# Mycobacterium lepraemurium and Mycobacterium leprae in Cultures of Mouse Peritoneal Macrophages (Preliminary Results)

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Among various mycobacterial infections murine leprosy seems to be the one that bears the closest resemblance to human leprosy, i.e., an infection caused by a noncultivable, intracellular, acid-fast bacillus having a very slow growth rate. Studies on the growth characteristics of Mycobacterium lepraemurium might furnish useful clues in organizing the search for the growth requirements of *Mycobacterium leprae*. During the past 10 years efforts have been made to grow M. lepraemurium in cultures of mouse peritoneal macrophages. Growth of the bacilli had been obtained only occasionally at the begin-Progressively, however, not only ning. were the macrophages maintained in good condition for a long period of time, but at present the growth of M. lepraemurium is observed in macrophage cultures with a growth rate as rapid as that in vivo in mice. A total of approximately 500 experiments have been performed for this study.

Trials on the growth of *M. leprae* in mouse peritoneal macrophages have been performed in Dr. Vernon Knight's laboratory, National Institute of Allergy and Infectious Diseases, during the past 3 years. Technic and requirements for the cultivation of *M. lepraemurium* have been adapted for these studies. It is recognized that the growth rate of *M. leprae* is much slower and requires an incubation temperature much lower than that of *M. lepraemurium*. For that reason more difficulties can be anticipated in studies of *M. leprae* than of *M. lepraemurium*. This report presents in brief: (1) various steps in the development of a cultivation method for mouse peritoneal macrophages and the growth characteristics of *M. lepraemurium* within them, and (2) a limited study on the growth of *M. leprae* in this cell system.

#### METHODS

Technics for cultivation of peritoneal macrophages of the mouse have been described elsewhere (<sup>2</sup>). Briefly, the peritoneal exudates of female mice (ex-breeders are preferred because of the larger abdominal cavity) of the general purpose strain at the National Institutes of Health were washed out with balanced salt solution (Hanks) in the early studies and with the chemically defined, protein-free medium NCTC 109 later. The cell suspension (5 ml. from each mouse) was introduced either into Leighton tubes containing an 8 x 22 mm. coverslip (1 ml. each tube) or into glass or plastic flasks. The medium was replaced 3 to 5 hours later. Occasionally the exudate cells were washed out with the complete medium without subsequent replacement. Pooled cell suspensions from several animals were often used. Cultures were maintained at 37.5°C in closed tubes in the early studies and in an atmosphere of 5 per cent CO<sub>2</sub>-air mixture in later experiments. The medium was changed twice a week.

In the early experiments mice were infected with *M. lepraemurium* intraperitoneally, and peritoneal exudate cells were harvested at various intervals after infection. In later experiments, macrophages were infected by adding the bacillary suspension either immediately after the harvest of peritoneal exudate or one day after cultivation.

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Culture medium. The media were made of various amounts of horse serum, balanced salt solution (BSS), Eagle basal medium, NCTC 109, beef embryo extract (BEE), and various amounts of the following supplements dissolved in BSS or NCTC 109: spleen homogenate (1 spleen in 5 ml.), liver homogenate (1:20), liver extract, glycerol, ferric nitrate and mycobactin (the latter in an ethanol or glycerol solution) (for details see below).

**Bacillary suspension.** This was prepared from leprotic lesions of the omentum or pelvic fatty pads of mice that had been infected 3 to 6 months previously with the Hawaiian strain of *M. lepraemurium*. A 1 per cent suspension was made by grinding a small piece of lesion with a few ml. of BSS or NCTC 109 in a Ten Broeck tissue grinder. Bacillary counts were made from smears of the suspension.

Enumeration of macrophages. The macrophages were counted in the square field of a 5 mm.<sup>2</sup> grill under a low power objective (10X). Counting was made along three parallel longitude lines in the coverslip. The total number of macrophages in the whole coverslip was calculated from the average number per square field multiplied by the following conversion factor:

Area of coverslip in  $mm^2$  176 = = 677 Area of square field in  $mm^2$  0.26

Schedule for recording growth of bacilli. Coverslips were stained on the day following inoculation (the zero day), and every 7 to 10 days or more thereafter. The simplified method of Hanks for estimating gross changes in a mycobacterial population by means of low power examination was used in some experiments (<sup>3</sup>). Slight growth was indicated by the fact that about 5 per cent of the macrophages contained visible bacilli (a visibly infected macrophage contains at least 25 bacilli). Moderate growth was denoted by 25 per cent visibly infected cells; growth by 50 per cent; and maximal growth by 75 per cent or more.

**Enumeration of intracellular bacilli**. Counting of intracellular bacilli can be done accurately only when the number of bacilli is small. The number of bacilli per

macrophage was recorded as the average for 100 infected macrophages, using the following scheme: the percentage of macrophages containing the following number of bacilli: 1-2, 3-5, 6-10, 11-20, 21-50, 51-100, 101-200, 201-400, 401-600, 601-800, etc. The total number of bacilli of the whole coverslip was calculated as follows:

Average number of macro- phages X 677 X per square field	Average number of bacilli per macro- phage	X	Percent- age of macro- phages with bacilli	
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Staining of acid-fast organisms. Coverslips were washed with 0.85 per cent NaC1 solution and fixed in Zenker fluid or Zenker-formol for 5 minutes. The coverslip was floated with the cell side down in the Ziehl-Neelsen stain, which had been heated just to boiling on a 3 x 1 inch glass slide. Care was taken to float the coverslip over a depth of stain and prevent cells from coming in contact with the glass slide. The coverslip was destained 5 minutes later with 1 per cent HCl in 95 per cent ethanol. Macrophages were stained with Harris hematoxylin for 30 to 60 seconds followed by an ammonia water wash.

#### RESULTS

### I. Steps in Development of the Growth of *M. lepraemurium* in Cultures of Macrophages

A. Macrophages infected in vivo. Peritoneal exudate cells obtained from animals that had been infected with murine leprosy were used in the beginning on the assumption that the host-parasite relationship would be in perfect condition when macrophages were infected long before they were transferred for in vitro cultivation. Different media containing various amounts of horse serum, BSS, Eagle basal medium, BEE, and/or mouse spleen homogenate were tried in the beginning. It was possible to maintain 29 out of 62 cultures for a period of 2 to 5 weeks, but only five cultures showed growth of the bacilli. These five cultures were maintained in a medium containing 95-96 per cent horse serum and 3-5 per cent of a 1:1 dilution of BEE. Media made with a lower content of horse serum did not support the growth of either the macrophages or the bacilli. This indicated that macrophages required a highly nutritious medium for their maintenance in good condition and for the promotion of mycobacterial growth. This was an interesting finding, since high serum concentrations, 50 per cent or more in the medium, have been reported to be toxic for cells in tissue cultures.

In the five cultures, the number of bacilli per macrophage increased 3 to 16-fold, with an average generation time of the bacilli of 11 days (6.2 to 14.6 days). The total number of bacilli in the whole slide increased 3 to 5-fold in three cultures and remained unchanged in the other two, despite a marked decrease in the population of macrophages in four out of five experiments (Table 1).

Elongation of *M. lepraemurium* was also observed in these cultures. This was evidenced by the longer average length of the bacilli after 2 to 5 weeks of cultivation (Table 1). Very long bacilli, up to 15 microns in length, were seen occasionally.

These observations appeared to be the first showing definite growth of M. lepraemurium in macrophage cultures-an important step toward the subsequent development of the present culture system; nevertheless, disadvantages of the in vivo infected cell model were obvious. First, the degree of phagocytosis (i.e., the percentage of macrophages containing bacilli) varied greatly among various experiments. These erratic in vivo increases and decreases in the degree of macrophage phagocytosis made determination and control of phagocytic rate and growth determination rather difficult. Second, the organisms were initially in a moderately long form and observation of extensive elongation was rather difficult. It was believed, therefore, that a system containing noninfected macrophages that could be infected subsequently with a controlled number of the shortest forms of the bacilli would be more conducive to the observation of both elongation and multiplication of the organisms.

**B.** Macrophages infected in vitro. (1) Forms of M. lepraemurium:—The shortest form of M. lepraemurium, about 1 micron in length, was observed to occur in mice about 3 months after infection  $(^1)$ . This finding led to the observation that organisms obtained from animals 3 to 6 months after infection showed substantial, measurable elongation during cultivation in macrophages *in vitro*.

(2) The standard infection:-Infection of macrophages was controlled by introducing a known number of short bacilli into a known macrophage population. For example, an infection with a 50 per cent phagocytosis rate and an average of 3 to 6 bacilli per macrophage was usually obtained by adding about  $1 \times 10^6$  bacilli per Leighton tube and replacing this suspension with medium 3 hours later.

(3) Medium 9:1 and  $CO_2$  incubator:— The effect of 10 different types of media on the growth of *M. lepraemurium* was tested in a total of 79 experiments. Growth of the bacilli, visible with the low power objective, was observed in 12 experiments: five with slight, two with moderate, three with marked and two with maximal growth. Although growth was observed in each of the 10 types of media, reproducible results were observed only in the last medium, which was made of 90 per cent horse serum and 10 per cent of a 1.5 dilution of BEE (designated Medium 9:1).

At this time the importance of maintaining a constant pH was noticed. All cultures were maintained in an atmosphere of 5 per cent CO<sub>2</sub>-air mixture, which regulated the medium to a constant pH of about 7.2. As a result of this arrangement, 20 out of 34 experiments showed visible growth. Bacillary elongation was also stimulated by this controlled pH. Elongation of bacilli was observed in all of the 33 experiments maintained in the CO<sub>2</sub> incubator, and the organisms were longer than those observed in the 79 experiments prior to the use of the CO<sub>2</sub> incubator.

(4) Variations in the growth rate of M. lepraemurium:—A wide range of growth of the bacilli was observed in a total of 36 cultures maintained in Medium 9:1 (Table 2). Growth patterns ranging from nega-

	Total no. bacilli		Average	number	of bacilli e of cultu	per macı re	ophage.		Generation time of
	in culture: Initial/ final X 105	1 day	7 days	9 days	14 days	21 days	28 days	35 days	bacilit, days
8 10	2.0/18.8	4.0	6.5	101	12.5	21.7	33.0	63.0	9.0
n	10.2/11.5	12.8		20.8	16.7	23.0	27.0	68.0	14.6
0 0	11.6/12.4 1.1/16.3	13.4 13.1	20.8		27.2 63.0	42.7			12.7 6.2
68	$\frac{1.9}{1.5}/ 0.9$	6.6 4.1	4.3	6.6	7.5 4.3	6.3 4.8	5.0		

TABLE 2. Comparison of the growth pattern of M. lepraemurium in cultures of macrophages maintained in 4 different media.

	Mediu	um 9:1		Medium 4.5.1 and	Medium 4:5:1
Growth of bacilli at 40th day	30 to 110 days	at 40th day %	Medium 4:5:1	liver homogenate, 5 mgm./ml. (30% NCTC 109 was replaced by BSS)	and liver 5 mgm./ml. g
None	36.1	75.0	12.2	12.5	0
Slight	33.3	14.0	63.4	37.5	0
Moderate	11.1	8.3	17.1	6.3	29.5
Marked	16.7	2.7	7.3	12.5	41.0
Maximal	2.8	0	0	31.2	29.5
No. of experiments	36	36	41	16	17

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tive to maximal were observed in a period of 30 to 110 days. When comparison was made at the 40th day of cultivation, no cultures showed maximal growth.

(5) Medium 4:5:1 and NCTC 109:-Efforts were then made to reduce the serum content of the medium, since it is known that certain batches of horse serum are toxic to tissue cells when used in high concentration. NCTC 109, a chemically defined medium containing 68 ingredients, was used successfully to reduce the serum content of the culture media to a low level while still maintaining the macrophages in good condition for a very long period of time (2). A medium made of 40 per cent horse serum, 50 per cent NCTC 109 and 10 per cent of a 1:5 dilution of BEE (in NCTC 109), and designated Medium 4:5:1, promoted faster growth rate of M. *lepraemurium* than that observed with the use of Medium 9:1 (Table 2). Medium 4:5:1 was then used as the basal medium to study the effect of various supplements on the growth of bacilli in the subsequent experiments.

NCTC 109 was also used to replace BSS for peritoneal exudate washings. This seemed to be an important step toward the maintenance of macrophages in good condition. The period needed for maturation of exudate cells into macrophages, as regularly observed with BSS washed exudate cells in the first 7 to 10 days of cultivation, was no longer observed. Active cells, with pseudopodia, were observed shortly after the cell suspension was placed in cultures, and all cells were in very good condition on the next day and thereafter. This seemed to indicate that leakage of some substances from the cells which might be of vital importance for the survival of macrophages, had occurred when the exudate cells were maintained in BSS; this leakage was apparently prevented by the more nutrient medium NCTC 109 (2).

(6) Liver homogenate: - Marked improvement in the growth of *M. lepraemurium* was observed when Medium 4:5:1 was supplemented with mouse liver homogenate, 5 mgm./ml. All cultures showed moderate to maximal growth (Table 2).

(7) NCTC 109 substitution: – Replacement of 30 per cent of NCTC 109 with BSS resulted in a marked decrease in growth of the bacilli (Table 2), indicating that the concentration of NCTC 109 is critical for maximal growth.

(8) Medium 199:-The effect of Medium 199, a chemically defined medium similar to NCTC 109, was studied by substituting it for the NCTC 109 used in Medium 4:5:1 and that used as washing fluid for the peritoneal exudate. Macrophages survived in this medium in fairly good condition for many weeks, but the growth of *M. lepraemurium* was much less than that in cells maintained in Medium 4:5:1 with NCTC 109.

(9) Liver extract:-Liver extract and its L fraction (Nutritional Biochemical Corp.) showed slightly higher activity than the mouse liver homogenate. The L fraction was preferred because of its solubility. Maximal activity of the L fraction was observed at a concentration of 3 to 4 mgm./ ml. It is of interest to note that growth of *M. lepraemurium* could now be evaluated in a short period of 28 days, instead of the 40 days used previously.

(10) Ferric nitrate:—Ferric nitrate stimulated the growth of *M. lepraemurium* at a concentration of 1 to 10  $\mu$ gm./ml. Cultures containing both the liver extract L fraction and ferric nitrate showed a growth rate much better than either of the two given alone.

(11) Glycerol and mycobactin:-Glycerol showed doubtful activity on multiplication of *M. lepraemurium*. Marked elongation and branching of the bacilli were observed more frequently when glycerol was added to the medium. Mycobactin was inactive.

(12) Culture at 30°C:-Cultures maintained in Medium 4:5:1, supplemented with liver extract L fraction, 1 mgm./ml., and ferric nitrate, 2  $\mu$ gm./ml., showed a marked increase in the number of macrophages at 37°C. At this temperature many small, inactive cells often accumulated on top of the monolayer of macrophages; however, when these cultures were maintained in a low temperature, 30°C., the monolayer remained in good condition for



FIG. 1. Growth of *M. lepraemurium* in serially transferred experiments. Two series are included. The cumulative increase in the number of bacilli per macrophage was  $1.1 \times 10^{9}$ -fold over a period of 35 weeks in Experiment 471, and  $3 \times 10^{7}$ -fold over a period of 29 weeks in Experiment 477. M<sup>+</sup> = mice infected with the bacilli harvested at that time and later showed growth of murine leprosy.

many weeks without the small cell accumulation. This was of interest because the macrophage cultures could now be maintained at a low temperature, a culture condition that had been difficult to achieve with other media. Since *M. leprae* is believed to favor the lower temperature, this cell model will make possible studies of the growth characteristics of the bacilli in this specific condition (see below).

(13) Rapid growth of M. lepraemurium in serially transferred cultures:— M. lepraemurium was grown in macrophages in Medium 4:5:1, supplemented with liver extract L fraction 2 mgm./ml. Serial transfers were made at intervals of 4 to 6 weeks by inoculation of the bacilli harvested from the previous cultures into cultures of new macrophages. Rapid growth of the organisms was observed regularly on each of the transfers. The cumulative increase in the number of bacilli per macrophage was  $1.1 \ge 10^9$ -fold over a period of 35 weeks in one series, and  $3.0 \ge 10^7$ -fold over a period of 29 weeks in another. In each series the generation time of *M. lepraemurium* was 8 to 9 days in the first few transfers and 7 to 8 days thereafter (Fig. 1). Maximal growth of the bacilli was observed at the end of 6 to 7 weeks for each transfer. All macrophages were filled with hundreds of organisms at this time (Figs. 2-5).

Mice infected with organisms harvested at the time of transfers showed development of murine leprosy (Fig. 2). A subcutaneous nodule, averaging about 0.5 gm. per mouse, was obtained in animals inoculated with 7 x 10<sup>6</sup> organisms in these experiments. On the other hand, *in vitro* growth of *M. lepraemurium* has never been observed in Loewenstein-Jensen medium inoculated with organisms that were harvested from various cultures in the past 10 years, including the harvests of the recent serial transfer experiments.

(14) Distribution of M. lepraemurium in macrophages:-The quantitative technic for evaluation of the growth of intracellular bacilli in the present study was based mostly on count of the number of bacilli in the host cell of the culture. It has been known that redistribution of intracellular organisms occurs in tissue cultures, especially in cells maintained in the plasma clot type of culture. In these cultures, organisms within dead macrophages remain in the clot and are engulfed by wandering cells with resulting accumulation of bacilli resembling an actual multiplication. This was not the case in monolayer cultures maintained in a liquid medium in which dead macrophages were set free and later removed with the frequent change of medium, i.e., every 3 to 4 days.

Redistribution may occur also as a result of the introduction of old host cells into a new host cell system. These older cells, in which numerous bacilli have been growing for a long period, soon die, releasing organisms which the new cells readily ingest, increasing the percentage of phagocytosis markedly. This problem is



eliminated with the present macrophage host-parasite model, since these cells are evenly infected throughout the culture and, furthermore, are maintained for a long period without transfer or addition of cells.

Table 3 gives data for the rate of phagocytosis of cultures maintained for a period of 28 days. Each rate represented an average from 25 experiments. In 28 days, the average number of bacilli per macrophage increased from 3.4 to 16.6 in the control medium and from 3.9 to 51.0 in the cultures with various supplements. The percentage of phagocytosis remained practically unchanged during this period of observation, indicating that redistribution of intracellular organisms was not a serious problem in the present cell system during a 28 day period.

Since the rate of phagocytosis remained unchanged and the cell population appeared stable in this host-parasite model in a period of 28 days, a quantitative evaluation of the growth of the parasites could be based on count of the number of intracellular bacilli alone within this period of time.

## II. Behavior of Mycobacterium leprae in Cultures of Mouse Peritoneal Macrophages

Experiments on the growth of *M. leprae* in cultures of macrophages were started about three years ago in a cooperative study with Dr. Richard C. Adler of Dr. Vernon Knight's laboratory. A few biopsy

Time after	Per cent wi	t macrophages th bacilli
infection, days	Medium 4:5:1	Medium 4:5:1 with various supplements <sup>n</sup>
1	68 <sup>b</sup>	65
7	67	69
14	64	63
21	61	67
28	62	74
No. of bacilli per macrophage:		
Initial	3.4	3.9
Final	16.1	51.0

TABLE 3. Distribution of M. lepraemurium in macrophage cultures in a period of 28 days.

<sup>a</sup>Supplements were liver extract L fraction, ferric nitrate, and/or glycerol.

<sup>b</sup>All data are averages of 25 experiments.

specimens obtained from patients in the National Institutes of Health were inoculated into cultures of mouse macrophages. Cultures of patient's blood and bone marrow cells, which had been parasitized with *M. leprae in vivo*, were also included. Most cultures were maintained for only a short period of time except for the cultures of mouse macrophages, which were maintained in fairly good condition for a period of about 10 weeks. There were no definite

(All bacilli in Figures 2-5 are acid-fast organisms stained with Ziehl-Neelsen followed by hematoxylin stain.)

Fig. 2. Growth of *M. lepraemurium* in a 40-day-old macrophage culture in one of the rapidgrowth experiments in the early part of this study.  $\times 210$ . Such rapid-growing culture was observed occasionally at that time. All macrophages are filled with acid-fast organisms.

FIG. 3. Magnification of the rectangular area of Fig. 2.  $\times 2,000$ .

FIG. 4. Growth of *M. leptaemurium* in a 42-day-old culture of the 7th transfer of serial transfer Experiment 471.  $\times$ 210. Such rapid-growing culture can now be obtained in every transfer of the serial transfer experiments.

FIG. 5. Magnification of the rectangular area of Fig.  $4 \times 2,000$ .

TABLE 4. Sources of biopsies of M. leprae.

Source of biopsy	No. of biopsy
Leprosy patients of:	
Dr. J. Convit, Venezuela	18
Dr. J. Barba Rubio, Mexico	4
Dr. V. Knight, N.I.H.	5
Dr. J. G. Tolentino from	
Philippines	12
Dr. S. J. Bueno de Mesquita	
from Surinam	6
Mice infected with M. leprae,	
Dr. C. H. Binford, AFIP	4
Total	49

signs of growth of *M. leprae* in any of these cultures.

Little work was done from the time of Dr. Adler's departure in July 1963 until February 1964. Since that time a total of 49 biopsy specimens of M. leprae have been obtained at intervals. The sources of these are shown in Table 4. Organisms obtained from these specimens were inoculated into mouse peritoneal macrophage cultures exclusively. In the early studies cultures were maintained at 37°C in Medium 4:5:1, supplemented with liver extract L fraction, 1 mgm./ml. A lower temperature, 30°C., has been employed during the past 6 months, with the Medium 4:5:1 supplemented with both the liver L fraction (1 mgm./ml.) and ferric nitrate (1 µgm./ml.) All cultures were maintained in a 5 per cent CO<sub>2</sub>-air mixture.

Up to the present time, growth of acidfast organisms has been observed in cultures inoculated with 11 biopsies obtained from patients of varying geographic origin: 5 from Venezuela, 2 from Surinam, 1 from Mexico, 1 from the Philippines, and 2 from Mexican patients at the National Institutes of Health.

In one experiment, numerous deeply stained solid, acid-fast bacilli, moderate in length but markedly broad in width, were observed all over the coverslip at the end of 10 weeks' cultivation. The average number of bacilli per macrophage increased from 12.0 to 28.2 at the end of 10 weeks. All these organisms were in solid form; in comparison, only 17 per cent of solid forms were seen in the original inoculum (Figs. 6-9). The average length of bacilli increased from 1 micron at the beginning to 1.9 microns at the end of 10 weeks. The total number of organisms was slightly more than double the original, although there had been some loss of the macrophage population (Table 5). The organisms remained in approximately the same condition until the 22nd week, when the culture deteriorated.

However, a few macrophages filled with numerous acid-fast organisms were observed at the end of 28 weeks in a culture inoculated at a later date (1 day later) with organisms obtained from the same biopsy. Transfer was made by infecting new macrophages with organisms harvested from the 28-week culture. Slow growth of bacilli was observed during a period of 10 weeks (Figs. 10, 11). A sec-

(All bacilli in Figures 6-11 are acid-fast organisms stained with Ziehl-Neelsen followed by hematoxylin stain. Organisms shown in these figures came from cultures infected with a biopsy specimen from a Venezuelan patient.)

FIG. 6. Smear of bacillary suspension made from the biopsy.  $\times 2,000$ .

FIG. 7. Macrophage with M. leprae the day after infection.  $\times 2,000$ .

FIG. 8. A macrophage containing many long, deeply stained, solid acid-fast bacilli from the 10-week-old culture.  $\times 2,000$ .

FIG. 9. A macrophage containing many deeply stained, solid acid-fast bacilli from the 10-week-old culture.  $\times 2,000$ .

FIG. 10. A macrophage containing numerous short acid-fast bacilli from the 28-week-old-culture, inoculated at a later date (1 day later) with organisms from the biopsy specimen used for the cultures shown in Figs. 7-9.  $\times 2,000$ .

FIG. 11. A macrophage containing many long, acid-fast bacilli from the 38-day-old culture of the first transfer from the 28-week-old culture shown in Fig. 10.  $\times 2,000$ .



Time of ultivation, days	Macro- phages with bacilli %	Average no. of bacilli per macrophage	No. of macro- phages per field	Total no. of bacilli in culture	Average length of bacilli, μ	Proportion of solid form of bacilli %
1	88	12.0	113	8.1 x 10 <sup>5</sup>	1.0	17
70	65	28.2	93	$1.8 \ge 10^{6}$	1.9	100

TABLE 5. M. leprae in cultures of mouse peritoneal macrophages.

ond transfer was made, and many long, solid, intracellular acid-fast organisms were found in the new culture. Continued observation is in progress.

Scattered macrophages containing short or elongated, solid, acid-fast organisms were observed in the other 10 cultures after 13 to 26 weeks of cultivation. The number of these macrophages ranged from a few to 30 in each coverslip, and the number of intracellular organisms ranged from a few to tens and hundreds per macrophage. The appearance of these organisms is shown in Figures 12-16. A total of four cultures are under transfer studies at the present time.

Lowenstein-Jensen medium has been used routinely for check of growth of contaminating organisms from all the biopsy specimens received. No growth of acidfast organisms has been observed so far. There was no growth in the medium inoculated with organisms harvested from the transfer experiment. toneal macrophages have been undertaken in this laboratory during the past 10 years. Growth of the bacilli was observed only occasionally at the beginning. Progress has been made through various steps of development. At present not only is the host cell, the macrophage, maintained in good condition for a long period of time, but the intracellular parasite, *M. lepraemurium*, has been growing as rapidly as *in vivo*.

Studies were made of the growth of *Mycobacterium leprae* in this cell system. Growth of acid-fast organisms was observed in cultures inoculated with eleven biopsy specimens obtained from patients of varying geographic origin: Venezuela, Surinam, Mexico and the Philippines. Whether or not these organisms are related to *M. leprae* is under investigation.

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#### SUMMARY

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(All bacilli in Figures 12-16 are acid-fast organisms stained with Ziehl-Neelsen followed by hematoxylin stain.)

FIG. 12. A macrophage containing many long, solid acid-fast bacilli from the 26-week-old culture. Biopsy specimen from another Venezuelan patient.  $\times 2,000$ .

FIG. 13. A macrophage containing numerous acid-fast bacilli from the 20-week-old culture. Biopsy specimen from a patient from Mexico.  $\times 2,000$ .

FIG. 14. A macrophage containing numerous acid-fast bacilli in the 20-week-old culture. Biopsy specimen from a Mexican patient at the National Institutes of Health.  $\times 2,000$ .

FIG. 15. A macrophage containing numerous long, acid-fast bacilli from the 13-week-old culture. Biopsy specimen from a patient from Surinam.  $\times 2,000$ .

FIG. 16. A macrophage containing numerous acid-fast bacilli from the 15-week-old culture. Biopsy specimen from a patient from the Philippines.  $\times 2,000$ .

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#### DISCUSSION

**Dr. Hart.** I think we can now decide that the cultivation of *M. lepraemurium* in tissue culture is significant for studies on *M. leprae*, and can say that we are in a highly encouraging stage. The discussion will be opened by Dr. Rees.

**Dr. Rees.** I believe the most outstanding feature in these two presentations lies in getting used to the time scale. Obviously these two groups of workers have accepted that leprosy bacilli exist in the bacillary form. They are slow growers and therefore special application must be made to the time scale. I am impressed by the fact that one experiment ran for 1,600 days! I shall pick on points from the foregoing two papers as far as they contribute to our main theme on the cultivation of leprosy bacilli.

It is of interest that both speakers used M. lepraemurium as a model in which to apply tissue culture methods and they have produced indisputable evidence that this organism can be grown successfully and maintained in cell culture. Using the rat fibroblast as host cell, tissue culture methods have been developed for producing in vitro large quantities of M. lepraemurium, and the methods could obviously be scaled up quite easily. It is of the greatest interest that tissue-culture grown M. lepraemurium, after months or years in cell culture, showed no changes in pathogenicity or virulence for animals, and that the generation time of the organism remained the same throughout. Furthermore, the tissueculture-grown bacilli could not be grown in a cell-free medium. Thus the bacillary population was stable and no variants were derived from mutations in vitro. Having once developed a suitable cell system for growing M. lepraemurium, the speakers investigated the influence of the extracellular environment (media) on intracellular growth. Both demonstrated conclusively that the extracellular environment could alter intracellular growth. For example, Miss Garbutt, using rat fibroblasts grown in human cord serum, obtained intracellular multiplication, whereas when human cord serum was replaced by newborn calf serum, no intracellular multiplication occurred. Dr. Chang, using mouse macrophages and high concentrations of horse serum, obtained intracellular multiplication of M. leprae*murium* and this multiplication could be enhanced or diminished by increasing or decreasing the concentration of horse serum, and, furthermore, the addition of mycobactin increased the yield of intracellular bacilli. These findings are in line with those obtained by Dr. Hanks and his colleagues on the intracellular growth of M. johnei in sheep macrophages. They found that maximum yields of M. johnei were obtained within the macrophages by the addition of mycobactin and other in vitro growth factors for this organism. Thus the growth within cells can be influenced by both the type of cell and its extracellular environment.

Miss Garbutt, in her tissue culture systems, demonstrated very clearly that certain soluble polysaccharides derived from the intracellularly growing *M. lepraemurium* diffused out of the cell and could be detected in the tissue culture medium in considerable quantities. It is known that in murine leprosy infections in animals and in human leprosy in man, heavy infections result in high titers of circulating antibody to mycobacterial polysaccharides. The tissue culture work has revealed a mechanism by which the soluble antigens readily diffuse out of the host cells into the tissue fluids and blood.

From these basic studies, using M. lepraemurium as a model, both speakers presented preliminary work indicating that they had successfully developed cell culture systems for the limited growth of M. leprae in vitro. These preliminary results are exciting and of the greatest importance. They offer for the first time clear-cut evidence that M. *leprae* can be cultured successfully in cells outside the animal body. I feel optimistic that cell cultures of this type will eventually be developed sufficiently for the large-scale production of M. *leprae*. The basic methods and technics that have opened up these possibilities have undoubtedly been developed from the use of M. *lepraemurium* as a model system.

Yesterday we had a very nice demonstration of how tissue culture studies and electron microscopy had enhanced our knowledge of the behavior and growth of rickettsiae. I believe that similar technics should be applied to M. leprae in cell cultures in order to provide us with precise knowledge on the growth of the bacilli within the intracellular environment. Presumably M. leprae are taken into cells in the same way as other particles and in the early phase are surrounded by a limiting membrane and exist in the classic type of phagosomes. This is the classic defense mechanism of a cell that normally leads to death of the invading organism. Yet within cells in which eventually M. leprae succeeds in multiplying, changes must take place that are advantageous to the organism and yet at the same time do not kill the host cell. It would seem to me that Miss Garbutt in particular has studied cell systems in which it should be possible to study at the level of the electron microscope the intracellular changes that are either favorable or unfavorable for the phagocytosed M. leprae.

**Dr. Hart.** Before the meeting is opened for general discussion I would like to call upon Professor Janssens.

**Professor Janssens.** I have always been very much interested in the presence of acid-fast bacteria in the lymph nodes of leprosy patients. I have the overall impression that this kind of material, which is very readily available and in easy supply almost everywhere, has not received the attention it deserves. This is the reason why I want to present very briefly what has been done by Jadin and Wery at our Prince Leopold Institute of Tropical Medicine.

## MULTIPLICATION OF MYCOBACTERIUM LEPRAE IN LYMPH NODE CELLS J. Jadin and M. Wery

In 1963, J. P. Delville succeeded in inducing multiplication of *M. leprae* in cells isolated from Kaposi's angiosarcoma. The authors have looked for a more readily available source of human cells, other than skin from leprosy patients, which is usually grossly superinfected.

Lymph nodes have always been an attractive source for the search for *M. leprae*, at least in Central Africa, where lymph node puncture is made routinely for several purposes. The lymph node juice is aspirated by sterile needle and syringe, and the  $\pm 0.025$  ml. liquid thus obtained is transferred into 10 ml. of Hanks' balanced salt solution enriched with glucose, lactalbumin hydrolysate, calf serum and hemoglobin. Bacterial overgrowth is controlled by addition of 200 units of penicillin per ml. of the prepared medium. The final dilution of the inoculum is 1:400.

After incubation for a fortnight at  $37^{\circ}$ C, numerous cells are found to contain typical globi. Serial transfer, at three week intervals, became possible, and has now been going on for 18 months. Out of the first tube up to 10-25 new ones may be inoculated from the sediment. The supernate, containing only a few organisms, is not suitable for multiplication.

Up to now, 130 specimens of lymph node puncture have been received from Leopoldville, Iyonda (Congo), Bamako (Mali) and Buenos Aires (Argentina), out of which 72 positive cultures were obtained. The failures are usually due to superinfection, but some tubes (1-2%) remained sterile and negative.

Several cell-lines have been established in Leighton tubes by trypsination of human skin on the synthetic TC Parker 199 medium with Hanks' balanced salt solution, human serum and chick embryo extract.

The day after this trypsination, the supernate is taken off and replaced by an M. *leprae* suspension in the same medium, originating from a lymph node culture, 1 tube being used for the inoculation of 5 Leighton tubes. After one week at 37°C, the liquid phase is removed and replaced twice weekly by fresh medium. From the seventh day on the cell multiplication is very active.

*M. leprae* multiplies after the first transfer and is maintained after a second; the globi of acid-fast organisms are seen near or in the nucleus. On the whole, the trypsination seems to be harmful to *M. leprae*.

In presenting this material I (P.G.J.) wanted to stress the possibilities for research offered by the acid-fast bacteria extracted out of the lymph nodes of leprosy patients.

**Dr. Imaeda.** During the past six years we have tried to cultivate *M. leprae* in tissue culture cells. The results are shown in Table 1. The commercial cell lines were cultivated in various culture media with *M. leprae.* Pooled lepromatous patients' sera were added in some experiments. This resulted in about 10 per cent increase of phagocytosis, possibly due to an immune adherence phenomenon. In these experiments, however, we could not observe significant growth of *M. leprae.* 

On the other hand, *M. lepraemurium* and *Mycobacterium* sp. isolated from human borderline leprosy patients and subsequently grown in hamster earlobes (by Dr. Convit's group), were grown in mice peritoneal macrophages maintained in culture media. Their generation time was 20 days under these culture conditions. However, mycobacteria isolated from human leprosy lesions and inoculated immediately into macrophages have never been grown in the same conditions as above (Table 1).

In several experiments, electron microscopy revealed intact morphology in *M. leprae* even after 270 days of cultivation, a fact suggesting that *M. leprae* still maintained its viability.

**Dr. Kirchheimer.** My question is directed to Miss Garbutt and it concerns her experiments with the Hansen bacillus. I might have missed what the incubation temperature was, but if I am to assume it was 34°C, did you try 37°C and was there any difference? And also, did the Hansen bacilli signal to you that they were going to multiply, i.e., did they elongate?

Cell line	Media	Observation period (days)	Intracellular growth	Mycobacteria from
B (Eagle)	Eagle HeLa, 5% horse serum	60	1	L.L.
B (Eagle)	Eagle HeLa, 5% horse serum	96	$-(\mathbf{B})$	L.L.
B (Eagle)	Eagle HeLa, 5% horse serum	70	1	L.L.
929	109, 5% horse serum	116	1	L.L.
929	BME, 10% calf, 5% LL sera	40	-(B)	1.1
929	BME, 10% calf, 2.5% inact. LL sera	42	2	1.1
929	BME, 10% calf, 0.1% LL sera	88	-(B)	BL.
929	BME, 10% calf, 0.1% LL sera	88		T.L.
929	BME, 40% horse serum	35	1	L.L.
929	BME, 30% calf serum	70	1	L.L.
eLa (Gey)	Eagle HeLa, 5% horse serum	26	1	L.L.
ver (Chang)	BME, 2% calf serum, 25 mgm.% DAP	340	1	1.1.
ver (Chang)	BME, 40% horse serum	270	-(B)	1.1.
ver (Chang)	BME, 20% human serum	30		L.L.
uman monocyte (J-111)	BME, 5% horse serum	340	1	L.L.
iman monocyte (J-111)	BME, 2% horse serum	350	I	L.L.
iman monocyte (J-111)	BME, 5% calf serum, 25 mgm.% DAP	40	1	. L.L.
ouse sarcoma-180	BME, 10% calf, 5% LL sera	50	I	L.L.
P. macrophage	109, 40% fetal bovine serum	32	Ĩ	B.L.
P. macrophage	109, 40% horse serum, 10% B.E.E.	112	-(B)	L.L.
P. macrophage	109, 40% horse serum, 5% B.E.E.	54	+	M. lepraemuriun
P. macrophage	109, 40% horse serum, 5% B.E.E.	54	+	Hamster-B.L.
P. macrophage	Puck, 40% bovine fetal serum	63	+	Hamster-B.L.
.P. macrophage	Puck, 40% bovine fetal serum	50	+	M. lepraemurium
3ME = basal medium, Eagle. LL sera = pooled lepromatous p nact. LL sera = inactivated lepi OAP = diaminopimelic acid. 3.E.E. = bovine embryo extract.	atients' sera. atients' sera. B.L. = Hamste bor Tembor	<ul> <li>macrophage: mice pe lepromatous leprosy.</li> <li>borderline leprosy.</li> <li>r-B.L. = hamster lesi derline leprosy patient derline leprosy patient</li> </ul>	ritoncal macrophag an caused by myco	e. bacteria isolated fro

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Miss Garbutt. We incubated our M. lepraemurium cultures at 34° and 37°C-it did not seem to make any difference. However, with M. leprae the cultures were put in the 37° incubator only for the initial four day infection period and then were transferred to 34°C. We have not used 37°C throughout. I could not answer your second question about the elongation. Doing total bacillary counts is extremely tedious; the numbers counted were very small and we were pleased even if we found short bacilli. We did not count any bacilli about which we were doubtful. If anything, I would think that the numbers reported are on the low side, because only respectablelooking bacilli were included.

**Dr. Morrison.** I would like to direct a question to Dr. Chang. I wonder if you have considered supplying mycobactin to your macrophage system in the form of ferric mycobactin, that is, mycobactin in the form of a hexadentate chelate with trivalent iron instead of in the metal-free form?

**Dr. Chang.** It is a good idea. We should have tried it, but we could not make it in our laboratory. We obtained mycobactin from Dr. Morrison either dissolved in alcohol or in glycerol. We did not get much growth with mycobactin alone, but we would like to try ferricmycobactin if you could make it.

Dr. Morrison. We will make it.

**Dr. Wayne.** Dr. Morrison asked half of my question. We have made some experiments with tubercle bacilli in the actively growing phase, to which we have added EDTA, the chelating agent, and got cessation of growth followed by lysis. However, this could have been prevented if the bacilli had been grown with additional citrate; on the other hand, if the citrate and the EDTA were *both* added *before* inoculation, there was no protection against the EDTA by the citrate. I was wondering, for this reason, just what the levels of citrate are in the various media that Dr. Chang used. **Dr. Chang.** We did not use citrate in the medium. We used heparin in NCTC 109 in the beginning for only 3 hours; then we washed it out and replaced it with normal medium. I do not think there was any citrate in the medium.

**Dr. Shepard.** Certainly, some congratulations need to be extended. It seems to be the start for the future of tissue culture work. No one knows where it will lead. It is the beginning, but it is a very beautiful beginning, and looks extremely promising. As to the temperature it may not be so important in tissue culture. When we worked with *M. marinum* and *M. balnei* and other tissue cells, we didn't seem to be so temperature-dependent. We had pretty good growth at higher temperatures, viz., 35-37°C. You have a double factor, viz., nutrition of the tissue culture cells as well as of the organism.

Dr. Hanks. Thank you, Dr. Shepard. I had hoped to be the first to congratulate Dr. Chang, Miss Garbutt and Dr. Rees on these significant accomplishments. It seems that with respect to future work we have vet another landmark. Among us we have learned that the chelate-requiring and "host-dependent" mycobacteria do not depend solely upon metabolites and components of host cells and that what they pick up from the extracellular medium is of major importance. I would like next to ask Miss Garbutt whether the several strains of cultivable mycobacteria emerged from the cell cultures early during the experiments or after considerable delay.

Miss Garbutt. They made their appearance at about three months.

**Dr. Hanks.** I trust you did not throw them out, as being of little interest, because you have here a parallel to one of the situations that has always complicated direct microbiologic work on bacilli recovered from leprosy patients.

**Dr. Hart.** We shall have to close the discussion now. 33, 3 (Pt. 2)

Møller-Christensen: Paleopathology

Dr. Binford. We have heard exciting new facts this morning on the growth of M. leprae in tissue cells. Dr. Møller-Christensen, Professor of Medical History at the University of Copenhagen, however, has unearthed new information on leprosy from graveyards in Denmark. By examining more than a thousand skeletons from medieval burying grounds of southern Denmark, he has found a lesion specific for leprosy in the skulls of leprosy patients who were buried somewhere between 1100 and 1200 A.D. In the introduction to a book that he published in 1961 under the title "Bone Changes in Leprosy," the pathologist, Professor Engelbreth Holm of the University of Copenhagen, said: "Indeed, it is very exceptional that a general practitioner without any special training in pathology, has managed to carry through this tremendous work. The excavation and examination of more than 1,000 skeletons from medieval monastery churchyards have been undertaken by Dr. Møller-Christensen personally, who in addition has succeeded in improving the technique even in this archaeological field. Also noteworthy is the fact that it has been possible, on the basis of medieval material, to provide brilliant osteoarchaeological and pathological-anatomical information about a disease which for a long time has been eradicated in Denmark, information which is of practical value in the world-wide anti-leprosy campaign."

I had the pleasure, last summer, of visiting Professor Møller-Christensen's Museum at the University of Copenhagen and seeing his material. We are much pleased that he brought some of his specimens with him. I understand he is going to donate these to the Medical Museum of the Armed Forces Institute of Pathology. He has set up an exhibit across the hall. I hope all of you, while at this meeting, will take advantage of this and look at it. We shall leave his publications with the exhibit, the one published in 1961, and an earlier one, "Ten Lepers from Naestved in Denmark," published in 1955. Professor Møller-Christensen, we are much pleased that you have come over here to bring us this message. His title is "Newer knowledge of leprosy through paleopathology."