; 10 mice per group sampled.
† Moist weight, corresponding to approximately 300 μg of dry weight and containing 4.6 × 10^7 viable BCG.

moist product to protect mice challenged with *M. tuberculosis* H37Rv to the same degree as those immunized with viable BCG.

The other portion of the moist BCG cell mass was lyophilized before an "oil disruption product" was prepared. This disruption product was highly potent in protecting mice against an aerosol challenge of virulent bacilli (Table 2). However, when this material was extracted with petroleum ether, it no longer produced a measurable degree of resistance in the aerosol challenge test, but the ability to induce resistance to massive intravenous challenge doses appeared unaltered. Thus, the "oil disruption product" prepared from lyophilized BCG was shown to differ from that prepared from moist BCG.

These data might be interpreted as indicating that the oil could be readily extracted from the disruption products of dry cells, whereas the moisture content of the wet cells interfered with the extraction. As a corollary to this, it might be hypothesized that the presence of only a small amount of oil was necessary to enhance the resistance to aerosol challenge produced by disrupted BCG.

Experiment III. To test the latter hypothesis, an "oil disruption product" prepared from moist cells was extracted first with petroleum ether and then with acetone. This procedure, which was expected to remove all but minute quantities of both water and oil, and also some lipoid material of bacterial origin, converted the "oil disruption product" to a dry powder. Data listed in Table 3 show that removal of oil by this method resulted in a complete loss of the capacity of the "oil disruption product" to protect mice against aerosol challenge, and that retreatment with oil not only restored but, as judged by viable-cell counts, actually increased the immunogenicity of this preparation.

Experiment IV. In a previous report (6), we compared the immunogenicity of an "oil disruption product" and of two Youmans-type protoplasmic particulate fractions, both prepared from the same lots of *M. tuberculosis* H37Ra and BCG, with that of a viable BCG standard vaccine. This particulate fraction, although protective in high doses (20 mg, moist weight) against massive intravenous infection, was not effective against aerosol infection. It remained to be determined whether treatment of the protoplasmic particulate fraction with oil would enhance its protective value in either the massive or aerosol challenge test.

Data in Table 4 indicate that no resistance against aerosol challenge was observed in mice vaccinated with unheated, oil-treated particulate fraction. Although oil treatment of the particulate fraction slightly increased the protective effect against large intravenous challenge doses, the

TABLE 2. *Protection of mice vaccinated with heat-sterilized fractions from BCG or with living BCG and challenged 4 weeks later with Mycobacterium tuberculosis H37Rv (experiment II)*

Vaccine	Intraperitoneal immunization, intravenous challenge with 3.2×10^7 *M. tuberculosis* H37Rv		Intravenous immunization, aerosol challenge with *M. tuberculosis* H37Rv		
				Results 30 days after challenge	
	Immunizing dose (dry wt)[a]	Survivors 30 days after challenge	Immunizing dose (dry wt)	No. with lung lesions/ no. of mice tested	Viable-cell count[b]
	μg	%	μg		
Prepared from moist cells					
"Oil disruption product," conventional	250 60	90 100	400	0/15	2.1×10^4
"Oil disruption product," extracted with petroleum ether	250 60	70 60	400	1/20	2.5×10^4
"Oil disruption product," extracted with petroleum ether, heated prior to extraction	250 60	70 95	400	2/20	1.5×10^4
Prepared from lyophilized cells					
"Oil disruption product"	250 60	40 75	400	0/20	6.3×10^2
"Oil disruption product," extracted with petroleum ether	250 60	50 60	400	12/19	1.1×10^6
Viable BCG standard vaccine	50[c] 12.5 (206,250 cells) 3.12 (51,550 cells) 0.78 (12,888 cells)	95 100 85 60	1,500[d]	2/18	1.5×10^4
Controls	None	5	None	40/40	1.1×10^6

[a] Twenty mice per dose.
[b] Median number of viable *M. tuberculosis* H37Rv per 100 mg of lung tissue, 10 mice per group sampled.
[c] Moist weight, 8.25×10^5 viable BCG.
[d] Moist weight, 4.2×10^7 viable BCG.

protection conferred even by very large doses never equalled that produced by 60 μg of "oil disruption product" (Table 2).

When we followed the procedures outlined by Youmans and Youmans (8) for preparing particulate fractions from disrupted BCG, such products usually contained viable cells. In those prepared for experiment IV, 1,000 to 2,000 viable BCG per 20-mg dose (moist weight) were counted. A similar fraction previously prepared from *M. tuberculosis* H37Ra contained even more viable cells (20,000 viable cells per 20-mg mouse dose; 6). According to Youmans and co-workers, this number of viable *M. tuberculosis* H37Ra is too small to produce a detectable response in "Strong A" mice (7, 9). However, Millman (5), using Youmans' test system, found that 40,000 viable *M. tuberculosis* H37Ra afforded resistance in CF_1 mice comparable to that of 20 mg of the particulate fraction reported by Youmans and co-workers (7–10). The CF_1 strain of mouse and the "Strong A" strain are reported to respond similarly to the particulate fraction (10). Millman (5) also noted that the immunizing potency of doses containing about 40,000 viable cells was destroyed by heating at 100 C for 30 min, a treatment which also destroyed the efficacy of the particulate fraction (8).

TABLE 3. *Protection of mice inoculated intravenously with heat-sterilized fractions from BCG or with living BCG, and challenged 4 weeks later with aerosol containing Mycobacterium tuberculosis H37Rv (experiment III)*

Vaccine	Immunizing dose (dry wt)	Results 30 days after challenge	
		No. with lung lesions/ no. of mice tested	Viable-cell count*
	μg		
"Oil disruption product," conventional, from wet cells	800	3/20	2.7×10^4
	200	2/20	1.2×10^5
"Oil disruption product," extracted with petroleum ether and acetone	800	20/20	7.4×10^6
	200	20/20	3.8×10^6
"Oil disruption product," extracted with petroleum ether, acetone, and then washed 3× with oil	800	0/20	2.5×10^2
	200	0/19	7.5×10^2
Viable BCG standard vaccine	200†	4/18	8.8×10^4
Controls	None	20/20	8.1×10^6

* Median number of viable *M. tuberculosis* H37Rv per 100 mg of lung tissue; 10 mice per group sampled.
† Moist weight, corresponding to approximately 40 μg of dry weight and containing 6.4×10^6 viable BCG.

TABLE 4. *Protection of mice vaccinated with particulate fraction from BCG or with living BCG and challenged 4 weeks later with Mycobacterium tuberculosis H37Rv (experiment IV)*

Vaccine	Intraperitoneal immunization, intravenous challenge with 1.1×10^8 H37Rv cells				Intravenous immunization, aerosol challenge with *M. tuberculosis* H37Rv		
	Immunizing dose (moist wt)	No. of mice	Survivors after challenge		Immunizing dose (moist wt)	Results 30 days after challenge	
			30 days	60 days		No. with lung lesions /no. of mice tested	Viable-cell count*
	μg		%	%	μg		
Particulate fraction	20,000	25	68	32	4,000	10/10	1.3×10^6
	5,000	25	60	24	2,000	10/10	—
	1,250	25	24	4	1,000	10/10	—
Particulate fraction washed with oil	20,000	25	76	48	4,000	10/10	3.9×10^6
	5,000	25	84	56	2,000	10/10	—
	1,250	25	44	16	1,000	10/10	—
Viable BCG standard vaccine	300†	23	100	78	200‡	0/10	1.3×10^4
Controls	None	25	16	0	None	10/10	5.9×10^6

* Median number of viable *M. tuberculosis* H37Rv per 100 mg of lung tissue, 10 mice per group sampled.
† Moist weight, 9.8×10^6 viable BCG.
‡ Moist weight, 6.5×10^6 viable BCG.

Data in Table 2, which agree with Millman's findings, indicate that a dose of BCG standard vaccine containing approximately 10,000 to 50,000 viable cells conferred measurable resistance in the Rocky Mountain Laboratory strain of mouse against massive intravenous challenge with *M. tuberculosis* H37Rv. Results of a separate experiment (Table 5) show that inocula containing

from 1,000 to 100,000 viable BCG produced immunity comparable to that given by 20 mg of the particulate fraction (40 to 60% survivors 30 days after challenge; 6–10). Also, as in the case of the particulate fraction (8), the activity of these vaccines containing a relatively small number of viable attenuated bacilli was completely destroyed upon heating at 80 C for 30 min (Table 5). These data, therefore, suggest that the resistance afforded mice by the protoplasmic particulate fraction prepared and tested in this laboratory was predominantly due to contaminating viable BCG or *M. tuberculosis* H37Ra.

Experiment V. This experiment was designed to extend some of the results of previous tests and to demonstrate whether the cell wall or the protoplasmic fraction contained the protective antigen that was activated by treatment with oil. The results listed in Table 6 confirmed that the resistance of mice to challenge by aerosol conferred by a conventional "oil disruption product" prepared from wet starting cells was retained in the preparation after much of the oil had been removed by refluxing with petroleum ether in the Soxhlet apparatus. Subsequent extraction with acetone, which removed water and permitted more of the oil to be extracted, resulted in a total loss of immunogenicity. The immunogenic potency of this extracted material could be restored by addition of only enough oil to form a paste.

TABLE 5. *Protection in mice inoculated intraperitoneally with 0 to 100,000 viable BCG and challenged intravenously 4 weeks later with Mycobacterium tuberculosis H37Rv (experiment IV)*

Viable BCG		No. of mice tested	Survivors*
Heat treatment for 30 min	No. of viable cells inoculated		
			%
None†	100,000	23	48
40 C	31,000	25	44
80 C	0	25	8
None†	12,000	24	63
40 C	4,600	25	55
80 C	—‡	—	—
None†	3,200	25	36
40 C	830	25	48
80 C	0	25	8
None†	20	25	16
Controls	0	25	4

* At 30 days after challenge with 1.4×10^8 *M. tuberculosis* H37Rv.

† Stored at 4 C.

‡ Material sterile, but no mice injected.

When the product of the initial extraction with petroleum ether in the Soxhlet apparatus was further exposed to petroleum ether after being thoroughly dispersed in a Sorvall Omni-mixer, the resulting material, although still a wet mass, had lost its capacity to protect mice in the aerosol challenge test. In contrast to the restoration by oil of the dry, acetone-extracted product, little if any immunogenic potency was restored to this wet, inactive sediment by mixing it with a similar amount of oil. A possible explanation for the inability of the oil to restore vaccine potency in this case is that the presence of water inhibits the penetration of the oil, thereby preventing its making contact with certain bacterial components, which obviously is necessary for the protective antigen to stimulate enhancement of resistance.

Washing lyophilized cell walls with oil or merely adding enough oil to the dry cell walls to make a paste brought about a striking enhancement of the protective properties of the vaccine; whereas treatment of the lyophilized protoplasm with oil had no significant effect. Data presented in Table 6 suggest that the cell wall contains the protective antigen, and that the most effective enhancement of the antigen is obtained when oil is added to dehydrated cell wall material.

Experiment VI. It has been shown previously (Larson and Ribi, *unpublished data*) that vaccines prepared from moist viable BCG cells which were first washed with oil, then suspended in Tween-saline and sterilized by heat, were no more effective in immunizing against aerosol challenge than were vaccines consisting of heat-sterilized BCG which had not been treated with oil. Inasmuch as the products obtained either by disruption in oil of lyophilized cells or by treatment of dried disruption products with oil were able to immunize mice against aerosol challenge with even greater efficiency than the conventional "oil disruption product" obtained by processing wet cells, it was considered important to examine the effect of oil when applied to lyophilized viable, instead of moist viable, BCG.

As in the previous experiments, all fractions described here were prepared from a single lot of starting cells. The data in Table 7 show that, in the aerosol challenge test, very little protection was afforded mice by lyophilized BCG whole cells which had been treated with oil and heat-sterilized, whereas the high protective ability of products prepared by disrupting lyophilized BCG in oil or by treating dried disrupted cells with oil was confirmed. In contrast, the results obtained with the test based on massive intravenous challenge showed that very small doses (62 μg) of heat-killed BCG, with or without the addition of oil, were as active as equal doses of "oil disruption product," and that the resistance produced

TABLE 6. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis H37Rv by intravenous vaccination with heat-sterilized fractions from BCG or with viable BCG (experiment V)*

Vaccine*	Results 30 days after challenge	
	No. with lung lesions/no. of mice tested	Viable-cell count†
"Oil disruption product" prepared from wet starting cells	1/20	3.1×10^3
↓ Extracted with petroleum ether in the Soxhlet apparatus	3/20	2.2×10^4
↓ Extracted with acetone and again with petroleum ether in the Soxhlet apparatus	20/20	2.6×10^6
↓ Paste in oil	0/20	7.7×10^3
↓ Mechanically dispersed and again extracted with petroleum ether	20/20	1.4×10^6
↓ Paste in oil	15/20	8.0×10^5
Cell walls, untreated	20/20	3.4×10^6
↓ Paste in oil	0/20	1.3×10^3
↓ Washed with oil	0/20	2.6×10^3
Protoplasm, untreated	20/20	1.8×10^6
↓ Paste in oil	20/20	4.4×10^5
Viable BCG standard vaccine	3/20	2.1×10^4
Controls	30/30	2.0×10^6

* Immunizing dose for all preparations was 300 μg (dry weight), with the exception of the BCG standard vaccine which was 200 μg (moist weight), containing 3.9×10^6 viable cells.

† Median no. of viable *M. tuberculosis* H37Rv per 100 mg of lung tissue; 10 mice per group sampled.

was retained after extraction of the oil, whereas such extraction resulted in loss of activity in the aerosol challenge test.

Based on data from this and previous experiments, it is suggested that the preparation of nonliving vaccines, which matched the viable BCG vaccine in ability to elicit immunity against experimental tuberculosis in mice, depended upon the presence of cell walls coated with oil. Furthermore, the cell walls appear to be active only when the cells have been ruptured. The results also suggest that the test system based on massive intravenous challenge is inadequate for evaluating the effectiveness of nonviable vaccines against experimental tuberculosis in mice.

DISCUSSION

This report deals with the preparation and evaluation of effective, nonliving, heat-stable vaccines against experimental tuberculosis in mice. Such vaccines may be prepared in any of the following ways: (i) by disrupting lyophilized BCG suspended in oil in a refrigerated pressure cell at 30,000 to 40,000 psi and collecting the oil-soaked sediment; (ii) by disrupting viable BCG suspended in water, lyophilizing the whole disruption product, and adding 4 or 8 drops of oil per 50 mg and mixing to form a paste; or (iii) by isolating the cell wall fraction from the "water disruption product" by centrifugation, lyophilizing it, and mixing the dry powder with 4 or 8 drops of oil to form a paste. The "oil disruption product" or the pastes are then suspended in 0.85% sodium chloride containing 0.2% Tween 80 to give a concentration of 5 mg (dry weight) per ml and heated at 65 C for 30 min. These stock vaccines may be diluted with saline to give the desired concentration per 0.2-ml dose.

In the pulmonary challenge test, the median number of viable *M. tuberculosis* H37Rv in lung

TABLE 7. *Protection of mice vaccinated with heat-sterilized fractions from BCG and challenged 4 weeks later with Mycobacterium tuberculosis H37Rv (experiment VI), results 30 days after challenge*

Vaccine	Intraperitoneal immunization, intravenous challenge with 8.5 × 10^7 *M. tuberculosis* H37Rv		Intravenous immunization, aerosol challenge with *M. tuberculosis* H37Rv	
	Immunizing dose (dry wt)*	Survivors	Immunizing dose (dry wt)	No. with lung lesions/no. of mice tested
	μg	%	μg	
Whole cells, lyophilized	250	92	400	18/20
	62	80	200	20/20
Whole cells, lyophilized and washed with oil	250	80	400	20/20
	62	76	200	19/20
"Oil disruption product," from lyophilized cells	250	96	400	0/20
	62	68	200	3/20
"Oil disruption product," extracted with petroleum ether	250	68	400	20/20
	62	80	200	20/20
"Oil disruption product," extracted with petroleum ether then washed with oil	250	64	400	1/20
	62	72	200	3/20
Viable BCG standard vaccine	100†	96	—	—
	25 (400,000 cells)	100	25‡	10/20
Controls	None	20	None	20/20

* Twenty-five mice per dose.
† Moist weight, 1.6 × 10^6 viable BCG.
‡ Moist weight, 0.4 × 10^6 viable BCG.

tissue of mice vaccinated with a few hundred micrograms of dried, oil-treated disruption products was roughly 3 to 4 logs lower than that of unvaccinated control mice, whereas only a 1 to 2 log lower count than that of the control mice was noted in mice receiving the viable BCG standard vaccine in amounts varying from 200 to 1,500 μg of moist weight (corresponding to approximately 40 to 300 μg of dry weight). Even though 50% end points have not yet been determined, it is evident that the immunogenicity of the conventional "oil disruption product" prepared from wet cells disrupted in oil was at least equal to that of the viable BCG standard vaccine, whereas the potency of dried, oil-treated disruption products was generally superior to either of these vaccines (Tables 1, 2, 3, and 6).

Viable BCG, when treated with oil, either as a moist mass or after lyophilization, and then heated, conferred no greater degree of protection to mice than did heat-killed, untreated cells. Since fractions containing cell walls, but not protoplasmic fractions, treated with oil produced effective immunity, it is suggested that an inner component of the cell wall is exposed to the oil during disruption of the cells and that the interaction of oil with this cell wall component activates a protective antigen. The fact that cell walls from bacilli disrupted in water became immunogenic when dried and treated with oil appears to dispose of our earlier speculation that disrupting the cells in oil might somehow prevent autoenzymes or degradative or denaturing forces from destroying the antigen.

The minimal quantity of oil required for an optimal response is not yet known, but is obviously small. A conventional "oil disruption product," prepared by disrupting moist cells in oil, can be extensively extracted with petroleum ether in the Soxhlet apparatus with only slight loss of activity. This suggests that residual moisture tends to prevent penetration of the extractant, thereby hindering removal of oil which had been forced into the cell walls by the high pressure of the disruption process. More vigorous methods of extraction gave products without prophylactic value to mice, presumably because of more effective removal of oil (Table 6). When the product was a dry powder, retreatment with oil fully restored immunogenicity. However, when the extracted product

was a moist mass, addition of oil only partially restored the potency to the vaccine. These results were confirmed in an experiment not described here, wherein treatment of moist cell walls with oil resulted in far less enhancement of immunogenic potency than when lyophilized cell walls were so treated. This may also explain why the vaccine potency of an "oil disruption product" prepared by disrupting dried cells in oil exceeded that of the conventional "oil disruption product" prepared from wet cells. As suggested earlier, direct contact between oil and antigen appears to be necessary to the effect; and the presence of water, under most circumstances, interferes with such contact. The consistent finding that moisture is deleterious to the enhancing action of the oil on vaccine potency, suggests that such enhancement may not be the same as that ordinarily produced by water-in-oil emulsions. The fact that small amounts of oil markedly increase protective effects without enhancing sensitization to tuberculoprotein (6) also speaks for a different kind of adjuvant effect.

Although complete extraction of oil from an "oil disruption product" resulted in loss of ability of the product to protect against aerosol challenge, it did not interfere with protection against massive intravenous challenge. In the massive challenge test, very small doses (62 μg) of heat-killed BCG sufficed to protect mice to a degree equal to that provided by similar doses of "oil disruption product," oil-treated cell walls, or viable BCG. Similar protection was obtained when the intravenous, instead of the intraperitoneal, route of immunization was used to assay samples of all types of materials described in this paper in the massive challenge test. Seemingly indisputable evidence has been presented in the literature to show that nonspecific factors participate in stimulating resistance in test systems based on massive intravenous challenge. Reports disclose enhancement of resistance by such nonspecific antigens as endotoxins from gram-negative bacteria or *Bordetella pertussis* vaccines (*see*, e.g., 1, 2, 5). These products, however, had no measurable immunizing effect when aerosol challenge was employed (4).

The test based on pulmonary challenge had been used to determine the value of viable attenuated tubercle bacilli as immunizing agents; the results were reliable and were not influenced by nonspecific antigens, and interference did not appear to be the mechanism of protection (4). Since oil-treated, disrupted BCG or *M. tuberculosis* H37Ra cells have been, so far, the only nonliving vaccines effective in this test, it would appear that the action of the oil is directed toward a cell wall antigen that is specific. Experiments planned or in progress include the testing of oil-treated cell walls from *M. tuberculosis* H37Rv, anonymous bacilli, *M. smegmatis*, *M. butyricum*, and species of *Salmonella* for their ability to stimulate resistance against challenge with *M. tuberculosis* H37Rv.

ACKNOWLEDGMENT

We are indebted to the Montana Tuberculosis Association for the technical assistance of Larry Ewalt during these studies.

LITERATURE CITED

1. DUBOS, R. J., AND R. W. SCHAEDLER. 1956. Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins. J. Exptl. Med. **104**:53–65.
2. DUBOS, R. J., R. W. SCHAEDLER, AND D. BÖHME. 1957. Effects of bacterial endotoxins on susceptibility to infection with Gram-positive and acid-fast bacteria. Federation Proc. **16**:856–857.
3. LARSON, C. L., E. RIBI, W. C. WICHT, R. H. LIST, AND G. GOODE. 1963. Resistance to tuberculosis in mice immunized with BCG disrupted in oil. Nature **198**:1214–1215.
4. LARSON, C. L., AND W. C. WICHT. 1962. Studies of resistance to experimental tuberculosis in mice vaccinated with living attenuated tubercle bacilli and challenged with virulent organisms. Am. Rev. Respirat. Diseases **85**:833–846.
5. MILLMAN, I. 1961. Nonspecific resistance to tuberculosis. Am. Rev. Respirat. Diseases **83**:668–675.
6. RIBI, E., C. L. LARSON, W. C. WICHT, R. LIST, AND G. GOODE. 1965. Resistance to experimental tuberculosis stimulated by fractions from attenuated tubercle bacilli. Proc. Soc. Exptl. Biol. Med. **118**:926 933.
7. YOUMANS, G. P., I. MILLMAN, AND A. S. YOUMANS. 1955. The immunizing activity against tuberculous infection in mice of enzymatically active particles isolated from extracts of *Mycobacterium tuberculosis*. J. Bacteriol. **70**:557–562.
8. YOUMANS, A. S., AND G. P. YOUMANS. 1964. Further studies on a labile immunogenic particulate substance isolated from *Mycobacterium tuberculosis*. J. Bacteriol. **87**:278–285.
9. YOUMANS, A. S., G. P. YOUMANS, AND I. MILLMAN. 1957. Immunogenicity of particles isolated from *Mycobacterium tuberculosis*. Proc. Soc. Exptl. Biol. Med. **96**:762–768.
10. YOUMANS, G. P., A. S. YOUMANS, AND K. KANAI. 1959. The difference in response of four strains of mice to immunization against tuberculous infection. Am. Rev. Respirat. Diseases **80**:753–756.