

# Growth of *Mycobacterium lepraemurium* in Cultures of Mouse Peritoneal Macrophages<sup>1</sup>

Y. T. CHANG, R. N. ANDERSEN, AND Z. VAITUZIS<sup>2</sup>

Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, and Leonard Wood Memorial (American Leprosy Foundation), Washington, D.C.

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Successful growth of *Mycobacterium lepraemurium* was observed in cultures of mouse peritoneal macrophages. The optimal host cell maintenance medium was composed of 40% horse serum, 50% of the chemically defined medium NCTC 109, and 10% of a 1:5 dilution of beef embryo extract, supplemented with both liver extract and ferric nitrate. Multiplication of the bacilli was observed in 1 week and maximal growth in 6 to 7 weeks. All macrophages were filled with tens to hundreds of the organisms in cultures showing maximal growth. Glycerol caused an increase in the normal length of *M. lepraemurium*, without a corresponding increase in the number of the bacilli. Elongation of *M. lepraemurium* was observed 3 or 4 days after infection. Rapid and uniform growth of *M. lepraemurium* was achieved in serially transferred cultures (subcultures). The cumulative increase of the number of intracellular bacilli was  $1.4 \times 10^{20}$ -fold in 14 transfers over a period of 68 weeks in one series, and  $10^{17}$ -fold in 12 transfers over a period of 56 weeks in another series. The generation time of *M. lepraemurium* was 7 days, a growth rate which approximates the fastest growth of the organisms in vivo. Organisms harvested from cultures at various stages of growth produced murine leprosy in mice, but showed no growth in bacteriological media. The present model offers an opportunity for studies on the host-parasite relationship without the complication of extracellular growth of the parasites.

Among various mycobacterial infections, murine leprosy seems to bear 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 intracellular requirements of *Mycobacterium lepraemurium* may furnish useful clues regarding those of *M. leprae*. Since the discovery of the bacillus by Stefansky (27), efforts have been made by many investigators to grow the organisms of murine leprosy in artificial media, without success (17). Attempts to grow the organisms in tissue cultures began with Zinsser and Carey (31), who observed successful proliferation of the bacilli in two cultures of explants from rat spleen. Fraser and Fletcher (quoted in 18) and Friedheim (7) were unable to confirm these results. Kudicke

and Vollmer (16) observed growth in tissue culture during the first 22 days after inoculation. Suwo and Kin (28) observed growth both in the cells and in the culture medium. On the other hand, Lowe and Dharmendra (18) were unable to obtain growth after an extensive trial in various tissue cultures. All of these studies used the plasma clot culture of explants of various tissues of rat, mouse, chick embryo, and mouse sarcoma. This type of culture has the disadvantage that any organisms liberated from the host cell will remain in the clot and may be picked up by the macrophages. Accumulation of organisms in cells by such rearrangement or redistribution of organisms may be mistaken for true bacterial multiplication.

Limited growth of *M. lepraemurium* was observed in 1958 by three groups of investigators (12, 26, 29), using monolayer cultures of mouse and rat spleen explants and of the peritoneal macrophages of rats. The host cells of these cultures were difficult to maintain in good condition, and in some experiments fresh noninfected cells had to be added periodically to maintain the cell population. Moreover, the importance of the

<sup>1</sup> Presented in part at various meetings since 1958. A list of titles of these reports and meetings at which they were presented is cited in the proceedings of LWM-AFIP Conference on Research Problems in Leprosy, Washington, D.C., 11-14 May 1965 (Intern. J. Leprosy 33:586-598, 1965).

<sup>2</sup> Present address: Department of Microbiology, University of Maryland, College Park.

length of the organisms inoculated had not yet been emphasized. The inoculation material was obtained from animals at the time the organisms were apparently in the long forms (3). It seems possible that the long-form bacilli which had been kept in poor host cells may have split into several short ones, resembling actual bacterial multiplication. Limited growth was also observed in established cell cultures, e.g., L cells (22, 29). Growth of the organisms was never reported to have occurred in subculture in any of these studies.

Successful growth of *M. lepraemurium* was observed in subcultures by Garbutt, Rees, and Barr (9) and Rees and Garbutt (25), using the 14pf strain of rat fibroblasts. Growth occurred in subcultures only when a large portion of cells of the original culture was transferred into fresh fibroblasts. Since fibroblasts grow too fast at 37 C, the culture was maintained at 34 C. Bacterial counts of the whole culture were used for evaluation of growth because the bacterial distribution in fibroblasts was not even. The organisms showed a rather slow growth rate, with a generation time of about 15 days (8).

The growth studies of *M. lepraemurium* being reported here began in 1955. Macrophages were used as host cells because this type of cell multiplies very slowly and any change in the bacillary population can be observed readily. Since the growth of *M. lepraemurium* is slow, long-term cultivation of macrophages is necessary. Efforts have been made along this line during the past 11 years. Growth of the bacilli was obtained only occasionally in early experiments. Progressively, however, not only were the macrophages maintained in good condition for a long period of time, but at present the growth of *M. lepraemurium* is observed in this cell system at a generation time of 7 days, approaching the fastest growth of the bacilli in animals.

#### MATERIALS AND METHODS

**Materials.** Filtered horse serum, medium 199, NCTC 109 (Hanks) balanced salt solution (BSS), Eagle's basal medium, and phenol red solution were obtained from Microbiological Associates, Inc., Bethesda, Md.; beef embryo extract (BEE; lyophilized 2-ml vials), from Difco Laboratories, Inc., Detroit, Mich.; liver extract and liver extract L fraction, from Nutritional Biochemicals Corp., Cleveland, Ohio; ferric nitrate, from Fisher Scientific Co., Silver Spring, Md.; heparin, from Hynson, Westcott & Dunning, Inc., Baltimore, Md. Mycobactin was kindly supplied by N. E. Morrison, Leonard Wood Memorial Laboratory, Johns Hopkins School of Hygiene, Baltimore, Md.

Leighton-type culture tubes (16 by 150 mm) with metal closures and Pasteur pipettes (bent tip) were

obtained from Bellco Glass Inc., Vineland, N.J.; Gold Seal microcover slips (8 by 22 mm; no. 2 thickness, changed to no. 1 thickness later), from Clay-Adams Inc., New York, N.Y.

**Cultivation of macrophages.** Techniques for cultivation of peritoneal macrophages of the mouse have been described elsewhere (4). Briefly, the peritoneal exudate of mice was washed out through a small incision just below the xiphoid process. In the early studies, the washing fluid was BSS containing 1:20,000 heparin and, later, the chemically defined, protein-free medium NCTC 109 with heparin. The cell suspension (5 ml from each mouse) was introduced into Leighton tubes containing an 8 by 22 mm cover slip (1 ml each tube). The NCTC 109 medium was replaced with complete medium 3 hr 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 C in closed tubes in the early studies, and in an atmosphere of 5% CO<sub>2</sub>-air 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 (the "in vivo-in vitro" experiments). In later studies, macrophages were infected by adding a bacillary suspension 1 day after cultivation of macrophages in tissue culture.

**Culture medium.** The media were made of various amounts of horse serum, BSS, Eagle's basal medium, medium 199, NCTC 109, BEE, and various amounts of the following supplements: spleen homogenate, liver homogenate, liver extract, glycerin, ferric nitrate, and mycobactin.

The contents of one vial of BEE were dissolved in 10 ml of BSS in the early studies and in NCTC 109 later, to make a 1:5 dilution. Centrifugation was sometimes necessary to eliminate undissolved lumps. The solution was used immediately after preparation.

Spleen homogenate was prepared aseptically by grinding one fresh mouse spleen in 5 ml of BSS in a Ten Broeck tissue grinder. The homogenate was frozen and thawed to break any intact cells present. The supernatant fluid from this preparation was kept at 4 C and used within 1 week. Liver homogenate was prepared in a similar manner and diluted 1:20 with BSS (with NCTC 109 later).

Liver extract and liver extract L fraction were dissolved in NCTC 109 as a 1% solution and sterilized by membrane filtration (Millipore Corp., Bedford, Mass). Ferric nitrate was dissolved in water as a 0.1% solution and sterilized by membrane filtration. Glycerol was dissolved in water as a 40% solution and sterilized by autoclaving. Mycobactin was dissolved in 40% glycerol and sterilized by autoclaving.

Although various types of media were used in the present study, most experiments were performed with the three media of the following composition.

**Medium 9:1.** This medium was made of 90% horse serum and 10% of a 1:5 dilution BEE (in BSS).

**Medium 4:5:1.** The effect of various supplements was studied in this basal medium, composed of 40%

horse serum, 50% NCTC 109, and 10% of a 1:5 dilution of BEE (in NCTC 109).

*Medium 4:4:1:1.* This was made of 40% horse serum, 40% NCTC 109, 10% of a 1:5 dilution of BEE, and 10% liver extract L fraction (1% in NCTC 109).

Penicillin (100 units per ml) was added to all media.

*Bacillary suspensions.* Suspensions were prepared from the leptotic 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 1:30 suspension was used for the infection of mice, 0.5 ml per mouse, injected intraperitoneally, for the "in vivo-in vitro" experiments. This suspension was made by grinding a piece of lesion with BSS in a Ten Broeck tissue grinder.

Bacterial counts of suspensions were made by counting 100 square fields (a 5-mm<sup>2</sup> grid in the ocular) in a 18-mm smear made by a 2-mm platinum loop in the early experiments (2). The pin-head technique (11) was used in later experiments.

*Enumeration of macrophages.* Macrophages were counted in a square field with 10× objective. Counting was made along three parallel longitudinal lines on the cover slip. The total number of macrophages on the whole cover slip was calculated from the average number per square field multiplied by the conversion factor: area of cover slip (in mm<sup>2</sup>)/area of square field (in mm<sup>2</sup>) = 176/0.26 = 677.

*Schedule for recording growth of bacilli with the low-power objective.* The simple method of Hanks (10) for low-power examination was used in some experiments. Slight growth was indicated by the fact that about 5% of the macrophages contained visible bacilli. (A visibly infected macrophage contains at least 25 bacilli as observed with a 10× objective.) Moderate growth was denoted by 25% visible infected cells, marked growth by 50%, and maximal growth by 75% 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. This is determined by randomly observing 100 macrophages on the cover slip and recording each as containing the following numbers: 1-2, 3-5, 6-10, 11-20, 21-50, 51-100, 101-200, 201-400, 401-600, 601-800, etc. An actual determination is presented in the following example: if the percentages for the 100 macrophages are 50, 30, 15, 3, and 2, in respect to the categories 1-2, 3-5, 6-10, 11-20, and 21-50, then the average number of organisms per macrophage will be:  $[(50 \times 1.5) + (30 \times 4) + (15 \times 8) + (3 \times 15) + (2 \times 35)]/100 = 4.3$ .

The total number of bacilli of the whole cover slip was calculated as follows: average number of macrophages per square field  $\times$  677  $\times$  average number of bacilli per macrophage  $\times$  percentage of macrophages with bacilli.

In the later part of the present study, the cell population of cultures had been kept in a fairly stable condition, and the rate of phagocytosis had been maintained unchanged within a certain period of

time. The intracellular bacterial count was then derived solely from the number of organisms per macrophage.

*Enumeration of organisms in the whole culture.* All macrophages in Leighton tubes were harvested with a rubber policeman. Organisms in the discarded medium after each change of medium were collected by centrifugation. A homogenate of the cell suspension was made in a Teflon Ten Broeck tissue grinder. Bacterial counts were made by the pin-head technique of Hanks et al. (11). Counts of the culture at 1 day and at the time of termination of the experiment were recorded. The total bacterial count included the organisms from the culture vessel and those collected from the discarded medium.

*Measurement of the length of a bacillus.* Measurement of the length of a bacillus was made with a scale in the ocular, each division being 0.55  $\mu$ . The length of the bacillus was recorded as the average for 100 bacilli, based on the following schemes: 1-3, 4-5, 6-7, 8-10, etc. For example, if the percentages are 60, 30, 6, and 4, respectively, then the length of the bacillus will be  $[(60 \times 2) + (30 \times 4.5) + (6 \times 6.5) + (4 \times 9)]/100 \times 0.55 = 1.8 \mu$ .

*Generation time of *M. lepraemurium*.* The generation time of bacillary growth was calculated according to the log base 2 method of Finney, Hazlewood, and Smith (5). Since multiplication of the bacilli does not occur in a period of 1 day, and since phagocytosis is complete in 1 day, the day 1 record was used as the day zero data for calculation of the generation time.

*Staining of macrophages and acid-fast organisms.* Cover slips were washed with 0.85% NaCl and fixed in Zenker solution for 5 min. The cover slip was floated with the cell side down in Ziehl-Neelsen stain, which had been heated just to boiling on a glass slide (about 2.5 by 7.5 cm). Care was taken to float the cover slip over a depth of stain to prevent cells from coming in contact with the glass slide. The cover slip was destained 5 min later with 1% HCl in 95% ethyl alcohol. Macrophages were stained with Harris hematoxylin for 30 to 60 sec, followed by a 0.3% ammonia-water wash.

## RESULTS

*In vivo-in vitro experiments, 1955-1958.* Peritoneal exudate cells from animals infected with murine leprosy were used in the beginning of the experiments, on the assumption that the host-parasite relationship would be nearly perfect when macrophages were infected long before they were transferred for in vitro cultivation. Different media, containing various amounts of horse serum, BSS, Eagle's basal medium, BEE, and mouse spleen homogenate, were tried. The composition of these media has been reported in a previous paper (4) as media numbers 1 to 6. It was possible to maintain 29 of 62 experiments for a period of 2 to 5 weeks, but only 5 experiments showed growth of the bacilli. These five experiments were maintained in a medium containing 95 to 97% horse serum and 3 to 5% of a

1:1 dilution of BEE (medium 6). Media made with a lower concentration of horse serum, or with a high serum concentration without BEE, 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 bacterial growth. In five experiments with growth of bacilli, the number of bacilli per macrophage increased 3- to 16-fold, with an average generation time for the bacilli of 12 days. The total number of bacilli in the whole cover slip increased 3- to 15-fold in three experiments and remained unchanged in the other two, despite those with a marked decrease in the population of macrophages. No growth was observed in experiments treated with isoniazid or in those infected with autoclave-killed bacilli (Table 1).

Elongation of *M. lepraemurium* was observed in four cultures of the first five experiments (Table 1). Very long bacilli, up to 15  $\mu$  in length, appeared occasionally. Thus, for the first time in the history a definite growth of *M. lepraemurium* (elongation accompanied by multiplication) was observed in tissue culture.

However, disadvantages of the in vivo-infected cell model were obvious. First, the percentage of macrophages containing bacilli varied greatly. These erratic variations made evaluation of bacillary growth rather difficult. Second, the organisms were initially in moderately long form, and observation of extensive elongation was difficult. It was believed, therefore, that a system containing noninfected macrophages which could be infected subsequently with a controlled number of the shortest forms of the bacilli would facilitate the observation of both elongation and multiplication of the organisms.

*Macrophages infected in vitro.* Known numbers of macrophages were infected with known num-

bers of short organisms. Selection of the latter was based on Chang's observation (3) that the length of *M. lepraemurium* in mice is maximal between 4 days and 9 weeks, then slowly decreases to minimal (about 1  $\mu$ ) by 16 weeks. Cultures usually were infected with bacilli obtained 3 to 6 months after infection. With approximately  $10^6$  macrophages and  $6 \times 10^5$  bacilli per Leighton tube, replacement of the bacterial suspension with fresh medium after 3 hr resulted in about 50% infected cells and an average of three to six bacilli per cell. In the experiments evaluated by the low-power-objective technique, a heavier infection was obtained by employing  $10^6$  bacilli per tube. Maximal infections were produced by leaving the bacilli and the complete medium in the culture for 3 days, to ensure complete phagocytosis.

Series I comprised 79 experiments on various types of media. Growth of *M. lepraemurium* was observed in cultures maintained in these media (Table 2). In a total of 79 experiments performed over a period of about 2 years, only 12 experiments showed growth of the bacilli. The extent of growth ranged from slight to maximal. The observation period covered 29 to 100 days. In cultures showing maximal growth, all host cells were studded with acid-fast organisms. Although growth was observed in various types of medium, consistent results were obtained only in a medium containing 90% horse serum and 10% of a 1:5 dilution of BEE, designated here as medium 9:1 and previously (4) as medium 7.

Series II comprised 36 experiments, each in the presence of CO<sub>2</sub>, comparing medium 9:1 versus medium 4:5:1. The importance of maintaining the medium at a pH of 7.2 with a 5% CO<sub>2</sub>-air mixture, as observed for the long-term cultivation of macrophages (4), also appeared to be significant for the growth of the bacilli. Further-

TABLE 1. Growth of *Mycobacterium lepraemurium* in macrophages infected in vivo (in vivo-in vitro experiments)

Expt	Days	Macrophages with bacteria (initial/final)	No. of bacteria/macrophage (initial/final)	No. of macrophages/field (initial/final)	Total no. of bacteria in the cover slip ( $\times 10^6$ )	Generation time <sup>a</sup>	Avg length of bacteria
		%				days	$\mu$
35	35	33/45	4.0/63.0	250/98	2.2/18.8	8.8	2.4/2.7
44	28	40/56	8.0/22.2	93/75	2.0/6.3	19.0	2.6/1.9
45	35	78/86	12.8/68.0	149/29	10.1/11.5	14.5	2.6/3.0
79	21	98/100	13.4/42.7	133/43	11.8/12.4	12.6	1.9/3.1
88	14	89/98	13.1/63.0	14/39	1.1/16.3	6.2	1.6/3.3
44 <sup>b</sup>	28	39/29	6.6/5.0	106/89	1.9/0.9		1.7/1.9
46 <sup>c</sup>	21	41/20	4.1/4.8	133/60	1.5/0.4		1.1/1.1

<sup>a</sup> Calculated from the average number of bacilli per macrophage.

<sup>b</sup> Isoniazid (4  $\mu$ g/ml) was added to the medium throughout this experiment.

<sup>c</sup> Autoclave-killed *M. lepraemurium* was used in this experiment.

TABLE 2. Growth of *Mycobacterium lepraemurium* in macrophages infected in vitro

Growth <sup>a</sup>	Expt	Cells infected initially	Medium <sup>b</sup>	Observation period (days)
Maximal	164	76	HS (40%), S, E	73
	224	28	HS (90%), E	100
Marked	169	77	HS (40%), S, E, Eagle	41
	209	78	HS (80%), S, E	60
	226	30	HS (90%), E	85
Moderate	95	80	HS (90%), S	49
	113	75	HS (95%), S	34
Slight	120	68	HS (90%), S	34
	153	70	HS (60%), S, 109	29
	205	70	HS (60%), S, E, L, Eagle	90
	206	81	HS (40%), S, E, L, Eagle	85
	228	82	HS (90%), E	45

<sup>a</sup> Growth was evaluated with the low-power objective.

<sup>b</sup> HS, horse serum; S, spleen homogenate; L, liver homogenate; E, BEE (1:5 dilution); 109, NCTC 109; Eagle, Eagle's basal medium.

more, a medium containing a low concentration of horse serum was desired, since certain batches of horse serum are harmful to tissue cultures when used in high concentrations (1). This was made possible by substituting the chemically defined medium NCTC 109 (19) for a portion of the serum. In addition, the peritoneal exudate was harvested with NCTC 109 instead of BSS. A medium consisting of 40% serum, 50% NCTC 109, and 10% of a 1:5 dilution of BEE, designated here as medium 4:5:1 and previously (4) as medium 13, was compared with medium 9:1 for the growth of *M. lepraemurium* in a period of 40 days. Growth, from slight to marked, was observed in 33 of the 36 experiments (92%) with medium 4:5:1, but in only 9 experiments (25%) with medium 9:1. Elongation of bacilli was observed in all 36 experiments with medium 9:1, including those showing no visible growth with the low-power-objective technique (Table 3).

*Factors affecting growth of M. lepraemurium in macrophages.* Medium 4:5:1 was next used as the basal medium to study the effect of various supplements on the growth of the bacilli. The effect of mycobactin, glycerol, liver homogenate, liver extract, ferric nitrate, and various combinations of these substances was studied. A study was also made on the effect of another chemically defined medium (medium 199) as a replacement for NCTC 109.

The above experiments were performed with an observation period of 40 days, a length of time necessary for the evaluation of bacterial growth by the low-power-objective technique. In the following experiments, with the exception of those

TABLE 3. Comparison of experiments yielding growth of *Mycobacterium lepraemurium* in macrophages during 40 days of maintenance in medium 9:1 and medium 4:5:1 in the presence of CO<sub>2</sub>

Growth	Cells infected initially (medium 4:5:1)/medium 9:1	Medium 4:5:1 <sup>a</sup>	Medium 9:1 <sup>a</sup>	Elongation of bacteria in medium 9:1 <sup>b</sup>
	%			
Marked...	80.0/78.0	8.3	2.7	1.8
Moderate...	78.6/79.7	19.4	8.3	1.1
Slight....	75.6/76.6	63.9	14.0	1.2
None.....	79.3/72.6	8.3	75.0	0.8

<sup>a</sup> Results expressed as percentages of experiments (36 experiments in each medium).

<sup>b</sup> Figures represent average increase over initial length of bacilli.

performed with glycerol and liver homogenate (see below), a more accurate method for evaluation, the enumeration of intracellular organisms, reduced the observation period to 4 weeks. Since variations of growth rate occurred in different experiments, all data were obtained from an average of five experiments.

*Mycobactin.* Mycobactin, a growth factor isolated from *M. phlei*, is essential for the growth of *M. johnei* in vitro (6). It was of interest to determine whether mycobactin might have a stimulating effect on the growth of *M. lepraemurium*. Since mycobactin was dissolved in glycerol, studies on the effect of the latter were also included. As shown in Fig. 15, mycobactin showed

no activity, and, as shown here and also below, glycerol was not stimulatory. Studies were also made with mycobactin dissolved in ethyl alcohol (0.2% in the medium). No stimulating activity was revealed by either of these compounds.

**Glycerol.** Each of 10 experiments consisted of two sets of cultures, one with glycerol and the other a control. The number of bacilli, judged by the low-power-objective technique, showed no marked difference between the two sets. Although the average length increased from 1.5  $\mu$  initially to 2.4  $\mu$  at 30 days, some bacilli attained a length as great as 15  $\mu$  (Fig. 5).

**Liver homogenate and liver extract.** The effect of mouse liver homogenate was first studied with the low-power-objective technique in 16 experiments maintained in medium 4:5:1 in which 30% of NCTC 109 was replaced by BSS. Half of the experiments showed moderate, marked, or maximal growth. When the complete medium 4:5:1 was used, in another 17 experiments, all showed from moderate to maximal growth.

The effect of liver homogenate was compared with that of a commercially available liver extract and its soluble L fraction. The latter was used for titrations of activity. As evaluated by counting intracellular organisms over a period of 28 days, the activities (per milliliter) were as follows: mouse liver homogenate <1 mg of liver extract = 1 mg of its L fraction <3 mg of L fraction = optimal (Fig. 16).

**Ferric nitrate.** Recently, ferric nitrate was found to possess a stimulating action on the growth of *M. johnei* in macrophage cultures (30). Ferric nitrate (1 to 100  $\mu\text{g/ml}$ ) had a definite stimulatory effect on the growth of *M. lepraemurium*; 4 to 10  $\mu\text{g/ml}$  was optimal (Fig. 15).

**Combination of liver extract L fraction, ferric nitrate, and glycerol.** Although combinations of the L fraction of liver and glycerol, or iron plus glycerol, were stimulatory, the addition of glycerol to combinations of L fraction and iron did not further enhance growth (Fig. 17).

**Medium 199.** Medium 199 is a chemically defined medium widely used in tissue culture (21). Cultures were established in medium 4:5:1 in which NCTC 109 was replaced by medium 199. Peritoneal exudate was also harvested with medium 199 instead of NCTC 109. Although macrophages could be maintained in good condition in this medium for many days, the growth of *M. lepraemurium* was significantly less than that observed with medium 4:5:1 containing NCTC 109. Supplementation with ferric nitrate, liver extract L fraction, or a combination of the two, did not promote an improvement in the growth of the bacilli.

**Growth rate of *M. lepraemurium*.** The growth

rate of *M. lepraemurium* was variable in different experiments. A typical example of these variations is shown in Fig. 17, representing results of the five experiments performed with combinations of liver extract, ferric nitrate, and glycerol. Such inconsistencies were observed throughout the observation period of the cultures.

Comparison of the growth rates of organisms was made in the five experiments which showed the highest rate, i.e., cultures supplemented with iron and L fraction of liver. The successive generation time at 7-day intervals over a period of 28 days was 7.0, 9.9, 10.5, and 11.8 days for the control experiments, and 9.0, 8.0, 7.5, and 6.8 days for the supplemented. However, a more uniform generation time (12.0 to 13.4 days) was obtained at the intervals when the calculation was based on a larger number (25) of control experiments. An average of 12.6 days was obtained for *M. lepraemurium* with medium 4:5:1.

**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 mg/ml in the earlier transfers and 1 mg/ml later). Serial transfers were made at intervals for 4 to 5 weeks (occasionally 3 or 6 weeks) by inoculation of bacilli harvested from the mature cultures into cultures of new macrophages. Two series of transfers were carried on, each having been initially infected with organisms obtained from a different mouse. The cumulative growth curves are shown in Fig. 18. The average generation time of *M. lepraemurium* was 7 days.

Growth was slightly slower in the original cultures and a few early transfers, with generation times of 8 to 10 days. Faster growth was observed after four to five transfers, and the generation time eventually decreased to 5 to 7 days. The number of bacilli per macrophage increased 10- to 41-fold at the end of each transfer, with a cumulative increase of  $1.4 \times 10^{20}$ -fold in 14 transfers over a period of 68 weeks in one series, and  $10^{17}$ -fold in 12 transfers over a period of 56 weeks in the other. Variations in the rate of growth that had been evident with organisms from the animals were no longer observed. With such rapid growth, multiplication of organisms could be easily recognized at the end of 3 weeks, with an eightfold increase in the number of bacilli.

Cultures allowed to surpass the usual period of time before transfer demonstrated that maximal growth of the bacilli occurred at the end of 6 to 7 weeks. At this time, most macrophages were filled with tens to hundreds of organisms. Organisms harvested after many transfers produced typical murine leprosy (Fig. 18).

A series of photomicrographs showing the

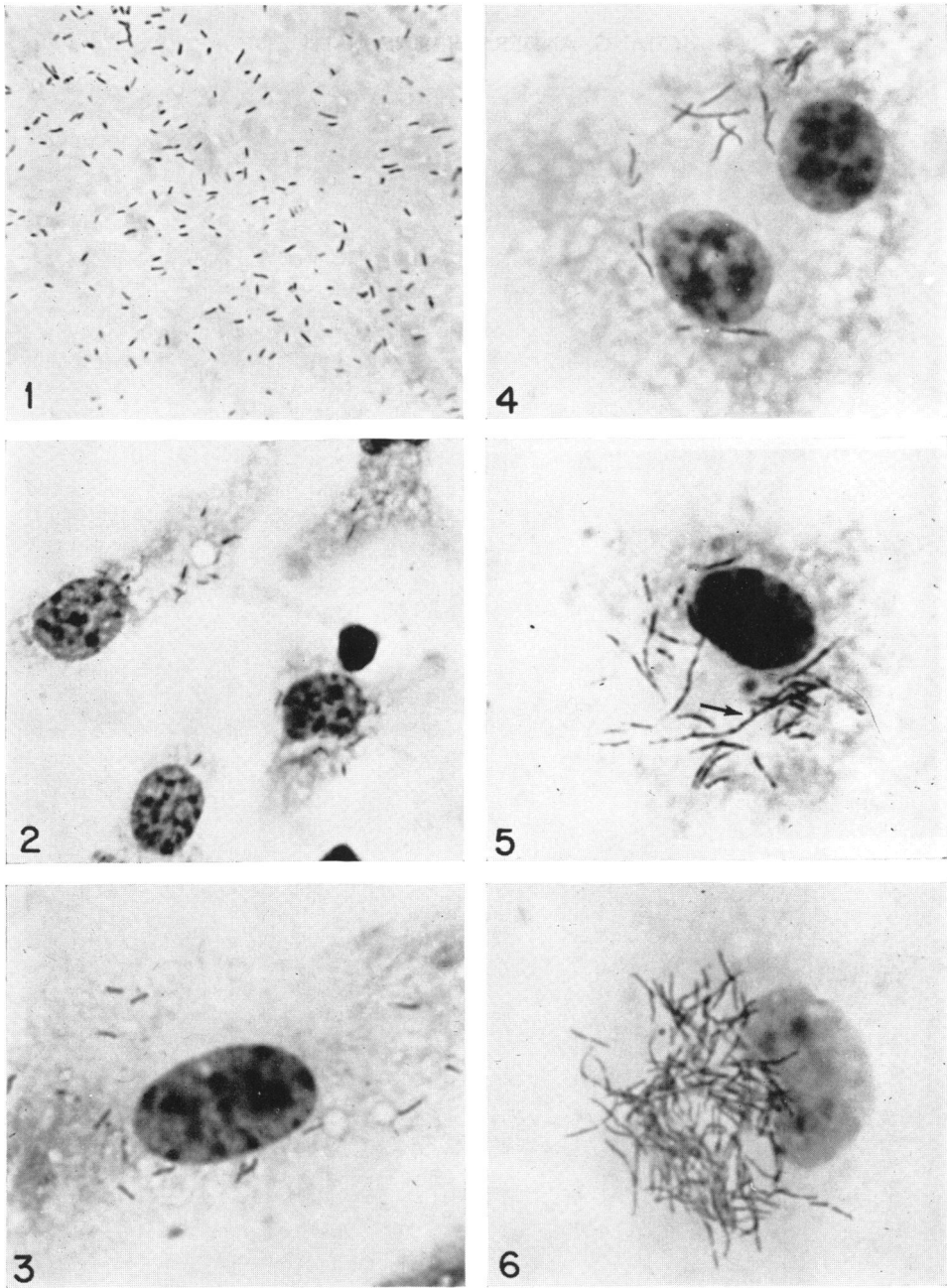


FIG. 1. Smear of bacillary suspension made from the omental lesions of a mouse infected with *Mycobacterium lepraemurium* 4 months previously.  $\times 1,150$ . (All bacilli in Fig. 1-14 are acid-fast organisms stained with Ziehl-Neelsen followed by hemotoxylin stain.)

FIG. 2. Macrophage with *M. lepraemurium* 5 hr after infection.  $\times 1,150$ .

FIG. 3. Macrophage with autoclave-killed *M. lepraemurium* in a 100-day-old culture. There was no growth of the organisms.  $\times 1,150$ .

FIG. 4. Macrophage with *M. lepraemurium* in a 20-day-old culture, showing elongation of the organisms.  $\times 2,000$ .

FIG. 5. Macrophage with *M. lepraemurium* in a glycerol-treated, 40-day-old culture. The arrow shows a very long bacillus with two tiny buddings, each originating from a dense granule of the bacillus.  $\times 2,000$ . This picture was overdeveloped to show the faintly stained organisms.

FIG. 6. Growth of *M. lepraemurium* in a macrophage in a 42-day-old culture of the 17th transfer of one serial-transfer experiment, showing both elongation and multiplication of the organisms. Note the good appearance of the large nucleus.  $\times 2,000$ .

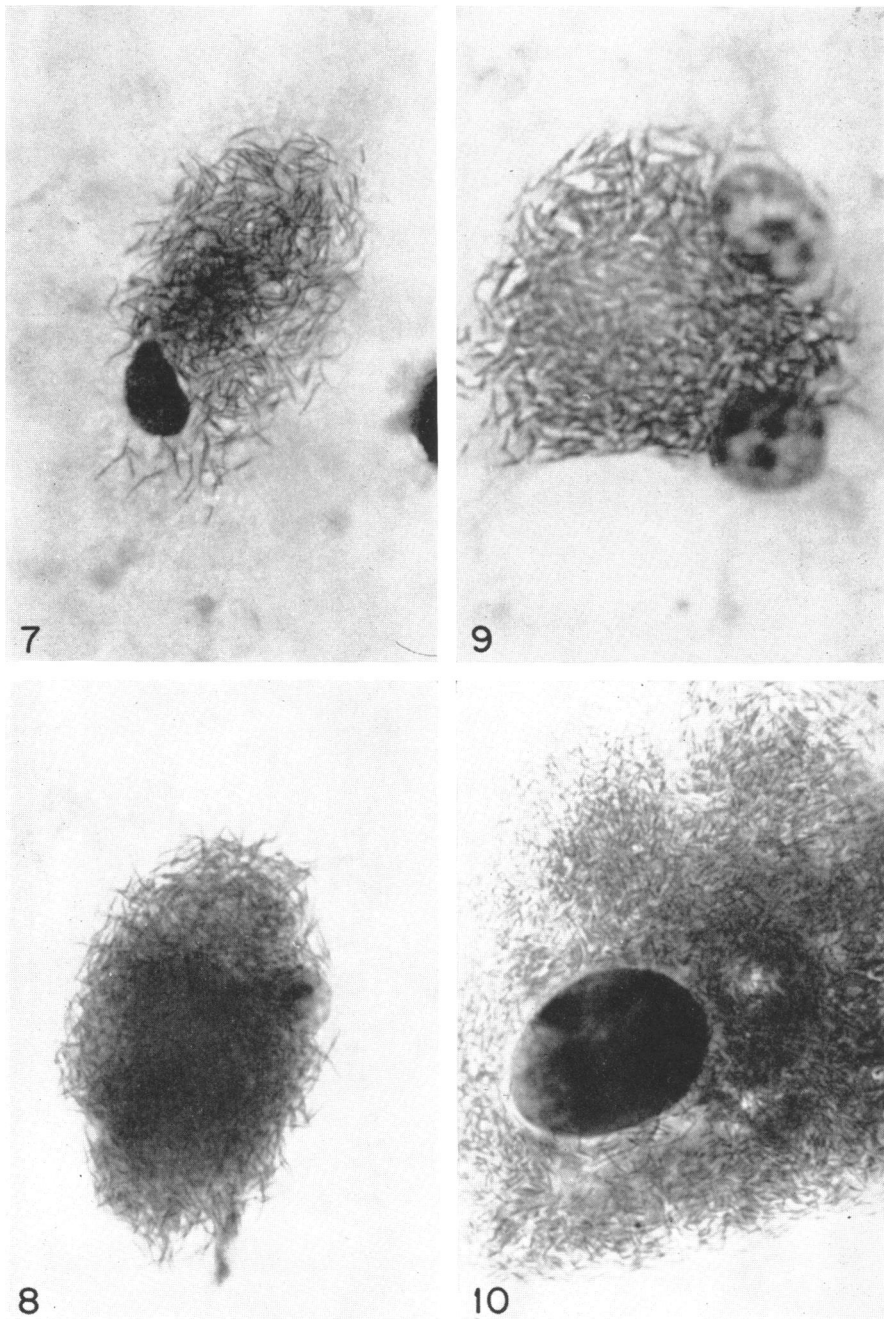


FIG. 7. Macrophage with numerous long *Mycobacterium lepraemurium* cells in a 100-day-old culture. The nucleus is pycnotic.  $\times 1,150$ .

FIG. 8. Macrophage with *M. lepraemurium* in a 100-day-old culture. Two small nuclei are surrounded by bacilli.  $\times 1,150$ .

FIG. 9. Macrophage with numerous organisms from a 28-day-old culture in the 13th transfer of one serial-transfer experiment. Note the two big nuclei.  $\times 2,000$ .

FIG. 10. Part of a large macrophage containing numerous short bacilli and a distinct nucleus; 40-day-old culture. The picture is overdeveloped to show the appearance of the short organisms.  $\times 1,150$ .



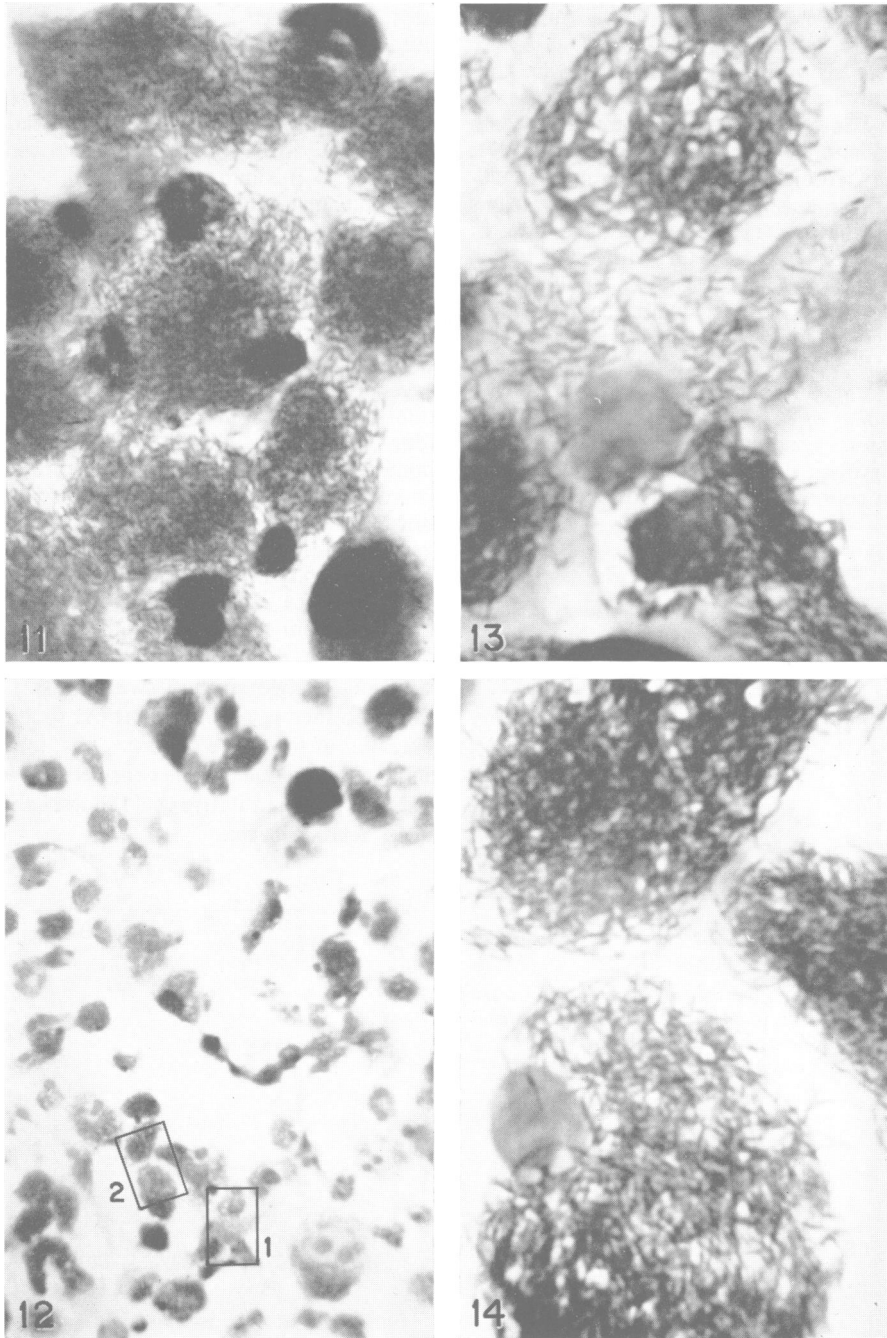


FIG. 11. Growth of *Mycobacterium lepraemurium* in macrophages in a 40-day-old culture. A cluster of macrophages, each containing hundreds of bacilli. Several nuclei are pycnotic and located on one side of the host cells.  $\times 2,000$ .

FIG. 12. Growth of *M. lepraemurium* in a 40-day-old culture in one of the rapid-growth experiments in the early part of this study.  $\times 210$ . Rapidly growing cultures, which were observed only occasionally at that time, can now be obtained in every transfer of the serial-transfer experiments. All macrophages are filled with acid-fast organisms.

FIG. 13. Enlargement of rectangular area 1 of Fig. 12.  $\times 2,000$ .

FIG. 14. Enlargement of rectangular area 2 of Fig. 12.  $\times 2,000$ .

appearance of *M. lepraemurium* at various stages of growth are shown in Fig. 1-14.

**Failure to grow on bacteriological media.** Cultivable acid-fast organisms have been frequently isolated by investigators as contaminants from tissues of animals with murine leprosy (17). Lowenstein-Jensen medium was routinely inoculated with organisms harvested from various cultures in the past 11 years, including those of the transferred cultures. Growth of acid-fast organisms has never been observed. Similar negative results were obtained with the occasional inoculation of four other media used for the growth of acid-fast organisms, i.e., Dorset, Petroff, Petragnani, and A.T.S.

**Distribution of *M. lepraemurium* in cultures of macrophages.** Quantitative evaluation of the bacillary growth in the present study was based mainly on counting the number of bacilli in host

cells. It has been known that redistribution of intracellular organisms occurs in tissue cultures, especially in cells maintained in plasma clot culture. This situation is avoided with monolayer cultures, in which the majority of dead macrophages and their contents are removed during renewals of medium.

Despite marked multiplication of the organisms within macrophages, the percentage of cells with bacilli remained nearly constant over a period of 28 days (Table 4). Thus, since the cell populations in this model are relatively stable, quantitations could be based on counts of intracellular bacilli.

To test this point, a comparison was made between the random intracellular counts and the total bacterial counts (in the whole culture) in four experiments. The latter were made from a homogenate of 5 to 10 pooled cultures. These counts included organisms collected from the discard after each change of medium. The average generation time, calculated from the intracellular count, was 5.9 days; that from the total bacterial count was 7.1 days. The slightly higher figure obtained from total count might be explained by the fact that the organisms eliminated with the changing of medium underwent no further division, subtracting slightly from the multiplication potential of the entire culture. Thus, intracellular enumeration alone may be considered as a reliable criterion for quantitative evaluation of the growth of *M. lepraemurium*.

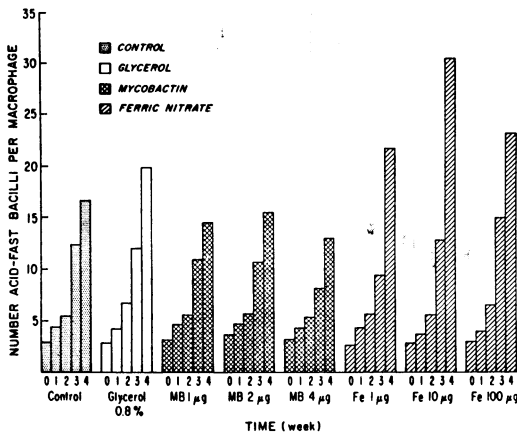


FIG. 15. Effect of glycerol, mycobactin, and ferric nitrate on the growth of *Mycobacterium lepraemurium* in macrophages. The concentrations are per milliliter of the medium. Data are averages of five experiments.

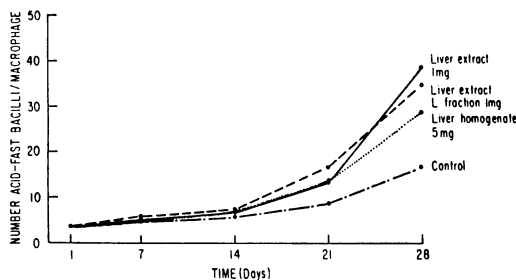


FIG. 16. Comparison of the effect of mouse liver homogenate, liver extract, and liver extract L fraction on the growth of *Mycobacterium lepraemurium* in macrophages. The concentrations are per milliliter of medium. Data are averages of five experiments.

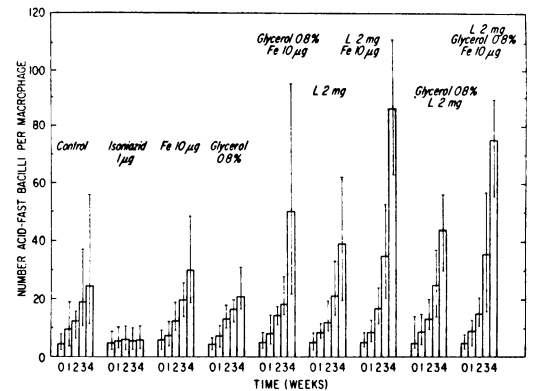


FIG. 17. Effect of various combinations of liver extract L fraction, ferric nitrate, and glycerol on the growth of *Mycobacterium lepraemurium* in macrophages. Empty bar represents the average number of organisms of five experiments. The line on the bar represents the highest and lowest number of organisms observed in these experiments. L = liver extract L fraction. Fe = ferric nitrate. The concentrations are per milliliter of medium.

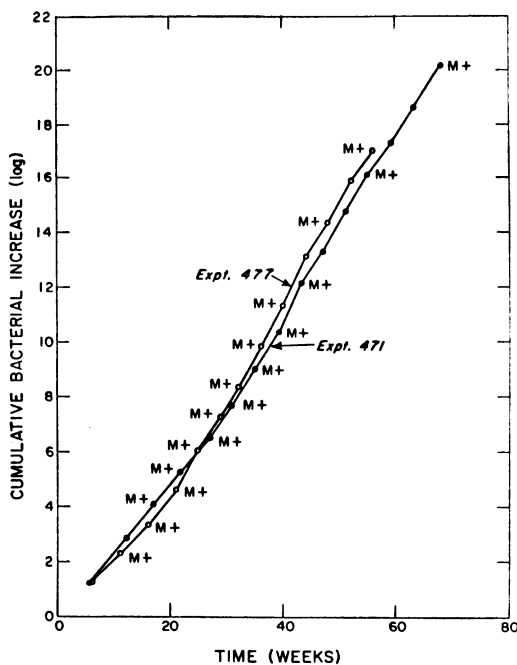


FIG. 18. Multiplication of subcultured *Mycobacterium lepraemurium*. Cumulative growth curves of two serial-transfer experiments. M+ denotes growth of murine leprosy in three mice inoculated subcutaneously with organisms harvested at the time of the indicated transfer. A leproma weighing 0.3 to 0.5 g was observed in "M+" animals in a period of 90 days.

TABLE 4. Proportions of infected cells<sup>a</sup>

Age of culture	Medium 4:5:1		Medium 4:5:1 with various supplements	
	No. of bacteria per macrophage	Macrophages with bacteria	No. of bacteria per macrophage	Macrophages with bacteria
days		%		%
1	3.4	68	3.9	65
7		67		69
14		64		63
21		61		67
28	16.1	62	51.0	74

<sup>a</sup> Data are averages based on the control cultures and the supplemented cultures which gave the highest counts in each of 25 experiments on factors that stimulate intracellular growth.

#### DISCUSSION

The main advantage of the present host cell-parasite system is that the host cells can be maintained in a monolayer in the same vessel for weeks

or months. Observation can be made in these cells without periodic transfers into new vessels, in contrast with ordinary cell lines. This does not mean that individual host cells remain stable. A constant replacement of senile host cells by a slow rate of reproduction of younger macrophages has been considered to exist in this cell system (4).

At least three factors may cause the disappearance of macrophages. The decrease in number during the period of maturation, which had been observed when peritoneal exudate cells were harvested with BSS instead of NCTC 109, has been described (4). The second factor is crowding. As macrophages grew larger, they exceeded the available space. This was evident in that healthy cells could be found in the discarded medium. These cells grew well when transferred to new vessels. The third cause was the death of the macrophages because of parasitism by *M. lepraemurium*. When macrophages were loaded with hundreds of organisms, their nuclei were usually pushed to one side and appeared pycnotic. These cells first became inactive, then aggregated in clumps of various sizes, before finally floating off the surface of the cover slips. Such clumps were frequently observed just before fixation of the cover slips for staining. Thus, the in vitro system is quite different from the situation in the animal, in which case macrophages loaded with acid-fast organisms accumulate and remain in tissues for long periods of time.

Since the disappearance of macrophages by crowding-out occurs prior to maximal infection, the losses of infected and noninfected cells are apparently on a random basis. Therefore, the percentage of cells and bacilli remains unchanged in the culture. After the stage of crowding-out, the population of macrophages remains relatively stable as long as the host cells remain unaffected by marked parasitization.

Elongation of bacilli has been recognized as a sign of growth of *M. tuberculosis* (20). The elongation of *M. lepraemurium* was observed by one of us (YTC) as the first definite sign of growth of this organisms in tissue culture. Rabson (23) confirmed this observation in a cell culture of mouse lymphoma.

Elongation of *M. lepraemurium* was detected as early as 3 or 4 days after infection. An average increase of two to three times the original length was observed in 1 to 2 weeks in cultures infected with the short organisms. A lesser degree of elongation was observed in the serially transferred cultures, since the organisms were already in moderately long form at the time of transfer. Glycerol seems to stimulate the bacillary growth

but inhibits division. The mechanisms of this action require elucidation.

Irregularly stained bacilli were seen frequently in cultures with a fast rate of bacillary growth. Organisms with faintly stained ends or with an isolated acid-fast dot at one or both ends were often observed. Chains of several acid-fast masses, stained faintly in some areas but deeply in others, were also noticed. More solidly stained organisms were observed when cultures were maintained for a longer time. It seems likely that the irregularly stained organisms are chiefly the young forms of *M. lepraemurium*, and that these became solid on maturation. Details will be reported elsewhere.

An unusual phenomenon was observed in cultures of the later part of the serial transfer experiments. The organisms appeared frequently in bundles consisting of a few to tens of even hundreds of bacilli. This phenomenon, known as globus formation, has been considered as a specific characteristic of the growth of human leprosy bacillus. Studies are in progress on factors which may provoke this globus formation. Details will be reported later.

In regard to the staining technique of Ziehl-Neelsen, the warm method was found better than the cold method. In the early part of this study, the cold method of Kinyoun (15) was used. Unstained bacilli were observed in the macrophages as white, rod-shaped, empty spaces, which reappeared as acid-fast rods when restained by the warm method.

The growth rate of *M. lepraemurium* varies with the type of maintenance medium used for the macrophages. Medium 4:5:1 gave better growth than medium 9:1, probably owing to improved maturation. Liver homogenate and liver extract, which greatly improved the growth of *M. lepraemurium*, also improved the appearance of the host cells. Ferric nitrate promoted an increase of the number of *M. lepraemurium* and occasionally the number of macrophages in the cultures. It seems likely that these substances stimulate the growth of *M. lepraemurium* both directly and indirectly.

The diversities in the growth rate of *M. lepraemurium* when inocula were obtained from animals at various stages of infection were most likely due to a lack of uniformity in the viability of bacilli at different phases of growth in vivo. No lag periods occurred in cultures inoculated with organisms harvested from the serially transferred cultures. Such bacilli are probably in the logarithmic phase of growth and ready for multiplication immediately after introduction into the new host cells. Furthermore, the host cells seem to have ready for immediate utilization all the nutrients required for bacillary propagation.

With these rapidly growing cultures, data on host-parasite relationships can be collected within periods of 3 to 4 weeks. Since the growth of *M. lepraemurium* is exclusively intracellular, a cell model is now available for studies of host-parasite relationships without interference by extracellular growth.

The generation time of *M. lepraemurium* has been studied in animals in several laboratories, by use of bacterial counting methods for growth evaluation. A generation time of 10.7 days was observed in mouse spleen by Chang (2), 7 to 10 days in mouse testes by Hayashi and Nakayama (13), 7 to 8 days in rat testes by Hilsen and Elek (14), 10 days in mouse testes by Hilsen and Elek (14), and 12 to 15 days in the liver and spleen of mouse and rat by Rees (24). The generation time of *M. lepraemurium* observed in macrophage cultures in the present study was 7 days. This approximates the fastest growth rates in the animal host.

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