Restored Pathogenicity of Attenuated *Mycobacterium lepraemurium* in Mice[1]

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Mycobacterium lepraemurium (*Mlm*) grow well in tissue culture cells derived from mouse foot pads ([4]), and in cultures of mouse, human or chick tissues grown under contact-inhibited conditions ([7]). Bacilli grown under these conditions retain their ability to produce lepromas when injected into mice, i.e., they retain their pathogenicity. *Mlm* also grow on Ogawa egg-yolk medium ([8]) and bacilli which produce rough colonies on Ogawa egg-yolk medium retain their pathogenicity for mice ([2]). After serial passage on Ogawa egg-yolk medium, the colony morphology of *Mlm* changes from rough to smooth, and bacilli isolated from smooth colonies have lost their ability to produce full-size lepromas in mice, i.e., these bacilli have become attenuated ([3]).

In this paper we have studied the ability of attenuated *Mlm* to regain their pathogenicity for mice after long-term culture in tissue culture cells grown under contact-inhibited conditions. We have shown that adaptation of attenuated *Mlm* to tissue-culture cells results in at least partial restoration of their pathogenicity.

MATERIALS AND METHODS

Mice. Specific pathogen free (SPF), female, C57BL/6AJc1 mice, 8 to 10 weeks of age, were obtained from Shizuoka Laboratory Animals Center, Hamamatsu City, Japan. Female CBA mice, 8 to 10 weeks of age, were bred in our laboratory.

Cells. Two cell lines were used. The A31 cell line is recloned from BALB/c 3T3 cells, and the 20 cell line, which lacks thymidine kinase, is derived from Fisher rat skin. Both cell lines were cultured in 35 mm diameter plastic plates (Falcon Plastics, Oxnard, California, U.S.A.) in a humidified 5% CO_2 incubator at 35°C. Both of these cell lines show a high degree of contact inhibition so that cell monolayers can be kept for long periods of time at very low saturation densities. The A31 cells were cultured in HAM-F12 medium (Nissui Co., Tokyo, Japan) containing 2% v/v fetal calf serum (Hyclone; Sterile Systems, Inc., Logan, Utah, U.S.A.), 5% v/v horse serum (Nakarai Chemical Co., Ltd, Kyoto, Japan), 100 units/ml penicillin (Meiji, Seiyaku, Tokyo), and 0.3 μg/ml amphotericin B (Fungizone®) (GIBCO Laboratories, Grand Island, New York, U.S.A.). The 20 cells were cultured in Dulbecco Minimal Essential Medium (MEM) (Flow Laboratories, Inc., Rockville, Maryland, U.S.A.) containing 5% v/v fetal calf serum and penicillin and Fungizone® as above.

Bacilli. Four sources of *Mlm* were used. a) The original Hawaii strain of *Mlm* freshly isolated from lepromas from CBA mice was designated *Mlm-in vivo*. b) Bacilli derived from rough colonies of *Mlm* after the second cultural passage on Ogawa egg-yolk medium were designated as *Mlm*-Ogawa-2nd, c) and those from rough colonies after the fifth passage as *Mlm*-Ogawa-5th. d) Bacilli derived from smooth colonies from cultures on Ogawa egg-yolk medium had been cultivated on this medium for over 10 years by serial passage at two-month intervals. These bacilli were designated as *Mlm*-Ogawa-10Y.

Infection of the tissue cultures. Suitable numbers of *Mlm* were added to the cell monolayers and allowed to incubate with the cells for 3 to 4 days. Thereafter the medium was changed twice weekly. To count the bacilli at the time the cells were harvested, the infected cells were washed 5 times

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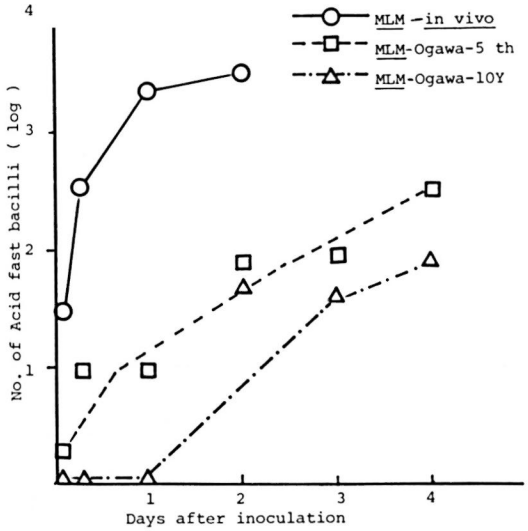

FIG. 1. Adherence ratio of *Mlm* to A31 cells.

with Dulbecco phosphate buffered saline (PBS), trypsinized (2 min, room temperature, 0.25% trypsin solution; Nakarai Chemical Co.), suspended in 5 ml PBS, and sonicated for 60 sec (Ohtak Works Sonicator, 30 watts, with cooling) to disrupt the infected cells and release the bacilli. The number of acid-fast bacilli was then counted by a modification of Shepard's method (9). In instances in which there was low frequency of infection of the host tissue culture cells by bacilli, the infected cells were washed 5 times with PBS, fixed in absolute methanol for 10 min, and stained by the Ziehl-Neelsen method on a hotplate at 50°C for approximately 1 hr. Two hundred cells were examined randomly microscopically to estimate the number of bacilli.

Pathogenicity and interference testing. To test for pathogenicity of the bacilli, *Mlm* were inoculated into mice in the foot pads or subcutaneously in the chest. The animals were then followed for increases in the number of bacilli or the formation of lepromas. To measure interference between attenuated and pathogenic bacilli, tissue culture cells or mice were simultaneously infected with *Mlm*-Ogawa-10Y and *Mlm-in vivo.*

RESULTS

Adherence of *Mlm* to A31 cells. Monolayers of A31 cells with 2×10^5 cells per 35 mm diameter plate were infected with 2×10^6 *Mlm.* After incubation for periods from 2 hr to 96 hr, free bacilli were removed by washing the monolayers 5 times with PBS. The cells were then fixed with methanol for 10 min, stained by the Ziehl-Neelsen method, and the number of bacilli remaining associated with the cells in the monolayer counted microscopically as described above. As shown in Figure 1, *Mlm-in vivo* adhered best, followed by *Mlm*-Ogawa-5th, followed by *Mlm*-Ogawa-10Y. Thus, the ability of *Mlm* to adhere to A31 cells decreased with the number of passages of the bacilli on Ogawa egg-yolk medium.

Growth in tissue culture cells. *Mlm-in vivo, Mlm*-Ogawa-5th, and *Mlm*-Ogawa-10Y were inoculated into monolayers containing approximately 2×10^5 A31 cells. The bacilli were added to each plate and allowed to incubate for 4 days. After washing, the plates inoculated with *Mlm-in vivo* showed approximately 16 intracellular bacilli per cell, while those inoculated with *Mlm*-Ogawa-5th and *Mlm*-Ogawa-10Y showed approximately 600 and approximately 170, respectively (Table 1). After 26 or 38 days of incubation, the infected cells were trypsinized and disrupted by sonication, and the bacilli were counted. *Mlm-in vivo* and *Mlm*-Ogawa-5th multiplied under

TABLE 1. *Relative growth ability of* Mlm-in vivo *and* Mlm-*Ogawa in tissue culture cells.*

	Host cells	Age (days)	No. AFB[a]		Increase (fold)	Doubling time[b] (days)
			Initial	Final		
Mlm-in vivo	A31	26	3.3×10^6	6.8×10^7	20.6	7
Mlm-Ogawa-5th	A31	26	1.2×10^8	8.8×10^8	7.3	9
Mlm-Ogawa-10Y	A31	38	3.4×10^7	2.6×10^7	0.8	—

[a] Intracellular number of AFB was scored. Initial = number of intracellular AFB after 4 days of infection. Data given represent arithmetic means of observation on 3 plates in each experiment.

[b] Doubling time (dt) in days calculated according to the formula $dt = (t_2 - t_1 \text{ in days})/\log_2(AFB_2/AFB_1)$.

TABLE 2. *A comparison of the growth of* Mlm-*Ogawa-10Y adapted to cells in A31 cells.*

	Age (days)	No. AFB		Increase (fold)	Doubling time (days)
		Initial	Final		
Mlm-Ogawa-10Y	38	3.4×10^7	2.6×10^7	0.8	–
Mlm-Ogawa-10Y	64	2.6×10^8	1.5×10^9	6.7	28
Mlm-Ogawa-10Y A31-2M	30	1.1×10^8	1.8×10^9	18.0	8

these conditions with doubling times of 7 and 9 days, respectively. On the other hand, no intracellular multiplication of *Mlm*-Ogawa-10Y was seen (Table 1).

We then attempted to adapt *Mlm*-Ogawa-10Y to A31 cells by incubating the bacilli in cell culture for a longer period of time. As shown in Table 2, after 64 days' incubation without subculture, limited multiplication of *Mlm*-Ogawa-10Y could be seen in A31 cells. These bacilli were designated as *Mlm*-Ogawa-10Y-A31-2M (*Mlm*-Ogawa-10Y which had been cultured in A31 cells for approximately 2 months) and were used to inoculate fresh A31 monolayers. These adapted bacilli multiplied well in the fresh A31 cells, showing a doubling time of 8 days which was comparable to the doubling time of *Mlm-in vivo* (Table 2).

Pathogenicity of *Mlm*-Ogawa-10Y. Kawaguchi, *et al.* ([3]) have shown that *Mlm* isolated from rough colonies on Ogawa egg-yolk medium produce lepromas in mice after inoculation. On the other hand, *Mlm* isolated from smooth colonies are not able to induce lepromas in CBA mice but are able to do so in BALB/c mice.

We have confirmed these findings in our laboratory, and have shown that bacilli isolated from rough colonies on Ogawa egg-yolk medium (after 5 or 8 passages) were able to produce lepromas in CBA mice, and that bacilli isolated from these lepromas could be passaged into new CBA mice and would again produce lepromas in the recipient animals. In contrast, *Mlm* isolated from smooth colonies on Ogawa egg-yolk medium (*Mlm*-Ogawa-10Y) were not capable of producing lepromas in CBA mice but did induce lepromas in BALB/c animals. It was not possible, however, to passage the bacilli from the lepromas produced by smooth colonies of *Mlm* in BALB/c mice into either new CBA or new BALB/c mice (unpublished data, not shown). Thus *Mlm*-Ogawa-10Y do not show strong pathogenicity for mice.

Pathogenicity of *Mlm*-Ogawa-10Y adapted to A31 cells. As described above, CBA mice did not develop lepromas following inoculations with *Mlm* isolated from smooth colonies on Ogawa egg-yolk medium (*Mlm*-Ogawa-10Y). *Mlm*-Ogawa-10Y were cultured in A31 monolayers for 4 months and were designated as *Mlm*-Ogawa-10Y-A31-4M. These bacilli were then used to inoculate fresh A31 cells and CBA mice. *Mlm*-Ogawa-10Y-A31-4M multiplied in A31 cells as well as *Mlm-in vivo* (data not shown), and were capable of producing lepromas in CBA mice (Fig. 2, Table 3) although the lepromas which were produced were smaller than those caused by *Mlm-in vivo*. These observations would suggest that the ability to replicate in A31 cells is not well correlated with the ability of *Mlm* to induce lepromas in mice.

Mlm-Ogawa-10Y-A31-4M were inoculated into the foot pads of CBA mice and then passaged into new CBA mice after 5, 8, and 15 months. These bacilli were designated *Mlm*-Ogawa-10Y-A31-4M-CBA-5M, -CBA-8M, and -CBA-15M, respectively. The bacilli harvested after 5 and 8

FIG. 2. *Mlm*-Ogawa-10Y-A31-4M in CBA mice 14.5 months after inoculation.

TABLE 3. *Pathogenicity of* Mlm *in CBA mice.*

	Inoculum period (mos.)	Leproma on chest	Swelling foot pad (mm)[a]
Mlm-Ogawa-10Y	12	–	0
Mlm-Ogawa-10Y	19	–	0.8
Mlm-Ogawa-10Y A31-4M	9	+[b]	
Mlm-Ogawa-10Y A31-4M	15	+, ++[c,d]	
Mlm-Ogawa-10Y A31-4M CBA-5M	10	+	
Mlm-Ogawa-10Y A31-4M CBA-8M	16	+	0.3
Mlm-Ogawa-10Y A31-4M CBA-15M	5	++	
Mlm-Ogawa-10Y A31-4M CBA-15M	8	+++[e]	
Mlm-Ogawa-10Y A31-4M nu/nu-8M	16	+++	2.6

[a] Arithmetic means of observation on 4 mice.
[b] + = nodule formation on chest.
[c] ++, +++ = leproma formation.
[d] 0.8 g (largest one).
[e] 7.8 g.

months produced small lepromas in new CBA mice, but only after long periods of time (approximately 2 years). On the other hand, bacilli harvested after 15 months (*Mlm*-Ogawa-10Y-A31-4M-CBA-15M) rapidly produced large lepromas after passage into new CBA mice (Table 3). These results suggest that the pathogenicity of attenuated *Mlm* can be gradually restored with time in passage in either tissue culture cells or in mice.

Mlm-Ogawa-10Y-A31-4M were inoculated into the foot pads of nude mice (BALB/c-nu/nu). After 8 months, the inoculated foot pads enlarged (3.7 mm foot pad thickness); the bacilli were harvested (designated as *Mlm*-Ogawa-10Y-A31-4M-nu/nu-8M) and passaged into CBA mice. These bacilli produced large lepromas in the recipient CBA mice, and the lepromas were larger than those caused by similar bacilli passaged for the same period of time in CBA mice (Table 3). These results would suggest that attenuated *Mlm* regain their pathogenicity more rapidly when passaged in immunodeficient hosts than when passaged in immunocompetent mice.

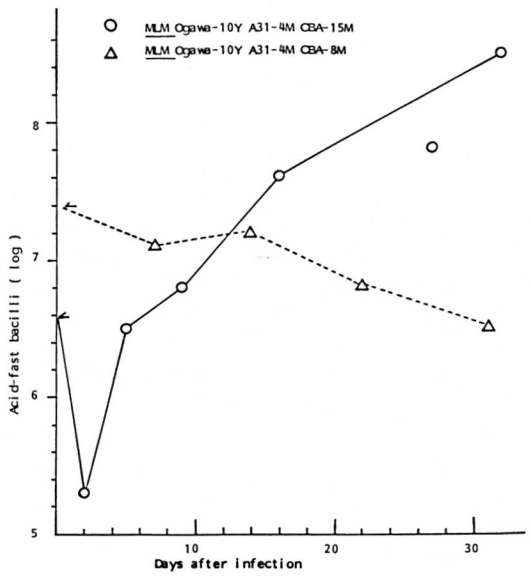

FIG. 3. Growth ability of *Mlm* in A31 cells.

Colony morphology of mouse-adapted *Mlm*-Ogawa-10Y. Cultures of *Mlm*-Ogawa-10Y-A31-4M-CBA-8M and -CBA-15M were set up on Ogawa egg-yolk medium. Both isolates produced smooth-type colonies after 1 month of incubation. After approximately 8 months of incubation, rough-type colonies were observed in the cultures of -CBA-15M although the morphology of the colonies was not typical and could represent mixtures of smooth and rough colonies. Cultures of -CBA-8M showed only smooth colonies throughout.

Growth of mouse-adapted *Mlm*-Ogawa-10Y in A31 cells. *Mlm*-Ogawa-10Y-A31-4M were inoculated into the foot pads of CBA mice, harvested 10 months and 15 months later (designated *Mlm*-Ogawa-10Y-A31-4M-CBA-10M and -CBA-15M, respectively), and used to infect fresh A31 monolayers. The -CBA-15M bacilli multiplied in the tissue culture cells but the -CBA-10M bacilli did not (Fig. 3).

TABLE 4. *Interference test of* Mlm *in tissue culture cells.*

	Host cells	Age (days)	No. AFB		Increase (fold)	Doubling time (days)
			Initial	Final		
Mlm-in vivo	20	15	1.0×10^8	3.6×10^8	3.6	8
Mlm-Ogawa-10Y	20	15	6.1×10^7	4.5×10^7	0.7	—
Mlm-in vivo + *Mlm*-Ogawa-10Y	20	15	1.0×10^8	2.8×10^8	2.8	10

Interference in the growth of *Mlm-in vivo* by *Mlm*-Ogawa-10Y. We tested the hypothesis that *Mlm*-Ogawa-10Y might interfere with the growth of *Mlm-in vivo* by co-cultivation in tissue culture and by co-inoculation into mice. In this experiment we utilized the 20 cell line for tissue culture. As seen in Table 4, the growth of *Mlm-in vivo* was not inhibited by simultaneous infection of the tissue culture cells with *Mlm*-Ogawa-10Y.

To test the possibility of interference *in vivo, Mlm-in vivo* and *Mlm*-Ogawa-10Y were inoculated subcutaneously (s.c.) into the chest of CBA mice and intraperitoneally (i.p.) into C57BL/6 mice, alone and in combination. In CBA mice, the rates of enlargement of the subcutaneous lepromas and the survival times of the animals were the same in those receiving *Mlm-in vivo* alone and in those receiving the mixture of *Mlm-in vivo* and *Mlm*-Ogawa-10Y. Similarly, the survival times in C57BL/6 mice infected i.p. were the same with *Mlm-in vivo* alone or with the mixture (Table 5). These results show that *Mlm*-Ogawa-10Y do not interfere with the growth of *Mlm-in vivo,* either in tissue culture or in mice.

Pathogenicity of large doses of *Mlm*-Ogawa-10Y. As shown in Table 5, C57BL/6 mice infected i.p. with 30×10^8 *Mlm*-Ogawa-10Y alone survived for over 450 days. At 15 months following inoculation, these mice were sacrificed and examined for evidence of infection. Many globi of acid-fast bacilli were found in the spleens and mesenteric lymph nodes in these animals, suggesting that *Mlm*-Ogawa-10Y were able to multiply in these mice after large i.p. inoculations. To clarify this point, we repeated the experiment by inoculating another group of three C57BL/6 mice i.p. with 30×10^8 *Mlm*-Ogawa-10Y. In this experiment, the mice died with heavy murine leprosy 276, 316, and 323 (mean = 305) days after inoculation, indicating that large doses of *Mlm*-Ogawa-10Y i.p. are able to multiply in C57BL/6 mice.

DISCUSSION

It is well known that hereditary modifications can be induced in viruses by expos-

TABLE 5. *Interference test of* Mlm *in mice.*

	Mice	Inoculum		Survival time[a] (days)	
		Site[b]	Size ($\times 10^8$)		
Mlm-in vivo	CBA	s.c.	3	217, 239, 246	(234)
Mlm-Ogawa-10Y	CBA	s.c.	30	>360, >360, >360[c]	(>360)
Mlm-in vivo + *Mlm*-Ogawa-10Y	CBA	s.c.	3 + 30	217, 240, 240	(232)
Mlm-in vivo	C57BL/6	i.p.	3	114, 133, 133	(124)
Mlm-Ogawa-10Y	C57BL/6	i.p.	30	>450, >450, >450[d]	(>450)
Mlm-in vivo + *Mlm*-Ogawa-10Y	C57BL/6	i.p.	3 + 30	114, 133, 131	(126)

[a] Numbers in parentheses are averages for survival days of 3 mice.
[b] s.c. = subcutaneous injection; i.p. = intraperitoneal injection.
[c] After about 1 year, these mice died with cancer.
[d] All mice were sacrificed after 15 months.

ing the virus to an environment different from its native surroundings. This has been shown, for example, in avian sarcoma viruses. The avian sarcoma virus B77 replicates well in chick embryo cells, and transforms these host cells to form colonies. On the other hand, the B77 virus does not replicate well in quail embryo cells, even though it also transforms these cells to form colonies. The B77 virus can be adapted to quail embryo cells by prolonged incubation in these cells, after which the virus begins to replicate in quail embryo cells. Once adapted to quail embryo cells in this fashion, the virus has been shown to lose its ability to replicate in chick embryo cells and to show antigenic differences from the original virus ([6]).

A number of changes occur in *Mlm* following long-term passage in culture on Ogawa egg-yolk medium. These long-term passaged bacilli (*Mlm*-Ogawa) show different colony morphology and different pathogenicity than *Mlm* freshly isolated from animals ([3]). In comparison with freshly isolated bacilli, *Mlm*-Ogawa show extremely rich superoxide dismutase activity (degradation of active oxygen) ([1]) and somewhat enhanced oxygen consumption with NADH (adaptation to aerobic condition) ([5]). On the other hand, the mycolic acid patterns of *Mlm* derived from smooth colonies are indistinguishable from those of *Mlm* derived from rough colonies on Ogawa egg-yolk medium (Kusaka, T., Nomaguchi, H., Miyata, Y., and Mori, T., unpublished observations).

We have passaged *Mlm* on Ogawa egg-yolk medium in our laboratory for more than 10 years. The pathogenicity of these bacilli has become attenuated. We have attempted to restore the pathogenicity of these attenuated bacilli by adapting them to growth in tissue culture cells. The present results suggest that this is possible, and that further passage in mice of these bacilli which have been adapted to tissue culture cell growth results in even more restoration of pathogenicity.

Another possibility for restoring pathogenicity in these attenuated bacilli is suggested by the experiment in which large doses of *Mlm*-Ogawa-10Y were given i.p. to C57BL/6 mice. These mice, receiving 5 mg wet weight or 3×10^9 bacilli, developed heavy murine leprosy at approximately 300 days after inoculation. It might be that such large numbers of attenuated *Mlm* can simply multiply in these mice or, as suggested by Yamaura, *et al.* ([10]) for *Mlm-in vivo,* large numbers of bacilli given i.p. to C57BL/6 mice may induce a depressed cell-mediated immunity in these animals which would then allow the attenuated bacilli to multiply and eventually to regain their pathogenicity.

The basis for the loss of pathogenicity of *Mlm* isolated from smooth colonies on Ogawa egg-yolk medium is not known. Two possibilities come to mind: The attenuated bacilli may not be incorporated well into host cells, or the attenuated bacilli may be more antigenic and induce more protective immunity in mice than freshly isolated organisms. Regarding the first possibility, as shown in Figure 1, *Mlm*-Ogawa-10Y seem to be incorporated into A31 cells in tissue culture although not as well as *Mlm-in vivo.* On the other hand, it could be that the attenuated bacilli are only adhering to the cells in culture and not being adequately incorporated for intracellular growth.

Regarding the second possibility, further studies are necessary to determine the antigenicity of *Mlm*-Ogawa-10Y in inducing cell-mediated immunity in mice. Large numbers of *Mlm*-Ogawa-10Y did not interfere with the growth of *Mlm-in vivo* in mice. Smaller inocula would be needed in order to study the antigenicity of these attenuated organisms since, as mentioned above, large inocula may be immunosuppressive. Thus, on the basis of the present experiments, it is not possible to determine the mechanism of the loss of pathogenicity of *Mlm* following long-term growth on Ogawa egg-yolk medium.

Of particular interest in the present studies was the observation that *Mlm*-Ogawa-10Y which had been adapted to tissue culture cells multiplied as readily as freshly isolated *Mlm* in these cells but, at the same time, these adapted bacilli were not able to grow as readily in mice as the freshly isolated organisms. These findings would suggest that the intracellular environment for the growth of *Mlm* in mice is somehow different from the intracellular environment for the growth of the bacilli in tissue culture cells.

SUMMARY

The ability of *Mycobacterium lepraemurium* (*Mlm*) to adhere to A31 cells in culture decreased with the number of passages of the bacilli on Ogawa egg-yolk medium. Pathogenic *Mlm* consistently grew in tissue culture cells but growth was not seen with attenuated *Mlm* isolated from a smooth colony. After prolonged incubation, attenuated *Mlm* became adapted to tissue culture growth. The pathogenicity of the attenuated bacilli was restored partially by the adaptation to tissue culture cells and restored almost completely by passage in mice. After restoration of pathogenicity by these methods, the *Mlm* formed rough-type colonies on Ogawa egg-yolk medium although the colonies were not completely of the rough type. Attenuated *Mlm* did not interfere with the growth of *in vivo*-derived *Mlm* in tissue culture or in mice.

RESUMEN

La capacidad del *Mycobacterium lepraemurium* (*Mlm*) para adherirse a células A31 en cultivo, disminuyó conforme aumentó el número de pases en el medio de Ogawa con yema de huevo. Los *Mlm* patogénicos crecieron consistentemente en las células cultivadas mientras que los *Mlm* atenuados aislados de colonias lisas no lo hicieron. Después de una incubación prolonga da, los *Mlm* atenuados llegaron a adaptarse y pudieron crecer en los cultivos celulares. La patogenicidad de los bacilos atenuados se restauró parcialmente siguiendo a su adaptación en los cultivos celulares y se recuperó totalmente por pases en ratones. Después de la restauración de la patogenicidad por estos métodos, el *Mlm* formó colonias que no fueron totalmente del tipo rugoso en el medio de Ogawa con yema de huevo. Los *Mlm* atenuados no interfirieron con el crecimiento en cultivos celulares o en el ratón, de *Mlm* recién aislados de animales infectados.

RÉSUMÉ

La capacité de *Mycobacterium lepraemurium* (*Mlm*) à adhérer aux cellules A31 en culture diminue avec le nombre de passages des bacilles sur un milieu d'Ogawa au jaune d'oeuf. Les *Mlm* pathogènes poussent régulièrement dans les cellules de cultures de tissu; par contre on n'observe aucune croissance de bacilles *Mlm* atténués isolés d'une colonie lisse. Après incubation prolongée, les *Mlm* atténués s'adaptent et poussent en culture de tissu. La pathogénicité des bacilles atténués peut être rétablie en partie par l'adaptation aux cellules de cultures de tissu, et rétablie presque complètement par passage chez la souris. Lorsque la pathogénicité est rétablie par cette méthode, les *Mlm* s'organisent en colonies du type rugueux sur le milieu au jaune d'oeuf d'Ogawa, encore que les colonies ne sont pas toutes de ce type. Les bacilles *Mlm* atténués n'inhibent pas la croissance de *Mlm* obtenu *in vivo* tant dans les cultures de tissu que chez la souris.

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