Limited Multiplication of *Mycobacterium lepraemurium* in Parabiotic Culture, as Influenced by Osmolarity of an Alkaline-Galactomannan Medium

LASZLO KATO AND BELA GOZSY

*Institut de Microbiologie et d’Hygiène de l’Université de Montréal, Montreal, Quebec, Canada*

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**ABSTRACT**

KATO, LASZLO (Institut de Microbiologie et d’Hygiène de l’Université de Montréal, Montreal, Quebec, Canada), AND BELA GOZSY. Limited multiplication of *Mycobacterium lepraemurium* in parabiotic culture, as influenced by osmolarity of an alkaline-galactomannan medium. J. Bacteriol. 91:1859–1862. 1966.—Limited multiplication of *Mycobacterium lepraemurium* has been achieved in an alkaline (pH 8.4) galactomannan-containing medium, when cultivated alone or in parabiosis with a feeder strain, *Torula minuta*. Hyperosmolarity (NaCl, 2.0%) enhanced multiplication in both cases. With 2.0% NaCl in the medium, selective lysis of the feeder cells occurred, without damage to *M. lepraemurium*. Multiplication depends more on the physical properties (viscosity, hyperosmolarity, and alkaline pH) of the medium than on its chemical composition. The described conditions are proposed as a model for cultivation trials with other “apparently noncultivable” microorganisms.

Many microorganisms, multiplying abundantly in the host, have not yet been successfully cultivated in vitro because of the lack of sufficient knowledge concerning growth factors they require or metabolic pathways they use. It is well known that, in the murine leprosy granuloma, *Mycobacterium lepraemurium* multiplies in the connective-tissue histiocytes, while mast-cell herds join them in the formation of a typical lesion (9, 10).

The investigations of the host-parasite relationship in this particular infection have led to a long series of trials concerning the in vitro reconstitution of the biosphere, in which the bacilli multiply when in the natural host (8). If mucopolysaccharides of specific chemical and physical characteristics were added in critical concentration to a synthetic basal medium, a limited multiplication of *M. lepraemurium* occurred; this was further enhanced when these same bacilli were grown in the presence of *Torula minuta* (7).

The medium used has unusual characteristics. First, it must contain galactomannan or any other straight-chain β-(1-4)-mannan with single α-(1-3)-galactoside side chains. Second, the medium must be strongly buffered at pH 8.4.

The polysaccharide was not metabolized by the bacilli, but appeared to be necessary for a high viscosity upon which the in vitro growth of the organisms depends. The high alkalinity of the environment required in vitro remains an unexplained growth-promoting condition, since it is known that, in the host cell, *M. lepraemurium* multiplies in an acidic biosphere represented by cytoplasm of the perivascular histiocytes. It was assumed that the alkaline-galactomannan medium (AGM) either prevents the effect of toxic factors upon the bacilli or facilitates transport of nutrients through a presumably poorly permeable cell membrane (5); this seems to reflect upon the physical properties of the medium rather than upon its chemical constituents.

In this contribution, additional changes were made in the physical properties of the medium, with the hope that an increase in the rate of multiplication might result.
MATERIALS AND METHODS

The constituents of the growth medium were as follows: NaCl, 8 g; KCl, 0.4 g; MgSO₄, 0.2 g; CaCl₂, 0.14 g; Na₂HPO₄, 0.06 g; NaHCO₃, 0.08 g; ferric ammonium citrate, 50 mg; CuSO₄, 0.1 mg; ZnCl₂, 0.1 mg; asparagine, 2 g; casein hydrolysate (NZ-amine type), 1 g; galactomannan, 1.5 g; tris(hydroxymethyl)-aminomethane (Sigma 7–8), 1.8 g. All constituents of the medium were weighed and accumulated in a large mortar. Galactomannan was a food-grade “Jaguar A20A” from Stein Hall & Co., Inc., Long Island City, N.Y.

The ingredients were thoroughly ground in the mortar, and the resulting fine powder was added to 50 ml of distilled water in a Waring Blender and homogenized at 2-min intervals for 3 to 20 sec at low speed; 950 ml of distilled water was then poured into the Waring Blender, and the mixture was again homogenized three times, 20 sec each time. The pH was adjusted to 8.4 with HCl or Sigma 7–8 (Sigma Chemical Co., St. Louis, Mo.). The suspension was left to hydrate overnight at 4°C. The resulting viscous medium was warmed in a water bath to 60 to 65°C, and filtered through rapid filter paper. Different amounts of NaCl crystals were dissolved in the clear AGM medium to obtain the desired hyperosmotic media, containing 15, 20, 25, or 30 g per liter of NaCl. The clear solution, cooled to room temperature, was adjusted again to pH 8.4. A 9-ml amount of the medium was distributed into each of the 50-ml screw-cap tubes, then autoclaved for 15 min at 15 psi. The medium was stored at 4°C and could be sterilized only once.

M. lepraemurium (Hawaiian strain) was transmitted regularly at 4-month intervals. The subcutaneous rat leprosy granuloma was harvested aseptically, and standard bacillary suspensions were prepared as previously described (7).

The stock strain of T. minuta was maintained on solid Sabouraud medium at room temperature. The contents of a 2-mm loopful of T. minuta cells, from the pink surface growth, were homogenized in 50 ml M. lepraemurium suspension. The 9-ml samples of AGM were inoculated with a 1-ml suspension of M. lepraemurium, with or without T. minuta, in 0.9% saline containing 30 units per ml of sodium penicillin G. Cultures were incubated at 34°C and shaken once every day. Counts of the bacilli in the cultures were made every 14th day by use of the micropoint techniques of Hanks, Chatterjee, and Lechat (6), as adapted to the present purposes (7). Criteria for multiplication were set in a previous communication (7). Occasionally the cultures were reinjected into rats, and animals were observed for the development of the characteristic subcutaneous rat leprosy granuloma.

RESULTS

As shown in Fig. 1, T. minuta cells multiplied at a limited rate in the AGM with 0.8% NaCl concentration. When the osmotic condition was raised to 1.5% NaCl, multiplication was even less during the first 2 weeks, after which lysis occurred as judged from the decrease in the number of cells as time went on. Further increases of osmotic pressure lead to greater lysis, being nearly complete in 3% NaCl. When examined with a phase microscope, most of the Torula cells from media with high concentrations of NaCl appeared swollen. These morphological changes occurred within 24 to 48 hr after incubation.

When M. lepraemurium was incubated alone into the AGM, a limited multiplication occurred at 0.8 to 1.5% NaCl concentration. By increasing the salt concentration to 2.0%, the rate of multiplication was considerably higher; however, at 2.5 and 3.0% NaCl concentration, insignificant or no multiplication occurred. Results are shown in Fig. 2. The morphology of the bacilli was well preserved in the media containing 2% or less of NaCl.

In the parabiotic culture, when M. lepraemurium was inoculated simultaneously with T. minuta, the feeder cells multiplied at rates comparable to those shown in Fig. 1. However, multiplication of M. lepraemurium was considerably enhanced (Fig. 3); 2.0% NaCl supported maximal growth. Under these conditions, very few T. minuta cells escaped lysis, and the multiplication of M. lepraemurium was logarithmic for 8 weeks, at which time a plateau was reached. At 0.8, 1.5, or 2.0% NaCl concentration, morphology of M. lepraemurium was well preserved. At 2.5% NaCl concentration, there was still a limited multiplication, and with 3.0% NaCl in the AGM no multiplication of M. lepraemurium was registered. Although the bacilli in 3% NaCl appeared to be shorter, no significant lysis occurred.
limited multiplication of *M. lepraemurium* has long been considered a "noncultivable" microorganism; nonetheless, a limited multiplication was reported in tissue cultures (1, 2). This bacillus has therefore been generally considered as a metabolically deficient strain dependent on host cells. Its hydrogen-transfer capacity is extremely low (3). Our knowledge concerning its nutritional and energy requirements is practically nil, and no acceptable data are available about its respiratory or metabolic pathways. Efforts to cultivate this bacillus may then be compared to pioneering on *terra incognita*.

To achieve in vitro cultivation of *M. lepraemurium*, many chemical and physical factors have been studied. Multiplication occurred only when the physical properties of the medium were modified (7). The present experiments bring forth further evidence that specific physical environmental conditions seem necessary for the utilization of essential nutrients by this "obligate intracellular parasite." In the natural host, the rat leprosy lesions occur in a biosphere which is characterized by a critical and constant viscosity of the pericapillary connective-tissue ground substances. These conditions are simulated in the medium by a viscous polysaccharide environment of galactomannan.

The hyperosmotic property of the medium is not extreme, but is far from the natural conditions in the host. High NaCl concentrations enhance multiplication when *M. lepraemurium* is cultivated alone, and are still more advantageous in the parabiotic culture with *Torula* cells. These results show that a high osmotic pressure enhanced the multiplication of *M. lepraemurium*. We can offer no explanation for this, except to suggest that membrane structure or function, or both, may somehow be involved. In the parabiotic culture, there is another element involved. As shown by the results obtained, the feeder cells (*T. minuta*) do not multiply under the hyperosmotic conditions which facilitate maximal multiplication of *M. lepraemurium*; on the contrary, a rapid lysis of the inoculated *Torula* cells occurs. Thus, the synthetic medium is supplemented with the intracellular constituents of the *Torula* cells: their respiratory and metabolic enzyme system, vitamins, nutrients, and growth factors which are stored in the pools of intact *Torula* cells. With lysis of the feeder cells, AGM becomes an artificial intracellular biosphere, a cell-free environment which contains intracellular constituents of the feeder cell, thus permitting the direct utilization of essentials by the metabolically deficient parasite. How long after lysis of *Torula* cells the intracellular entities are maintained *ad integrum* in the AGM is an important question, but the high salt concentration and alkaline pH may exert a protective effect at least on many of them for a limited time.

The beneficial effect of high NaCl concentration

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**Fig. 2.** Multiplication of Mycobacterium lepraemurium in alkaline-galactomannan medium plotted against time (in weeks), as influenced by different concentrations of NaCl.

**Fig. 3.** Multiplication of Mycobacterium lepraemurium (inoculated simultaneously with *Torula minuta* into alkaline-galactomannan medium) plotted against time (in weeks), as influenced by different concentrations of NaCl.

At 4 and 8 weeks, rats were injected subcutaneously with samples from both cultures containing 2.0% NaCl. Characteristic rat leprosy lesions developed within 12 weeks. The same cultures were inoculated into Dubos, Sauton, and Loewenstein media. There was no multiplication during the 60 days of incubation.

**Discussion**

*M. lepraemurium* has long been considered a "noncultivable" microorganism; nonetheless, a limited multiplication was reported in tissue cultures (1, 2). This bacillus has therefore been generally considered as a metabolically deficient strain dependent on host cells. Its hydrogen-transfer capacity is extremely low (3). Our knowledge concerning its nutritional and energy requirements is practically nil, and no acceptable data are available about its respiratory or metabolic pathways. Efforts to cultivate this bacillus may then be compared to pioneering on *terra incognita*.

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The beneficial effect of high NaCl concentration
is not due to changes in the Na-Ka ratio. Unpublished results show that multiplication occurred when the ratio was within the limits of 8:1 to 8:0.1.

The fact that the observed multiplication is optimal at a slightly alkaline pH remains unexplained. In the cytoplasm of histiocytes, *M. lepraemurium* multiplies in an acidic environment, which does not exclude the possibility of a second preference for multiplication in an environment other than in the host. Maximal hydrogen-transfer capacity of *M. lepraemurium* was found by Hanks (4) to take place at slightly alkaline pH with a second preference at a weakly acidic value.

Though the unlimited cultivation of *M. lepraemurium* has not been achieved, information has been gained to the effect that a noncultivable microorganism is not necessarily dependent on chemical host factors, but can be forced to a limited in vitro multiplication by providing an appropriate physical environment. Such physical alterations in the medium can also produce selective lysis of feeder cells, thus facilitating direct utilization of intracellular constituents of the feeder cells by the parasite. The principles set forth here might serve as models for attempting the cultivation of other "noncultivable" microorganisms.

**Literature Cited**


