LIGHT AND ELECTRON MICROSCOPE STUDIES OF MYCOBACTERIUM-MYCOBACTERIOPHAGE INTERACTIONS

II. Electron Microscope Studies

KENJI TAKEYA, MASAATSU KOIKE, RYOICHI MORI, YASUMA YUDA, AND TADAO TODA

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

Received for publication January 22, 1959

In the previous experiments (Takeya et al., 1959b) the interactions of Mycobacterium avium with mycobacteriophage B-1 were studied by cytological techniques, and the latent period of the B-1 phage infecting the host cell was determined under the conditions used in the preparation for electron microscopy. The present paper deals with the results of experiments on the interactions of mycobacteriophage B-1 with Mycobacterium avium as revealed by electron microscopy.

MATERIALS AND METHODS

M. avium (Jucho strain) and mycobacteriophage B-1 were used throughout the studies. The preparation of the phage suspensions and media, and the basic techniques have been described in the first communication (Takeya et al., 1959b), except the technique for ultrathin sectioning. The morphology of mycobacteriophages B-2, B-3, A-2, A-3, A-4, A-5, A-6, and A-7 (isolated by Takeya and Yoshimura, 1957) was examined by means of the electron microscope. The specimens were air-dried or frozen-dried by Tokuyasu's technique (1966).

Techniques for ultrathin sectioning. Aerated, 3 per cent glycerol broth cultures of young host cells were infected with B-1 phage. At an appropriate time in the latent period, phage production was stopped by the addition of OsO₄ and cells were harvested by centrifugation. The cells were resuspended in isotonic potassium dichromate buffer solution containing 1 per cent OsO₄ and were fixed for 60 min at room temperature. After fixation the cells were dehydrated through a graded series of ethyl alcohol and then embedded with n-butyl methacrylate containing 2 per cent benzoyl peroxide. The preparations were maintained at 45 C for 16 hr for polymerization of the resin. Sections were cut with glass knives using a Porter-Blum ultramicrotome and were picked up on formvar-coated copper mesh screens for electron microscopy. The specimens were examined by a JEM 5C electron microscope equipped with a 50 μ aperture.

RESULTS

Morphology of mycobacteriophages. All 9 strains of phage examined were tadpole shaped (figures 1-4). The heads of the phage particles appeared as regular hexagons in frozen-dried specimens. In frozen-dried specimens of B-1 phage, the diameter of the head was measured to be 70 mμ and the tail 140 to 170 mμ in length and 10 mμ in width. At the distal end of the tail, an end piece was occasionally observed.

Adsorption of the phage to the host cell. The adsorption to the host cells of the phage particles by their tails was clearly observed in the frozen dried specimens (figure 4) and was sometimes observed even in air-dried specimens (figure 3). “Ghost” phage particles, which appeared to have injected phage-deoxyribonucleic acid (DNA) into the cell body, were occasionally found attached to the host cell (figure 5).

Morphological changes of the phage-infected cells during the latent period. Examination of the infected cells during the latent period failed to demonstrate the “morula” and “rosette” presented by Penso (1955) as the intermediate stages of mycobacteriophage production. On the other hand, granules resembling these structures were found in healthy mycobacteria (figures 11-14). Small dense granules in rosette-like arrangement (figure 11) and those vacuolized by intense electron bombardment (figure 12) look like “morula” and “rosette,” respectively. Apparent impurities attached to the bacterial cells and vacuolized by electron bombardment sometimes resembled “rosettes” (figure 14).

1 Presented at the 31st Annual Meeting of the Japan Bacteriological Society, Okayama, Japan, April 6, 1958.
At the end of the latent period, many seemingly mature phage particles were found to be clustered and localized in the host cell (figures 6 and 7). Paired fibrous structures were often seen within lysed cell bodies (figures 9 and 10). Although these structures were first suspected to be concerned in phage multiplication, it is now apparent that they are normal components of the mycobacterial cell because the same structures can be observed in healthy cells mechanically or chemically lysed (Takeya et al., 1958).

**Ultrathin sections of the phage-infected cells.** Ultrathin sections of uninfected normal mycobacterial cells indicated the existence of dense
Figure 4. Adsorption to a host cell of phage particles by their tails. *Mycobacterium avium* (Jucho strain)-B-1 phage system; frozen-dried, chromium-shadowed.

Figure 5. "Ghost phages" are seen attached to a host cell. *M. avium* (Jucho strain)-B-1 phage system; air-dried, chromium-shadowed.

Figure 6. An infected cell at the end of the latent period. *M. avium* (Jucho strain)-B-1 phage system; air-dried, chromium-shadowed.

Figure 7. A host cell 58 min after infection with phages. *M. avium* (Jucho strain)-B-1 phage system; air-dried, unshadowed.

Figure 8. Host cells lysed by phage infection. *M. avium* (Jucho strain)-B-1 phage system; air-dried, chromium-shadowed.
TAKEYA, KOIKE, MORI, YUDA, AND TODA

Figures 9 and 10. A host cell lysed by phage infection. Numerous paired fibrous structures are observable within the lysed cell body. *Mycobacterium avium* (Jucho strain)-B-1 phage system; air-dried, chromium-shadowed.

Figure 11. BCG (Bacillus Calmette-Guérin). Noninfected healthy cells; air-dried, unshadowed.

Figure 12. Same field as figure 11, after exposure to an intense electron beam; air-dried, unshadowed.

Figure 13. *Mycobacterium lepraemurium*. Noninfected healthy cell; air-dried, unshadowed.

Figure 14. *M. avium* (Jucho strain). Noninfected healthy cell; air-dried, unshadowed: g, large dense granule, the main component of which is believed to be polyphosphate.
Figures 15 to 18. Ultrathin sections of host cells 30 min after infection with phages. *Mycobacterium avium* (Jucho strain)-B-1 phage system.

Figure 19. Ultrathin section of a host cell 58 min after infection with phages. *M. avium* (Jucho strain)-B-1 phage system: cw, cell wall; cm, cytoplasmic membrane; m, mitochondria-like structure.

granules, nuclear sites, and mitochondria-like structures within the cytoplasm. The details of these structures will be reported elsewhere (Takeya *et al.*, 1959a; Koike *et al.*, 1959, unpublished data). The examination of the cells 10 min after infection failed to reveal any obvious morphological change. About 30 min after infection, radical changes were observed in the organization of the intracellular structures. Dense particles and doughnut-form particles, which correspond in shape and size to phage heads, were found in a localized focus where the density was lower than that of the cytoplasm (figures 15–18). These particles were often found entangled with irregu-
larly interwoven fine fibrils (figures 16 and 17). The dense particles appeared to be hexagon-shaped. These foci were often located near the nuclear vacuole. Ultrathin-sectioned cells at the end of the latent period presented themselves as seemingly lysed ghost cells (figure 19). This may have resulted from the premature lysis because phage-infected bacteria are believed to become gradually susceptible to lysis by OsO₄ (Maaløe et al., 1954). The dense granule and the mitochondria-like structure remained unchanged throughout the latent period, even in lysed cell bodies (figures 10 and 17).

**DISCUSSION**

Electron microscope studies of the mycobacteria-myco bacteriophage system were reported by Penso (1955) and Sellers et al. (1957). In their studies, however, the latent period was not measured exactly under the conditions used in their preparations for electron microscopy. In the first communication (Takeya et al., 1959b), the latent period for mycobacteriophage B-1 infecting *M. avium* (Jucho strain) was determined under the conditions used in the preparations for electron microscopy; hence, much more accurate results would be expected from the present examination.

Penso (1955) presented a unique hypothesis on mycobacteriophage production within the host cell based on his electron micrographs. According to Penso, the head and tail of the mycobacteriophage (Phagus lactiola) are filled with a number of small granules ("sporophages") which represent the genetic portion of the phage. The "sporophages" penetrate the cell and multiply, giving rise to the formation of moruliform bodies ("morula"). The separate components of the "morula" become vacuolized, multiply, and then turn into a group of ring-shaped particles ("rosette"). Each component later becomes a mature phage particle. In our experiments, the phage tail was also found to have a bead-like granular structure (figure 2), but this might have been artifact produced during the course of specimen preparation. The end piece of the phage tail, which has also been reported in mycobacteriophages by Sellers et al. (1957), may be an "attachment organ."

The examination of the phage-infected cells during the latent period failed to demonstrate the structures which correspond to the "morula" and the "rosette," in both unsectioned and sectioned preparations. During the latter half of the latent period, dense particles similar in shape and size to the phage head were found clustered in a focus or foci within the unsectioned and also the sectioned cells. This finding coincides with the results of staining DNA (Takeya et al., 1959b), which resembles T7 phage-infected cells of *Escherichia coli*, and shows that the mycobacteriophage production is a spatially organized process. All these facts indicate that Penso's hypothesis cannot be applied to the *M. avium*-mycobacteriophage B-1 system. Moreover, our experiments may suggest that the mycobacteriophage production in general is not a specially organized process similar to that of other living organisms, as has been postulated by Penso (1955), but a process essentially analogous to coliphage production. Granular structures found in the phage-infected mycobacterial cells should be carefully interpreted because structures resembling "morula" and "rosette" can often be observed in healthy mycobacterial cells.

The mitochondria-like structures observed in sectioned preparations have recently been considered to correspond to the mitochondria of plant and animal cells (Shinohara et al., 1958). The fact that the structures persist unchanged throughout the phage infection, and the results of the neotetrazolium staining of the previous experiment (Takeya et al., 1959b), suggests that the functioning of the mitochondria might be important to intracellular production of phage. The dense granules, which are believed to correspond to the metachromatic granules in the light microscope preparation (Glaeurt and Brieger, 1955; Takeya et al., 1959a), also appeared to remain unchanged throughout the phage infection.

The techniques used in fixation and embedding for the ultrathin sections used in this study appear to be unsatisfactory because the nuclear material was found conglomerated within the nuclear vacuole. Further studies of ultrathin sections with improved techniques will provide more accurate and detailed information.

**SUMMARY**

The interactions of *Mycobacterium avium* (Jucho strain) with mycobacteriophage B-1 were studied by means of the electron microscope. Mycobacteriophage B-1 was revealed to have
a hexagonal head and a slender tail. The end piece was occasionally observed. Examination of phage infected cells during the latent period revealed that the phage production was localized in a focus or foci within infected cells and Penso’s hypothesis on the production of mycobacteriophage could not be applied to the bacterium-phage system used in this experiment. The mitochondria-like structure found in the ultrathin sections was persistent and unchanged throughout the latent period.

REFERENCES
Penso, G. 1955 Cycle of phage development within the bacterial cell. Protoplasma, 45, 251-263.