Cultivation of *Mycobacterium X* from *Mycobacterium leprae*-infected Tissues in Propane-tetradecane-humic Acid Medium

TO THE EDITOR:

Ribulose diphosphate carboxylase (RuDC) was detected in cell-free extracts of *Mycobacterium leprae* [5]. This enzyme catalyzes the incorporation of C from CO_2 and CO_3 into organic substances in autotrophic soil microorganisms. RuDC in *M. leprae*, coupled with the absence of fumarate dehydrogenase and low levels of NADH oxidase, suggested that *M. leprae* might have autotrophic characteristics without being an autotroph per se. However, it was not possible to grow *M. leprae* on inorganic media (personal observation, unpublished data).

It is known that soil is richly populated with cultivable mycobacteria. Noncultivable species of mycobacteria were found by Kazda [6, 9] in the gray layer of sphagnum vegetation. In the formerly leprosy-endemic areas of Norway, Kazda [7, 8] isolated strains of mycobacteria which grow in a liquid medium extracted from the gray layer of sphagnum. These strains multiplied in the foot pads of mice, producing a pathology indistinguishable from that of *M. leprae*. Armadillo-derived *M. leprae* also multiplied three to four times in the sphagnum medium of Kazda, but not in the subcultures (personal observation, unpublished data). Straight-chain hydrocarbons, propane and tetradecane, supported growth of mycobacteria from *M. leprae*-infected tissues [3, 4]. Primary cultures and subcultures of mycobacteria were obtained in the Kazda sphagnum medium enriched with propane and tetradecane and inoculated with *M. leprae* from armadillo spleen (personal observation, unpublished data).

Sugars, free amino acids, and humic acid are the known components of decaying sphagnum vegetation. However, the growth promoting substrates in the Kazda sphagnum medium are not known. Although the gray layer has only traces of humic acid and humic acid is insoluble in the conditions under which the sphagnum medium is prepared, the light-brown color of the sphagnum medium suggested the presence of humic acid. Evidence is now available that humic acid alone does not promote growth of *M. leprae*, but the propane-tetradecane medium is a valuable one for the growth of mycobacteria from *M. leprae*-infected tissues if the medium is enriched with humic acid.

A technic to prepare water soluble humic acid has been developed in this laboratory. Fifty grams of commercial peat moss (Fafarde, Canada) was homogenized by Waring blender in one liter M/15 pH 7 Sørensen buffer, and adjusted to pH 7 four times in 24 hr. The homogenate was autoclaved for 1 hr and filtered while hot on gauze, then again on rapid filter paper. The filtrate was precipitated with an equal volume of acetone. The dark-brown precipitate was separated by centrifugation at 6000 rpm for 20 min. The supernatant was discarded. The paste was placed on filter paper to drain off excess acetone-water. The paste was kept in a closed container at 4°C. Care was taken not to let the paste dry, because it was learned that the dried humic acid powder becomes insoluble in water. The dry weight of a small portion of the paste was determined. Based on the dry weight content, the paste was diluted with distilled water to obtain a 5% humic acid concentration. This was dissolved by boiling, resulting in evaporation of the remaining acetone in the paste. The humic acid solution was sterilized in the autoclave for 40 min.

The propane-tetradecane humic acid medium contained in one liter distilled water: KH_2PO_4 7 g, Na_2HPO_4 0.5 g, $MgSO_4$ 0.1 g, NH_4SO_4 2 g, yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.) 0.1 g, and 5 ml humic acid solution. In each of the 50 ml screw-cap tubes 20 ml aliquots of the medium were distributed. The media were sterilized for 40 min in the autoclave.

Propane gas was dissolved in tetradecane. Ten ml tetradecane (USP grade) in a 50 ml screw-cap tube was autoclaved for 40 min. This was bubbled with 95% purity propane gas for 1 min under 1 lb/in^2 pressure at room temperature through a 1 ml serological pipette under aseptic conditions.

To each tube containing 20 ml sterile medium, 0.1 ml propane-tetradecane was added aseptically.

Each tube was inoculated with 2×10^5 cells of *M. leprae* isolated by partial purification from the spleen of *M. leprae*-infected armadillos. Following inoculation, the cultures were again bubbled for 10 sec under aseptic conditions with propane gas. Cultures were incubated at 32°C and shaken once weekly.

Increasing turbidity and numerical multiplication of cells were taken as the criteria for growth ([4]). Cells accumulated at the surface of the media in the form of a whitish emulsion-like veil. Bacilli were strongly acid fast. The cultures were transferred (1:10) into fresh media at 10-week intervals. The subcultures were growing at a rate comparable to the primary cultures. Cultures did not grow on Löwenstein, in Dubos media, nor in the presence of humic acid without propane tetradecane.

Mycobacterium X was previously isolated in a tetradecane medium from *M. leprae*-infected armadillos. These strains did not grow on Löwenstein or in Dubos media but produced the disease typical of *M. leprae* in the foot pads of mice. When inoculated into propane-tetradecane-humic acid media, the cultures grew at a faster rate compared to growth in the tetradecane media. Humic acid had a definite promoting effect on the growth of acid-fast bacilli from *M. leprae*-infected tissues. Four strains of *Mycobacterium X* and three strains of mycobacteria isolated from *M. leprae*-infected armadillo spleen are now growing regularly in subcultures on propane-tetradecane-humic acid media.

Comments. The biological oxidation of propane and tetradecane into ketones, aldehydes, alcohols, and acids is a well-documented, rich energy source for mycobacteria ([3, 4]). Little is known about the chemical structure of humic acid, a brown polymeric substance which occurs in the organic matter of soil and composts. Decomposed sphagnum, commercially available as peat moss, is extremely rich in humic acid ([1]).

While propane and tetradecane are preferential carbon and energy sources for mycobacteria, no evidence was obtained as to the mechanism involved in the growth-promoting effect of humic acid in the present experiments. The oxidative degradation of humic acid yields p-hydroxybenzaldehyde, syringealdehyde, vanillic acid, p-hydroxybenzoic acid, and 3, 5-dihydroxybenzoic acid ([1, 2]). These oxidative processes might account in part for the growth-promoting effect of humic acid. However, since humic acid alone did not permit multiplication without the energy rich propane and tetradecane in the media, humic acid or its oxidative degradation products might serve as growth factors, rather than carbon and energy sources, for the mycobacteria cultivable from *M. leprae*-infected tissues.

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