GROWTH CHARACTERISTICS OF TUBERCLE BACILLI AND CERTAIN OTHER MYCOBACTERIA IN HeLa CELLS

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PLATES 1 TO 4

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The growth of tubercle bacilli in tissue culture is an old observation, dating back to the earliest days of tissue culture. One hundred and thirty references are cited in Murray and Kopeck's Bibliography of Tissue Culture (1), and many of the earlier works have been discussed in recent years (2, 3). In the earlier tissue culture technics pieces of tissue were suspended in the medium or embedded in plasma clots, and the growth which followed was usually not as vigorous or consistent as that which follows the use of the monolayer technic in which the cells grow in a single layer on a glass surface. In the monolayer technic materials such as nutrients, metabolites, and infectious particles may exchange freely between the volume of the tissue culture fluid and the immediate environment of living cells.

It has been recently observed that cells of the strain HeLa (Gey) in monolayer engage in more active phagocytosis of tubercle bacilli when certain horse sera are incorporated into the tissue culture medium (4). The effect is a pronounced one, and it is possible to increase the proportion of cells that become infected in one day from one in several hundreds in 40 per cent human serum to half or more of the cells in 40 per cent horse serum. The phenomenon has been observed with monkey kidney cells and cells of strain KB (Eagle) and extends to non-viable particles, such as dead tubercle bacilli, carbon particles, and particles of diatomaceous earth. Other mycobacteria are also phagocyted under these conditions, so that it is possible to introduce various strains of mycobacteria into HeLa cells and observe their behavior in these cells of human origin.

With the technic used here it is usually possible to limit the new infection of cells to one particular day, so that the subsequent development of events is fairly uniform from one infected cell to the next. Such a uniformity is of considerable assistance in the interpretation of the experimental findings, for example, in judging the relative intracellular growth rates of two strains or in comparing the patterns of intracellular growth for two strains.

It was found in a general way that the mycobacteria with apparent patho-

genicity for the human species are able to multiply in HeLa cells, and that among the tubercle bacilli examined there is a direct correlation between the apparent pathogenicity of the strain for man and its growth rate in HeLa cells. In addition there is a helpful degree of individuality of the growth pattern according to mycobacterial species and strain.

Materials and Methods

The HeLa cells were received weekly in bottles from the George Washington Carver Foundation, Tuskegee, Alabama. In the present work the cells were pushed off the glass with a rubber policeman into the growth medium of Syverton *et al.* (5) and broken up with the use of a bulb and pipette. Trypsinization procedures are also satisfactory. The cell suspension was then diluted to contain about 200,000 cells per ml. by the addition of growth medium, and 1 ml. of the suspension added to each tube. Leighton tubes were used. They had flattened sides large enough to receive 8 × 30 mm. coverslips on which the cells settled and commenced to multiply. 2 days later the growth medium was removed and the coverslips and attached cells washed in the tube twice with 1 ml. of balanced salt solution (BSS) (6).

The medium used to promote phagocytosis and thus accomplish infection of the cells was then added in the amount of 1 ml. It consisted of 40 per cent horse serum in BSS, or 10 per cent horse serum in BSS containing the amino acids and vitamins recommended by Eagle (7). Not all horse sera tried were effective in the promotion of phagocytosis, but since an effective serum remained so for a period of at least 6 months, good specimens either of commercial origin or of our own collection were sought and relied on for work such as this. Although there have been variations between lots of serum taken from the same horse, there have been greater differences among individual horses. A workable procedure has been to search for a horse that gives a good serum and then to continue to use the same animal. In comparing serum samples, the number of cells per microscopic field that become infected after 24 hours in the medium containing the horse serum in question was compared to that of a known effective sample in the same test. The suspension of mycobacteria in 0.05 ml. was added shortly after the addition of the medium containing horse serum.

After 1 day in the horse serum medium, change was made to one consisting of 40 per cent human serum in BSS. Changes of medium were then made every 2 days as routine. The reasons for the use of human serum in the tissue culture medium after 1 day are that HeLa cells thrive better in media made from human serum, and most of the phagocytosis that will occur has been completed during the 1st day.

At appropriate intervals, for example, 1, 3, and 5 days after addition of the mycobacteria, the coverslips and cells still in the tube were washed twice with 1 ml. of BSS, and 2 ml. of neutral formalin were then added. After at least 3 hours of fixation, dehydration was performed in 50 and 95 per cent ethanol, and the coverslips removed from the tube to racks made of glass rods, where they were allowed to remain for at least 1 hour after they appeared dry. Carbolfuchsin was then added, and after 20 minutes washed off with water. Destaining was carried out with 1 per cent HCl in 95 per cent ethanol for a period that did not exceed 3 seconds. The cells were counterstained with Giemsa twice its usual strength at pH 6.5 for 5 minutes. After being washed and allowed to dry the coverslips were mounted on microscope slides.

The mycobacterial suspensions for infection of the tissue cultures were prepared from cultures in tween-albumin medium (Difco's Tb-tween), which had been inoculated 3 to 7 days previously from cultures maintained on Loewenstein-Jensen medium. Except when otherwise stated bacterial cultures were obtained from the Bacteriology Section of the Communicable Disease Center, Chamblee, Georgia. Incubation of bacterial cultures was at 37°C except for *Mycobacterium balnei*, *Mycobacterium marinum* and *Mycobacterium platypoecilus*, which were

grown at 35°C. The suspensions were prepared by stirring the liquid culture to suspend the sediment, allowing it to stand a few minutes to allow the larger clumps to settle, pipetting off the supernatant to another tube which was centrifuged at 3000 R.P.M. for 30 minutes, the supernatant discarded, the sediment resuspended in BSS by careful and forceful use of a bulb and pipette, and the turbidity adjusted by adding more BSS until there remained a faint but distinct turbidity which showed streaming when the suspension was stirred. Plate counts of such suspensions showed 2 to 5 $\times$ 10^5 viable units in the 0.05 ml. of inoculum. Turbidity of the streaming type was a constant characteristic of suspensions seen with the microscope to contain bacilli primarily in singles or small groups, and it was such suspensions that were especially favorable to phagocytosis. Larger clumps of bacteria gave turbidity which did not show streaming and resulted in a low rate of cell infection.

Since antibiotics were not employed as routine, bacteriologically sterile technic was used but tube mouths were only lightly flamed. Because of the risk of laboratory infection, all of the possibly dangerous operations were carried out in an infectious disease hood.

The technics described here eliminate much of the variability seen especially with plasma clot tissue cultures, but other types of variability should be recognized in interpreting results. Variations among infected cells in the same preparation are, of course, controlled by the examination of many infected cells on the coverslip. When comparing the results with two mycobacterial strains in the same experiment it is also necessary to remember that the progress of infection may be hastened if the number of bacteria phagocyted by a cell on the first day was particularly high. Such excessive initial infection to a cell occurs more frequently following the use of a bacterial inoculum which is more concentrated, or one in which there are more small clumps of bacilli, rather than singles. Thus it is important to compare the results at earlier stages, for example, at 1 and 3 days, and not to rely on the results seen at 5 days. In doubtful cases it is helpful to examine the slides after their labels have been covered with pieces of adhesive tape, and the slides mixed in a random fashion.

The illustrations presented were obtained with inocula diluted as described above. For reasons of economy the illustrations are largely confined to a few typical infected cells at one rather mature stage of development.

EXPERIMENTAL RESULTS

Tubercle Bacilli.—One day after adding a suspension of H37Rv strain of the human tubercle bacillus to the tissue culture fluid, cells become infected by phagocytosis (Fig. 1). Bacilli in singles or small clumps are taken into the cytoplasm, but bacterial growth is not yet apparent. At this stage the cells have been exposed to the bacilli in a medium containing horse serum. It is difficult to differentiate the various strains of mycobacteria by observations at this stage. 3 days after the addition of the bacterial suspension (2 days later than Fig. 1) bacterial multiplication is evident (Fig. 2) and at 5 days bacterial growth is pronounced and large intracellular cords can be seen (Fig. 3). The cords form in fantastic shapes in the central part of the cytoplasm and sometimes loop around the nucleus. This distinctive growth pattern has been seen in all cases with fully virulent laboratory cultures of human and bovine tubercle bacilli kept on Loewenstein-Jensen medium, and with freshly isolated cultures from human sputum. More significantly, it has been observed when tubercle bacilli were grown in HeLa cells directly out of tuberculous sputa without intermediate cultivation on bacteriological media. In each of 39 instances to date when

tubercle bacilli have been grown in HeLa cells by the direct inoculation of sputa into the tissue culture fluid, the pattern of intracellular growth has been indistinguishable from that illustrated here for strain H37Rv (8). This is interpreted to be the standard picture for fully virulent tubercle bacilli growing in HeLa cells. The low degree of cytopathic effect is notable, and even when the nucleus is pushed aside by the mass of bacterial growth only minimal changes in cytology were noted. Eventually the bacterial mass enters the extracellular fluid, and the infected cell dies, as shown in Fig. 4 at 7 days. Not much spread of the infection among cells seems to occur with tubercle bacilli, and if the inoculum is diluted so that only an occasional cell becomes infected, that cell goes through the same course of events without involving its neighbors. Finally, when the extracellular growth is prominent some infection of underlying cells appears to occur. Extracellular cords can be differentiated microscopically from intracellular cords by their greater density, apparently due to their greater thickness.

The Ravenel strain of the bovine tubercle bacillus grows very similarly to the human strains; Fig. 6 shows the result at 5 days. Although there is a tendency toward stiffer appearing cords with this strain, another strain marked Copenhagen was not distinguishable from human strains.

The avirulent variant H37Ra (9) multiplies more slowly in HeLa cells than H37Rv. A typical result at 5 days is shown in Fig. 7. BCG appears to grow at about the same rate as H37Ra, as can be seen in Fig. 8 taken at 5 days. This strain was isolated from BCG vaccine obtained from the Research Foundation, Chicago. The Birkhaug strain of BCG was not distinguishable. With both H37Ra and BCG there is a tendency for the inoculum to contain small clumps of bacilli, so that the amount of growth seen at 5 days in two experiments may vary. The pattern of bacillary growth always allowed differentiation of H37Ra from BCG.

The strains R1Rv and R1Ra (10) were kindly sent by Dr. William Steenken, Jr., of the Trudeau Laboratory. R1Rv is able to produce a chronic infection in normal guinea pigs, but is capable of causing progressive and fatal infections in silicotic guinea pigs. The variant R1Ra produces only a local, retrogressive lesion in normal or silicotic guinea pigs. When introduced into HeLa cells, R1Rv was observed to grow distinctly more rapidly than BCG or H37Ra, but less rapidly than the fully avirulent strains. No well developed intracellular cording was seen, but there was a clear tendency for parallel alignment. R1Ra, on the other hand, did not show clear evidence of growth even at 7 days in experiments in which H37Ra and BCG in parallel tubes showed their usual amounts of growth.

In Fig. 5 can be seen the result at 5 days with a strain of H37Rv which we maintained for several months by sometimes irregular transfers in tween-albumin medium. Though there is no tendency to form intracellular cords, the

growth rate is not noticeably less than H37Rv maintained on Loewenstein Jensen medium, so that intracellular cording apparently is not necessary for rapid intracellular growth of tubercle bacilli.

Six strains of INH-resistant human tubercle bacilli were studied.[1] All of these strains grew in HeLa cells at rates which could not be differentiated from H37Rv. None manifested the strong tendency for intracellular cording of Fig. 3, but all showed some tendencies for parallel alignment in amounts that bore no relationship to their reported pathogenicity for guinea pigs. A fourth strain of tubercle bacilli sent by Dr. Middlebrook was labelled Pearson I and described as being "pathogenic and not INH-resistant," and this strain also showed diminished intracellular cording. The significance of decreased intracellular cordings in strains that have been kept on laboratory media for some time is not clear at present. From the results here it would seem to bear no strict relationship to pathogenicity for guinea pigs, but the relationship to pathogenicity for man is not known.

Rapidly Growing, Acid-Fast Bacteria. Mycobacterium phlei, Mycobacterium smegmatis, and *Mycobacterium fortuitum*, cultures of which were kindly sent by Dr. Ruth E. Gordon of Rutgers University, New Brunswick, New Jersey, have been studied and their behavior will be reported in detail elsewhere (12). Due to their rapid growth in the tissue culture fluid, the experimental conditions used above had to be modified somewhat, *e.g.*, by the addition of streptomycin to the human serum media used after the bacilli had entered the cytoplasm, *i.e.*, after the 1st day. The doses of streptomycin used were much below that dose shown to be without effect on tubercle bacilli which had entered the cytoplasm of HeLa cells (13). In brief, the findings were that *M. phlei* and *M. smegmatis* gave no evidence of intracellular growth, whereas *M. fortuitum* did multiply in HeLa cells.

Mycobacteria with Optical Temperature below 37°C.—The tendency for leprosy to involve skin has been explained by many as the result of a temperature optimum of *Mycobacterium leprae* at some point below 37°C., so we were interested in observing the behavior of *Mycobacterium balnei*, which has caused chronic granulomas in skin abrasions received in certain swimming pools from which the organism could be isolated (14). *M. balnei* has an optimal temperature of 31–35°C. on bacteriological media. Cultures were obtained from the American Type Culture Collection and bore the designations BIV, V, X, and XVI of

[1] Two strains, Clift 1a, and Kowski were kindly sent by Major W. C. Morse of Fitzsimmons Army Hospital, Denver. Their INH resistance, catalase activity, and pathogenicity for guinea pigs were reported (11). Three strains sent by Dr. Gardner Middlebrook of the National Jewish Hospital, Denver, were marked "Pearson IIF, INH-resistant, catalase-positive, diminished pathogenicity; Pearson III, INH-resistant, catalase-negative, apathogenic; and Waites, INH-resistant, catalase-positive, pathogenic for guinea pigs." A strain of H37Rv, INH-resistant, was kindly sent by Dr. Steenken. Tests in this laboratory showed that INH resistance was still present.

Linell and Norden (14). Fig. 9 illustrates the rapid growth of strain X in HeLa cells at 35°C. A feature of its growth at 35°C. or lower is its tendency to involve more cells from day to day. Since this is especially seen in the neighborhood of heavily infected cells, the impression is gained that the bacilli are occasionally able to transfer to neighboring cells. The net result during the first 3 days is the appearance of some heavily infected cells along with a large increase in the number of cells with only a few bacilli (Fig. 9). BIV, V, and XVI could not be distinguished from X. *M. marinum* is not unlike *M. balnei* in its behavior on laboratory media, in that it also has an optimum below 37°C., and its growth is similar in appearance and rate of formation. It has been isolated from diseased tissues of cold blooded animals. A culture of *M. marinum* was kindly contributed by Dr. Gordon, who also sent a culture of a similar organism called *M. platypoecilus* (15). These two strains grew similarly to *M. balnei* in the cells, but showed some individual differences. *M. marinum* gave the most intracellular growth seen with any mycobacterium so far and seemed to enlarge the cell and push the nucleus to one end (Fig. 10). An increase in number of infected cells on successive days was not seen. The growth of *M. platypoecilus* in HeLa cells closely resembled both *M. balnei* and *M. marinum*, but the curved and bent form of the bacillus characteristically modified the growth pattern (Fig. 11). *M. balnei*, *M. marinum*, and *M. platypoecilus* also grew in the cells at 37°C., but less than at 35°C. The growth at 31 and 33°C. was similar to that at 35°C.

DISCUSSION

The early work with mycobacteria in tissue culture indicated only slight cytopathic effect of tubercle bacilli, and the present work confirms this finding. It is surprising to see how much of the cytoplasmic volume can become occupied by mycobacteria, with but slight effect on the morphology of the cell itself. The relative innocuousness of mycobacteria in tissue culture would seem to be a necessary concomitant of their plentiful growth in living cells.

A relationship between the formation of cords by strains of tubercle bacilli growing on bacteriological media and virulence of the stain was pointed out by Middlebrook, Dubos, and Pierce, who state that short but definite cords could be seen in the brains of mice infected with a culture of H37Rv, and that cords were readily demonstrated in the yolk sacs of chick embryos infected with virulent mammalian tubercle bacilli (16). Presumably these observations referred to extracellular growth. We are indebted to Dr. Jules Freund of The Public Health Research Institute of New York City for pointing out that A. Maximow in 1928 illustrated cord formation by tubercle bacilli growing in cells in tissue culture (24).

The HeLa cell–mycobacterium system shows some interesting similarities and dissimilarities to mycobacterial diseases in the intact animal. There is apparently no part played by hypersensitivity or humoral immunity. Two types

of resistance to mycobacterial infection seem to be manifested by these cells. One is the simple matter of presence or absence of phagocytosis; that is, it would be a function of the cell surface, and could be modified by the incorporation of equine or human sera in the tissue culture media. As was stated above, the same changes in tissue culture media affect phagocytosis in monkey kidney and KB cells in monolayer tissue culture. Whether similar changes can be effected in the intact animal remains to be seen. It is possible that the resistance of many cell types in the intact animal to agents to which the animal is susceptible, may be the result of the cells' failure to take the agent into their cytoplasm. That the interior of a cell may provide a favorable environment for the multiplication of an infectious agent, and yet escape infection is shown by the fate of HeLa cells in human serum medium containing tubercle bacilli (4).

A second type of resistance is encountered by the mycobacterium after it enters the cell and was manifested by the decreased growth of H37Ra and BCG as compared to H37Rv and other "virulent" mammalian tubercle bacilli, and by the lack of growth of *M. phlei* and *M. smegmatis* in the cells. This would seem to be related to the resistance of the intact animal to non-pathogens, and it is interesting to find it displayed by a cell in tissue culture. Similar results are common in virology in which the susceptibility of the intact animal to a particular virus is reflected by cells of the animal in tissue culture. It appears to be a useful property of cells such as the HeLa cell, since it may offer a means of testing virulence of mycobacteria for man.

The tubercle bacilli studied may be arranged in order of their respective rates of growth in HeLa cells: (*a*) H37Rv and other apparently fully virulent tubercle bacilli; (*b*) R1Rv; (*c*) H37Ra and BCG; and (*d*) R1Ra. This closely resembles the order found by Pierce, Dubos, and Schaeffer for the pathogenicity of the same strains in mice (17), and is the same as that manifested in guinea pigs (17). The virulence of the INH- resistant strains in mice and guinea pigs is, however, frequently much decreased, whereas in HeLa cells their rates of growth were not found to be different from fully virulent strains. Since the pathogenicity of an infectious agent for man is, however, not easily measurable, the ability of INH-resistant strains to produce disease in man is in dispute (18).

The most intracellular mycobacterial growth was observed with the cultures *M. balnei*, *M. marinum*, and *M. platypoecilus*, and this when the cell cultures were kept at 31–35°C. *M. balnei* has been observed in mice to involve preferentially the tissues where the temperature is lower, such as the scrotum, feet, and tail. The usual site of the lesions in humans infected with *M. balnei* is the skin of the elbows which has an average temperature below 37°C (19). The amount of intracellular growth seen with these mycobacteria with optima below 37°C. on bacteriological media would suggest the potential pathogenicity of all these strains for man in tissue with lower temperatures.

The similarity of behavior of these cultures in HeLa cells emphasizes their

close relationship, and would tend to make less important the differential criterion used to separate *M. balnei* and *M. marinum*; *viz.*, pathogenicity for mice (14).

Mackaness (20) and Suter (21) have in recent years shown that tubercle bacilli grow in rabbit and guinea pig macrophages which are maintained *in vitro*, and with their technics have studied many important problems of experimental tuberculosis. Since the macrophage in the body is an important cell in tuberculosis, the fate of mycobacteria in human macrophages rather than HeLa cells might be more prophetic of the outcome of human infections with the strain in question. Although we have not studied the macrophage systems of Mackaness or Suter in parallel with HeLa cell cultures, it seems clear that growth of the bacilli is more rapid in the HeLa cell. The HeLa cell is more convenient and differs from macrophages in being relatively sturdy and more active metabolically. In considerations of susceptibility to infectious agents of various types of cells in tissue culture it may be pertinent to consider the experiences with viruses. Knowledge of the cell type in which a virus propagates most actively in the intact animal does not necessarily allow the prediction of the cell type which will in tissue culture allow the most rapid growth of virus. The now classic example of this is the growth of poliovirus in tissue culture containing no nervous tissue (22). On the other hand, the species of animal giving origin to the tissue culture is of great importance in the case of viruses (23).

SUMMARY

By making use of the increased phagocytosis which follows the exposure of HeLa cells to tissue culture media containing selected horse sera, it was possible to introduce all of the mycobacterial species studied into the cells, where many of them proceeded to grow.

Fully virulent strains of tubercle bacilli filled much of the cytoplasm in a few days and formed characteristic cords not seen with other strains. The strains said to be less virulent, R1Rv, BCG, H37Ra, and R1Ra, grew less rapidly and in characteristic patterns. Their rates of multiplication in HeLa cells were in the order named and correlated well with their reported pathogenicity for mice and guinea pigs. Six INH-resistant strains grew at rates characteristic of fully virulent strains.

Among the "rapidly growing" species, *M. phlei* and *M. smegmatis* did not show evidence of growth in the cells, although *M. fortuitum* did.

Some strains with optimal temperatures on bacteriological media below 37°C, *M. balnei*, *M. marinum*, and *M. platypoecilus*, grew rapidly in HeLa cells, especially at temperatures of 31 to 35°C.

The growth patterns of the bacilli in HeLa cells appear sufficiently specific to be useful in differentiation among the mycobacteria.

The technical assistance of Mrs. Mary Eleanor Jones and of Miss M. Nannett Green is gratefully acknowledged.

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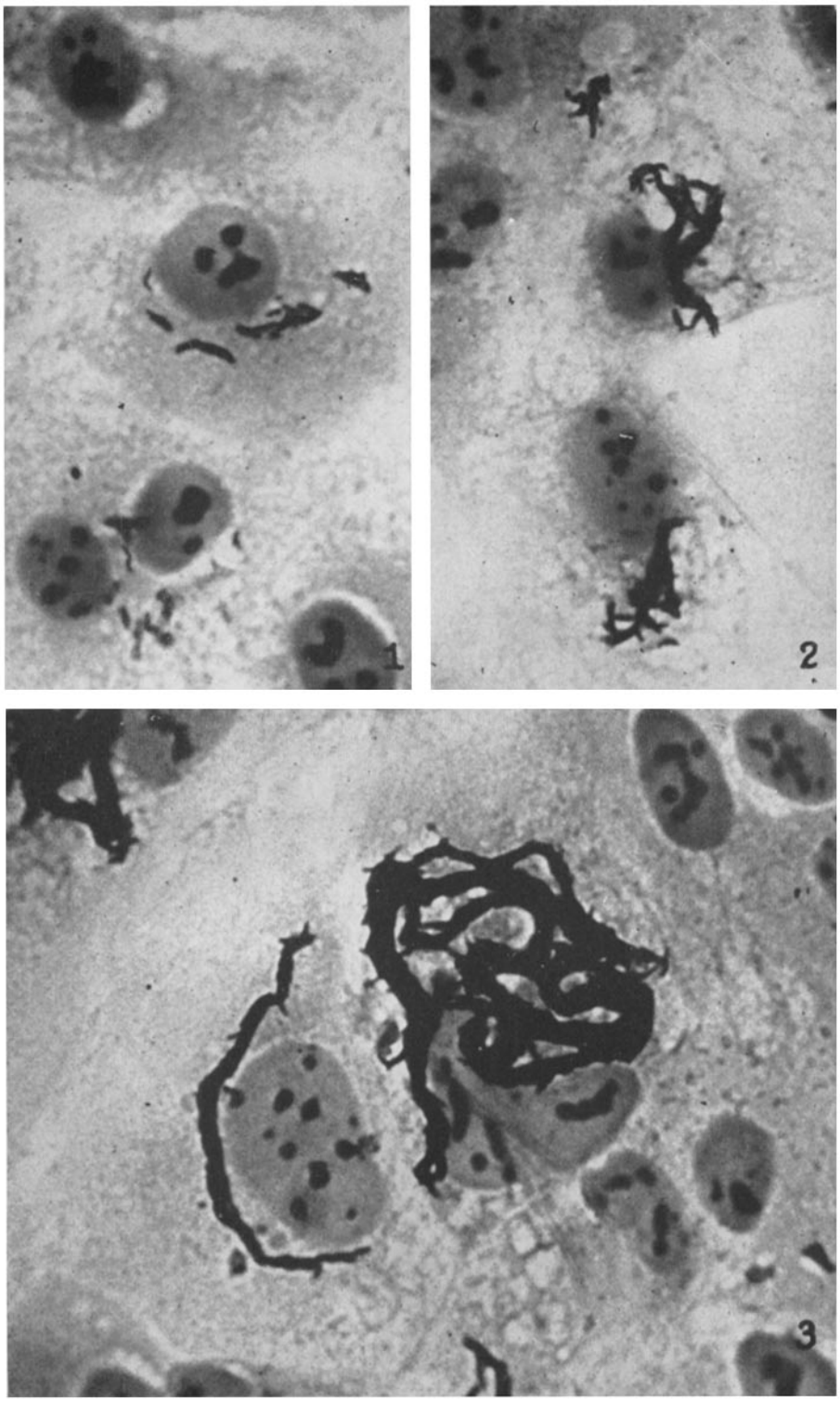
EXPLANATION OF PLATES

PLATE 1

FIG. 1. Human tubercle bacilli (H37Rv) phagocyted by HeLa cells in one day after addition of the bacilli to the tissue culture in a medium containing horse serum. At this stage, which is before much intracellular growth has occurred, different strains of tubercle bacilli are not easily differentiated. × 1000.

FIG. 2. Human tubercle bacilli (H37Rv) at 3 days, 2 days later than Fig. 1. Intracellular growth is apparent, and the bacilli are beginning to form cords. × 1000.

FIG. 3. Human tubercle bacilli (H37Rv) at 5 days. Well developed cords have been formed by the bacilli. All mycobacteria studied took up locations close to the nucleus. × 1000.



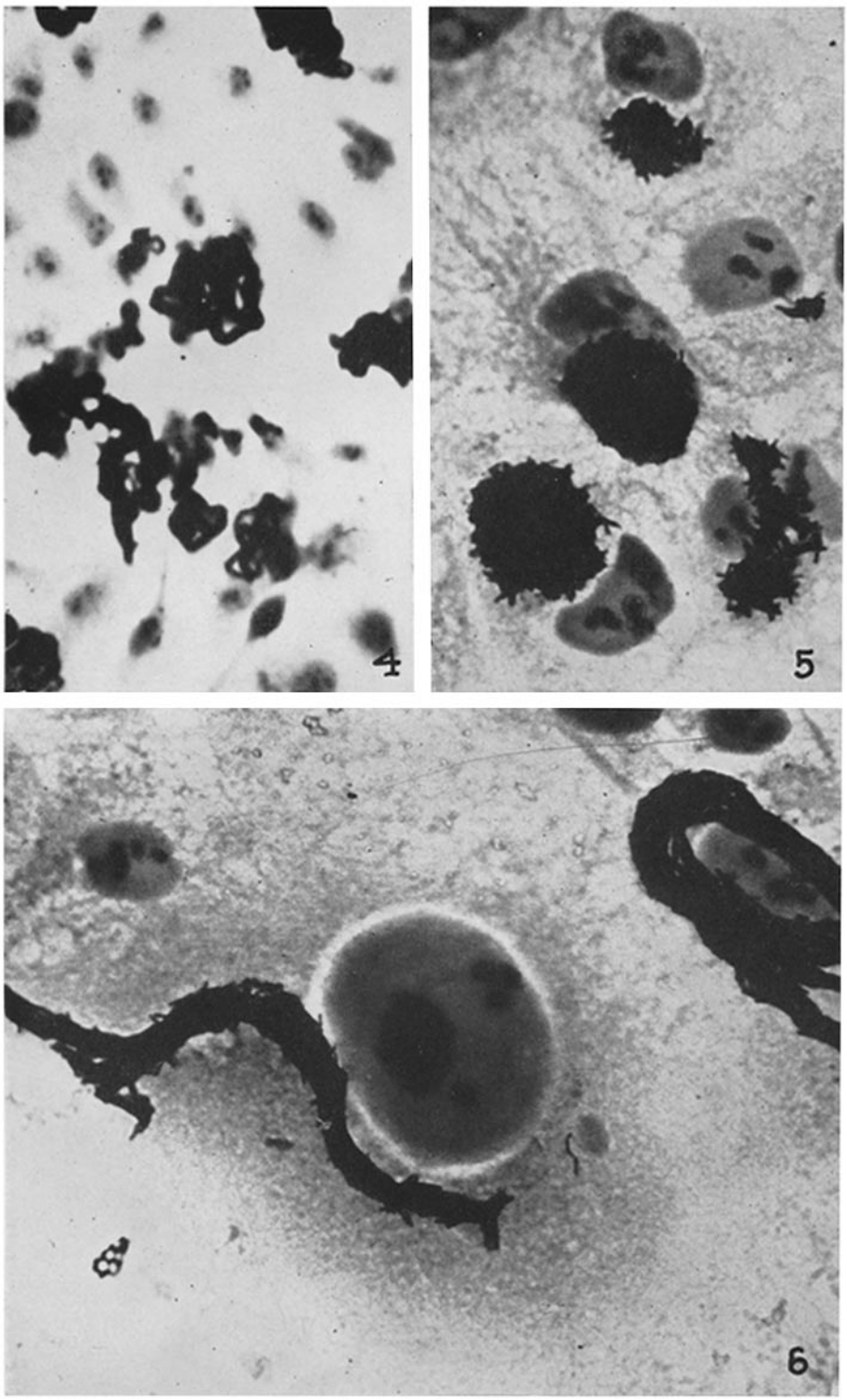
(Shepard: Mycobacteria in HeLa cells)

PLATE 2

FIG. 4. Human tubercle bacilli (H37Rv) at 7 days. The cells have been killed by the mass of intracellular bacilli which then continue to grow in the extracellular fluid. × 250.

FIG. 5. A substrain of H37Rv at 5 days. It no longer forms cords, but seems able to grow at about the same rate as typically virulent strains. × 1000.

FIG. 6. Bovine tubercle bacilli (Ravenel) at 5 days. Well developed cords are typical of virulent human and bovine strains. × 1000.



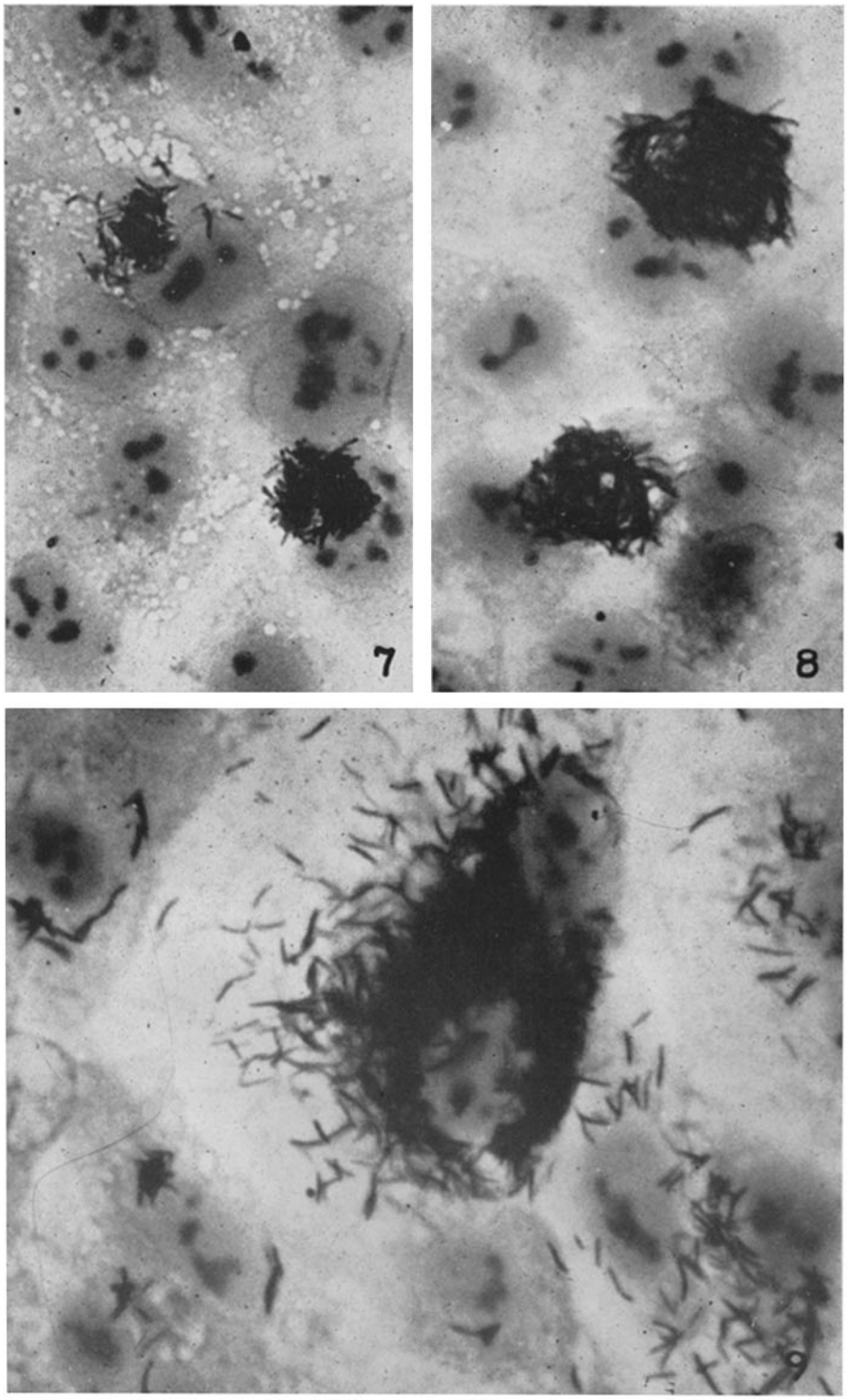
(Shepard: Mycobacteria in HeLa cells)

PLATE 3

FIG. 7. H37Ra, the avirulent variant of H37Rv, at 5 days. The amount of growth is much less than that seen with H37Rv, and the pattern of bacillary growth is distinctive. × 1000.

FIG. 8. BCG at 5 days. The amount of growth was about the same as that seen with H37Ra. The growth pattern of the bacilli is characteristic. × 1000.

FIG. 9. *M. balnei*, the cause of "swimming pool granuloma," 3 days at 35° C. There is rapid intracellular growth at this temperature and somewhat less at 37°C. × 1000.

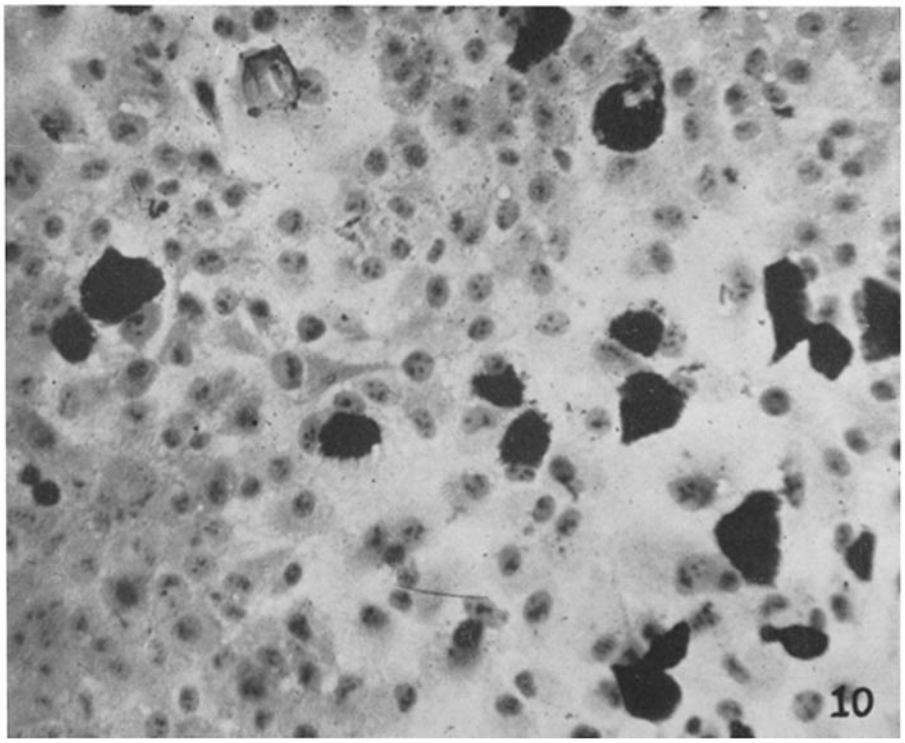


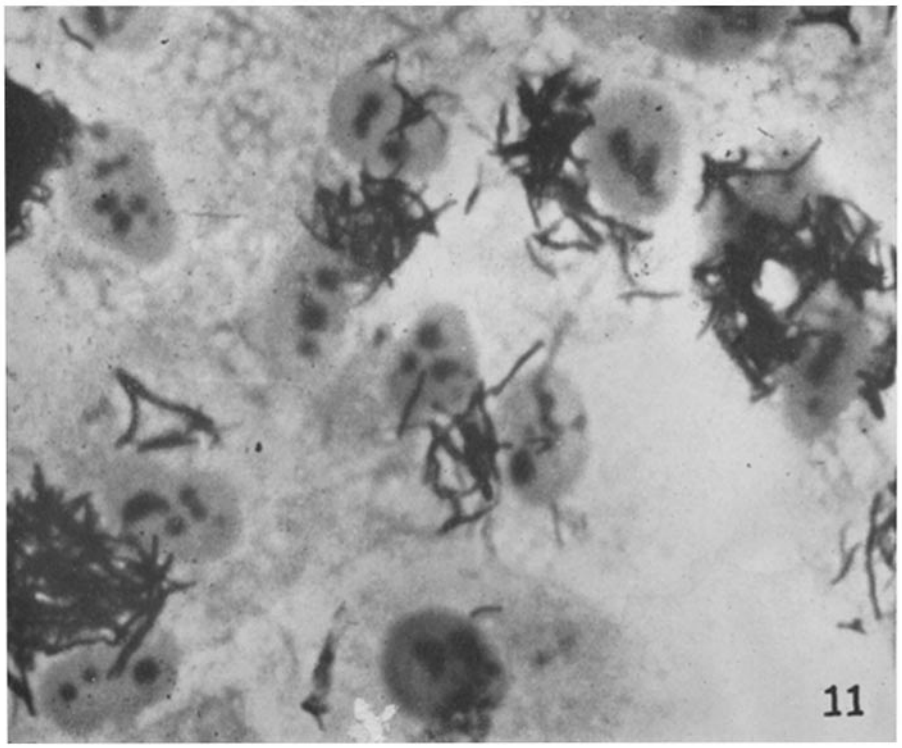
(Shepard: Mycobacteria in Hel ells)

PLATE 4

FIG. 10. *M. marinum*, 3 days at 35°C. Note the degree to which the cell has been filled by the bacterial growth so that the boundaries of the cytoplasm are marked off and the nucleus pushed aside. × 250.

FIG. 11. *M. platypoecilus*, 3 days at 35°C. × 1000.





(Shepard: Mycobacteria in HeLa cells)