COMMITTEE 2: ADVANCES IN THE MICROBIOLOGY OF *M. LEPRAE*

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The report summarizes the progress made in the field of general microbiology of *M. leprae* during the past five year period since the Ninth International Leprosy Congress in London in 1968. *M. lepraemurium* has been included in the review as an interim model for *M. leprae*. Progress has been made in four areas: cytology, metabolism, cultivation, and the identification of *M. leprae*.

CYTOLOGY OF *M. LEPRAE*

Morphologic Index (solid ratio). The utility of the Morphologic Index (MI), based on the proportion of solidly staining *M. leprae* cells has been exploited particularly to follow the initial antimycobacterial drug action in patients during chemotherapy. The present MI does not distinguish the infectious from the noninfectious patient. No unified opinion has been formulated regard-

ing the question whether the ratios of solid staining bacilli are associated with viability in the bacteriologic sense. Further studies appear necessary.

Pyridine-extractable acid-fastness. *M. leprae* in section for smears lose the property of acid-fastness, but not gram-positivity when extracted with pyridine. The acid-fastness of *M. lepraemurium*, *M. tuberculosis*, BCG and *M. intracellulare*, is not affected by this procedure. It has been suggested that a differentiation between *M. leprae* and other mycobacteria is possible by using Ziehl-Neelsen staining after extraction with pyridine. Further studies are needed with *M. ulcerans* and *M. marinum* from human lesions, with *in vitro* grown mycobacteria and with senescent populations of cultivable mycobacteria.

Electron microscopy. Studies with the combined use of electron microscopy and chemical and biological technics have been carried out. Miscellaneous information such as the band structure and peptidoglycolipid filaments on the surface of *M. leprae*, chemical components of the cell wall of *M. lepraemurium* and the electron-transparent capsule-like outer zone around the bacilli were obtained.

As a significant discovery, the mycolic acids in the cell wall of *M. lepraemurium* have been demonstrated to differ from corynemycolic or nocardic acids. The characteristic mycolic acid-arabinogalactan-murein in the cell wall of *M. lepraemurium* resembles that in other members of the genus mycobacterium such as *M. tuberculosis bovis* and strain BCG. The discovery of mycolic acid in *M. leprae* isolated from human tissues likewise indicates that this pathogen is a mycobacterium.

In the field of electron microscopy, further chemical and biochemical information would permit the interpretation of the relationship between structure and function or the physiologic state of *M. leprae*.

METABOLISM OF *M. LEPRAE*

The extraordinarily long generation time of 12-14 days in the mouse foot pad has been regarded as one of the characteristics of *M. leprae*. No information is available to explain this slow rate of metabolism. One difficulty in metabolic studies on *M. leprae* is that of obtaining adequate supplies of cells and a second is that of collecting all the bacilli as suspensions without tissue contamination.

Fragmented reports have appeared indicating the presence of various enzymes in *M. leprae*. Among these, *o*-diphenoloxidase has been suggested to be unique to *M. leprae*, being distinct from plant and mammalian enzymes. Concentrates of *M. leprae* prepared from lepromatous material actively oxidized, 3,4-dihydroxyphenylalanine (DOPA) to pigmented products. This specific metabolic activity has been proposed as an identification test for *M. leprae*.

Ribulose diphosphate carboxylase activity was demonstrated in the supernatant from disrupted *M. leprae* collected from lepromatous tissues. It appears to be important if confirmed, because this enzyme occurs otherwise only in autotrophic bacteria and green plants.

Recent evidence has suggested an incorporation of tritiated thymidine into leprosy bacilli in cultures of human lepromatous macrophages. If the observation is confirmed, this is an important advance, because it implies that the organisms were synthesizing DNA. Further studies of this type are needed.

Metabolism of *M. lepraemurium*. Since the last congress, knowledge of the metabolism of *M. lepraemurium* increased dramatically. The major advance is in our knowledge that the overall energetics of the organism operate independently from those of the host. It has been demonstrated that *M. lepraemurium* contains a cytochrome-linked pathway for oxygen utilization. Due to the limited rate of terminal electron transfer, the assimilation and oxidation of exogenous substrates occurs very slowly and does not result in marked stimulation of oxygen uptake.

Experiments using isotope-labeled substrates and cell-free extracts of *M. lepraemurium* have confirmed the following facts:

1. An extraordinarily slow rate of aerobic metabolism based upon a host-independent tricarboxylic acid (TCA) cycle. All enzymes of the TCA cycle have been demonstrated, the pyruvate and α-ketoglutarate dehy-

drogenases being rate limiting. Although the TCA cycle may contain an alternative pathway at the α-ketoglutarate step, a conventional TCA pattern of isotopic distribution arose during substrate oxidation.

2. There appears to be a lack of capacity to oxidize glucose, even though its incorporation into cellular material has been established.
3. Short-chain fatty acids, in contrast to medium-chain fatty acids, cannot be utilized for lipid synthesis.

Studies on energetics have been further advanced by the development of ultrasensitive methods for determining ATP (energy levels) in host-grown microbes. Host ATP has been eliminated and the quantitation of ATP refined to require only 1/60th the number of bacterial cells employed in the most sensitive methods hitherto available. The method has been applied thus far to demonstrate the potential losses of ATP during extraction, purification, prolonged refrigeration, and the growth potential of *M. lepraemurium* in cultivation studies. These methods have been designed to investigate the energetics of *M. leprae*.

CULTIVATION PROBLEM

Cultivation of *M. leprae*. Four cell-free systems have been described for the cultivation of *M. leprae in vitro*. These are: a) a U-tube divided by a fine sintered glass membrane and using a conventional medium for mycobacteria, b) an inorganic medium suitable for autotrophic bacteria, c) semi-soft agar media, and d) media enriched with substance of mycobacterial origin. These experiments have not been successfully repeated by other investigators.

Attempts at cultivation in cell cultures were carried out using cell lines of human origin, cell strains derived from human tissues, cell strains derived from animals, and mouse macrophages. No proliferation of the bacteria was noted.

Recently, evidence has been obtained regarding a limited multiplication of *M. leprae* within macrophages derived from human peripheral blood cells. The applicability of the method is restricted by limited survival of the host cells. The majority of host cells did not survive beyond 60-80 days in most cultures.

Cultivation of *M. lepraemurium*. Extracellular growth of *M. lepraemurium* was obtained in cell-impermeable diffusion chambers implanted in the peritoneal cavities of mice. The generation time observed was 11 days. The division time was shortened to 8 days when macrophages were included in the chambers.

Noteworthy reports on the growth of *M. lepraemurium* in cell-free medium have appeared recently and reproducible results have been obtained by some other investigators.

1. A pale yellow, R-type macroscopic colony was produced on 1% egg yolk solid medium after more than three months of cultivation at 37°C. Subcultures have been continued for 10-16 generations. Low plating efficiencies indicate a need for further studies.
2. From 30- 300-fold multiplication of *M. lepraemurium* (Hawaiian strain) was noticed at 30°C in Kirchner's medium containing calcium pantothenate, α-ketoglutaric acid, cytochrome c, hemin and l-cysteine. Successive cultivation through serial transfer is required. Corroboration is desired in order to determine if specific growth factors are needed.

ADVANCES IN IDENTIFICATION OF *M. LEPRAE*

The WHO Expert Committee on Leprosy 1970 presented a report on the identification of *M. leprae* as follows:

> "In all the cultivation work, it is important to prove the viability of the purported growth and to identify it by the methods now available (the inoculation of mouse foot pad, lepromin test, enzymatic studies of DOPA oxidation, and serological identification of nodular extract antigen)."

The pyridine sensitivity and the specific *o*-phenoloxidase in *M. leprae* have already been described.

It has been observed that leprosy nodular extract (NE) contained at least two antigens which have been differentiated from human serum proteins by the immunodiffu-

sion test. One of these antigens is a heat-stable polysaccharide, and the other is a heat-labile protein which give a single precipitation line with anti-NE serum absorbed with human serum. Inasmuch as this antigen is highly specific for *M. leprae*, serological identification of *M. leprae* by the technic of immunofluorescence should become practicable with antiserum prepared against the antigen.