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Application of Diffusion Chamber Technic to the Cultivation of *Mycobacterium lepraemurium* I. *In vivo* Studies[1]

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Numerous studies have so far been conducted on the cultivation of *Mycobacterium leprae* and *Mycobacterium lepraemurium*. However, no media-bound method of cultivation has been established.

A number of investigators have reported that *M. lepraemurium* may be cultivated by cell culture technics. The method employed by Chang ([1, 2, 3]), Chang and Neikirk ([4]), Chang, Anderson and Vaituzis ([5]), in which mouse peritoneal macrophages were employed, may be said to have contributed much to progress in the cultivation of *M. lepraemurium*.

In the present study *M. lepraemurium* enclosed in diffusion chambers implanted in the mouse peritoneal cavity gave evidence of *in vivo* extracellular growth. Additional studies observed the fate of *M. lepraemurium* when enclosed in diffusion chambers with either mouse peritoneal macrophages or LBu cells, which are the mouse stable strain cells.

MATERIALS AND METHODS

The diffusion chamber. This was made by bonding a plexiglass ring (outer diameter 14mm; inner diameter 10mm; thickness 2mm, Cat. No. PROO 014 01 Millipore Corp.) and millipore filters type GS (pore size $0.22\mu \pm 0.02\mu$) with MF cement (Cat. No. XX 70 000 00), Figure 13.

The rim bond of the ring and filters was carefully covered from the outside with MF cement to prevent leakage of bacteria and invasion of cells.

After injecting a 0.1 ml test specimen through a side hole in the ring, a plastic thread (Cat. No. PR00 000 00) which had been cut to about 12mm length, was inserted into the side hole and then the hole was completely sealed by means of MF cement (Cat. No. 70 000 01). The diffusion chamber and its plastic thread were sterilized with ethylenoxide gas before use.

Bacillary suspension. Murine lepromas were developed by serial subcutaneous passage of the Hawaiian strain of *M. lepraemurium* in F_1 mice obtained by mating a male of the C3H strain and a female ddO strain mouse. About one gram of aseptically removed leproma was minced and ground with mortar and pestle. Sterilized 1/75 M phosphate buffered saline PH 7.2 (PBS) was added in small quantities and mixed till an approximate 1:100 emulsion dilution was obtained.

The emulsion was centrifuged at 3000 rpm for 15 minutes. The supernatant was filtered through two sterilized filter papers and diluted appropriately with PBS to make a bacillary suspension of the desired concentration. Such bacillary suspensions contained 90% of single baccili. No mass aggregate of more than five bacilli was observed. Prior to use the bacillary suspension was divided into two parts. One was diluted with four percent calf serum PBS and the other with Medium 5:4:1 ([4]) (NCTC Medium 109, 50%; horse serum, 40%; 1:5 diluted bovine embryo extract, 10%). Each dilution was made in duplicate, one sample being used for counting the number of bacilli and the other was used as the experimental inoculum.

Host cells. Mouse peritoneal macrophages were obtained by injecting five milliliters of PBS into the peritoneal cavity of

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ten female mice of the ddO strain about four weeks after birth. The mice were supplied by the Breeding Station for Laboratory Animals, Osaka University. Three days after injection, the mice were sacrificed by luxation of the cervical vertebra. Then, after disinfecting the abdominal wall, the skin was dissected to expose the muscle wall, and eight milliliters of Medium 5:4:1 were injected into the abdominal cavity. Light aspiration and injection were repeated two to three times with the syringe, followed by aspiration of as much liquid as possible from the abdomen.

The liquid thus obtained was pooled in a centrifuge tube to an amount of 50 ml and then centrifuged at 800 rpm for ten minutes in the cold room at 4°C. The supernatant was discarded. The sediment was resuspended in Medium 5:4:1 in an amount about one-tenth that of the supernatant. The cell concentration was determined by counting in a hemocytometer. A suspension of over 10^6 mouse peritoneal macrophages per milliliter was usually obtained. Depending on intended use, the suspension of macrophages was further variously diluted with Medium 5:4:1.

LBu cells were furnished by Dr. Yoshio Okada, Department of Preventive Medicine of this Institute. This cell is a mutant of L cell devoid of thymidinokinase. It was cultured in a tissue culture bottle in a 20% calf serum NCTC Medium 109, yielding a monolayer sheet. The cell was detached from the glass walls by trypsinization, suspended in Medium 5:4:1, and after cell count, was put to use.

Animals and operation procedure. In addition to the four week old female mice of the ddO strain, conventional female guinea pigs weighing 400-500 gm were used for diffusion chamber implantation. Laparotomy was performed under ether anesthesia. The diffusion chamber was implanted as low in the abdominal cavity as possible and then the abdominal cavity was closed by a double layer of sutures.

After the test specimen was enclosed in the diffusion chamber, Medium 5:4:1 was poured in a Petri dish into which the diffusion chamber was dipped to prevent it from drying. If necessary the pH of Medium 5:4:1 was corrected to pH 7.4 by addition of PBS.

Method of observation. The animals were sacrificed by ether anesthesia and the diffusion chamber immediately taken out.

In the mice in most cases the diffusion chamber was found to be adherent to omentum or internal organs and it could be easily removed. At times it was adherent to the liver. The diffusion chambers were found to be coated with a thin connective tissue which was peeled off and removed. The chambers to be used for counting the number of bacilli were dipped in PBS. For the microscopic observation of the interior of the chamber it was immediately dipped in 10% solution of formalin for fixation.

In the case of the guinea pigs, the chambers were more often covered by omentum than in the case of the mice. This presented no particular difficulty in their removal from the abdominal cavity.

For counting the number of bacilli, the usual practice was to place the chamber in a homogenizer cup together with 5 ml of three percent calf serum PBS and to homogenize it until the ring was broken into small pieces. If needed, additional three percent calf serum PBS was added and then the modified method of Shepard ([12, 13]) was employed.

For microscopic examination the chamber was soaked in a ten percent formalin solution for more than three hours and rinsed thoroughly with running water. The filter was shorn off from the ring with sharp scissors and stained by the Ziehl-hematoxylin-Neelsen method (using 1:10 diluted Loeffler's solution). After the filter was sufficiently dried on a glass slide, it was made transparent by xylene and cedarwood oil. After cover slipping it was examined microscopically.

RESULTS

As shown in Table 1 and Figure 1, when *M. lepraemurium* only were enclosed in the diffusion chamber and implanted in the peritoneal cavity of the mouse, the maximum number of bacilli obtained after the

TABLE 1. *Number of* M. lepraemurium *in cell-free diffusion chamber implanted in mouse peritoneal cavity.*

Time after implantation	0	79 days	4 months	5 months	6 months
Number of bacilli per chamber	7.0×10^4	1.2×10^5	2.3×10^5	2.5×10^5	2.3×10^5
	6.4×10^4		1.9×10^5	4.1×10^4	9.0×10^4
	6.1×10^4		1.6×10^5	3.9×10^4	7.4×10^4
	4.3×10^4		1.5×10^5	2.6×10^4	10^4
	3.3×10^4		1.1×10^5		

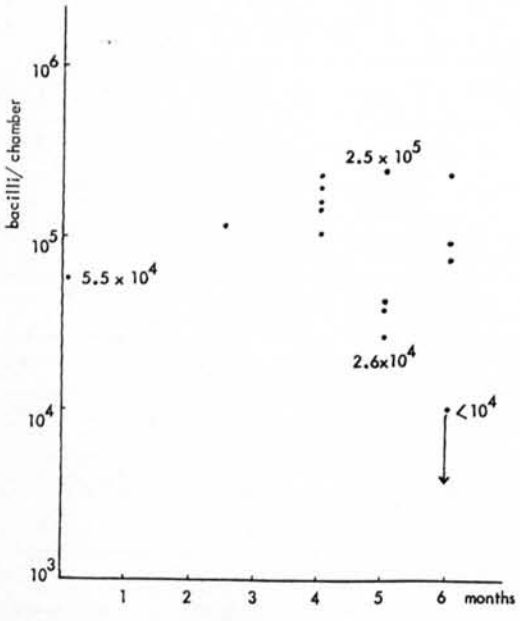

FIG. 1. Number of *M. lepraemurium* in cell-free diffusion chamber implanted in mouse peritoneal cavity.

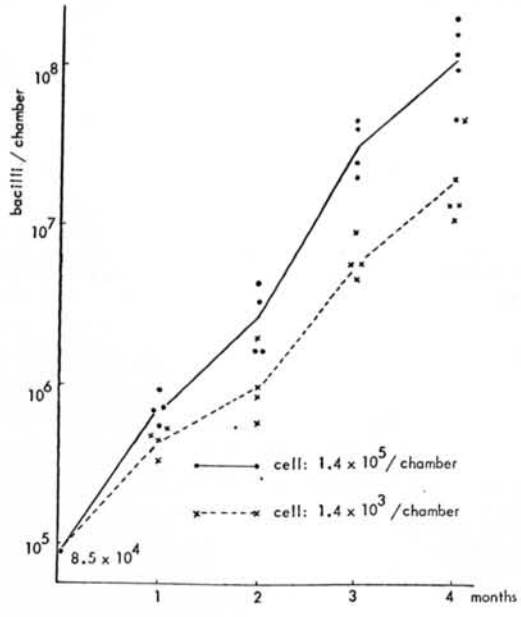

FIG. 2. Number of *M. lepraemurium* in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.

TABLE 2. *Number of* M. lepraemurium *in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.*

Time after implantation		0	1 month	2 months	3 months	4 months
Number of cells per chamber 1.4×10^5	Number of bacilli per chamber	8.5×10^4	5.4×10^5	1.6×10^6	1.9×10^7	4.5×10^7
			7.1×10^5	1.6×10^6	2.4×10^7	9.5×10^7
			7.2×10^5	3.3×10^6	3.9×10^7	1.2×10^8
			9.0×10^5	4.3×10^6	4.4×10^7	1.5×10^8
						2.0×10^8
Number of cells per chamber 1.4×10^3	Number of bacilli per chamber	8.5×10^4	3.3×10^5	5.8×10^5	4.4×10^6	1.1×10^7
			4.3×10^5	8.0×10^5	5.5×10^6	1.4×10^7
			4.7×10^5	9.8×10^5	5.5×10^6	1.4×10^7
			5.2×10^5	1.8×10^6	8.5×10^6	1.8×10^7
						4.6×10^7

TABLE 3. *Number of* M. lepraemurium *in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.*

Time after implantation		0	1 month	2 months	3 months	4 months
Number of cells: 14 per chamber	Number of bacilli per chamber	8.5×10^4	3.1×10^5 3.2×10^5 3.4×10^5 3.5×10^5	9.6×10^4 1.3×10^5 2.8×10^5 1.3×10^6	3.5×10^5 3.6×10^5 4.6×10^5 6.5×10^5	2.8×10^5 2.8×10^5 6.0×10^5 6.8×10^5 1.0×10^6
Cell-free control	Number of bacilli per chamber	8.5×10^4				5.0×10^4 5.0×10^4 5.3×10^4 1.2×10^5 1.6×10^5 2.0×10^5

implantation was 2.5×10^5/diffusion chamber after five months. This was less than five times the 5.5×10^4 bacilli present at the time of implantation. Thus, no significant multiplication of *M. lepraemurium* was found.

When both *M. lepraemurium* and mouse peritoneal macrophages were placed in the chambers and implanted in the mouse, there was a striking and an approximate logarithmic multiplication of bacilli (Tables 2, 3; Figs. 2, 3) when the number of mouse peritoneal macrophages per chamber was slightly over 1,000. The generation time of *M. lepraemurium*, when the initiating mouse peritoneal macrophage count was 1.4×10^5 per chamber, was found to be about 12 days, and when the count was 1.4×10^3 per chamber, the generation time was about 15 days.

When the mouse peritoneal macrophage count per chamber was 14 cells, the results obtained were just slightly better as compared with control chambers free of macrophages. As shown in Figures 4 and 5, when the number of macrophages was 1.4×10^5/chamber or 1.4×10^3/chamber, the cell density was noted not to be much different as far as the stained specimens were concerned after four months of implantation. It was concluded that cell multiplication had occurred. Also, as shown in Figures 6 and 7, multiplication of *M. lepraemurium* was seen in nearly all of the cells. However, when the number of macrophages was 14/chamber initially, the

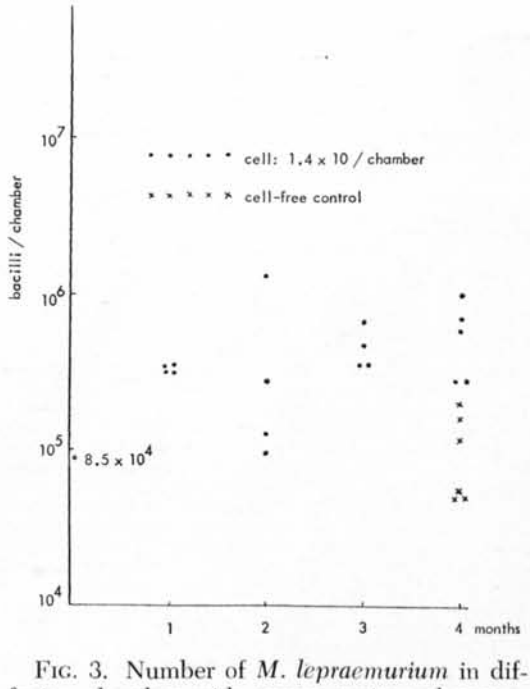

FIG. 3. Number of *M. lepraemurium* in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.

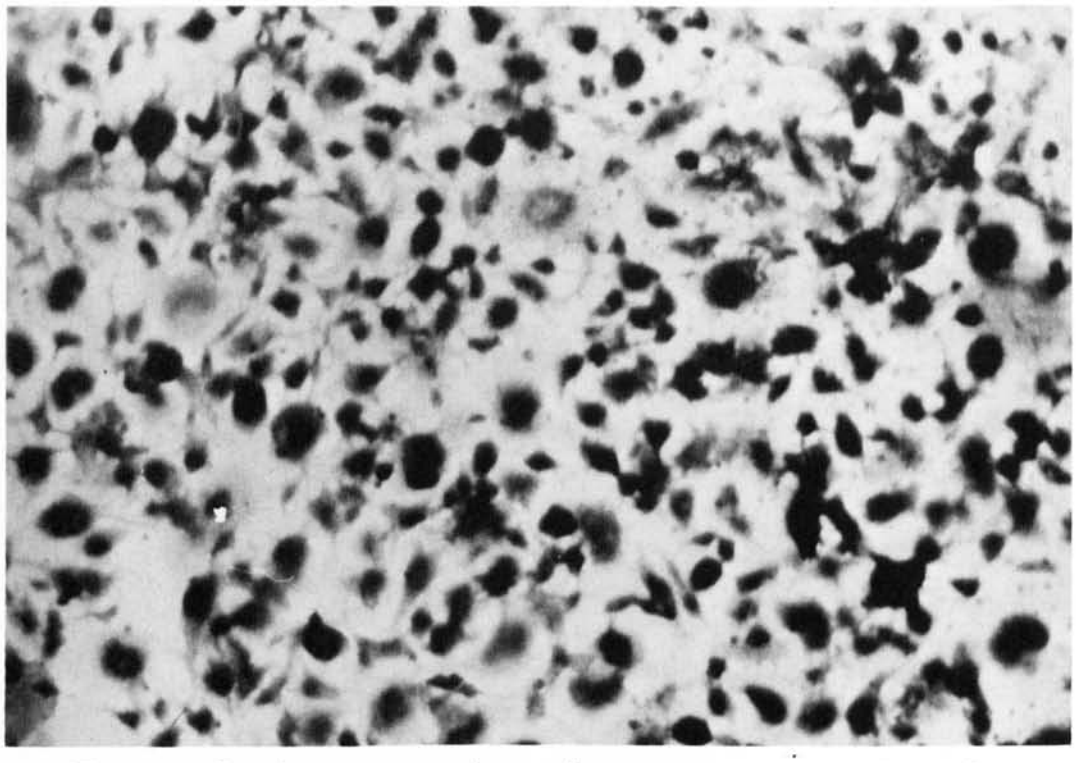

FIG. 4. Number of mouse peritoneal macrophage at start: 1.4×10^5/diffusion chamber. Magnification: 10×10. Four months after implantation.

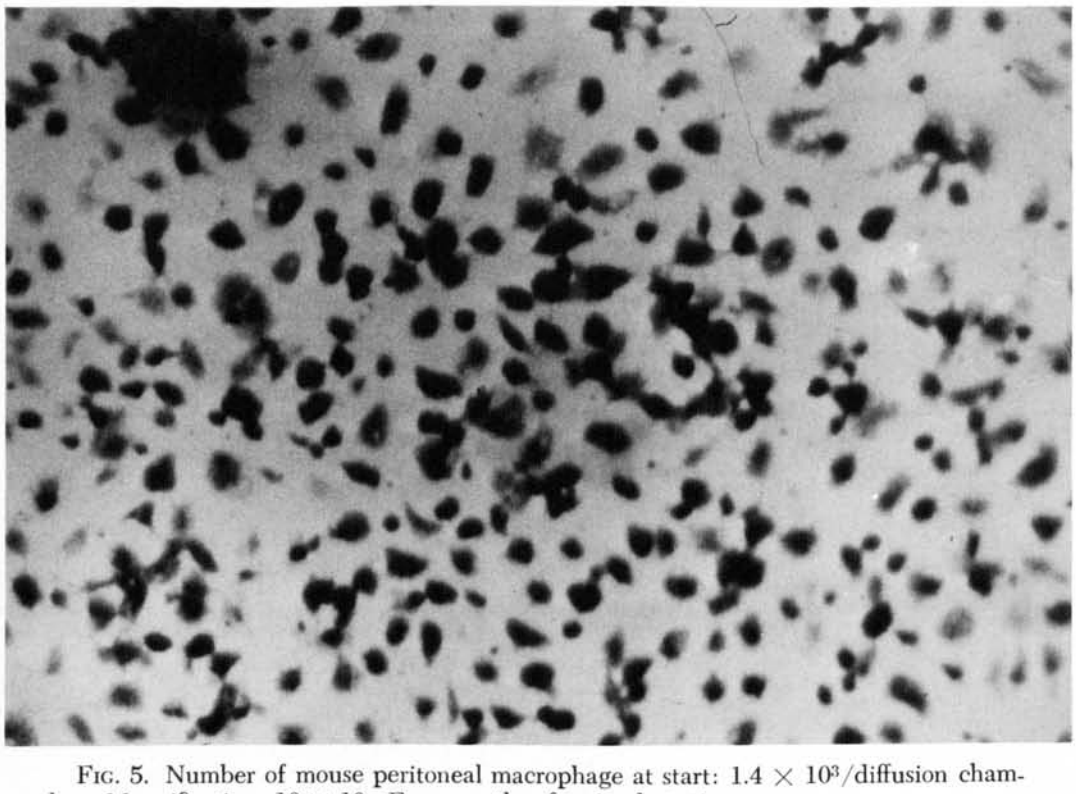

FIG. 5. Number of mouse peritoneal macrophage at start: 1.4×10^3/diffusion chamber. Magnification: 10×10. Four months after implantation.

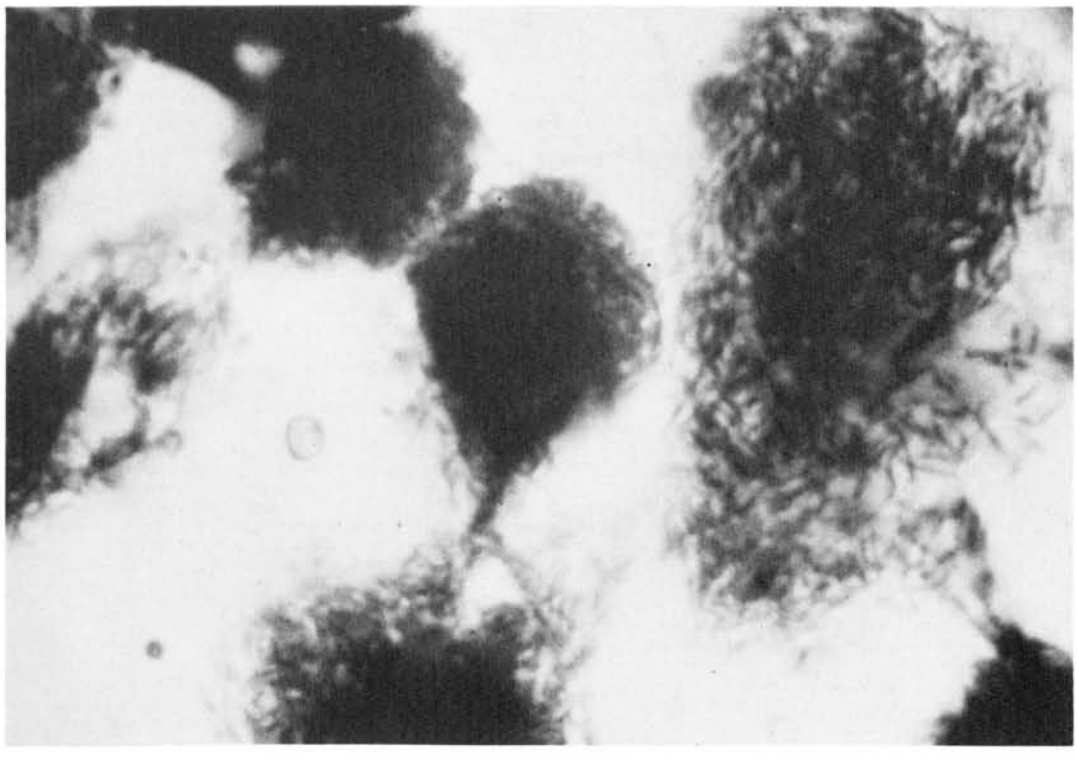

FIG. 6. Number of mouse peritoneal macrophage at start: 1.4×10^5/diffusion chamber. Magnification: 100×10. Four months after implantation.

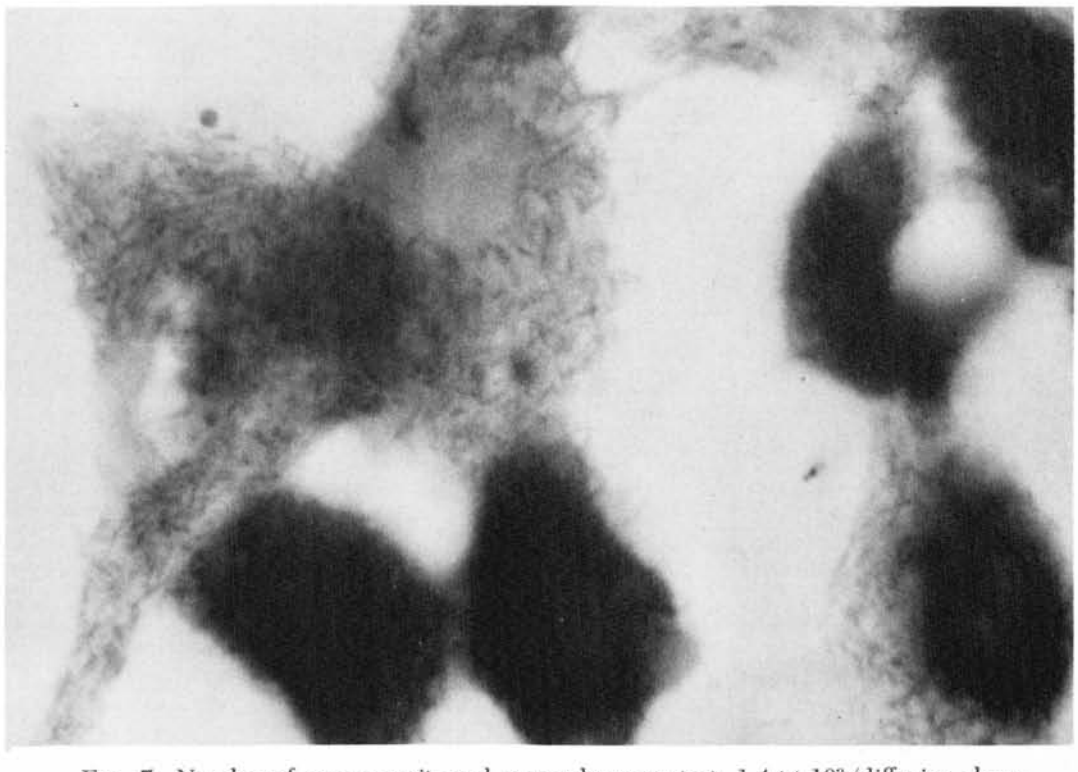

FIG. 7. Number of mouse peritoneal macrophage at start: 1.4×10^3/diffusion chamber. Magnification: 100×10. Four months after implantation.

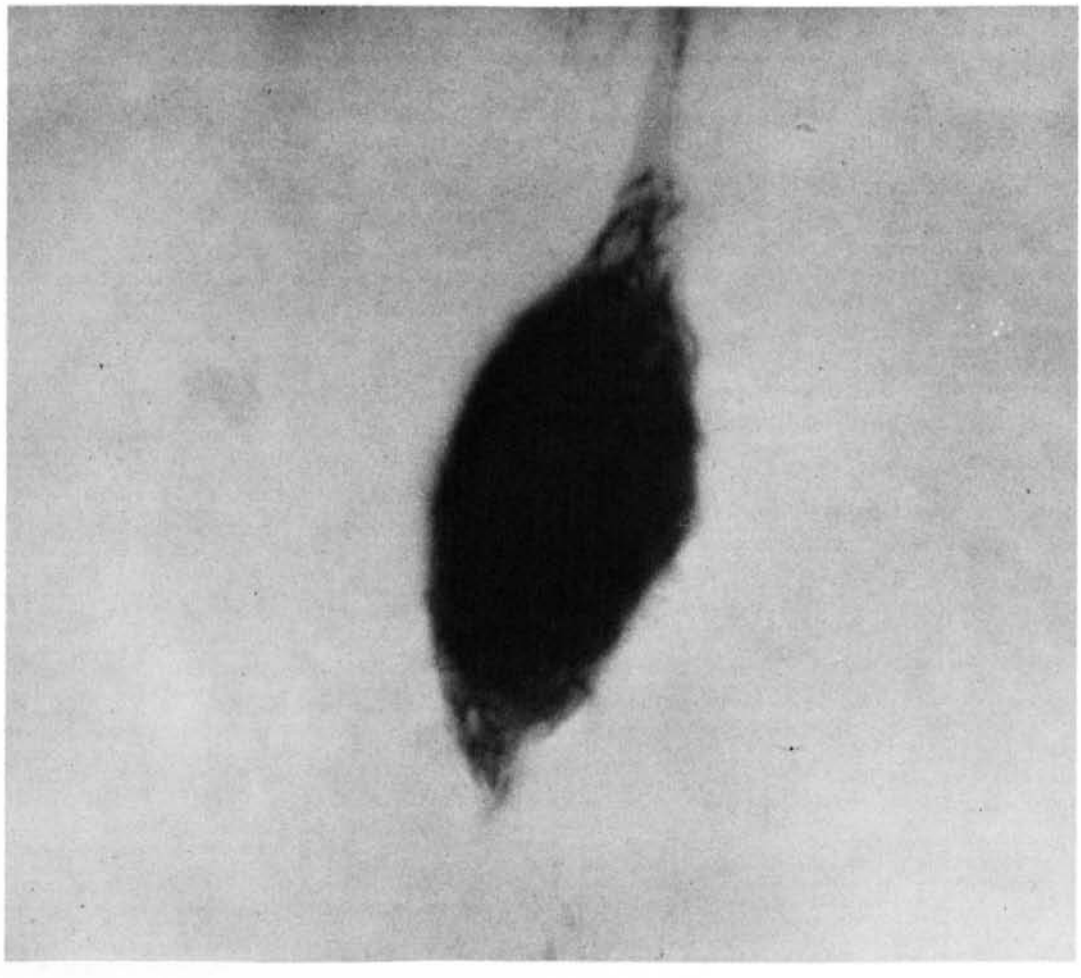

FIG. 8. Number of mouse peritoneal macrophage at start: 14/diffusion chamber. Magnification: 100 × 10. Four months after implantation.

stained specimen after four months of implantation presented quite a small number of cells. The cells seen were, however, filled with bacilli (Fig. 8).

In the fourth month of implantation, a single chamber, in which the number of bacilli was 1.2×10^8, was ground in a homogenizer with 5 ml of three percent calf serum PBS to dilute it 50 times. It was further diluted with three percent calf serum PBS by tenfold serial dilution to make a final dilution of 50,000 times. From the latter, 0.2 ml containing approximately 4.8×10^3 bacilli was inoculated subcutaneously into the abdominal wall of each of eight mice. All mice inoculated developed typical murine lepromas at their sites of inoculation by the fifth month after inoculation. At the same time, a 50-times dilution of the original suspension was inoculated in the amount of 0.1 ml in each of Ogawa's and Henjny's egg media and cultured at 37°C and 33°C. No growth developed in three months of cultivation.

Figure 9 indicates the number of *M. lepraemurium* which would possibly lead to a noticeable multiplication of bacilli when *M. lepraemurium* and mouse peritoneal macrophages are enclosed together in diffusion chambers and implanted in the peritoneal cavity of the mouse.

Thus, a suspension of 1.3×10^6 *M. lepraemurium* per milliliter was serially diluted tenfold and the suspension obtained at each stage was mixed with a suspension of 2.2×10^6/ml of mouse peritoneal mac-

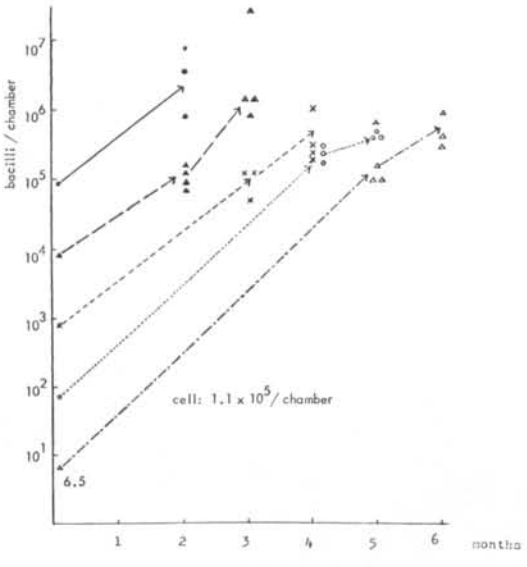

FIG. 9. Growth of *M. lepraemurium* in diffusion chamber with mouse peritoneal macrophage.

rophages in equal quantities and 0.1 ml of the mixture was enclosed in a chamber and implanted in the mouse peritoneal cavity. At the same time, a chamber containing macrophages only (1.1×10^5/ chamber) was implanted in the peritoneal cavity of mouse as control. As shown in Figure 9, even though the number of bacilli at start was less than ten cells (6.5/ diffusion chamber), a noticeable multiplication could be recognized after five months of implantation.

Figure 10 shows a stained specimen of the filter after five months of implantation when the number of bacilli initially was 6.5/chamber. Five *M. lepraemurium*-free control chambers were stained after six months of implantation and counted for bacilli. No acid-fast organisms were found.

At the start of these experiments, the same number of bacilli as the number placed in the chambers were inoculated

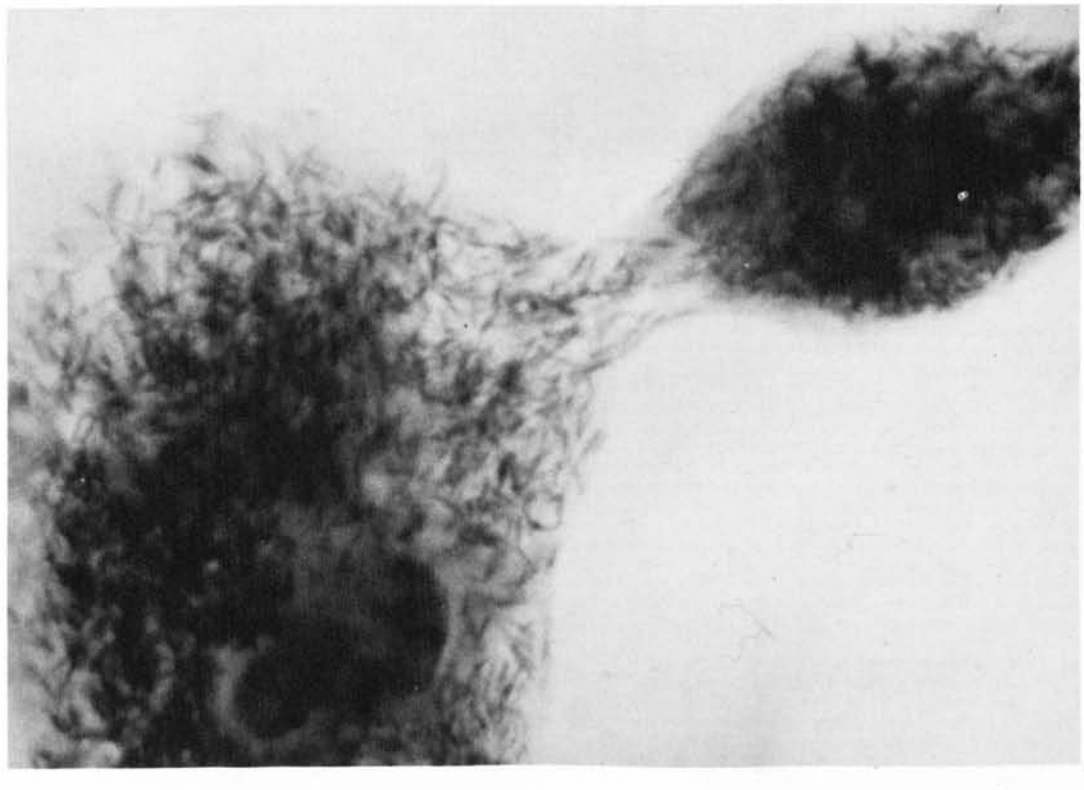

FIG. 10. Number of *M. lepraemurium* at start: 6.5/diffusion chamber. Magnification: 100×10. Five months after implantation.

subcutaneously into the abdominal wall of eight mice for each dilution. After six months the animals with more than 6.5×10^1 inoculum developed inoculation site lepromas in all the mice. In those groups receiving 6.5 bacilli, murine lepromas developed in the site of inoculation in five of the eight mice.

When *M. lepraemurium* and mouse peritoneal macrophages were enclosed in chambers and implanted in the peritoneal cavity of guinea pigs, after two months of implantation two of four chambers had 4.1×10^6 bacilli per chamber (about 18-times) and 2.2×10^6 bacilli per chamber (about 10-times) respectively as against 2.3×10^5 bacilli per chamber initially (Fig. 11). In the other two there were 3.9×10^5 and 2.8×10^5 bacilli per chamber respectively. This was not significantly different from the original inoculum. After three months there also was no significant increase in bacilli while a marked difference was noticed in the control group implanted in the peritoneal cavities of mice.

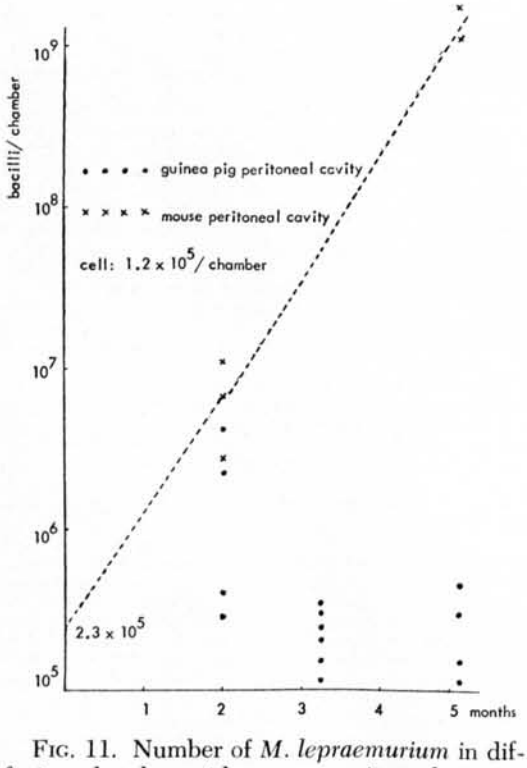

FIG. 11. Number of *M. lepraemurium* in diffusion chamber with mouse peritoneal macrophages implanted in mouse and guinea pig peritoneal cavity.

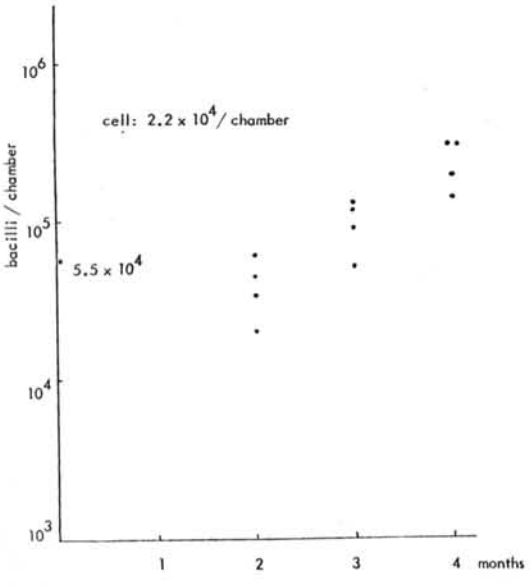

FIG. 12. Number of *M. lepraemurium* in diffusion chamber with LBu cells implanted in mouse peritoneal cavity.

Observation of the stained specimens of the filters after five months of implantation indicated a smaller number of cells in the guinea pig chambers as compared with the mouse while multiplication of *M. lepraemurium* in the cells could not be observed.

When *M. lepraemurium* and LBu cells, a stable strain cell derived from the mouse, were enclosed in chambers and implanted in the mouse peritoneal cavity as shown in Figure 12, four month's observation indicated no marked multiplication of bacilli. In the stained specimens of the filters, the cells clearly showed multiplication with cell sheets covering the inside of the chambers. These cell sheets were found to be well-maintained for as long as four months although multiplication of bacilli inside the cells could not be found.

DISCUSSION

The question as to whether or not *M. lepraemurium* can have *in vivo* extracellular growth in animals susceptible to this bacterium with only the body fluid as the source of nutrition, is highly interesting in relation to possible *in vitro* cultivation.

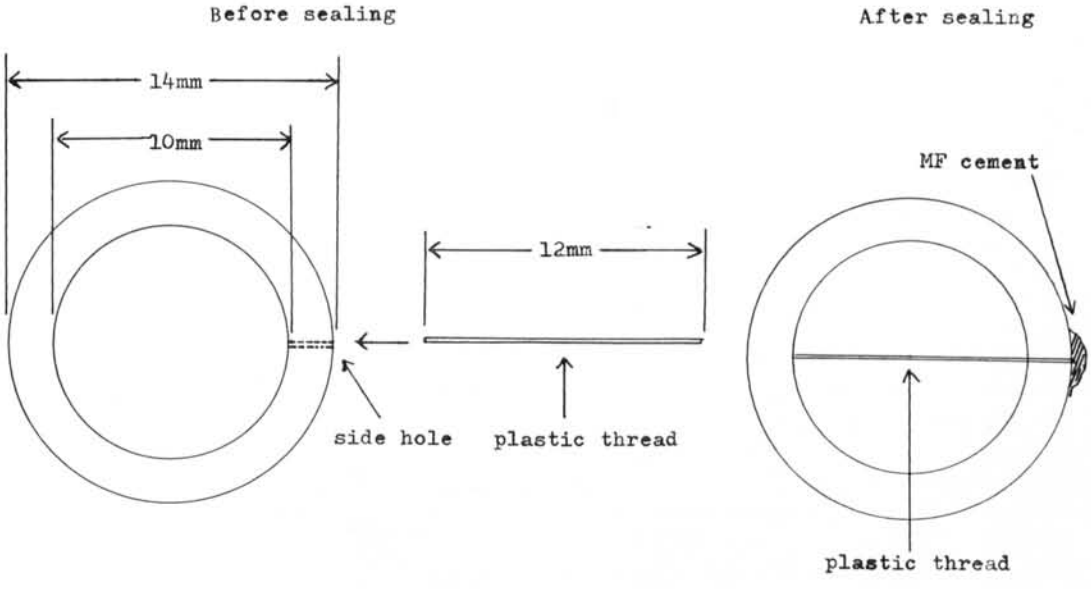

FIG. 13. Sketch of diffusion chamber.

The number of bacilli initially, as shown in Table 1, ranged from 7.0×10^4/chamber to 3.3×10^4/chamber; 5.5×10^4/chamber being the mean value. Since the maximum is smaller than 1.1×10^5/chamber, the lowest value found after four months, the number of initial bacilli as compared to the count after four months may be considered to be statistically significantly different. *In vivo* extracellular growth of *M. lepraemurium* in this case, if any, must be of a very limited extent. This is presumed to be due to the fact that the bacilli used as starting inoculum contained some bacilli having been in host cells just prior to division, or bacilli having some unidentified growth factors necessary for the multiplication of *M. lepraemurium*. Mouse peritoneal fluid alone is thought not to provide conditions adequate for multiplication of *M. lepraemurium*.

With respect to possible *in vivo* extracellular growth of *M. lepraemurium* in cell-free chambers, there is much room left for further study. In present experiments, however, marked growth of *M. lepraemurium* satisfying the two conditions of increase in the number of bacilli over 100 times and confirmation of a logarithmic growth phase could not be obtained.

Chang ([1, 2, 3]), Chang and Neikirk ([4]), and Chang, Anderson and Vaituzis ([5]) reported on the superiority of mouse peritoneal macrophage as the host cell for *in vitro* cell culture of *M. lepraemurium* and held that the generation time of *M. lepraemurium* within mouse peritoneal macrophages is about seven days while Yang and Lew ([15]), also acknowledged these macrophages as suitable host cells for *M. lepraemurium*.

The authors enclosed both *M. lepraemurium* and mouse peritoneal macrophages in diffusion chambers implanted in the mouse peritoneal cavity, and thereby were able to obtain a logarithmic growth of bacilli within the chambers. It was made clear that if the number of macrophages was about 1,000, significant bacillary growth occurred. The generation time of *M. lepraemurium* at the time of logarithmic growth was found to be 12 days and 15 days. The difference is thought to be due to the differences in the condition of *in vitro* cell cultures used by Chang *et al*, and in the *in vivo* experiment of the authors. The differences may also be related to the difference in the number of macrophages used.

The technic employed by the authors has a remarkable advantage for quantitative observation of *in vivo* growth of *M. lep-*

raemurium and hereafter, it is expected that the technic may be applicable to chemotherapeutic and immunologic studies of *M. lepraemurium*. Even though the number of bacilli enclosed in chambers was as low as less than ten, a marked multiplication of bacilli may be observed, so it would seem possible by this technic to identify easily and accurately whether or not the acid-fast organism latent in healthy animals as found by Nishimura *et al* (10), and Mori *et al* (9), was *M. lepraemurium* or not. And if multiplication in chambers was found, subsequent bacteriological examination of the organisms would be simple.

When *M. lepraemurium* and mouse peritoneal macrophages were enclosed in diffusion chambers and implanted in the abdominal cavity of the guinea pigs, no marked multiplication of bacilli could be seen. This is assumed to have been due to mouse peritoneal macrophages not finding favorable conditions in guinea pigs.

Garbutt *et al* (7, 8) Rees and Garbutt (11), and Garbutt (6), reported the possibility of *in vitro* cell culture of *M. lepraemurium* by strain cell 14 pf of rat fibroblast. Wallace *et al* (14), reported that *in vitro* cell culture of *M. lepraemurium* may be possible also in L-cells.

In the present experiments, LBu cells, which are a thymidinekinase-less mutant of L-cells, were used. These cells multiplied well in the chambers and could be maintained for four months, but they were unsuitable as host cells for *M. lepraemurium*. This is not only because this is a stable strain cell but also because it is a special mutant with different biological properties from the original L-cell.

SUMMARY

The diffusion chamber technic was adopted for *in vivo* extracellular growth of *M. lepraemurium* in mice, but when *M. lepraemurium* was enclosed in the diffusion chamber and implanted in the abdominal cavity of the mouse, no significant multiplication of *M. lepraemurium* could be seen in observations lasting for six months. On the other hand, when *M. lepraemurium* and mouse peritoneal macrophages were enclosed in diffusion chambers and implanted in the mouse peritoneal cavity, there occurred a logarithmic growth of *M. lepraemurium* and the generation time was 12 days and 15 days. It was found that these bacilli maintained infectivity for the mouse. When *M. lepraemurium* and mouse peritoneal macrophage were enclosed in diffusion chambers and implanted in guinea pig peritoneal cavities, no marked multiplication of *M. lepraemurium* could be seen. LBu cells, the thymidinekinase-less mutant of L-cells, were also used in these experiments. This cell is unsuitable as the host cell for *M. lepraemurium*.

RESUMEN

Se utilizó la técnica de la cámara de difusión para el crecimiento extracelular in *vivo* del *M. lepraemurium* en ratones. Pero cuando el *M. lepraemurium* se introducía dentro de la cámara de difusión y se implantaba en la cavidad abdominal del ratón, no se observaba una multiplicación significativa del *M. lepraemurium* en períodos de seis meses. Por otra parte, cuando se colocaban dentro de las cámaras de difusión *M. lepraemurium* y macrófagos peritoneales de ratón y leugo se implantaba la cámara dentro de la cavidad peritoneal del ratón, se producía un crecimiento logarítmico del *M. lepraemurium* con un tiempo de generación de 12 días y de 15 días. Se encontró que estos bacilos mantenían su infectividad para el ratón. Cuando el *M. lepraemurium* y los macrófagos peritoneales de ratón se colocaben dentro de la cámara de difusión y luego se implantation en la cavidad peritoneal de cobayos, no se observaba una multiplicación significativa del *M. lepraemurium*. También se ultilizaron en estos experimentos células LBu, que son mutantes sin timidinoquinasa de las células L. Estas células no son adecuadas como células huésped para el *M. lepraemurium*.

RÉSUMÉ

La technique de diffusion en chambre a été utilisée en vue d'étudier la croissance extracellulaire *in vivo* de *M. lepraemurium* chez la souris. Lorsque *M. lepraemurium* était introduit dans la chambre de diffusion, qui était alors implantée dans la cavité abdominale de la souris, aucune multiplication significativa de ce microorganisme n'a pu être mise en évidence lors

d'observations qui se sont poursuivies pendant six mois. Par ailleurs, lorsque *M. lepraemurium* était introduit dans la chambre de diffusion, avec des macrophages péritonéaux de la souris, et implanté dans la cavité péritonéale de cet animal, on a observé une croissance logarithmique de *M. lepraemurium*, dont le temps de génération se situait à 12 et à 15 jours. On a constaté que ces bacilles gardaient leur pouvoir infectieux pour la souris. Lorsque *M. lepraemurium* et des macrophages péritoneaux de la souris étaient introduits dans les chambres de diffusion, qui étaient alors implantées dans la cavité péritonéale de cobayes, aucune multiplication notable de *M. lepraemurium* n'a pu être mise en évidence. Des cellules LBu, qui sont des mutants de formes-L dépourvus de thymidinekinase, ont également été utilisées dans ces expériences. Cette cellule s'est révélés ne pas convenir comme cellule hôte de *M. lepraemurium*.

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