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Studies on the Immunological Properties of the Cell Wall-, Particle- and Soluble-Fractions of Sonicated BCG Cells^{*1, *2}

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SUMMARY

An investigation has been made of the immunological properties of the cell wall-, particle- and soluble-fractions isolated from sonicated BCG cells in a sufficiently pure state for immunological studies. The cell walls were the most active of the fractions tested in inducing tuberculin hypersensitivity in guinea pigs. The results also suggest that cell walls may be involved in the development of an acquired resistance of mice to tuberculous infection. The ability to produce a humoral antibody in rabbits and to react with immune sera, was tested in each of the isolated cell fractions. Much cross reaction was observed among these fractions. Further studies have been made on the serological "specificity" of the test fractions by the adsorption test.

INTRODUCTION

With the development of the electron microscopy and the application of biochemical and biophysical techniques to the study of the microbial structures, it has become possible within recent years to isolate and characterize various subcellular structures of microbial cells. The flagella, cell walls, protoplasmic membrane, nuclear body and other structures of many microorganisms have been isolated in a native and sufficiently pure state and the properties and functions of these preparations have been investigated from various points of view (Spooner and Spooner, 1956; Gunsalus and Stanier, 1960).

In the author's laboratory *Mycobacterium* has been studied from the following two aspects, 1) the isolation and characterization of some subcellular structures of the cells and 2) the search for lytic enzyme active on the cell walls of *Mycobacteria*.

In our preceding papers reports were made on the chemical composition of the cell wall and other fractions isolated from mechanically disintegrated BCG cells and on the isolation of bacteria capable of lysing BCG cell walls (Kotani *et al.*, 1959a, b). The present report gives data on the immunological properties of cell fractions isolated by differential centrifugation of disintegrated BCG cells.

^{*1} A preliminary report of some of this work was presented at the 34th Annual Meeting of the Japanese Association for Tuberculosis, Tokyo, April, 1959.

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MATERIALS AND METHODS

1. *BCG cells*

Washed cells of BCG grown as surface pellicles on Sauton media for 9 to 12 days at 37°C were used. The cells were stored in the frozen state in a dry ice box. Details of the growth conditions and method of harvesting the cells were given in the previous paper (Kotani *et al.*, 1959a).

2. *Test animals*

Guinea pigs, each weighing about 300 g and giving no positive reaction to a 10-fold dilution of Sauton Old Tuberculin, were used in the experiments on the sensitizing antigenicity of cell fractions. In the experiments on the ability of the fractions to enhance resistance to tuberculous infection, use was made of mice of stock ddO and strain CF #1, 5 to 7 weeks old at the time of the first immunizing injection. Stock ddO was supplied from the Central Breeding Station of Experimental Animals, Osaka University, and strain CF #1 was obtained through the courtesy of Takeda Pharmaceutical Industries, Ltd. Rabbits weighing about 2.5 kg were used as test animals for production of humoral antibodies after injection of test fractions.

3. *Tuberculin*

Old Tuberculin used in intracutaneous tests was prepared at this laboratory from the filtrate of about a 7 week old culture of *Mycobacterium tuberculosis* (strain Aoyama B) grown on Sauton medium and a preliminary potency test of the preparation was made according to the descriptions in "Minimum Requirement of Biological Products" (Ministry of Health and Welfare, Japanese Government, 1954).

4. *Rupture of BCG cells*

A Raytheon 50 watt, 9-Kc, Magnetostriction Oscillator (model S-102A) was used. The conditions selected are described later.

5. *Preparation of immunizing antigen*

Prescribed amounts of lyophilized preparations of each of the cell fractions and whole cells were suspended by vigorous mixing in an adjuvant consisting of liquid paraffin, Arlacel A (Mannide monolate, Atlas Powder Co.) and distilled water in the ratio of 2:1:1. The suspensions were sterilized by heating at 65°C for one hour and were used at immunizing antigens throughout the experiments.

6. *Analytical procedures*

The methods employed for chemical analyses of the cell wall fractions were essentially the same as those described in the previous paper (Kotani *et al.*, 1959a).

7. *Measurement of contamination of the cell wall fractions with acid-fast bacilli.*

The degree of contamination with acid-fast bacilli in the disintegrated BCG cell suspensions taken just before trypsin treatment (cf. Table 1) were examined as follows. A fixed volume of test specimen was smeared evenly as a disc (1 cm diameter) on a glass slide and the smear was stained by Ziehl-Neelsen's method. The total number of acid-fast bacilli found in 11 microscopic fields, which were selected at equal intervals along the diameter of stained smears, was counted under the microscope and was compared with that in a control smear made in the same way from a BCG cell suspension containing a known dry weight of the cells per ml. The degree of contamination of the specimens with acid-fast bacilli was determined on the basis of the above comparisons and was expressed as mg dry weight of contaminating bacilli per 100 mg of cell wall fraction obtained from the disintegrated cell suspension examined.

To check the purity and to examine the morphological features of each of the cell fractions, specimens of each cell fractions were taken just before lyophilization and mounted on collodion films. The preparations were shadowed with chromium at an angle of 15 degrees to the plane of the film and examined under a Japan Electron Optics Laboratory electron microscope, model JEM-5G.

8. *Methods for testing the ability of the cell fractions to enhance the resistance of mice against tuberculous infection*

A single cell suspension of a highly virulent *Mycobacterium bovis* (strain Ravenel) was prepared according to the conventional technique, from 9 day old cultures on 1 per cent KH_2PO_4 -Ogawa's whole egg medium or Dubos' medium. This was used as the challenging inoculum. Control and immunized mice to be tested were infected through the tail vein with 0.2 ml of the above suspension and were sacrificed at intervals after the infection for examination of the number of infected bacilli in the various organs. Each of the excised organs was mixed with 4 per cent NaOH solution in a total volume of 5 ml (10 ml in the case of the liver) and the mixture was homogenized in a Waring blender (Tokyo Bunka Kizai Co.) at 10,000 rpm for 3 minutes. The number of viable bacilli in the bacillary suspensions used for challenge and in the organ homogenates obtained as above, was determined by the conventional quantitative culture method using 3 per cent KH_2PO_4 -Ogawa's medium.

9. *Serological tests of the antigenicity of the cell fractions in vivo and in vitro*

The ability of each of the cell fractions to produce humoral antibodies in rabbits and to react with immune serum were determined by the complement fixation test. The concentration of each of the cell fractions giving the highest antibody titer in the reaction with homologous immune serum was determined by the box titration method and suspensions at the concentrations thus selected (0.05 mg, 0.1 mg and 0.1 mg per ml of the cell wall-, particle- and soluble-fractions, respectively) were used as antigens. 0.25 ml aliquots of each of the antigen suspensions, serial two-fold dilutions of test serum specimens, complement (2.5 hemolytic units) and 3 per cent ox erythrocyte suspension sensitized with 7 units of hemolysin were used. The reaction mixtures, other than the hemolytic system, were incubated for complement fixation at 4°C for 12 hours and then at 37°C for 30 minutes. The sensitized erythrocyte suspensions were then added and the mixtures were incubated further at 37°C for one hour to allow hemolysis. The results were measured immediately. A 0.85 per cent saline solution containing 0.16 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per ml was used throughout the serological experiments.

RESULTS

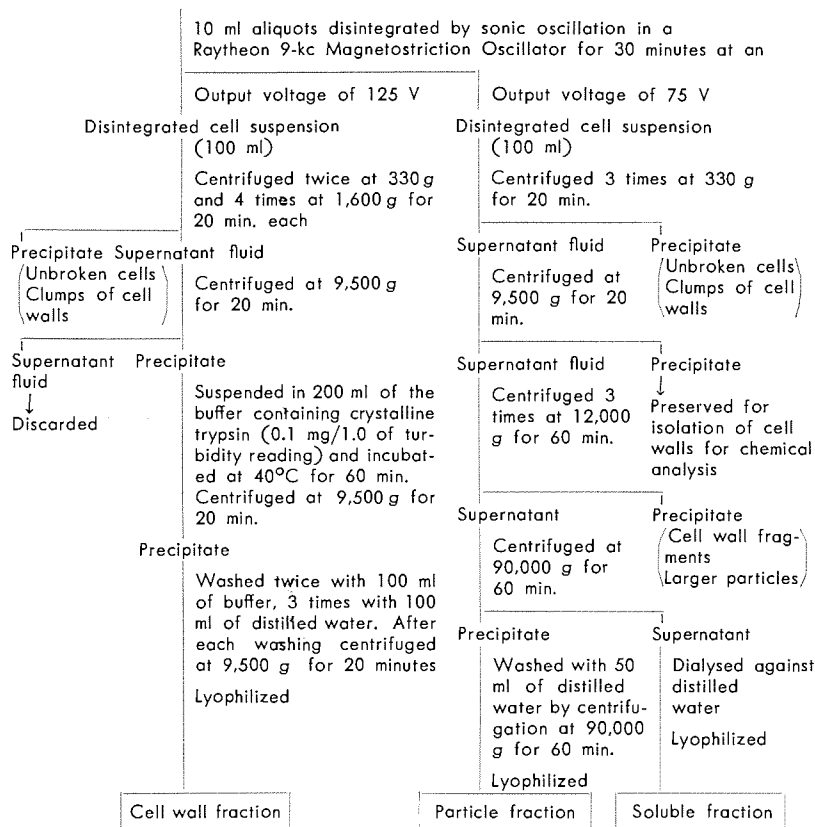
1. *Method of isolation of the cell wall-, particle- and soluble-fractions from sonicated BCG cells in a sufficiently pure state for use in immunological experiments*

The methods used in the present study for disrupting BCG cells and for isolation of each of the cell fractions from disintegrated cells are essentially the same as those described in the previous paper (Kotani *et al.*, 1959a). However some modifications have been made because even slight contamination of test cell fractions with acid-fast bacilli or with other fractions, which is negligible in chemical analyses, complicates the results of immunological experiments and make interpretation difficult.

The methods used in the present experiments are illustrated in Table 1. The main differences between the procedures employed in this and the previous study are as follows: 1) in preparing the cell wall fractions, the output voltage at which the sonic oscillator was run was raised from 75 volt to 125 volt and low

Table 1. Method of isolation of cell wall-, particle- and soluble-fractions of BCG for use in the immunological study

BCG cell suspension (2 g wet weight/10 ml of 0.067 M phosphate buffer, pH 7.8)



speed centrifugations to remove unbroken cells was repeated twice at 330 *g* and four times at 1,600 *g* for 20 minutes instead three times at 330 *g* for 20 minutes, and 2) the particle fractions were obtained by centrifuging the supernatant fluid of disintegrated cell suspensions which were centrifuged three times instead of once at 12,000 *g* for 60 minutes to minimize contamination of this fraction with cell wall fragments.

The typical electron microscopic appearance of each of the cell fractions thus obtained is shown in Figs. 1, 2 and 3. Table 2 presents the results of chemical analyses made on the cell wall fraction, together with the yield of the cell materials and the degree of contamination by unbroken acid-fast bacilli. The corresponding figures given by cell wall preparations isolated in a study on their chemical composition are quoted from the previous paper and also shown in this Table.

As shown in Fig. 1, the cell wall fraction shows no definite membraneous

Table 2. A comparison of the properties of the cell wall preparations isolated in the present and the previous study

Cell wall preparations	Yield %*1	Contami- nation with un- broken cells %*2	Chemical composition %*3						
			Total N	Total P	Sugars*1				Total lipides
					Reducing substance	Hexose	Pentose	Hexo- samine	
For immunolo- gical study	0.8 } 4.4	<0.008	2.7	0.28	23.0	11.4	12.6	4.6	55.6
For chemical study*1	7 } 15	0.8 } 3.0	3.0	0.36	21.2	10.2	11.7	3.7	64.4

*1 Quoted from the report of Kotani *et al.* (1959a)

*2 Percentage of initial BCG cells by dry weight

*3 Percentage of isolated cell wall preparation

*4 Reducing substance, hexose, pentose and hexosamine are expressed as glucose, galactose, arabinose and glucosamine-HCl, respectively.

structures, so that it is difficult to judge by electron microscopy whether it is really a cell preparation or not. Table 2 indicates, however, that this fraction is almost identical in chemical composition with the cell walls isolated in the previous study, showing a definite membranous morphology, and is practically free from unbroken cells. Electron microscopy also reveals that contamination of the fraction with unbroken cells or electron dense cytoplasmic materials is negligible. It may be concluded therefore that the cell wall fractions isolated as described in Table 1 are satisfactory for the present investigation.

Figs. 2 and 3 show that at least morphologically the particle- and soluble-fractions prepared as described above are sufficiently pure and homogeneous.

2. Ability of the cell fractions to induce tuberculin hypersensitivity

Lyophilized preparations of each of the cell fractions and whole cells suspended in the adjuvant at a concentration of 10 to 40 mg per ml were used as sensitizing antigens. Groups of 3 or 4 guinea pigs were injected in the right thigh subcutaneously with 0.1 ml of the various suspensions. The control group was injected with 0.1 ml of the adjuvant alone. Tuberculin sensitivity was tested in these animals at intervals after sensitization by intracutaneous injection of 0.1 ml of a 10- and/or 100-fold dilution of Sauton Old Tuberculin. The size of the redness and the intensity of the induration of the reaction were measured 24, 48 and 72 hours after injection of tuberculin.

The results of two typical experiments are recorded in Tables 3 and 4. It may be seen that guinea pigs sensitized with the cell wall fraction give a definite positive tuberculin reaction comparable in intensity to that exhibited by animals injected with whole cells. The particle- and soluble-fractions, on the other hand, do not induce any definite tuberculin hypersensitivity, at least under the present experimental conditions.

Table 4 shows results on the tuberculin test in guinea pigs sensitized with a cell wall fraction which had been extracted thoroughly with a chloroform-methanol (2:1) mixture at room temperature as described in the previous paper. The sen-

Table 3. Ability of each of the cell fractions to induce tuberculin hypersensitivity in guinea pigs (Experiment 1)

Guinea pig No.	Days after sensitization	Reactions measured 24 hrs after an intracutaneous injections of tuberculin*1*2						
		Guinea pigs sensitized with						
		Whole cells 1 mg*3	Cell wall fraction 1 mg	Particle fraction 1 mg		Soluble fraction 1 mg		Adjuvant only
		$\times 100^{*4}$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$
1	24-25	++ 21 $\times$ 26	+	— (6 $\times$ 6)*5	— 0 $\times$ 0	— 2 $\times$ 2	— 0 $\times$ 0	— 1 $\times$ 1
2		++ 19 $\times$ 17	+	— 2 $\times$ 2	— 1 $\times$ 1	— 3 $\times$ 3	— 0 $\times$ 0	— 0 $\times$ 0
3			+	— 3 $\times$ 3	— 2 $\times$ 2	— (13 $\times$ 10)	— (13 $\times$ 10)	
1	49-56	+++ (C.B.) 35 $\times$ 22	+	+	—	—	—	—
2		+++ (C.B.) 26 $\times$ 19	++ (C.B.) 31 $\times$ 17	— 17 $\times$ 17	— 3 $\times$ 3	— 5 $\times$ 4	— 0 $\times$ 0	— 0 $\times$ 0
3			++ 28 $\times$ 20	— 7 $\times$ 6	— 0 $\times$ 0	— 22 $\times$ 15	— 2 $\times$ 2	

*1 Intensity of induration

*2 C. B. : Central bleeding

Size of redness (mm $\times$ mm)

*3 Dry weight of the preparations injected / guinea pig

*4 The dilution of Old Sauton Tuberculin

*5 The boundary of redness was indefinite

Table 4. Ability of each of the cell fractions to induce tuberculin hypersensitivity in guinea pigs (Experiment 2)

Guinea pig No.	Days after sensitization	Reactions measured 72 hrs after an intracutaneous injection of tuberculin*1*2							
		Guinea pigs sensitized with							
		Cell wall fraction				Particle fraction		Defatted cell walls*5	
		1 mg*3		4 mg		4 mg		4 mg	
		$\times 10^{*4}$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$
1	27-31	+	+	++	+	—	—	+	—
		20 $\times$ 19	15 $\times$ 14	15 $\times$ 14	(15 $\times$ 14)	5 $\times$ 5	(6 $\times$ 6)	12 $\times$ 12	6 $\times$ 6
2		+	+	++	++	—	—	++	+
		10 $\times$ 10	16 $\times$ 14	16 $\times$ 12	13 $\times$ 12	6 $\times$ 6	4 $\times$ 4	13 $\times$ 14	9 $\times$ 9
3	27-31	+	++	++	+	±	—	+	—
		9 $\times$ 9	14 $\times$ 14	20 $\times$ 19	13 $\times$ 12	13 $\times$ 12	8 $\times$ 6	17 $\times$ 15	10 $\times$ 9
4	27-31	+	++						
		15 $\times$ 16	(25 $\times$ 23)						
1	57-63	+	+	+	—	—	—	±	±
		12 $\times$ 16	12 $\times$ 12	13 $\times$ 12	6 $\times$ 5	5 $\times$ 3	0 $\times$ 0	13 $\times$ 13	8 $\times$ 9
2		+	+	++	±	—	—	++ (C.B.)	+
		9 $\times$ 8	10 $\times$ 10	16 $\times$ 15	7 $\times$ 7	10 $\times$ 9	0 $\times$ 0	21 $\times$ 13 (8 $\times$ 8)	13 $\times$ 10
3	57-63	++	+	++	+	—	—	±	+
		10 $\times$ 9	10 $\times$ 8	13 $\times$ 11	11 $\times$ 10	8 $\times$ 7	2 $\times$ 2	11 $\times$ 11	12 $\times$ 10
4	57-63	++	+						
		18 $\times$ 15	10 $\times$ 7						

*1~*4 Symbols the same as in Table 3.

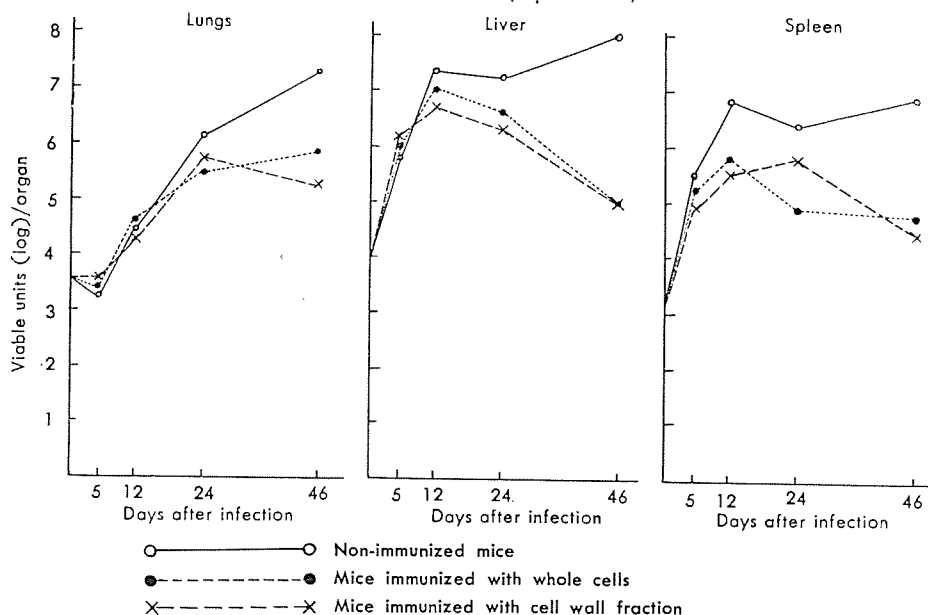
*5 The cell wall preparation extracted thoroughly with a chloroform-methanol mixture (2:1).

sitizing antigenicity of BCG cell walls, as shown in this Table, is not significantly decreased by removal of the lipids extractable with neutral organic solvents. It is of interest and worth further study that the free lipid component, which amounts to nearly 40 per cent of the weight of the cell walls, does not seem to play any important part in induction of tuberculin hypersensitivity.

3. Ability of the cell fractions to enhance the resistance of mice to tuberculous infection

Groups of mice of strain CF#1 or stock ddO were each immunized with 0.1 ml of the cell fractions suspended in the adjuvant. Controls received adjuvant only. Immunizing injections were repeated after 2 or 3 weeks. Five to eight weeks after the second injection, immunized and control mice were challenged by an intravenous injection through the tail vein of 0.2 ml of a single cell suspension of a highly virulent *Myc. bovis*, strain Ravenel. At various intervals after the challenge, the mice were killed by a blow on the head and the number of infected bacilli in the spleen, liver, lungs and kidneys was determined as described in the Methods section. The results obtained with mice immunized with each of the cell fractions and whole cells were compared with those of control animals. The presence or absence of a statistically significant difference between the number of infected bacilli recovered from the organs of immunized and control ani-

Fig. 1. Ability of the cell fractions to enhance the resistance of mice to tuberculous infection (Experiment 1)



Mice of stock ddO (♂) were immunized twice with an interval of 15 days. 34 days after the second immunization they were challenged with 8.6×10^6 viable units of a highly virulent *Myc. bovis*, strain Ravenel. Two mice from each group were examined on the days indicated in the Figure.

Table 5. Ability of the cell fractions to enhance the resistance of mice against tuberculous infection (Experiment 2)

Mice immunized with	Number of mice examined	Days after challenge	Number (log) of infected bacilli recovered from			
			Lungs	Liver	Spleen	Kidneys
—	2	0	3.20	4.45	5.21	0.94
None	5	31	5.65	5.75	4.67	3.74
Whole cells (3 mg × 2)	5		4.92*	5.13*	3.32**	2.60**
Cell wall fraction (" ")	5		5.45	5.23*	3.46**	2.77**
Particle fraction (" ")	5		5.40	5.51	4.40	2.93*
Soluble fraction (" ")	5		5.58	5.29	4.30	3.31
None	3	68	5.83	4.82	4.23	3.92
Whole cells (3 mg × 2)	4		6.21	4.49	3.58	2.48
Cell wall fraction (" ")	4		5.25*	4.30	3.87	2.26*
Particle fraction (" ")	4		6.16	4.78	3.50	4.43
Soluble fraction (" ")	3		6.30	4.28	3.40	3.59

Mice of strain CF#1 (♀) were immunized twice at an interval of 20 days. 41 days after the second immunization they were challenged with 3.2×10^5 viable units of a highly virulent *Myc. bovis*, strain Ravenel.

*P<0.05 **P<0.01

imals was provisionally tested by the method of Masuyama (1949).

The results of three typical experiments, together with the experimental conditions used are presented in Fig. 4 and Tables 5 and 6. The number of bacilli recovered from the organs of mice immunized with cell walls or whole cells tends to be lower than that of those in the corresponding organs of control mice. A rather larger number of bacilli were found in the mice immunized with the particle fraction or with the soluble fraction than in those immunized with the cell walls.

Therefore the cell wall fraction may be mainly involved in the development of an acquired resistance to tuberculous infection. It should be pointed out, however, that in the present experiment resistance resulting from immunization with whole cells was not so great as expected.

4. Ability of the cell fractions to produce humoral antibodies and to react with immune sera

Three rabbits in each group were injected subcutaneously twice with an interval of 17 days between injections with 0.3 ml of one of the cell fractions suspended in the adjuvant. Nineteen days after the second injection, serum specimens were taken by cardiac puncture and were inactivated by heating at 60°C

Table 6. Ability of the cell fractions to enhance the resistance of mice against tuberculous infection

Mice immunized with	Number of mice examined	Days after challenge	Number (log) of infected bacilli recovered from		
			Lungs	Liver	Spleen
	2	0	1.76	4.10	3.03
None	4		3.60	5.13	4.85
Whole cells (4 mg × 2)	4		2.10**	4.75	4.08*
Cell wall fraction (1 mg × 2)	4	15	2.73*	5.03	4.44
Particle fraction (1 mg × 2)	4	17	2.84	5.11	4.33
Soluble fraction (2 mg × 2)	4		3.23	4.89	4.09*
None	6		3.95	3.49	2.89
Whole cells (4 mg × 2)	4		3.23*	2.50*	2.43
Cell wall fraction (1 mg × 2)	5	78	3.93	3.13	2.78
Particle fraction (1 mg × 2)	5	80	5.06	3.68	3.21
Soluble fraction (2 mg × 2)	5		3.93	3.11	2.64

Mice of stock ddo (♀) were immunized twice with an interval of 23 days. 58 days after the second immunization they were challenged with 1.3×10^4 viable units of a highly virulent *Myc. bovis*, strain Ravenel.

* $P < 0.05$ ** $P < 0.01$

for 20 minutes. The antibody content of these specimens was determined by the complement fixation test, the various cell fractions as antigens. Results were expressed in terms of the highest dilution of the test serum showing a definite complement fixation.

As shown in Table 7, the cell wall- and particle-fractions are good antigens, both in producing humoral antibodies and in reacting as complement fixing antigens with immune serum. The activity of the soluble fraction is weak in both tests.

As a considerable degree of cross reaction between the cell fractions and heterologous immune sera was found, the serological relationship of the cell fractions was examined by adsorption with homologous and heterologous antigens, as follows. As adsorbing antigen, saline suspensions of both the cell walls and particles and a solution of soluble-fraction (2 mg, 2 and 10 mg, 5 mg per ml, respectively) were used. A specimen of pooled serum taken from rabbits immunized with the cell wall- or particle-fractions was used. 0.5 ml aliquots was mixed with 1.5 ml of each of the adsorbing antigens. After incubation at 37°C for 2 hours, the mixture was centrifuged at 900 *g* for 10 minutes and was again mixed with the same antigen as used in the first adsorption. The mixture was incubated

Table 7. Ability of the cell fractions to produce humoral antibodies in rabbits and to react with immune sera

Sera from guinea pigs immunized with	Rabbit No.	Titer* of the complement fixing antibody reacting with		
		Cell wall fraction	Particle fraction	Soluble fraction
Cell wall fraction	1	512	512	64
	2	2048	1024	256
	3	1024	1024	128
Particle fraction	1	256	256	64
	2	512	512	256
	3	256	256	64
Soluble fraction	1	16	32	16
	2	32	32	8
	3	32	32	4
None	1	<4	<4	<4
	2	<4	<4	<4
	3	32	4	<4
	4	<4	<4	<4
	5	4	8	<4
	6	<4	<4	<4

*The highest dilution of test serum showing a definite complement fixation.

Table 8. Serological relation of the cell wall-, particle- and soluble-fractions examined by adsorption experiments

Immune sera	Doses of adsorbing antigens mg/ml serum	Titer* of the complement fixing antibody reacting with		
		Cell wall fraction	Particle fraction	Soluble fraction
Immune serum against cell wall fractions (pooled) adsorbed with				
None	—	1024	1024	16
Cell wall fraction	14	<8	64	<8
Particle fraction	14	128	32	—
" "	70	<8	8	<8
Soluble fraction	35	256	512	8
Immune serum against particle fraction (pooled) adsorbed with				
None	—	512	512	32
Cell wall fraction	14	<8	128	8
Particle fraction	70	<8	<8	<8
Soluble fraction	35	16	64	<8

*The highest dilution of test serum showing a definite complement fixation.

at 37°C for 2 hours and at 4°C for a further 24 hours and then centrifuged at 80,000 *g* for one hour to remove the added antigens as completely as possible. The supernatant was used as the adsorbed serum.

The results of complement fixation tests made on these adsorbed sera are presented in Table 8. Although no definite conclusion can be made, these results may be interpreted as follows: 1) the particle fraction has a relatively specific antigenic substance which is not present, at least not in large quantity, in the other cell fractions and 2) in the particle fraction, there is a considerable amount of the cell wall fragments or of the antigens shared by the cell walls. It should be pointed out in this connection, that the particle fraction used in this experiment was unfortunately obtained from a supernatant which was centrifuged only once at 12,000 *g* for 60 minutes (cf. Table 1).

Although the experiment described above gives some information on the serological relation of each of the cell fractions, results are only preliminary. Further experiments are needed to elucidate these matters.

DISCUSSION

From sonicated BCG cells, various subcellular fractions were isolated in a sufficiently pure state for immunological investigations. Their immunological activity was examined in various ways. Only the reports of Crowle (1954, 1958), Youmans *et al.* (1955, 1957) and Ribí *et al.* (1959) deal systematically with the immunological properties of cell fractions isolated from physically disintegrated *Mycobacterial* cells.

There is a considerable degree of agreement between the results obtained in the present study and those reported by Crowle. He tested preparations of physically disrupted tubercle bacilli and found the insoluble residue or bacillary hulls elicited statistically significant "immunity" in guinea pigs, moderate antibody formation and consistent tuberculin allergy. Bacillary constituents soluble in borate-citrate buffer, on the other hand, induced conspicuous antibody formation and some tuberculin allergy, but no "immunity". Unfortunately, because Crowle's original paper (1954), presented as a Doctoral dissertation at Stanford University, was not available, the authors do not know whether the insoluble residue employed as an immunizing antigen in his study consisted exclusively of cell walls or whether his preparation was contaminated with unbroken acid-fast cells.

The results reported by Youmans and his colleagues seem to be somewhat different from those presented in this study. Although they claimed that they have isolated from strains H37Ra and BCG a particle fraction which can immunize mice against infection with virulent tubercle bacilli, the high lipid content of their preparations and their methods of isolation of the fraction suggest that it may have been heavily contaminated with cell walls.

The finding of Ribí *et al.*, that cell walls isolated from *Mycobacterial* cells induce hypersensitivity is consistent with the results presented. It should be pointed out, however, that the cell wall preparations used in their study were isolated from *Mycobacterium butyricum*, not from a mammalian *Mykobacterium* and the hypersensi-

tivity induced was tested with either the cell walls or protoplasm obtained from the same organism.

The results of the present study indicate that the cell walls are the most active structural units of BCG cells, both in inducing tuberculin hypersensitivity and in enhancing the resistance of mice to tuberculous infection. However, the active factor of BCG cells responsible for the induction of acquired resistance to tuberculous infection or of tuberculin hypersensitivity, does not seem to be exclusively localized in the cell walls. If the active factor were localized exclusively in the cell walls, the potency of a given amount of the cell wall preparation should be equal to that of about four times as many whole cells, because the cell walls of bacteria constitute 20 to 25 per cent of the whole cell weight. However, in fact, the activity of the cell wall fractions was somewhat inferior to that of the whole cells, even on the same weight basis.

Another interesting point demonstrated in the present study is that BCG cell walls retain the ability to induce tuberculin hypersensitivity, even after the exhaustive removal of their free lipid component which constitutes nearly 40 per cent of the weight of the walls. Since the study of Raffel *et al.* (1955) showed that waxes soluble in chloroform without hydrolysis play an important part in induction of a delayed type, tuberculin hypersensitivity, the above findings call for fresh experiments to resolve the problems of tuberculin hypersensitivity. Studies are in progress on the effect on the ability of the cell wall fractions to induce tuberculin hypersensitivity of treatment with an extract of L₁ bacterium (cf. Kotani *et al.*, 1959b), egg white lysozyme and Nagarse (a crystalline protease produced by *Bacillus subtilis*).

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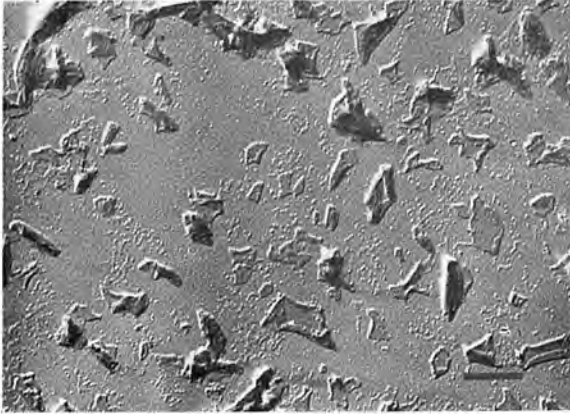


Fig. 2 Cell wall fraction

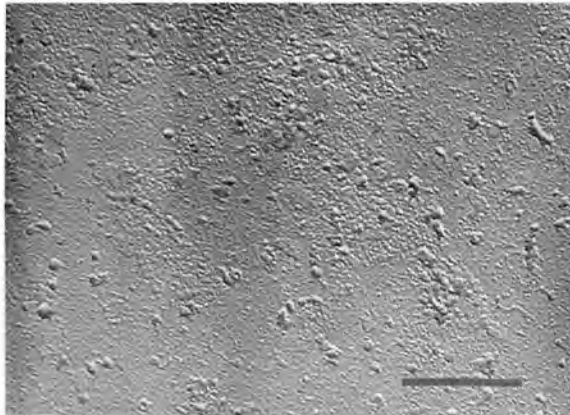


Fig. 3. Particle fraction

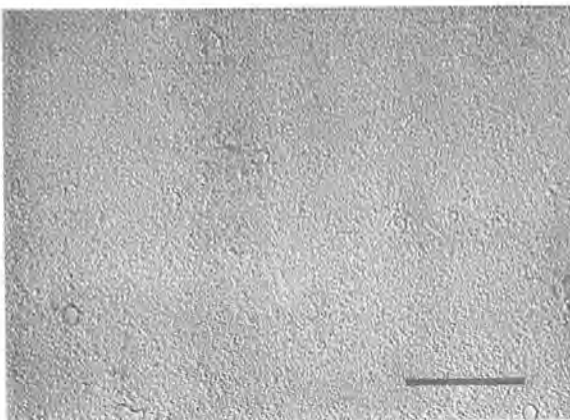


Fig. 4. Soluble fraction

Figs. 2-4: Electron micrographs of the cell fractions.

Samples were mounted on a collodion film, shadowed with chromium and examined with a Japan Electron Optics Laboratory electron microscope, model JEM-5G. Scale: 1μ