Attempts to Cultivate and Determine the Maximum Period of Viability of *Mycobacterium leprae* in Tissue Culture[1, 2]

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Although *Mycobacterium leprae* was the first mycobacterium to be discovered and was one of the earliest bacteria to be linked specifically to human disease, less is known about it than about any other member of the group. Before 1965, all efforts to cultivate it—either in artificial media or in tissue culture—had failed. At that time, Garbutt ([3]) reported the successful cultivation of *M. leprae* in tissue cultures of both a strain of human embryonic lung diploid cells and a cell line of rat fibroblasts, thus spurring renewed interest in the cultivation of this organism *in vitro*.

In 1970, however, McRae and Shepard ([4]) reported that they had been unable to demonstrate multiplication of *M. leprae* either in the same line of rat fibroblast cultures or in a variety of cultures derived from tissues of man, mouse, and cold-blooded animals. Although some of their experiments lasted for as long as 463 days, during which time acid-fast bacilli were usually detectable, no viable organisms were detected after 50 days in culture.

In our studies, we have not attempted to repeat the work of Garbutt. However, her report encouraged us to investigate further the possibility of cultivating *M. leprae* in a number of mammalian cell cultures, including those from both human and murine sources. Our initial goal was to find tissues, preferable from cooler sites on the body, that could be maintained in culture for long periods of time with minimal changes of nutrient fluid. These conditions, plus the obvious fact that the cells had to phagocytize *M. leprae* were judged to be the most important prerequisites for the cultivation of these organisms. Although many tissues were tested for their ability to support multiplication of *M. leprae*, this report deals only with those that were able to phagocytize these organisms. No evidence of multiplication of *M. leprae* was found in any of the tissues investigated. However, the results of detailed studies carried out to determine the length of time *M. leprae* remained viable in the various tissues are of interest and are presented herein.

MATERIALS AND METHODS

The cell cultures used in these experiments are listed in Table 1. Primary monolayer cultures of testis from adult BALB/c mice and from normal and thymectomized adult Buffalo rats were prepared in this laboratory. After removal of the capsule, the cells were treated with 0.2% trypsin to produce a single cell suspension. They were then washed with phosphate-buffered saline, resuspended in culture medium and distributed to culture vessels. Primary cultures of BALB/c mouse eye were treated in a similar fashion after removal of the vitreous humor. In some instances, secondary and tertiary cultures were made from the testes and up to five passages were made on the eye cultures.

A culture of human foreskin fibroblasts in its tenth passage was provided by Dr. T. Merigan of Stanford University Medical Center. A human sarcoma in its fifth tissue culture passage was obtained from Dr. R. S. Chang, University of California, Davis. Cultures of human embryonic skin-muscle, human embryonic finger tip and human amnion were obtained from commercial sources. Passages of these cultures were made in the same fashion as the cultures from our own laboratory.

1 Received for publication 29 June 1972.
2 This investigation was supported by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (Grant No. AI-08417), Bethesda, Md. 20014.
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TABLE 1. *Tissue cultures investigated for their longevity and ability to phagocytize* M. leprae.

Culture origin	No. of expts.	Passage levels used[a]	Maximum duration of expts. in a single passage (days)	Percent phagocytosis[b]
Mouse testis	14	1 to 3	210	70
Mouse eye	5	1 to 5	120	63
Rat testis	7	1	110	23
Human foreskin	3	12 to 20	116	40
Human embryonic skin-muscle	2	1	120	39
Human embryonic finger tip	2	5	300	40
Human amnion	2	1	150	46
Human leucocytes	6	1	210	100
Human sarcoma	3	7 to 12	413	54
Human leproma	4	1 to 3	300	84

[a] All of these were monolayer cultures except the human leproma, which were initially organ cultures prepared from skin of untreated lepromatous patients. Fibroblastic outgrowths from the original explants were passaged as monolayer cultures.

[b] Average of several cultures determined by counting 100 cells one month post-infection. Phagocytosis studies on human leproma cultures were carried out on the passaged fibroblastic cells, which, on initial passage, were stained to determine the percent containing phagocytized acid-fast bacilli.

Human leucocyte cultures were prepared from 50 ml of venous blood from healthy donors, using the sedimentation method of Chang (1).

Depending on the growth characteristics of the various cells, they were prepared in concentrations ranging from 6×10^5 per ml to 10^7 per ml and seeded into Leighton tubes, 35- and 65-mm Petri dishes, and 25-cm² T-flasks. Incubation was carried out at both 31°C and 34°C. In the initial experiments, the nutrient media employed for all but the leucocyte cultures was Eagle's minimum essential medium containing 5% inactivated fetal bovine serum and 100 units/ml penicillin. The Petri dish cultures were incubated under conditions of 100% humidity and 5% CO_2. In later experiments, L-15 (Leibovitz) medium containing 10% inactivated fetal bovine serum was utilized because it contained neither glucose nor bicarbonate. The Petri dish cultures could then be grown and maintained in a free gas exchange with the atmosphere in an incubator with a controlled relative humidity of 70%.

The leucocytes were cultivated in medium #199 containing 20% fetal bovine serum.

Organ cultures were prepared from lepromas of untreated patients with lepromatous leprosy. Adipose tissue was dissected away, and the skin was cut into 1- to 2-mm pieces. The pieces were distributed into Falcon organ culture dishes, 3-4 per dish.

The inocula of *M. leprae* were obtained from the foot pads of BALB/c mice, and also from testes and foot pads of thymectomized, antithymocytic serum-treated rats. The inocula were tested on appropriate media to exclude the presence of cultivable mycobacteria. The *M. leprae* were partially purified and concentrated before being inoculated into tissue culture. A preliminary centrifugation was carried out at 850 g for five minutes. This did not remove significant numbers of acid-fast bacilli (AFB), but did remove most of the tissue particles. The supernate was then centrifuged at 3,200 g for 30 minutes at 5°C. The pellet was resuspended in tissue culture medium to the desired concentration, and the organisms were stained and counted by the method of Shepard and McRae (6). Cultures were inoculated with 1×10^6 to 6×10^6 AFB, after which they were incubated.

Cultures were examined microscopically several times a week. Nutrient fluids were changed either when they became excessively acid, when the cell sheets showed

signs of cytotoxicity, or when the sheets started to detach from the glass. These fluids were subjected to sonic vibration for two minutes, using the cup horn of a Bronwill Biosonik III. This permitted the disruption of any cells present in the nutrient fluid to be carried out in a sealed tube under aseptic conditions without affecting the viability of the *M. leprae*. The organisms were counted; if sufficient numbers were present, they were either diluted or concentrated for inoculation into both hind food pads of BALB/c mice (5×10^3 to 1×10^4 organisms per foot pad in 0.03 ml of Hanks' solution). Six months after inoculation, the foot pads were harvested ([5]) and the AFB were counted to determine if the inoculum had been viable. Occasionally, if the initial harvest was negative, the harvest was repeated at eight and twelve months after inoculation.

Cultures of organs previously infected *in vivo* were additionally treated by one of two methods. At varying periods after initiation of the culture, cell sheets were trypsinized, following which the cells and fluids were centrifuged at 3,200 g for 30 minutes at 5°C. The pellet was resuspended in Hank's solution in 10% of the original volume. Sonication was carried out as above, the total number of AFB was determined, and the suspension was diluted and inoculated into mice. In addition to being inoculated into animals, the organisms on some occasions were also inoculated into the same kind of cultures from which they had been harvested.

In the second method, the trypsinized cells were counted and half of them were disrupted to determine the number of AFB in the culture. The organisms were then passed to new uninfected cultures of the same type with the expectation that the *M. leprae* would be phagocytized and replicate in the growing monolayer. The undisrupted half of the culture was passaged either alone or in combination with new uninfected cells, also of the same type. The latter procedure generally was carried out when the original cells were primary cultures, because they grew with difficulty in second passage.

All cultures contained 9×22 mm coverslips, which were removed at intervals to determine both the percentage of cells infected and the numbers of organisms per cell.

RESULTS

All cultures used for these studies were tested two to four weeks after infection for their ability to phagocytize *M. leprae*. The results (Table 1) indicate wide variation, ranging from 23% to 100%. There was also variation in phagocytosis within each cell type, dependent to some extent on temperature of incubation. At 31°C, phagocytosis was slower, taking about four weeks to reach maximum, and tended to be slightly less than at 34°C, at which temperature maximum phagocytosis was reached about two weeks after infection.

As indicated in Table 1, the largest group of experiments were carried out in cultures of adult BALB/c mouse testis, because preliminary experiments indicated that both primary and secondary cultures from this tissue could be maintained for long periods of time with infrequent changes of media. In a typical experiment carried out at 31°C, 60-mm Petri dishes, each containing 10^7 testis cells infected with 4.39×10^6 *M. leprae*, were maintained for 210 days. Using L-15 medium with 10% fetal bovine serum, changes of medium were necessary only at intervals ranging from 55 to 93 days. When the fluids were first changed 55 days after infection, they contained floating cells that had detached from the monolayer (4.2×10^4/Petri dish). Fifty-eight percent of these, or 2.4×10^4, contained phagocytized AFB, averaging 23 organisms per cell. This gave a total of 5.5×10^5 AFB in the floating cells. The fluids themselves averaged 4.5×10^5 AFB/Petri dish for an average of 1×10^6 AFB/Petri dish in detached cells and fluid (vs 4.39×10^6 inoculated). There was no indication, either at that time or subsequently, that multiplication of the organisms had occurred.

An identical experiment was carried out at 34°C. The first change of nutrient fluids was on the 57th day after infection. These fluids also contained cells detached from the monolayer, 54% (3.43×10^4) of which contained phagocytized AFB. There was

an average of 16 organisms per cell or 5.5 $\times$ 10^5/Petri dish in the detached cells. The fluids themselves contained only 5.7 $\times$ 10^4 organisms per dish. On the 77th day after infection, coverslips were removed from these Petri dishes. On the average, 82% of the cells contained phagocytized AFB, with an average of 40 organisms per cell. On the 100th day after infection, secondary cultures were prepared containing a mixture of 1.8 $\times$ 10^4 cells from the original culture plus 10^6 uninfected testis cells derived directly from mice. From the previous calculations indicating 82% of the cells to be infected, the new cultures should have contained 1.48 $\times$ 10^4 cells infected with 5.9 $\times$ 10^5 *M. leprae*. Furthermore, of the total cells (both old and new), only 1.4% should have been infected. However, 31 days later, coverslips from these cultures revealed that 62% of them contained phagocytized AFB, with an average of seven organisms per cell. On the 98th day, coverslips from these cultures were again examined, but there was little change; 78% of the cells contained phagocytized AFB, with an average of eight organisms per cell. A redistribution of the *M. leprae* must have occurred, probably through death and subsequent lysis of heavily phagocytized cells. Unfortunately, these cultures had to be terminated on the 101st day because they became contaminated with a mold. At that time, the AFB in both fluids and cells were counted. There was an average of 2.01 $\times$ 10^5 AFB per dish. This compares with the estimated 5.9 $\times$ 10^5 organisms they contained when started 101 days earlier and the 4.39 $\times$ 10^6 *M. leprae* inoculated into the original cultures 201 days earlier.

In addition to the experiments described above, five experiments were carried out using testis from BALB/c mice previously infected *in vivo*. With this single exception, the procedure was identical to that used with testis cultures infected *in vitro*. In one experiment, testes were removed 131 days after they had been inoculated with 5 $\times$ 10^3 *M. leprae*. Counts done on several testes at that time revealed an average of 1.1 $\times$ 10^5 AFB per testis. The cultures, containing 10^7 cells/dish and incubated at 31°C, were maintained in primary passage for 208 days, during which period only three changes of fluid were made—on the 17th, 72nd, and 119th days after initiation of the culture. On the 208th day, the cultures were trypsinized and contained an average of 2.25$\times$ 10^5 viable cells. As in the previous experiments, the old cells (2.25 $\times$ 10^5) were mixed with new cells (2.08 $\times$ 10^6) obtained directly from uninfected mice. These cultures were in turn maintained for another 85 days, at which time they too had to be discarded because of mold contamination.

Similar results were obtained with replicate cultures incubated at 34°C except that fluid changes were made at intervals of 31 to 64 days. The primary cultures were maintained for 208 days. At that time, passage was made on the cultures as described above. The secondary cultures were maintained for an additional 102 days, for a total of 310 days in the two passages.

Coverslips from all of the cultures of testis infected *in vivo* were removed at intervals. Of the many hundreds of cells examined, only a few contained AFB, and never more than a few organisms per cell. Although the nutrient fluids were concentrated tenfold prior to staining and examination, they contained only occasional AFB. At the time the cultures were terminated, no AFB could be found in the culture.

All the tissues reported in this study were maintained after infection with *M. leprae* for at least 110 days in a single passage and up to 635 days in multiple passages. In no instance was there indication that multiplication of *M. leprae* had occurred. Although organisms were recovered during most of the period of observation, there was a gradual decrease, especially in cultures that were serially passed. The mouse eye cultures were maintained up to 330 days after infection, covering five passages, but no viable organisms were found after the first passage. Likewise, the human sarcoma cultures were terminated after six passages, covering a period of 635 days, but no viable organisms could be detected by the 28th day after infection. Furthermore, *M. leprae* were diluted out and disappeared from the culture after four

TABLE 2. *Viability of* M. leprae *in various tissue cultures as tested in BALB/c mice.*

Cell culture	Maximum period of viability of *M. leprae* (days)[a]	Results of inoculating *M. leprae*-containing tissue culture fluids into BALB/c mice[b]		
		Number inoculated	Number recovered	Net increase (−fold)
Mouse testis	28	1×10^4	2.4×10^5	24
	28	5×10^3	1.7×10^5	34
	118	1×10^4	7.8×10^4[c]	7.8
Testis (normal rat)	28	5×10^3	6.4×10^5	128
Testis (neonatally thymectomized rat)	28	5×10^3	1.3×10^5	26
	74	1×10^3	1.9×10^4	19
Mouse eye	91	6.7×10^3	7.1×10^4[d]	10.6
Human leucocyte	64	1×10^4	1.2×10^5	12

[a] Each figure represents separate cultures. Fluids taken after indicated period or from other cultures at 28 days after infection contained no viable *M. leprae*.

[b] Indicated number of *M. leprae* from tissue culture were inoculated into each hind foot pad and harvested 6 to 12 months later.

[c] These organisms were inoculated into second group of mice; they showed a 364-fold increase when foot pads were harvested after 6 months.

[d] These organisms also were inoculated into second group of mice; they showed a 34-fold increase after 6 months.

passages covering a period of one year.

During the period following infection with *M. leprae*, the cultures were monitored at frequent intervals to determine the length of time viable AFB could be detected. The results are presented in Table 2. Although many more fluids were tested, the data are given only for those that contained viable AFB for a minimum of 28 days.

Among the cultures of human tissues, no viable *M. leprae* were recovered from foreskin, embryonic skin-muscle, embryonic fingertip, amnion, and sarcoma by the 20th day after infection. Although all of the organ cultures made from lepromas of untreated patients contained viable AFB at the time the cultures were initiated, no viable organisms were recovered by the 28th day of culture. Several serial passages were made on the fibroblastic outgrowth that arose from the skin explants. A coverslip from one of these passage cultures revealed that 84% of the cells contained an average of five AFB. However, they were never shown to be viable. In one instance it was possible to recover viable *M. leprae* from a culture of human leucocytes 64 days after infection.

Viability of *M. leprae* was maintained for 28 to 118 days in separate cultures of the murine tissues. Since viability was determined by inoculating organisms from tissue culture into mouse foot pads and demonstrating an increase in number, it was decided at the outset that a tenfold increase in the number of organisms in the mouse foot pads would be considered as evidence of viability. However, in two instances where the bacillary increases in mouse foot pads were 7.8-fold and 10.6-fold, the organisms harvested from the foot pads were passaged to a second group of mice; multiplication in the passage mice occurred, demonstrating that the inoculum from tissue culture had indeed contained viable organisms. The organisms harvested from mice inoculated with eye culture material 91 days after infection of the culture increased 34-fold in the second group of mice. The organisms derived from the 118-day fluids of the testis culture, after

passage to the second group of mice, showed a 364-fold increase.

DISCUSSION

The results of this study to date, although disappointing in the sense that we have been unable to demonstrate multiplication of *M. leprae in vitro,* are encouraging in other respects. We have shown that tissues from adult mice, rats and humans can be maintained for up to ten months in single passage, with only infrequent changes of nutrient fluids. Murine testes, which are known to be highly susceptible *in vivo* to infection with *M. leprae* (2), appear to be particularly well-suited to long-term culture at relatively low temperatures (31°C to 34°C). It is therefore noteworthy that *M. leprae* survived in cultures of mouse testis for 118 days. Although this is an isolated observation and probably represents survival of only a very small percentage of *M. leprae,* it should also be pointed out that in two other instances, survival in murine tissues was recorded for 74 and 91 days. It is interesting to note that fluids from the testis cultures that yielded viable AFB 118 days after infection had also been tested previously 68 days after infection, and although at that time they contained AFB, these were not viable. An explanation of this phenomenon is not readily available. The data presented suggest that only a small percentage of *M. leprae* survive longer than two to four weeks after infection of the culture and only a small percentage of the tissue culture cells contain viable organisms. At a given time, although the culture might contain viable organisms, they might be undetected unless by chance the tissue culture fluids contained viable organism-containing cells that had become detached from the monolayer. It was rather surprising that although the organ cultures of human lepromas were maintained under conditions more closely approximating the *in vivo* situation, they were unable to support *M. leprae* in a viable state after the initial culture period.

Because of these results, we have extended our investigation of cultures derived from organs known to support good multiplication of *M. leprae in vivo.* Currently under study are cultures of ears, testes and foot pads of normal and neonatally thymectomized Lewis rats. Cells from these organs can also be carried in long-term culture. An added advantage is that these organs can be obtained for cultures from infected rats during logarithmic multiplication of *M. leprae,* yielding cultures that initially contain maximal numbers of viable organisms. In these cultures we will be able to study additional factors that contribute to the maintenance of viability of *M. leprae in vitro,* with the hope that those cultural conditions that promote maintenance of viability will lead us to those conditions necessary for multiplication of *M. leprae in vitro.*

SUMMARY

Repeated long-term attempts have been made to cultivate *M. leprae in vitro* in cultures derived from tissues of man, mouse and rat. These attempts were uniformly unsuccessful. However, it was possible to demonstrate that most of these tissues could be maintained from 110 to 413 days in a single passage with only minimal changes of nutrient fluids. When these tissues were infected with *M. leprae* and maintained at either 31°C or 34°C, phagocytosis was highly variable, ranging between 23% and 100%. In these experiments, cultures of murine origin appeared to be a more favorable milieu than cultures of human tissue for maintaining viability of *M. leprae* over extended periods of time. Seven of the eight experiments in which *M. leprae* survived from 28 to 118 days were in murine tissue cultures.

RESUMEN

Se han realizado repetidos intentos a largo plazo para cultivar el *M.leprae in vitro,* en cultivos derivados de tejidos de hombre, ratón y rata. Estos intentos fracasaron uniformemente. Sin embargo, fué posible demostrar que la mayor parte de estos tejidos podían ser mantenidos desde 110 hasta 413 días en un pasaje único, con solamente cambios mínimos de los fluidos nutritivos. Cuando estos tejidos fueron infectados con *M.leprae* y fueron mantenidos a 31° C. o a 34° C., la fagocitosis fué altamente variable, con un rango entre

23% y 100%. En estos experimentos, los cultivos de origen murino parecieron ser un medio más favorable que los cultivos de tejidos humanos para la mantención de la viabilidad del *M.leprae* a lo largo de períodos extensos. Siete de los ocho experimentos en los cuales el *M.leprae* sobrevivió desde 28 hasta 118 días fueron realizados en cultivos de tejidos murinos.

RÉSUMÉ

On a procédé à des essais répétés sur de longues périodes, en vue de cultiver *M.leprae in vitro* dans des cultures provenant de tissus humains, de tissus de la souris et de tissus du rat. Ces essais se sont révélés uniformément des échecs. Pourtant, il a été possible de montrer que la plupart de ces tissus pouvaient être maintenus, de 110 à 413 jours, dans un simple passage avec des modifications très minimes des liquides nutrients. Lorsque ces tissus sont infectés par *M.leprae* et maintenus à 31° C ou à 34° C, on a constaté que la phagocytose était fort variable, variant de 23 pour cent à 100 pour cent. Au cours de ces expériences, les cultures d'origine murine se sont révélées constituer un milieu de culture plus favorable, que les cultures de tissus humains, en ce qui concerne la viabilité de *M.leprae* pour des périodes de temps prolongées. Sur huit expériences au cours desquelles *M.leprae* a survécu pendant 28 à 118 jours, sept d'entre elles avaient été menées sur des cultures de tissus murin.

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