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A Scotochromogenic Slow-Growing Mycobacterium Probably the Etiologic Agent of Rat Leprosy[1]

Laszlo Kato and Muhammed Ishaque [2]

Pioneering in the metabolism of noncultivable mycobacteria, Gray ([5]), Hanks ([6]), and Hanks and Gray ([8]) searched systematically for substrates as prospective energy sources for *M. leprae (Ml)* and *M. lepraemurium (Mlm)*. After several years of investigations, they concluded that *Ml* and *Mlm* cannot derive energy from substrates which cultivable mycobacteria use as energy sources for growth, multiplication and expression of virulence. It was advocated, and quite generally accepted, that both of these mycobacteria are metabolically deficient and depend on the host to supply most of the necessary molecules to build up their cellular constituents. This view was supported by the findings by many investigators that *Ml* and *Mlm* are deficient in enzymes which play an important if not key role in energy metabolism and respiration. Most of the studies were made with *Mlm* because these cells are readily available in large quantities from infected rats. It was reported that a key enzyme of the tricarboxylic acid cycle, α-ketoglutaric dehydrogenase, is absent in *Mlm*, that they also have low NADH oxidase activity and that cytochrome c is absent in these cells ([18, 19]).

In contrast to these findings, we advocated the antipode of this often accepted concept. We have shown that at least *Mlm* is metabolically competent, having a complete functional tricarboxylic acid cycle and a well-functioning uninterrupted electron transport chain; cytochrome c included ([10, 16]). With improved methods, we have shown the presence of α-ketoglutarate dehydrogenase, NADH oxidase and cytochrome c in *Mlm*. Our investigations clearly showed that host grown *Mlm* can oxidize at least four groups of substrates. One is complex, heat stable: yeast extract. The other is succininc acid, and a third group of molecules possessing SH groups as a common denominator ([13]). Oleic acid, lanolin (oleum eucerini) and Tween 80 were similarly oxidized by these cells. Some of these substrates are sources of energy since substrate oxidation was coupled with oxidative phosphorylation ([1]). We concluded that *Mlm* has all the prerequisites for independent growth outside the host and we ventured to predict that these "noncultivable mycobacteria" might be later recognized *in vitro* as fast-growing microorganisms ([16]).

We are now able to report that, in the presence of yeast extract, succinate (Na) or cysteine, respectively as energy sources and glycerol as the source of carbon, relatively fast-growing scotochromogenic acid-fast microorganisms resembling *M. scrofulaceum* are regularly cultured from murine lepromas. When injected into albino rats, the cultures retain infectivity, though of a lowered pathogenicity, but still provoking a limited disease typical of murine leprosy.

MATERIALS AND METHODS

Mycobacterium lepraemurium. The Hawaiian strain of *Mlm* has been passaged at three to six month intervals for 25 years in Sprague-Dawley female rats. A three month old rat leproma was removed aseptically and cut into small pieces with scissors. The minced tissue was washed with distilled water, saline solution and a pH 6.8, M/15 phosphate buffer solution to remove excess blood. The tissue was homogenized in a Vortex homogenizer at 12,000 rpm, three times for twelve seconds. The homogenate was filtered through a sterile nylon stocking. The filtrate was centrifuged at 2,500 rpm for five minutes. The supernatant was centrifuged for ten minutes at 6,000 rpm. The latter supernatant was then discarded and the sediment was resuspended in the PO_4 buffer solution and the suspension was adjusted with the same buffer to approximately 300 Klett units.

Chemicals. Yeast extract "Difco" was used. All chemicals, except the common salts, were purchased from Sigma Chemical

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[2] L. Kato, M.D., Professor and Head; M. Ishaque, Ph.D., Associate Professor, Hansen Chair of Research, Bacteriology Research Center, Institut Armand-Frappier, University of Quebec, Ville de Laval, P. Que., Canada.

Company, N.J. Lanolin (oleum eucerini) was kindly donated by Smith and Nephew Co. of Canada. Dextran was obtained through the courtesy of Abbott Laboratories of Canada.

Phosphate buffer solution. High potassium, low sodium buffer was used to simulate the intracellular environment. The 0.066 M PO_4 buffer contained: KH_2PO_4, 8.2 gm and Na_2HPO_4, 0.5 gm in one liter of distilled water. This solution at a pH of 5.5 was selected because previous experiments showed optimal endogenous respiration, substrate oxidation and growth at this acidic pH value.

Preparation of the culture medium. The following substrates were added respectively to the culture media as expected sources of energy. A constant concentration of 2 gm per liter was used: yeast extract powder "Difco"; sodium succinate; L-cysteine; penicillamine; Tween 80; oleic acid; lanolin (oleum eucerini).

The oleic acid solution contained oleic acid (Merck), 48 ml and NaOH N/20, 40 ml, homogenized in a mortar, adjusted to pH 5.6. Glycerol, 30 ml per liter was the only source of carbon in all media. L-asparagin, 2 gm per liter was the nitrogen source in some of the media.

Solid media contained 3% purified agar-agar (Difco). After sterilization, media were solidified in an inclined position at room temperature. Semisolid media contained 1.5% agar-agar.

The media were distributed into test tubes or flasks as desired. Nine milliliter amounts were distributed into 50 ml screw cap tubes or 100 ml into 400 ml flat culture-flasks.

All media were sterilized in an autoclave for 30 minutes and those which contained hyaluronic acid for 20 minutes. After sterilization the final pH was 5.5 ± 0.2. The addition of sera or serum albumin had an insignificant effect on the pH.

The sterlized media were cooled at room temperature and then 5% sterile serum albumin fraction V or different animal sera were added under aseptic conditions. Sera were sterilized by filtration.

DUBOS LIQUID MEDIUM was prepared from Dubos Broth Base (Difco) with Tween 80 and bovine serum albumin fraction V added.

DUBOS SOLID MEDIUM was prepared from Dubos Oleic Agar Base (Difco) and contained serum albumin fraction V.

LOWENSTEIN MEDIUM was prepared from Lowenstein Medium Base (Difco).

Media were inoculated with 0.5 ml of the standard bacillary suspension containing *Mlm* isolated from rat lepromas and 200 units/ml penicillin G. sodium were added to the inoculated media.

Growth conditions. All cultures were incubated at 34°C. Tubes were shaken vigorously immediately after inoculation and once daily. The inoculated liquid media were placed in a maximal possible inclined position so as to assure the greatest possible contact of the liquid surface with air.

The hot Ziehl-Neelsen technic was used to study morphology and acid-fastness. Counterstaining was done with methylene blue at pH 10 as by Skinsnes *et al* ([22]).

Effect of polysaccharides and their constituents on in vitro growth. A series of liquid media were prepared containing connective tissue mucopolysaccharides: heparin, hyaluronic acid and chondroitin sulfate, respectively. A highly branched bacterial mucopolysaccharide, dextran, having an average molecular weight of 75;000, was also utilized. Two polysaccharides of vegetable origin, purified agar and galactomannan, were also included as well as ovomucoid from egg white. The following constituents of polysaccharides were tested: D-glucuronic acid, D-galactosamine, N-acetyl-D-glucosamine and D-mannose. Each of these substances were added, respectively, to the yeast extract-glycerol medium in a constant concentration of 100 mg per 100 ml. Each 50 ml screw cap tube contained 10 ml of the media. These were sterilized in an autoclave for 25 minutes. Media were kept at +4°C and were used within four weeks.

Infectivity of the cultures. Female Sprague-Dawley rats weighing 50 to 60 grams were infected with the bacilli from the third subculture. During the second week of the logarithmic growth phase, the culture was vigorously shaken and 0.3 ml of the culture was inoculated subcutaneously into the scapular region in each rat. Animals were kept on a standard Purina Chow diet and water *ad libitum* at a constant temperature of 22°C with constant humidity.

RESULTS

Growth in the presence of oxidizable substrates. Seven substrates were oxidized by

TABLE 1. In vitro *multiplication of acid-fast bacilli in a liquid medium containing different sources of energy, nitrogen and carbon in the presence or absence of sheep serum. Media were inoculated at 0 time with* M. lepraemurium *isolated from rat lepromas. Increasing turbidity was registered as +, ++, +++; heavy growth as ++++ and abundant growth as ‡‡‡‡.*

Source of carbon	: glycerol 30 gm/l	—	Gl	—	Gl	—	Gl	—	Gl
Source of nitrogen	: L-asparagin 2 gm/l	—	—	Asp	Asp	—	—	Asp	Asp
Sheep serum	: 10% v/v	—	—	—	—	Se	Se	Se	Se
Growth in six weeks with oxidizable substances as energy sources									
Yeast extract	: 2 gm/l	±	++	±	±	++	++++ ++++	++++ ++++	++++ ++++
Succinate Na	: 2 gm/l	±	+	+	+	++	++++	++++	++++
L-cysteine	: 2 gm/l	±	±	±	±	+++	++++	++	+++
Penicillamine	: 2 gm/l	±	±	±	±	++	+++	+	+++
Tween 80	: 2 gm/l	±	±	±	±	±	±	±	±
Oleic acid	: 2 gm/l	±	±	±	±	±	±	±	±
Lanolin	: 2 gm/l	±	+	+	+	++	++++	++++	++++
PO_4 buffer control		±	±	±	±	±	±	±	±
Lowenstein-Jensen medium		No growth during 60 days							
Dubos liquid and solid media		No growth during 60 days							

Mlm isolated from rats. These were incorporated respectively in a series of liquid culture media as prospective sources of energy. Some of the media were prepared with added glycerol as carbon source and/or with added L-asparagin as source of nitrogen. Each series of cultivation trials was performed with or without sheep serum added. Results are summarized in Table 1. The turbidity which developed in six weeks after incubation was recorded. These are estimated designations of growth in order to visualize *in vitro* multiplication of the acid-fast bacilli. In each case, Ziehl-Neelsen staining and phase contrast examination confirmed that turbidity was due to bacillary multiplication and not to precipitation. A sign ± designates a turbidity equal to the heat killed control. Increasing turbidity was registered as +, ++, +++, heavy growth as ++++ and extremely heavy growth as ‡‡‡‡. Table 1 shows that no multiplication occurred in the presence of any of the oxidizable substrates with or without glycerol and/or asparagin added. Tween 80, oleic acid and lanolin did not support growth when sheep serum was added to the same variations of culture media. With succinate, L-cysteine, penicillamine and yeast extract, growth occurred only in the presence of sheep serum and mainly when glycerol was added as a source of carbon. Table 1 also shows that succinate, cysteine, penicillamine and yeast extract provided some source of carbon and sheep serum, some source of nitrogen. It also became evident that asparagin as an additional source of nitrogen did not improve growth in the media. No growth occurred during 60 days of incubation when *Mlm* isolated from the rat were inoculated directly into Dubos medium, liquid or solid, or on Lowenstein-Jensen media.

Table 1 summarizes the results after six weeks of incubation; however, measurable growth already occurred within two weeks in all the media which later showed heavy turbidity. These will be presented in separate tables and in Figure 1.

From the results we concluded that:

1. The medium of choice contained in one liter of distilled water: KH_2PO_4, 8.2 gm; Na_2HPO_4, 0.5 gm; yeast extract "Difco," 2 gm; glycerol, 30 gm; and 10% sheep serum added aseptically. (Medium KI-1)

2. The medium of second choice contained the same substances with Na succinate added instead of yeast extract. (Medium KI-2)

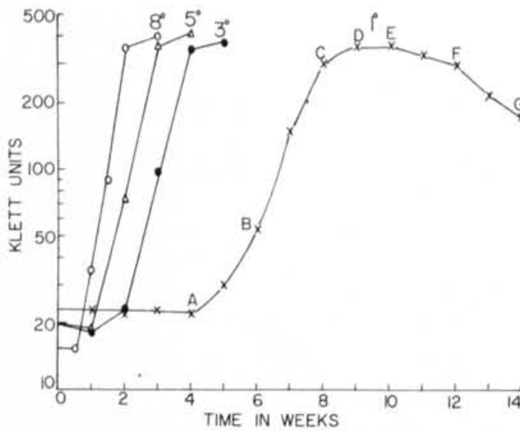

FIG. 1. Kinetics of *in vitro* growth of scotochromogenic culture. The primary culture was inoculated at 0 time with *M. lepraemurium* isolated from rat leprotic nodules. Subcultures were transferred into the homologue media from the primary culture. A progressive shortening of the latency growth period in the subcultures indicates slow adaptation to extracellular substrates.

3. The medium of third choice contained the same substances but had L-cysteine added instead of yeast extract. (Medium KI-3)

In the above three media, strong acid-fastness was registered after three weeks of incubation in the primary culture.

With 3% agar-agar added to the KI-1, 2 and 3 media, visible but weak growth occurred at the end of the sixth week. Bacilli were strongly acid-fast and the surface growth on the agar media showed the presence of a scotochromogenic yellowish pigment.

From the third subculture and at the end of the sixth week, Lowenstein-Jensen and Dubos liquid and solid media were inoculated with bacilli grown on KI-1 liquid and solid media. Visible scotochromogenic growth occurred at 34°C after three weeks of incubation.

Cultures obtained on KI-1 as well as Lowenstein-Jensen and Dubos media are now regularly transferred and maintained on the homologue media.

Growth in the yeast extract, glycerol, sheep serum media (KI-1) was measured in a Klett spectrophotometer and the results for the primary culture are presented in Figure 1. Changes in optical density were plotted against time in days. The initial phase (A) was of a relatively long latency period lasting close to five weeks. This was followed by a seven day long positive growth acceleration phase (B). An abundant growth was observed at the end of the relatively short logarithmic growth phase (C). This was followed by a negative growth acceleration (D) and a maximum stationary phase (E). Accelerated death phase (F) and logarithmic death phase (G) were of a relatively slow rate. The presented logarithmic growth curve is representative of one of the several cultures. Variations of ± 20 days in the initial phase were measured with different inocula.

Bacilli freshly isolated from the rats were also inoculated into Dubos and Lowenstein media. No growth occurred during two months observation period.

During the third week of the logarithmic growth phase, 0.2 ml of the culture was transferred into 10 ml homologue liquid medium. Figure 1 also shows the obtained logarithmic growth curves in the third, fifth and eighth subcultures. A short lasting latency period and initial growth acceleration phase was followed by a relatively sharp logarithmic growth phase. The growth curve followed the known pattern of slow-growing mycobacteria. Cultures are now regularly transferred during the logarithmic growth phase into the homologue media. Subcultures are obtained regularly and with ease. Due to the adaptation to the extracellular existence and to the artificial culture medium, the latency period is shortened and the logarithmic growth rate is somewhat faster in the subcultures.

With 3% agar added to the KI-1 medium, semisolid agar slants were prepared with 10% sheep serum added. Poor but visible growth occurred after five to eight weeks incubation. Growth was scotochromogenic and bacilli were strongly acid-fast.

All cultures were scotochromogenic whether in the bottom of the liquid media or on the surface of the solid media. Most of the bacilli retain acid-fastness during the latency period. During the growth acceleration phase and the first week of the logarithmic phase, however, most of the bacilli were nonacid-fast and relatively short. Full acid-fastness was regained only at the end of the logarithmic growth phase.

Growth on media with polysaccharides. In the second subculture, a heavy growth was visible after three weeks incubation. The cul-

TABLE 2. *Growth of scotochromogenic bacilli in media containing yeast extract (YE), glycerol (Gl) with serum albumin (SA) and different polysaccharides or their constituents added respectively. Horse serum (HS). Media were inoculated from the second subculture of the scotochromogenic strain isolated from rat lepromas. Estimation of growth as in Table 1.*

Medium	Growth in four weeks
YE—Gl	++++
YE—Gl—HS	++++
YE—Gl—Heparin	±
YE—Gl—Hyaluronic acid	±
YE—Gl—SA	+
YE—Gl—SA—Chondroitin—SO_4	++++
YE—Gl—SA—Ovomucoid	++++
YE—Gl—SA—D-glucuronic acid	++++
YE—Gl—SA—D-galactosamine	++++
YE—Gl—SA—D-glucosamine	++++
YE—Gl—SA—N-ac-D-glucosamine	++++
YE—Gl—SA—D-mannose	++++
YE—Gl—SA—Agar	+++
YE—Gl—SA—Dextran	++++
YE—Gl—SA—Galactomannan	++++
Heat-killed *M. lepraemurium* in YE—Gl	±

ture was centrifuged, washed twice in phosphate buffer and resuspended in yeast exact-glycerol medium. The suspension was adjusted to 300 Klett units. From these suspensions, 0.2 ml was inoculated into each of the media containing the mucopolysaccharides, their constituents and appropriate controls. Tubes were incubated in a maximally inclined position at 34°C. They were shaken once daily. Results are shown schematically in Table 2. In four weeks, heavy growth occurred in the yeast extract-glycerol media with or without horse serum added. The growth was strongly inhibited by serum albumin, heparin and hyaluronic acid, respectively. The other tested mucopolysaccharides (chondroitin sulfate, ovomucoid, dextran, agar and galactomannan) and their constituents did not have an inhibitory effect on the growth of the scotochromogenic cultures.

Glycerol, succinate and penicillamine as carbon sources. Since yeast extract was oxidized ([13]) by host grown *Mlm,* this heat stable complex can be considered as a prospective energy source for *in vitro* growth. A constant 0.2% solution of yeast extract (Difco) was prepared in the pH 5.6 phosphate buffer solution. A series of media were prepared containing Na succinate, 1 gm per liter; penicillamine, 1 gm per liter; and glycerol, 30 gm per liter, respectively. Media were autoclaved for 30 minutes in 15 ml screw cap tubes containing 10 ml of each medium. Tubes were inoculated from the third subculture, two weeks old, 0.2 ml into each tube from a suspension adjusted to 300 Klett units.

Table 3 shows a schematic representation of the obtained growth after four weeks incubation at 34°C. Yeast extract alone or supplemented with horse serum did not promote growth. Similarly, succinate and penicillamine did not support the growth in the yeast extract-horse serum media. An abundant growth was obtained in the yeast extract, horse serum and glycerol containing media in four weeks.

The effect of different animal sera on the bacillary growth. Horse, calf, sheep or goat serum were added aseptically and respectively to the yeast extract-glycerol medium. After

TABLE 3. *Growth of scotochromogenic acid-fast bacilli in media containing yeast extract (YE) with or without horse serum (HS) and different substrates as prospective sources of carbon. Glycerol (Gl) was the only substrate which promoted multiplication. Media were inoculated from the third subculture of a scotochromogenic strain isolated from rat lepromas. Estimation of growth as in Table 1.*

Medium	Growth in six weeks
YE	±
YE—HS	±
YE—HS—Na succinate	+
YE—HS—Penicillamine	+
YE—HS—Glycerol	++++
Heat-killed *M. lepraemurium*	±

TABLE 4. *The effect of different animal sera on the bacillary growth in a media containing yeast extract (YE) and glycerol (Gl). Inoculum and estimation of growth as in Table 1.*

Medium	Growth in two weeks	Growth in six weeks
YE—Gl	+	++++
YE—Gl—Horse serum	++	++++
YE—Gl—Calf serum	++	++++
YE—Gl—Sheep serum	++++	++++
YE—Gl—Goat serum	++++	++++
Heat-killed *M. lepraemurium* in YE—Gl	±	+

two weeks of incubation, each of the four animal sera potentiated bacillary growth in the yeast extract-glycerol medium. Highest growth rate was obtained with sheep and goat serum, somewhat less with calf, and even less with horse serum. After six weeks of incubation, however, the same abundant growth was obtained with any of the sera as estimated by the naked eye. Results are shown in Table 4.

Reproducible cultivation. Over a period of 12 months ten rats were sacrificed, one each from ten different colonies of infected rats. Subcutaneous lepromas were removed from each of the ten rats. From the isolated acid-fast bacillary suspension, the same scotochromogenic cultures were obtained in the KI-1 liquid media. Only from three of the ten lepromas was growth obtained on KI-1 solid media.

Growth on Lowenstein-Jensen media. Lowenstein-Jensen media was inoculated from the third subculture, transferring 0.2 ml of the culture during the logarithmic growth phase in KI-1 medium. A scotochromogenic, yellowish, smooth surface growth was visible after 16 days of incubation at 34°C. The cultured cells were strongly acid-fast and easily subcultured on Lowenstein-Jensen media.

Infectivity of the cultures in rats. Bacilli from the third subculture in KI-1 liquid media during the logarithmic growth phase and also from the surface of the Lowenstein-Jensen medium subcultures were harvested. Cells were washed once, resuspended in the phosphate buffered solution and adjusted to 200 Klett units. From this suspension, 0.5 ml was injected subcutaneously into Sprague-Dawley rats weighing 60 to 70 grams. Ten rats were injected with each of the cultures respectively. In four months, huge palpable subcutaneous tumors developed in control rats at the site of injection with bacilli isolated from rat lepromas. At autopsy, the typical visible "pathology" of rat leprosy was observed. In rats which were injected subcu-

taneously with bacilli from cultures on KI-1 or Lowenstein media, the observed "pathology" was different in shape and size. Instead of the huge ovoid shaped lepromas, the flesh colored lepromas were flat, nonnecrotic and had a diameter of two to six centimeters. The leprous tissue was less than one to two millimeters thick. Impression smears showed connective tissue histiocytes parasitized with acid-fast rods. Very few lymphocytes and polymorphonuclear leucocytes were seen. No visible lesions were found in the organs. The spleen was somewhat swollen. Six of ten rats injected with bacilli from the KI-1 medium and eight of ten rats injected with the bacilli from the Lowenstein medium subcultures developed similar "pathology" of murine leprosy. Bacilli isolated from the subcutaneous lepromas were inoculated into Lowenstein-Jensen media. No growth occurred in six weeks.

DISCUSSION

Hanks and Gray ([8]) and recently Pattyn ([21]) strongly emphasized the need for metabolic studies leading to the cultivation of *Ml* and *Mlm*. Hanks ([7]) noted that the hydrogen transfer capacity and infectivity of host-grown murine leprosy bacilli was well-preserved in the presence of yeast extract. A systematic search for prospective substrates as energy source for multiplication of *Mlm* led to the finding that heat stable yeast extract is enzymatically oxidized by host-grown *Mlm* ([13]). The oxidation was coupled with oxidative phosphorylation ([1]). These findings prompted large scale cultivation trials using powdered, heat-resistant yeast extract as a source of energy. Using the Warburg manometric technics and oxygen monitoring device, we found that optimal oxidation occurred at a pH value ranging from pH 5.5 to pH 7.0 in a phosphate buffer solution with a high potassium, low sodium ratio. The simplest possible and most promising medium was with the addition of glycerol as the carbon source since glycerol is an excellent and most widely used ingredient as a source of carbon in most of the conventional media on which cultivable mycobacteria are cultured. Addition of any ingredient as a source of nitrogen into the media seems to be unnecessary since yeast extract is a most abundant source of nitrogen with its high content of amino acids and low molecular weight peptides. The addition of trace elements to the medium seems also to be unnecessary since yeast extract contains most of the metals necessary for the growth of mycobacteria. Our theoretical considerations led to the formulation of a most simple medium containing yeast extract and glycerol. Due to the heat stable ingredients, the medium can be sterilized by autoclaving. Instead of yeast extract, other oxidizable substrates such as Na-succinate or L-cysteine permitted similarly excellent growth of the bacilli in the media. A logarithmic growth rate was obtained by adding, in aseptic conditions, horse, bovine or goat, but preferentially sheep serum to the medium.

It is safe to state that, in this simple medium, yeast extract provides the source of energy and nitrogen as well as metals and trace elements. Glycerol is the source of carbon. Yeast extract might also contribute growth factors.

Gray ([5]), Hanks ([7]), and Hanks and Gray ([8]) reported that, in addition to yeast extract, bovine serum albumin preserved the hydrogen transfer capacity and infectivity of *Mlm*. Serum albumin is also a regular ingredient of culture media for mycobacteria. The role of serum albumin in the media is not clear. It is believed to protect the cells from toxic ingredients or toxic metabolites ([2]). Based on Hanks' observations, Skinsnes and coworkers ([22]) also supplemented their LA-3 medium with serum albumin on which they successfully cultured mycobacteria from human leprotic nodules. In our experiments, bovine serum albumin definitely inhibited growth of *Mlm* in the primary culture during 60 days of the acid-fast microorganisms grown on yeast extract-glycerol media. The *in vitro* growth of cultivable species of mycobacteria is enhanced, or at least not altered, by bovine serum albumin ([9]). It is known that all the scotochromogenic mycobacteria multiply abundantly in the presence of bovine serum albumin.

Summarizing ten years of work into the cultivation of *M. lepraemurium*, we reported ([11, 14]) that heparin and a protein-bound mucopolysaccharide from rat tail tendon were the only ingredients used in more than 8,000 variations of culture media which permitted a limited multiplication and the prolonged preservation of the healthy morphology of these noncultivable microorganisms. These

observations led to a detailed search for prospective nutrients among more than 100 mucopolysaccharides for growth, multiplication, and retention of virulence of *Mlm* ([11]). Among the numerous polysaccharides listed, *Mlm* multiplied in an alkaline medium containing galactomannan ([11]). Though the multiplication was logarithmic for a short time, we were unable to subculture *Mlm* in the homologue media. In later experiments, we were also unable to detect an increase of endogenous respiration or oxidative phosphorylation in the presence of any of the mucopolysaccharides tested. Our present results show that, in the yeast extract-glycerol medium, none of the three tested mucopolysaccharides of vegetable origin, dextran, galactomannan or agar, enhanced or inhibited the growth of the bacilli.

In the experiments of Duran-Reynals, hyaluronic acid inhibited the multiplication of an intracellular parasite ([3]). In our presented experiments, hyaluronic acid prepared from umbilical cord as well as umbilical cord extract and another connective tissue mucopolysaccharide, heparin, inhibited growth and multiplication in the primary culture of *Mlm*. Other connective tissue mucopolysaccharides, chondroitin sulfate and ovomucoid, had no inhibitory effect. This is in sharp contrast with our working theory presented earlier. We advocated at that time that, since human and murine leprosy bacilli grow abundantly in the connective tissue histiocytes, they must utilize for growth, multiplication and virulence, substrates which are present in the connective tissue matrix ([14]). We suspected, at that time, the protein-bound carbohydrates, acid mucopolysaccharides, the biological amines present in mast cells as histamine, serotonin and catecholamines as prospective substrates for *in vitro* growth of *Mlm* on media enriched with connective tissue constituents ([14]). Then, Skinsnes and his co-workers discovered that *Ml* isolated from the host is provided by the enzyme β-glucuronidase for the cleavage of hyaluronic acid. Subsequently, they reported ([22]) the successful cultivation of *Ml* on a hyaluronic acid-based medium. The obtained culture was scotochromogenic and a slow-growing species of mycobacteria identified by Pattyn ([20]) as *M. scrofulaceum*. Using a modified LA-3 medium, we isolated a similar scotochromogenic strain from an untreated lepromatous leprosy patient in Dakar ([15]). In cooperation with Mankiewicz ([17]), we were able to confirm the findings of Pattyn that both the Honolulu and the Dakar strains are slow-growing scotochromogenic mycobacteria having all the characteristics of *M. scrofulaceum*. We are, however, unable to share the conclusion drawn by Pattyn ([12, 20]). In view of our present findings, we are rather inclined to debate that *Ml* and *Mlm* once adapted for multiplication outside the host on artificial media, might belong to the scotochromogenic species probably resembling *M. scrofulaceum*. In this respect, it is interesting to note that both cultures, isolated by the Skinsnes group and the Montreal team from human leprotic nodules, are scotochromogenic and so is *M. marianum*. We are now able to culture regularly from rat lepromas, strains of mycobacteria which are scotochromogenic, thus forming a yellow pigment in the presence or absence of light.

There is, however, a basic difference between the scotochromogens isolated from rat lepromas. The human strains were isolated as primary cultures in media containing hyaluronic acid and serum albumin ([15, 22]), while both bovine serum albumin and hyaluronic acid are extremely toxic for the rat bacilli in the primary culture. Already, our earlier studies indicated the toxicity of serum albumin for *Mlm* freshly isolated from the host ([4]). Animal sera or serum albumin were the *sine qua non* and the absolute necessity for the growth of primary cultivation of the human strains ([15, 22]). The rat strain is easily cultured from the host on artificial media in the absence of animal sera but supplemented with yeast extract. Once adapted to life outside the host, both the human and the rat strains grow abundantly on conventional culture media such as the Lowenstein-Jensen medium used for the cultivation of other mycobacteria. Both the human and the rat strains are scotochromogenic and biologically closely related and identifiable as belonging to the *M. scrofulaceum* subspecies ([17, 20]).

Once adapted to growth outside the host, the *Mlm* cultures also grow in the presence of hyaluronic acid, heparin or serum albumin, substances which were toxic in the primary culture.

When the cultures isolated from the rat lepromas on KI-1, 2, or 3 media and also

TABLE 5. *Sensitivity of four scotochromogenic cultures to different antituberculosis drugs. The scotochromogenic cultures were isolated from rat leproma (RAT), from human lepromatous leprosy nodules in our laboratories (DAKAR), and isolated by Skinsnes* et al *(HI-75) and an authentic culture of* M. scrofulaceum. *(R = Resistant; S = Sensitive)*

PAS	1 μg/ml	5 μg/ml	10 μg/ml	Mycobacteria
	R	90% R	60% R	RAT
	R	R	R	DAKAR
	R	R	90% R	HI-75
	R	R	R	SCROFULACEUM
Streptomycin	1 μg/ml	5 μg/ml	10 μg/ml	
	R	90% R	75% R	RAT
	R	95% R	95% R	DAKAR
	R	R	95% R	HI-75
	R	R	R	SCROFULACEUM
INH	.2 μg/ml	1 μg/ml	5 μg/ml	
	R	R	90% R	RAT
	R	R	R	DAKAR
	R	R	R	HI-75
	R	R	R	SCROFULACEUM
Ethanbutol	4 μg/ml	8 μg/ml	16 μg/ml	
	S	S	S	RAT
	R	R	R	DAKAR
	R	R	R	HI-75
	R	R	R	SCROFULACEUM
Rifampicin	.2 μg/ml	.5 μg/ml	1 μg/ml	
	S	S	S	RAT
	S	S	S	DAKAR
	S	S	S	HI-75
	S	S	S	SCROFULACEUM

when adapted to the Lowenstein-Jensen medium, were reinjected into rats, a limited disease was reproduced in the animals resembling the "pathology" of rat leprosy. The final identification and classification of the obtained cultures remain to be investigated and confirmed. It can be stated, however, that the described mycobacteria:

1. Can be regularly cultured from rat lepromas.

2. They grow at a logarithmic rate on a simple medium which provides one of the specific sources of energy, nitrogen, carbon and probably growth promoting factors.

3. The bacilli are strongly acid-fast when fully grown and can be easily and *ad infinitum* subcultured in the homologue media.

4. When adapted they can be easily subcultured on conventional culture media for mycobacteria, i.e., Lowenstein-Jensen medium.

5. The cultures grown on KI-1 medium, once adapted to the Lowenstein-Jensen medium, reproduce the specific disease known as murine leprosy. The subcultures retain infectivity with considerably reduced pathogenicity.

Table 5 shows the sensitivity and resistance of the obtained cultures to antibacterial agents ([17]). The scotochromogenic cultures

isolated from rat lepromas, the cultures obtained from human leprous tissue (Dakar and HI-75) as well as an authentic strain of *M. scrofulaceum* showed resistance to streptomycin. The same antibiotic is inactive *in vivo* against human and murine leprosy. The cultures are resistant to INH and PAS, substances which have no therapeutic effect in human and murine leprosy. All the cultures were extremely sensitive *in vitro* to rifampicin, the most effective drug known so far for leprosy. Sensitivity to antibacterial agents is a basic genetic characteristic of microorganisms. The parallelism found between *in vitro* sensitivity of the four scotochromogenic cultures and the effect of the drugs on the disease merits special attention.

SUMMARY

Culture media were prepared in which yeast extract, succinate and L-cysteine, respectively, served as a source of energy. These substrates were oxidized by *M. lepraemurium* as measured with manometric technics. Glycerol was the only source of carbon in the media. Bacilli were isolated from subcutaneous lepromas of infected rats. After five weeks of latency, a heavy growth developed during a logarithmic growth phase lasting about ten days in media containing any of the substrates for energy generation with glycerol added. In phosphate buffer solution, at pH 5.5, optimal growth occurred when incubated at 34°C. Bovine, horse, goat and sheep serum respectively enhanced *in vitro* multiplication. Hyaluronic acid, heparin and serum albumin were toxic and inhibited growth in the primary *M. lepraemurium* cultures. When host-grown murine leprosy bacilli were inoculated into Lowenstein or Dubos media no growth occurred.

The cultures were scotochromogenic and produced a yellow pigment. Young cultures were nonacid-fast. Full acid-fastness developed during the logarithmic growth phase.

The strains were easily subcultured not only in the homologue media but became rapidly adapted to new substrates. They then became adapted to and grew in the presence of hyaluronic acid, heparin and serum albumin as well as on Lowenstein and Dubos media. With subsequent subculturing, the latency period became as short as two days followed by three to four days of logarithmic growth. The primary cultures and their subcultures on the homologue media retained specific infectivity for rats but lost their pathogenicity and virulence considerably.

RESUMEN

Se prepararon medios de cultivo que contenían, respectivamente, extracto de levaduras, succinato o cisteína, como única fuente de energía. Empleando técnicas manométricas se encontró que el *M. lepraemurium* fue capaz de utilizar todos estos substratos. La única fuente de carbono en el medio fue el glicerol. Los bacilos se aislaron de los lepromas subcutáneos de ratas infectadas. Después de 5 semanas de latencia se desarrolló un crecimiento abundante durante una fase de crecimiento logarítmico que duró aproximadamente 10 días. Esto sucedió en los medios conteniendo cualquiera de los substratos energéticos y glicerol. En una solución reguladora de fosfatos, pH 5.5, el crecimiento óptimo ocurrió cuando la incubación se hizo a 34°C. Los sueros de bovino, de caballo, de cabra, o de carnero, incrementaron la multiplicación del bacilo *in vitro*. El ácido hialurónico, la heparina y la seroalbúmina resultaron tóxicos e inhibieron el crecimiento de los cultivos primarios del *M. lepraemurium*. No se obtuvo crecimiento cuando los bacilos de la lepra murina crecidos en su huésped se inocularon en los medios de Lowenstein o de Dubos. Los cultivos fueron escotocromógenos y produjeron un pigmento amarillo. Los cultivos jóvenes no fueron resistentes al alcohol ácido pero esta resistencia al alcohol acidulado se desarrolló plenamente durante la fase de crecimiento logarítmico.

Las cepas se pudieron subcultivar con facilidad en el medio homólogo y también se adaptaron rapidamente a crecer en presencia de otros substratos (ácido hialurónico, heparina y seroalbúmina), y aún en otros medios (Lowenstein y Dubos). En los subcultivos posteriores el periodo de latencia se acortó mucho (llegando a ser hasta de 2 días) y fue seguido por 3 ó 4 dias de crecimiento logarítmico. Los cultivos primarios y sus subcultivos en el medio homólogo retuvieron su infectividad específica en las ratas pero perdieron considerablemente su patogenicidad y virulencia.

RÉSUMÉ

Des milieux de culture ont été préparés dans lesquels l'extrait de levure, le succinate et la L-cystéine ont servi respectivement comme source d'énergie. Ces substrats furent oxydés par *M. lepraemurium* tels que mesurés par des techniques manométriques. Le glycérol était la seule source de carbone dans les milieux. Les bacilles étaient isolés à partir de lépromes sous-cutanés des rats infectés. Après cinq semaines de latence, une bonne poussée s'est développée au cours de la

phase logarithmique de croissance qui a duré environ dix jours. Ceci avait lieu dans les milieux contenant n'importe lequel des substrats source d'énergie accompagné de glycérol. Dans le tampon phosphate, pH 5.5, la croissance optimale avait lieu lorsqu'incubé à 34°C. Les sérums de boeuf, de cheval, de chèvre et de mouton de façon respective stimulaient la multiplication *in vitro*. L'acide hyaluronique, l'héparine, l'albumine sérique étaient toxiques et inhibaient la croissance dans les cultures primaires de *M. lepraemurium*. Lorsque les bacilles de la lèpre murine cultivés dans l'hôte étaient inoculés sur les milieux Lowenstein ou Dubos, aucune poussée n'avait lieu.

Les cultures étaient scotochromogènes et produisaient un pigment jaune. Les cultures jeunes étaient non acido-résistantes. L'acido-résistance était maximale au cours de la phase logarithmique.

Les souches étaient facilement sous-cultivables non seulement sur milieu homologue, mais s'adaptaient rapidement à de nouveaux substrats. Les souches se sont adaptées et ont poussé en présence d'acide hyaluronique, d'héparine et d'albumine sérique et aussi sur les milieux Lowenstein et Dubos. Avec les sous-cultures, la période de latence diminuait jusqu'à deux jours, suivie par trois à quatre jours de croissance logarithmique. Les cultures primaires et leurs sous-cultures sur milieu homologue avaient conservé une infectiosité spécifique pour les rats, mais avaient perdu considérablement de leur pathogénicité et de leur virulence.

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