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Immunologic Significance of the Cell Wall of Mycobacteria. (24011)

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Separation of bacterial cells into their major morphological elements (cell wall and internal protoplasm) by a method first described by Salton and Horne(1) offers a new approach to antigenic analysis of bacteria. When aqueous suspensions of bacteria are vibrated with minute glass beads, the rigid cell wall cracks, and protoplasm is liberated in a dispersed state. Cell walls are then separated from protoplasm by differential centrifugation. Examination of a fungus and several bacteria by this technic showed that important antigens are situated in the cell wall(2-6).

Whereas the procedure of Salton and Horne is directly applicable for fractionation of certain bacterial species, it cannot be successfully applied to mycobacteria unless special measures and precautions are taken. To achieve selective disruption of mycobacteria, suspensions of washed cells must be monodispersed when exposed to mechanical vibration, and products of disruption must also be maintained in a dispersed state to allow final separation of cell wall from protoplasm.

Methods. *Mycobacterium butyricum* and 2 strains of *M. tuberculosis* (BCG and H37 Ra) were studied. *M. butyricum* was grown at 37°C for either 3 days on solid Meadow agar medium(7) or for 24 hours in liquid Meadow medium. BCG and H37Ra cultures were grown in stationary liquid Dubos Tween-albumin medium(8) for 8 to 10 days at 37°C. Bacterial cells were collected and washed by centrifugation in cold water containing 0.5% Tween. Washed bacilli could be adequately redispersed in aqueous Tween. *M. tuberculosis* grown on solid or liquid media in which granular growth occurred did not disperse adequately in Tween solution. All procedures described below were performed at 4°C. Suspensions of dispersed washed bacteria were diluted with Tween solution to a concentration having an optical density of 700 mμ in a Klett-Summerson nephelometer with filter No. 420. Five ml of suspension mixed with 5 g Cataphote No. 12 glass beads were shaken in the Mickle tissue disintegrator for 10-second periods for a total shaking time of 4 minutes. Agitation was discontinued between each of these periods for 10 to 30 seconds to avoid raising the temperature of the suspension. About half the cells were broken after this mechanical treatment. It must be emphasized that products of the Mickle process have a marked tendency to clump during vibration and prolonged treatment will produce aggregates to such an extent that cell walls cannot be obtained. Under the experimental conditions employed, a vibration period of 4 minutes was usually suitable. However, the optimum vibration time for different lots of cell suspension must be individually determined by electron microscopy of samples taken from the Mickle tubes. Difficulties regarding aggregation were not encountered during mechanical disintegration of other bacteria.* The mixture of nondispersible aggregates, intact cells, cell walls, and protoplasm was allowed to stand until the glass beads settled. The suspension was then decanted into conical 12-ml tubes, centrifuged, and supernatants containing protoplasm were removed. All cen-

* We recently observed that a commercial pressure cell can be utilized in place of the Mickle apparatus to increase the yield of cell-wall material.

trifugations were performed at 2,000 g for 60 minutes. The sediment in each tube was washed twice by thorough resuspension in Tween solution and centrifugation, then resuspended in Tween solution and allowed to stand overnight or until the aggregates settled. Following centrifugation of resultant supernatant suspensions, the sediments were resuspended in distilled water and pooled. Up to this point in the separation, the sediment obtained from each centrifuge tube of crude Mickle product had to be processed individually. Also, detergent is necessary until protoplasm and nondispersible aggregates are removed, after which distilled water must be used for a suspending medium. The sediment obtained from centrifuging the aqueous pool consisted of a translucent upper layer of cell walls and an opaque lower layer of unbroken cells. Repeated resuspensions and centrifugations of the upper layer in water, until only one layer was discernible, resulted in complete separation of cell walls from intact cells. Electron micrographs revealed that cell walls of mycobacteria obtained by the Mickle process were not fragmented, but were merely cracked at one end of the rod and that these cell-wall preparations contained little or no protoplasmic remnants (Fig. 1).

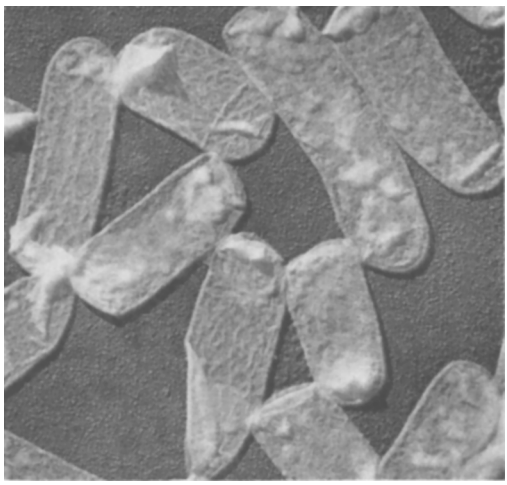

FIG. 1. *Mycobacterium butyricum*, cell-wall preparation, × 25,000.

As in earlier studies with *Bacterium tularense*(9), fractions were tested for biological activity by intradermal injection of rabbits. Firm, raised, smooth nodules were produced with preparations of mycobacteria grown on either solid or liquid media and killed by heat, formaldehyde, or ether.

Results. Rabbits were injected intradermally with preparations obtained from strain H37Ra. Preparation No. 1 consisted of whole organisms (1 mg/ml) in Tween solution containing 0.4% formalin. No. 2 of whole organisms (1 mg/ml) in Tween solution saturated with ether. No. 3 of protoplasm (3 mg/ml) in 0.2% formalin, and No. 4 of cell walls (0.2 mg/ml) in 0.4% formalin. Each of 2 rabbits received 0.2-ml amounts of a series of 2-fold dilutions in 0.85% NaCl solution from undiluted to 1:128 of each of the 4 preparations. Lesions were produced with whole organisms and cell walls but not with protoplasm. In the first rabbit, 1.6 μg of whole organisms (Nos. 1 and 2) and 0.3 μg of cell walls (No. 4) produced lesions. In the second, similar amounts of whole organisms (Nos. 1 and 2) and 1.25 μg of cell walls (No. 4) produced reactions.

In another experiment similar results were obtained with BCG. Each of 3 rabbits was injected at different sites with 0.1-ml amounts of serial dilutions of suspensions containing cell walls and of suspensions containing protoplasm in initial concentrations of 0.2 mg/ml and 1.40 mg/ml, respectively. In each animal 0.6 μg of cell walls produced lesions, while 140 μg of protoplasm did not.

Protoplasm and cell walls of *M. butyricum* also were tested. Protoplasm was given in 0.1-ml quantities of serial twofold dilutions containing from 140 μg to 2.2 μg and cell walls in dilutions containing 40 μg to 0.6 μg. Three animals injected only with protoplasm failed to react, and 2 animals given only cell walls developed lesions at sites of injection with 1.25 μg or 2.5 μg of material. Four other rabbits received protoplasm and cell walls at different sites. In none of them did protoplasm produce an effect, whereas each animal reacted to a dose of 1.25 μg of cell walls.

After 22 days, the 6 rabbits which had received cell walls of *M. butyricum*, the 3 rabbits which received an initial injection of protoplasm of *M. butyricum*, and 3 normal

animals were injected into separate areas of the skin with 40 μg of cell walls and 40 μg of protoplasm of homologous organisms. At 48 hours postinjection, protoplasm had caused little reaction at the site of inoculation, whereas cell walls had produced severe reaction in the 6 animals that had received an initial injection of cell walls. The lesions were red and edematous and varied from 20 x 7 to 38 x 35 mm in diameter. The 3 animals that had received protoplasm as the initial inoculum developed flat lesions varying from 7 x 7 to 13 x 13 mm in diameter following the second inoculation of cell walls, although the 3 normal animals failed to respond. These results demonstrate that cell walls contain fractions capable of inducing hypersensitivity of the delayed type and of eliciting skin reactions in sensitized animals.

Initial lesions produced by mycobacteria injected into the skin of rabbits do not appear until after 4 or 5 days. In this respect these organisms differ from *B. tularense* and *Hemophilus pertussis* suspensions which produce visible effects in rabbits within 24 hours(10). Initial and sensitivity reactions produced by fractions of mycobacteria can be differentiated by their time of appearance. Details of these and other studies will be discussed later.

Summary. A technic was described for separation of cell wall and internal protoplasm from cells of *Mycobacteria*. Data are presented which demonstrated that cell walls produced lesions when injected intradermally into rabbits, whereas protoplasm failed to produce these lesions. Cell walls were also shown to be capable of inducing hypersensitivity of the delayed type. Separation of these morphological elements resulted in definite separation of one element possessing certain biological activities from material not possessing these characteristics. Such initial fractionation of the cell should facilitate purification and characterization of substances which elicit typical tissue responses to infection with tubercle bacilli.

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Serological Studies on Hemolytic Antigen from Leptospira. (24012)

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A hemolytic (HL) procedure(1), which was a modification of the hemagglutination test of Chang and McComb(2), was reported from this laboratory. More recent reports (3,4) describe the preparation of this HL antigen from non-pathogenic *Leptospira biflexa*, its standardization and stabilization, and its evaluation in serodiagnosis of human leptospirosis. This evaluation demonstrated the generic specificity of the HL antigen, as well as its marked sensitivity with rabbit and human antisera, and suggested that currently used microscopic agglutination tests could be advantageously supplanted by the HL procedure in serodiagnosis of human leptospirosis. In view of its potential usefulness, further serological studies on HL antigen were carried out.

Materials and methods. HL antigen was prepared from *L. biflexa* as previously de-