Attempts to Cultivate *Mycobacterium lepraemurium* in Cell-Free Media

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To students of physiopathology rat leprosy is a chronic inflammatory process specific for connective tissue. Wherever lesions occur—in the viscera, muscles, sensory organs, genitalia, or the skin—whether as incipient or fully developed granulomata at the site of experimental infection or in metastatic lesions, the disease is limited to the connective tissue, particularly that close to veins and capillaries([2,16]). Two characteristic types of cells participate in the granuloma formation: connective tissue histiocytes, which harbor the parasite, and connective tissue mast cells([12,13]), which synthesize most of the "building blocks" of the connective tissue and such mediators of capillary permeability as histamine and serotonin. Mast cells initiate the acute inflammatory response, and produce heparin and a series of enzymes([1,14,15]). Thus, experimental murine leprosy, while not a connective tissue disease, is one involving that tissue exclusively. The relation of lesions to the biosphere in which they occur has been investigated in our laboratories. Activation of capillary endothelium([12]), capillary permeability([3,9]), phagocytic function([7]), reticuloendothelial response([6,8,9]), and mast cell response([11,13]), have been systematically explored during evolution of the disease from the moment of infection until death of the animals. In the course of these studies a working theory emerged, viz., that the connective tissue environment is a reservoir where substances are hidden which murine leprosy bacilli can utilize for growth, multiplication, and virulence.

Taking advantage of available advanced data in the field of connective tissue chemistry, the author initiated a search for prospective nutritives *in vitro* among components and degradation products of connective tissue cells, fibers and ground substances, including the inflammatory exudate as well as structurally or physically related substances, the latter being explored more intensely, as the naturally occurring elements of the connective tissue.

In the first ten years, more than 8,000 *in vitro* variants investigated remained sterile. Negative results were published elsewhere([10]). During this period of disappointment, only two substances were found to promote growth, and that limited, viz., heparin and rat-tail tendon extract. The latter was identified as a mucopolysaccharide. With both macromolecules, a two- to three-fold multiplication occurred in BSS/serum albumin and, although no further multiplication of *M. lepraemurium* was counted, the morphology and staining properties of the bacilli were well preserved for several months. The observation

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that the two macromolecules permitted a limited, insignificant but definite multiplication, was meager encouragement, but a path to follow. Connective tissue is extremely rich in polysaccharides and here were found two connective tissue mucopolysaccharides that promoted at least a single generation. In our investigations of connective tissue response to experimental rat leprosy, we found that the incipient lesion and the growing granuloma are surprisingly rich in mast cells, some histologic fields resembling mast cell tumor([13]). Again, it must be pointed out that in the rat mast cells synthesize heparin and mucopolysaccharides for the connective tissue. These facts considered, further investigations were prompted to explore in detail the vast field of connective tissue mucopolysaccharides, and mucopolysaccharides of plant and bacterial origin as nutritional or physical environmental factors for *in vitro* multiplication of *M. lepraemurium*.

A working theory emerged from the concept that the disease occurs in the connective tissue. This hypothesis was reconsidered; the disease occurs in the biosphere richest in mucopolysaccharides. For the past five years, the effect of a huge number of mucopolysaccharides on multiplication of *M. lepraemurium* was explored. Results are reported in this communication.

MATERIALS, METHODS AND RESULTS

Mycobacterium lepraemurium. The Hawaiian strain of rat leprosy was transmitted regularly, at four month intervals, into the scapular region of young Wiersing rats. The four month old granuloma was harvested aseptically and homogenized three times for 20 seconds in a Virtis homogenizer with distilled water. The bacilli were washed once, twice, three times, or four times, by centrifugation, care being taken to separate bacilli from tissue debris and particles other than the bacilli. The paste was homogenized, diluted in saline, and standardized, using the microspot technic of Hanks and associates([5]). Penicillin G sodium was added to the suspension to a concentration of 30 units/ml. From this, 1 ml. was added to each of several tubes containing 9 ml. of the medium. Thus, the inoculated medium contained 2 x 10^4 bacilli per microspot, corresponding to an average of 30 x 10^6 cells per ml., and 3 units/ml. of penicillin. Cultures were incubated at 37°C, 34°C, and room temperature. To prevent evaporation of the media, 50 ml. screw-cap tubes were used, sealed with parafilm and kept in high-humidity breeding chambers.

Criteria for multiplication. 1. Appearance of growing colonies, consisting of acid-fast bacteria, which would not grow on Dubos and Kirschner liquid media.

2. Changes in turbidity of the liquid media. In constant films, a considerable increase in number of bacilli when compared with the controls. Such visible multiplication can be established by an experienced investigator if there is at least a 2 to 3 times multiplication of the inoculum.

3. Counting of *M. lepraemurium*. In earlier experiments by the red blood cell method, while later the microspot technics of Hanks and associates([5]) were used, with the following adaptation for our specific needs:

(a) Declumping was omitted, since in the media used *M. lepraemurium* does not form clumps, and the bacillary suspension is easily dispersed by shaking the tubes for 40 seconds.

(b) Dilution with phenol-water-serum was omitted, since the medium contains a mucopolysaccharide, thus facilitating retention of films on slides.

(c) Staining of films was omitted and bacilli were counted with the phase contrast microscope, a prerequisite being that the test suspension was free from nonacid-fast contamination.

The procedure in fully developed form is as follows:

1. The bacillary suspension is homogenized by shaking the test tube vigorously for 40 seconds. When dilution was necessary, the homolog medium was used as diluent.

2. An aliquot of 0.05 ml. from the dispersed sample is transferred to a spot plate.

3. The sample is stirred with a small (0.53 mm. diameter) pinhead (pin mounted in a wooden handle) and 8 spots of the sample are transferred by stamping on the

respective sites of a slide, using the templates.

4. Films are allowed to dry at room temperature, and then the slides are placed in a Coplin jar of formalin vapor for 3 minutes.

5. The formalin is evaporated by placing the slides on a hot plate (60-70°C) for 1 minute.

6. A loopful of formalin is centered on the underside of a cover glass, which is then put over the films, assuring gentle and even spreading of formalin between slide and cover glass.

7. Counting is made immediately by phase contrast microscopy, using the 36-square ocular grid on films that have uniform diameters and perfect rims.

The total number of bacilli is counted in all adjacent square fields (16 small squares/field) of one diameter of the microspot. From this figure, the total number of bacilli per spot is calculated as by Hanks and associates([5]).

In the experiments here reported the total number of bacilli/ml. was not calculated. Instead, liquid media were inoculated in such a way that, when the above named counting technics were used, the average number of bacilli per microspot was as close as possible to 2×10^4.

Occasionally the cultures were reinjected into rats and development of the characteristic granuloma was observed.

TABLE 1. *Effect of* in vitro *solutions on the multiplication (37° C, 26 days) and on the morphology (37°, 90 days) of* M. lepraemurium.

Substances	Mgm. per cent	Multiplication after 26 days	Morphology in 90 days		
			Acid-fast	Preserved	Debris
Hanks solution		−	+	+	−
Hanks + heparin	100	±	+	+	−
Hanks + serum albumin + heparin	50-100	+	+	+	−
Saline	8.5	−	+	+	−
Saline + $CuSo_4$	0.01		+	+	−
Saline + ferric ammonium citrate	5		+	+	−
Saline + $ZnCl_2$	0.01		+	+	−
Saline + asparagin	200		+	+	−
Saline + NZ amine −B (Casein)	200		+	+	−
Saline + glycerol	500		+	−	+
Saline + Na penicillin G.	500μ/ml.		+	−	±
	50μ/ml.		+	+	±
	5μ/ml.		+	+	−
Hanks + asparagin + heparin	200-100	+	+	+	−
Hanks + NZ amine −B (Casein) + heparin	200-100	+	+	+	−
Hanks + asparagin + NZ amine −B (casein) + heparin	200-100-100	+	+	+	−
Hanks + $CuSo_4$ + ferric ammonium citrate + $ZnCl_2$	0.01-5-0.01	−	+	+	−
Dubos-Tween, serum albumin		−	±	−	+
Dubos, no Tween, serum albumin		−	+	+	±
Dubos Tween + heparin	100	−	±	−	+
Dubos, no Tween, serum albumin, heparin	100	−	+	±	+
Dubos salts only + heparin	100	−	+	+	±
Sauton		−	+	±	+
Sauton + heparin	100	−	+	−	±

EXPERIMENTAL STUDY

Effect of mineral elements, carbon and nitrogen sources and constituents of the Dubos medium on the morphology of *M. lepraemurium* and on the growth-promoting effect of heparin. Table 1 shows that, during 90 days of incubation at 37°C, the morphology of *M. lepraemurium* was well preserved in Hanks solution. Bacilli were sound looking, retained acid-fastness, and were not reduced to debris. Dubos-Tween-serum albumin and Sauton medium had a deteriorating effect on *M. lepraemurium*. Much debris appeared in the presence of Tween, glycerol, and 500 units/ml. of penicillin, and also with the salts of Dubos medium. It is noteworthy here, as in later experiments, that in the presence of low ionic strength phosphate buffer, the morphology was better preserved than in high ionic strength phosphate. Cu, Fe and Zn salts permitted readily observed appearance of bacilli during the 90-day observation period. The experiments here reported at least showed which substances should be avoided in an empiric base, and gave some indication for formulating a tentative basic medium, which fulfills the principium of *non nocere*.

The basal medium was composed as follows:

NaCl	8 gm.
KCl	0.4 gm.
$MgSO_4$	0.2 gm.
$CaCl_2$	0.14 gm.
Na_2HPO_4 $(2H_2O)$	0.06 gm.
KH_2PO_4	0.06 gm.
$NaHCO_3$	0.08 gm.
Ferric ammonium citrate	0.05 gm.
$CuSO_4$	0.1 mgm.
$ZnCl_2$	0.1 mgm.
Asparagine	2.0 gm.
N-Z-amine	1.0 gm.
H_2O	*ad* 1,000 ml.

Screening of mucopolysaccharides for promoting growth of *M. lepraemurium*. As the size of the heparin molecule is estimated to be as large as 200 anhydrohexose units, it is improbable that it can be utilized by *M. lepraemurium*. Rather it is suspected that its molecular shape, configuration, or some physical properties, facilitate multiplication by adsorbing toxic substances, protecting the bacterial surface, and permitting utilization of inorganic or organic nutritives. Nor should we exclude the possibility that enzymatic processes can break down from this macromolecule such units as might be employed by *M. lepraemurium*. The building units of heparin, however, as seen from Table 2 (D-glucuronic acid and D-glucosamine) did not substitute the growth promoting properties of heparin. It seemed logical, therefore, to investigate systematically whether other polysaccharides have effects similar to, or even superior to those of heparin. Sugar polymers of different size and structure, like homoglycans, di-, tri-, tetra-, penta- and hexa-heteroglycans, were submitted for screening. In each group, branched and linear, purified, hydrolyzed and partially hydrolyzed polymers were included. In most cases the composition, structure and molecular weight of the materials was known.

The substances tested were hydrated in a constant concentration of 1 mgm./ml. in the basal medium for 24 hours, and then centrifuged for 20 minutes at 15,000 rpm., adjusted to pH 7.0, and sterilized by autoclave for 10 minutes. Inoculation was performed as previously, and eight parallel inoculated media were examined for multiplication of the inoculum at 21 to 26 days, with the phase contrast microscope. Because of the great number of test substances involved, exact countings were not made. Instead, an arbitrary sign, "+," signified definite multiplication, and "±" represented a well-preserved morphology, but doubtful multiplication. Without exception all experiments were "double blind." The substances were obtained by code number. The media were discarded after the short test period because of lack of space and time.

Table 2 enumerates the polysaccharides tested and the results obtained. It is instructive to read, here, that a series of polymers promoted *M. lepraemurium* growth in the basal medium. None of the active polysaccharides resembled heparin in structure, shape or size, nor did they contain amino-sugar units or glucuronic

TABLE 2. *Effect of mucopolysaccharides on the* in vitro *multiplication of* M. lepraemurium *(activity +, ±, —; incubation 37°C, 30 days). Solutions were dissolved in the basal medium (BM).*

Agents	Activity	Approx. composition (per cent)	Est. molecular weight
Jaguar S-1	+	D-galactose 33 D-mannose 67	220,000
Supercol G	+	" " "	"
Supercol F	+	" " "	"
Supercol S-2	+	" " "	"
Ext. Jaguar A-20-A	+	" " "	"
Ext. Supercol G	+	" " "	"
Ext. Supercol GF	+	" " "	"
Ext. Supercol F	+	" " "	"
Ext. Supercol S-2	+	" " "	"
Ext. red kidney beans	—	—	—
Ext. *Crotolaria intermedia*	±	D-galactose 28 D-mannose 64	—
Ext. locust bean	—	D-galactose 17-25 D-mannose 73-83	310,000
Ext. alfalfa	±		—
Ext. *Espina corona*	—	—	—
Ext. *Cassia nodosa*	—	—	—
G.Mills XO-200	—	Bisulfite adducts of galactomannan	—
Ext. G.Mills XO-200	—		—
G.Mills XO-201	—		—
Ext. G.Mills XO-201	—		—
Torula minuta	+	Unidentified polysaccharide	—
Ext. tamarind gum	+	D-galactose 17 D-glucose 53 D-xylose 30	—
Karaya gum	—	D-galacturonic acid 43 D-galactose 14 L-rhamnose (partially acetylated) 15	9,500,000
Ext. myprosa	—	Mannose 40	24,000,000
Myprosa	—	Glucose 20 Potassium gluconate 20 Acetyl 20	24,000,000
Lambda carrageenin	—	D-galacto-pryanose-4-sulfate	100,000 800,000
Pectin, citrus & apple	—	Chiefly partially methoxylated poly-galacturonic acids	150,000 300,000
Dextran	—	D-glucose	20,000-250-000 20,000,000

TABLE 2.—*Continued.*

Agents	Activity	Approx. composition (per cent)	Est. molecular weight
Powdered psyllium Ext. powdered psyllium	—	Chiefly L-arabinose 14	—
Blond powdered psyllium	—	D-xylose 80	
Ext. psyllium seed	—		
Ext. flaxseed	—	D-xylose, L-galactose L-rhamnose, L-arabinose & D-galacturonic acid	—
Ext. quince seed	—	Cellulose and a readily hydroloyzed polysaccharide (arabinose & aldobiuronic acids)	—
Hemicellulose B	—	—	—
Hydrolized guaran w-010-I	—	—	—
Hydrolized guaran w-010-II	—	—	—
Hylrolized guaran w-100-I	—	—	—
Hydrolyzed guaran W-100-II	—	—	—
Hydrolyzed guaran w-100-III	—	—	—
Guaran sulfate %S=0.08 D.S.=1.48	—	—	—
%S=12.8 D.S.=1.03	—	—	—
Supercol GF	+	D-galactose 33 D-mannose 67	220,000
Ext. flamboyant seed	—	—	—
Ext. *Crotolaria spectibilis*	—	—	—
Ext. fenugreek seed	+	D-mannose 71 D-galactose 45	—
Ext. tara seed	+	D-galactose 26 D-mannose 71	—
Paloverde	+	D-galactose 22 D-mannose 73	—
Ext. paloverde	+	D-galactose 22 D-mannose 73	—
Flame tree seed	+	D-galactose 19 D-mannose 79	—
Ext. flame tree seed	+	D-galactose 19 D-mannose 79	—
Huizache	+	D-galactose 28 D-mannose 69	—
Ext. huizache	+	D-galactose 28 D-mannose 69	—
Tamarind gum	+	D-galactose 17 D-glucose 53 D-xylose 30	—
Stractan	—	Arabinose 14 Galactose 86	—

TABLE 2.—*Continued.*

Agents	Activity	Approx. composition (per cent)	Est. molecular weight
Ghatti gum	—	D-glucuronic acid 12 D-galactose 27 D-mannose 8 L-arabinose 41 Calcium salt	12,000
Amylose sulfate S=7.25%	—	Linear chain of D-glucopyranosyl units	—
Algin sulfate S=0.8%	—	Linear chain of alginic acid (anhydro-D-manuronic and L-glucuronic acids)	—
Mannotriose (B-D-1,4)	—	—	—
6-o-d D-galactopyranosyl-B-D-manopyranose		—	—
Kappa carrageenin	—	36 anhydro-D-galacto-pyranose units linked to D-galactopyranose 4-sulfate	100,000 800,000
Synthetic cellulose polymers			
Hydoxyethylcellulose (Cellosize wp 40)	—	Substituted cellulose	
Methylcellulose 15 CPS	—	Repeating units of methylated condensed glucose (cellulose)	
NA carboxymethyl-cellulose 12 MP	—	RO CH_2 COO Na Cellulose structure	
Galactose	—		
Mannose	—		
Fractionation and degradation of Jaguar A-20-A			
Ext. jaguar A-20-A	+		
" " " +0.02% H_2O_2	—		
" " " +0.04% H_2O_2	—		
" " " +0.08% H_2O_2	—		
" " " +0.1% H_2O_2	—		
" " " +0.5% H_2O_2	—		
" " " +1.0% H_2O_2	—		
A-20-A (Ext.) hot acetone	—		
20% acetone fraction of A-20-A	+		
30% acetone fraction of A-20-A	+		
A-20-A (Ext.) cold alcohol	—		
20% ethanol fraction of A-20-A	±		
30% ethanol fraction of A-20-A	+		
40% ethanol fraction of A-20-A	—		

acid. Whereas heparin is a sulfated polysaccharide, these active substances are not. It is, however, characteristic that all the compounds that induced limited multiplication of *M. lepraemuium* were galactomannans (GM), but only those were active which had a molecular weight of about 200,000 (polydisperse polymers). All active polysaccharides were straight-chain mannans with galactose side chains. In all active substances the mannose units are joined by beta (1-4) glycosidic linkages and the galactose branching is accomplished through an alpha (1-6) linkage. The structure can be schematized as:

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  G   G   G
  |   |   |
M-M-M-M-M-M
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There were but two exceptions to this rule. First, the tamarind gum polysaccharide was used, which is composed of galactose, glucose, and xylose units; even this polymer resembles the GM in that it is a straight chain to which galactose side branches are attached, as well as xylose. Secondly, an unidentified *Torula minuta* polysaccharide promoted growth of *M. lepraemurium*.

Partially and completely hydrolized GM possessed no growth-eliciting properties, and galactose and mannose were also inactive. Nor was there promotion of growth with sulfated galactomannan (GM) or with H_2O_2-treated degradation. Wherever a galactomannan enhanced growth activity, it was independent of the origin, the plant or seed from which it was prepared.

Since GM is widely used as industrial gum, a commercial preparation manufactured from guar seed endosperm, of constant physical and chemical properties, was selected for further experiment, viz., Jaguar A-20-A (Stein Hall & Co. Inc., Long Island City, N.Y.). GM is highly viscous at low concentrations, maintains its properties over a wide pH range, and is nonionic. It is easily hydrated and compatible with electrolytes, and resists sterilization in the autoclave for 10 minutes.

Optimal concentration of GM, optimal pH range and optimal composition of the basal medium. Incubation temperature. The basal medium, containing GM (BM-GM),[2] was prepared as follows. All components of the basal medium, sufficient for one liter of solution were weighed separately and thoroughly ground and mixed in a mortar or ball mill. The resulting fine powder was dissolved in warm distilled water and the pH adjusted with NaOH or HCl to 7.2. Aliquots of GM (Jaguar A-20-A) were added to 50 ml. of the basal solution in a Waring blender and homogenized 3 times for 20 seconds with low speed, at 2 minute intervals. Then the remaining 950 ml. of distilled water was poured into the blender. The mixture was again homogenized 3 times for 20 seconds, and the pH adjusted to 7.2 after the first and third homogenization. The suspension was hydrated at 4°C for 10 hours and shaken occasionally. Subsequently the viscous solution was heated in a water bath to 70-80°C and filtered through rapid filter paper. The clear solution, cooled to room temperature, was adjusted to the desired pH values. Nine ml. of the solution was distributed into each of the 50 ml. screw cap tubes, and then autoclaved for 10 minutes and inoculated with standard suspension of *M. lepraemurium* as above.

Tables 3, 4 and 5 show that there is a critical range of GM concentration that is optimal for promoting multiplication. Similarly, a pH value of 6.8 resulted in optimal preservation of healthy morphology, an optimal multiplication[3] and take, in the reinoculated rats after 30 days of cultivation. Both 37°C and 34°C incubation temperatures permitted multiplication. On the basis of the present observations, all further experiments were performed in the basal medium (BM) containing 1.5 percent GM at pH 6.8, and incubated at 34°C.

When 1.5 percent GM was added to the Dubos, Kirschner, or Sauton media adjusted to pH 6.8, and the inoculated media were incubated at 34°C, no multiplication occurred. In all three media much acid-fast debris was found at 30 days, a fact thus

[2]A limited supply of the medium can be obtained without charge from the author.

[3]At that time the alkaline range was not even considered for testing.

TABLE 3. *Estimated multiplication of* M. lepraemurium *in liquid basal medium (BM) containing different concentrations of galactomannan (pts./1000 GM), and morphology of* M. lepraemurium *after 30 days incubation at 37°C. Rats were infected after 30 days incubation.*

pts./1000 GM concentration in BM	Multiplication of *M. lepraemurium* in 30 days	Morphology in 30 days			Take in rats 30 days 37°C
		Acid-fast	Preserved	Debris	
0.5	—	+	+	—	1/6
1.5	+	+	+	—	6/6
3.0	+	+	+	—	4/6
6.0	—	+	+	—	0/6
0.0	—	+	+	—	0/6

TABLE 4. *Effect of pH on the multiplication, morphology and infectiveness of* M. lepraemurium *in liquid basal medium (BM) containing 1.5 pts./1000 galactomannan (GM) at 37°C, 30 days.*

BM-GM 1.5 pts./1000 pH	pH in 30 days	Multiplication of *M. lepraemurium* in 30 days	Morphology in 30 days			Take in rats 30 days 37°C
			Acid-fast	Preserved	Debris	
7.5	7.5	±	+	+	—	0/6
7.0	6.8	+	+	+	—	6/6
6.5	6.2	+	+	+	—	2/6
6.0	6.0	±	+	+	—	2/6
5.0	5.1	—	+	+	—	0/6
4.0	4.15	—	+	+	—	0/6
3.5	3.4	—	+	+	—	0/6

TABLE 5. *Effect of incubation temperature on multiplication and morphology of* M. lepraemurium *in liquid basal medium (BM) containing 1.5 pts./1000 galactomannan (GM).*

BM-GM 1.5 pts./1000 pH 6.8 Incubation temp. °C	Multiplication of *M. lepraemurium* in 30 days	Morphology in 30 days		
		Acid-fast	Preserved	Debris
37°C	+	+	+	—
34°C	+	+	+	—
20±1.0°C	±	+	+	—

confirming again our previous observations that the constituents of these media are toxic for *M. lepraemurium*.

Meanwhile, a discouraging observation was made. *M. lepraemurium* grown in BM-GM was centrifuged and reinoculated in the same medium, which was freshly prepared. In the subculture no multiplication whatsoever was registered. On the contrary, most of the transferred bacilli were reduced within 48 hours to acid-fast debris, and mainly to small coccoid forms.

TABLE 6. *Multiplication and morphology of* M. lepraemurium *when incubated for 30 days at 37°C and pH 6.8 in different media containing 1.5 pts./1000 galactomannan (GM).*

Media + GM 1.5 pts./1000 pH 6.8	Multiplication of *M. lepraemurium* in 30 days	Morphology in 30 days		
		Acid-fast	Preserved	Debris
BM+GM	+	+	+	—
Dubos+GM no Tween	—	+	—	+
Sauton+GM	—	±	—	+
Kirschner+GM	—	+	—	+

It was suspected that GM is not the sole factor responsible for multiplication, and that the original suspension of *M. lepraemurium* contained some host factors necessary for the multiplication observed. To clarify this, BM-GM was inoculated with the unwashed homogenized granuloma and with *M. lepraemurium* suspensions washed 1, 2, 3 and 4 times in 50 ml. saline per 1 gm. of rat leprosy granuloma. After 30 days' incubation at pH 6.8 and 34°C the tissue debris did not permit estimation of multiplication in tubes inoculated with unwashed granuloma suspension. The usual multiplication occurred in media inoculated with bacilli washed only once, but there was no increase in the number of bacilli when the suspension of *M. lepraemurium* washed two, three and four times was used for inoculation.

***Torula* polysaccharide and living *Torula minuta* promote multiplication of *M. lepraemurium*.** As shown in Table 2, a polysaccharide prepared from *Torula minuta* cultures promotes multiplication of *M. lepraemurium*. To investigate this observation further *Torula minuta* was cultivated at 34°C in liquid Sabouraud medium for 4 days. The cultures were centrifuged and the supernatant was precipitated with an equal volume of acetone. Precipitation was repeated twice with acetone from aqueous solution. The powder was washed with acetone and dried at room temperature, then hydrated and dissolved in the BM, a procedure that resulted in a viscous solution, similar to GM. *M. lepraemurium* was inoculated in the BM containing 1.5 pts./1000 of the unidentified *Torula* polysaccharide. Multiplication comparable to that in BM-GM occurred when the mixture was incubated at 34°C and pH 6.8.

Torula minuta was cultivated on the surface of solid Sabouraud medium at 34°C for 3 days. A loopful of the abundant surface-grown, rose colored colonies was homogenized in 50 ml. standard suspension *M. lepraemurium*, and BM-GM media were inoculated with the *Torula-M. lepraemurium*-mixed suspension. When incubated at 34°C *Torula minuta* cells multiplied abundantly in 48 hours. When examined at 14 and 28 days, it was found that *M. lepraemurium* were present in usually high number. The same experiment was repeated several times and it was established that, with simultaneous inoculation, *Torula minuta* influenced favorably the multiplication of the bacilli in the BM-GM, but not in the BM alone. In both cultures, the pH rose to 8 in 2 days and to 8.4 in 4 days.

The independent multiplication of *M. lepraemurium* and dependent growth of *M. lepraemurium* with living *Torula minuta* cells. Tubes of BM-GM adjusted to pH 8.2 with Tris(hydroxymethyl) aminomethane (Sigma 7-9), were inoculated with standard suspension of *M. lepraemurium*. Other tubes of the same media were inoculated with *M. lepraemurium* suspension plus living *Torula minuta* cells. All cultures were incubated at 34°C, and at two week intervals the numbers of bacilli per microspot were counted separately in each of the 8 tubes with *M. lepraemurium* and 8 tubes with *M. lepraemurium* plus *Torula* cells. At 6 and 12 weeks rats were injected subcutaneously with samples from both types of culture. In Figure 1 the average number of bacilli per microspot is plotted

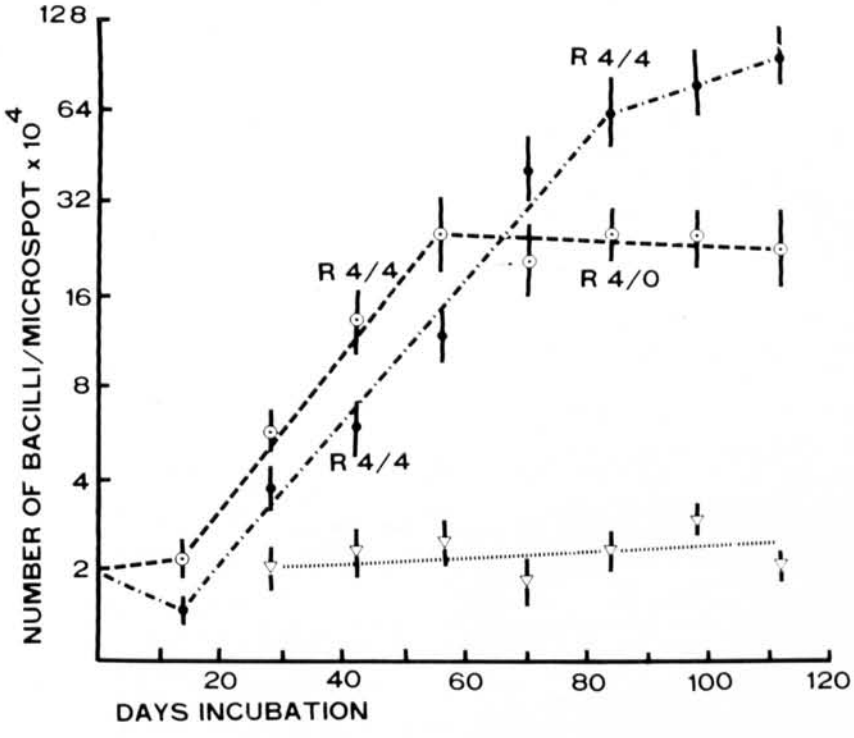

FIG. 1. Cumulative MLM/microspot $\times 10^4$ plotted against days of cultivation in liquid basal medium containing 1.5 pts/1000 galactomannan at 34°C (⊙ – ⊙ – ⊙) and at + 2°C (△ . . . △), as well as in parabiosis with *Torula minuta* (• – . – •). Reinoculation into rats: R. Medium was adjusted to pH 8.2 with Tris(hydroxymethyl) aminomethane.

against time in days. A 9-fold multiplication occurred in the BM-GM medium in 56 days, and a 32-fold multiplication was counted when *M. lepraemurium* was cultivated simultaneously with *Torula minuta* in the same medium. In both cases a latency period was followed by a logarithmic phase and the multiplication ceased in a plateau. The multiplying bacilli formed small clumps in which bacilli were ranged side by side, rather than end-to-end. In the media sedimentation occurs within a few hours.

DISCUSSION

Limited multiplication of *M. lepraemurium* occurred in a cell-free medium in the presence of a mucopolysaccharide of characteristic physical and chemical properties. By no means can we consider this phenomenon as an independent growth. When media were inoculated with suspensions of *M. lepraemurium* that were washed more than once, there was no multiplication, a fact suggesting that host factors, transferred into the medium, were essentially responsible for the multiplication obtained. Unsuccessful attempts were made to procure subcultures when bacilli were transferred into fresh media during the logarithmic phase of growth. This observation is further evidence that multiplication in the first cultures was of a tissue-dependent nature. It is further improbable that *M. lepraemurium* of dense and least permeable capsular surfaces could use the huge linear molecule of GM directly as a nutritive. It can also be excluded that the GM macromolecule is hydrolyzed or otherwise split enzymatically in the medium and that the resulting small polymers or monomers are utilized, since none of the chem-

ically or enzymatically degraded products of GM promoted multiplication. Even the naturally occurring low molecular weight galactomannans of the same structure were ineffective. It was apparently necessary that critical physical (colloidal) conditions be created in the nutrient media in order to obtain multiplication. As seen in our experiments, multiplication occurred only with GM of critical molecular weight (200,000) and with critical viscosity in the medium: 1-3 pts./1000 GM in the solution. Higher or lower viscosity grades of the same galactans, as well as higher or lower concentrations than 1-3 pts./1000 of the same polysaccharide, resulted in no multiplication. These observations suggest that a critical viscosity of the mucopolysaccharide solution as a physical entity facilitates utilization of small molecules by rat leprosy bacilli of dense, and the least permeable membrane([4]).

When *M. lepraemurium* multiplies in the host cell, the lesion occurs in a biosphere where conditions are closely similar to the GM-containing media, ensuring a critical and constant viscosity of the pericapillary connective tissue ground substance.

When *M. lepraemurium* was grown in parabiosis with *Torula minuta*, the multiplication was still limited, but more abundant, again only in the presence of GM in a critical viscosity environment. This latter observation at least permits expression, though with reservation and caution, of two factors involved in dependency: a physical environmental condition seems necessary for the utilization of essentials, and a deficient system is necessary to derive from parabiotic, living cells, or subcellular elements. In order to obtain growth, though limited, these conditions can be simulated by a viscous polysaccharide environment, a galactomannan and parabiotic culture with living *Torula minuta* cells.

SUMMARY

Limited multiplication of *M. lepraemurium* was observed in a cell-free liquid medium containing a critical concentration of a mucopolysaccharide, galactomannan. This limited multiplication was enhanced by growth in parabiotic cultures with *Torula minuta*. The growth was dependent on host factors transferred with the inoculum and/or on systems furnished by the parabiotic cells. Under optimal conditions a 32-fold multiplication was counted when the inoculum was incubated at 34°C in a liquid alkaline medium adjusted to pH 8.2 with Tris(hydroxymethyl) aminomethane.

Acknowledgments. Although the investigations here reported were not supported by any research grants, the Institute has provided unlimited support. The Dean, Dr. A. Frappier, has always remained faithful to his principle that a researcher must be permitted to pursue his natural curiosity. Especial thanks are due to my assistants, and to Dr. T. C. Grubb (Vick Divisions Research), who diligently and tirelessly searched for rare and innumerable sugars.

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Dr. Weiser. Thank you, Dr. Kato. Our next discussant is Dr. Hilson.

Dr. Hilson. I think I should perhaps explain first that the work that I am going to mention briefly was actually carried out at St. George's Hospital Medical School in London, which is my more usual environment.