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Mycobacterium leprae, Probably a Microbe-dependent Microorganism

TO THE EDITOR:

Experimental evidence supports the recently advanced concept that *Mycobacterium leprae* is probably a unique microbe-dependent microbe ([2, 4, 6]). Results led to the formulation of a new culture medium, permitting *in vitro* multiplication of bacilli derived from armadillos infected with *M. leprae* ([3, 4]). The proposed multifactorial culture medium (MFM) was based on the recently acquired knowledge that getting *M. leprae* to grow is a multifactorial problem ([1, 3]). The slow growth of the bacilli in a deep liquid medium, however, was difficult to assess and not satisfactory for practical purposes as a tool for diagnosis, drug sensitivity, metabolic studies, and as a pharmacological model for detecting new antileprosy drugs. Experiments were aimed at achieving visible growth on solid media, accelerating growth rate, increasing yield, and providing further evidence for the validity of the claim that getting *M. leprae* to grow is dependent on a supply of growth factors (mycobactin and exochelin) from secondary mycobacteria. Results just available now bring further evidence that growth of those bacilli from armadillos infected with *M. leprae* is dependent on the above growth factors, but that a still-unidentified third growth factor might contribute to considerable yield of cell mass, and finally that a visible growth can be achieved on the surface of agar slants of the multifactorial media.

An iron-poor Sauton-Tween liquid medium contained asparagine 4 g, citric acid 2 g, K_2HPO_4 0.5 g, $ZnSO_4$ 0.04 g, $MgSO_4$ 0.4 g, glycerol 40 ml, and Tween 80 10 ml in 1 liter of distilled water. It was adjusted to pH 7.0 with NH_4OH. The solution was distributed 200 ml/flask and autoclaved for 30 min. The media were inoculated with *M. phlei* and incubated at 37°C until a thick, heavy growth developed on the surface of the medium. The flasks were sonicated in a Bronsonic sonicator (20–90 kilocycles frequency) for 10 min. The cultures were autoclaved for 30 min, and filtered while hot through Whatman 802 filter paper. The filtrate was adjusted to pH 5.8 with KH_2PO_4. Na thioglycolate 1.5 g, thioctic acid 0.05 g, and β-cyclodextrin 1.5 g were collected in a mortar, mixed, and ground for 5 min. The fine powder was dissolved in the mortar with small amounts from the filtrate and added to the collected filtrate. Ferric ammonium citrate 0.05 g and $MgSO_4$ 0.1 g were also dissolved in the filtrate. The solution was readjusted to pH 5.8 and distilled water added to the volume of 1 liter. Readjustment to pH 5.8 was required; 500 ml of the liquid MFM was autoclaved 30 min. In 500 ml of the medium 15 g agar (Difco) was dissolved and 20 ml was distributed into each 50-ml screw-cap tube. Autoclaved for 30 min, the media were cooled to make agar slants.

Armadillo-grown *M. leprae* cells were collected, prepared, and semi-purified aseptically, using the liquid MFM. The cell suspension was centrifuged at 3000 rpm for 15 min. Two 5-mm diameter loopsful of the

thick sediment was evenly spread on the center of the agar slant in an area of about 5 cm^2. The heavy inoculum was needed to obtain growth in the primary culture. Growth became visible in 2 to 3 weeks of incubation at 34°C. The inoculum increased in thickness during the following 3 months as a light beige, creamy, semiglossy mass. Subsequent subcultures were obtained on the same medium, but a heavy transfer was needed to obtain comparable growth. The cells were strongly acid-fast, arranged in clumps resembling globi in smears of lepromatous leprosy lesions. Pyridine extraction abolished acid-fastness but not gram-positivity of the cells. The cells did not grow on Löwenstein, in Dubos, or 7H10 liquid media. No growth was obtained either on the MFM agar slants if media were prepared without sonication of the *M. phlei* culture in the iron-poor Sauton-Tween media.

It is known that both mycobactins and exochelins are secreted by mycobacteria into Sauton-Tween 80 liquid media ([5]). Under microaerophilic conditions, these two factors of iron acquisition and transport were sufficient to support multiplication of bacilli derived from armadillos infected with *M. leprae* ([3, 4]). However, prolonged sonication of the growth factor donor *M. phlei* culture was probably necessary to release either increased amounts of mycobactin and/or exochelin, or eventually other growth factors to support growth under strictly aerobic conditions on the solid multifactorial medium, as described in the present communication.

Results are not sufficient to claim the successful cultivation of *M. leprae,* neither are the cultures sufficiently identified as authentic *M. leprae.* It is therefore proposed to designate the obtained strains as *M. leprae*(?). Results however do strengthen the concept that *M. leprae* might be a microbe-dependent microbe and that donor cells, if sonicated, supply growth factors probably other than mycobactin and exochelin. There are indications that these bacilli are not microaerophilic as often suggested for *M. leprae.* Further cultivation trials are warranted and proposed on the above-described multifactorial medium agar slants.

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