GROWTH CHARACTERISTICS IN HEla CELLS OF THE RAPIDLY GROWING ACID FAST BACTERIA, MYCOBACTERIUM FORTUITUM, MYCOBACTERIUM PHLEI, AND MYCOBACTERIUM SMEGMAtIS

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With the techniques found suitable for the growth of tubercle bacilli in HeLa cells (Shepard, 1955, 1957a) little extracellular bacterial growth occurs when virulent strains are used. When rapidly growing mycobacteria (Gordon and Smith, 1953, 1955) are introduced into the tissue cultures in the same concentration, bacterial growth in the extracellular tissue culture fluid is rapid and soon results in death of the HeLa cells.

To be able to observe whether the well known saprophytes Mycobacterium phlei and Mycobacterium smegmatis and the potentially pathogenic Mycobacterium fortuitum could grow in HeLa cells, it was necessary to modify the procedures in two respects. The first was the addition of 500 μg streptomycin/ml of tissue culture fluid after the bacilli had been phagocytosed by the HeLa cells, so that the growth of bacilli remaining outside the cells would be suppressed. It had previously been shown that concentrations of streptomycin up to 2,000 μg/ml were without effect on intracellular growth of tubercle bacilli (Shepard, 1957b). The second modification was introduced because observations of stained smears revealed that many large clumps of organisms were present in the bacterial inocula prepared from the rapidly growing mycobacteria when the method found suitable for tubercle bacilli was followed (Shepard, 1955, 1957a). This change consisted of filtering the bacterial suspension through filter paper (Fenner, 1951).

The classification of the rapidly growing acid fast bacteria, M. phlei, M. smegmatis, and M. fortuitum, has been placed on a firm basis by the studies of Gordon and Smith (1953, 1955). Wells et al. (1955) studied the pathogenicity in animals of a culture of M. fortuitum which they had isolated from a patient with cellulitis and lymphadenitis. A distinctive disease picture was produced in mice following intraperitoneal injection of this culture, as well as of 8 of 9 cultures of M. fortuitum which these workers received from Dr. Gordon.

MATERIALS AND METHODS

The procedure followed was that described elsewhere (Shepard, 1955, 1957a) with the modifications given below. The medium used to promote phagocytosis of the mycobacteria was, in most instances, 10 per cent of a suitable horse serum in BSS (Hanks and Wallace, 1949) to which were added the amino acids, vitamins, and glutamine recommended by Eagle (1955). The mycobacterial cultures which had been grown in media of the Tween-albumin type for 48 hr were centrifuged, resuspended in 2 ml of BSS, filtered through Whatman no. 1 filter paper, and then diluted until they showed only faint turbidity of the streaming type when stirred. Filtering was carried out through 7 cm circles which had been folded in eighths and inserted into 18 by 150-mm test tubes, the tightness of the fit being sufficient to hold the papers near the tops of the tubes. Sterilization was by autoclave, and just before use the papers were moistened with BSS. In this procedure it is necessary to observe carefully that the fluid added to the filter paper does not escape over the sides of the cone through folds in the paper.

The mycobacterial suspension was added in 0.05-ml amounts to the tissue cultures which now contained 1 ml of medium containing horse serum. The following day the coverslips with their attached cells were washed twice with 1 ml of BSS, and 1 ml of 40 per cent human serum in BSS was added. Changes of culture fluid were then carried out every two days. Streptomycin was added only to the medium containing human serum. At appropriate intervals the coverslips with their attached cells were washed in BSS,
Figure 1 (left). Mycobacterium phlei strain 451 one day after being added to HeLa cells. The bacilli have been phagocytosed. Enlargement 310 X in all figures.

Figure 2 (right). Mycobacterium phlei strain 451 at three days. Although phagocytosed, the bacilli do not show growth.

Figure 3 (left). Mycobacterium smegmatis strain 470 at one day. Phagocytosis has taken place.

Figure 4 (right). Mycobacterium smegmatis strain 470 at three days. No bacterial growth.
fixed in formalin, and stained by a cold acid fast procedure with Giemsa as a counterstain.

Three strains of *M. phlei* were studied, one of which was received from the Bacteriology Section of the Communicable Disease Center (CDC) in Chamblee, Georgia, and two from Dr. Ruth M. Gordon of Rutgers University. Of the latter two no. 451 was isolated from soil and no. 479 from bovine mastitis. Three strains of *M. smegmatis* were studied, one from the Bacteriology Section of CDC and two from Dr. Gordon, no. 454 from soil and no. 470 from bovine mastitis. The three strains of *M. fortuitum* studied were all from Dr. Gordon, no. 389 from barnyard soil, N8573 which was isolated by Wells from human cervical adenitis, and no. 435 from sputum. The cultures from Dr. Gordon have been described (Gordon and Smith, 1955).

**RESULTS**

In figures 1–4 results with *M. phlei* and *M. smegmatis* are shown. Although these organisms were phagocytosed by the HeLa cells, no bacillary growth ensued. The illustrations cover only the first three days after infection, but other experiments carried out over longer periods (up to nine days) have consistently failed to show any evidence of growth by these organisms in HeLa cells. Cellular infection was much more frequent than is revealed by the illustrations due to the limitations of depth of focus and of reproduction in black and white. Three strains of *M. phlei* and three of *M. smegmatis* were studied and none showed evidence of growth.

In distinct contrast were the results with *M. fortuitum*, which are illustrated in figures 5–9. On the first day after addition of the bacterial inocula, bacterial growth was manifested by the large numbers of bacilli within the cells. An accompanying change was a marked increase in

*Figure 5* (upper). *Mycobacterium fortuitum* strain 389 at one day. Phagocytosis has occurred and intracellular growth is manifested by the large numbers of cytoplasmic organisms and an increase in their average length.

*Figure 6* (center). *Mycobacterium fortuitum* strain 389 at two days. Further intracellular growth and decreased average length of bacilli.

*Figure 7* (lower). *Mycobacterium fortuitum* strain 389 at three days. Further intracellular growth. Occasional clubbing is seen at this stage with all strains.

Figs. 5-7
average length of the bacilli. Such lengthening was not observed with *M. phlei* and *M. smegmatis* within cells. Examination of smears of the bacterial inocula introduced into the culture did not serve to differentiate *M. fortuitum* from *M. smegmatis* and *M. phlei* at that stage.

On the following two days there was a further growth of *M. fortuitum* in the cells. This was accompanied by a shortening of the organisms, many of which grew in dense masses in which the resolution of individual organisms required careful focusing. A cytopathic change also resulted, consisting of an aggregation of red staining material within the cell in the vicinity of the bacterial mass. In cells in which bacterial growth was more advanced, there was also a folding up and withdrawal of cell edges, nuclear pyknosis, and finally death and sloughing of the cell. Other mycobacteria grown in HeLa cells to date, namely, tubercle bacilli and organisms of the *M. marinum-M. balnei* group, have produced a cytopathic effect so slight that it may be only the result of mechanical interference with cell function by the mass of bacteria.

No important differences were seen among the strains of *M. fortuitum* except that strain 435 showed rather frequent branching (figure 9). This was seen especially on the first day when the bacilli were well spread out and did not appear to result from accidental superposition of neighboring bacilli. Similar branching was not observed with the other strains. The table of Gordon and Smith (1955) indicates that this strain had been suspected of being a species of Nocardia.

**DISCUSSION**

It is to be noted that the origin of the strains, whether from soil or diseased tissue, did not affect the results here seen with any of the three species of mycobacteria. The growth of strains of *M. fortuitum* was rapid enough in HeLa cells to be obvious in one day. This species could thus be differentiated at this stage from strains of *M. phlei* and *M. smegmatis*, which were also phagocytosed but showed no signs of growth. On the second and third day the growth of all three of these species in the extracellular tissue culture fluid was enough to destroy the tissue culture, unless streptomycin was present. The drug protected the HeLa cells and made it possible to observe that, although *M. fortuitum* continued to grow until death of the infected cells was produced, neither *M. phlei* nor *M. smegmatis* showed any growth even though they appeared to be lying in the cytoplasm of the HeLa cell for days afterward.

This difference between *M. fortuitum*, thought to be pathogenic for humans, and *M. phlei* and *M. smegmatis*, considered to be nonpathogenic, is evidence that the resistance of the intact animal against these nonpathogenic species is manifested also by a cell line in tissue culture. A similar
relationship between pathogenicity and rate of growth in HeLa cells was seen among strains of tubercle bacilli of different degrees of virulence (Shepard, 1957b).

The ease of visualization of mycobacteria makes it possible to work out some of the mechanisms affecting their growth in cells in a way that would be difficult with smaller infectious agents such as viruses. Thus, it could be seen that absence of phagocytosis was the reason for the failure of tubercle bacilli to show growth in HeLa cells kept only in media containing human serum (Shepard, 1955). The demonstrability of mycobacteria also made it possible to observe that the slow growth in HeLa cell cultures of tubercle bacilli of modified virulence, such as H37Ra and BCG, as compared to the more rapid growth of fully virulent strains, was the result of slow multiplication in the cytoplasm of the individual infected cell, and was not due to delayed or inefficient cellular infection or release. In the present studies the failure of *M. phlei* and *M. smegmatis* to show growth could be associated with the cytoplasmic environment.

An increase in size is a usual concomitant to early bacterial growth and the increase in length of mycobacteria early in their growth cycle has been made use of by Fenner (1956) in studies of *M. ulcerans*. The increase in length is considerably greater with *M. fortuitum* than with the other mycobacteria studied in HeLa cells.

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**SUMMARY**

Techniques are described for the study of the rapidly growing acid fast bacteria in HeLa cells. Bacilli of the nonpathogenic species *Mycobacterium phlei* and *Mycobacterium smegmatis* when introduced into HeLa cells remained in the cytoplasm for days without signs of growth. In contrast, the growth of *Mycobacterium fortuitum* in the cytoplasm was rapid enough to be visible in one day. Growth continued in the succeeding days and was accompanied by a cytopathic effect.

**REFERENCES**


