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EFFECT OF MITOCHONDRIAL STABILIZERS ON THE IMMUNOGENICITY OF THE PARTICULATE FRACTION ISOLATED FROM *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

YOUMANS, ANNE S. (Northwestern University Medical School, Chicago, Ill.), AND GUY P. YOUMANS. Effect of mitochondrial stabilizers on the immunogenicity of the particulate fraction isolated from *Mycobacterium tuberculosis*. J. Bacteriol. 87:1346-1354. 1964.—A number of substances which have been used to stabilize mammalian mitochondrial preparations were tested to determine whether they would similarly affect the immunogenicity of a particulate fraction prepared from ruptured viable attenuated mycobacterial cells. The use of 0.44 M sucrose and the presence of 3×10^{-2} M $MgCl_2$ during the preparatory processes markedly increased the immunogenicity of the particulate fraction. The increase was so great that immunogenic preparations were then consistently obtained which, in adequate dosage, were more immunogenic in CF-1 male mice than were viable attenuated mycobacterial cells. On the other hand, adenosine triphosphate (ATP), citrate, and polyvinylpyrrolidone when present during the preparatory processes reduced the immunogenicity. The addition of $MgCl_2$, ethylenediaminetetraacetate, or ATP to the particulate fraction after it had been prepared did not increase its immunogenicity. When the particles were prepared in the 0.44 M sucrose buffer alone, incorporated in Freund's adjuvant, and injected intraperitoneally, immunogenicity was increased. However, this increase was not significantly greater than that obtained when the particles were prepared in the sucrose buffer containing $MgCl_2$. The immune state engendered in mice by the intraperitoneal injection of the particulate fraction persisted for at least 12 weeks.

Youmans, Millman, and Youmans (1955), Youmans, Youmans, and Millman (1957), and Kanai and Youmans (1960) reported that a labile immunogenic particulate fraction could be obtained by high-speed centrifugation from ruptured viable mycobacterial cells. Initially (Youmans et al., 1955; Millman and Darter, 1956; Darter and Millman, 1957), the enzymatic activity of these particles suggested that they might be the bacterial equivalents of mammalian mitochondria. Later, Youmans and Youmans (1964), because of the difficulty which had been encountered in obtaining uniformly active immunogenic preparations, investigated the stability of the immunogenic property under a variety of conditions.

In general, the capacity to elicit an immune response to severe pulmonary tuberculous infection in mice was adversely affected by the same conditions which have been found to affect adversely the structure, and therefore function, of mammalian mitochondria. For example, the effect of temperature on the immunogenic activity of the particulate fraction was marked. As the temperature was raised above 4 C, the immunogenic activity was inactivated irreversibly. Furthermore, this inverse relationship between temperature and immunogenic activity was linear. Slater and Cleland (1952) found that 52% of the α-ketoglutaric oxidase activity from rat heart muscle sarcosomes was destroyed at 15.5 C and 94% at 25 C, indicating the lability of these structures. Later these same investigators (Cleland and Slater, 1953*a*) observed that sarcosomes spontaneously transformed 15 times faster at 17 C than at 2 C. Moreover, mammalian mitochondria, if allowed to stand at 25, 30, or 37 C in solutions which do not contain stabilizing compounds, become swollen within a few minutes, resulting in damage to the mitochondrial structure (Harman and Kitiyakara, 1955; Lipsett and Corwin, 1959; Tapley, 1956; Lehninger and Remmert, 1959; Lehninger, 1959; Kaltenbach and Harman, 1955; Cleland, 1952; Ernster and Löw, 1955).

The immunogenic activity of the particulate fraction also was decreased by changes in the hydrogen ion concentration from the optimal pH of 6.8 to 7.0 (Youmans and Youmans, 1964). This decrease was irreversible, since neutraliza-

tion of the more acid or alkaline preparations did not restore activity. The particulate fraction also tended to shift the pH toward the acid side, especially in the most alkaline solutions. Harman and Kitiyakara (1955) observed that mitochondria prepared from pigeon skeletal muscle also tended to shift the more alkaline solutions (pH 9.0) toward neutrality and acted, therefore, somewhat like buffers. In addition, the size and structure of the mitochondria changed according to the pH of the medium; the change in size was partly reversible, but the change in shape was irreversible. Cleland (1952) found that the hydrogen ion concentration had a pronounced effect on permeability of sarcosomes to KCl.

Other conditions which adversely affect the immunizing potency of the particulate fraction (Youmans and Youmans, 1964) also adversely affect the biochemical or cytological characteristics of mammalian mitochondria; for example, (i) the presence of the detergent sodium lauryl sulfate (Frederic, 1954), (ii) the use of a Waring Blendor (Harman and Kitiyakara, 1955; Kielley and Kielley, 1953; Dounce, 1955), (iii) dialysis against hypotonic solutions, and (iv) a delay in the handling of the ruptured-cell preparations (Lehninger, 1959; Dounce et al., 1955). In addition, Brodie and Gray (1957) isolated particles, which they felt resembled mammalian mitochondria, from broken *Mycobacterium phlei* cells. The enzymatic activity of these structures was adversely affected by many of the same conditions which reduced the immunogenic potency of the particulate fraction.

The question of whether microorganisms contain intracytoplasmic structures similar to mitochondria has not been resolved. Some investigators (Marr, 1960; Mitchell, 1959; Mitchell and Moyle, 1951, 1956; Abrams and McNamara, 1962; Hughes, 1962; Stanier, 1954) reported that the particulate fractions obtained from microorganisms appear to be derived from the plasma (cellular) membrane, and that microorganisms probably do not have structural units similar to mammalian mitochondria. However, others (Shinohara, Fukushi, and Suzuki, 1957; Toda et al., 1957; Chapman, Hanks, and Wallace, 1959; Sato, 1963; Mudd, 1956) have visualized by electron microscopy intracellular structures which they think may represent the equivalent of mitochondria. More recently, Novikoff (1961) and Mudd (1962) suggested a relationship between cytoplasmic granules and the plasma membrane.

Regardless of the nature of the particles found in the immunizing fraction, the similar lability of the particulate fraction and of mammalian mitochondria under a variety of conditions suggested that agents which were used to stabilize mitochondrial preparations might have a similar action on the immunogenic property of the particulate fraction.

MATERIALS AND METHODS

The methods employed in this study were similar to those described previously (Youmans and Youmans, 1964); however, some modifications in the preparation of the particulate fraction were made to take advantage of information obtained during the course of the present study. Most of these will be described in Results.

Briefly, in all the experiments the immunizing particulate fraction was prepared from the attenuated H37Ra strain of *Mycobacterium tuberculosis*. The cells were grown, collected at 3 weeks, and ruptured in cold (0 to 4 C) French pressure cells as described (Youmans and Youmans, 1964). However, in the present experiments the pressure in the pressure cells was increased and maintained between 13,000 and 16,000 psi, at which level it was found that 90 to 100% of the tubercle bacilli were broken. Pressures higher than this appeared to give a lower yield of the particulate fraction. Whether the particles were solubilized or were broken into such small fragments that they remained suspended in the supernatant fluid after 3 hr of centrifugation at 144,000 $\times$ g is not known.

The ropey cellular material was ejected from the pressure cells through a short "carrying" tube into a small beaker containing 5 to 10 ml of sucrose-buffer slush. Chopped ice surrounded the beaker. The broken cellular mass was poured into another chilled beaker, also in an ice bath, and kept in a refrigerator until time of centrifugation. Before centrifugation, which was started as soon as possible, the broken cellular mass was stirred gently with a glass rod. All centrifugations were done in a model L Spinco ultracentrifuge at temperatures between 0 and 4 C.

It was found that with the improved breakage of whole cells fewer low-speed centrifugations were necessary to free the material of whole cells. The first centrifugation, therefore, was done at 15,000 rev/min (26,390 $\times$ g) for 30 min. An acid-

fast stain of this sediment revealed pale-blue broken cell walls, deeper blue granules, and any acid-fast bacilli which might be present. The supernatant fluid then was centrifuged at 20,000 rev/min (46,900 × *g*) for 15 min, and an acid-fast stain of a smear from this sediment showed no whole cells, and only a very faint blue background. The supernatant fluid then was finally centrifuged at 40,000 rev/min (144,000 × *g*) for 3 hr. This supernatant fluid was discarded, and the small yellow gelatinous pellet was weighed, collected, and diluted with 0.01 M phosphate buffer (pH 7.0) as described earlier (Kanai and Youmans, 1960; Youmans and Youmans, 1964) so that each ml contained 100 mg of particulate fraction. Acid-fast stains of smears made from this material showed a blue rather homogeneous background and no intact organisms. Thus, the time necessary to prepare the particulate fraction has been reduced greatly.

The methods used in the experiments employing mice were the same as previously reported (Youmans and Youmans, 1964) except that CF-1 mice instead of Strong A mice were used. The reason for this change was that, even though both strains of mice responded to immunization equally well with the particulate fraction, the CF-1 strain developed an appreciably higher degree of immunity when immunized with viable attenuated cells (Youmans, Youmans, and Kanai, 1959; Kanai and Youmans, 1960). It was felt, therefore, that the use of CF-1 mice would constitute a more sensitive and critical evaluation of the immunizing capacity of the particulate material. An equivalent response to immunization with viable cells and an immunizing fraction in CF-1 mice would be of much greater significance than an equivalent response to the two agents in Strong A mice. The method used for the evaluation of the immune response in the CF-1 mice was the same as was given in detail by Youmans and Youmans (1957). For each vaccine preparation, 30 or 40 CF-1 male mice were vaccinated intraperitoneally, except as otherwise noted. After 4 weeks, the mice were challenged intravenously with 1.0 mg of a fine suspension of the highly virulent strain, H37Rv, of *M. tuberculosis*. Those mice which survived for 30 days were considered to be immune. In each experiment, control mice were vaccinated with 1.0 mg of a suspension of the H37Ra cells from which the particulate fraction was made. All vaccines were kept cold and were handled as described before (Youmans and Youmans, 1964).

Statistically, the significance of differences between groups of mice was calculated by use of the chi-square test.

TABLE 1. *Immunogenicity of particulate fraction prepared in different sucrose buffers*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Sucrose buffer (0.25 M)	20.0	271	102	37.6
	5.0	224	44	19.6
Sucrose buffer (0.44 M)	20.0	701	372	53.1
	5.0	667	224	33.6
Sucrose buffer (0.44 M)	20.0	139	66	47.5
plus PVP† (7.3%)	5.0	87	27	31.0
Sucrose buffer (0.88 M)	20.0	151	71	47.0
	5.0	156	55	35.3
H37Ra cells	1.0	897	579	64.5
None	0.0	909	59	6.5

* S-30 = number of mice which survived > 30 days.

† PVP = polyvinylpyrrolidone.

RESULTS

In the past, 0.25 M sucrose solutions were made in 2 × 10^{-4} M phosphate buffer (pH 7.0), and the cells were broken in this solution. However, the immunogenic activity was found to vary widely from time to time and was not as high as that obtained with whole cells in CF-1 mice (Kanai and Youmans, 1960; Youmans et al., 1959). Therefore, higher concentrations of sucrose and 7.3% polyvinylpyrrolidone (PVP; Novikoff, 1956) were added to the 2 × 10^{-4} M phosphate buffer (pH 7.0) to determine whether more active and stable immunogenic preparations could be obtained (Table 1). The number of mice varied for each vaccine preparation because the data were pooled from different experiments in which a given substance was used.

The vaccine prepared in the 0.44 M sucrose buffer protected a significantly ($p = <0.001$) greater number of mice than did that prepared in the 0.25 M sucrose buffer. The higher concentration of sucrose, therefore, was used in all subsequent experiments. The use of 0.88 M sucrose or the addition of PVP to the 0.44 M sucrose buffer did not increase the immunogenicity of the particles over that obtained with the 0.44 M sucrose solution alone.

Since 1.0 mg of whole cells still immunized

CF-1 mice to a significantly ($p = <0.001$) greater degree than did 20.0 mg of the particulate fraction, the possibility existed that the cell wall of the whole cells might have an adjuvant or protective effect on the labile immunogenic material present inside the cell, thereby permitting the production of a higher degree of immunity. The effect of an adjuvant on the particulate fraction, therefore, was tried to determine whether it might increase the immunogenicity of the particles or whether it would inactivate the labile immunogenic substance.

Particulate fraction was prepared in the 0.44 M sucrose buffer as usual, but was diluted so that 1 ml contained 200.0 mg instead of 100.0 mg. It was incorporated then into Freund's adjuvant (two parts mineral oil, one part aquaphor, one part particulate fraction). Because of the oily nature of the adjuvant, the materials were at room temperature when mixed together in a mortar with a pestle, and when injected into the mice; however, at all other times the vaccine mixture was kept at 0 to 4 C.

The pooled results of two such experiments are shown in Table 2. Freund's adjuvant not only did not decrease immunogenicity, but it significantly ($p = <0.005$) increased the immunogenic activity of the particulate fraction. A 20-mg amount of this preparation protected mice as well as did 1.0 mg of the viable whole cells; however, on a quantitative basis whole cells still were immunogenically more active.

Magnesium has been found to help stabilize preparations of mammalian mitochondria (Harman and Kitiyakara, 1955; Lipsett and Corwin, 1959; Tapley, 1956; Ernster and Löw, 1955); therefore, the effect of magnesium ions on the stability of the immunogenic moiety in the particulate fraction was investigated. Three concentrations of $MgCl_2$ were used (3×10^{-2}, 10^{-3}, and 2×10^{-4} M) in the basic 0.44 M sucrose buffer. A portion of cells was broken in each solution, and also in each experiment a portion of cells was ruptured in the 0.44 M sucrose buffer which did not contain $MgCl_2$. The particulate fractions were prepared as usual; pooled data from several experiments are shown in Table 3.

It is evident from Table 3 that 20.0 mg of the particulate fraction prepared in the sucrose buffer containing 3×10^{-2} M $MgCl_2$ possessed a significantly ($p = <0.001$) higher degree of immunogenicity than was obtained with whole cells or with the particulate fraction prepared in the 0.44 M sucrose buffer alone. The immunity produced was also higher ($p = 0.05$) than that obtained with the 2×10^{-4} M $MgCl_2$ sucrose preparation and that ($p = <0.001$) obtained with the 10^{-3} M $MgCl_2$ sucrose preparation. Although the presence of magnesium did not increase significantly the immune response obtained with the 5.0-mg vaccinating dose, it did increase ($p = <0.001$) the response in mice vaccinated with 1.0 mg of the particulate fraction.

The data obtained with a 1.0-mg dose prepared in the magnesium sucrose buffer are especially interesting, in that the addition of magnesium ions increased the immunogenicity of the 1.0-mg vaccine dose in three of four experiments. The percentages of survival of the mice were 71.4,

TABLE 2. *Effect of Freund's adjuvant on the immunogenicity of particulate fraction*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Particulate fraction	20.0	60	32	53.3
	5.0	87	32	36.8
Particulate fraction in Freund's adjuvant	20.0	57	45	78.9
	5.0	86	51	59.3
H37Ra cells	1.0	58	41	70.7
None	0.0	49	8	16.3

* S-30 = number of mice which survived > 30 days.

TABLE 3. *Effect of magnesium ions on the immunogenicity of particulate fraction*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Sucrose (0.44 M)	20.0	300	156	52.0
	5.0	309	117	37.9
	1.0	114	13	11.4
Sucrose (0.44 M) plus $MgCl_2$ (3×10^{-2} M)	20.0	349	245	70.2
	5.0	136	63	46.3
	1.0	114	40	35.1
Sucrose (0.44 M) plus $MgCl_2$ (10^{-3} M)	20.0	113	57	50.4
	5.0	116	31	26.7
Sucrose (0.44 M) plus $MgCl_2$ (2×10^{-4} M)	20.0	93	56	60.2
	5.0	83	37	44.6
H37Ra cells	1.0	580	324	55.9
None	—	595	39	6.6

* S-30 = number of mice which survived > 30 days.

TABLE 4. *Immunogenic effect of 20.0 mg of particulate fraction prepared in 0.44 M sucrose plus 3 × 10^{-2} M $MgCl_2$, and of 1.0 mg of H37Ra cells*

Date of vaccination	Particulate fraction			H37Ra cells			Controls		
	No. of mice	No. of S-30 mice*	Percentage of S-30 mice	No. of mice	No. of S-30 mice	Percentage of S-30 mice	No. of mice	No. of S-30 mice	Percentage of S-30 mice
4-19-63	29	20	69.0	29	23	79.3	30	2	6.7
5- 3-63	25	13	52.0	30	7	23.3	30	0	0.0
6-14-63	30	19	63.3	28	3	10.7	28	0	0.0
6-21-63	25	16	64.0	29	12	41.4	30	1	3.3
7-12-63	38	21	55.3	28	14	50.0	29	0	0.0
7-19-63	29	23	79.3	29	21	72.4	29	1	3.4
8- 9-63	28	21	75.0	28	21	75.0	28	1	3.4
8-16-63	30	16	53.3	29	18	62.0	30	2	6.6
8-30-63	28	26	92.9	28	20	71.4	30	1	3.3
9- 4-63	29	23	79.3	29	10	34.5	29	0	0.0
9-11-63	29	22	75.9	29	22	75.9	28	1	3.6
9-18-63	29	25	86.2	30	26	86.6	30	3	10.0
Total	349	245	70.2	346	197	56.9	351	12	3.4

* S-30 = number of mice which survived > 30 days.

TABLE 5. *Effect of ATP and magnesium on the immunogenicity of particulate fraction*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Prepared in 0.44 M sucrose	20.0	96	45	46.8
	5.0	93	28	30.1
$MgCl_2$ (10^{-3} M) added	20.0	86	46	53.4
	5.0	86	30	34.8
$MgCl_2$ (10^{-3} M) and ATP (0.015 M) added	20.0	96	39	40.6
	5.0	96	34	35.4
H37Ra cells	1.0	95	58	61.0
None	0.0	98	6	6.1

* S-30 mice = number of mice which survived > 30 days.

26.7, 6.9, and 37.0. In the same experiments, the percentages of mice vaccinated with the 20.0-mg dose which survived for 30 days were 92.9, 79.3, 75.9, and 86.2; therefore, there was a rough correlation between the 20.0- and 1.0-mg doses. Far larger differences occurred with the 1.0-mg dose than with the 20.0-mg dose. This suggests that the 1.0-mg dose may be a more sensitive indicator now than the 20.0-mg dose for the determination of the effect of compounds on the stability of the immunogenic substance.

Currently, 3 × 10^{-2} M $MgCl_2$ is added routinely to the 0.44 M sucrose buffer; Table 4 gives the results obtained in the last 12 experiments. These data are given not only to show that consistently active preparations of the particulate fraction can be obtained but that the results were more uniform in the mice vaccinated with the particulate fractions (coefficient variation = 18.73%) prepared in the magnesium and sucrose buffer than were those obtained with the whole-cell vaccines (coefficient variation = 56.53%). Also, from this table a correlation could be made between the activity of the particulate fraction and the whole cells from which it was made. An analysis of these data shows a moderately high (+0.69) linear correlation between the immunogenic activity of the two vaccines. The immunogenic activity of the whole cells, therefore, probably does, in part, influence the activity of the particulate fraction made from those cells.

Adenosine triphosphate (ATP) and magnesium ions were examined for their combined effect on the immunogenic activity of the particulate fraction, since these two substances have been found to affect favorably the stability of mammalian mitochondria (Cleland and Slater, 1953*a*; Tapley, 1956; Lehninger, 1959; Ernster and Löw, 1955). The pooled results of three experiments obtained in mice vaccinated with particulate fraction made with and without the addition of 0.015 M ATP are given in Table 5. The immunogenic activity of the particulate fraction was decreased ($p = 0.05$) in the presence of this concentration of ATP.

Since citrate also had been found to help

stabilize the structure of mammalian mitochondria (Cleland and Slater, 1953*a*; Lipsett and Corwin, 1959; Tapley, 1956), its effect on the immunogenic activity of the particulate fraction was determined. Citrate, in a concentration of 10^{-2} M, was added to the 0.44 M sucrose buffer, and the particulate fraction was prepared. The results of one experiment (Table 6) show that citrate in this concentration significantly ($p = 0.01$) lowered the immunogenic activity. Citrate as a chelating agent appeared to have no stabilizing effect on the immunogenicity of the particulate fraction.

In all experiments, as described in Materials and Methods, the final dilution prior to injection of the particulate fraction was made with 0.01 M phosphate buffer (pH 7.0) alone. The possibility existed that the addition to the phosphate buffer of certain compounds which have been found useful in maintaining the structure of mammalian mitochondria would result in a more stable and, therefore, more active immunogenic substance. The compounds which were added individually to the phosphate buffers were ethylenediaminetetraacetic acid (EDTA), 10^{-2} M (Cleland, 1952; Cleland and Slater, 1953*a*, *b*); ATP, 10^{-2} M; and $MgCl_2$, 3×10^{-2} M. The particulate fraction was prepared in 0.44 M sucrose-magnesium buffer, and tubes containing the pellets which were obtained after the 144,000 × *g* centrifugation were divided into groups. The pellets were collected and diluted with one of the specific buffers. Since the immunogenic activity was quite high if the mice were injected with 20.0 mg of the particulate fraction, the more sensitive test of injecting 1.0 mg of the particulate fraction also was employed. The results obtained in the mice vaccinated with these preparations are given in Table 7.

The best diluent of the particulate fraction at this time still appears to be 0.01 M phosphate buffer alone, since the greatest number of mice survived after vaccination with this preparation. The immunogenic activity was significantly greater ($p = 0.01$) than was obtained with whole cells or with $MgCl_2$ ($p = 0.005$). The vaccine diluted with phosphate buffer alone did not differ significantly from the ATP buffer preparation ($p = 0.20$), and only slightly from the EDTA buffer preparation ($p = 0.05$). Although the activity was low in the mice injected with 1.0 mg of the "phosphate buffer" particulate fraction, there was a significant difference ($p = 0.005$) between these mice and the nonvaccinated control mice. Because of these findings, particulate fraction was prepared also in a more highly buffered (0.01 M phosphate buffer) sucrose-magnesium chloride solution. The data from two experiments showed that the immunogenic activity was slightly less when the particulate fraction was prepared with the higher concentration of phosphate.

From preliminary experiments, the immunogenic activity in mice vaccinated with the particulate fraction prepared in 0.44 M sucrose buffer alone lasts at least 12 weeks, since 50% of the mice survived for 6 weeks and 56.6% survived for 12 weeks. Experiments are being done now in

TABLE 6. *Effect of citrate on the immunogenicity of the particulate fraction*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Sucrose (0.44 M) plus $MgCl_2$ (3×10^{-2} M)	20.0	38	21	55.3
Sucrose (0.44 M) plus Na citrate (10^{-2} M)	20.0	20	4	20.0
H37Ra cells	1.0	28	14	50.0
None		29	0	0.0

* S-30 mice = number of mice which survived > 30 days.

TABLE 7. *Effect of $MgCl_2$, EDTA, and ATP on the immunogenicity of particulate fraction when added to the phosphate buffer diluent prior to vaccination*

Immunizing prepn	Amt injected	No. of mice	No. of S-30 mice*	Percentage of S-30 mice
	mg			
Phosphate buffer (0.01 M)	20.0	58	45	77.6
	1.0	59	10	16.9
Phosphate buffer (0.01 M) plus $MgCl_2$ (3×10^{-2} M)	20.0	60	32	53.3
	1.0	58	7	12.1
Phosphate buffer (0.01 M) plus EDTA (10^{-2} M)	20.0	57	35	61.4
	1.0	57	3	5.3
Phosphate buffer (0.01 M) plus ATP (10^{-2} M)	20.0	57	37	64.9
	1.0	59	9	15.3
H37Ra cells	1.0	58	32	55.2
None	—	57	1	1.8

* S-30 = number of mice which survived > 30 days.

which mice have been vaccinated with the particulate fraction prepared in the magnesium chloride sucrose buffer, and these also will be challenged at intervals.

DISCUSSION

It has been shown with mammalian mitochondria that if the concentration of sucrose is much lower than 0.25 M, swelling of the mitochondrion may occur. Since moist H37Ra cells must be used in the preparation of the particulate fraction, the amount of moisture present will vary from week to week, and the final sucrose concentration of the broken cell mass might be lower than 0.25 M. It was found that by employing 0.44 M sucrose the immunogenicity of the particulate fraction was increased significantly over that obtained with 0.25 M sucrose and that the higher concentration (0.88 M) of sucrose did not further increase the immunogenicity. These results would suggest certainly that a hypertonic sucrose solution does help stabilize the immunogenic moiety in the particulate fraction.

Although in these experiments the immunogenic activity was consistently higher than had been obtained previously, the particulate fraction still did not immunize as well as whole cells in the CF-1 mice. Magnesium ions were added, therefore, to the 0.44 M sucrose buffer. The immunogenic activity of the particulate fraction was increased to such an extent that in 12 consecutive preparations an average of 70.2% of the mice lived longer than 30 days, and in two experiments over 90% of the mice lived longer; this compares with an average 56.9% survival rate in mice which had been vaccinated with whole cells. Moreover, these results were obtained without the use of Freund's adjuvant. A 20-mg amount of particulate fraction immunized mice to a significantly greater degree than did 1.0 mg of H37Ra. Moreover, for the first time, an immune response was obtained with a 1.0-mg dose of the particulate fraction, and in one experiment this was as active as the 1.0-mg dose of whole cells.

These results would suggest that generally, on a weight basis, viable cells, through some unknown mechanism, produce a higher immune response in mice than can be obtained with the particulate fraction. However, the one experiment in which 1.0 mg of the particulate fraction immunized mice as well as did whole cells suggests that the particulate fraction when fully stabilized will immunize mice to the same degree as whole cells even when the two vaccines are compared on a quantitative basis. In addition, purification procedures, now underway on the particulate fraction, may result in purer preparations, so that perhaps less material will be needed to immunize.

Other compounds known to help stabilize mitochondrial preparations, such as ATP, PVP, citrate, and EDTA, were found to have no additional stabilizing effect on the immunizing substance in these experiments; in some cases, they were found to be detrimental.

The data given in this paper afford no definitive information regarding the nature of the immunizing substance, as to whether it is a mitochondrial structure, a plasma-membrane component, or some other cell inclusion. Studies now in progress on the separation of the components of the ruptured mycobacterial cell mass may be more revealing.

Mice, therefore, can be immunized with (i) a heat-stable cell-wall preparation which produces a low degree of immunity (Kanai and Youmans, 1960), and (ii) a very labile substance present in the particulate fraction which in sufficient quantity will produce, under appropriate conditions, an immune response equal to or greater than that obtained with whole cells. Killed whole-cell vaccines will give only the former low response (Youmans and Youmans, 1957); therefore, an immunizing fraction of maximal potency can be obtained only from live mycobacterial cells.

These data show that with the increase in stability of the particulate fraction, and thereby in its immunogenicity, progress has been made toward the development of a nonviable, nonallergenic (Kanai, Youmans, and Youmans, 1960) vaccine against tuberculosis.

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