Formation of Protoplasts from Mycobacteria by Mycobacteriophage.*† (24300)

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Isolation of both enzymatically active and immunogenically active particles from tubercle bacilli has been reported(1,2,3,4). Isolation of these intracellular units, resembling mitochondria in many respects, was obtained by grinding whole washed cells with abrasives in a ball mill for as long as 18 hours. This procedure was drastic but necessary since other means resulted in failure(5). Attempts to correlate particulate fractions with some intracellular structure(2,3) have met with criticism since, as has often been pointed out, cell walls as well as cell membranes could be ground to heterogeneous particulates resembling in size and sedimentation rate the particles referred to as mitochondria(6). The solution to this problem with tubercle bacilli would be to use less drastic means for liberation of intracellular components. The conversion of tubercle bacilli to protoplasts is the obvious answer but unfortunately this group of microorganisms is resistant to the lytic action of lysozyme(7) and therefore, cannot be converted by this means.

This report will describe a method successfully used in this laboratory for conversion of different strains of tubercle bacilli to protoplasts by use of concentrated mycobacteriophage.

Materials and methods. For stock suspensions of phage§ trypticase soy agar plates were seeded with sufficient numbers of the avirulent *Mycobacterium tuberculosis* strain 607 and phage to produce confluent lysis in 24 hours at 37°C. The surface from 25 such plates was washed with 0.85% NaCl and the pooled washings filtered through Berkefeld filters.

An adaptation procedure was necessary in order to produce phage active in forming protoplasts of the different strains tested. It was found that phage propagated on strain 607 had little or no activity against the H37Ra, BCG‖, and H37Rv mycobacterial strains while phage propagated on strain H37Ra was active against strains BCG and H37Rv as well. The following procedure was followed. Two 4 liter Erlenmeyer flasks each containing 2 liters of Proskauer and Beck medium were inoculated with 0.075 mg of strain 607 or H37Ra (determined by centrifugation in a Hopkins tube) and 1 ml of stock 607 phage containing 3 x 10^4 phage. This represented a phage bacteria ratio of approximately 1-100. The flasks were then stoppered with rubber stoppers containing cotton adapters. These were placed on a shaking machine housed in a 37°C incubator and the flasks were agitated gently for 48 hours when strain 607 was used and for 3 weeks when strain H37Ra was used. Periodically aliquots from the strain 607 flasks were removed and titered by plaque count against the 607 strain. The H37Ra strain was titered in the same manner only once at the end of the 3 weeks incubation period and only against the H37Ra strain. The culture medium was then filtered through Berkefeld filters, shell frozen in lyophilizing flasks and evaporated under vacuum for 24 hours. This produced a 10-fold concentration (4 liters to 400 ml). The 400 ml were then dialyzed at a temperature of 5°C against 0.01 M phosphate buffer at pH 7.0 (3 changes over a period of 24 hours) and lyophilized to dryness. The dry material was resuspended in a desired volume of 10% sucrose containing 0.1% $MgSO_4$. The number of cells to be converted to protoplasts determined final volume

* A preliminary report was given at the 58th Meeting of Soc. Am. Bact., Chicago, April 30, 1958.

† This investigation was aided by research grant from Illinois Tuberculosis Assn.

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§ Obtained from Dr. S. Froman, Dept. of Bacteriology, Univ. of California, Los Angeles.

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of phage concentrate. As a rule 10 ml was the final volume chosen. These produced an estimated phage concentrate of 10^8. This count was estimated entirely on the basis of volume since it was found that the treatment necessary for concentration inactivated approximately 90% of the original phage particles. This was evidenced by a reduction in the formation of plaques after incubation with either the 607 or H37Ra strain of tubercle bacilli as host. Four ml of the phage concentrate was added to 0.01 mg wet weight of bacterial cells suspended in 6.0 ml of 10% sucrose containing 0.1% $MgSO_4$. This allowed a phage bacteria ratio of 1000:1. The mixed suspension was incubated in a water bath at 37°C and examined in wet mount continuously for 5 minutes and every 15 minutes thereafter for a total of 60 minutes.

Light micrographs were taken with a Spencer dark M objective (N.A. 1.25) at 1/100 sec. using Adox KB 14 35 mm film. For electron micrographs a microdrop of suspension obtained at specified times was placed on a formvar grid and allowed to air dry. This was then washed by adding and withdrawing a drop of distilled water from a dropper 10 to 20 times. The prepared grids were then examined in the EMU-3C electron microscope.

Results. Fig. 1-7 represent photomicrographs of the 607 strain of tubercle bacilli. After 15 minutes of contact with phage concentrate at 37°C changes in mycobacterial cell morphology occurred (Fig. 1, 2, 5). At the start of observation the cells appeared more refractive than normal cells (Fig. 1) with a surrounding halo of brilliant light. Although swelling was evident after only 5 minutes it was very pronounced after only 15 minutes. Swelling began either at the ends or in the center of individual cells. Often, as a result of swelling at one end only, the cell would appear to be bent at right angles or to assume the appearance of a figure eight (Fig. 2). The figure eight type would always remain constricted in the center and eventually form 2 protoplasts (Fig. 3). Where only one end would swell the other end would remain attached (Fig. 3). At the end of 60 minutes complete protoplasting was evident (Fig. 4). The individual protoplasts remained surrounded by a halo of light making it difficult to visualize the true outline of the cell. Fig. 6 shows 2 protoplasts surrounded by debris and/or phage particles. The photograph appears blurred due to the presence of sucrose which was difficult to remove completely without destroying the labile unfixed protoplasts. While the technic used did not allow detection of phage particles attached to cell walls it is felt that newly reported fixation procedures(8) will permit this to be accomplished.

Similar results were obtained with the H37Ra, H37Rv and BCG mycobacterial strains.

Discussion. The results reported confirm those of Carey *et al.*(9) of formation of *Escherichia coli* protoplasts by the mass absorption of inactive phage. Although the preparation of their phage concentrate differed from that reported here the results are essentially the same.

Of interest is the fact that the phage, which was non-specific in its ability to lyse different strains of tubercle bacilli, as shown by formation of plaques on Petri plates, should require a period of adaptation to be capable of causing formation of protoplasts of the H37Ra, H37Rv and BCG strains of tubercle bacilli. The reason for this is still obscure but may reflect differences in cell wall composition between the two groups of mycobacteria or differences in phage particles. If the latter is correct the adaptation period was a period of selection where only those phage particles active against strains H37Ra, H37Rv and BCG were selected.

While it appears likely that the protoplasts were caused by mass adsorption of inactive mycobacteriophage causing a weakening and dissolution of cell walls it also remains a possibility that some component(s) liberated from the phage by the concentration treatment was responsible. Whatever the mechanism, the fact remains that the ability to convert mycobacterial cells to protoplasts will simplify future studies on relation of intracellular structure and function among the

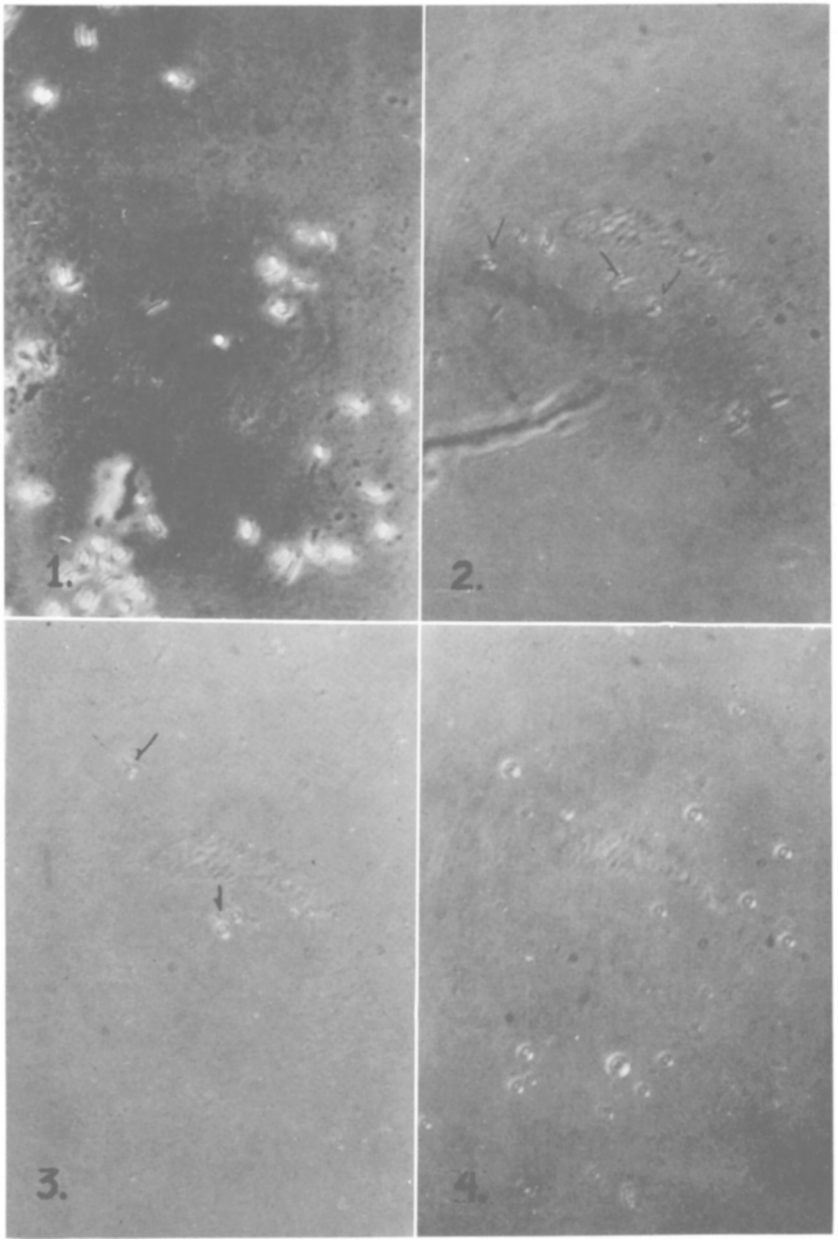

FIG. 1. *Mycobacterium tuberculosis* strain 607 after 15 min. contact with phage concentrate. Individual cells highly refractive and swelling evident either at one end or both ends of cell. A normal cell (*n*) visible for comparison. × 3300.

FIG. 2. *M. tuberculosis* strain 607 after 15 min. contact with phage concentrate. Figure eight and bent forms visible. × 3300.

FIG. 3. Same after 30 min.

FIG. 4. Same after 60 min.

mycobacteria.

Summary. Various mycobacterial cells were converted to protoplasts by treatment with a mycobacteriophage concentrate. The technic involved production of sufficient adapted phage by growth either in the 607 or H37Ra strains of tubercle bacilli. Further concentration was necessary in order to obtain a

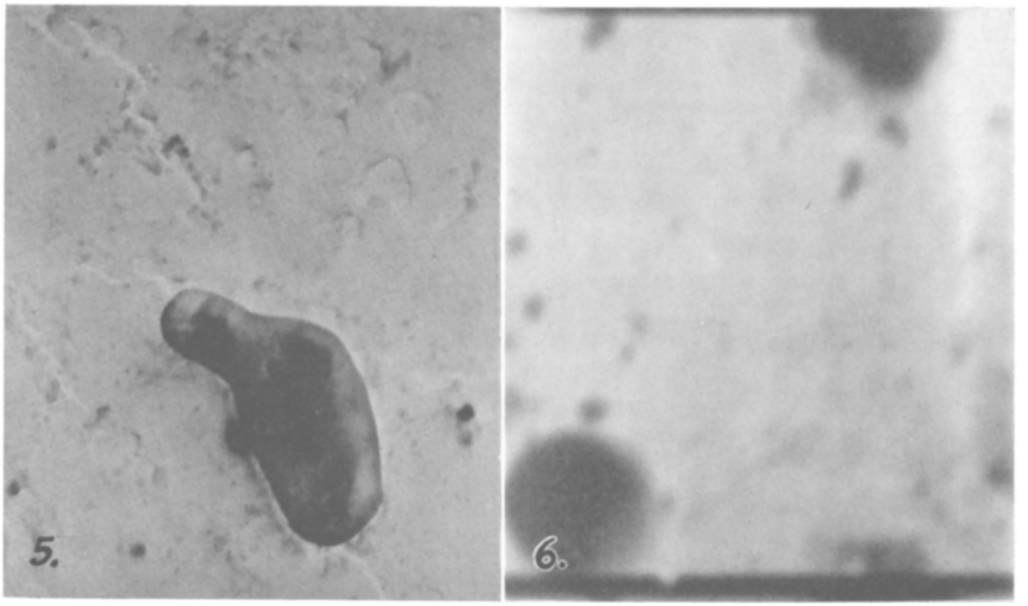

FIG. 5. Swollen cell of *M. tuberculosis* strain 607 after 15 min. contact with phage concentrate. × 36514.

FIG. 6. Protoplasts of *M. tuberculosis* strain 607 after 60 min. contact with phage concentrate. × 25025.

sufficiently high phage bacteria ratio. This was accomplished by lyophilization. The significance of the reported findings is discussed.

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Received July 10, 1958. P.S.E.B.M., 1958, v99.

o-Hydroxyphenylacetic Acid Excretion in the Phenylalanine Tolerance Test For Carriers of Phenylketonuria.* (24301)

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The recessive gene responsible for phenylketonuria is associated in the heterozygous parents with an abnormally low tolerance for ingested phenylalanine(1) and an elevated fasting level of plasma phenylalanine(2). This accumulation of phenylalanine in the apparently normal carriers of the disease is referable to a partial impairment of phenylalanine hydroxylase, the enzyme whose ab-

* Support is acknowledged from U. S. Atomic Energy Comm., to New England Deaconess Hospital and U. S. Public Health Service.