A COMPARISON OF THE GROWTH OF SELECTED MYCOBACTERIA IN HeLa, MONKEY KIDNEY, AND HUMAN AMNION CELLS IN TISSUE CULTURE

By CHARLES C. SHEPARD, M.D.

(From the Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Montgomery, Alabama)

PLATES 28 TO 30

(Received for publication, August 5, 1957)

The cytoplasm of HeLa cells provides a favorable site for the rapid multiplication of tubercle bacilli and several other species of mycobacteria pathogenic for humans (1). The purpose of the present study was to learn the comparative suitability for the growth of mycobacteria of some other human and simian cells growing in tissue culture of the monolayer type. Human amnion and monkey kidney cells were selected for comparison with HeLa cells for reasons which included their sturdiness and availability as well as their differing degrees of metabolic activity.

As had been found with HeLa cells (2) the phagocytic activity was a function of the serum constituent of the culture fluid, and media were worked out which provided for entrance of the mycobacteria into all three types of cells with sufficient frequency to allow a satisfactory comparison of the growth rates of mycobacteria. The mycobacteria chosen for the comparison consisted of strains of tubercle bacilli of original and reduced pathogenicity, and four other species of mycobacteria pathogenic for humans. They varied widely in their generation times in bacteriological media, and in the disease picture which they had produced in humans. These studies have been made with the view of orientating attempts at cultivating the human leprosy bacillus in cells in tissue culture and some preliminary results are described, which are as yet inconclusive.

Materials and Methods

The tissue culture methods employed were basically those in common use at present in virus work. In general, work with mycobacteria requires cells in optimal nutrition, and the so called "maintenance media" usually do not provide cells in maximal condition. However, since the presence of human serum in the media does not appear to reduce growth of the mycobacteria in the cells, it was possible to employ media containing human serum at all times except on the "day of infection," and thus to have cells in more active physiological condition. The cells were grown on 8 × 30 mm. coverslips in Leighton tubes.

HeLa cell cultures were started from bottle cultures which had been grown 4 days in daily changes of medium. It is necessary that the cells be well flattened out on the glass, and the employment of young and well nourished bottle cultures as sources of cells for the

tube cultures has ensured this. Cultures of *rhesus* monkey kidney were prepared by overnight trypsinization at 4° (3). Human amnion cultures were trypsinized at room temperature for 6 to 8 hours with a minimum of stirring. After trypsinization both monkey kidney and human amnion cells were sedimented once at 600 R.P.M. and resuspended in growth medium, and the medium was changed the next day. The number of cells used to start the cultures, the constitution of the growth medium, and the frequency of its change are shown in Table II. The lesser metabolic activity of the human amnion cells allowed less frequent changes of medium. Usually HeLa cell cultures were ready for use in 2 days, and monkey kidney and human amnion in 5 days. Bacterial contamination was guarded against by the use of sterile technics wherever possible. Penicillin and streptomycin were added to the growth medium used for monkey kidney and human amnion cultures the first few days. The antibiotics appeared to be adequately removed after the double washing with BSS (Hanks's balanced salt solution) which was carried out to remove growth medium before the "infection medium" was added. Potentially dangerous operations were performed in an infectious disease hood.

TABLE I

The Cells, Their Initial Inocula, and Their Media

Cell	Initial inoculation	"Infection" media	"Growth" medium
	cells/tube		
HeLa	200,000	Ho 10 in Eagle's	Hu 40 q. 2d.
Monkey kidney	300,000	Ho 5 in 0.5 per cent LAH	Hu 40 q. 2d.
Human amnion	700,000	Ho 10 in Eagle's	Hu 20 q. 3d.

Hu 40 signifies 40 per cent pooled human sera plus 60 per cent BSS; Ho 10 in Eagle's, 10 per cent of selected horse serum in BSS containing the amino acids and vitamins recommended by Eagle (4); 0.5 per cent LAH, 0.5 per cent of enzymatic hydrolysate of lactalbumin + 2 per cent horse serum; q. 2d., every 2 days.

The "infection media" found best for the three cells are also shown in Table I. In general, a particular lot of horse serum found to be favorable for the promotion of phagocytosis of HeLa cells, was studied at different concentrations in the media of the monkey kidney and human amnion cells, and the amount of phagocytosis of tubercle bacilli observed (5). The most favorable concentration may be found somewhat different with other lots of horse serum. The amount of phagocytosis in the "infection medium" was always much greater than that seen in the "growth medium" and this made it possible to be reasonably certain that the microscopic picture subsequently seen represented intracellular development since the desired day of infection.

Mycobacterial inocula were prepared from growth in tween-albumin media (Difco's Tb-Tween). The cultures are listed in Table II, and when used as inocula were 6 to 7 days old, except those of *Mycobacterium fortuitum* which were 2 days old, and *Mycobacterium ulcerans* which were several weeks old. They were grown at 37°, except for *Mycobacterium balnei* (33°) and *Mycobacterium ulcerans* (31°). The cultures were stirred, allowed to settle a few minutes, the supernates removed, and centrifuged, the sediments resuspended in BSS by forceful aspiration with bulb and pipette, filtered through Whatman No. 1 paper, and adjusted to a turbidity of the streaming type which was barely visible with tubercle bacilli and several times stronger with other species. Since *M. ulcerans* did not pass the filter paper well it was sometimes not filtered. Microscopic examination of smears showed the mycobacteria to be

present mostly in singles or small clumps. The control of this point is essential. The inocula were added in 0.05 ml. amounts to the 1 ml. of tissue culture media employed at all times.

At intervals coverslips were washed, fixed in neutral formalin, stained with carbol fuchsin, destained with 1 per cent HCl in ethanol for less than 3 seconds, and counterstained lightly with Giemsa. The intervals chosen were usually 1, 3, and 5 days except with *M. fortuitum* and *M. balnei* in which cases 1, 2, and 3 days were more appropriate. As will be described, *M. ulcerans* required much longer periods of observation.

Conclusions concerning relative amounts of growth were based on long continued observation of coverslip preparations removed at appropriate intervals during the course of several experiments with each type of tissue culture. The illustrations are of necessity limited to

TABLE II

The Mycobacteria Employed

Name	Description
1. H37Rv	Fully virulent human tubercle bacillus
2. H37Ra	Avirulent variant of H37Rv
3. BCG	Isolated from vaccine from Chicago Research Institute
4. R1Rv	Human tubercle bacillus of reduced virulence
5. Kowski	INH-resistant human tubercle bacillus
6. *M. balnei*	Strain X, isolated from human with "swimming pool granuloma"
7. *M. fortuitum*	N8573, isolated from human with chronic cellulitis and lymphadenitis
8. "Yellow bacillus"	Barbee, photochromogenic "atypical" acid-fast from human pulmonary disease
9. *M. ulcerans*	Strains RS, isolated from human with tropical ulcer

Nos. 1 through 7 were received through the courtesy of the donors described previously (1, 9).

Nos. 8 and 9, respectively, were obtained through the kindness of Dr. Ann Pollack, now at Albert Einstein Medical School, New York, and Dr. Frank Fenner, Australian National University, Canberra, Australia.

INH stands for isoniazid.

several typical examples of infected cells. The rate of intracellular growth has also been estimated in terms of generation times for some of the strains of tubercle bacilli.

RESULTS

Tubercle Bacilli.—Figs. 1 to 3 show H37Rv after 5 days of growth in the three types of tissue culture. Roughly the same amount of growth was seen in HeLa and monkey kidney cells but distinctly less in human amnion cells. H37Ra and BCG grew considerably less rapidly than H37Rv in all three types of tissue cultures, and again most slowly in human amnion cells. R1Rv grew more rapidly than H37Ra in all three types of cells and most slowly in human amnion cells (Figs. 4 to 6). In HeLa cells the difference in rates of growth could be easily seen but the complicating factors of cell type and size discussed below

make a difference of the same order of magnitude more difficult to observe in the other two types of cells.

The arrangement of the strains of tubercle bacilli in order of their rates of growth in HeLa cells (1), is thus the same as that in monkey kidney and human amnion. The order is the same as that described for mice and guinea pigs by Pierce, Dubos, and Schaeffer (6). The rate of growth of the INH-resistant strain, Kowski, was not distinguishable from H37Rv in any of the types of tissue cultures, but an exact comparison was not possible since the bacterial groups it formed were less compact than H37Rv. Since there was a possibility that the bacilli growing in the cells were not truly INH-resistant, some observations were carried out in tissue cultures whose media contained 1 μg. of INH per ml. The growth of strain Kowski was not affected in cells of any of the three types of tissue cultures by this concentration of INH, although the multiplication of H37Rv could be prevented by much lower levels of INH (5, 7).

Other mycobacteria—M. fortuitum grew more rapidly in HeLa and monkey kidney than in human amnion cells. *M. balnei*, which is closely related to *M. marinum*, was studied at 33° in all three cells and it showed the same order of growth (Figs. 7 and 8). The "yellow bacillus" showed the same differences in growth rate in the three cell types.

M. ulcerans.—This slow growing microorganism has an optimal temperature on bacteriological media at 30–33°. When inoculated into monkey kidney tissue cultures, intracellular growth became obvious after about 10 days (Figs. 9 and 10). The intracellular growth was sometimes arranged in packets, but showed no cording. Extracellular cords became visible in the stained preparations at about 15 days and could also be seen at this time by low power examination of living preparations in their tubes. Although some lengthening of bacilli has been observed in HeLa and human amnion cells, obvious intracellular growth has not been seen. The influence of cell size on these observations is discussed below.

Comparison of the amount of intracellular growth of *M. ulcerans* at 33 and 35° showed no distinct differences, although the cells appeared to maintain their condition better on prolonged incubation at the lower temperature. Intracellular growth of the organism was not observed in duplicate tubes of monkey kidney cells when the medium used was LAH (0.5 per cent lactalbumin hydrolysate in BSS + 2 per cent horse serum).

X-Irradiation.—Studies were made of the effect on mycobacterial growth of x-irradiation of the cells, usually 2 days before their infection. HeLa cells were studied more extensively in experiments which made use of all members of the battery of mycobacteria, and pre-irradiation in doses of 100, 500, and 1,000 r. Doses of 5,000 r were also studied with most of the mycobacteria listed in Table I. Some experiments were carried out with cellular infections on the 9th postirradiation day. As Puck and Marcus (8) have reported, uniform popula-

tions of giant cells were seen following doses of 1,000 r or greater, and although these allowed the development of large intracellular mycobacterial groups, as shown in Fig. 11, the great size of the groups was thought to be the result of a greater original inoculum per cell consequent to the large surface area. When the inoculum was diluted until the infecting dose per cell was the same as that observed with non-irradiated cells, the bacterial groups which subsequently developed were no larger in irradiated than in non-irradiated cells.

Most of the mycobacteria of Table I were also studied in monkey kidney cells which had previously received 100, 500, 1000, and 2500 r. The medium employed was LAH. Human amnion cells were given 100, 500, or 1000 r before infection. No giant cells developed following irradiation, nor were significant differences from non-irradiated controls seen in the subsequent development of mycobacteria in either of these cell types.

The Influence of Cell Size.—Some of the cells in tissue culture were so small that mycobacterial growth reached the periphery of the cell in the early stages. For example, in human amnion cells and the smaller monkey kidney cells the cords of strain H37Rv reached the outer region of the cell in only a few generations, and the impression was gained that further bacterial growth was then inhibited. The longer generation time of H37Rv after the 3rd day in monkey kidney cells (Table III) is thought to be the result of the influence of cells of small size, and larger monkey kidney cells with fully developed bacterial patterns could be found by longer searching. In HeLa cell cultures that were more than a week or 2 old, the cells were crowded and small, at least in lateral dimensions, and intracellular growth appeared to be inhibited under these conditions also. An inhibiting effect of small cell size was noted with the longer mycobacteria, such as *M. balnei*, the "yellow bacillus," and *M. fortuitum* on the 1st day.

The Influence of Cell Type.—Although HeLa and human amnion cultures consist of a comparatively uniform cell population, the monkey kidney cultures contain many types of cells. In young monkey kidney cultures nests of more frequently infected cells were often observed. They contained pale, vacuolated cytoplasm, as seen in Fig. 5, and observation of mycobacterial growth tended to emphasize this type of cell. Infection of all morphological types of monkey kidney cells took place, and there was no obvious difference in rates of growth in the early stages. However, the experimental system is such that observation of susceptibility of cells is centered on the cells that phagocytosed mycobacteria on the day of infection.

Generation Times.—The increase in size of the intracellular groups from day to day of such organisms as H37Rv and H37Ra is thought to be their chief manifestation of growth for the following reasons: (*a*) the growth occurs in firm clumps containing bacilli in characteristic arrangement, *i.e.*, H37Rv grows with parallel alignment and in cords, (*b*) phagocytosis of new bacilli after the

1st day would be at a minimum due to the change from "infection" to growth medium," and (*c*) there is no marked tendency for change in the total number of infected cells with passing time. To estimate the generation time the number of bacilli in at least 50 bacterial groups was counted with the 97 × oil immersion objective, on the coverslips removed at 1, 3, and 5 days, and the generation time (doubling time) calculated which would account for the observed increase in average size of bacterial group. The results are recorded in Table III. The generation time of H37Rv in HeLa cells and monkey kidneys was estimated to be about 1 day. The figure for monkey kidneys after the 3rd day is closer to 2 days, and this increase is thought to arise from the limitations imposed by

TABLE III

Estimated Generation Times of Strain of Tubercle Bacilli in the Three Types of Tissue Culture Cells

Inoculum	H37Rv		H37Ra		Tuberculous sputa	
Days studied	1–3	3–5	1–3	3–5	1–3	3–5
HeLa, H37Rv 1:1	1.1	0.9	2.3	2.2	1.8	1.0
" , H37Rv 1:5	1.1	1.2			(Ave-	(Ave-
MK, Hu 40	1.0	1.9	5.8	3.5	rage)	rage)
" , LAH	1.2	1.9	>20	4.1		
Am	1.4	1.4	11.3	3.9		
"	1.4	1.6	4.3	>20		

The estimates are based on the increase in size of intracellular bacterial groups between the 1st and 3rd, and the 3rd and 5th day after the bacilli were added to the tissue culture. The results given for H37Rv in HeLa cells were obtained in an experiment comparing two different dilutions of the same inoculum, those for monkey kidney are from an experiment comparing the two growth media indicated, and those for human amnion cells are from two different experiments separated by an interval of 5 months. The results were tubercle bacilli from sputa are reported in more detail elsewhere (12, 5).

the proportion of small cells in the monkey kidney cultures, as mentioned above. The generation time of H37Rv in human amnion cells was about 1.5 days. The generation time of H37Ra in all three types of cells was considerably greater. The estimates of generation times that exceed 5 days are thought to be much less reliable than those of short periods.

The results comparing LAH medium with Hu 40 on monkey kidney cells indicate faster mycobacterial growth in cells in the latter medium. The promotion of mycobacterial growth in monkey kidney cells by Hu 40 was much more pronounced in the case of *M. ulcerans*, which was not observed to grow at all unless the medium was Hu 40. This medium markedly promotes the growth and acid production of monkey kidney cells themselves also.

Estimates of growth rates of other species and strains were not reliable when

carried by the method described, primarily because the bacterial groups were not sharply delimited.

Mycobacteria Growth Patterns.—The mycobacterial growth patterns show a high degree of specificity for the strains and species when grown in HeLa cells (1, 9). Although the mycobacterial strain or species showed the same characteristics when growing in monkey kidney or human amnion cells, the specificity was not so well developed. In monkey kidney cells the variation in cell types and sizes made the growth patterns less uniform, although the full development of patterns could be observed in the larger cells. In human amnion cells the small size of the cells and slowness of mycobacterial growth resulted in poorly developed patterns.

Human Leprosy Bacilli.—M. leprae from the skin of patients with lepromatous leprosy have been purified by a process involving centrifugal washing in solutions of bovine albumin (5) and added to the tissue cultures. Ingestion of bacilli was satisfactorily frequent and cultures have been followed for extended periods, usually at 33 to 35°. HeLa cell cultures could be kept for 1 to 2 months, but after the first 2 weeks they became so crowded with cells that observations of the bacilli in the cytoplasm were made difficult. The giant cells produced by irradiation were more suitable for observations of intracytoplasmic organisms, but significant changes in bacilli were not seen during the life of these cells, which was 3 to 4 weeks. Human amnion cells did not become overcrowded and have been kept for periods exceeding 6 months while still containing acid-fast bacilli. Monkey kidney cells have not become crowded either, and cultures with many cells containing leprosy bacilli have now been kept for several months. Whether multiplication has occurred is difficult to decide but definite changes in the bacterial population have not been observed. Since growth of *M. leprae* might be expected to be very slow, the experiments so far completed, although extensive, must be considered preliminary and inconclusive.

DISCUSSION

The results seen earlier with HeLa cells (1, 9) are reflected in these comparative studies with monkey kidney and human amnion cells. It is possible to generalize the results in terms of factors that appear to govern the growth rate of mycobacteria in these tissue culture cells of human and simian origin.

First is a factor reflecting the pathogenicity of the strain in the intact animal. Thus the lineal arrangement of the strains H37Rv, R1Rv, H37Ra, and BCG in order of their pathogenicity for mice and guinea pigs as reported by Pierce, Dubos, and Schaeffer (6) is the same as the order of their growth rates in HeLa, monkey kidney, and human amnion cells. In bacteriological media the rates of growth of these four strains of tubercle bacilli do not differ greatly from each other.

Another illustration of this factor is the failure of *M. phlei* and *M. smegmatis*

to grow in the cytoplasm of HeLa cells, whereas *M. fortuitum* grows rapidly in this location (9). The growth rates in bacteriological media of the latter three species are rapid and approximately equal.

A second factor affecting intracellular growth rates can be perceived when the tubercle bacilli of modified virulence and the saprophytes are eliminated from consideration. The following arrangement: *M. fortuitum*, *M. balnei*, and the "yellow bacillus" not far from *M. tuberculosis*, is in order of their growth rates in HeLa, monkey kidney, and human amnion cells in tissue culture and is the same as that found for these species in bacteriological medium in the absence of cells. The additional pathogen, *M. ulcerans*, which was observed to grow only in monkey kidney cells, grew more slowly there than any other of these species, and it also grew the most slowly of all in bacteriological media. Although the experiments with *M. ulcerans* were carried out principally at 33°, comparative studies with H37Rv at 33° showed that although it grew more slowly in cells at 33° than at 37° it did not grow as slowly as *M. ulcerans*. The chronicity of the human disease caused by these mycobacteria is also reflected in the arrangement above.

A third factor is related to the type of cell in tissue culture. Each pathogenic species of mycobacteria grew at roughly the same rates in HeLa and monkey kidney, but distinctly more slowly in human amnion cells. The possibility suggests itself that the rate of growth of the pathogenic mycobacterium is related to the metabolic activity of the cell, since in the media employed the HeLa and monkey kidney grew at about the same rate, whereas human amnion cells grew much more slowly. Eagle has recently found generation times of about 1 day for both HeLa and monkey kidney in media of optimal nutrition (10). Another indication of the metabolic activity of the tissue culture is the frequency with which the medium must be changed. This had been found to be 2 days for HeLa and monkey kidney and 3 days for human amnion cells, before the growth rates of the mycobacteria had been learned.

Other factors affecting growth rates of mycobacteria emphasized by previous studies have been the presence or absence of phagocytosis (2), and the temperature of the cell (1).

Although the tissue culture system would appear to offer attractive possibilities for the study of effects of x-irradiation on factors of host resistance, no effects were noted with the mycobacteria studied other than the effect related to the greater initial inoculum per cell as a consequence of the greater surface area of the giant HeLa cells. The doses of x-irradiation used appeared large enough to prevent cellular growth after the limited amount of multiplication that occurred in the first few days following irradiation, although the cells continued almost normal acid production in the tissue culture fluid. It should also be noted that x-irradiation has been reported to raise, lower, and leave unchanged the resistance of the intact animal to tuberculosis (11). The studies

by Puck and Marcus of x-irradiated HeLa cells noted an apparent decrease in resistance to Newcastle disease virus which was thought to be due possibly to the greater cell surface (8).

SUMMARY

HeLa, monkey kidney, and human amnion cells in tissue cultures were compared as sites for the multiplication of strains of tubercle bacilli or original and reduced pathogenicity, and for several other species of mycobacteria capable of causing disease in humans.

The arrangement of the pathogenic species in order of their growth rates in HeLa cells was *Mycobacterium fortuitum*, *Mycobacterium balnei*, and the "yellow bacillus," followed closely by the tubercle bacillus. This order was also correct for these species in monkey kidney and human amnion cells, and is the same as that seen in bacteriological media.

The arrangement of the strains of tubercle bacilli in order of their growth rates in all three types of cells was: H37Rv, then R1Rv, and lastly H37Ra, which multiplied about as slowly as BCG. An INH-resistant strain grew about as rapidly as H37Rv.

Growth of the pathogenic species occurred at about the same rates in HeLa and monkey kidney cells, but was distinctly slower in human amnion cells, which are less active metabolically.

Irradiation of the cells in doses up to 5000 r did not affect the subsequent growth of mycobacteria in them.

Preliminary experiments with human leprosy bacilli indicate that they can be introduced into these cells in high numbers and that the bacilli then persist for the life of the cells.

The technical assistance of Mrs. M. Nannett Jackman and Mrs. Mary E. Jones is gratefully acknowledged.

BIBLIOGRAPHY

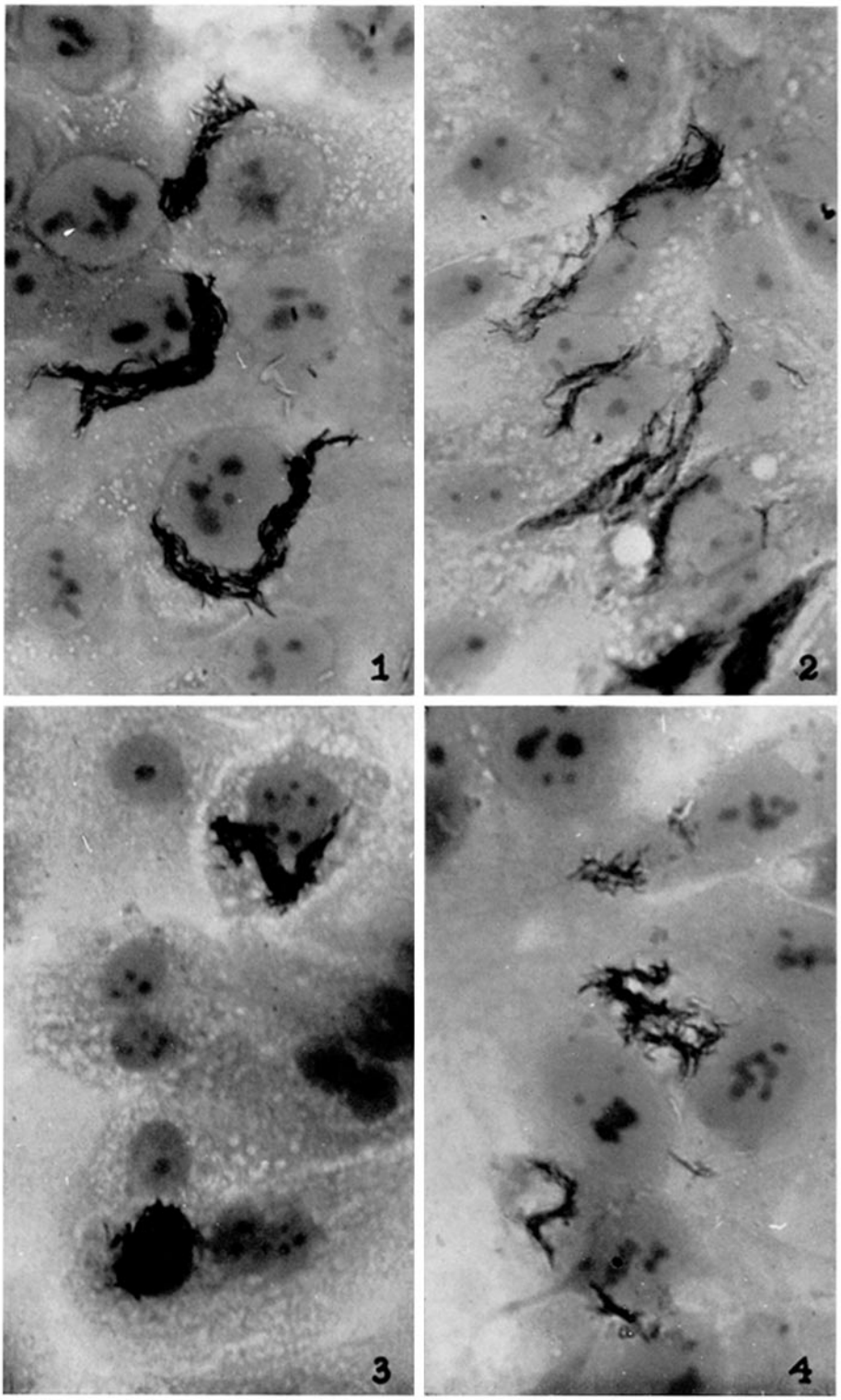
1. Shepard, C. C., *J. Exp. Med.*, 1957, **105**, 39.
2. Shepard, C. C., *Proc. Soc. Exp. Biol. and Med.*, 1955, **90**, 392.
3. Bodian, D., *Virology*, 1956, **4**, 575.
4. Eagle, H., *J. Exp. Med.*, 1955, **102**, 37.
5. Data in preparation.
6. Pierce, C. H., Dubos, R. J., and Schaeffer, W. B., *J. Exp. Med.*, 1953, **97**, 189.
7. Shepard, C. C., *J. Bact.*, 1957, **73**, 494.
8. Puck, T. T., and Marcus, P. I., *J. Exp. Med.*, 1956, **103**, 653.
9. Shepard, C. C., *J. Bact.*, 1957, **73**, 722.
10. Eagle, H., personal communication.
11. Taliaferro, W. H., and Taliaferro, L. G., *J. Immunol.*, 1951, **66**, 181.
12. Shepard, C. C., *Bact. Proc.*, 1957, 82.

EXPLANATION OF PLATES

PLATE 28

FIGS. 1 to 3. Human tubercle bacilli (H37Rv) 5 days after inoculation into HeLa, monkey kidney, and human amnion cells, respectively. × 825.

FIG. 4. R1Rv, a strain of tubercle bacilli of modified virulence, 5 days after inoculation into HeLa cells. × 825.

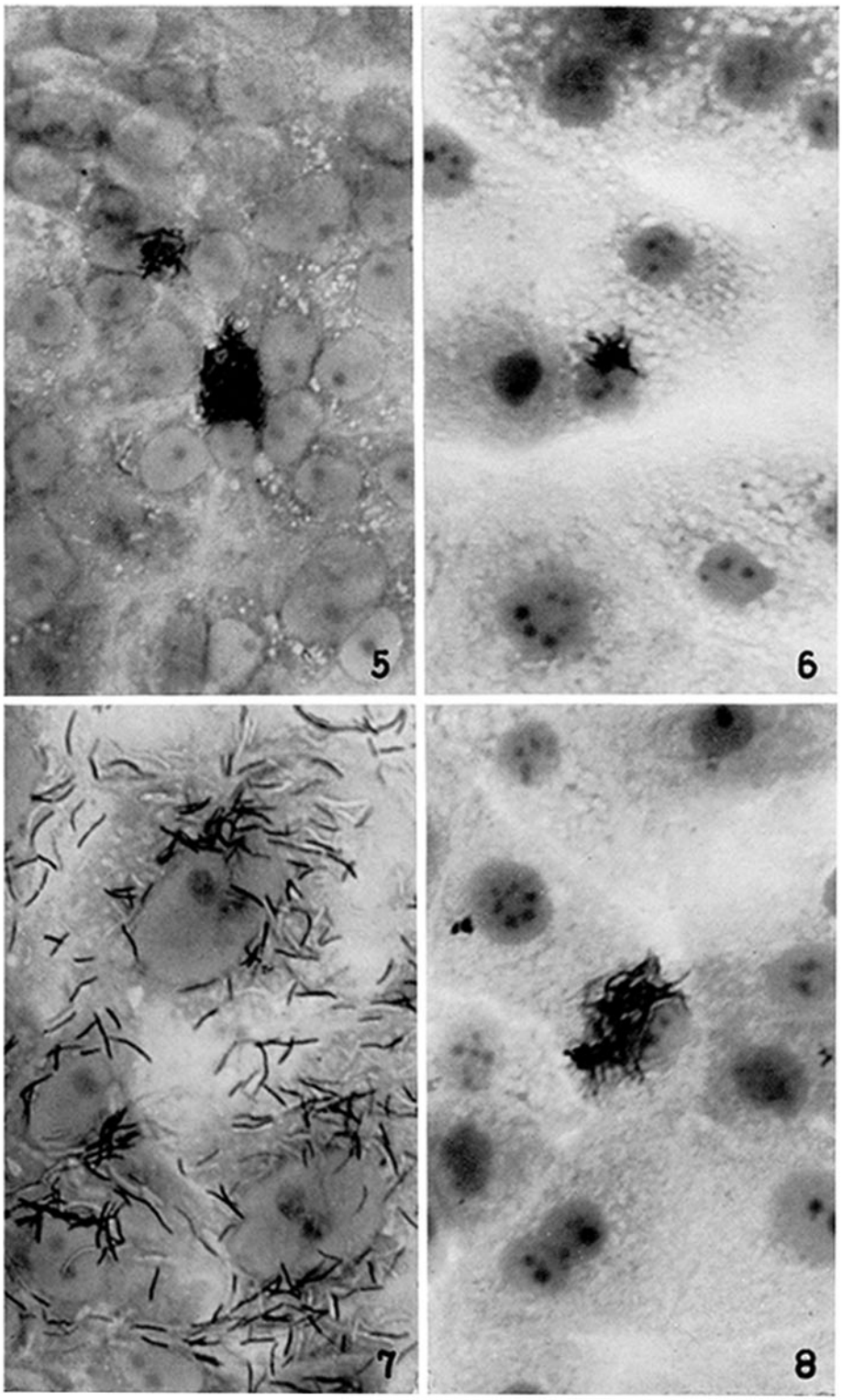


(Shepard: Growth of mycobacteria in tissue culture)

PLATE 29

FIGS. 5 and 6. R1Rv 5 days after inoculation into monkey kidney and human amnion cells, respectively. × 825.

FIGS. 7 and 8. *M. balnei* (strain X), the cause of "swimming pool granuloma," 3 days after inoculation into monkey kidney and human amnion cells, respectively. × 825.



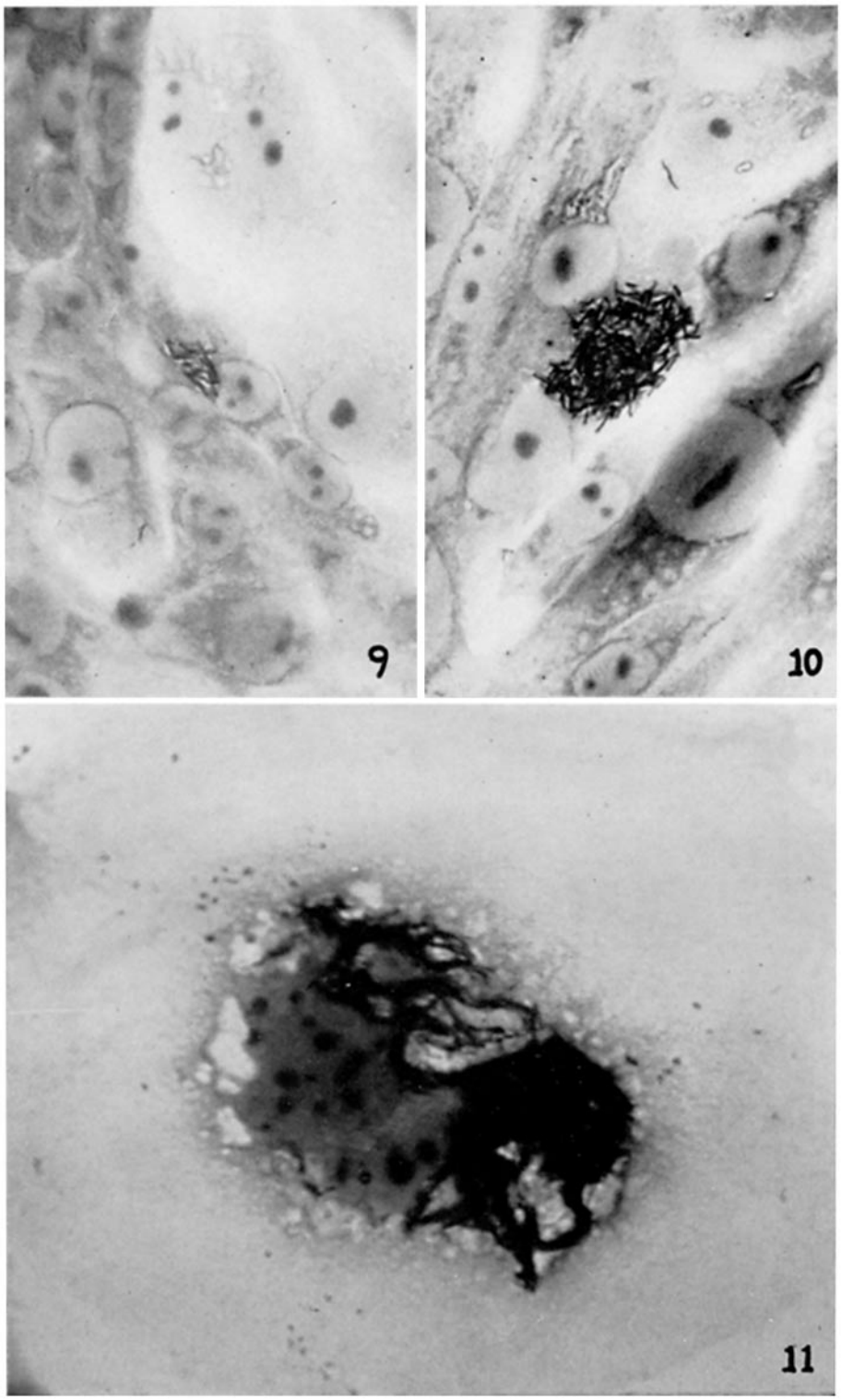
(Shepard: Growth of mycobacteria in tissue culture)

PLATE 30

FIG. 9. *M. ulcerans* (strain RS), which causes skin ulcers, 4 days after inoculation into monkey kidney cultures. No apparent growth at this time. × 825.

FIG. 10. The same after 10 days, the earliest time at which obvious intracellular growth was seen. A slow increase in size of intracellular bacterial groups was observed in the succeeding days. × 825.

FIG. 11. An x-irradiated HeLa cell 5 days after inoculation with tubercle bacilli (strain H37Rv). 1,000 r were given to the cells 2 days before inoculation with bacilli. × 825.



(Shepard: Growth of mycobacteria in tissue culture)