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Immunogenicity of Cell Walls from Various Mycobacteria Against Airborne Tuberculosis in Mice[1]

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Protective potency of oil-treated cell walls of various mycobacteria against airborne infection of mice with a few cells of *Mycobacterium tuberculosis* H37Rv was compared with that of viable BCG. Although less potent than BCG cell walls, the cell walls of atypical mycobacteria of Runyon's groups I to IV protected against challenge by aerosol to some degree. Protection afforded by cell walls of H37Rv and of the avirulent mutants H37Ra and Washington II was comparable to that provided by BCG cell walls. However, cell walls of a highly virulent strain of *M. bovis* (Bovinus I) provided the best protection yet achieved. Present evidence suggests that protective substances are shared by all mycobacteria but in differing amounts; the relationship between virulence and immunogenicity has yet to be clarified.

It has been reported that lyophilized cell walls of *Mycobacterium bovis*, strain BCG, and *M. tuberculosis*, strain H37Ra, ground to a paste with a small quantity of mineral oil or the synthetic hydrocarbon 7-*n*-hexyloctadecane and then suspended in saline containing 0.2% Tween 80, stimulated resistance in mice to aerosol challenge with a few viable cells of *M. tuberculosis* H37Rv (10, 12). This type of immunity was shown to persist for at least 6 months (1). Although cell walls protected mice against challenge by either the intravenous or respiratory route, the aerosol challenge test has been adopted as the method of choice for the evaluation of experimental vaccines because (i) it closely simulates natural infection of man, and (ii) only mycobacteria or appropriate mycobacterial fractions have been found to enhance resistance to pulmonary infection (11), whereas nonspecific materials such as endotoxin (3, 7, 11), ferritin (I. Millman, *personal communication*), and *Escherichia coli* ribosomes (9) promote resistance to intravenous challenge with virulent tubercle bacilli.

Recently it was learned that in an effective vaccine the cell walls are layered on the surface of minute oil droplets (2). Any factor or treatment which inhibited the association of cell walls with oil droplets, such as the presence of a large quantity of the emulsifying agent Tween 80 or prior extraction of the cell walls with ether, ethyl alcohol, and chloroform, also reduced or abolished the potency of the vaccine (9). Even though we now know that in a potent vaccine, the mycobacterial cell walls are on the surface of the oil droplets, contrary to our earlier concept that the oil coated the cell wall, we have continued to use, for the sake of convenience, the expression "oil-treated cell walls."

This study was undertaken to determine the degree of immunity conferred by cell walls from human and bovine strains of tubercle bacilli with varying degrees of virulence and from atypical mycobacteria of Runyon's groups I to IV.

MATERIALS AND METHODS

Mycobacteria. The strains of atypical mycobacteria were supplied by R. Bönicke, Forschungsinstitut Borstel, Borstel, Germany. Cultures of *M. tuberculosis*, strains Washington II and H37Rv (used for the preparation of cell walls, but not for the challenge infection), and *M. bovis*, strain Bovinus I, were obtained from the Robert Koch Institute, Berlin, Germany. *M. bovis*, strain Vallée, was obtained from Th. Schliesser, University of Munich, Munich, Germany. *M. tuberculosis*, strain R_1Rv, obtained originally from the Trudeau Institute, Saranac Lake, New York, was supplied by W. R. Barclay, University of Chicago, Chicago, Ill. *M. tuberculosis*, strain H37Ra, also obtained from the Trudeau Institute, has been maintained at the Rocky Mountain Laboratory. The Pasteur Institute strain of BCG (1173 P2), maintained at the Rocky Mountain Laboratory, was used for the preparation of BCG cell walls.

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Cultivation. All strains were maintained on Hohn or Löwenstein-Jensen medium with the exception of BCG, which was maintained on Sauton's potato medium. They were subcultured twice in Sauton's liquid medium, with the exception of strain Bovinus I which was subcultured in Long's medium. For vaccine production, each strain was grown for 10 to 18 days as a pellicle on the appropriate medium, and the cells were harvested on a sterile gauze filter, supported by a stainless-steel screen, and washed twice with distilled water.

Preparation of cell walls. Cell walls from all strains were prepared by the method described previously for BCG (10). The degree of cell disruption was estimated by electron microscopy.

Preparation of vaccines. Vaccines of all cell wall preparations were made by the method described earlier (1), except that the vaccines were used within 24 hr of preparation and were not frozen. The viable BCG standard vaccine was supplied through the courtesy of Sol Roy Rosenthal, University of Illinois, Chicago, and prepared for use as described previously (10).

Protection test. The test was performed as previously described (10). Briefly, 3-week-old female white mice reared at the Rocky Mountain Laboratory were inoculated intravenously with 0.2 ml of the experimental vaccine and were challenged 1 month later in a Middlebrook chamber (6) with an aerosol containing *M. tuberculosis* H37Rv. The substrain of H37Rv used for the aerosol challenge was maintained at the Rocky Mountain Laboratory on Hohn medium and passed through guinea pigs annually. Autopsies, lung cultures, and sometimes spleen cultures were performed 1 month after challenge infection.

RESULTS

Immunogenicity of cell walls from atypical mycobacteria. Oil-treated cell walls from representative mycobacteria of Runyon's groups I to IV were compared with oil-treated BCG cell walls and viable BCG for their ability to protect mice against aerosol challenge with *M. tuberculosis* H37Rv (Table 1). As judged by the number of mice with grossly visible tubercles in the lungs and by the median count of viable H37Rv cells per 100 mg of lung tissue, at the dose level of 400 μg, the cell wall vaccine prepared from *M. kansasii* (group I) provided the best protection of all the vaccines containing atypical mycobacterial cell walls. The 400-μg dose protected nearly as well as the 200-μg dose of BCG cell walls, but the 200-μg dose of *M. kansasii* cell walls was markedly inferior to the equivalent dose of BCG cell walls. However, for the 400-μg dose a 2 log lower count (3.4×10^2) than for the 300-μg dose of viable BCG (1.9×10^4) and a 4 log lower count than for the unvaccinated controls were noted. Higher doses of viable BCG usually do not significantly increase this order of protection (10). The next best protection was afforded by cell walls from *M. avium* and from the Battey bacillus (group III); they also protected mice as well or better than did viable BCG, whereas the protection afforded by cell walls from *M. aquae* (group II) was comparable to that afforded by viable BCG. Cell walls prepared from *M. smegmatis*, classified with the saprophytic mycobacteria in Runyon's group IV, provided the lowest degree of protection; the median count was only 1 log lower than that of the controls. Results from other experiments (*not shown*) demonstrated that oil-treated cell walls from other mycobacteria of group IV, *M. fortuitum* and *M. phlei*, also protected to the same low degree as did *M. smegmatis*.

TABLE 1. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis H37Rv by intravenous inoculation with oil-treated cell walls from atypical mycobacteria*

Cell walls	Runyon's group	Immunizing dose[a] (μg)	Results 1 month after challenge: No. with lung lesions/no. of mice tested	Results 1 month after challenge: Median count/0.1 g of lung[b]
M. kansasii	I	400	2/20	3.4×10^2
		200	4/20	1.1×10^4
M. aquae var. *ureolyticum*	II	400	1/20	1.5×10^4
		200	3/20	1.8×10^4
Battey bacillus	III	400	0/18	1.7×10^3
		200	0/20	2.9×10^3
M. avium	III	400	0/19	3.4×10^3
		200	0/20	8.0×10^3
M. smegmatis	IV	400	9/20	1.4×10^5
		200	17/20	1.8×10^5
BCG	—	200	0/20	3.2×10^2
Viable BCG standard	—	300	1/20	1.9×10^4
Controls	—	—	30/30	2.1×10^6

[a] All in terms of dry weight except the viable BCG standard. The viable BCG standard contained 4.8×10^6 viable units.

[b] Median count of viable cells of *M. tuberculosis* H37Rv for 10 lungs.

As shown by Barclay et al. (2), a particular association of oil and cell walls is essential for a BCG cell-wall vaccine to be effective. Consequently, the vaccine of low potency made with cell walls of *M. smegmatis* was examined for the stability of this association in the diluted emulsion. The oil droplets were coated with cell-wall material, and it would appear, therefore, that the low protective potency of cell walls from saprophytic mycobacteria is due to lower immunogenicity rather than to insufficient coating of the droplets (Fig. 1).

Immunogenicity of cell walls from M. tuberculosis. Cell-wall vaccines prepared from the avirulent mutants H37Ra and Washington II, from the attenuated strain R_1Rv, and from the

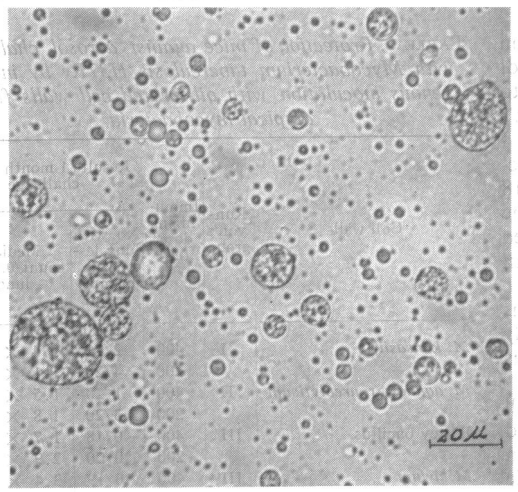

FIG. 1. *Oil-in-water emulsion with Mycobacterium smegmatis cell walls, showing emulsified oil droplets coated with cell walls and fragments.* × *675.*

virulent strain H37Rv all afforded protection superior to that provided by the optimal dose of the viable BCG standard vaccine (Table 2). The difference between the median counts of H37Rv cells in the lungs of mice vaccinated with viable BCG and of unvaccinated controls was about 2 logs, whereas a difference of up to 4 logs can be noted between counts of viable H37Rv in cell-wall vaccinated and control mice.

Immunogenicity of cell walls from M. bovis. Results obtained with oil-treated cell walls from the attenuated strains BCG and Vallée and from the virulent strain Bovinus I are compared in Table 3. It is evident that cell walls from strains BCG and Vallée provided protection comparable to that afforded by strains of *M. tuberculosis* (Table 2); those from the virulent strain Bovinus I provided the best protection yet achieved by any antituberculosis vaccine tested in our laboratory. In the latter case, no lungs of mice vaccinated with a dose of only 75 μg of these cell walls had any visible tubercles, and the median count was only 48 viable cells of H37Rv per 100 mg of lung tissue. In the majority of mice vaccinated with the 150-μg dose, virulent tubercle bacilli could not be detected in either the lungs or spleens 1 month after infection. All unvaccinated control mice had many large tubercles in their lungs and a median count of 5.3×10^6 viable H37Rv per 100 mg of lung tissue. That early dissemination of the tuberculous lung infection was prevented by these cell-wall vaccines and by viable BCG was evidenced by the absence of tubercle bacilli in the spleens of vaccinated mice, whereas 3.7×10^4 H37Rv cells were found per 0.1 g of spleen from control animals 1 month after challenge.

Lungs of mice vaccinated with 100 μg of oil-treated cell walls of the virulent strain Bovinus I

TABLE 2. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis H37Rv by intravenous inoculation with oil-treated cell walls of avirulent, attenuated, and virulent strains of M. tuberculosis*

Cell walls	Immunizing dose[a] (μg)	Results 1 month after challenge: No. with lung lesions/no. of mice tested	Results 1 month after challenge: Median count/0.1 g of lung[b]
Strain H37Ra, avirulent	450	0/15	8.5×10^1
	150	1/15	9.7×10^2
Strain Washington II, avirulent	450	0/20	1.9×10^2
	150	2/20	6.2×10^3
Strain R_1Rv, attenuated	300	0/19	2.8×10^3
	150	2/20	6.8×10^3
Strain H37Rv, virulent	300	0/20	1.8×10^2
	150	0/20	3.0×10^2
Viable BCG standard	300	2/20	1.7×10^4
Controls	—	30/30	1.2×10^6

[a] All in terms of dry weight except the viable BCG standard. The viable BCG standard contained 5.1×10^6 viable units.

[b] Median count of viable cells of *M. tuberculosis* H37Rv for 10 lungs.

TABLE 3. *Protection of mice against aerosol challenge with Mycobacterium tuberculosis H37Rv by intravenous inoculation with oil-treated cell walls of attenuated and virulent strains of M. bovis*

Cell walls	Immunizing dose[a] (μg)	Results 1 month after challenge: No. with lung lesions/no. of mice tested	Results 1 month after challenge: Median count/0.1 g: Lung	Results 1 month after challenge: Median count/0.1 g: Spleen[b]
Strain BCG, attenuated	300	0/20	3.8×10^2	<5
	150	0/20	9.0×10^2	
Strain Vallée, attenuated	300	0/20	1.4×10^3	
	150	0/20	1.2×10^4	
Strain Bovinus I, virulent	150	0/20	<10	<5
	75	0/20	4.8×10^1	
Viable BCG standard	300	1/20	1.2×10^4	<5
Controls	—	30/30	5.3×10^6	3.7×10^4

[a] All in terms of dry weight except the viable BCG standard. The viable BCG standard contained 6.2×10^6 viable units.

[b] Median count of viable cells of *M. tuberculosis* H37Rv or 10 lungs or spleens.

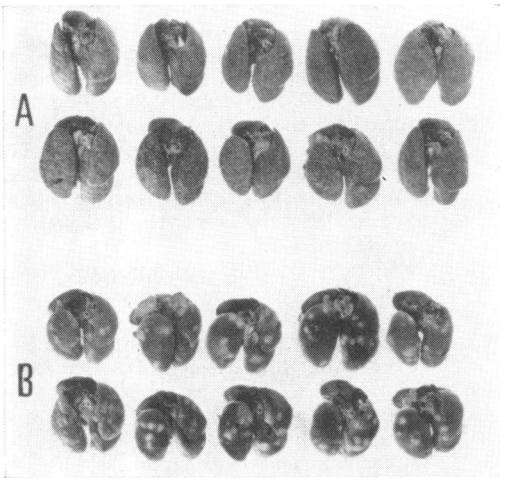

FIG. 2. *Lungs of mice removed 30 days after challenge by aerosol with H37Rv. (A) Mice vaccinated intravenously, 30 days prior to challenge, with 100 μg of oil-treated cell walls of Mycobacterium bovis strain Bovinus I. (B) Unvaccinated control mice.*

(group A) and of unvaccinated control mice (group B) 1 month after aerosol challenge are shown in Fig. 2. All lungs of the unvaccinated control mice have large nodular tubercles up to 3 mm in diameter, whereas no tubercles can be observed in lungs of mice which were vaccinated with the cell walls.

DISCUSSION

The results of these experiments suggest that all species of mycobacteria so far tested share in their cell wall a common factor which enhances resistance of mice to pulmonary infection with virulent tubercle bacilli. To achieve resistance against this type of infection, it is necessary to treat the mycobacterial cell walls with small amounts of mineral oil. The specificity of this test system was shown previously, inasmuch as oil-treated cell walls from unrelated species such as *Brucella abortus*, *E. coli*, *Listeria monocytogenes*, *Salmonella typhimurium* (11), and *Corynebacterium parvum* (Tarmina et al., *unpublished data*) failed to stimulate resistance to aerosol challenge of mice with virulent tubercle bacilli.

Although less potent than oil-treated BCG cell walls, the oil-treated cell walls of atypical mycobacteria of Runyon's groups I to IV had a measurable protective effect. The efficacy of cell walls from *M. kansasii* was notable (group I): 400 μg of these cell walls were as potent as 200 μg of BCG cell walls (*see* Table 1). Similarly, Larson and Wicht (5) found that viable *M. kansasii* and H37Ra given to mice by the respiratory route were equally effective against aerosol challenge with H37Rv. However, in their experiments, viable mycobacteria of groups II, III, and IV were not effective when administered by aerosol. Results of protection tests in other laboratories, experiments in which mice were inoculated with various living mycobacteria of groups I to IV and challenged intravenously with large doses of H37Rv, were strikingly similar to our results with the aerosol challenge test. Youmans et al. (15) found viable *M. kansasii* superior to viable mycobacteria of Runyon's groups II, III, and IV. Organisms of group II were found less effective than those of group III, and mycobacteria of group IV failed to enhance resistance. Siebenmann and Barbara (13), using the same test system in mice, found a similar order of vaccine potency of viable atypical mycobacteria.

This ranking of various atypical mycobacteria in order of vaccine potency has also been demonstrated in survival tests with guinea pigs by Freerksen (4), Palmer and Long (8), and Vandiviere (14). Accordingly, against subcutaneous or intraperitoneal challenge, *M. kansasii* was most effective (although less so than BCG), whereas strains of Runyon's group IV afforded the lowest degree of protection. From the results of these experiments with different host species and different methods of infection and evaluation, it is tempting to conclude that the same immunogenic factor or factors that are responsible for enhancement of resistance to infection with virulent tubercle bacilli produced by viable vaccines of mycobacteria are operative when corresponding oil-treated cell walls are used for immunization.

In this study, cell walls from *M. smegmatis* or *M. phlei* were found to coat mineral oil droplets readily (Fig. 1), but the protection afforded was less than that of BCG cell walls. In view of the results of comparative protection tests with viable typical and atypical mycobacteria discussed above and with oil-treated cell walls of such mycobacteria reported in this paper, it would appear that the lower vaccine potency of cell walls from atypical mycobacteria is due to lower inherent immunogenicity of the cell walls rather than to insufficient coating of the oil droplets. Whether this proposed common protective factor is of the same chemical nature and present in different quantities in various mycobacterial species or whether there exists a system of chemically related factors which differ in potency has yet to to be determined.

The determination of whether lungs do or do not contain grossly visible tubercles is not very subjective when an adequate dose of cell walls

is given (*see* Fig. 2). However, difficulties in reading the macroscopic result may arise when less active vaccines or low doses are given, since, in these cases, only a few tubercles close to 0.5 mm in diameter may develop.

The lungs and spleens of the majority of mice which had been inoculated with 150-μg doses of oil-treated cell walls from the virulent strain Bovinus I (Table 3) did not contain detectable numbers of viable H37Rv 1 month after aerosol challenge. The question arises whether "absolute immunity" was achieved; i.e., whether the estimated 30 to 50 invading H37Rv cells which reached the lung tissue had been destroyed. Preliminary results of a current test designed to study the duration of immunity achieved with this particular vaccine revealed the presence of H37Rv cells in lungs and spleens 4 months after aerosol challenge. It may well be that our culture technique was insufficient to detect the few bacilli in the lung tissue 30 days after challenge. It is also conceivable that some of the invading bacilli had survived in other organs in which the defense (immune response) was less effective, thus enabling multiplication, followed by redissemination into spleen and lung tissue, to occur.

In the search for a more effective nonliving prophylactic against tuberculosis, the finding that oil-treated cell walls from the virulent strain Bovinus I had greater protective potency than did oil-treated BCG cell walls or viable attenuated mycobacteria was encouraging. The hope for practical use of such a vaccine, however, depends upon the outcome of efforts to eliminate the excessive granulomatous response (1, 2) without impairment of the ability to enhance resistance to tuberculous infection.

LITERATURE CITED

1. Anacker, R. L., W. R. Barclay, W. Brehmer, C. L. Larson, and E. Ribi. 1967. Duration of immunity to tuberculosis in mice vaccinated intravenously with oil-treated cell walls of *Mycobacterium bovis* strain BCG. J. Immunol. **98**:1265–1273.
2. Barclay, W. R., R. Anacker, W. Brehmer, and E. Ribi. 1967. Effects of oil-treated mycobacterial cell walls on the organs of mice. J. Bacteriol. **94**:1736–1745.
3. 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.
4. Freerksen, E. 1959. Der Superinfektionsschutz bei der Tuberkulose. Deut. Med. Wochschr. **84**:1533–1540, 1617–1619.
5. Larson, C. L., and W. C. Wicht. 1963. Resistance to infection with virulent tubercle bacilli in mice immunized with viable *Mycobacterium balnei* and unclassified mycobacteria administered aerogenically. Am. Rev. Respirat. Diseases **88**:456–461.
6. Middlebrook, G. 1952. An apparatus for airborne infection of mice. Proc. Soc. Exptl. Biol. Med. **80**:105–110.
7. Millman, I. 1961. Nonspecific resistance to tuberculosis. Am. Rev. Respirat. Diseases **83**:668–675.
8. Palmer, C. E., and M. W. Long. 1966. Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. Am. Rev. Respirat. Diseases **94**:553–568.
9. Ribi, E., R. L. Anacker, W. R. Barclay, W. Brehmer, G. Middlebrook, K. C. Milner, and D. F. Tarmina. 1968. Structure and biological function of Mycobacteria. Ann. N.Y. Acad. Sci., *in press*.
10. Ribi, E., R. L. Anacker, W. Brehmer, G. Goode, C. L. Larson, R. H. List, K. C. Milner, and W. C. Wicht. 1966. Factors influencing protection against experimental tuberculosis in mice by heat-stable cell wall vaccines. J. Bacteriol. **92**:869–879.
11. Ribi, E., W. Brehmer, and K. C. Milner. 1967. Specificity of resistance to tuberculosis and salmonellosis stimulated in mice by oil-treated cell walls. Proc. Soc. Exptl. Biol. Med. **124**:408–413.
12. Ribi, E., C. Larson, W. Wicht, R. List, and G. Goode. 1966. Effective nonliving vaccine against experimental tuberculosis in mice. J. Bacteriol. **91**:975–983.
13. Siebenmann, C. O., and C. Barbara. 1964. Immunologic relationships between typical and atypical mycobacteria as studied by means of the mouse protection test. Am. Rev. Respirat. Diseases **89**:20–28.
14. Vandiviere, H. M. 1968. Antituberculosis vaccines other than BCG. Bull. Intern. Union Tuberc., *in press*.
15. Youmans, G. P., R. C. Parlett, and A. S. Youmans. 1961. The significance of the response of mice to immunization with viable unclassified mycobacteria. Am. Rev. Respirat. Diseases **83**:903–905.