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Attempts at Cultivation of *Mycobacterium leprae* in Macrophages from Susceptible Animal Hosts[1]

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Numerous efforts worldwide have been devoted to the *in vitro* cultivation of *Mycobacterium leprae* since its discovery by Armauer Hansen at the end of the last century. Yet 100 years later this organism remains uncultivable and we are dependent upon experimental animals for the demonstration of growth of *M. leprae*. Many workers have examined tissue culture systems in an attempt to find a suitable *in vitro* environment for *M. leprae*. Fieldsteel and McIntosh ([6]) used a range of cell lines derived from human, rat, or mouse tissue but found no evidence of multiplication. Matsuo and Utsunomiya ([12]) developed a mouse foot pad cell line which they were able to maintain for several years. However, both these systems are unsatisfactory. Although they meet the requirement for a host cell which can be maintained in culture for long periods [a feature necessitated by the fact that *M. leprae* has a very long mean generation time, estimated at 10–14 days ([11])], the cultures have to be divided, resulting in dilution of the number of bacilli in culture. In contrast, Chang and Neikirk ([3]) used mouse peritoneal macrophages, and Samuel, *et al.* ([19]) used peripheral blood monocytes derived from healthy donors and from tuberculoid and lepromatous leprosy patients. Since these cultures are not cell lines, the problem of cell division does not arise but, in general, cultures were of only 60–80 days' duration which may not be sufficient time for detection of growth. Garbutt ([7]) reported some multiplication of *M. leprae* in rat fibroblasts and a human diploid cell line, but Shepard and McRae failed to confirm this work ([14]).

In 1976, Kohsaka in Japan ([10]) and Colston and Hilson ([5]) in this department independently showed that *M. leprae* grow to much greater numbers in the nude athymic mouse than in its euthymic counterpart. Growth of *M. leprae* may be expected to reach 10^{10} bacilli/gram of tissue with extensive dissemination to superficial tissue, lymph nodes, liver, and spleen. *M. leprae* is naturally an intracellular organism, being found commonly in macrophages, and it has been shown to grow well in the athymic nude mouse. Since there may be inherent differences between macrophages from athymic and euthymic mice, we decided to investigate nude mouse macrophage cultures infected *in vitro* with *M. leprae*. We also cultured macrophages from the nude athymic rat and from the armadillo, thus focusing our efforts upon cells from donor species that have been shown to develop lepromatous infection ([4, 9]).

In 1964, Chang described a method for the long-term cultivation of mouse peritoneal macrophages ([2]) and used it to demonstrate the intracellular growth of *M. lepraemurium* ([3, 21]), the causal organism of rodent leprosy and, at that time, another uncultivable organism. Based on this method, we maintained nude mouse macrophages infected with *M. leprae* for periods of up to at least 200 days in an attempt to demonstrate intracellular growth or division of this organism.

MATERIALS AND METHODS

Animals

Nude rats, on a random outbred background, were supplied by the Laboratory Animal Centre, Carshalton, Surrey, England. Nude mice were bred in St. George's Medical School Animal Unit onto a random outbred background. They were maintained in isolator conditions and were colonized with a resident gut flora. Nude mouse macrophages were derived from these animals

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unless otherwise stated. Germ-free nude mice were also supplied by the Laboratory Animal Centre, Carshalton, and used immediately. Armadillo tissue was kindly provided by Dr. R. J. W. Rees of the Department of Leprosy and Mycobacterial Research, Medical Research Council, London.

Macrophage cultures

All procedures were carried out aseptically. Macrophages were washed from the unstimulated peritoneal cavity, using NCTC 109 medium (Difco) containing 10 IU/ml preservative-free heparin + 100 μg/ml ampicillin. A volume of 5 ml was used for mice and 15 ml for rats, with about 4 ml and 10 ml, respectively, being recovered by withdrawal through a wide-gauge needle (21 gauge) into a plastic syringe. These suspensions consistently gave counts of 2–4 × 10^6 peritoneal cells per ml, about 50% of which were judged to be macrophages after Leishman's staining [a finding already reported by Nogueira, *et al.* ([15]) and McLeod and Remington ([13]) in normal mice. Our findings also agree with Rao, *et al.* ([18]) who found no difference in the yield of peritoneal macrophages from normal and nude mice]. It was assumed that any adherent non-macrophage cells would be removed from the culture during the washing procedure and subsequent medium changes. Cell suspensions were dispensed at 1 ml/tube into plastic, flat-based, tissue culture tubes (A. S. Nunc, Kamstrup, Denmark, supplied by Gibco Ltd., Uxbridge, England). After overnight culture, the supernatant was removed and replaced with complete NCTC 109 medium containing 40% horse serum, 2% beef embryo extract, and ampicillin (100 μg/ml), after washing 2 times with phosphate buffered saline.

Bone marrow macrophages were harvested from the femora. These were dissected out and cleaned of as much muscle and tissue as possible. Each femur was then removed by cutting below the knee joint (to give a closed end) and at the top of the shaft of the bone to give an open end, with as great a length of bone as possible. The bones were placed in a Petri dish containing cold medium. Using a 25 gauge needle inserted into the marrow cavity, medium (2 ml for mouse, 7 ml for rat, 15 ml for armadillo) was gently passed through the cavity washing out the bone marrow. The bone marrow cell aggregates were broken up by aspirating them through a 25 gauge needle, and the cell suspension was dispensed, 1 ml/tube as above. The cells were not counted since bone-marrow macrophages are immature cells and cell division was expected. The medium used for bone-marrow culture was the complete medium described above supplemented with 10 IU/ml preservative-free heparin. It was not changed for several days so that macrophages had ample time for adherence. Subsequent media did not contain heparin.

These cultures were maintained in complete medium, which was replaced every 14 days at 1 ml/tube, following the method of Chang ([2]), and cultures were incubated at 35°C in 5% CO_2 in air. In some experiments, 40% fetal calf serum replaced the horse serum and some cultures were incubated at 30°C.

Infection of cultures

M. leprae suspensions were derived from a) human biopsies taken from untreated lepromatous patients. These were transported to our laboratory on ice in a thermos flask and processed immediately on arrival (approximately three days after excision of the biopsy); b) freshly harvested foot pads from nude mice infected with 4.7 × 10^4 *M. leprae*/foot pad 17 months previously; and c) the spleen of an armadillo infected with 5 × 10^8 *M. leprae* intravenously 18 months previously. This tissue had been stored at −70°C.

Tissues were homogenized in glass grinders in saline containing 0.1% albumin, and bacilli were washed and counted using the method of Holmes and Hilson ([8]) and diluted as required, the final dilution being made in complete medium. The morphological index (MI) of *M. leprae* suspensions was assessed at this stage. *M. lepraemurium* suspensions (Douglas strain) were prepared by the same method from spleens of infected mice.

The cultures were infected by removing old medium and replacing with 1 ml of medium containing the required number of *M. leprae*. They were incubated overnight to allow phagocytosis to take place, washed to remove unphagocytosed bacilli, and replenished with complete medium. At this point, three tubes were sampled to give base-line

counts of *M. leprae*, the remaining cultures being maintained as long as possible.

Maintenance and harvest of cultures

Pooling method. Old medium was removed every 14 days, pooled in a reservoir to be stored at −20°C for future counting, and replaced by 1 ml fresh culture medium. Thus a pool of old medium was stored separately for each culture. At the end of the experiment (or at an intermediate time of harvest), macrophage cultures were subjected to 30 sec ultrasonication at 800 kHz (Luziesa, Paris, France). This breaks up the host cells without damaging acid-fast bacilli ([1]). The contents of the tube, plus the pooled supernatants from all previous medium changes, were then mixed and spun at 2640 × *g* for 60 min to spin down *M. leprae*. The supernatant was discarded, the pellet resuspended in 1 ml albumin saline and the number of *M. leprae*/ml counted ([8]). The base-line count was the number of *M. leprae*/ml counted from three tubes immediately after the phagocytic period.

Subculture method. In this method, macrophage cultures were infected as above, but the infecting medium was not removed until the time of medium change—every 14 days. This medium, containing nonphagocytosed *M. leprae*, was pooled from all cultures in the experiment, spun at 2640 × *g* for 60 min, and the pellet resuspended in 1 ml culture medium which was used to infect another macrophage culture. Thus, at each medium change the number of cultures increased by one. The base-line was the original number of cultures (usually 3) times the number of *M. leprae*/culture, and at the end of the experiment all tubes were sonicated and pooled to give a total count of *M. leprae* recovered from an enclosed system.

Cultures were observed at regular intervals using an inverted microscope and were terminated if cells appeared to be rounding off, or if a reduction in cell numbers was observed.

RESULTS

After an initial period, attempts at maintaining long-term macrophage cultures were successful, some cultures being maintained for over 200 days and the majority for 100 days or more. Cultures were terminated either because of contamination or because the cells appeared to be rounding up or losing adherence. Otherwise continued microscopic observation consistently showed numerous adherent, spreading macrophages which were maintained for as long as possible.

Forty-four macrophage cultures were examined for their ability to support *M. leprae in vitro*. Of these, 14 showed an increase in counts ranging from 1.1- to 4.5-fold (mean 2.5-fold) at the end of the culture period. It is unlikely that these figures represent growth or division of *M. leprae*. Increases could be due to the breaking up of globi or to fragmentation of acid-fast rods. Decreases may be explained by the death and disintegration of bacilli and the accompanying loss of acid-fastness. Also, loss of bacilli may occur during centrifugation. Furthermore, numerous bacilli would be exocytosed into the medium and pooled and macrophages, especially those with a heavy bacterial load, are likely to lose their adherence and be lost from the culture; both of these factors decrease the number of potentially viable bacilli remaining in the culture system to multiply.

Fourteen different *M. leprae* suspensions derived from human biopsies were used to infect cultures. No correlation was observed between the behavior of these suspensions in macrophage cultures and their growth in the normal mouse foot pad. However, these suspensions showed a correlation between their MI and the increase in numbers of bacilli recovered at the end of a culture period—suspensions with a low MI showed no increases; whereas some of these with an MI of 10% or more showed increases (Kendall's rank correlation, $p = 0.01$).

No significant benefit was derived from modifying culture conditions in terms of incubation at 30°C instead of 35°C, or replacement of horse serum by fetal calf serum (which is likely to contain fewer factors that might be a source of macrophage activation).

Nude mouse macrophages

Pooling method. Results of *M. leprae* infection of nude mouse macrophages cultured using the pooling method are shown in Tables 1 and 2. Variations in the size of the inoculum or the source of *M. leprae* produced no significant changes in the numbers of *M. leprae* recovered, even after culture periods of 200 days; nor was there any

TABLE 1. M. leprae *recovered after* in vitro *cultivation in nude mouse peritoneal macrophages—pooling method.*

Inoculum (*M. leprae*/ml)	*M. leprae* recovered/ml[a]		Duration of culture (days)
	Base-line	Final count	
Armadillo 1.0×10^7	6.1 ± 4.9	9.1 ± 4.4	40
Human 1.0×10^4	0.28 ± 0.25	0.17[c]	46
		0.1[c]	64
		0.32 ± 0.25	113
Human 1.0×10^6	2.8 ± 0.28	2.3 ± 0.85	112
		1.8 ± 0.1	165
		2.2[c]	172
Nude MFP[b] 1.0×10^7	4.0 ± 1.3	9.4 ± 3.2	67
		5.0[c]	177
Human 1.0×10^6	1.6 ± 1.1	4.7 ± 2.8	60
Human 6.4×10^6	8.6 ± 3.1	7.0[c]	84
Human 1.0×10^6	1.9 ± 1.6	0.96 ± 0.24	67[d]
Human 1.0×10^6	2.6 ± 0.3	2.0 ± 0.6	145[e]
Human 1.0×10^7	9.4 ± 6.5	14.0 ± 3.0	175[d]
M. lepraemurium 1.0×10^7	0.24 ± 0.16	180 ± 150	60

[a] Each figure represents the mean of three cultures $\times 10^5 \pm 1$ standard deviation.
[b] MFP = mouse foot pad.
[c] Only 1 harvested.
[d] These cultures were maintained in medium containing 40% fetal calf serum instead of 40% horse serum.
[e] This culture was maintained at 30°C instead of 35°C.

difference observed between bone-marrow and peritoneal macrophages, or when cultures were incubated at 30°C or in the presence of fetal calf serum. By contrast *M. lepraemurium* showed tenfold increases after 60 days in culture. Human-derived *M. leprae,* killed by exposure to 2.5 M rads γ-irradiation from a cobalt^{60} source, showed little change in numbers recovered after a 104-day culture period.

Subculture method. Results of four experiments using the subculture technique showed a drop in the numbers of *M. leprae* recovered (Table 3). In one experiment where the same inoculum was also used for a pooling experiment, the former method showed a drop in the numbers of *M. leprae* recovered after 93 days; whereas in the latter, a 2.3-fold increase was observed. In one experiment, cultures were maintained at 30°C for 30 days after which *M. leprae* recovery was decreased by nearly fourfold. It may be that these decreases are due to loss of bacilli during centrifugation. In a control experiment using *M. lepraemurium,* a 16.6-fold increase was observed after 120 days.

Germ-free nude mouse macrophages

Because it has been shown that macrophages from germ-free mice are not as activated as those from nude mice colonized with a resident gut flora ([18] and Sharp and Colston, unpublished data), it seemed possible that germ-free nude mouse macrophages might provide a more acceptable environment for *M. leprae* growth than those from colonized nudes. Therefore, some macrophage cultures were derived from nude mice raised in germ-free conditions and cultured using the pooling method. In appearance, these cells were no different from those from colonized nude mice and the numbers of *M. leprae* removed after culture periods of 113 days and 165 days were similar to the base-line counts (Table 4). No difference was apparent in cultures main-

TABLE 2. M. leprae *recovered after* in vitro *cultivation in nude mouse bone-marrow macrophages—pooling method.*

Inoculum (*M. leprae*/ml)	*M. leprae* recovered/ml[a]		Duration of culture (days)
	Base-line	Final count	
Human 1.0×10^6	7.0 ± 8.6	3.9 ± 4.4 3.9 ± 1.1	33 140
Human 1.0×10^6	2.4 ± 0.72	1.9[c] 2.3 ± 0.99 1.3[c]	50 113 211
Human 1.0×10^6	2.0 ± 1.0	4.0 ± 1.7 1.9 ± 0.7 1.6[c]	112 165 172
Human 1.0×10^5	0.24 ± 0	0.72[c]	157
Nude MFP[b] 1.0×10^7	6.7 ± 6.0	2.4 ± 0.28 6.1 ± 3.5	106 191
Human 1.0×10^6	2.4 ± 6.4	6.1[c] 1.5 ± 0.35	120 160
Human 6.4×10^6	11.0 ± 2.3	50.0 ± 5.2	60
Human 6.4×10^6	11.0 ± 5.9	6.3 ± 2.9	140
Human 1.0×10^6	3.0 ± 0.9	1.7 ± 0 2.4 ± 0	80 220
Human 1.0×10^7	15 ± 9.6	12 ± 1.2	175[d]
M. lepraemurium 1.0×10^7	17 ± 8.0	270 ± 40	60

[a] Each figure represents the mean of three cultures × 10^5 ± 1 standard deviation.
[b] MFP = mouse foot pad.
[c] Only 1 harvested.
[d] This culture was maintained in medium supplemented with 40% fetal calf serum instead of 40% horse serum.

tained in the presence of fetal calf serum, or at 30°C.

Nude and normal rat macrophages

Macrophages from normal rats were cultured in three experiments; those from athymic nude rats, in two experiments. All macrophage cultures were infected with 1.0×10^6 human-derived *M. leprae*/tube. They were maintained using the pooling method for up to 193 days, during which time they remained adherent and spreading, although not as elongated as mouse macrophages. None of the cultures showed any significant change in the numbers of *M. leprae* recovered (Table 5).

Armadillo macrophages

Macrophages harvested from the femoral bone marrow of armadillos were difficult to culture without contamination. This was partly due to the fact that several hours elapsed in transit between the time of death and the macrophage harvest. Since these animals are not easily available, there was little opportunity to develop optimal conditions for culturing their macrophages. However, on two occasions, bone-marrow macrophages were successfully cultured for 100 and 130 days.

A third culture was successfully established and infected *in vitro* with 1×10^6 *M. leprae*/ml. A base-line count showed 2.0×10^5 *M. leprae*/ml phagocytosed, and after a period of 81 days 3.2×10^5 *M. leprae*/ml were recovered.

A culture of armadillo bone-marrow macrophages infected *in vivo* was also maintained for 90 days (see results under Infection *in vivo*).

Infection *in vivo*

Although macrophages from animals infected with *M. leprae* were harvested on

TABLE 3. M. leprae *recovered from macrophages by the subculture method.*

Macrophage source	Inoculum (AFB/ml)[a]	AFB/ml		Duration of culture (days)
		Base-line	Final count	
Nude mouse peritoneal	Human *M. leprae* 1.0×10^6	6.0×10^6	1.9×10^6	157
Nude mouse peritoneal	Nude mouse foot pad 1.0×10^7	6.9×10^7	3.2×10^6	93
Nude mouse bone marrow	Human *M. leprae* 1.0×10^7	3.0×10^7	3.5×10^6	137
Nude mouse bone marrow	Human *M. leprae* 1.0×10^6	8.0×10^6	2.3×10^6	70
Normal mouse peritoneal	Human *M. leprae* 1.0×10^6	4.0×10^6	9.4×10^5	120
Normal mouse peritoneal	Human *M. leprae* 1.0×10^7	4.0×10^7	2.2×10^6	167
Normal mouse peritoneal	*M. lepraemurium* 1.0×10^7	6.0×10^6	1.0×10^8	120

[a] AFB = acid-fast bacilli, i.e., *M. leprae* or *M. lepraemurium.*
[b] This culture was maintained at 30°C instead of 35°C.

several occasions, only two of these produced successful cultures of adherent cells already containing bacilli. Often, although suspensions of macrophages harvested contained bacilli, the cells themselves were less adherent than their uninfected counterparts and long-term cultures could not be established.

Bone-marrow macrophages were cultured from an armadillo which had been infected with 5×10^8 *M. leprae* iv, 19 months previously and which showed symptoms of a well-developed systemic infection. Base-line counts showed 1.8×10^6 *M. leprae*/ml. After 60 days of culture, 3.8×10^6 *M. leprae*/ml were counted: assuming a 10% viability and about four generations, each of 14 days, this count is as expected although it does not allow for any lag period. It is possible that a lag phase is not necessary since the bacilli are already accommodated in their intracellular environment in contrast to the *in vitro* infection where a period of adjustment may be envisaged. However, after 90 days 3.0×10^6 *M. leprae*/ml were counted, a much lower figure than the 2.5×10^7/ml theoretically expected.

Peritoneal macrophages were harvested from a nude mouse infected with 4.7×10^4 *M. leprae*/foot pad 22 months previously.

TABLE 4. *Recovery of* M. leprae *from macrophage cultures from germ-free athymic nude mice.*

Macrophage source	Inoculum (*M. leprae*/ml)	*M. leprae*/ml[a]		Duration of culture (days)
		Base-line	Final count	
Bone marrow	Human 10^6	6.7 ± 2.7	5.0 ± 2.4	165[b]
Peritoneal	Human 10^6	3.5 ± 0.85	3.0 ± 0.7	165[b]
Bone marrow	Human 10^6	5.8 ± 0.9	3.8 ± 0.97	113
Peritoneal	Human 10^6	4.4 ± 0.7	3.8 ± 0.46	113

[a] Each figure represents the mean of three cultures $\times 10^5 \pm 1$ standard deviation.
[b] These cultures were maintained in medium supplemented with 40% fetal calf serum instead of 40% horse serum, and at a temperature of 30°C instead of 35°C.

TABLE 5. *Recovery of* M. leprae *from macrophages cultured from athymic nude rats and normal rats.*

Macrophage source	Inoculum (*M. leprae*/ml)	*M. leprae*/ml[a]		Duration of culture (days)
		Base-line	Final count	
Normal rat peritoneal	Human 1.0×10^6	2.4 ± 0	1.6 ± 0.4	53
Normal rat peritoneal	Human 1.0×10^6	2.0 ± 0.1	1.6[b]	50
			2.1 ± 0.1	94
			2.2 ± 0.4	120
Normal rat bone marrow	Human 1.0×10^6	3.9 ± 1.1	3.6 ± 2.5	94
			1.6 ± 0.9	193
Nude rat peritoneal	Human 1.0×10^6	2.1 ± 1.2	1.7 ± 0.2	94
Nude rat bone marrow	Human 1.0×10^6	3.2 ± 0.8	3.3 ± 0.1	103

[a] Each figure represents the mean of three cultures × 10^5 ± 1 standard deviation.
[b] Only 1 harvested.

Ziehl-Neelsen staining showed about 30% of cells containing 1–5 or more *M. leprae*. A base-line harvest gave a count of 4.8×10^4 *M. leprae*/ml and after a culture period of 195 days, 1.9×10^5 *M. leprae* were recovered.

DISCUSSION

In this paper we describe attempts to cultivate *M. leprae* in a long-term macrophage culture system. In our experiments we have introduced two factors which might conceivably have enhanced opportunities for the intracellular growth of *M. leprae*. These were intended to overcome the problems met by earlier workers in terms of the requirement for a long-term but nondividing cell culture, and to give preference for cell donors which are susceptible to progressive *in vivo* infection. Using the culture methods described by Chang [2], we were able to maintain our macrophage cultures for over 200 days, thus providing the advantages of long-term culture without the problems posed by a dividing cell line. In addition, our macrophages were derived from congenitally athymic nude mice and rats, and from armadillos, in all of which *M. leprae* infection has been shown to disseminate and grow up to very high levels [4, 5, 9].

It was of particular interest to compare macrophages from different sites. North [16] has shown that the development of cell-mediated immunity (which is invoked during *M. leprae* infection) is accompanied by an influx into tissues of blood-derived mononuclear cells, precursors of which are to be found in the bone marrow. It is these cells which are active at inflammatory sites and which may be the prime hosts for intracellular organisms. Whereas peritoneal macrophages have already differentiated, those from bone marrow still have the potential to mature *in vitro*. The implied differences between these two cell types might be expected to influence the intracellular behavior of *M. leprae*. In spite of these modifications we were unable to demonstrate the growth of *M. leprae* in any of our experiments. About one third of our cultures showed increases in the numbers of *M. leprae* recovered at the end of a culture period, but it is unlikely that this represents growth. A more likely explanation is that increases are due to the fragmentation of clumps of bacilli which are difficult to eliminate from an infecting suspension. On the other hand, it is possible that technical problems concerning losses during centrifugation, and adherence of bacilli to culture vessels, contributed to our inability to recover all bacilli from our cultures. The fact that macrophages from three different susceptible hosts showed similar results emphasizes the discrepancy between *in vivo* and *in vitro* conditions for the growth of *M. leprae*. This organism thrives in macrophages of nude mice, nude rats, and armadillos. In nude animals, this growth is attributed to a general T lymphocyte deficiency [5], but this is not so with armadillos, which are immunologically intact [17]. Failure of *M. leprae*

to grow *in vitro* in macrophages from these animals could be due to a) deprivation of one or more essential nutrients in *in vitro* culture or b) the possibility that macrophage microbicidal mechanisms are suppressed *in vivo* in susceptible animals allowing growth to occur; whereas this suppression is absent *in vitro* and the cultured macrophages are able to express stasis or killing.

Although we have insufficient evidence at present, it may be that infection *in vivo* is a critical factor. *In vivo* conditions may suppress the bactericidal activity thought to take place around the time of phagocytosis, and once *M. leprae* is established in its intracellular environment it may be indifferent to *in vitro* or *in vivo* conditions.

It would appear that the differences demonstrated in macrophage activity of germ-free nude mice and nude mice with a resident gut flora ([18] and Sharp and Colston, unpublished data) are not expressed in long-term cultures, since macrophages from germ-free animals gave results similar to those from the more active macrophages harvested from colonized nude mice.

Our results showed that *M. lepraemurium*, used as a positive control, is able to multiply in our system.

It would be of interest to know whether the viability of *M. leprae* is changed during *in vitro* culture. It is known that *in vivo M. leprae* "persist" in macrophages for a very long time ([20]). Thus, it is possible that a) the majority of bacilli recovered from a long-term culture are dead although remaining acid fast; b) that *M. leprae* are indifferent to their *in vitro* intracellular environment and that their viability remains unchanged; or c) that some dead bacilli have been degraded and cleared by the macrophages but, due to a certain amount of growth, the total count remains about the same. Several experiments are in progress to investigate the fate of *M. leprae* in *in vitro* cultures, and they will be reported in a subsequent paper.

SUMMARY

Macrophages from nude mice, nude rats, and armadillos were cultured *in vitro* and examined for their ability to support *Mycobacterium leprae*. No significant growth of this organism was observed after over 200 days of culture. No significant benefit was derived from modifying culture conditions or from variations in the source of macrophages or the source of *M. leprae*.

RESUMEN

Se cultivaron *in vitro* macrófagos de ratones desnudos, de ratas desnudas y de armadillos, y se examinó su capacidad para permitir el crecimiento del *Mycobacterium leprae*. No se observó un crecimiento significativo de este organismo después de 200 días de cultivo. Tampoco se observó un efecto benéfico significante al modificar las condiciones del cultivo, al variar la fuente de macrófagos, o al variar la fuente del *M. leprae*.

RÉSUMÉ

On a cultivé *in vitro* des macrophages de souris glabres, de rats glabres, et de tatous; ces macrophages ont été examinés en vue de voir dans quelle mesure ils permettent la croissance de *Mycobacterium leprae*. Aucune croissance significative de cet organisme n'a été observé après plus de 200 jours de culture. Aucun bénéfice significatif n'a été obtenu par la modification des conditions de cultures ou en faisant varier la source de macrophages ou la source de *M. leprae*.

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REFERENCES

1. Brown, C. A. and Brown, I. N. *Mycobacterium bovis*, BCG. Modulation of murine antibody responses: Influence of dose and degree of aggregation of live or dead organisms. Br. J. Exp. Pathol. **63** (1982) 133–143.
2. Chang, Y. T. Long term cultivation of mouse peritoneal macrophages. J. Natl. Cancer Inst. **32** (1964) 19–35.
3. Chang, Y. T. and Neikirk, R. L. *Mycobacterium lepraemurium* and *Mycobacterium leprae* in cultures of mouse peritoneal macrophages. Int. J. Lepr. **33** (1965) 536–598.
4. Colston, M. J., Fieldsteel, A. H., Lancaster, R. D. and Dawson, P. J. The athymic rat: Immunological status and infection with *Mycobacterium leprae*. Abstract in Int. J. Lepr. **47** (1979) 679.
5. Colston, M. J. and Hilson, G. R. F. Growth of *Mycobacterium leprae* and *Mycobacterium marinum* in congenitally athymic (nude) mice. Nature **262** (1976) 399–401.
6. Fieldsteel, A. H. and McIntosh, A. Attempts to cultivate and determine the maximum period of viability of *Mycobacterium leprae* in tissue culture. Int. J. Lepr. **40** (1972) 271–277.
7. Garbutt, E. W. Studies on *M. lepraemurium* and *M. leprae* in tissue culture. Int. J. Lepr. **33** (1965) 578–585.

8. Holmes, I. B. and Hilson, G. R. F. The effect of rifampicin and dapsone on experimental *Mycobacterium leprae* infections: Minimum inhibitory concentrations and bactericidal action. J. Med. Microbiol. **5** (1972) 251–261.
9. Kirchheimer, W. F. and Storrs, C. E. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. Int. J. Lepr. **39** (1971) 693–702.
10. Kohsaka, K., Mori, T. and Ito, T. Lepromatoid lesion developed in nude mice inoculated with *Mycobacterium leprae*. Lepro **45** (1976) 177–187.
11. Levy, L. Studies of the mouse foot-pad technique for cultivation of *Mycobacterium leprae*. 3. Doubling time during logarithmic multiplication. Lepr. Rev. **47** (1976) 103–106.
12. Matuso, Y. and Utsunomiya, S. Attempts at cultivation of *Mycobacterium leprae* in cell cultures. Jpn. J. Microbiol. **20** (1976) 471–473.
13. McLeod, R. and Remington, J. S. Studies on the specificity of killing of intracellular pathogens by macrophages. Cell. Immunol. **34** (1977) 156–174.
14. McRae, D. H. and Shepard, C. C. Attempts to cultivate *Mycobacterium leprae* in tissue culture. Int. J. Lepr. **38** (1970) 342–343.
15. Nogueira, N., Gordon, S. and Cohn, Z. *Trypanosoma cruzi:* Modification of macrophage function during infection. J. Exp. Med. **146** (1977) 157–171.
16. North, R. J. The relative importance of blood monocytes and fixed macrophages in the expression of cell mediated immunity to infection. J. Exp. Med. **132** (1970) 521–534.
17. Purtilo, D. T., Walsh, G. P., Storrs, E. E. and Gannon, C. The immune system of the nine-banded armadillo (*Dasypus novemcinctus*, Linn.). Anat. Rec. **181** (1975) 725–734.
18. Rao, G. R., Rawls, W. E., Perey, D. Y. and Tompkins, W. F. Macrophage activation in congenitally athymic mice raised under conventional or germ-free conditions. J. Reticuloendothel. Soc. **21** (1977) 13–20.
19. Samuel, D. R., Godal, T., Myrvang, B. and Song, Y. K. Behavior of *Mycobacterium leprae* in human macrophages *in vitro*. Infect. Immun. **8** (1973) 446–449.
20. Tomon, K. Bacterial persisters in leprosy. Int. J. Lepr. **49** (1981) 205–217.
21. Yamagami, A. and Chang, Y. T. Growth of *Mycobacterium lepraemurium* in cultures of macrophages obtained from various sources. Infect. Immun. **17** (1977) 531–534.