Effect of Mycobacterial Cell Components upon Susceptibility of Mice to Infection with *M. tuberculosis*.* (30616)

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We have reported upon the immunizing activity of a number of mycobacterial cell components obtained by differential centrifugation of the mass resulting from the mechanical rupture of viable cells of the attenuated H37Ra strain of *Mycobacterium tuberculosis* (1-6). In these studies the immune response of the mice was, in most cases, tested 4 weeks after vaccination. The major immunizing effect was obtained with a particulate fraction of intracellular origin and the immunizing substance now has been localized in the ribosomal subfraction(6).

It is well recognized that viable attenuated mycobacterial cells are highly effective immunizing agents against tuberculosis both in experimental animals and in man, when exposure to virulent tubercle bacilli occurs some days or weeks following vaccination; heat-killed attenuated mycobacterial cells are far less effective(7,8).

Recently, however, we have shown that when virulent tubercle bacilli are mixed with 2 to 4 times as many viable attenuated mycobacterial cells and the 2 injected intravenously simultaneously into mice, resistance to infection by the virulent cells is greatly reduced(9). In fact, mixing and simultaneous injection is not necessary, either the attenuated or the virulent strain can be injected intravenously, and, providing the cells of the other strain are injected within about 10 minutes, the same marked decrease in resistance will be manifest. Heat-killed attenuated mycobacterial cells are far less effective for the elicitation of this phenomenon(9). Schaedler and Dubos(10) noted a reduction in resistance of mice to infection with *Staphylococcus aureus* and *Mycobacterium fortuitum* when heat-killed mycobacterial cells, or a methanol extract of mycobacteria, was injected simultaneous with, or just before, the infecting agent.

Subsequent investigations have revealed that lipopolysaccharide extracted from *E. coli* when mixed with virulent tubercle bacilli and injected into mice did not result in increased susceptibility to tuberculous infection making it unlikely that a similar substance in attenuated mycobacterial cells might be responsible (11).

We have attempted to define further the nature of the mycobacterial resistance lowering factor (MRLF) by mixing virulent tubercle bacilli with the components obtained by differential centrifugation from viable attenuated cells ruptured by mechanical means, and injecting these mixtures intravenously into mice. This paper reports the results of this study.

Methods and materials. The attenuated H37Ra, and the virulent H37Rv mycobacterial strains were employed. These were cultivated at 37°C as pellicles on a modified Proskauer and Beck medium and the cells harvested 2 to 3 weeks after inoculation. The composition of the culture medium and the methods of preparation and standardization of the cell suspensions have been published(12).

The methods used for obtaining the fractions of the cells of the H37Ra strain also have been published in detail(3-6). Briefly, however, viable attenuated mycobacteria were ruptured in 0.44 M sucrose—3×10^{-2} M $MgCl_2$—2×10^{-4} M phosphate buffer solution in cold French pressure cells at between 15,000 and 17,000 psi. The broken cell mass was centrifuged at 26,390 $\times$ *g* for 15 minutes. This removed most of the unbroken cells and cell walls. The supernatant was then centrifuged at 46,900 $\times$ *g* for 15 minutes, the scant sediment discarded, and the supernatant recentrifuged at 144,700 $\times$ *g* for 3 hours. These pellets constituted the cell-free particulate fraction (A). The particulate fraction was resuspended in the sucrose-$MgCl_2$-phosphate buffer solution. The resuspended

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TABLE I. Effect of Mycobacterial Cell Fractions upon Susceptibility of Mice to Infection with *M. tuberculosis.*

	Whole cells and fractions inj*	Amt (mg)†	No. of mice	Median survival time in days (95% confidence limits)	% Reduction of median survival time
1.	H37Rv	1	79	17.5 (17.0-18.0)	
2.	H37Rv + H37Ra	1 4	81	11.5 (11.1-11.9)	34
3.	H37Rv + A	1 4	79	17.0 (16.5-17.5)	3
4.	H37Rv + B	1 4	78	17.0 (16.6-17.4)	3
5.	H37Rv + C	1 4	30	16.0 (15.4-16.5)	9
6.	H37Rv + D	1 4	30	16.0 (15.4-16.5)	9
7.	H37Rv + E	1 4	75	18.0 (17.4-18.5)	0
8.	H37Rv + B + C	1 2 2	29	16.0 (15.3-16.7)	9
9.	H37Rv + B + D	1 2 2	29	17.0 (16.4-17.6)	3
10.	H37Rv + C + D	1 2 2	30	17.0 (15.8-18.2)	3

* A = particulate fraction; B = 20,360 × *g* fraction; C = 56,550 × *g* fraction; D = 144,000 × *g* (ribosomal) fraction; E = cell walls.
† Moist weight.

particulate fraction was fractionated by centrifuging first at 20,360 × *g* for 1 hour to obtain a cytoplasmic membrane fraction (B); the supernatant fluid from this was centrifuged at 56,550 × *g* for 1 hour to obtain the large (70-180 mμ) enzymatically active particles as described by Brodie and Gray (13), (C); and the supernatant fluid from this was centrifuged at 144,000 × *g* for 3 hours to sediment the ribosomal fraction (D). These fractions were resuspended in 0.01 molar phosphate buffer solution following each centrifugation and mixed with the virulent H37Rv cells just prior to injection into the mice. All procedures were done at 0-4°C.

Mycobacterial cell walls were prepared from the residue obtained following the first centrifugation (26,390 × *g* by repeated washing as described by Kanai and Youmans(1) fraction (E).

Resistance of the mice to infection was measured by keeping a record of the day of death of each mouse and then calculating the median survival time and the significance of differences using the methods recommended by Litchfield(14).

Results. Several experiments were performed in which the various mycobacterial fractions were mixed, either alone or in combination, with 1 mg of the virulent H37Rv strain and the mixture injected intravenously into mice. In none of the experiments was there convincing evidence that any of the fractions contributed significantly to the decreased resistance to infection produced by the viable cells from which the fractions were prepared. Since these experiments differed only in that they were conducted at different times, the data have been pooled in Table I. Although some of the mice injected with the fractions and mixtures showed at the 5% level a significantly reduced median survival time, in no case did the decrease approximate that produced by viable cells, leaving, therefore, the reality of the differences in doubt.

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TABLE II. Effect of Mycobacterial Cell Fractions upon Susceptibility of Mice to Infection with *M. tuberculosis.*

	Whole cells and fractions inj*	Amt (mg)†	No. of mice	Median survival time in days (95% confidence limits)	% Reduction of median survival time
1.	H37Rv	3‡	30	7.0 (6.1-8.1)	
2.	H37Rv + H37Ra	3 4	24	4.0 (3.4-4.7)	43
3.	H37Rv + A	3 4	29	4.8 (4.4-5.3)	32
4.	H37Rv + B	3 4	29	7.8 (7.3-8.3)	0
5.	H37Rv + C	3 4	30	6.6 (5.8-7.6)	6
6.	H37Rv + D	3 4	30	8.0 (7.3-8.8)	0
7.	H37Rv + E	3 4	30	7.0 (6.4-7.6)	0
8.	H37Rv + B + C	3 2 2	30	4.2 (3.9-4.5)	40
9.	H37Rv + B + D	3 2 2	28	4.2 (3.8-4.7)	40
10.	H37Rv + C + D	3 2 2	30	4.7 (4.3-5.1)	33

* A = particulate fraction; B = 20,360 × *g* fraction; C = 56,550 × *g* fraction; D = 144,000 × *g* (ribosomal) fraction; E = cell walls.
† Moist weight.
‡ Estimated.

experiment the mice received more than the 1 mg dose of H37Rv cells that had been planned. Although the exact infecting dose is unknown the value of the data is in no way impaired, since the important consideration is the degree to which the agents produced a deviation in the median survival time from that produced by the infecting agent alone. As a matter of fact, since there is a linear relationship between the median survival time and the logarithm of the infecting dose(15), it is possible to estimate that each mouse received approximately 3 mg H37Rv cells. The data from this experiment are shown in Table II. One of the agents, the particulate fraction, and all of the combinations of fractions, produced a reduction in median survival time which did not differ significantly from that produced by the viable cells. Particulate fraction is a mixture of fractions B, C, and D, therefore, its effectiveness when used alone would be expected.

Another experiment was performed in which a measured 4 mg infecting dose of the H37Rv strain of *M. tuberculosis* was used. It was known that this would give an infection of even shorter duration than that seen in the previous experiment. These results are shown in Table III and provide general confirmation. As before, the viable H37Ra cells produced approximately the same degree of reduction in survival as did the particulate fraction. The B fraction appeared to reduce the median survival time but statistically, this survival time does not differ significantly from that produced by either the H37Rv alone or the H37Rv plus viable H37Ra cells. Also, this same fraction in combination with fraction D did not significantly decrease resistance to infection in this experiment.

Discussion. These results show that the mycobacterial resistance lowering factor is located among the intracellular components of the mycobacterial cell. The active particulate fraction consists of a mixture of cytoplasmic membrane fragments, larger enzy-

TABLE III. Effect of Mycobacterial Cell Fractions upon Susceptibility of Mice to Infection with *M. tuberculosis.*

	Whole cells and fractions inj*	Amt (mg)†	No. of mice	Median survival time in days (95% confidence limits)	% Reduction of median survival time
1.	H37Rv	4	30	4.3 (3.9-4.6)	
2.	H37Rv + H37Ra	4 4	27	2.7 (2.0-3.6)	37
3.	H37Rv + A	4 4	30	3.3 (3.0-3.7)	23
4.	H37Rv + B	4 4	25	3.5 (2.9-4.2)	19
5.	H37Rv + C	4 4	27	4.0 (3.7-4.3)	7
6.	H37Rv + D	4 4	29	5.2 (4.6-5.8)	0
7.	H37Rv + E	4 4	30	4.1 (3.8-4.5)	5
8.	H37Rv + B + C	4 2 2	30	3.2 (2.8-3.5)	26
9.	H37Rv + B + D	4 2 2	30	4.0 (3.6-4.5)	7
10.	H37Rv + C + D	4 2 2	28	3.0 (2.5-3.7)	30

* A = particulate fraction; B = 20,360 × *g* fraction; C = 56,550 × *g* fraction; D = 144,000 × *g* (ribosomal) fraction; E = cell walls.
† Moist weight.

matically active particles and ribosomes(12). The subfractions of the particulate fraction were, except for one instance, inactive alone but activity was restored when any 2 were combined. This last phenomenon can be accounted for by postulating at least 3 substances which must be present in order to give the resistance lowering effect, or, it can be accounted for by assuming that the state of aggregation of the particles when mixed is the essential feature. The answer must await further investigation.

In order to elicit the resistance lowering effect of the particulate fraction and of the combinations of subfractions a much larger infecting dose of *M. tuberculosis* had to be used. When viable attenuated mycobacterial cells are injected into animals they are killed and broken down at a slow exponential rate, and several weeks may elapse before all are destroyed(16). Therefore, with these cells the resistance lowering intracellular components would be released at a constant but slow rate, and would exert their effect over a longer period. However, these same intracellular substances when artificially removed from the mycobacterial cell and injected, may be very rapidly degraded by the host, and exert a resistance lowering effect for only a short period. The marked reduction in the time of the tuberculous infection which is accomplished by increasing the infecting dose of virulent cells could, possibly, permit the resistance lowering materials to act over a proportionately greater period.

It is of interest that the active resistance lowering fraction is also the fraction which we have shown contains an important immunizing constituent of attenuated mycobacterial cells. This may be purely coincidental, but, it is not unlikely that the acquired immune mechanism, whatever it may be, would be directed toward that constituent of the mycobacterial cell that was most injurious to the host. The immunizing portion of the particulate fraction has been found to reside in the ribosomal fraction but an adjuvant property of the membraneous material pres-

ent in the particulate fraction is essential for marked immunogenicity(6). Freund's incomplete adjuvant will replace the 'natural' adjuvant for the production of immunity, but attempts to determine whether Freund's incomplete adjuvant would contribute to the resistance lowering action of the ribosomal fraction have been frustrated by the fact that mice will not tolerate Freund's incomplete adjuvant given intravenously.

The way in which the mycobacterial intracellular particulate materials lower resistance is not known. Presumably the cells of the reticuloendothelial system are affected in some manner which makes them less capable of inhibiting the multiplication of virulent cells. This action differs from that of the endotoxins obtained from Gram negative bacteria since *E. coli* lipopolysaccharide under these conditions will not lower resistance of mice to tuberculous infection(11).

Summary. When virulent mycobacteria are mixed with large numbers of viable attenuated mycobacterial cells and injected intravenously into mice the susceptibility to tuberculous infection is markedly increased. By mixing different mycobacterial components, prepared by differential centrifugation of mechanically ruptured attenuated mycobacterial cells, and injecting these into mice, the resistance lowering factor was localized in the intracellular particulate material which sedimented at 144,000 $\times$ *g*. Subfractions, prepared by differential centrifugation, of this particulate fraction, were usually inactive but gained resistance lowering activity when any two were recombined. The particulate fraction is composed of cell membranes, enzymatically active particles and ribosomes.

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Detection of Gonococcal Antibody.* (30617)

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Since asymptomatic gonorrhea is a continuing public health problem, especially in females, a serologic test readily applicable to large-scale detection of the disease could be a useful adjunct to presently available culture and fluorescent antibody (FA) procedures. Serologic procedures currently available for detection of gonococcal antibody appear to be nonspecific, especially with respect to meningococcal antibody and/or insufficiently correlated with the presence or absence of human

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