XIV INTERNATIONAL LEPROSY CONGRESS REPORTS OF THE WORKSHOP COMMITTEES

WORKSHOP 1: MICROBIOLOGY

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The progress made on various aspects was reviewed as follows:

Purification of *M. leprae*. Armadillo-derived *Mycobacterium leprae* continue to be used for various biochemical, structural and antigenic studies. A modified hybrid protocol (containing alkaline treatment and 30% Percoll density gradient-1/79 & 1/77) especially aiming to remove pigmented host material and useful for purification of *M. leprae* armadillo liver has been developed. In addition, an assay based on the estimation of arabinose content to check the purity (mycobacterial content) of bacilli after purification has been described. However, it was felt that the effect of gamma irradiation on various viability markers/tests and integrity of DNA (which may influence the PCR signals) is not fully understood. Further, no objective techniques to monitor the contamination of purified *M. leprae* with soluble host material and other mycobacteria are available.

Cultivation. Attempts to grow *M. leprae* in modified conventional/earlier described media (7H9, Dubos and DH) as well as unconventional (simple media for chemoautotrophs) have been described over the past 5 years. Chemoautotrophic nocardioform (CAN) organisms from *M. leprae* infected tissues have been repeatedly isolated, subcultured and have been shown to have resemblances to *M. leprae* by enzymatic, chemical (mycolates and PGL), antigenic (lepromin), 36-kDa PCR and pathogenicity (mouse mutilation) criteria. Further, coccoid, mycelial, cystic and spore-like forms (nocardioform arthrospores and blastospores) in the growth cycle of the leprosy bacillus have been postulated in recent reports. These investigations need to be pursued further and isolates taxonomically fully characterized. There were presentations which showed that PCR is being proposed to be used as a taxonomic tool. However, keeping in mind the danger of "carry over," the need to try other genomic markers—DNA/DNA, DNA/RNA hybridization (overall and using specific probes), as well as RFLP analysis—was suggested.

Also the necessity of having confirmed "pure" and "viable" organisms in the cultivation attempts was emphasized.

Physiology/Biochemistry. Studies to investigate the physiology of *M. leprae* by biochemical and molecular approaches were reported during this 5-year period. Exochelins and ferritins have been identified in *M. leprae*. Further studies have shown that while purine biosynthesis is undetectable in *M. leprae*, this organism is capable of pyrimidine biosynthesis and scavenging. *M.*

leprae has been shown to have the capacity to utilize/hydrolyze several host lipids. Acetate has not been found to be incorporated in *M. leprae,* possibly because of the absence of phosphotransacylase; fatty acid synthases have been detected although at low activity. In contrast, fatty acid elongates were readily detectable in *M. leprae.* Phospholipids have been observed to be hydrolyzed by *M. leprae,* but this does not obviously damage the host membranes. The organisms have been reported to be capable of utilizing acylglycerols (using a model but not, so far, using natural substrates). Nothing yet is known about whether *M. leprae* can use sphingolipids which are the major neural lipids. Suggestions for the design of drugs against PGL and LAM biosynthesis and immunotherapy to block the entry and prevent persistence in Schwann cells and macrophages have been made.

During this 5-year period, further proteins of *M. leprae* were identified and for many, their functions have been deduced. Their genes have been cloned and sequenced to varying extents. The important ones are: 10 kDa (groES); LSR; 28 kDa (SOD); 28 kDa (IRG); major cell wall proteins (histone derived from host tissue); a major membrane protein (homology with bacterioferritin) and six "less abundant" proteins (two possibly virulence factors showing homology with alkyl hydroperoxide reductase and a thiosulfate sulfurtransferase, a third with a LT/LIC ribosomal protein homolog). Some of these proteins may have a role in the response of organisms to oxidative/other stresses and also in possible virulence.

Several recent studies have focused on the physicochemical factors important in the growth of *M. leprae.* Using bioluminescence, ^{3}H-thymidine uptake and other parameters, several physicochemical factors such as nutrients (gelatin, pyruvate, malate, silicone, fossil fuel derivatives, purines, urea, glycerol, asparagine, etc.) and physical conditions (lower pH 6–6.5, temperatures of 30°C–33°C, lower oxygen levels) have been identified as possibly relevant for the *in vitro* growth of *M. leprae.* These factors and appropriate procedures such as the processing of specimens, addition of large quantities of lipids as cyclodextrin complex (sphingolipids, palmitic acid), etc., were suggested to improve the ATP synthesis/possible growth in future studies. The need to analyze the experiences with other difficult-to-grow mycobacteria to identify critical factors possibly important for *M. leprae* was emphasized.

Sequencing of genes of *M. leprae.* Information about several gene sequences of *M. leprae* has become available, and on the basis of published data about ribosomal RNA genes, the leprosy bacillus has been shown to belong to the slow-growing mycobacterial cluster. In addition, the genome sequencing project on *M. leprae* has been progressing and about 15% of the genome has already been sequenced. The information generated has been reported to be complementary to data emerging about several genes coding for structural proteins of *M. leprae.* If present funding levels are maintained, the entire genome of *M. leprae* could be sequenced in about 3 years.

Gene probes/amplification methods. During this period, several gene probes and gene amplification techniques to detect *M. leprae* gene sequences have been developed, and there are some others under development. Among the important gene amplification techniques are those targeting 18 kDa, 36 kDa, 65 kDa, ribosomal RNA and repetitive DNA sequences. Rapid genetic techniques also are being developed to detect mutations conferring resistance to rifampin in *M. leprae.* Data about the application of different PCR assays in the clinical specimens as well as standard specimens (including IMMLEP trials) show that the techniques are practicable. These assays appear to be generally sensitive for multibacillary cases; whereas sensitivity is about 50% for smear-negative paucibacillary cases. The optimum methodology for specimen collection, storage, extraction and criteria for positivity (whether by EB/autoradiography) needs to be refined further in greater detail for their application in clinical diagnosis and epidemiology.

***In vitro* estimation of viability.** In the absence of an acceptable definition of "cultivable unit" of *M. leprae,* all other criteria continue to be indirect. Data discussed showed that *in vitro* methods are proving to be useful for screening agents without en-

countering the problems of pharmacokinetics of mice. In addition to earlier discussed methods such as morphological index, electronmicroscopy, FDA-EB staining, metabolite/substrate uptake assays, various macrophage assays, newer or modified techniques based on biochemical, bioluminescent and molecular approaches have been described as alternatives to mouse foot pads. Data presented about the application of FDA-EB staining, bacillary ATP measurement, Na+/K+ ratio measurement by LAMMA, radiorespirometry (BACTEC/Buddemeyer systems) and gene probe/amplification (limiting dilution PCR, different quantitative PCRs, ribosomal RNA-based systems) show that several approaches can be useful for monitoring the responses to chemotherapy, drug screening and *in vitro* measurements of metabolic status of *M. leprae*. Since these methods assess different aspects of viability, even more than one method may be useful/necessary for a particular purpose. Using radiorespirometry, newer compounds active against *M. leprae* have been reported which have been shown later to be promising in clinical trials. Also, LAMMA, other uptake assays, ATP decay assays have been shown to be useful for drug screening. Follow-up studies are required to adapt and assess these methods for their ultimate clinical or laboratory application.

Possible environmental sources of *M. leprae*. There appear to be relationships between the distribution of fossil fuels and the endemicity of leprosy which need to be epidemiologically investigated. The new technologies will provide the tools necessary to investigate further the possibility of an environmental reservoir for *M. leprae*.

Areas of future research.

a) As a result of our experience with tuberculosis where, in addition to cultivation, rapid and alternative methods are also required, it is therefore felt that parallel efforts on cultivation as well as development and application of alternate methods for viability, detection and identification characteristics of *M. leprae* in the patients, environment and laboratory should be continued.

b) Molecular approaches to fill the critical gaps in the physiology/biochemistry, structural aspects, drug resistance and virulence factors need to be tried in future studies.

c) Studies on the development and application of gene probes/gene amplification methods to diagnose and investigate the epidemiology of leprosy need to be given special attention.

d) In addition to already known taxonomic characteristics, newer molecular tools should be used to establish the identity of any "cultivable" form of *M. leprae*.

e) The demands for the bacillus and its component parts for various purposes remain high, and it will still be important to find the answers to several questions (for example, even when the genome sequencing project is complete, it will still be necessary to know which genes are expressed). To meet these research demands, "pure" *M. leprae* will need to be provided in the coming years and the supplies from armadillos/nude mice should continue to be a priority.