LIGHT AND ELECTRON MICROSCOPE STUDIES OF MYCOBACTERIUM-
MYCOBACTERIOPHAGE INTERACTIONS

I. LIGHT MICROSCOPE STUDIES

KENJI TAKEYA, RYOICHI MORI, NORITAKA NAKASHIMA, MASAATSU KOIKE, AND
TADAO TODA

Department of Bacteriology, School of Medicine, Kyushu University, Fukuoka, Japan

Received for publication January 22, 1959

Several mycobacteriophages active against mycobacteria have been isolated by Takeya and
Yoshimura (1957) using the soil-enrichment technique. Among these phages, B-1 was found to have the broadest host range including virulent strains of tubercle bacilli. Among
the Mycobacterium between mycobacteriophage containing approximately 10^8 plaque-forming particles per ml was used for the experiment. For cytological study the preparation technique of Hillier et al., (1948) was employed. Host cells were grown on a collodion film overlying a 3 per cent glycerol agar plate. After cells divided and grew into a microcolony of 10 to 50 cells, they were infected with a drop of the phage. Though the multiplicity of infection could not be determined exactly, the conditions of the experiments provided multiple infection. At appropriate times the cells were fixed with 0.07 per cent OsO₄ or with 10 per cent formalin + 2 per cent K₂Cr₂O₇. The film was then placed afloat on distilled water for 1 to 2 hr to dialyze and remove excess salts and other impurities. Finally the film was taken on a glass slide for examination with a light microscope or on a copper mesh screen for electron microscope examination.

The lysis of host cells caused by phage infection was followed under the phase contrast microscope by the use of an improved technique of Takeya et al., (1952). The technique is outlined as follows. A thin layer of 3 per cent glycerol agar medium is spread on a cover glass and covered with a collodion film. The host cells are next inoculated onto the collodion film, the cover glass is placed on a hollow-ground slide, and fixed there with wax. The slide is then incubated at 37 C. After an appropriate time, the cover glass is removed from the slide and the cells on the collodion film are infected with phage by placing a drop of phage suspension on the film. The cover glass is returned to the hollow-ground slide, and observed continuously under the phase contrast microscope.

Giemsa stain. The preparations were twice treated with 70 per cent alcohol for 5 min each time and were digested with HCl at 60 C for 15 min. They were then stained with a Giemsa solution (2 drops per ml of 0.06 M Sorensen buffer, pH 7.0) for 10 min and washed with tap water.

Neotetrazolium stain. Unfixed cells were suspended in heart infusion broth containing 0.05 per cent neotetrazolium chloride and incubated for 10 min at 37 C. After fixation with 0.07 per cent OsO₄, impression smears were made on glass slides and stained with 0.5 per cent malachite green in aqueous solution as a counter stain. The concentration of neotetrazolium chloride used in this stain proved to be sufficient to inhibit the growth of the host cell.

Metachromatic granule stain. The preparations were stained by Neisser’s acidified methylene blue for 20 min followed by a counter stain of 0.5 per cent malachite green in aqueous solution.

Thionine stain. The preparations were twice treated with 70 per cent alcohol for 5 min each time and stained with 0.01 per cent thionine for 10 min.

1 Presented at the Ninth Annual Meeting of the Kyushu Branch of the Japan Society for Tuberculosis, Kagoshima, Japan, September 14, 1957.
Figure 1. Lysis of Mycobacterium avium (Jucho strain) caused by B-1 phage infection. The host cells were grown on collodion film overlying 3 per cent glycerol agar. After infection with phage, cells were continuously observed under the phase contrast microscope (1380X): a. 30 min. after infection; b. 45 min; c. 65 min, several cells began to lyse; d. 68 min; e. 71 min; f. 75 min; g. 85 min; and h. 137 min.
RESULTS

Growth curve studies. The latent period of B-1 phage infecting the host cells grown on solid media was determined by continuously observing the process of bacterial lysis due to phage infection under the phase contrast microscope (figure 1). The results of this experiment are shown in figure 2, together with one example of the one step growth curve of the same phage infecting the host cells grown in heart infusion broth. The total number of cells in several microcolonies was counted before infection with the phage and the decreasing number of cells after infection was plotted against the time scale. This curve, designated as the “lysis curve,” showed that the latent period of M. avium (Jucho strain)-mycobacteriophage B-1 system on solid media was 60 to 65 min. The “rise” period was measured to be 20 to 30 min. The generation time of the host cells under the same cultural conditions was approximately 140 min.

The length of the latent period obtained by the one step growth experiment in heart infusion broth coincides exactly with that determined from the “lysis curve.” Therefore, the specimens for light microscope study were thereafter prepared by making impression smears from the broth culture by the technique of Murray et al. (1950).

Light cytology of cells infected with B-1 phage.

Figure 3 illustrates the sequence of deoxyribonucleic acid (DNA) stain following the infection of the host cells with B-1 phage. In young uninfected host cells, 2, 4, and occasionally 8, chromatinic bodies were observed (figure 3a). These chromatinic bodies were found to be reduced in number and increased in size from 10 to 20 min after infection (figure 3b and c). Later they converged into one or two large masses of chromatin (figure 3d-f). The cells which contained one large central chromatinic mass became swollen and spindle-shaped. One hundred cells on successive preparations were counted and the distribution of cell types was plotted as shown in figure 6. The changes produced in the chromatinic substance are illustrated in this figure. Cells stained with thionine showed the reverse picture, the chromatinic bodies appearing as unstained areas in a basophilic cytoplasm.

Usually, young uninfected host cells contained 2 to 5 neotetrazolium-reducing granules and 2 to 4 metachromatic granules. Figure 4 illustrates the neotetrazolium-stained cells and figure 5 those stained with Neisser’s methylene blue at 0, 30, and 60 min after infection. There was no apparent alteration in the size, number, and localization of neotetrazolium-reducing granules and of metachromatic granules during the course of infection. Metachromatic granules were often observ-

![Figure 2](Image)

**Figure 2.** "Lysis curve" and one step growth curve of Mycobacterium avium (Jucho strain) infected with B-1 phage. "Lysis curve" was obtained on 3 per cent glycerol agar. The one step growth curve was obtained in heart infusion broth. The length of the latent period obtained in each of the two examples of the "lysis curve" coincides with that determined from the one step growth curve.
Figure 3. HCl-Giemsa stain of *Mycobacterium avium* (Jucho strain) infected with B-1 phage (5400X). a. Before infection; b. 10 min after infection; c. 20 min; d. 30 min; e. 40 min; and f. 60 min.

Figure 4. Neotetrazolium stain of *M. avium* (Jucho strain) infected with B-1 phage (3700X). a. Before infection; b. 30 min, and c. 60 min, after infection.

Figure 5. Metachromatic stain of *M. avium* (Jucho strain) infected with B-1 phage (3000X). a. Before infection; b. 30 min, and c. 60 min, after infection.
Figure 6. Graph showing the distribution of cell types during infection with B-1 phage. Granular cells: cells with large chromatinic bodies more than 1 μ in size. Cells of intermediate type: cells with intermediate-sized chromatinic bodies, 0.5 to 1 μ in size. Normal cells: cells with small chromatinic bodies, approximately 0.5 μ in size. The numbers on the curves represent the mean number of chromatinic bodies per cell.

able even after the lysis of bacteria caused by phage infection.

DISCUSSION

Escherichia coli-coliphage interactions have been studied by cytological techniques. Chromatin staining during T-even phage infection has revealed that disruption of nuclear chromatin is followed by a distribution of the chromatin along the periphery of the infected cells, and then the cells become filled with irregularly dispersed granular chromatin (Luria and Human, 1950; Murray et al., 1950; Beutner et al., 1953). On the other hand, the behavior of chromatin within the mycobacterial cells infected with mycobacteriophage has not yet been reported. The present examination revealed that, following infection, the chromatinic bodies became fewer and larger, finally fusing into one or two large masses of chromatin. This change in chromatinic substance coincides with that in the case of E. coli infected with T7 phage (Luria and Human, 1950) and suggests that B-1 phage production within the mycobacterial cells is a spatially organized process.

The latent and the rise periods of B-1 phage-M. avium (Jucho strain) system were determined to be 60 to 65 min and 20 to 30 min, respectively, from the "lysis curve," which was obtained by continuous observations under the phase contrast microscope, and also from the one step growth curve. These values are somewhat less than those obtained by Bowman (1958) with the D29 phage-Mycobacterium ranae system. The fact that the latent period was measured from the "lysis curve," under the conditions used in the modified preparation technique of Hillier et al. (1948) for electron microscopy, will make it possible to study more accurately the host cell-phage interaction by means of electron microscopy, since this technique is known to have many advantages (Beutner et al., 1953).

In the present study the neotetrazolium-reducing granules, which are believed to represent the bacterial mitochondria (Mudd, 1956), were found to persist unchanged throughout infection. This result coincides with findings of Hartman et al. (1953) that 2,3,5-triphenyltetrazolium reductase activity remained constant during the latent period of T2r infection of E. coli. The main component of the metachromatic granule is apparently polyphosphate (Winkler, 1953; Glauert and Brieger, 1955; Mudd et al., 1956, 1958; Takeya et al., 1959). It has recently been shown that "high energy" phosphate is stored as polyphosphate in microorganisms and serves as an "energy pool" (Yoshida, 1953; Kornberg et al., 1956; Kornberg, 1957). Accordingly, the behavior of metachromatic granules during the latent period was investigated in this experiment but no significant change in number and size of the granules was observed.

SUMMARY

The latent period of mycobacteriophage B-1 on host strain Mycobacterium avium (Jucho strain) grown on a 3 per cent glycerol agar medium was found to be 60 to 65 min using the "lysis curve," which was determined by continuous observations of phage-infected cells under the phase contrast microscope.

Cytological examination of the host cells during the latent period revealed that the neotetrazolium-reducing granules and metachromatic granules were not altered in size, number, and localization, but the chromatinic substance changed in a way similar to that with Escherichia coli strain B infected with T7 phage.
REFERENCES


