SYMPOSIUM ON RELATIONSHIP OF STRUCTURE OF MICROORGANISMS TO THEIR IMMUNOLOGICAL PROPERTIES[1]

II. Host-Reactive Properties of Cell Walls and Protoplasm from Mycobacteria

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Introduction

The structural elements with which this presentation is concerned are those obtained by disrupting viable mycobacterial cells and separating the disruption products by differential centrifugation. The soluble portion is considered to be protoplasm and contains all portions of the cells except the cell walls. The cell walls obtained after disruption of cells in aqueous suspension appear to be essentially pure when examined by electron microscopy. The biological methods employed in this laboratory for examination of the primary fractions include: (i) production of primary lesions in the skin of normal rabbits and guinea pigs, (ii) elicitation of delayed skin reactions in sensitized animals, (iii) production of allergic encephalomyelitis in guinea pigs, and (iv) immunization of mice against infection with virulent tubercle bacilli.

Cummins and Harris (2) obtained cell walls of various organisms, including mycobacteria, by disrupting them in a Mickle apparatus. Prior to disruption, the cells were killed in 0.5% formalin. To harvest pure cell walls from the mixture of broken cells, it was necessary to treat with 0.5% KOH in ethanol for 48 hr and wash with alcohol followed by distilled water. Ribi et al. (17) described a method whereby cell walls and protoplasm could be obtained from viable mycobacteria suspended in Tween 80 and water and shaken with minute glass beads in a Mickle apparatus. After repeated washing, the cell walls were found to be intact except for ruptured ends. They contained little or no protoplasmic remnants (Fig. 1). The use of the method was limited by the restricted amounts of material which could be pocessed. Subsequently, Ribi et al. (18) devised a method employing a pressure cell with a cooled orifice for rupture of large quantities of organisms. By differential centrifugation of the effluent, cell walls and protoplasm could be obtained in good yield and a high degree of purity. A typical cell-wall preparation obtained in this manner is shown in Fig 2. Kanai and Youmans (4) used a pressure cell system and a modification of the method of Lamanna and Mallette (7) to isolate various morphological fractions from mycobacteria.

Reactions in the Skin of Normal Animals

Serial twofold dilutions of cell walls, whole cells, or protoplasm in saline were injected, in 0.2-ml quantities, into the skin of rabbits and guinea pigs, and the areas of injection were observed at suitable intervals (9). Protoplasm, in doses up to 1000 μg, produced only transient redness and edema. Cell walls produced raised, hard, red areas which appeared in about 4 to 5 days. The latter lesions varied from 5 to 25 mm in diameter depending upon the amount of cell walls administered, persisted for 4 to 5 weeks, and healed without leaving a scar. Typical lesions produced in a normal rabbit by cell walls from *Mycobacterium butyricum* are shown in Fig. 3.

[1] This symposium was held at the Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, 7 May 1963, under the sponsorship of the Division of Medical Microbiology and Immunology, with Edgar Ribi as convener.

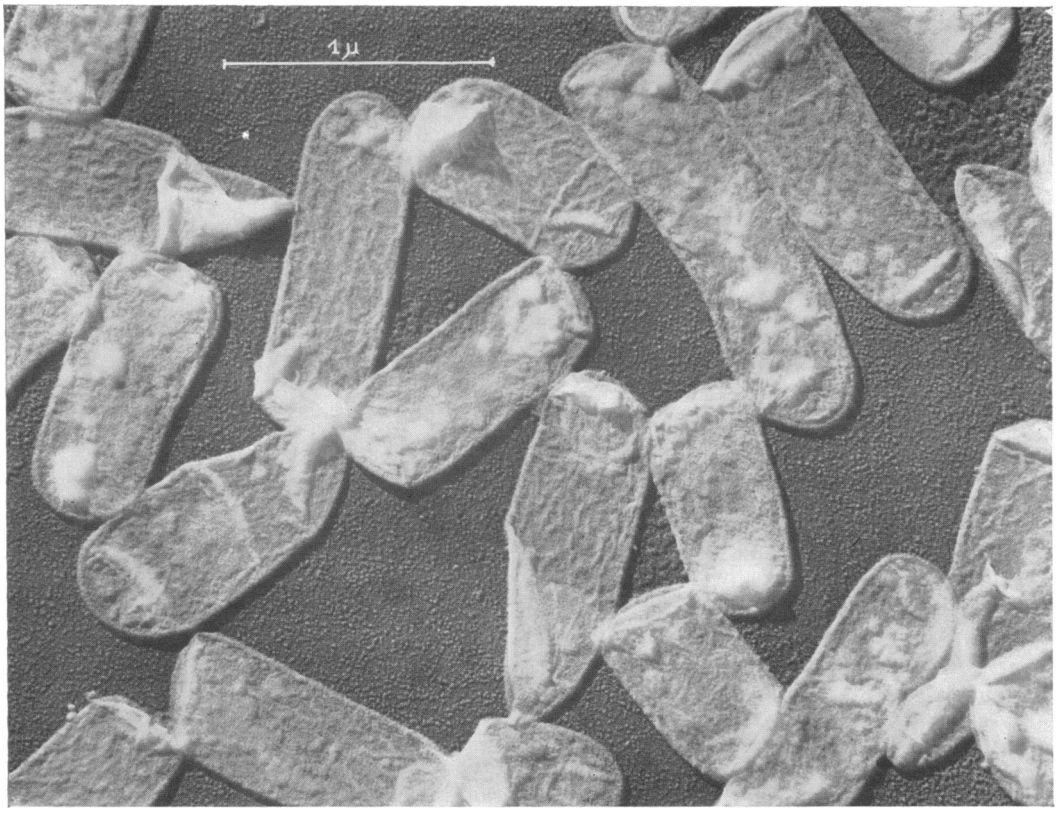

FIG. 1. *Cell-wall preparation from Mycobacterium butyricum disrupted in the Mickle apparatus.*

Cell walls usually were effective in a dose of 1 μg; whole cells were effective in a somewhat higher dose, roughly proportional to the amount of inert protoplasm they contained. Only minor differences were noted in reactions produced by cell walls of the various bacterial species tested (*M. tuberculosis*; anonymous bacilli groups I,II, and IV; *M. butyricum*, *M. phlei*; *and M. smegma tis*). The response of guinea pigs to intradermal injection of cell walls and whole cells was less consistent than that of rabbits, and about four times as much material was ordinarily required.

Such observations indicate that a technique which separates acid-fast organisms into different morphological fractions by physical means also efficiently separates the cell into components producing different biological reactions in normal animals. These results were confirmed by Kanai et al. (5), and appear to be a confirmation in morphological terms of the earlier results obtained by Raffel (16).

Whole cells or cell walls of many kinds of microorganisms have a primary inflammatory effect on the skin of normal animals (6, 8, 10, 14). In several instances, this is attributable to endotoxin, but lesions produced by *Bacterium tularense* (*Francisella tularensis*), for example, cannot be related to such a substance (8), and the responsible factor in mycobacteria has not been identified with certainty although waxes from the cell wall are believed to play a role.

DELAYED HYPERSENSITIVITY

It is now well known that killed tubercle bacilli are capable of inducing delayed hypersensitivity in animals, and it seems that protein and an accessory factor are needed to induce this state. The latter may be related to Wax D.

The materials usually employed to elicit reactions in previously sensitized animals are "old tuberculin" (OT) and "purified protein protein derivative" (PPD), which are derived from heated filtrates of tubercle bacilli grown on liquid media for prolonged periods. PPD,

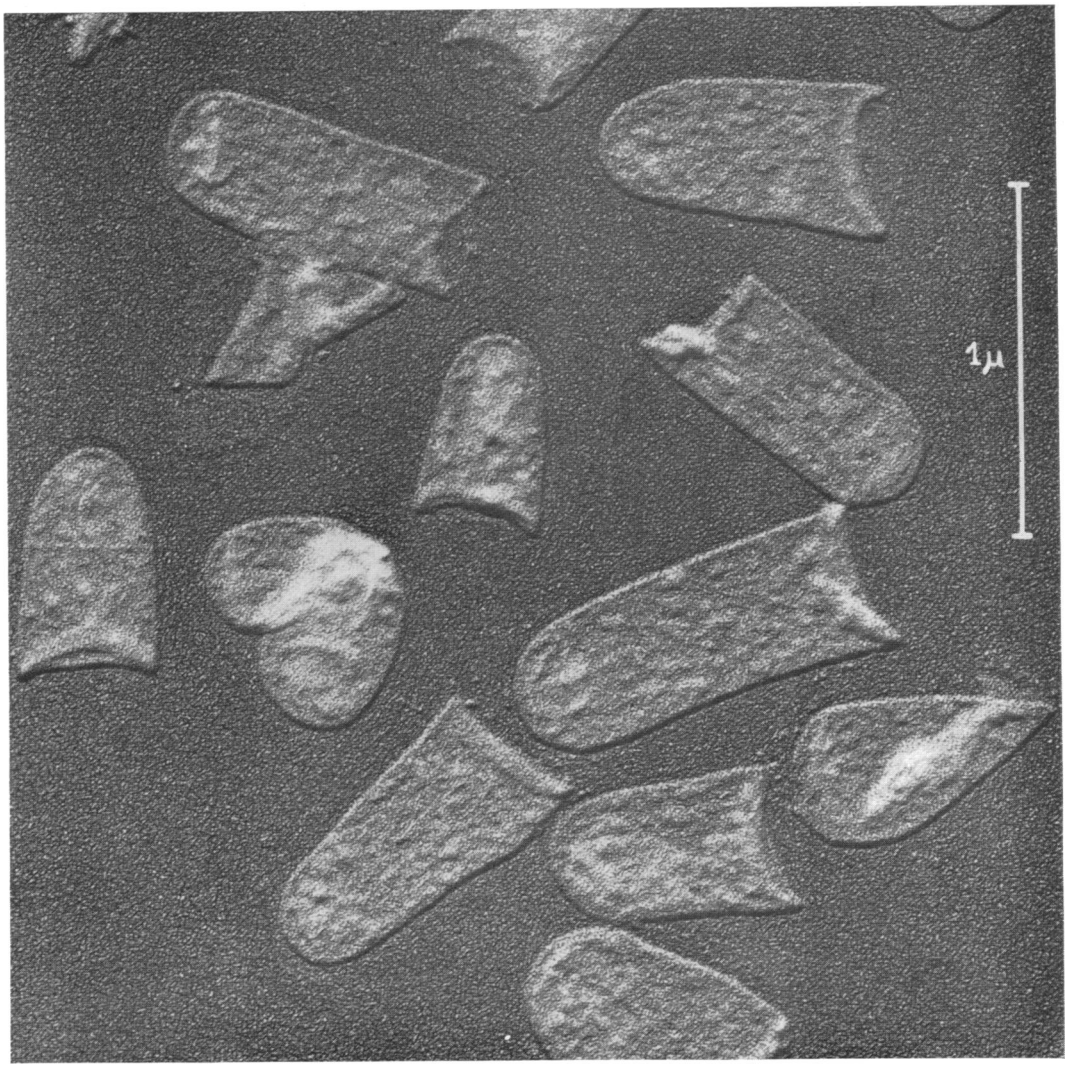

FIG. 2. *Cell-wall preparation from Mycobacterium tuberculosis (BCG) disrupted in a Sorvall Refrigerated Cell Fractionator.*

which elicits responses in animals sensitized with any of several species of mycobacteria, is only a relatively pure product, since it contains a number of identifiable polysaccharides and proteins. The need for skin test antigens capable of allowing differentiation of infections due to typical and atypical tubercle bacilli has become increasingly apparent and has aroused considerable interest. Magnusson (13) reported that it is possible to differentiate delayed hypersensitivity reactions induced in experimental animals by specific sensitins of tubercle bacilli (avian and mammalian), *M. balnei*, and the anonymous bacilli. Studies by Affronti (1), Edwards et al. (3), and Sartwell and Dyke (19) indicated that differentiation of various infections by acid-fast bacilli can be made on the basis of skin reactions. These methods, however, involve difficulties which arise from the fact that size of reaction is the criterion employed for differentiation. Our studies of cell walls and protoplasm from mycobacteria have indicated that use of these physically separated fractions may have a special value for the purpose.

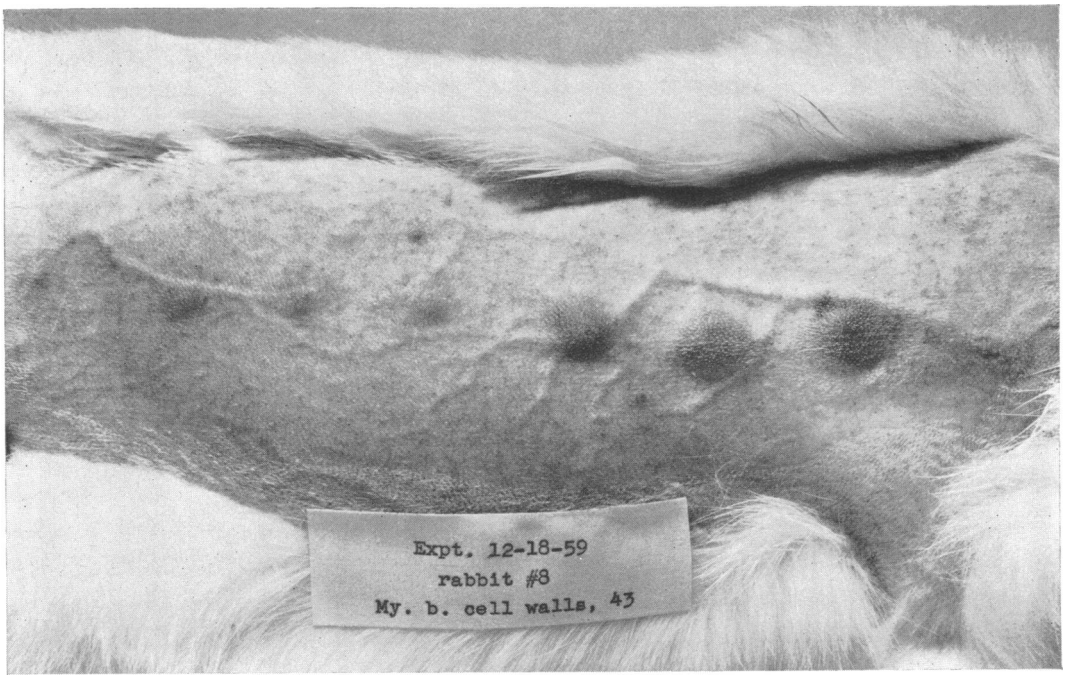

FIG. 3. *Skin lesions in a normal rabbit 5 days after intradermal injection of 0.2 ml of nine serial twofold dilutions of cell walls of Mycobacterium butyricum, starting with 40 μg. The least dose which produced a lesion was 0.6 μg.*

Table 1 illustrates the interrelationships between two species of mycobacteria (*M. tuberculosis* and *M. butyricum*). Morphological fractions were tested for their ability to induce primary lesions and the state of hypersensitivity and to elicit hypersensitivity reactions in sensitized animals. It may be seen that cell walls, like whole bacteria, are active in all reactions, and that they elicit hypersensitivity reactions in animals sensitized with heterologous materials. Protoplasm is inactive in most respects but has the faculty of eliciting delayed skin reactions in specifically sensitized animals only.

Lesions produced after injection of cell walls of *M. butyricum* into animals sensitized with 2 μg of homologous cell walls are shown in Fig. 4. Serial twofold dilutions, beginning with 10 μg, were employed; the last of the 12 lesions was produced by 0.005 μg of cell-wall material. Lesions thus provoked by cell walls or whole cells developed within 1 or 2 days and were hard, raised, and deep red or purple. After 4 or 5 weeks, they faded and left scars. Lesions elicited by protoplasm also appeared within 2 days but were less indurated and disappeared within the next 2 days without leaving scars.

Representative data from an experiment demonstrating that protoplasm cannot sensitize but can elicit the reaction of hypersensitivity appear in Table 2. At 33 days after the sensitizing injections, the rabbits were reinjected with doubling dilutions of the fractions, ranging from 10 to 0.02 μg. Doses of 0.02 μg of cell walls and 0.04 μg of protoplasm produced lesions within 2 days in animals sensitized with cell walls. Animals which had been previously injected with large amounts of protoplasm were negative, as were the two control rabbits. After 4 days, the lesions provoked by protoplasm had disappeared or were fading. As expected, rabbits 3 and 4 and and the control animals had by this time developed primary lesions at sites of injection with cell walls.

The lack of specificity of reactions elicited by cell walls was evidenced by the fact that animals sensitized with cell walls of *M. butyricum* and tested with cell walls of *M. butyricum* and *M. tuberculosis* reacted to the same amount of cell

TABLE 1. *Dermal reactivity in rabbits produced by cell walls and protoplasm from representative species of mycobacteria*

Species	Fraction	Induction of		Elicitation of hypersensitive reaction in animals sensitized with	
		Primary lesion	State of hypersensitivity	BCG*	*M. butyricum**
M. tuberculosis (BCG)	Cell walls	+	+	+	+
	Protoplasm	0	0	+	0
M. butyricum	Cell walls	+	+	+	+
	Protoplasm	0	0	0	+

* Whole cells or cell walls.

FIG. 4. *Skin lesions in a sensitized rabbit 3 days after intradermal injection of 0.2 ml of 12 serial twofold dilutions of cell walls of Mycobacterium butyricum, starting with 10 μg. The smallest dose (0.005 μg) produced a response, and no end point was obtained. The rabbit was sensitized 5 weeks before the skin test by intradermal injection of 2 μg of cell walls of M. butyricum.*

walls of either organism. Similar results were obtained when cell walls of *M. tuberculosis* were used to sensitize animals. Likewise, no evidence of specificity was obtained when cell walls of *M. phlei*, *M. smegmatis*, or H37Ra (*M. tuberculosis*) were employed. In contrast, when protoplasm was used as a provoking reagent, the reactions were highly specific, showing no crossing unless extremely large amounts of heterologous materials were injected. This remarkable specificity of homologous protoplasm is demonstrated in Fig. 5. The rabbit was sensitized with cell walls of *M. tuberculosis* and tested with preparations of protoplasm from the same organism and from *M. butyricum* in doses of 0.8, 4, 20, and 100 μg. No reactions developed at sites of injection of heterologous protoplasm on the right side of the figure, but, on the left, all

TABLE 2. *Sensitivity to cell walls and protoplasm of BCG among rabbits initially injected with these fractions*

Rabbit no.	Sensitizing (single injection)		Provoking*		
	Preparation	Dose (μg)	Preparation	Effective dose (μg)	
				2 days	4 days
1	Cell walls	10	Cell walls	≦0.02	≦0.02 (≦0.005)†
			Protoplasm	0.04	—‡
2	Cell walls	20	Cell walls	≦0.02	≦0.02
			Protoplasm	0.04	0.08 (fading)
3	Protoplasm	880	Cell walls	—	2.5
			Protoplasm	—	—
4	Protoplasm	880	Cell walls	—	1.25
			Protoplasm	—	—
5	Controls		Cell walls	—	0.6
6			Protoplasm	—	—

* Rabbits injected intradermally at different sites with 0.2-ml volumes of twofold dilutions of the fractions. Doses ranged from 10 to 0.02 μg. Test carried out 33 days after initial injection.

† Value from another experiment (see Fig. 4).

‡ Negative.

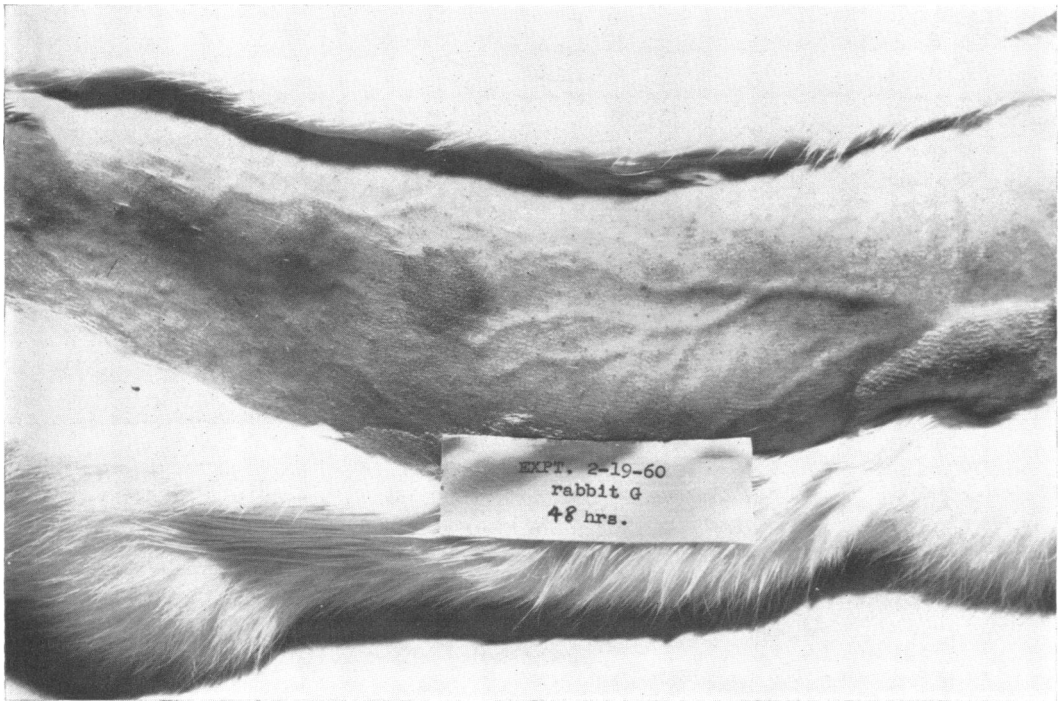

FIG. 5. *Reactions in the skin of a rabbit sensitized by intradermal injection of cell walls of Mycobacterium tuberculosis (BCG) and tested with 0.8, 4, 20, and 100 μg of protoplasm of BCG and M. butyricum. No reactions were noted at the sites of injection of heterologous protoplasm (right), but reactions occurred at the sites of injection of homologous protoplasm (left). The 0.8-μg dose produced the lesion at the extreme left.*

four doses of homologous material produced typical lesions. The reverse situation was also demonstrated. Specific reactions also were obtained by injection of protoplasm from strain BCG into guinea pigs infected with virulent tubercle bacilli, while injection of comparable amounts of protoplasm of *M. butyricum* produced only equivocal responses or none. Animals sensitized with cell walls of *M. smegmatis* and *M. phlei* also responded to injection of specific protoplasm but responded poorly or not at all to nonspecific material.

Preliminary studies indicated that rabbits sensitized with cell walls of BCG responded to to intradermal injection of protoplasm of BCG, *M. kansasii*, and group II and group IV anonymous bacilli in such a way as to suggest that protoplasm of group IV is closely related to that of BCG, protoplasm of group II is somewhat less closely related, and protoplasm of *M. kansasii* is only remotely related to that of BCG.

Studies were made of protoplasms of BCG and groups I, II, and IV anonymous bacilli by precipitin tests with rabbit immune serum prepared by injection of the animal with heat-killed virulent bovine tubercle bacilli suspended in mineral oil. Osserman's method (15) for identification of specific precipitin bands was employed. It was shown that group IV and BCG protoplasm shared two antigens and BCG protoplasm contained an additional antigen not shared by group IV bacilli (Fig. 6). Group II protoplasm shared a single antigen with BCG protoplasm and group I protoplasm had no common antigen with BCG. Absorption of antibody with protoplasms gave similar results.

These findings show that cell walls and protoplasm differ in respect to their ability to induce and provoke delayed hypersensitivity. PPD contains eight antigens which react with the serum used in our studies. Since some of these were identical with components in extracts of whole cells, which were not found in protoplasm, it is concluded that PPD contains material from the cell wall. The facts that protoplasm contains fewer antigens than culture filtrates and that at least one of these shows a high degree of species specificity suggest the use of protoplasm as starting material for isolation of specific skin test antigens.

The results of our studies of the role of protoplasm and cell walls in delayed hypersensitivity were substantiated by observations of Kanai, Youmans, and Youmans (5). They found, further, that trypsin, pepsin, and ribonuclease do not affect the allergenicity of cell-wall preparations. Extraction with alkaline ethanol destroys the activity, presumably by extracting a lipoprotein. White et al. (21) found that Wax D of human bacilli serves as an adjuvant in the production of delayed hypersensitivity, while Wax D from other organisms is not effective. A wide variety of heat-killed acid-fast bacilli were, however, effective. Cell walls of acid-fast bacilli

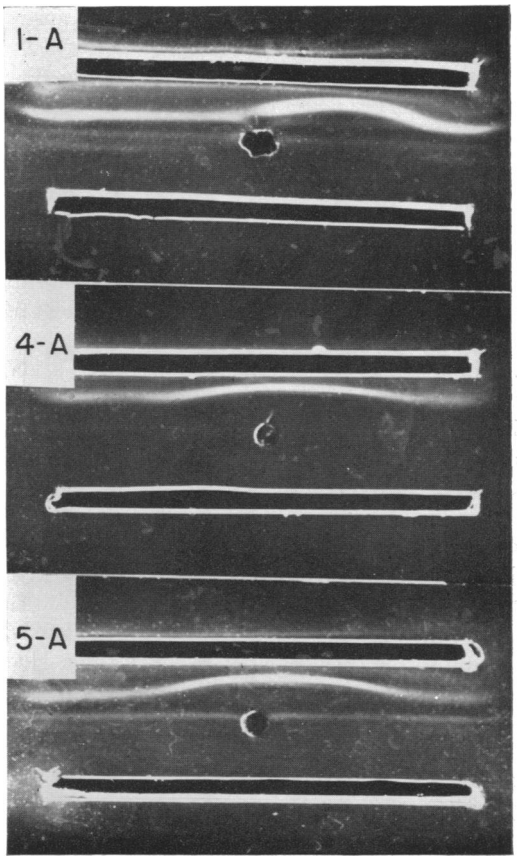

FIG. 6. *Immunoelectrophoretic studies of BCG and group IV protoplasm. The constituents of each test were as follows. In 1A, the well contained BCG-61 protoplasm, the lower trough contained PPD, and the upper trough contained anti-BCG serum; 4A had group IV protoplasm, BCG-61 protoplasm, and anti-BCG serum, respectively, and 5A had group IV protoplasm, PPD, and anti-BCG serum, respectively, in the well, the lower trough, and the upper trough.*

must, therefore, contain all the active components present in whole heat-killed cells because various members of the group, in addition to the human tubercle bacillus, give rise to delayed hypersensitivity.

ALLERGIC ENCEPHALOMYELITIS

The role and the mode of action of complete Freund's adjuvant in the production of allergic encephalomyelitis is still one of the mysteries surrounding this experimental disease. It is generally accepted that oil enhances the sensitizing ability of killed mycobacteria by increasing the cellular response to the killed organisms. The tubercle bacilli, in turn, are responsible for eliciting a specific type of cellular response. The only bacillary fraction that has been successfully substituted for killed whole cells in producing allergic encephalomyelitis is Wax D prepared from human strains. While Wax D from bovine strains, such as BCG, and from saprophytic mycobacteria, is not effective, killed whole cells or these organisms are known to be active in this test. Mycolic acid is the dominant constituent of the wax fraction. Since Wax D preparations from bovine strains do not contain amino acids, which are found in Wax D from human strains, it has been suggested that in addition to mycolic acid the presence of a peptide or protein is essential for adjuvant activity.

We have examined the adjuvant activities of cell walls and protoplasm prepared from H37Ra, BCG and *M. butyricum* in a test designed to give reasonably precise median effective doses suitable for comparing the potencies of the fractions. Guinea pigs were inoculated in either the nuchal area or the foot pad with a single dose of water-in-oil emulsion containing homologous brain and the mycobacterial fraction to be tested. The minimal criterion for a "positive" guinea pig was paresis for 2 successive days. Only a few positive animals met this minimal requirement. The great majority became paralyzed or died soon after onset of paresis. This criterion was adopted after several years correlation of observation with histopathological examination. Representative results of an experiment in which H37Ra was employed by nuchal route injection are listed in Table 3. The median effective dose (AED_{50}) was of the order of 0.1 mg for both killed whole cells and cell walls of H37Ra. Essentially the same results were obtained with similar preparations of BCG and *M. butyricum*. In only one of seven preparations was the protoplasmic fraction found to be active at the highest dose levels tested (1.0 and 2.0 mg). There was no discernible difference in potency of the preparations when they were sterilized by boiling for 1 hr or by the addition of 0.4% phenol, or when Tween 80 was present. Also, there was no difference in effectiveness of the materials when suspended in the aqueous or oil phase of the adjuvant mixture.

By the foot pad route, approximately one-tenth the amount of killed whole cells and cell walls sufficed to produce an effect comparable with that given by injection into the nuchal area. Results of such tests obtained with cell-wall preparations are shown in Table 4; AED_{50} values

TABLE 3. *Allergic encephalomyelitis produced in guinea pigs by injection of water-in-oil emulsions containing brain and killed whole cells, cell walls, or protoplasm from Mycobacterium tuberculosis H37Ra into the nuchal area*

Amt of microbial preparation injected (mg)	Results*		
	Killed whole cells	Cell walls	Protoplasm
2.0	—	—	0/10
1.0	9/10	10/10	0/10
0.5	8/10	10/10	0/10
0.1	5/10	5/10	0/10
0.02	0/10	0/10	0/10
AED_{50} (mg)	0.14	0.10	>2

* Number of animals with encephalomyelitis/number tested.

TABLE 4. *Allergic encephalomyelitis produced in guinea pigs by injection of water-in-oil emulsions of brain and mycobacterial cell walls into the foot pad*

Cell-wall dosage (mg)	Results*		
	BCG	H37Ra	H37Ra†
0.1	—	—	9/10
0.05	8/10	10/10	—
0.01	9/10	8/10	5/10
0.005	3/10	6/10	—
0.001	1/10	1/10	1/10
AED_{50} (mg)	0.01	0.005	0.01

* Number of animals with encephalomyelitis/number tested.

† Supernatant (60,000 × *g*, 2 hr) of cell walls disrupted in a sonic oscillator.

of the order of 0.01 mg were obtained for cell-wall preparations.

When purified cell walls suspended in water were solubilized in a sonic oscillator, the soluble material retained in the supernatant after it was centrifuged at 60,000 × *g* for 2 hr was, on a weight basis, as active as were cell walls which had not been solubilized (shown in Table 3). This water-soluble fraction, which was also highly active in producing primary and secondary lesions upon intradermal injection into rabbits, has not been chemically analyzed as yet. It will be of interest to determine what constituents this fraction may have in common with Wax D.

Immunity to Infection with Virulent Tubercle Bacilli

Many experimental systems employed to study resistance to tuberculous infection in mice are based upon the use of large numbers of organisms in the challenge dose, an abnormal route of administration of the challenge organisms, and death rates to measure the degree of resistance. In many of the systems, nonspecific antigens often produce evidence of resistance. A method was devised to eliminate some of these objections (11). Briefly, it includes (i) administration of vaccine to mice either intravenously or by aerosol, (ii) challenge by the pulmonary route with small numbers of virulent organisms 4 weeks later, (iii) notation of the number of pulmonary tubercles, and (iv) determination of the number of virulent organisms in the lungs and spleens of vaccinated and control mice sacrificed 4 weeks after their exposure to virulent bacilli. In tests to determine the value of viable attenuated organisms (BCG or H37Ra) as immunizing agents, the method gave reliable results and was not influenced by nonspecific antigens. No evidence of interference could be demonstrated.

In this protection test, killed whole cells, cell walls, protoplasm, and total break-up products obtained after rupture of bacilli in aqueous media were ineffective. These materials had been sterilized by chemical agents. Recently, we found that whole cells killed by heating at 65 C for 30 min were somewhat immunogenic. Further studies suggested that disruption of organisms in oil might yield immunogenic materials.

The following experiment demonstrates the efficacy of this procedure. Viable BCG cells grown on Dubos medium at 37 C for 11 days were suspended in light mineral oil (Drakeol) and disrupted at a pressure of 43,000 psi at 5 to 10 C. The effluent was washed three times in oil by centrifugation at 20,000 × *g* for 1 hr. The final sediment was resuspended in oil and stored for 90 days at 2 C. The suspension was then centrifuged at 2500 × *g* for 1 hr, and the sediment was resuspended in saline containing 0.02% Tween 80 and heated at 65 C for 30 min. This dead material consisting of cell walls contaminated with some protoplasm was termed "oil disruption product." Heat-killed whole cells and cell walls from organisms disrupted in water ("water cell walls") were used as controls. All antigens were from the same pool of organisms. Mice were injected intravenously with varying amounts of the antigens and tested according to the method described.

The results of the experiment are shown in Fig. 7. A dose response was noted in the data obtained from cultures of the spleen. The number of virulent bacilli present in the tissues was, at each dose level of oil vaccine, about 2 logs less than that noted with killed whole cells. Based on results obtained from cultures of the spleen and lungs, the degree of resistance produced by the "oil disruption product" was similar to that produced by the live attenuated organisms. The average figures obtained in the last five experiments with live attenuated organisms as vaccine showed the following numbers of virulent organisms per gram in mice (immunized and nonimmunized, respectively): spleen, 14×10^2 and 31×10^4; lungs, 12×10^4 and 40×10^6. At the 200 μg level, these figures for the group vaccinated with "oil disruption product" were: spleen,

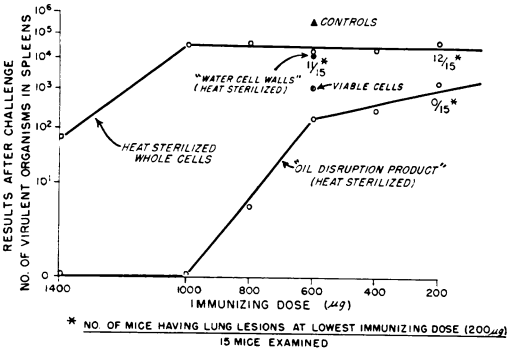

FIG. 7. *Protection of mice against air-borne challenge with virulent tubercle bacilli (H37Rv) by intravenous vaccination with products from BCG ruptured in oil and with heat-killed BCG.*

19×10^2 and 40×10^4; lungs, 89×10^3 and 14×10^6.

The number of mice with lung lesions was compared with that noted in mice vaccinated with living BCG (Paris strain). Among mice vaccinated with 50 μg of BCG, 9% had pulmonary lesions; with 5 μg, 31% had pulmonary tubercles after challenge with H37Rv. Comparable figures for mice vaccinated with killed whole cells, "water cell walls," and "oil disruption product" in this experiment were 30, 73, and 0%, respectively.

These results demonstrate the possibility of immunizing mice with organisms disrupted in oil. It is not known why oil preserves the antigen. It is tempting, however, to consider that a water-soluble, heat-labile enzyme capable of destroying the antigen is released when organisms are disrupted in water, but, when the bacilli are disrupted in oil, the enzyme is incapable of acting on the antigen. Other possibilities may be considered, but only further study will answer this interesting question.

Heat-killed whole cells are more immunogenic than cells killed by formalin or phenol, a fact which suggests that heating may destroy factors harmful to essential antigen. Kanai and Youmans (4) attributed resistance to intracellular particles which can be sedimented by centrifugation at $145{,}000 \times g$ for 3 hr. These particles lose their antigenicity when heated at 98 C for 1 hr. It will be recalled that our material was sedimented by centrifugation at $2500 \times g$. There is, therefore, a considerable difference in the size of the immunogenic materials studied by our group and by the other group. It is also of interest to note that about 5 mg of the fraction used by Kanai and Youmans were necessary to produce resistance, whereas only 200 μg of the "oil disruption product" produced significant resistance.

Of considerable interest are results obtained from experiments in which mice were immunized with living anonymous bacilli or H37Ra against infection with virulent tubercle bacilli (12) and in which rabbits sensitized with dead tubercle bacilli were tested for sensitivity to protoplasm of BCG and anonymous bacilli, groups I, II, and IV. Both H37Ra and group I bacilli gave rise to effective resistance to infection (Table 5). Groups II, III, and IV organisms failed to protect the animals. Rabbits sensitized with BCG reacted to an equal degree to injections of 10 μg of protoplasm of BCG and group IV organisms, to a lesser extent to protoplasm of group II bacilli, and minimally, if at all, to that of group I bacilli (Table 6).

TABLE 5. *Immunity to infection with Mycobacterium tuberculosis (H37Rv) conferred on mice by immunization with living H37Ra or anonymous bacilli*

Organism of vaccine	Immunity produced
M. tuberculosis (H37Ra)	++++
Anonymous bacilli (group I)	++++
Anonymous bacilli (group II)	0
Anonymous bacilli (group III)	0
Anonymous bacilli (group IV)	0

TABLE 6. *Hypersensitive reactions provoked in rabbits sensitized with killed BCG by protoplasms of BCG and anonymous bacilli groups I, II, and IV*

Protoplasm obtained from	Delayed reaction
M. tuberculosis (BCG)	++++
Anonymous bacilli (group I)	?
Anonymous bacilli (group II)	++
Anonymous bacilli (group IV)	++++

If, as seems probable, the cell walls contain the antigens responsible for production of resistance, the above results indicate that cell walls and protoplasm may be endowed with different types of specificity. Cell walls of group I bacilli contain antigens in common with cell walls of BCG, but group I protoplasm differs from that of BCG. Cell walls of group IV organisms, however, show little relationship to those of BCG, but protoplasms obviously share similar antigens.

SUMMARY AND CONCLUSIONS

When acid-fast bacilli are disrupted by mechanical means and separated into soluble (protoplasm) and insoluble (cell-wall) fractions by differential centrifugation, it is possible to demonstrate biological differences in the fractions. Cell walls are capable of producing lesions in the skin of normal animals, inducing delayed hypersensitivity, provoking relatively nonspecific delayed reactions, replacing killed whole bacilli in Freund's adjuvant, and, presumably, producing resistance to infection. Protoplasm fails to produce lesions in the skin of normal animals, to induce delayed hypersensitivity, to act as

adjuvant, and to immunize mice against infection. Protoplasm does, however, provoke relatively specific delayed reactions in previously sensitized animals. In addition to these differences, Suter and Kirsanow (20) showed that cell walls of BCG are capable of increasing the reactivity of mice to endotoxin, whereas protoplasm does not produce this phenomenon.

Literature Cited

1. Affronti, L. F. 1959. Purified protein derivatives (PPD) and other antigens prepared from atypical acid-fast bacilli and *Nocardia asteroides*. Am. Rev. Tuberc. Pulmonary Diseases **79**:248–295.
2. Cummins, C. S., and H. Harris. 1958. Studies on the cell-wall composition of *Actinomycetales* and related groups. J. Gen. Microbiol. **18**:173–189.
3. Edwards, L. B., C. E. Palmer, L. F. Affronti, L. Hapwood, and P. Q. Edwards. 1960. Epidemiologic studies of tuberculin sensitivity. II. Response to experimental infection with mycobacteria isolated from human sources. Am. J. Hyg. **71**:218–241.
4. Kanai, K., and G. P. Youmans. 1960. Immunogenicity of intracellular particles and cell walls from *Mycobacterium tuberculosis*. J. Bacteriol. **80**:607–614.
5. Kanai, K., G. P. Youmans, and A. S. Youmans. 1960. Allergenicity of intracellular particles, cell walls, and cytoplasmic fluid from *Mycobacterium tuberculosis*. J. Bacteriol. **80**:615–621.
6. Lackman, D. B., J. F. Bell, C. L. Larson, M. L. Casey, and W. W. Benson. 1959. An intradermal test for Q fever in man. Arch. Inst. Pasteur Tunis **36**:557–569.
7. Lamanna, C., and M. F. Mallette. 1954. Use of glass beads for the mechanical rupture of microorganisms in concentrated suspensions. J. Bacteriol. **67**:503–504.
8. Larson, C. L. 1946. A skin reaction in rabbits produced by intradermal inoculation of suspensions of killed *Pasteurella tularensis*. Public Health Rept. U.S. **61**:1797–1806.
9. Larson, C. L., E. Ribi, W. Wicht, and R. List. 1961. Skin reactions produced in rabbits by cell walls and protoplasm of *Mycobacterium tuberculosis* and *M. butyricum*. Am. Rev. Respirat. Diseases **83**:184–193.
10. Larson, C. L., E. Ribi, K. C. Milner, and J. E. Lieberman. 1960. A method for titrating endotoxin activity in the skin of rabbits. J. Exptl. Med. **111**:1–20.
11. Larson, C. L., and W. C. Wicht. 1962. Studies of resistance to experimental tuberculosis in mice vaccinated with living attenuated tubercle bacilli and challenged with virulent organisms. Am. Rev. Respirat. Diseases **85**:833–846.
12. Larson, C. L., and W. C. Wicht. 1963. Resistance to infection with virulent tubercle bacilli in mice immunized with viable *Mycobacterium balnei* and atypical acid-fast bacilli administered aerogenically. Am. Rev. Respirat. Diseases **88**:456–461.
13. Magnusson, M. 1961. Specificity of mycobacterial sensitins. I. Studies in guinea pigs with purified "tuberculin" prepared from mammalian and avian tubercle bacilli, *Mycobacterium balnei*, and other acid-fast bacilli. Am. Rev. Respirat. Diseases **83**:57–68.
14. Munoz, J., E. Ribi, and C. L. Larson. 1959. Antigens of *Bordetella pertussis*. I. Activities of cell walls and protoplasm. J. Immunol. **83**:496–501.
15. Osserman, E. F. 1960. A modified technique of immunoelectrophoresis facilitating the identification of specific precipitin arcs. J. Immunol. **84**:93–97.
16. Raffel, S. 1948. The components of the tubercle bacillus responsible for the delayed type of "infectious allergy." J. Infect. Diseases **82**:264–293.
17. Ribi, E., C. L. Larson, R. List, and W. C. Wicht. 1958. Immunologic significance of the cell wall of *Mycobacteria*. Proc. Soc. Exptl. Biol. Med. **98**:263–265.
18. Ribi, E., T. Perrine, R. List, W. Brown, and G. Goode. 1959. Use of pressure cell to prepare cell walls from mycobacteria. Proc. Soc. Exptl. Biol. Med. **100**:647–649.
19. Sartwell, P. E., and L. M. Dyke. 1960. Comparative sensitivity of college students to tuberculin PPD-S and PPD-B. Am. J. Hyg. **71**:204–211.
20. Suter, E., and E. M. Kirsanow. 1961. Hyperreactivity to endotoxin in mice infected with mycobacteria. Induction and elicitation of the reaction. Immunology **4**:354–365.
21. White, R. G., H. Bernstocky, R. G. S. Johns, and E. Lederer. 1958. The influence of components of *M. tuberculosis* and other mycobacteria upon antibody production to ovalbumin. Immunology **1**:54–66.