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# *In Vitro* Cultivation of Leprosy Bacilli on Hyaluronic Acid Based Medium 1. Preliminary Report [1,2]

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The efforts that have been made to cultivate *M. leprae in vitro* during the century since its discovery are too extensive to be reviewed in this report. A nice, interpretive summary has recently been presented by Yoshie ([26]). Essentially, the results have been so unrewarding as to have led to the designation of this pathogen by many investigators as an uncultivable mycobacterium, probably very fastidious in its growth requirements and quite probably an obligate intracellular parasite. The pattern of lesion distribution in leprosy patients has also led to a strongly held hypothesis that its growth is low temperature dependent.

Recent histochemical studies ([12, 14, 22, 23]) in this laboratory have led to concepts quite contrary to these, based on findings that concentrations of *M. leprae* in the human host are associated with the presence of acid mucopolysaccharides of the host (Color Plate A). Mouse inoculation of *M. leprae* demonstrated that hyaluronic acid (HA) applied to the inoculum and inoculation site will promote growth of *M. leprae* in the mouse abdominal wall and peritoneum, neither of which areas have the postulated required low temperature and neither of which support *M. leprae* growth in immunologically intact mice. Extracellular bacilli were abundant and after one year of such treatment nerve invasion by bacilli was noted. It was also found that a 0.1% saline solution of HA will support viable bacilli for some weeks in the refrigerator or 37°C incubator but only minimal proliferation. After several trials with various solid media fortified with hyaluronic acid, a rather simple liquid medium was devised which has been designated the Leprosy Atelier Culture Medium No. 3 (LA-3). The purpose of this presentation is to describe prolific growth of *M. leprae* (and also *M. lepraemurium*) in this medium and to demonstrate the reasonable probability that the claimed *M. leprae* culture is indeed this organism.

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## MATERIALS AND METHODS

**Leprosy Atelier Culture Medium No. 3 (LA-3).** This medium was deliberately crafted on the basis of three major considerations:

1. The determination that HA is a suitable basic growth promoter for *M. leprae* in mice and presumably is an adequate energy source ([12, 13, 22]).
2. Hanks' finding, in working on the measurement and preservation of metabolic activity of *M. lepraemurium*, that a combination of bovine albumin plus yeast supplement gave optimum results ([6, 8]).
3. The recognition that pH requirements would be of considerable significance. It having been determined that *M. leprae* utilizes $\beta$-glucuronidase (GAG) and its HA metabolism, the effective pH range of this enzyme (pH 4.5 optimum to pH 7.0) was an important consideration. However, many other enzymes such as those involved in phosphorylation operate best at a higher pH than GAG. A compromise of pH 6.24 was empirically determined to be suitable.

The LA-3 medium, having the following composition, thus became the culture medium on which *M. leprae* as well as *M. lepraemurium* are now cultivated in this laboratory.

*The composition of the medium.*

1. 0.066 M phosphate buffer pH 6.24 — 81 ml
2. Glycerin — 3 ml
3. Hyaluronic acid (sodium salt, grade-III-S, from human umbilical cord, Sigma) — 100 mg
4. Bovine serum albumin (Cohn Fraction V, Sigma) — 6 gm
5. Fresh yeast extract (aseptic, Microbiological Associates, Inc., Bethesda, Maryland) — 16 ml
6. Potassium penicillin G (Eli Lilly and Co. Suspended in factory made sodium-citrate buffer 0.6 ml) — 20,000 unit

*Preparation of the medium.*

Solution A: Mix 31 ml of the phosphate buffer, 3 ml of glycerin and 100 mg of hyaluronic acid and autoclave at 15 lb for 15 minutes.

Solution B: Dissolve 6 gm of bovine serum albumin in 50 ml of the phosphate buffer.

Solution C: (Culture Medium): Mix A and B and add 16 ml of fresh yeast extract and 20,000 unit of penicillin. The whole medium is passed through a Seitz filter and refrigerated until used.

Cultivation has been in 4–6 ml quantities of medium in Falcon polypropyline, capped, 17 × 100 mm culture tubes, or capped glass culture tubes, at 37°C in standard incubators with no special attention to atmosphere. After some experience the glass tubes are preferred. Some parallel cultures are maintained at 25°C. The culture tube caps are loose enough to provide aeration.

**LA-3 medium as plate cultures (LA-3P).** The above prepared medium was prepared for plate use by incorporating 2% reagent grade agar or 0.5% agarose.

**Dubos liquid medium + 0.1% HA.** The standard Dubos (oleic acid-albumin) media to which 0.1% HA is added.

**Electron microscopy.** In as far as feasible, cultures were monitored periodically by electron microscopy utilizing a Zeiss EM-9S2 instrument. The laboratory is literally built around this instrument so as to facilitate its constant use in association with, and as an extension of, other laboratory technics.

For the most part, whole bacilli were placed in suspension on carbonized, Formvar (polyvinyl formaldehyde plastic) coated grids. Complete drying of the suspension usually resulted in too heavy concentrations of bacilli or in the presence of interfering concentrations of media ingredients. Intermediate, sub-drying periods of sedimentation were not found helpful. Accordingly, the excess of the suspension droplet on the grid was removed carefully with the edge of a filter paper, the grid was allowed to dry and then fixed over osmium tetroxide vapor. Negative staining with phosphotungstic acid and shadow casting with the use of a Denton Vacuum DV-515 Automatic Evaporator were selectively employed.

**Electron transport activity in leprosy bacilli by electron microscopy ([7, 8, 15, 16]).** Bacilli were suspended in peptone broth (Supplemented Peptone Broth, Blood Culture Medium. Becton, Dickinson & Co.). A small droplet of the suspension was placed on a Formvar supported grid to form a "bead" extending to the edge of the grid. This droplet was touched with the edge of a Whatman #1 filter paper to remove most of the excess liquid and the grid then permitted to dry. The grid, bacilli down, was next placed in a solution of 20 mg% lysozyme in 0.066 M

phosphate buffer, pH 6.4, at 37° for 30–60 minutes. Brief rinsing in 0.1 M Na-cacodylate buffer with 7% sucrose was followed by floating the grid, bacilli down, on a solution of 0.1% tellurite in extract + 6% albumin for 37°C for a minimum of 15-20 hours. Again the grid was rinsed in 0.1 M Na-cacodylate buffer with 7% sucrose and allowed to dry. (*Note:* to strengthen the Formvar film, the grid can be carbon coated following the above procedure.)

Controls used for the tellurite reaction have consisted of boiling for 15–30 minutes, potassium cyanide 2 mM for the whole incubation period and sodium azide 2 mM for the total incubation period. These methods all kill the reaction, supporting the concept that the method as used is a valid indication of electron transport activity and thus of mycobacterial viability.

**Acid-fast stain for M. leprae.** These attempts at cultivating *M. leprae* have necessitated a review of acid-fast staining methods and their evolution as applied to this pathogen. The subject is too extensive for complete review in this presentation but several pertinent points require attention.

It seems evident that, in general, the methods of staining have been directed at increasing the demonstration of acid-fastness in order to better evaluate the numbers of bacilli present since *M. leprae* is recognized to be relatively "weakly" acid-fast. Thus, sulfuric acid rather than hydrochloric acid in combination with ethanol has been recommended as being less vigorous in decolorization. Formalin is a reducing fixative for proteins and possibly also for lipids over a long period of fixation and under these conditions acid-fastness may have to be "restored." The Fite-Faraco method ([4, 11]) was an "improvement" in that cotton seed oil (an oxident) was introduced into the staining procedure to "restore" acid-fastness and staining was enhanced.

Wade's ([24]) studies were directed at "preserving" acid-fastness by utilizing a mixture of paraffin oil and aviation gasoline, or rectified turpentine, thus apparently increasing resistance to decolorization. The currently favored treatment with peanut oil in many laboratories, including ours, apparently achieves its superior demonstration by oxidative effects. The periodic acid treatment technic of Nyka ([18]) clearly is in a similar category.

Ironically, these improved methods have, perhaps, helped obscure significant features of the life cycle of this pathogen as now revealed by *in vitro* cultivation studies. In the present studies, the older Ziehl-Neelsen method utilizing Löeffler's methylene blue, operative at pH 10, has been of correlative value in providing a better evaluation of cultivation progress and as correlative with viability studies employing the hydrogen transfer capacity by the formation of formazan from the tetrazolium salt as delineated above.

**M. leprae inocula.**

SA-75— Saigon, 33 years, male, untreated LL. Biopsy 2-15-75.

SA-74— $C_3H$ mouse, one year passaged with hyaluronic acid, mixed inoculum from two Saigon, five Hong Kong and two Seoul untreated LL patients. Biopsies: June, 1974.

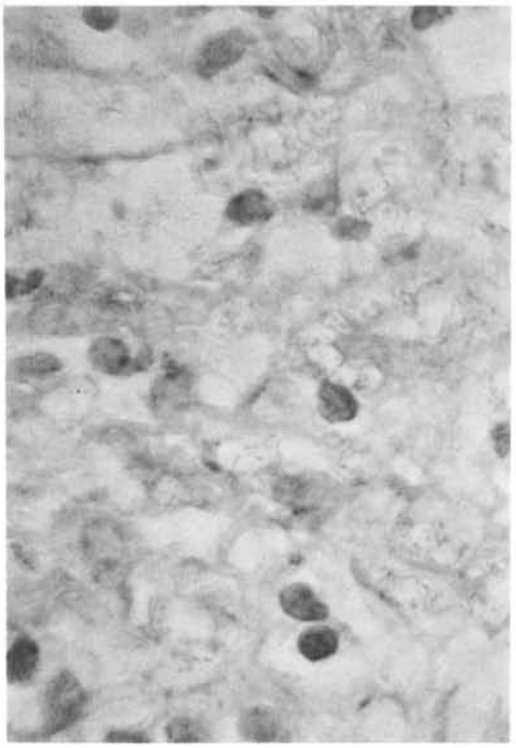
HK-74— Swiss mouse, one year passaged with hyaluronic acid, mixed inoculum from five Hong Kong and two Seoul untreated LL patients; as SA-74 but without Saigon material.

HK-75— Hong Kong, 49 years, male, untreated LL. Biopsy 7-4-75.

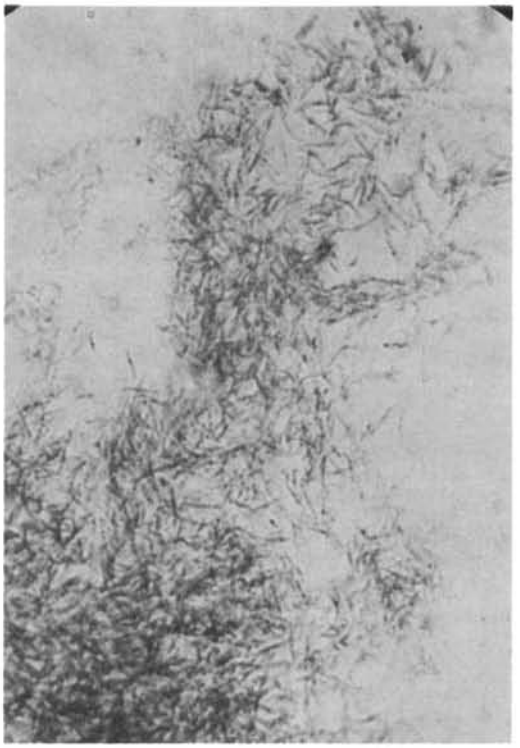
HI-75— Philippine, two year Hawaii immigrant, 76 years, male. Recently untreated, apparently relapsed LL. Biopsy 9-6-75.

MO-75— Molokai patient with diagnosis of LL in 1936; male, 56 years. Continuous sulfones since 1946. Recent years BI negative until August 1975 relapse. Presumed DDS resistant. Biopsy 9-19-75.

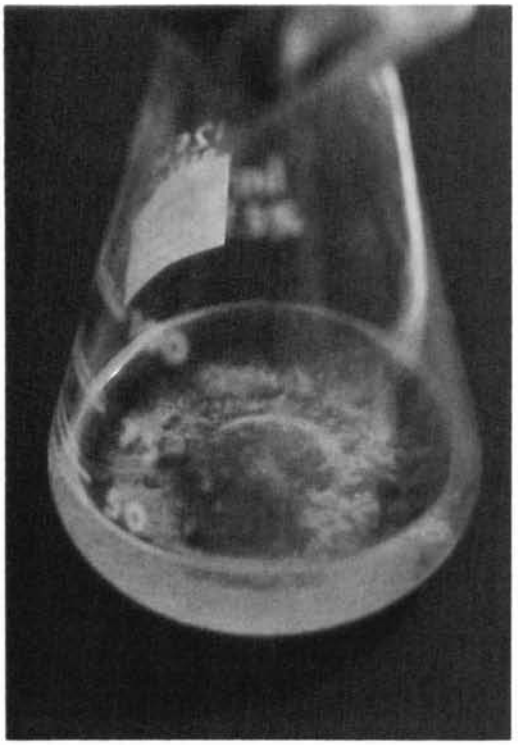
Skin biopsies were obtained in each case under aseptic conditions and kept under refrigeration at 4°C or on wet ice during transport until arrival at the laboratory. This period varied from one day to two weeks. The tissues were then minced and ground in 1/15 M phosphate buffer, pH 6.24, by means of a Potter-Elvehjem homogenizer for five to ten minutes till completely homogenized. After filtering through gauze, the suspension was centrifuged at 1,000 rpm (200 g) for five minutes. Resuspended and centrifuged twice again at 1,000 rpm and 2,000 rpm (800 g). The final pooled supernatant was then centrifuged at 10,000 rpm



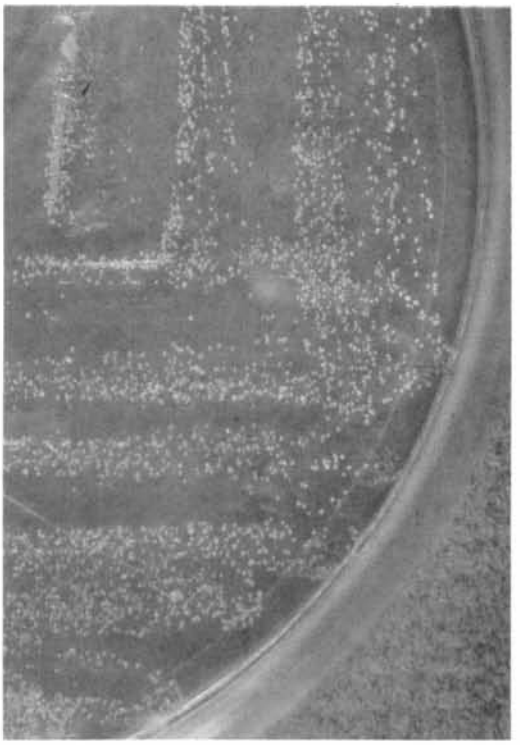
COLOR PLATE A. Combined Mowry/acid-fast stain of LL skin lesion with bacilli concentrated in AMPS (blue).



COLOR PLATE B. Acid-fast bacilli from LA-3P colony. 24 days. ×400.



COLOR PLATE C. Bacillary sediment in LA-3 medium, aerated b.i.d., 16 days.



COLOR PLATE D. Colonies, LA-3P, 19 days.

(12,000 g at 10°C). Thereafter, the centrifuged residue was treated for 30 minutes at room temperature with mixed equal volumes of 4% NaOH and 2.9% sodium citrate solution for sterilization of possible contaminating micro-organisms. Then the suspension was again centrifuged at 10,000 rpm (10°C), resuspended in 1/15 M phosphate buffer solution and the process repeated three times with the final residue being suspended in a small volume of phosphate buffer or in 0.1% hyaluronic acid in normal saline.

Sterile utensils were used throughout and all procedures, except grinding and centrifugation, were carried out in an ultraviolet light sterilized microbiology hood.

**Immuno-fluorescence identification of M. leprae.** The problems arising in the identification of cultivated bacilli as *M. leprae* are considered separately ([21]). It is evident that cultivation raises problems in identification not previously considered in depth. Attempts at such consideration led to the conclusion that an immuno-fluorescent technic was necessary and could provide definitive identification.

An immuno-fluorescent technic was worked out and is presented as a separate communication ([13]) in this issue of the JOURNAL.

Mycobacterial isolates, cultures and subcultures were monitored by this technic.

## RESULTS

**Bacillary growth.** The first specimen (SA-74), on the basis of prior experience, was in part inoculated into the abdominal wall of $C_3H$ female mice which subsequently received 0.1% HA intraperitoneally weekly for one year. There then was extensive growth of bacilli at the local inoculation site, with nerve invasion, and intraperitoneally. Significant numbers of bacilli were extracellular, suggesting that extracellular growth and proliferation was taking place. These bacilli were harvested from the tissues as above and inoculated on the LA-3 medium which was now available. It now grows in this medium as one strain.

The remainder of this first specimen was utilized for initial growth trials on a battery of standard media normally used for initial growth trials for cultivating acid-fast bacilli (Tarshish, Ogawa, Wallenstein) but in this instance fortified with HA in a concentration of 0.1%. Though there was some thin surface growth, particularly with the Tarshish medium, the cultivated strain was eventually lost by inadequate growth conditions and in contaminants.

The second specimen (HK-74) was inoculated into abdominal walls of Swiss female mice which subsequently also received 0.1% HA intraperitoneally at weekly intervals for one year. It was also inoculated on the previously most successful of the HA fortified media, Tarshish. It had been found that HA is markedly hydrophilic and solid media surfaces tended to dry out. Therefore, the solid medium was now used to coat standard glass microslides which had been cut in half longitudinally. These "media-slides" with inoculated bacilli were inserted into culture tubes containing 0.1% HA in saline and the cultures were thus kept moist and bathed in this solution. A faint surface growth, without distinct colonies, developed as it had previously, but it was noted that a higher concentration of bacilli eventually were found free in the HA/saline solution and it was determined that they could be maintained for some time in this solution and appeared to proliferate slightly in that a fine precipitate of bacilli eventually developed in the bottom of the tube. If Hanks' Balance Salt Solution was used in place of saline, the results were poor, probably because of binding of HA by calcium. The indicator in this BSS solution showed no change, indicating absence of acidic change which usually accompanies active bacterial growth metabolism. These findings led to the determination that *M. leprae* can be maintained viably for some weeks either by incubation or ice box storage in the 0.1% HA in normal saline solution.

All strains of bacilli from the above described sources now grow well on the LA-3 medium and all present the same growth characteristics save for SA-75 which was lost to overgrowth by another acid-fast organism.

*M. lepraemurium* (Hawaiian strain) is now also cultivated in this medium. Its growth characteristics are under evaluation and will not be included in this presentation. They differ sufficiently from those of *M. leprae* to permit separate identification.

**Growth pattern.** In HA-saline suspension the bacilli present as weakly acid-fast short rods or as protoplast-like granules, often conglutinate.

Growth in the LA-3 medium was initially slow despite heavy initial inoculation, requiring up to six weeks for a sediment to develop in the test tube. Secondary inoculation into larger quantities of medium in an Erlenmeyer flask with aeration by b.i.d. agitation to a surface froth, yield good turbidity with heavy bacillary precipitate in two weeks (Color Plate C).

**Growth morphology in LA-3 medium.** Details relating to possible life cycle, growth under variant conditions and means of multiplication will be separately reported.

Briefly, on LA-3 medium the bacilli grow relatively slowly but provide a reasonably heavy precipitate as well as dispersion throughout the medium in six weeks on primary culture. Some differences in the rapidity of this growth are seen depending on whether the inoculum is derived from the phosphate buffer or from the HA-saline suspension. The bacillary precipitate is finely granular with a feathery adherence when it is gently shaken up.

Initially, the bacillary forms present as individual or clumped protoplast-like bodies or short rods which are not acid-fast. Gradually, acid-fast forms appear and by six weeks of cultivation the majority of the culture suspensions present as acid-fast rods.

When cultures are grown in 50 ml of LA-3 in 125 ml Erlenmeyer flasks with twice a day aeration as described, growth morphology differs. Growth is more rapid and elongate forms which are acid-fast predominate. In general the morphology more closely resembles that seen on the LA-3P plate cultures.

**Growth morphology on LA-3P medium.** Growth on the solid medium form is considerably more rapid than in the liquid medium and much more rapid than expected from that described for *M. leprae* growth in the mouse foot pad ([20]).

By 24 hours, somewhat vague, colony-like amorphous shapes can be seen with the microscope using a 5× macrotessar lens, a 10× occular and reflected light. By 48 hours distinct colonies are formative and grayish-white. By five days, the colonies are larger, though still microscopic in size, but are beginning to show a brownish yellow pigmentation. By the second to third week, the colonies are clearly macroscopic and become orange-yellow (Color Plate D).

The earliest growth forms, at about five days, thus far picked up from these cultures tend to show elongated, non-acid-fast forms with suggestions of some branching. Portions of them may be irregularly acid-fast. At the time the fully formed microscopic yellowish colonies are developed by about 19 days (Color Plate D), the colonies consist of acid-fast rods and long or filamentous shapes (Color Plate B). Some of this appearance of elongation is due to end-to-end bacilli in abundance.

**Viability of cultivated bacilli.** Preliminary studies of bacillary suspensions isolated from LL biopsies and of cultured suspensions have been made by combining the electron-microscopic tellurite viability technic with acid-fast staining. Drops of bacillary suspension placed on EM grids were prepared as described for the tellurite procedure. After EM determination of positive reaction, the EM grid preparations were acid-fast stained. Experience to the present suggests that the non-acid-fast bacillary forms yield the tellurite positive reaction whereas well stained acid-fast forms may not. At what stage of development this occurs is not clear and the study is still in process.

**Immuno-fluorescence identification.** Mycobacterial isolates, primary cultures and subcultures have been monitored for *M. leprae* specificity with the results being as presented in Table 1, which refers to cultures on LA-3 liquid medium.

Culture on LA-3P plates also show immuno-fluorescence in early stages. At three weeks the colony bacilli no longer yield the immuno-fluorescent reaction according to preliminary determinations. These studies are still in process.

**Dubos medium + HA.** Rather belatedly we found that *M. leprae* will grow also in this medium apparently at about the same rate as in LA-3. This is under further investigation. We have not had opportunity to attempt direct inoculation from patient isolate to this medium.

## DISCUSSION

Alleged *in vitro* cultivation of *M. leprae* is based chiefly on the following six determinations.

1. Pathologic and experimentally determined rationale for the essential *M. leprae* nutrient requirement ([12, 14, 22]).
2. Several cultures having the same characteristics have been isolated from LL

TABLE 1. *Immuno-fluorescence reaction to* M. leprae *specific antibody.*

| Strain | Original isolate | | 1° culture | Subculture |
|---|---|---|---|---|
| | human | mouse | | |
| SA-75 | O[a] | + | O[a] | +[b] |
| SA-74 | O[a] | + | + | + |
| HK-74 | O[a] | + | + | + |
| HK-75 | + | | + | abandoned |
| HI-75 | + | | + | + |
| MO-75 | + | | + | + |

[a] Technic not available at that stage.
[b] An associate contaminant mycobacterium was revealed by the technic.

patients widely separate in time and by geography.

3. Failure of culture isolates to subculture on the usual media employed in the cultivation of mycobacteria at both 37°C and room temperature.
4. 1° cultures in liquid medium successfully transferred to 2° liquid medium and to 2° agar medium plates.
5. Bacillary isolates and bacilli of 1° and 2° liquid medium cultures, as well as LA 3P cultures, all stain with pooled LL serum, FITC coupled, *M. leprae* specific antibody with which a broad range of other mycobacteria do not react.
6. *M. lepraemurium* also presents good growth on this medium.

The growth of *M. lepraemurium* on the media described is, of course, not definitive evidence of *M. leprae in vitro* cultivation, but it is supportive in so far as the cultivation problems of both pathogens have long been regarded as related and attempts at cultivation of *M. lepraemurium* have been widely undertaken in attempts to achieve a model for the cultivation of *M. leprae.*

Additional support for this concept is to be found in the recent cultivation attempts by Nakamura ([17]) as related to *M. lepraemurium.* In these efforts a liquid medium designated as ND-5 was devised. This was based on the Dubos medium with supplements including, significantly, goat serum. The results were notably better than with the previous NC-5 medium, possibly because goat serum may be especially rich in acid-mucopolysaccharides; at least so suggests our atavistic memory though we have been unable to track down its source.

Again, comparison does not necessarily establish fact, but Table 2 states approximately the supportive lines of evidence available at the times of first presentation of growth of *M. leprae* in the mouse foot pad, in the armadillo and in the present alleged *in vitro* cultivation. This does indicate that the allegation is not lightly made.

The finding that *M. leprae* grows more rapidly on the media plate than in the liquid medium suggested the need for greater aeration of the latter and the enhanced growth in aerated LA-3 is supportive of this observation.

The less than optimal response in the non-aerated liquid medium seems to be revealing with respect to the pathogen life cycle adaptability under adverse circumstances. This may be of value in understanding features relating to the pathogenesis of the mouse food pad growth as well as the human disease. Thus, the mouse and its foot pad must be regarded as a hostile environment permitting limited *M. leprae* proliferation and presenting the additional problem of what happens to the inoculum immediately after injection. Thus, Desikan ([3]) as well as Levy ([10]) and others have been unable to account for the disappearance of *M. leprae* inoculated into mouse foot pads since acid-fast bacilli are not found there for the first three months after inoculation and cannot be accounted for by search of other tissues and organs. It would appear that a strong possibility is that the inoculated bacilli either revert to non-acid-fast forms or are torn apart by the defense mechanisms to liberate non-acid-fast but reproductive capable forms. The acid-fast stains employed would not reveal these forms, particularly if they were not looked for.

TABLE 2. *Evidence status at time of initial presentation.*

| | Mouse foot pad[a] -Shepard | Armadillo[b] Kirchheimer & Storrs | Cultivation OKS & Collaborators |
|---|---|---|---|
| Repeatability & multiple successes by primary author(s) | + | ○ One animal only | + |
| Nerve involvement | ○ | + | ± Not directly applicable[c] |
| DOPA reaction | ○ Not available | + | +[d] |
| Prior experimental supporting data and published rationale | ○ | ○ | + |
| Immunologic identity determination of bacilli concerned | ○ | ○ | + |
| Failure of mycobacterial isolate to grow on conventional media | + | + | + |
| Transmission or transfer success from one medium or animal to another | + | ○ | + |

[a] Shepard, C. C. J. Exp. Med. **112** (1960) 445-454.
[b] Kirchheimer, W. F. & Storrs, E. E. Int. J. Lepr. **39** (1971) 693-702.
[c] Two cultured *M. leprae* strains were first passaged through mice which were given hyaluronic acid weekly during passage. The lesions in these mice showed nerve involvement.
[d] Two ingredients of the medium used yield + reactions. At present this is not acceptable as identifying evidence.

The limited proliferation of *M. leprae* in the mouse foot pad has most commonly been regarded as due to some limiting immune development. However, with weekly injections of hyaluronic acid into mice inoculated with *M. leprae* suspensions directly from LL patients, we noted considerably more foot pad proliferation to the point where gross alteration in the size of the foot pad was evident. This suggests that the limitation of foot pad growth may be, at least in part, due to nutritional deficiency from exhaustion of locally available acid-mucopolysaccharide. Histochemical determinations on a group of surplus inoculated foot pads should quickly determine whether or not this is the case.

The possibility of variant *M. leprae* forms has been repeatedly raised in the past, perhaps most recently by Barksdale and associates (1). Indeed, careful acid-fast staining of human biopsy material often reveals, even in the same globi, methylene blue-staining rods intermixed with the acid-fast forms. According to preliminary determinations, largely based on the apparent validity of the tellurite reduction reaction as a measure of viability, there is a strong likelihood that it is these non-acid-fast forms that are viable. At what stage they lose viability is not clear, but by the time they are strongly acid-fast they often no longer give the reaction. Also, the bacilli grown on the LA-3P medium yield immuno-fluorescent reaction in the early colony stages but, according to preliminary observations, seem to lose this capacity in the well-developed, three to four week old, yellow colonies composed entirely of acid-fast bacilli. It is known (2) that peroxidation of lipids may be injurious to proteins. In the plate cultures oxidation opportunity is "abnormal" as compared to conditions of *M. leprae* life in human host tissues. Nevertheless, the same process may well occur due to the considerable variety of oxidases present in these tissues (27) and Fisher and Barksdale (5) may well have been prescient in suggesting that these acid-fast forms, at least in their later stages, are "mummified remains of once viable bacte-

ria." It is not unlikely that the whole concept of the Morphologic Index will require re-evaluation.

Among the many questions and possibilities raised by this alleged cultivation of *M. leprae* is the puzzling one relating to why this organism, which apparently possesses the enzymatic capacities for utilizing the citric acid energy cycle, is unable to utilize directly the simple carbohydrate elements of this cycle for its growth requirements and apparently specifically demands the complex acid-mucopolysaccharides, particularly hyaluronic acid, as its metabolic starting point.

The answers to this question are, of course, not yet known but three pertinent considerations may provide a starting point for solving the question.

The first consideration is that life consists of substance as well as energy. It is conceivable that the leprosy bacilli require the acid-mucopolysaccharides as a basis for essential structural components. Electron micrographs on variant growth conditions to date suggest variation in cell wall structure.

A second consideration refers to the fact that the chemical structures of the long chained acid-mucopolysaccharides provides relatively condensed nutrient/energy sources as compared to the simple sugars because of their relatively high potential oxygen availability in the unit saccharides.

The third concept has phylogenetic or ontogenetic implications. The numerous similarities between the mycobacteria and fungi are the basis for their being classified together in the order *Actinomycetales,* apart from the true bacteria which are listed in the order *Eubacteriales.* The range of fungi is vast with a broad pathogenic range extending from plant to mammalian tissues. Polysaccharides such as the mannans, and, in some instances acid-mucopolysaccharides, are present in great quantities in the higher order of plants in which the majority of the plant polysaccharides are components of the cell walls, being components of cellulose, xylans, etc., where some fungi also grow. The chitin found in sea animals, fungi, and insects resembles cellulose in its chemical and biological properties ([19]) and is the core structure of gram positive substance. *Aspergillus niger,* the usual cause of otomycosis which may occasionally be found in other human lesions, for example, contains enzymes capable of degrading cellulose and xylan ([25]). There seems here to be an inadequately explored phylogenetic relationship between the mycobacteria and the fungi which may have taxonomic significance and may be productive of clues as to the origins of mycobacterial parasites of man and of *M. leprae* in particular.

## SUMMARY

*In vitro* cultivation is reported of *Mycobacterium leprae* on a medium (designated LA-3) based on hyaluronic acid with additional ingredients of yeast extract, bovine albumin and glycerin together with phosphate buffer. The medium is also incorporated with agar or agarose (designated LA-3P) to serve as culture plates.

Initial growth in LA-3 in test tubes required about six weeks but subsequently this was speeded up to about two weeks utilizing larger quantities of media with aeration by shaking twice a day.

Growth on LA-3P yields numerous small orange-yellow colonies in two to three weeks.

Facets of the emerging aspects of the life cycle of *M. leprae* under cultivation are given preliminary report.

The bases for the allegation of *M. leprae* identity of the cultured bacilli are essentially the following six determinations.

1. Pathologic and experimentally determined rationale for the essential *M. leprae* nutrient requirement.
2. Several cultures having the same characteristics have been isolated from LL patients widely separate in time and by geography.
3. Failure of culture isolates to subculture on the usual media employed in the cultivation of mycobacteria at both 37°C and room temperature.
4. 1° cultures in liquid medium successfully transferred to 2° liquid medium and to 2° agar medium plates.
5. Bacillary isolates and bacilli of 1° and 2° liquid medium cultures all stain with pooled LL serum, FITC coupled, *M. leprae* specific antibody with which a broad range of other mycobacteria do not react.
6. *M. lepraemurium* also presents good growth on this medium.

## RESUMEN

En este trabajo se describe el cultivo *in vitro* de *M. leprae* sobre un medio (designado LA-3) basado en el ácido hialurónico, extracto de levadura, albúmina bovina, glicerina y buffer de fosfato. El medio es también preparado con agar o agarosa (designado LA-3P) para servir como placas de cultivo.

El crecimiento inicial en LA-3 en tubos de ensayo requirió alrededor de seis semanas pero luego el tiempo fue reducido a dos semanas utilizando cantidades mayores del medio con aeración mediante la agitación efectuada dos veces al dia.

El medio LA-3P permite el crecimiento de numerosas colonias amarillo-anaranjadas en dos o tres semanas.

Las fases de los aspectos emerjentes del ciclo de vida del *M. leprae* bajo cultivo se describen en forma preliminar.

El alegato de identidad del *M. leprae* en los cultivos se basa esencialmente en las siguientes determinaciones:

1. Criterio patológico y experimental relativo a los requerimientos nutricionales del *M. leprae.*
2. Varios cultivos con similares características han sido aislados de pacientes con LL en diversas oportunidades, a intervalos prolongados de tiempo y en distintas areas geográficas.
3. Incapacidad de subcultivar cultivos aislados en los medios usuales empleados en el cultivo de micobacterias a temperatura ambiente o a 37°C.
4. Cultivos primarios en medio líquido exitosamente transferidos a secundario medio líquido y a secundario medio en placas de agar.
5. Todos los aislados bacilares y bacilos de los medios líquidos de cultivo primario y secundario se tiñen con suero LL, FITC acoplado, y anticuerpo específico de *M. leprae;* mientras que una gran variedad de otras micobacterias no reacciona.
6. *M. lepraemurium* también presenta buen crecimiento en este medio.



## RÉSUMÉ

On rapporte les résultats de la culture *in vitro* de *Mycobacterium leprae* sur un milieu désigné LA-3, composé d'acide hyaluronique additionné de divers ingrédients, à savoir de l'extrait de levure, de l'albumine bovine, et de la glycérine, avec un tampon phosphate. On incorpore également au milieu de l'agar ou de l'agarose (désigné sous les sigles LA-3P) pour servir de support de culture.

La croissance initiale en milieu LA-3 en tubes à essai a pris environ six semaines. Ultérieurement, on est cependant parvenu à accélérer la croissance et à réduire cette période à environ deux semaines, en utilisant de plus grandes quantités de milieu et en l'aérant en le secouant deux fois par jour.

La croissance sur milieu LA-3P a livré de nombreuses petites colonies de couleur jaune-orangée, en deux à trois semaines.

Les divers aspects qui apparaissent ainsi concernant le cycle vital de *M. leprae* en culture font l'objet d'un rapport préliminaire.

Les arguments qui permettent de soutenir que les bacilles cultivés sont identiques à *M. leprae* reposent essentiellement sur les six déterminations suivantes:

1. La détermination des exigences nutritives essentielles de *M. leprae* sur des bases de pathologie expérimentale.
2. Plusieurs cultures ayant les mêmes caractéristiques ont été isolées de malades LL vivant dans de endroits entièrement séparés au point de vue géographique, et à des moments différents.
3. Les échecs enregistrés dans les essais de transfert de la culture sur des milieux habituels généralement employés pour cultiver les mycobactéries, tant à 37°C qu'à la température ambiante.
4. Le fait que des primo cultures en milieu liquide ont pu ensuite être transférées avec succès à un second milieu liquide ainsi qu'à des plaques de milieu sur agar.
5. Les isolats bacillaires, de même que les bacilles recueillis dans les cultures sur milieux liquides signalés ci-dessus en 1 et en 2 du paragraphe 4, se colorent toutes avec des pools de sérum LL couplés au FITC, qui constituent un anticorps spécifique pour *M. leprae* avec lequel une grande variété d'autres mycobactéries ne réagissent point.
6. *M. lepraemurium* présente également une croissance satisfaisante sur ce milieu.



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