THE IMMUNIZING ACTIVITY AGAINST TUBERCULOUS INFECTION IN MICE OF ENZYMATICALLY ACTIVE PARTICLES ISOLATED FROM EXTRACTS OF *MYCOBACTERIUM TUBERCULOSIS*[1]

GUY P. YOUMANS, IRVING MILLMAN, AND ANNE S. YOUMANS

Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois

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It long has been recognized that an appreciable degree of acquired immunity to tuberculosis can be engendered in experimental animals with vaccines composed of living virulent, living attenuated, or living avirulent tubercle bacilli (Rich, 1951; Swedberg, 1951; Dubos *et al.*, 1953; Raffel, 1953). A much smaller amount of immunity also can be produced by injection of the same organisms killed by heat or chemicals (Rich, 1951; Swedberg, 1951; Solotorovsky *et al.*, 1951; Dubos *et al.*, 1953). However, none of the components or fractions prepared from tubercle bacilli has been found to have an immunogenic activity which approximates that produced with whole living cells. As pointed out by Rich (1951) knowledge of the nature of the immunizing substance would not only be of great importance in prophylactic immunization but would be of equal importance for the light it would shed upon the mechanism of acquired resistance to tuberculosis.

Hypothetically, inability of an extractable component of the tubercle bacillus to stimulate the establishment of appreciable immunity can be accounted for in several ways. For example, immunogenic activity may be dependent upon the integrity of some antigenic complex in the tubercle bacillus which is destroyed by even mild chemical manipulation. The much greater immunizing potency of living tubercle bacilli, suggests that the production of an immune reaction may depend upon the elaboration of an immunizing substance *in vivo* by metabolizing mycobacterial cells.

Recent work in this laboratory has resulted in the isolation from the avirulent H37Ra strain of *Mycobacterium tuberculosis* var. *hominis* of a red fraction containing very small enzymatically active non-acid fast particles which possess many of the characteristics of mitochondria (Millman and Youmans, 1955). It will be shown in the present report that this fraction containing these enzymatically active particles and another fraction containing similar but smaller enzymatically active particles were the only fractions obtained from mycobacterial cells capable of immunizing mice to subsequent infection with a highly virulent strain of *M. tuberculosis* var. *hominis*. Furthermore, the degree of immunity produced was comparable to that produced by whole living cells of the same culture from which the particles were isolated.

METHODS

Extracts of the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* were prepared from 20- to 30-day-old cultures grown on the surface of modified Proskauer and Beck medium (Youmans and Karlson, 1947). One gram (dry weight) of microorganisms was ground at 4 C for 18 hours in a ball mill with powdered glass in a solution of 0.25 M sucrose dissolved in 0.01 M phosphate buffer, pH 7.0. The details of this procedure have been published elsewhere (Millman and Youmans, 1955). Table 1 gives the details of the subsequent treatment of the ground mycobacterial cells.

Survival of mice following injection of the various fractions of tubercle bacilli was tested by a procedure which had previously been developed for determining the activity of chemotherapeutic agents (Youmans and Youmans, 1951). Inbred mice of the Strong A strain weighing 18 to 22 g were distributed randomly 10 to a cage. Twenty mice then were injected intraperitoneally with each immunizing agent contained in a volume of from 0.2 to 0.5 ml. The twenty normal controls were distributed into cages at the same time. Twenty-eight days later all mice were challenged intravenously with 1.0 mg wet weight of the virulent H37Rv strain of *M. tuberculosis* var. *hominis*. The mice were examined and deaths recorded daily. Each mouse was autopsied and

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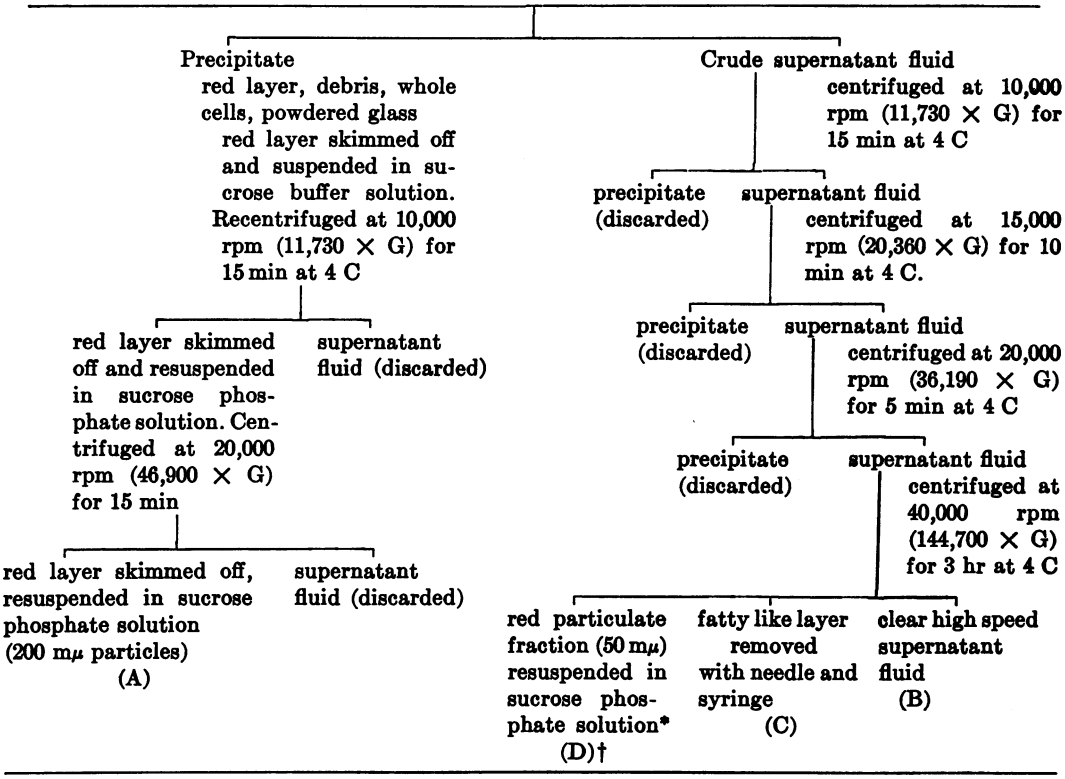
TABLE 1

The thick paste obtained after grinding was diluted with sucrose buffer solution* and centrifuged at 5000 rpm for one hour



* In some experiments only 0.25 M sucrose (no electrolyte) was employed.

† Resuspended and washed 3 times with 0.25 M sucrose solution with or without phosphate buffer by centrifugation at 40,000 (144,700 × G) for 3 hours.

the presence or absence of characteristic pulmonary lesions recorded. This step is essential, since nontuberculous deaths, unless detected, affect the validity of the data.

The immune response of the mice was assessed by comparing the survival times of the immunized and nonimmunized animals. Previous experience has shown that the death of mice immunized with living and heat killed mycobacteria when subsequently challenged with 1.0 mg of the H37Rv strain lacks the uniformity previously demonstrated for nonimmunized mice (Youmans and Youmans, 1955, *unpublished data*). Instead of following a normal frequency distribution pattern over a period of about 30 days as do the deaths of the control mice, the deaths of the immunized animals are distributed over a period of 150 days or longer. Furthermore, a proportion of the mice, which in different experiments may vary from 10 to 50 per cent, die within the first 30 days whereas the remaining 50 to 90 per cent of the mice die during the next 70 to 120 days. It can be seen from this description that the distribution of deaths of the immunized mice is markedly skewed. A similar distribution of deaths of immunized mice can be noted in the data published by others (Swedberg, 1951; Bloch and Segal, 1955). Since the mice which die before 30 days do not differ appreciably in their resistance to tuberculosis from the nonimmunized controls the degree of the immune response has been assessed by noting and comparing the number of animals in each group which live longer than 30 days. A detailed analysis of the response of immunized mice to infection with virulent tubercle bacilli is in preparation and will be published later.

The number of tubercle bacilli in vaccines composed of living H37Ra cells and in the extracts, was determined by one or both of the following two methods: (a) Enumeration of viable particles by the plating and colony counting technique recommended by Fenner (1951), and (b) by direct microscopic counts. The latter were carried out by spreading 0.01 ml of the preparation to be examined over an area of 1.0 sq cm on a glass slide, by Ziehl-Nielson staining and by recording the number of organisms in several hundred oil immersion fields.

RESULTS

To date six different extracts of the H37Ra strain of *M. tuberculosis* have been divided into fractions by high speed centrifugation and the fractions tested for immunogenic activity. These fractions include the particulate red fraction obtained at relatively low centrifugal speed and composed of particles in the neighborhood of 200 mμ in diameter (Millman and Youmans, 1955) (table 1, fraction A). A clear straw-colored supernatant fluid remaining after centrifugation at 40,000 rpm (144,700 × G) for three hours (table 1, fraction B). An oily or "fat" like fraction found floating upon the supernatant following centrifugation at 40,000 rpm (144,700 × G) for three hours (table 1, fraction C). Finally, a red colored fraction composed of nonacid fast particles approximately 50 mμ in diameter obtained as a deposit following centrifugation for three hours at 40,000 rpm (144, 700 × G) (table 1, fraction D).

Except for the two particulate red fractions (A, 200 mμ, and D, 50 mμ) none of these fractions had any significant immunizing action in mice. The degrees of immunity produced by the particulate red fractions and by control injections of 1.0 mg of living H37Ra cells are compared in table 2. It is evident that the immune response obtained with five of the six particulate red fractions was either equal to or superior to that found in mice injected with the living cells of strain H37Ra.

Examination of Ziehl-Nielson stained smears revealed, however, that particulate red fraction

TABLE 2

The immunizing effect of particulate red fractions obtained from H37Ra cells against tuberculous infections in mice

Material Injected	Number of Mice			Number of Tuberlcle Bacilli in Material Injected
	Days of survival			
	<30	>30	% >30	
Red fraction A	10	10	50.0	Not counted but numerous
None	20	0	0.0	
Red fraction DA	6	10	62.5	Not counted but numerous
Living H37Ra cells	11	9	45.0	
None	13	0	0.0	
Red fraction D_1	10	9	47.4	20,000
Living H37Ra cells	11	8	42.1	
None	20	0	0.0	
Red fraction D_2	17	3	15.0	8,000
Living H37Ra cells	8	11	57.9	
None	18	2	10.0	
Red fraction D_3	9	11	55.0	40,000
Living H37Ra cells	13	5	27.77	
None	20	0	0.0	
Red fraction D_4	9	11	55.0	8,000
Living H37Ra cells	10	10	50.0	
None	20	0	0.0	

TABLE 3

The immunizing effect of different numbers of living M. tuberculosis H37Ra cells against tuberculous infections in mice

Wet Weight of Tubercle Bacilli	Number of Tubercle Bacilli by Plate Counts*	Number of Cells by Direct Microscopic Count	Number of Mice		
			Days of survival		
			<30	>30	% >30
mg					
1.0	40,660,000†	29,150,000‡	8	11	57.9
0.2		17,381,000§	12	7	36.8
0.02		1,610,000	17	3	15.0
0.002		260,000	19	1	14.5
0.0002			18	0	15.0
0.00002			19	1	13.5
0.000002			17	3	17.0
None			18	2	10.0

* The figure in this column actually represents the number of viable particles since all suspensions of tubercle bacilli contain clumps of cells in addition to single organisms.

† Standard deviation = ±6,782,000.

‡ Standard deviation = ±8,350,000.

§ Standard deviation = ±8,738,000.

A and fraction DA (mixture of fractions D and A, table 2) contained a large number of whole mycobacterial cells. These cells may have been responsible for the immunizing activity noted. Similar examination, however, revealed that fraction D_1, D_2, D_3 and D_4 prepared by fractional high speed centrifugation contained very few whole mycobacterial cells in the immunizing dose (table 2).

Table 3 reveals the results of an experiment in which the immunizing ability of small numbers of living mycobacterial cells was measured. No statistically significant degree of immunity, as indicated by the χ^2 test, was produced by the injection of approximately 1,610,000 cells. Active fractions D_1, D_3, and D_4 therefore contained too few cells to account for the high degree of immunity produced.

The inactivity of fraction D_2 may be accounted for by the fact that the particles were washed in 0.25 M sucrose solution which contained no phosphate. A subsequent experiment has revealed that omission of phosphate from the suspending fluid resulted in loss of immunogenic activity. Whether this loss of immunogenic activity was due to the absence of phosphate ion or due to the absence of buffering capacity has not been determined. Loss of immunogenic activity also has been observed following extraction of the particles with ether or acetone.

DISCUSSION

The significant feature of these results is the production in mice of an immune state, equivalent to that produced by the injection of living organisms, following the injection of a fraction of the tubercle bacillus containing too few whole cells to elicit any immunity.

In contrast to other bacterial vaccines, the immunologically active fraction consisted of a suspension of very small enzymatically active particles which were mechanically separated from the mycobacterial cells using methods and conditions carefully designed to yield cellular components which have been altered as little as possible. The results of published studies have shown that the larger particles are highly active enzymatically (Millman and Youmans, 1955). They apparently possess a full complement of the enzymes of Kreb's tricarboxylic acid cycle and a complete cytochrome system. The smaller particles obtained after prolonged centrifugation at 40,000 rpm also are active enzymatically. Detailed results of studies on the enzymatic activity of these smaller particles (50 mμ) will be published separately. In view, however, of the similarity in appearance and physiological properties and the tendency of the smaller particles to agglutinate, it seems reasonable to assume that the larger

particles (fraction A) actually consist of agglomerations of the small particles (fraction D).

There is some evidence also which suggests that the integrity of these particles and possibly the maintenance of their physiological activity may be essential for immunogenicity. Immunogenicity was lost when the particles were extracted with acetone or ether and was absent unless they were suspended in a medium containing potassium phosphate.

The possibility that immunogenicity might depend upon some substance adsorbed to the surface of these small particles also must be considered. In several experiments the washings from these particles were found to be immunologically inactive but this evidence is inconclusive since the active substance might be strongly bound. The immunogenic activity also might be related to the antigenicity of some other unidentified substance or of some complex which is destroyed when the particles disintegrate. Although no evidence bearing on these points is available at the present time it is difficult to visualize a mechanism whereby antibody specific for small intracellular structures could penetrate the bacterial cell wall in order to combine and produce an effect deleterious to the microorganism.

Actually, the immunogenicity of these small enzymatically active structures, considered in conjunction with the complete lack of any evidence that specific antibody plays a role in immunity to tuberculosis, strongly suggests that classical immune mechanisms involving antibody are not involved in immunity to tuberculosis.

Application of the immunogenicity of the particulate red fraction to the protection of human beings against tuberculosis appears somewhat remote at the present. Time will be needed first to determine methods for the preparation of these particles completely free of whole cells and in greater yield; to define the conditions under which the immunogenicity is maintained maximally for the longest period of time; and to determine the optimal immunizing dose and the allergenicity of the particles. Studies also should be conducted to determine whether similar particles from other species and varieties of mycobacteria, including the virulent human variety, might be even more suitable as immunizing agents.

Finally, even though the immunogenicity of the enzymatically active particles isolated from extracts of the H37Ra strain provides an adequate explanation for the greater immunizing power of living tubercle bacilli, as compared with tubercle bacilli killed with chemicals or by heat, we can not assume that such particles comprise the only immunogenic substance present in mycobacterial cells, especially since heat killed tubercle bacilli do produce a small but significant amount of antituberculous immunity in mice (Swedberg, 1951; Solotorovsky *et al.*, 1951; Dubos *et al* 1953).

SUMMARY

Small enzymatically active nonacid fast particles possessing many of the characteristics of mitochondria have been isolated by ultracentrifugation from extracts prepared by grinding whole living cells of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra in sucrose phosphate solution. These small enzymatically active particles when injected intraperitoneally into mice stimulated in these animals the production of a degree of immunity which was equal to that produced by a 1.0 mg immunizing dose of whole living H37Ra cells. Two other fractions, a straw-colored supernatant fluid and a "fatty" fraction, were devoid of immunogenic activity.

The significance of these findings and their relation to the problem of the nature of acquired immunity to tuberculosis is discussed.

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