Application of Diffusion Chamber Technic to the Cultivation of Mycobacterium lepraemurium

I. In vivo Studies

Tonetaro Ito and Yoshiharu Kishi

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 techniques. 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 L929 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. PRO 014 01 Millipore Corp.) and millipore filters type GS (pore size 0.2μm ± 0.02μm) 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. PL00 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 ethylene oxide gas before use.

Bacillary suspension. Murine lepromas were developed by serial subcutaneous passage of the Hawaiian strain of M. lepraemurium in F1 mice obtained by mating a male of the C3H strain and a female db1O 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 bacilli. 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 (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...
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 ligation 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 \(10^7\) 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 wall 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-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 cedar wood 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. leprae murium in cell-free diffusion chamber implanted in mouse peritoneal cavity.

<table>
<thead>
<tr>
<th>Time after implantation</th>
<th>0</th>
<th>79 days</th>
<th>4 months</th>
<th>5 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of bacilli per chamber</td>
<td>$6.4 \times 10^6$</td>
<td>$6.1 \times 10^6$</td>
<td>$4.3 \times 10^6$</td>
<td>$3.3 \times 10^6$</td>
<td>$5.5 \times 10^4$</td>
</tr>
<tr>
<td>Number of bacilli</td>
<td>$7.0 \times 10^6$</td>
<td>$1.2 \times 10^6$</td>
<td>$2.3 \times 10^6$</td>
<td>$2.5 \times 10^6$</td>
<td>$2.3 \times 10^6$</td>
</tr>
</tbody>
</table>

Fig. 1. Number of M. leprae murium in cell-free diffusion chamber implanted in mouse peritoneal cavity.

Fig. 2. Number of M. leprae murium in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.

Table 2. Number of M. leprae murium in diffusion chamber with mouse peritoneal macrophages implanted in mouse peritoneal cavity.

<table>
<thead>
<tr>
<th>Time after implantation</th>
<th>0</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells per chamber</td>
<td>$8.5 \times 10^4$</td>
<td>$5.4 \times 10^4$</td>
<td>$1.6 \times 10^4$</td>
<td>$1.9 \times 10^4$</td>
<td>$4.5 \times 10^4$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$1.4 \times 10^5$</td>
<td>$7.1 \times 10^4$</td>
<td>$1.6 \times 10^4$</td>
<td>$2.4 \times 10^4$</td>
<td>$9.5 \times 10^4$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$1.4 \times 10^5$</td>
<td>$7.2 \times 10^4$</td>
<td>$3.3 \times 10^4$</td>
<td>$3.9 \times 10^4$</td>
<td>$1.2 \times 10^5$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$1.4 \times 10^5$</td>
<td>$9.0 \times 10^4$</td>
<td>$4.3 \times 10^4$</td>
<td>$4.4 \times 10^4$</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$8.5 \times 10^4$</td>
<td>$3.3 \times 10^4$</td>
<td>$5.8 \times 10^4$</td>
<td>$4.4 \times 10^4$</td>
<td>$1.1 \times 10^5$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$4.3 \times 10^5$</td>
<td>$8.0 \times 10^4$</td>
<td>$5.5 \times 10^4$</td>
<td>$5.5 \times 10^4$</td>
<td>$1.4 \times 10^5$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$4.7 \times 10^5$</td>
<td>$9.8 \times 10^4$</td>
<td>$5.5 \times 10^4$</td>
<td>$1.8 \times 10^5$</td>
<td>$1.8 \times 10^5$</td>
</tr>
<tr>
<td>Number of cells per chamber</td>
<td>$5.2 \times 10^5$</td>
<td>$1.8 \times 10^4$</td>
<td>$8.5 \times 10^4$</td>
<td>$4.6 \times 10^5$</td>
<td>$4.6 \times 10^5$</td>
</tr>
</tbody>
</table>
implantation was $2.5 \times 10^6$/diffusion chamber after five months. This was less than five times the $5.5 \times 10^4$ 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 $1.4 \times 10^5$ per chamber, was found to be about 12 days, and when the count was $1.4 \times 10^4$ per chamber, the generation time was about 15 days.

When the number of macrophages was $14/\text{chamber}$ initially, 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 $1.4 \times 10^5/\text{chamber}$ or $1.4 \times 10^4/\text{chamber}$, 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 $14/\text{chamber}$ initially, the...
FIG. 4. Number of mouse peritoneal macrophage at start: $1.4 \times 10^5$/diffusion chamber. Magnification: $10 \times 10$. Four months after implantation.

FIG. 5. Number of mouse peritoneal macrophage at start: $1.4 \times 10^5$/diffusion chamber. Magnification: $10 \times 15$. Four months after implantation.
Fig. 6. Number of mouse peritoneal macrophage at start: $1.4 \times 10^5$/diffusion chamber. Magnification: $100 \times 10$. Four months after implantation.

Fig. 7. Number of mouse peritoneal macrophage at start: $1.4 \times 10^5$/diffusion chamber. Magnification: $100 \times 10$. 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 $1.2 \times 10^8$, 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 $4.8 \times 10^6$ 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 Henney’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. leprae murium which would possibly lead to a noticeable multiplication of bacilli when M. leprae murium and mouse peritoneal macrophages are enclosed together in diffusion chambers and implanted in the peritoneal cavity of the mouse.

Thus, a suspension of $1.3 \times 10^9$ M. leprae murium per milliliter was serially diluted tenfold and the suspension obtained at each stage was mixed with a suspension of $2.2 \times 10^8$/ml of mouse peritoneal mac-
Ito & Kishi: Cultivation of Mycobacterium leprae murium

roplages 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 × 10⁷/ 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. leprae murium-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.
subcutaneously into the abdominal wall of eight mice for each dilution. After six months the animals with more than $6.5 \times 10^5$ 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 $M. leprae murium$ 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 $4.1 \times 10^5$ bacilli per chamber (about 18-times) and $2.2 \times 10^6$ bacilli per chamber (about 100-times) respectively as against $2.3 \times 10^5$ bacilli per chamber initially (Fig. 11). In the other two there were $3.9 \times 10^5$ and $2.8 \times 10^5$ 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.

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 $M. leprae murium$ in the cells could not be observed.

When $M. leprae murium$ 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 $M. leprae murium$ 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.
Before sealing | After sealing

The number of bacilli initially, as shown in Table 1, ranged from $7.0 \times 10^4$/chamber to $3.3 \times 10^5$/chamber, being the mean value. Since the maximum is smaller than $1.1 \times 10^6$/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 vitro 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 vitro 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 technique employed by the authors has a remarkable advantage for quantitative observation of in vitro growth of *M. lepraemurium*.
raeaeurium and thereafter, 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 Nishinara et al. (18), and Mori et al. (19), 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. (10, 11), and Garbutt (14), reported the possibility of in vitro cell culture of M. lepraemurium by strain cell 14 pf of rat fibroblast. Wallace et al. (21), reported that in vitro cell culture of M. lepraemurium may be possible also in L-cells.

In the present experiments, LBa 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 vitro 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. LBa 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 periodos de seis meses. Por otra parte, cuando se colocaban dentro de las cámaras de difusión M. lepraemurium y macrófagos peritoneales de ratón y luego 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 colocaban dentro de la cámara de difusión y luego se implantaban en la cavidad peritoneal de cobayas, no se observaba una multiplicación significativa del M. lepraemurium. También se utilizaron en estos experimentos células LBa, que son mutantes sin timidinoquinasa de las células L. Estas células no son adecuadas como células huésped para el M. lepraemurium.

**RESUME**

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 significative de ce microorganisme n'a pu être mise en évidence lors
d'observations qui se sont poursuivies pendant six mois. Par ailleurs, lorsque M. leprae­
muriar1129 était introduit dans la chambre de diffusion, avec des macrophages périto­
eloires de la souris, et implanté dans la cavité péritonéale de cet animal, on a observé une croissance logarith­mique de M. leprae­muriar1129, 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. lepra­
e­mur11ar1129 et des macrophages péritonéaux 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. leprae­mur11ar1129 n'a pu être mise en évidence. Des cellules LBu, qui sont des mutants de formes-L depourvus de thy­
nical assistanc e. 

Acknowledgements. This investigation re­
du M. leprae­mur11ar1129. 

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