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The Growth and Drug Sensitivity of *M. lepraemurium* by Tissue Culture Applying Monolayer and Agar Suspension Technique[1]

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We have shown that *M. lepraemurium* could grow well in an established cell line of A31 cells ([2-5]). The A31 cell is a recloned cell line from Balb/c 3T3 ([3]). This cell line shows a high degree of contact inhibition so the cell monolayer can be kept for a long time at a very low saturation density in monolayer culture.

M. leprae or *M. lepraemurium* have long doubling times; therefore in order to demonstrate their multiplication in tissue culture, the host cells must be kept for a long period without cell division. It is difficult to keep ordinary cell lines healthy for long periods of time without cell division.

The soft agar technique is the established method for the selective assay of transformed cells ([5]). The transformed cells are capable of forming colonies in soft agar medium, but normal cells are not. The normal cells remain as single cells or may form minute colonies undergoing one to three divisions in soft agar medium. These observations prompted us to apply this method to stop the cell division or form minute colonies of ordinary normal cells to act as host cells for *M. leprae* or *M. lepraemurium*. Transformed cells also may be used as host cells for these bacilli by adjusting the concentration of agar to produce a more solid phase so that the plating of the transformed cells results in minute colonies in the agar.

In this paper, we describe the growth and the drug sensitivity of *M. lepraemurium* in A31 cells using monolayer cell cultivation and the growth of *M. lepraemurium* in mouse, human, and chick cells by agar suspension culture.

MATERIALS AND METHODS

Cells. The A31 cell line and the A31 cell line transformed by polyoma virus (A31-pv) were kindly supplied by Dr. A. Hakura, Department of Tumor Viruses, Research Institute for Microbial Diseases, Osaka University. These cell lines were cultured in 60 mm diameter Falcon plastic plates with F12 medium supplemented with 10% calf serum and 100 u per ml of penicillin in a 5% CO_2 chamber at 37°C.

The human neuroblastoma cell line was furnished by Dr. S. Matsumoto, Research Institute for Viruses, Kyoto University. This cell line was cultured as above with RPMI-1640 medium containing 10% fetal bovine serum and 100 u per ml of penicillin.

The foot pad cells from Balb/c-nu/nu (fp-nude) and chick embryo cells were isolated in our laboratory and cultured as above with F12 medium supplemented with 10% calf serum and 100 u per ml of penicillin.

Infection with bacilli. Murine lepromata were developed by serial subcutaneous passage of the Hawaiian strain of *M. lepraemurium* in C3H mice. About 1 g of aseptically removed leproma was minced and ground in a mortar. The emulsion was diluted 1:100 with medium and allowed to stand for about one hr. The bacterial suspension used for this experiment was prepared from the supernatant by making a suitable dilution with medium. To inoculate into the cell cultures, 0.1 ml of the diluted supernatant was added to the monolayer of cells in 35 mm diameter Falcon plates. Af-

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TABLE 1. *Growth of* M. lepraemurium *in A31 cells by monolayer culture.*

Exp. No.	Age of culture (days)	No. of AFB inoculated per plate $\times 10^6$	No. of AFB[a] intracellular per plate initial/final $\times 10^6$	Bacterial increase (fold)	Doubling[b] time (days)
1	40	—	11/4800	440	4.5
2	42	90	68/8700	130	6.0
3	47	40	27/960	36	9.3
	80[c]	40	27/19,000	704	9.5

[a] The data given represent arithmetic means of observation on three plates in each experiment.
[b] Doubling time (dt) in days calculated according to the formula:

$$dt = \left(t_2 - t_1 \text{ in days}\right) \Big/ \left(\log_2 \frac{AFBt_2}{AFBt_1}\right).$$

[c] On day 47, the infected cells were transferred into two plates, and the cultivation was continued.

ter three to four days of incubation with bacilli, the medium was changed twice a week. A conditioned medium was used for the A31 cells and F12 medium with 5% serum used for the A31-pv, human neuroblastoma, fp-nude, and chick embryo cells until bacterial enumerations could be made.

The conditioned medium was prepared as follows: A31 cells in 80% growth on a Roux bottle were cultured with F12 medium containing 20% calf serum for 60 hr. The culture medium was removed and diluted with an equal volume of F12 medium without serum, and this served as the conditioned medium.

Agar suspension culture. The agar suspension culture techniques of MacPherson and Montagnier ([5]) were modified as follows: Base layers of 3 ml of medium containing 10% calf serum and 0.5% w/v Difco Noble Agar were set up in 60 mm Falcon plates. A second layer was then added consisting of cells infected with *M. lepraemurium* in 3 ml of medium containing 10% calf serum and either 0.3% agar or 0.5% agar. The plates were then incubated at 37°C in a 5% CO_2 chamber. After 20 days, the plates were overlaid with medium containing 10% calf serum and 0.3% agar, and the cultivation was continued until the bacilli were counted.

Counting of bacilli. With monolayer cultures, the infected cells were trypsinized, suspended in 5 ml of medium, sonicated for one min to release the bacilli from the cells, and the number of bacilli was scored by a modification of the method of Shepard ([9]). In instances in which there was a low frequency of infection of host cells by bacilli, the plastic plates with cells were washed with phosphate buffered saline (PBS), fixed with methanol for ten min, and stained by the Ziehl-Neelsen method on a hot plate (about 50°C). Two hundred cells were randomly examined microscopically, and the number of bacilli was scored.

With agar suspension cultures, the whole agar medium was transferred to a test tube, and about 30 ml of medium was added. The agar suspension was mixed well with a pipette and centrifuged at 10,000 rpm 12,000 × g for 20 min. The aggregate of cells in the bottom of the tube was resuspended in 10–15 ml of medium, sonicated for two min to release the bacilli from the cells, and the bacilli counted.

Autoradiography. The method of Kato, *et al.* ([4]) was modified as follows. Tritiated thymidine (6-^{3}H-thymidine, sp act 5.0 Ci mmol) was used. After labeling with ^{3}H-thymidine, the plate was fixed with methanol for ten min, treated with 2% perchloric acid (PCA) at 4°C for 40 min to remove acid soluble molecules, and then rinsed three to five times with distilled water. Two ml of Sakura NR-MI emulsion in the sol phase at 45°C for 40 min was poured into the plates and then quickly decanted. The plates were stored for four days at 4°C in a dark chamber. The chamber humidity was maintained at about 40% with silica gel. The plates were developed with Konidol-X for four to five min at 20°C, fixed with Konifix for ten min at 20°C, washed gently, dried in an air-cooler, and stained by the Ziehl-Neelsen method at room temperature.

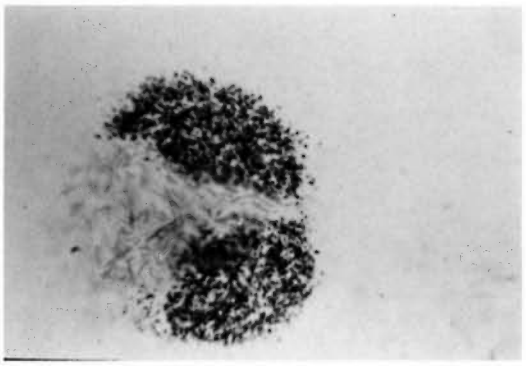

FIG. 1. Autoradiography of cells infected with *M. lepraemurium*. Grains are clearly concentrated in nucleus of cell but not in bacilli in this experiment.

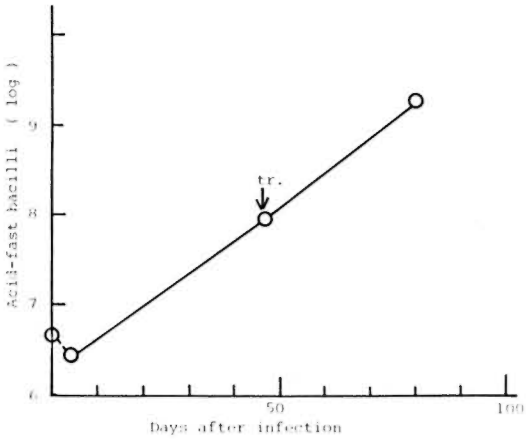

FIG. 2. The serial transfer of *M. lepraemurium*. On day 47, the host cells of bacilli were transferred into two plates (tr). On day 80, the bacilli in the two plates were enumerated.

RESULTS

Growth of *M. lepraemurium* in A31 cells. The A31 cells in monolayer containing 2×10^5 cells per 35 mm Falcon plate were inoculated with 4×10^6 *M. lepraemurium* (20 bacilli per cell). Free bacilli were removed by washing with medium after four days of incubation, and the number of bacilli remaining associated with the cells was counted and found to be 2.7×10^6. In another experiment, 9×10^6 *M. lepraemurium* were inoculated (45 bacilli per cell), and 6.8×10^6 bacilli were incorporated into the cells after four days (Table 1). These results show that about 70% of inoculated bacilli are phagocytized by the cells at bacilli/cell ratios of 20–45 under these conditions.

The phagocytized bacilli increase in number in the cytoplasm of the cells. The growth efficiencies of *M. lepraemurium* are summarized in Table 1. There was a consistent 130- to 704-fold increase in yield of bacilli after 40 to 80 days of cultivation, giving doubling times of 4.5 to 9.5 days.

A31 cells (2×10^5) were inoculated with 2×10^5 *M. lepraemurium* (bacilli/cell ratio of 1:1). The number of cells having phagocytized bacilli was scored after two weeks by microscopic examination of 200 cells at random. Eighty percent of the cells were infected with bacilli, and 20% were not. After four weeks of infection, 20% of the cells remained noninfected even though 6% of the cells were now filled with well elongated bacilli. After six weeks of infection, globi-like formations of bacilli were found in 3% of the cells, and only 3% of the cells were noninfected. These observations suggest that cell to cell infection does not occur with *M. lepraemurium* in the early stages of the *in vitro* infection but that it may occur at later stages when bacilli burst out from cells containing globi-like formations of bacilli.

The sensitivity of A31 cells to *M. lepraemurium*. From six to 6×10^4 bacilli were inoculated into A31 monolayers, each containing 2×10^5 cells. *M. lepraemurium* were enumerated after one month of cultivation. As shown in Table 2, bacillary multiplication occurred even with the minimal inoculum size of only six bacilli per plate,

TABLE 2. *Sensitivity test of A31 cell to* M. lepraemurium.

No. of bacilli inoculated[a]	60,000	6000	600	60	6
No. of cells infected[b]	32,120	9240	5335	715	110

[a] This number of bacilli were inoculated into A31 monolayer in 35 mm Falcon plate.

[b] These cells were infected with more than ten bacilli after one month of cultivation. The data represent arithmetic means of observations on three plates in each experiment.

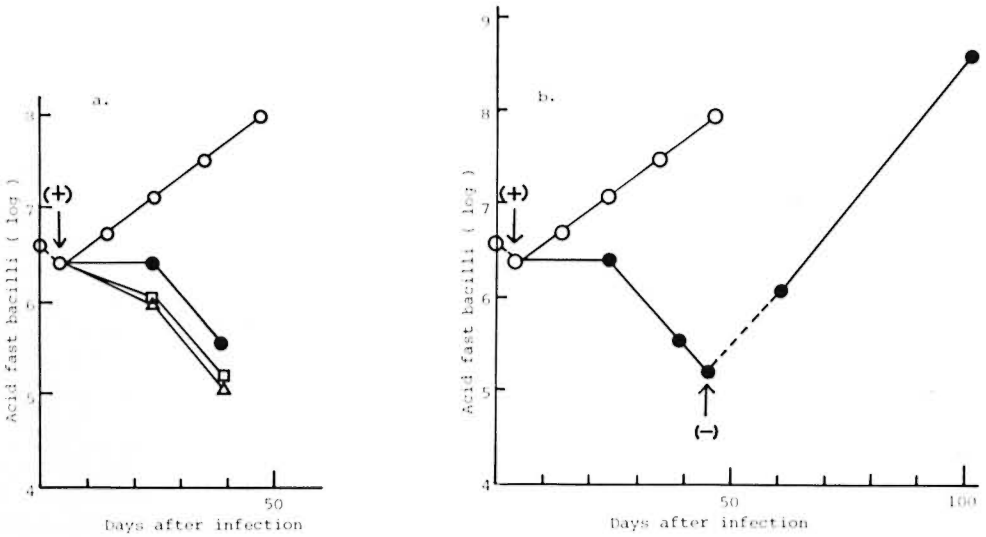

FIG. 3a. The effect of drugs in the growth of *M. lepraemurium*. Each drug was added on day 4 after inoculation of bacilli as indicated by the arrow. ○, control; ●, streptomycin 100 μg/ml; △, rifampin 5 μg/ml; □, INH 5 μg/ml.

FIG. 3b. The effect of streptomycin 100 μg per ml on the growth of *M. lepraemurium*. Streptomycin was removed on day 41 of the drug treatment as indicated by the arrow, and the culture was continued in drug free medium.

there being 110 cells infected with more than ten bacilli per cell after one month of incubation. These results show that A31 cells are very sensitive to infection with *M. lepraemurium*.

Autoradiography of A31 cells infected with *M. lepraemurium* and serial transfer of *M. lepraemurium*. Matsuo has reported ([6]) that mouse foot pad cells infected with *M. lepraemurium* have the ability to replicate. We studied the ability of our cells infected with *M. lepraemurium* to undergo cell replication by autoradiography. A31 cells were infected with *M. lepraemurium* at bacilli/cell ratios of ten. After one month of cultivation, the cells were trypsinized, transferred to two plates, and labeled with 2 μCi per ml of ^{3}H-thymidine for 20 hr. Autoradiography was then performed. As shown in Fig. 1, grains representing ^{3}H-thymidine are clearly concentrated in the nuclei of the cells infected with bacilli, indicating that these cells are synthesizing DNA. This experiment shows that cells infected with bacilli have the ability to undergo cell replication.

After 47 days of infection with *M. lepraemurium* at a bacilli/cell ratio of 20, the infected cells were transferred to two plates. The cultures were continued for a total of 80 days. The growth curve of the bacilli is shown in Fig. 2. These results show that the intracellular bacilli continue to multiply well with no apparent loss of bacilli due to the host cell transfer.

Growth of *M. lepraemurium* in agar suspension culture. We compared yields of bacilli in A31 cells in agar suspension culture and in monolayer culture at 37°C. A31 cells in monolayer were inoculated with bacilli at bacilli/cell ratios of about ten. After three days, the infected cells were trypsinized and suspended in medium. Equal volumes of the suspension were plated into medium for monolayer culture and into agar medium containing final concentrations of 0.33% of 0.5% agar for agar suspension cultures. After 50 days of cultivation, an increase in the number of bacilli was observed in the soft agar cultures (both in 0.33% and 0.5% agar) and that increase was essentially the same as in monolayer culture (Table 3). Bacterial multiplication was also observed in A31-pv, fp-nude, human neuroblastoma, and chick embryo cells using 0.5% agar suspension cultures (Table 3). These results applying the soft agar technique show that *M. lepraemurium* grow well not only in

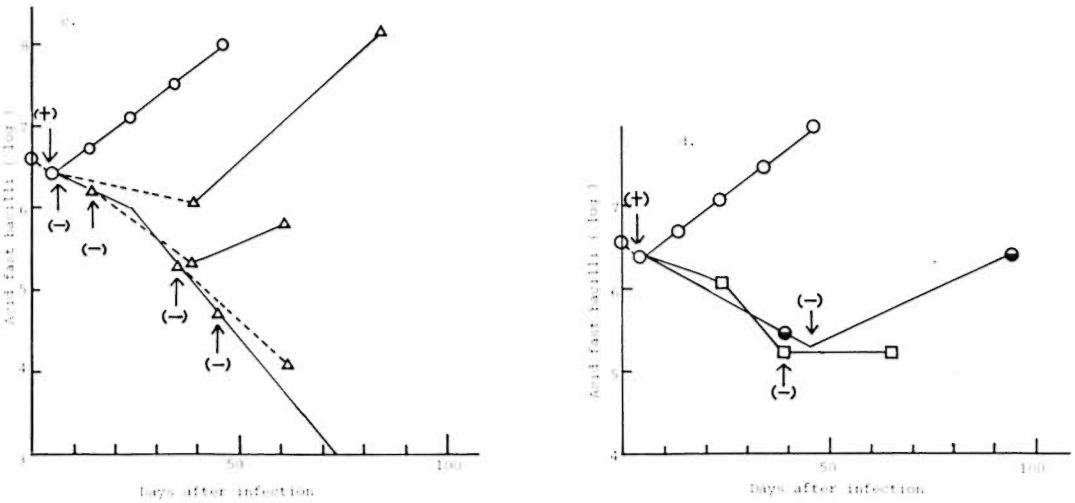

FIG. 3c. The effect of rifampin 5 μg per ml on the growth of *M. lepraemurium*. Rifampin was removed on day 2, 10, 31 or 41 of treatment as indicated by the arrow, and each culture was continued in drug free medium.

FIG. 3d. The effect of INH and clindamycin on the growth of *M. lepraemurium*. INH was removed on day 35, and clindamycin was removed on day 41 of the treatment, and the culture was continued in drug free medium. □, INH 5 μg/ml; ◒, clindamycin 25 μg/ml.

mouse cells but also in human and chick cells *in vitro*.

Drug sensitivity of intracellular *M. lepraemurium* in A31 cells in monolayer culture. The suppressive activity of streptomycin on the growth of *M. lepraemurium* in macrophage culture was studied by Chang [1]. We studied the effects of streptomycin, isonicotinic acid hydrazide (INH), rifampin, and clindamycin on the growth of *M. lepraemurium* in A31 cells in monolayer culture. The A31 cells in monolayer were inoculated with 4×10^6 *M. lepraemurium*. After four days of incubation, the cells were washed with medium to remove the nonphagocytized bacilli, and streptomycin 100 μg, INH 5 μg, clindamycin 25 μg, or rifampin 5 μg per ml was added to the medium. These drug concentrations were near the maximum concentrations which did not produce toxic effects on the host cells. During exposure to drugs, the bacilli gradually lost acid-fastness. Bacterial enumerations were based on the number of bacilli which showed clear acid-fastness in this experiment. Each of the drugs suppressed bacterial multiplication under these conditions (Fig. 3a).

After approximately 40 days' drug treatment, the cultured cells were transferred to

TABLE 3. *Growth of* M. lepraemurium *in cells by monolayer and agar suspension culture.*

Exp. no.	Method	Age of culture	No. of AFB initial/final	Doubling time
1	Monolayer A31	50 days	$5.2 \times 10^5/1.4 \times 10^8$	6.2 days
	Agar 0.33% A31	50	$5.2 \times 10^5/7.5 \times 10^7$	6.9
	0.50% A31	50	$5.2 \times 10^5/2.5 \times 10^8$	5.6
	0.50% A31-pv	50	$1.0 \times 10^6/1.4 \times 10^8$	7.0
	0.50% fp-nude	50	$7.1 \times 10^5/1.1 \times 10^8$	6.8
2	0.50% A31	30	$7.2 \times 10^7/1.4 \times 10^9$	7.1
	0.50% Chick embryo	30	$4.2 \times 10^7/3.0 \times 10^8$	10.7
	0.50% Human neuroblastoma	30	$5.2 \times 10^7/4.3 \times 10^8$	10.0

new plates. The new cultures were also treated with the same drugs. Marked suppression of bacillary growth, similar to the activity shown in the original cultures, was observed. This indicated that resistance of the intracellular *M. lepraemurium* to these drugs did not develop during the 40 days of treatment.

The antibiotics were removed at intervals during the cultivation by changing to drug-free media and continuing the cultures. As shown in Fig. 3b, streptomycin was removed from the medium after 41 days of drug treatment (after 45 days of cultivation since the cultures were carried out initially with drug free medium for four days). After the removal of the streptomycin, the intracellular bacilli began to increase in number without a lag period. As shown in Fig. 3c, rifampin was removed from the culture medium after two days, ten days, and 41 days of treatment, and the cultures were continued with drug-free medium. Regrowth of the intracellular bacilli occurred after lag periods in the cultures which had been treated with the drug for two days and ten days, but this was not observed in the cultures which had been treated with the drug for 41 days. The plates treated with rifampin for 41 days were continued in cultivation for 56 more days in drug-free medim, but at the end of that time no bacilli were seen in the cell culture. Clindamycin was removed after 41 days of treatment, and the intracellular bacilli began to grow without a lag period (Fig. 3d). Intracellular bacilli which had been treated with INH for 35 days showed regrowth after a lag period (Fig. 3d).

DISCUSSION

The A31 cell is a cloned cell line and provides a good tool to analyze host-parasite relationships. We have demonstrated by autoradiographic techniques that the cell, when infected with *M. lepraemurium*, has the ability to undergo cell division. On the other hand, cells infected with bacilli did not produce foci of multilayered growth on a background of contact-inhibited A31 cells in monolayer; in other words, we could not demonstrate *in vitro* the granulomatous lesion as it is seen in the natural host.

In agar suspension culture, *M. lepraemurium* can grow in human and chick cells *in vitro*. These observations suggest that *M. lepraemurium* has the ability to infect and grow in various strains of cells *in vitro*. On the other hand, it is known that *M. lepraemurium* does not grow well in monkeys and birds *in vivo* [7].

The agar suspension culture technique is very convenient for the cultivation of *M. lepraemurium* in tissue culture since this method does not require that the medium be changed very often, it being necessary to only overlay with agar medium at intervals of about 20 days. Bacilli are not lost when they burst from cells because they are retained in the agar. As described in the present results, a number of ordinary cell lines and transformed cell lines are capable of serving as host cells for *M. lepraemurium* using agar suspension culture techniques. In monolayer culture, the fp-nude cells have come off the plates during the cultivation, and the transformed cells (A31-pv and human neuroblastoma cell lines) continued to multiply even with low concentrations of serum and at low temperatures. Thus, these cell lines are not suitable in monolayer cultures for the cultivation of *M. lepraemurium*. In monolayer cultures, A31 cells do not remain in good condition for more than two months without transfer of the cells. On the other hand, A31 cells in agar remained viable for more than 5 months as shown by their ability to absorb neutral red. These observations should provide useful clues for the cultivation of *M. leprae*. We are investigating the possibility of culturing *M. leprae* by applying agar suspension culture techniques.

SUMMARY

M. lepraemurium grow well in a Balb/c 3T3 recloned cell line (A31). In monolayer culture, the average generation time of *M. lepraemurium* in A31 cells was 5.3 to 9.4 days at 37°C. A31 cells are very sensitive to infection with *M. lepraemurium*. Bacterial increases were readily apparent 30 days after inoculating 2×10^5 A31 cells in monolayer culture with only six bacilli. The intracellular bacilli were well transferred without apparent losses by host cell transfer.

The growth of intracellular bacilli was inhibited by streptomycin 100 μg/ml, clindamycin 25 μg/ml, INH 5 μg/ml, and rifampin

5 μg/ml. When streptomycin or clindamycin was removed from the culture medium after 41 days of treatment and the cultivation continued in drug-free medium, the intracellular bacilli began to multiply once more without a lag period. When the intracellular bacilli were treated with INH for 35 days or rifampin for ten days, growth resumed, but only after lag periods after removal of these drugs.

We utilized agar suspension techniques for the cultivation of host cells for *M. lepraemurium* because normal cells or transformed cells ceased undergoing cell division and remained healthy for long periods of time in agar medium. *M. lepraemurium* grew well in A31, A31 transformed by polyoma virus, nude mouse foot pad, chick embryo, and human neuroblastoma cells, utilizing the agar suspension technique. The agar suspension cell culture method should provide useful clues for the cultivation of *M. leprae*.

RESUMEN

El *M. lepraemurium* crece bien en la linea celular A31 reclonada a partir de Balb/c 3T3. El tiempo promedio de generación del *M. lepraemurium* en el cultivo en monocapa de las células A31 fue de 5.3 a 9.4 días a 37°C. Las células A31 resultaron muy susceptibles a la infección con el *M. lepraemurium*. El incremento en el número de bacterias fue claramente evidente 30 días después de haber inoculado 2×10^5 células A31 con tan sólo seis bacilos. Los bacilos intracelulares pudieron transferirse bien a cultivos frescos, sin pérdidas aparentes.

El crecimiento de los bacilos intracelulares fue inhibido por estreptomicina (100 μg/ml), clindamicina (25 μg/ml), INH (5 μg/ml) y rifampina (5 μg/ml). Cuando se removió la estreptomicina o la clindamicina, después de 41 días de tratamiento de los cultivos con la droga y éstos se continuaron en medio libre de droga, los bacilos intracelulares empezaron a multiplicarse sin un periodo lag de adaptación. Cuando los bacilos intracelulares se trataron con INH por 35 días o con rifampina por diez días, el crecimiento recomenzó, al remover las drogas, sólo después de ciertos periodos lag. La técnica para el cultivo en agar de las suspensiones celulares se utilizó porque tanto las células normales como las células transformadas suspenden su división celular y permanecen saludables por periodos prolongados de tiempo en el medio con agar. El *M. lepraemurium* creció bien en las células A31, en las células A31 transformadas por el virus del polioma, en las células del cojinete plantar del ratón desnudo, del embrión de pollo y de neuroblastoma humano. La técnica del cultivo en agar de las suspensiones celulares podría dar información importante para el cultivo del *M. leprae*.

RÉSUMÉ

M. lepraemurium pousse bien dans une lignée cellulaire reclonée Balb/c 3T3 (A31). Dans les cultures en couche unique, le temps de génération moyen de *M. lepraemurium* dans les cellules A31 était 5,3 à 9,4 jours à la température de 37°C. Les cellules A31 étaient très sensibles à l'infection par *M. lepraemurium*. Une augmentation bactérienne était déjà apparente 30 jours après l'inoculation de 2×10^5 cellules A31 en couche unique, ne contenant que six bacilles. Les bacilles intracellulaires pouvaient être transférés de façon satisfaisante sans que se manifeste une perte apparente des bacilles à la suite du transfert des cellules hôtes.

La croissance des bacilles intracellulaires était inhibée par la streptomycine à la dose de 100 μg/ml, de clindamycine à la dose de 25 μg/ml, et d'INH à la dose de 5 μg/ml, et de rifampine à la dose de 5 μg/ml. Lorsque la streptomycine ou la clindamycine était retirée du milieu de culture, après 41 jours de traitement, et que la culture était poursuivie dans un milieu sans médicament, les bacilles intracellulaires commençaient à se multiplier une fois de plus, sans période de latence. Lorsque les bacilles intracellulaires étaient traités par l'INH pendant 35 jours ou par la rifampine pendant dix jours, la croissance reprenait après que ces médicaments aient été supprimés, mais toutefois seulement après une certaine latence.

On a utilisé des techniques de suspension sur agar pour la culture de cellules hôtes de *M. lepraemurium*, car sur milieu avec agar, les cellules normales ou les cellules transformées cessent les divisions cellulaires en cours et demeurent normales pour de longues périodes de temps. *M. lepraemurium* a montré une croissance excellente dans les cellules A31, dans les cellules A31 transformées par le polyoma virus, dans le coussinet plantaire de la souris glabre, dans l'embryon de poulet, et dans des cellules de neuroblastome humain, en utilisant la technique de suspension avec agar. La méthode de culture cellulaire en suspension dans l'agar devrait fournir des éléments utiles pour la culture de *M. leprae*.

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