leprosy functional activity of NK was decreased as compared to healthy donors and inactive patients (p<0,05), being higher in the last group (p<0,01). The decreased NK activity in active leprosy patients might be a consequence of NK-depletion due to massive antigenic load. Among possible causes of NK-activity in cured patients M.leprae persistence in body tissues might be supposed. In leprosy patients significantly increased frequency of HLA-B7 antigen was observed. Besides, the association between HLA-B7 antigen and low level of NK-cytotoxicity was found out suggesting a genetic determination of functional deficiency of NK in leprosy. NK-activity correlates with leprosy status and, alongside with other indices, might be used for assessment of immune state and effectiveness of therapeutic regimens.

MICROBIOLOGY

MI1

A MOLECULAR ANALYSIS OF MYCOBACTERIAL ANTIGENS WHICH STIMULATE γδ T CELLS

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Most T lymphocytes in human peripheral blood (hpb) express the αβ T cell receptor (TCR). T cells expressing the γδ TCR account for less than 10% of CD3+ hpb T cells. Several microorganisms, including mycobacteria, have been shown to produce a marked in vitro expansion of γδ T cells.
The nature of the γδ stimulatory molecule(s) is controversial. In this study we have used a variety of fractionation methods to identify these molecules, and to charactarise the γδ T cell response.
We find that virtually all individuals tested show a stimulation of Vγ9 γδ T cells when hpb are incubated in the presence of low molecular weight (<5kDa) fractions of mycobacteria, and that there are at least five low molecular weight molecules, all very close in molecular nature, involved in this stimulation.
The γδ T cell response to these molecules has been further charactarised in terms of the lymphokine profile, the involvement of the TCR, and the requirement for antigen processing.

MI2

N-TERMINAL AMINO ACID SEQUENCING OF Mycobacterium leprae PROTEINS: DEFINITION OF THE L12 RIBOSOMAL PROTEIN

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The high abundance of some specific polypeptides in armadillo-derived Mycobacterium leprae has permitted their purification in enough quantities to perform their complete amino acid sequence (see Pessolani et al., abstract this Congress). In anticipation of the conclusion of the biochemical definition of such proteins, the following approach has recently been undertaken in other to define the minor proteins of the leprosy bacillus: (1) fractionation of the bacterial proteins by SDS-PAGE or two-dimensional gel electrophoresis; (2) transference of proteins onto polyvinylidene difluoride (PVDF) membranes and subsequent N-terminal amino acid sequencing by automated Edman degradation; (3) cloning and sequencing of the genes that code for these proteins by using oligonucleotides derived from the amino acid sequences. The N-terminal amino acid sequences of two polypeptides present in extracts of whole cells and of four polypeptides present in the cytosolic fraction of the bacteria were obtained so far. A search in a protein sequence data bank indicated that a 15 kDa cytosolic protein shares 65% homology in a 17 amino acid stretch with the N-terminal region of the Streptomyces griseus L12 ribosomal protein, probably constituting the M. leprae L12 homolog. Two independent approaches are currently being undertaken in order to clone and sequence the gene that codes for the M. leprae L12 ribosomal protein: (1) amplification of the gene by using oligonucleotide primers derived from the N-terminal amino acid sequence, and from phylogenetically conserved amino acid sequences derived from the L12 protein of other bacterial species; (2) cloning of an approximately 4.0 kb EcoRI fragment from the M. leprae genomic DNA that hybridizes with a pool of degenerate oligonucleotides derived from the N-terminal amino acid sequence. In addition to contributing to the understanding of the physiology of mycobacterial ribosomes, the characterization of the M. leprae L12 ribosomal gene may favor the cloning of genes commonly arranged in the same operon, such as the gene that codes for the β subunit of RNA polymerase, the well-known target of the drug rifampicin.
(Work supported by NIH, NIAID Contract NO1 AI-05074.)

MI3

DETECTION OF MYCOBACTERIUM LEPRAE DNA BY PCR IN SKIN SCRAPINGS AND NASAL SECRETIONS FROM MULTIBACILLARY AND PAUCIBACILLARY LEPROSY PATIENTS.

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Detection and species identification of various difficult-to-grow mycobacteria have improved as a result of developments in DNA amplification tests. We have shown that M. leprae DNA can be detected by PCR amplification in extracts from human skin and that 99% of untreated, multibacillary (MB) patients and approximately 50% of AFB-negative, paucibacillary patients (PB) tested positive for M. leprae. Since routine diagnosis of leprosy does not rely on examination of skin biopsy material, but, is limited to clinical observation of the patient and microscopic examination of skin scrapings for acid-fast bacilli (AFB), we tested the utility of PCR to detect M. leprae in skin scrapings and compared these results with PCR reactivity of biopsies from the same patients. Another anatomical location, postulated as a site for initial entry and eventual dissemination of M. leprae in untreated patients, is the nasal mucosa. Nasal secretions were collected from leprosy patients and tested by PCR for M. leprae and compared with the results from skin scrapings and skin biopsies. Nasal secretions and skin scrapings were collected on cotton swabs and scalpel blades, respectively, and placed into 1.0 ml each of sputolysin containing Tween 20 (0.05%). The particulate fraction was recovered by centrifugation and resuspended in 100 ul of deionized water and frozen for subsequent analysis by PCR. Results of samples from 7 of 7 MB and 1 of 2 PB patients showed a direct correlation between PCR positivity of the skin biopsy and the skin scrapings taken from at least one site. Six of 7 (MB) and 1 of 2 (PB) nasal secretions tested positive by PCR. Preliminary results suggest that PCR testing of routine, clinically available samples may be useful in diagnosing and monitoring leprosy.

MI4

EVALUATION OF THE POLYMERASE CHAIN REACTION AS A TOOL FOR LEPROSY DIAGNOSIS

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The identification of *M.leprae* is difficult, partly due to the inability of the bacillus to grow *in vitro*.The current diagnosis of leprosy is

based on microscopic detection of acid fast bacilli in tissue smears, in combination with histopathology and clinical evaluation. Because of the large incubation period of leprosy, and the lack of a reliable test for detection of a subclinical infection, we aimed at the development of a sensitive and specific assay for the detection of *M.leprae* , based on the amplification of bacterial DNA through PCR and hybridization. Oligonucleotides for the amplification of a repetitive sequence, specific for *M.leprae,* were synthesized and PCR amplification was optimised. A hybridization assay augmented the sensitivity and specificity of the test. Different types of clinical samples such as blood, lymph, skin biopsy, nasal secretion and hair bulbs were collected from leprosy patients. Processing of these samples was optimized and inhibitors of the PCR reaction inactivated. A *M.leprae* specific DNA amplification was obtained for each of the samples and 79 patients with a defined clinical spectrum were analysed by gel electrophoresis and hybridization. Results assessed the possibility of using lymph, blood or hair bulb material for diagnosis of both multibacilary and paucibacilary patients in early stages of the disease and, additionally, use of nasal secretion for monitoring the therapeutic regimens.

This project was supported by UNDP/WHO Special Program for Research and Training on Tropical Diseases (TDR).

MI5

NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION (NASBA) FOR IDENTIFICATION AND VIABILITY ASSESSMENT OF *M.LEPRAE* IN SKIN BIOPSY SPECIMENS

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NASBA is an isothermal amplification technique which does not depend on specialized equipment. We have selected a primerset in a highly conserved region of the 16S rRNA sequence allowing amplification of a 200 nt target which comprises a variable region. For identification of the amplified RNA of the mycobacteria, species-specific probes to this variable region were chosen and used in an Enzyme-Linked Gel Assay (ELGA). NASBA was shown to be sensitive and specific for identification of mycobacterial infections in biological samples. Identification of M.leprae in skin biopsy specimens could be performed in less than one day. An advantage of this in vitro RNA amplification technique is that it offers a tool for assessing viability of the mycobacteria. Using in vitro killed *M.smegmatis,* we found a correlation between viability of mycobacteria and the degradation of 16S rRNA. Detection of *M.leprae* DNA (PCR) and 16S rRNA (NASBA) in skin biopsy specimens of multibacillary patients showed that during treatment *M.leprae* rRNA disappears faster than DNA. Thus, detection of rRNA through NASBA might provide an objective means of assessing the bacterial load and efficacy of the therapy.

MI6

DETECTION OF *M. LEPRAE* BY THE POLYMERASE CHAIN REACTION IN NASAL SWABS OF PATIENTS AND THEIR CONTACTS

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Nose swabs from 4 PB and 8 MB patients and their contacts were tested for the presence of *M. leprae* by two PCR: one amplifying a gene coding for the species specific rRNA, and a second amplifying a specific repetitive sequence.

Examinations were done monthly after the start of treatment, in PB cases during 4 months, in MB patients for periods of either 12 or 41 months. Sample preparation was by freeze-boiling. The inclusion of an internal control allowed the detection of inhibitors of the reaction, which were present in 30 % of the samples. Positive results were obtained in 1.9 % and 7.9 % of contacts of PB and MB respectively. Since this difference is not significant, these infections were probably community acquired.

MI7

COMPUTER RECOGNITION OF POSSIBLE FUNCTIONAL OR STRUCTURAL SITES ON MYCOBACTERIUM LEPRAE DNA

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Groups working on sequences of nucleic acids contributed a large amount of data stored in data banks. Several mycobacterial nucleotide sequences are also available through computer networks.

We applied a new computer methodology (1), able to recognize guanine and cytosine-rich zones on nucleotide sequences, to genomic DNA sequences obtained from MycDB (Mycobacterium Database).

The following M. leprae DNA sequences were analysed: 65 kDa, 36 kDa, 28 kDa and 18 kDa. Guanine and cytosine-rich regions were found on all genes within 500 bases from the start of the coding sequence.

The identified zones could be related to functional or structural features of the genes.

(1) Arrigo, P., et al., CABIOS, 7, 353 (1991).

MI18

DETERMINATION OF THE ADENYLATE ENERGY CHARGE (AEC)

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The adenylate energy charge (AEC) defines the proportion of energy-rich adenine nucleotides refered to the total amount of adenine nucleotides. The ratio is given by the equation AEC = (1/2 ATP + ADP)/(ATP + ADP + AMP). The determination of the AEC is - in contrast to the determination of the ATP content - independent of the number of bacterial organisms and well reproducible as shown in a variety of publications for different bacterial genera and also for eucaryotic cells. For mycobacterial species only a few data have been published so far. AEC data determined for several untreated mycobacterial species, including *M.leprae* derived from armadillo material, will be presented and compared with literature data. The influence of different drugs and drug concentrations on *M.tuberculosis* and *M.smegmatis* have been monitored via AEC measurements, demonstrating the general applicability of the method for the determination of drug effects and with that for drug screening. Furthermore, the AEC data derived in these experiments were compared with the results from the mass spectrometric determination of the medians of the intrabacterial Na^+,K^+-ratios of the same bacterial populations resulting in a linear correlation between these two parameters. Furthermore, a correlation between the AEC and the percentage of viable organisms was found. The latter will be shown to be dependent of the bacterial species but independent of the mode of drug action. We will discuss the technical details of the method with particular emphasis on the non-cultivable species *M.leprae* and the implications of the method for *in vitro* drug screening and *in vivo* therapy control.

MI9

A SIMPLE AND RAPID TECHNIQUE FOR THE DETECTION OF RIFAMPICIN RESISTANCE IN *Mycobacterium leprae*

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Rifampicin is the backbone of the current multidrug therapy used for the treatment of leprosy. It has been shown recently, in a study of lepromatous leprosy patients who had relapsed after rifampicin monotherapy, that drug resistance stems from missense mutations in the *rpoB* gene of *Mycobacterium leprae*, encoding the β-subunit of the essential enzyme, RNA polymerase. All of the mutations were found within a short region and similar mutations have been found in the *rpoB* gene of rifampicin-resistant *Mycobacterium tuberculosis*.

A rapid test for rifampicin-resistance would represent a valuable tool for leprosy control programmes. Such a test, based on the polymerase chain reaction and single stranded conformation polymorphism analysis, has been developed. Less than 48 hours are required to obtain the results directly from biopsies and this approach represents an attractive alternative to mouse footpad inoculation.

MI10

MOLECULAR CHARACTERIZATION OF RIFAMPIN RESISTANCE IN PATHOGENIC MYCOBACTERIA

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The majority of mutations which result in the rifampin-resistant phenotype in prokaryotes have been mapped to a 250 bp region of the rpoB gene encoding the B-subunit of the DNA-dependent RNA polymerase. Recently, the rpoB gene of *Mycobacterium leprae* has been sequenced (S. Cole, Pasteur Institute). Using the *M. leprae* rpoB sequence we have synthesized PCR primers which amplify a 644 bp fragment encoding region 4. Using nested sequencing primers and a direct DNA sequencing protocol, we have determined the nucleic acid sequence and deduced amino acid sequence of this region in several rifampin-sensitive (Rif-s) and one rifampin-resistant (Rif-r) strains of *M. leprae*. The DNA sequence of the Rif-s strains contain 100% homology with that reported by S. Cole. The sequence of the Rif-r strain was found to contain a point mutation at base number 1274 resulting in a change from a serine residue found in Rif-s strains to a leucine residue in the amino acid sequence of the Rif-r strain. Comparison of secondary structure predictions for deduced a.a. sequences from Rif-r and Rif-s rpoB polypeptides using the Chou-Fasman and Hopp-Woods algorithms showed that the serine to leucine substitution profoundly increased the hydrophobicity of this region, possibly contributing to the antibiotic resistance observed in this strain. A point mutation in the same codon in a *Salmonella typhimurium* changes the serine residue to a phenylalanine in the Rif-r mutant strain. This mutation has been characterized as the genetic basis of rifampin resistance in this mutant. Several other Rif-r strains of *M. leprae* are being sequenced to establish the potential number of mutations conferring resistance in this species. The sequence of the rpoB gene in region 4 of *M. tuberculosis* is also being obtained using a similar strategy.

MI11

CLONING AND CHARACTERIZATION OF THE GENES CODING FOR THE 85-COMPLEX ANTIGENS OF *MYCOBACTERIUM LEPRAE*

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The genes conding for antigens 85-A, 85-B and 85-C have been isolated from a λ-Dash::*M.leprae* genomic library screened with a labelled DNA fragment containing part of the *M.tuberculosis* 85-A coding sequence. This is the first report on the sequence of the full complement of 85-complex genes in *M.leprae*. The 85-A DNA coding sequence is 990bp long, the 85-B coding sequence 981bp long and the 85-C coding sequence 999bp long. The deduced amino acid sequences are 330, 327 and 333 residues long, respectively, with predicted molecular weights for the mature proteins of 31.0, 30.0 and 31.5 kDa. Comparison to other genes of the 85-complex and hydrophobicity analysis suggest the presence, in the protein sequences, of signal peptide regions. The *M.leprae* DNA coding sequences share 82.3% (85-A), 78.9% (85-B) and 84.4% (85-C) homology to the corresponding *M.tuberculosis* genes. These *M.leprae* proteins are now being expressed in *Escherichia coli* with the aim of obtaining large amounts of protein for functional and immunological studies.

This project received financial support from the UNDP/WHO Special Program for Research and Training in Tropical Diseases (TDR) and from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

MI12

STRAIN DIFFERENCES IDENTIFIED WITHIN *MYCOBACTERIUM LEPRAE*.

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Random amplified DNA (RAPD) was generated by the polymerase chain reaction (PCR) using an arbitrarily chosen 10-mer oligonucleotide. DNA patterns were also generated by PCR using primers based on the inverted sequences of enterobacterial repetitive intergeneric consensus (ERIC) and repetitve extragenic palindromic (REP) elements. The polymorphic patterns of *M. leprae* DNA shown by the three methods were clearly distinguishable from those of other (myco)bacteria. Thirteen isolates of *M.leprae* were investigated. The DNA patterns of these isolates were compared with that of DNA provided by the WHO, which served as a reference. Of the 13 isolates, one was from a naturally infected armadillo and 12 were from armadillos, experimentally infected with bacilli from multibacillary leprosy patients from different parts of the world. The isolates also differed in the number of passages made in armadillo. Distinct differences were observed in two isolates in both RAPD, ERIC- and REP-PCR; in one additional isolate only the RAPD-PCR pattern differed from the WHO-reference; different ERIC patterns were furthermore observed in two isolates and REP-PCR showed differences in two isolates.These preliminary results suggest that strain diffences do exist within *M.leprae*, but we did not find any relation with geographical origin.

MI13

POLYMERASE CHAIN REACTION FOR THE DETECTION OF MYCOBACTERIUM LEPRAE IN SKIN TISSUE

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We have tested several sets of primers for the amplication of a 530 bp and 372 bp fragment of genes specifically encoding for 36 kDa,18 kDa, and 65 kDa protein of M. leprae respectively. All were found to be very specific and sensitive for the detection of M.leprae organisms in suspensions. We have used the primers S 13, S62 which amplified a 530 bp fragment of the 36 kDa protein gene of M.leprae in PCR technique on fresh and paraffinized skin sections. The results

showed that PCR is much more powerful in specfic determination of M.leprae organisms in skin tissue as compared with conventional acid-fast staining method. Hybridization of the amplified fragments with digoxigenin-labelled 1 kb 36 kD gene probe increased the sensitivity of detection by 30% . A quantiative test to determine the proportion of viable bacilli in biopsy specimens by the PCR as compared with mouse footpad inoculation test is being carried out.

MI14

APPLICATION OF A POLYMERASE CHAIN REACTION FOR DETECTION OF MYCOBACTERIUM LEPRAE IN NASAL SWAB SPECIMENS

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Little is known about the scope and importance of subclinical infection or carriage of M.leprae. Given that multibacillary patients and nasal carriage of M.leprae are likely associated with transmission of leprosy and treated patients are not likely transmitters of the disease, as a first step developing a test, we decided to examine rates of nasal carriage in treated and untreatedmultibacillary patients by the polymerase chain reaction(PCR). In this study, PCR is based on the selective amplification of a 530-bp fragment of the gene encoding the proline-rich antigen of M.leprae was used to detect M.leprae in nasal secretions collected on swabs from 16 treated and 22 untreated multibacillary patients. To prevent false positive amplifications, dUTP and uracil-N-glycosylase were adopted. To minimize false negative results, due to inhibitory components from nasal mucosa, swabs were treated with quanidinium thiocyanate(GuSCN) or DMSO. It was found that the 59.1%(13/22) untreated MB patients were positive compared to 18.8%(3/16) treated MB patients. The reduced nasal carriage in treated patients is consistent with reports of decreased transmission in families of treated MB patients. These findings suggest that the PCR may be useful for studying transmission of M.leprae by comparing nasal carriage rates in families of treated and untreated MB patients

MI15

ENHANCED CHEMILUMINESCENT ASSAY (ECL) : A HIGHLY SENSITIVE METHOD FOR THE DETECTION OF M.LEPRAE INFECTION IN NASAL SECRETIONS

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The role of nasal carriage in the transmission of leprosy has always been considered in studies relating to the development of rapid and simple means of detecting M.leprae infection. Using non-invasive collection method, two test are available; the PCR method and a monoclonal test. The PCR has greater sensitivity but is very expensive, the monoclonal test is cheaper but is constrained by unstable color development thus making the interpretation of reactions difficult. In addition, data cannot be documented for the substrate fades with time. Recently, a non-radioactive labeling and detection system have been used to improve sensitivity of antigen detection in Western blot systems. For the first time, we have adapted the Enhanced Chemiluminescent Assay (ECL) and were able to detect M.leprae and its antigen, phenolic glycolipid-I (PGL-I) in nasal secretions of our patients using our monoclonal antibody,DZ-1. Thirty one leprosy patients were tested, 24 were MBs and all were test positive; of the 6 PBs, three were test positive and three were negative and one Neural type was slightly positive. Thirty nine individuals free of leprosy were used a controls and all but 2 were test negative indicating the need to improve the specificity of the ECL which can easily be done without loss of sensitivity. Our data have shown that the ECL is highly sensitive. The most beneficial aspect of the test is the ability to keep a permanent record of the result on x-ray film. Therefore,the ECL has the potential use as a screening test to detect early leprosy, to study transmission, and disease reactivation.

MI16

UTILIZACION DE 3 SISTEMAS DE PCR PARA LA DETECCION DE ADN DE M. LEPRAE EN MUESTRAS QUIMICAS.

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Se ensayaron 3 sistemas de PCR para la detección de ADN de M. leprae en muestras de suero y linfa de pacientes en los cuales se sospechaba la posibilidad de estar infectados con M. leprae porque tenian títulos elevados de anticuerpos en el suero contra este microorganismo.

En el suero no se detectó la presencia de las secuencias de Ácidos nucléicos que estos sistemas amplifican mientras que en la linfa los resultados no permiten detectar bacilos cuando la baciloscopía es negativa.

MI17

MECHANISM OF ENERGY TRANSDUCTION IN MYCOBACTERIUM LEPRAE

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Adenosine triphosphate (ATP) constitutes the "molecular energy currency" in living cells. Energy released by the hydrolysis of the ATP drives life processes, including synthesis of nucleic acids and transport of metabolites across cell membranes. Most organisms synthesize their own ATP through the enzymatic degradation of utilizable substrates. Why Mycobacterium leprae is dependent on the host cells for its survival and proliferation has not yet been explained satisfactorily.

Free-living bacteria hydrolyze phosphorylated organic compounds extracellularly and transport the organic portion and the phosphate moiety in two separate steps; the compounds are resynthesized intracellularly. Obligate intracellular parasites generally transport high-energy molecules in the intact state from the host cells. M. leprae prepared from FRESH tissues of experimentally-infected armadillos or nude mice readily took up [2,8-^{3}H] ATP. Initially, there was a rapid electrostatic binding which is energy-independent. This ionic phase was followed by an energy dependent phase that was abolished by metabolic inhibitors. In a competition assay, unlabeled ATP or ADP inhibited transport of ^{3}H-ATP by M. leprae; adenosine or PO_4 had little effect. Evidently, the organism takes up unhydrolyzed ATP by an active transport process. The bacteria possessed an E_1 E_2 ATPase that creates a trans-membrane potential driving transport of solutes into cells, but not an F_0 F_1 ATPase that catalyzes ATP synthesis. This dependence of M. leprae on the host for high energy compounds may be a reason for its failure to grow in culture media.

MI8

CLONING AND SEQUENCING OF THE *TUF* GENE CODING FOR THE ELONGATION FACTOR TU OF *MYCOBACTERIUM LEPRAE*

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The *Tuf* gene encoding the elongation factor Tu (EF-Tu) of *Mycobacterium leprae* has been cloned and sequenced. Part of the gene fragment (-COOH terminal) was isolated from clone R7 obtained from the lambda gt11 library. The full gene was identified by colony hybridization of the *M. leprae* genomic DNA. Nucleotide sequence determination revealed that gene contains a coding region of 1188 bp with GUG as start codon. A putative Shine Dalgarno sequence is located 8 bp upstream. The deduced amino acid sequence has 396 residues with a molecular weight of 43.6 kDa. Computer analysis showed that the GDP or GTP binding sites are located at amino acid positions 19-26, 83-87, and 138-141.

Comparison of *M. leprae* EF-Tu amino acids with that of other species revealed 95.2%, 79.6%, 74.5%, and 74.7% homology with *M. tuberculosis, Micrococcus luteus, Escherichia coli,* and *Salmonella typhimurium,* respectively. Mitochondrial EF-Tu of *Saccharomyces cerevisiae* (62.7%) and chloroplast EF-Tu of *Arabidopsis thalina* (65.6%) are some of the eukaryotic EF-Tus' showing strong homology with that of *M. leprae.* Southern hybridization of *M. leprae Tuf* gene with genomic DNA of slow growing and fast growing mycobacteria and related species like *Corynebacterium fascians* and *Nocardia asteriodes* suggests that the gene is highly conserved among these species.

MI19

APPLICATION OF LASER MICROPROBE MASS ANALYSIS TO *IN VITRO* DRUG SCREENING

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The susceptibility of *M.leprae* to different anti-leprosy agents was assessed in a newly developed *in vitro* drug screening system. The drug effects on the physiological state of isolated *M.leprae* are monitored by the mass spectrometric analysis of the ratio of intrabacterial concentrations of sodium and potassium ions of single bacterial organisms (Na^+,K^+-ratio). The rational behind this approach is the ability of all living unimpaired cells, bacteria as well as eucaryotes, to accumulate K^+ and exclude Na^+ by energy-demanding processes and to use the transmembrane gradient as energy storage for several central metabolic processes, e.g. transport. We could show that the intrabacterial Na^+,K^+-ratio is a sensitive indicator for the physiological state -i.e., viability - of the bacteria and that changes of this value correlate with those observed by means of established microbiological techniques. For *in vitro* drug screening, armadillo-derived *M.leprae* were incubated at 32°C in modified - increase of malachite green content - Middlebrook 7H9 for 2-3 weeks in the presence of different drugs and their combinations. Changes in the distributions of the intrabacterial Na^+,K^+-ratios reflect time- and concentration-dependences of drug effects for those drugs which do not interfere with DNA-replication and multiplication. Moreover, it renders important information on the combined action of different drugs in terms of antagonism or synergism.

MI20

LABELING OF THE 65 KDA HEAT SHOCK PROTEIN IN *MYCOBACTERIUM LEPRAE* AND OTHER MYCOBACTERIAL SPECIES.

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We have used immungold ultracytochemistry to label *in situ* the 65 kDa heat shock protein on *Mycobacterium leprae* and other mycobacterial species (*M. tuberculosis, M. avium* and *M. smegmatis*). Leprosy bacilli were observed by thin section electron microscopy in liver samples of infected armadillo and in skin biopsy tissues of humans with Hansen's disease, both before and after strating chemotherapy.

We found that virtually all leprosy bacilli showed positive labeling by immunogold spheres coupled with polyclonal antibodies against the recombinant 65 kDa antigen of *M. bovis* BCG expressed in E. coli (the recombinant protein and thge specific antibodies were a gift of Dr. J. D. A. van Embden, national Institute of Public Healthn and Environmental Protection, Bilthoven, The Netherlands). Interestingly, the immunocytochemical marking was still present in debris of dead *M. leprae* bacilli detected in the infected tissues. This indicates that the antigenicity of the 65 kDa heat shock protein of leprosy bacilli may persist well beyond the loss of viability of the mycobacteria. Since there is evidence that the 65 kDa mycobacterial antigen may be involved in autoreactive phenomena, which are often described in patients with mycobacteriosis, our results suggest that remnants of mycobacteria may be the cause of autoaggressive disorders in patients that have been "cured" from Hansen's disease.

We have also investigated the subcellular distribution of the 65 kDa antigen in cellular fractions of cultured *M. smegmatis* and *M. avium*. Our data, obtained by immunogold cytochemistry and immunoblotting, revealed that this heat shock element is present in all of the fractions studied (cytosol, membrane and cell wall) which may explain the *in situ* detection of the antigen in cell wall debris of mycobacteria.

MI21

EXTRACORPOREAL VIABILITY OF M.LEPRAE

Sreevatsa

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M.leprae available for laboratory research are very limited. Extracorporeal bacilli survive for a few days. However, while processing or handling tissues or bacteria for various experiments a good proportion of the organisms are often lost. At the same time viability of M.leprae may also be affected. Therefore studies were carried out to assess whether various methodological procedures adopted while conducting experiments or while maintaining bacilli under different conditions, affected the number of organisms made available or their viability.

M.leprae obtained from human or armadillo sources were subjected to several methodological procedures and also were maintained under various conditions of temperatures. Bacilli subjected to all these procedures were inoculated into hind foot-pads of Balb/c mice.

Results of mice foot-pad harvests showed that decontamination affected the viability whereas centrifugation and purification did not show any effect. Bacilli survived upto varying periods from 7 to 90 days under different conditions of maintenance and preservations. Thirty minutes of Ultra violet rays exposures killed all bacilli. Where as disinfectants took longer time to kill all bacilli. However, 70 per cent of alcohol was effective in killing bacilli within 15 minutes.

MI22

ULTRASTRUCTURAL PARAMETERS INDICATING VIABILITY OF M. leprae AFTER CHEMOTHERAPY.

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M. leprae causative organism for leprosy, can be identified by light microscope using, Fite-Faraco stain but it is not possible to clearly deliniate its morphology. Therefore, the ultrastructural characteristics of M. leprae obtained from lepromatous leprosy cases both untreated and treated with MDT (Rifampicin, Clofazimine and Dapsone, WHO Regimen) were studied and observations were compared with light microscopic findings. Skin biopsies of two untreated ($BI=6^+$) Ridley Scale and three treated ($BI=3^+$) lepromatous leprosy cases were examined. The treated patients had MDT (WHO regimen) for 6 months to 1 year. A uniform suspension was put on the collodion coated copper grid and examined under electron microscope (H-300) after carbon coating in vaccum under high pressure.

Ultrastructural studies of M. leprae in untreated cases revealed that bacilli were solid staining but of different length, thickness and some of these had banded structure. Under light microscope, bacilli in treated cases appeared to have irregularly fragmented cytoplasm with degenerated cell wall. However, under electron microscope these bacilli were found to be completely filled with cytoplasm like a solid bacillus but were of shorter length.

This is more so important because there is a need to define structural criteria and viability of the M. leprae organism, during the treatment of bactericidal and antimycobacterial drugs.

MI23

ASSESSING THE VIABILITY OF MYCOBACTERIUM LEPRAE ON 28 CASES ON MDT BY THE FDA/EB STAINING TECHNIQUE

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The authors evaluated the FDA/EB staining technique in assessing the viability of mycobacterium leprae in skin smears taken regularly from 28 previously untreated MB cases in the period of MDT. According to the theory of the FDA/EB staining technique, green cells were deemed to be viable, red as dead, and dual stained bacilli as viable. The results of this study showed that the average percentage of green cells was 42.75%(a range of 7.5% to 88%) before MDT, 20.46 %after 1 to 2 months' MDT, 14.79% after 3 months', 5.66% after 6 months ' 1.15% after 18 months' and 0.35% after 24 months' MDT. The authors believed that FDA/EB staining technique was accurate, simple, convenient, and feasible. The percentage of green cells could be used as one of valuable indicators in assessing the effectiveness of MDT and in predicting the possibility of relapse. The authors also observed that green cells were still found in 4 cases after 24 months' MDT, indicating they were in need of a certain period of continued intensive treatment. Compared with the findings of this mehtod, BI and MI values were not considered reliable in the assessment of the viability of Mycobacterium leprae.

MI24

DETECTION OF 16S rRNA FOR VIABILITY ASSESSMENT OF MYCOBACTERIA

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Previously we developed a specific polymerase chain reaction (PCR) for detection of *M.leprae* DNA in clinical specimens. Correlation between the amount of PCR product formed and the viability of bacilli in human skin biopsy specimens has been found. The amount of 16S rRNA in the bacteria may be a better indicator of the metabolic state of the mycobacteria, rather than the DNA content. Nucleic Acid Sequence Based Amplification (NASBA), an RNA-amplification technique, was used to test this hypothesis. An *in vitro* system for killing mycobacteria was developed to assess this hypothesis. Therefore, *M.smegmatis* was exposed to the antimycobacterial drugs rifampicin and ofloxacin. Growth and viability of the bacteria was determined by measuring respectively the optical density (OD) and the amount of colony forming units (CFU). Both parameters were compared to the presence of DNA (PCR) and rRNA (NASBA) in the bacilli. Exposure of *M.smegmatis* to rifampicin and ofloxacin showed that there was a loss of viability after 1 day. These resuts were compared to the DNA and RNA content of the mycobacteria. Although no decrease in the DNA content was seen, a slide decrease in 16S rRNA content was observed after 1 day and total clearance of the 16S rRNA was seen after 5 days. These results suggest that the 16S rRNA content reflects the viability of mycobacteria in an *in vitro* system.

MI25

THE EFFECT OF ELECTROCHEMICALLY ACTIVATED LIQUIDS ON MYCOBACTERIAL VIABILITY

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To stimulate the growth of some species of slow-growing mycobacteria, liquid media, modified with a cathode fraction of electrochemically activated salt solutions, were used. In addition, the effect of an anode fraction of the same solution on mycobacterial growth was studied. Standard Sauton's medium was taken for control. Slow growing species, such as M.avium, M.tuberculosis, M.01 and M.011 (growing strains isolated from LL patients), M.lufu and M.leprae (isolated from 3 LL patients and not growing on standard nutrient media). Viability of cultivable mycobacteria was checked by their growth on Loewenstein-Jensen medium, and M.leprae - by multiplication in mice foot-pads. Cultivable strains at a dose of $5x10^6$ microorganisms per ml were inoculated into test media, and 3, 5, 7 and 9 days later the material was transferred into a solid medium. The results of the experiment were judged by the amount of colony-forming units in a solid medium. M.leprae were introduced into cathode and anode fractions of salt solution at a dose of 10^6 - 10^7 microorganisms per ml and incubated at 20oC and 37oC. In 1, 2, 3 and 4 weeks of incubation M.leprae were inoculated into mice foot-pads. Cultivation of slow growing mycobacteria on cathode-containing media resulted in augmented mycobacterial growth. With anode fraction mycobacteria did not grow. M.leprae remained viable after their incubation in a cathode fraction of the salt solution as long as for 1-4 weeks, evidenced by the development of infection in mice.

MI26

A RAPID IN VITRO MICROASSAY FOR THE VIABILITY AND DRUG SENSITIVITY OF M. LEPRAE

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We have developed an in vitro assay for the viability of M. leprae based on the levels of mRNA detected by reverse transcription linked PCR amplification. The hypothesis is that because of the short halflife of procaryotic mRNA, dead or drug treated bacilli will have reduced levels or no mRNA and the measurement of mRNA levels can be used as an estimate of viability after drug treatment. mRNA is extracted from M. leprae bacilli by sonication in guanidinium thiocyanate and purification in CsCl gradients. cDNA is synthesised from total RNA with reverse transcriptase using a 3' gene specific primer for the 70kDa antigen (DnaK homolog) and PCR amplified after addition of a 5' primer. Bands of the correct size and restriction polymorphism patterns are obtained indicating that mRNA can be extracted from M. leprae and that it can be detected by RT-PCR. False positives due to contaminating genomic DNA were excluded by DNase 1 treatment. Using a recombinant truncated artificial mRNA template for the 70kDa gene mRNA, the RT-PCR assay can detect down to 1fg of mRNA: equivalent to 10^3 bacilli. Currently a two log fold killing can be detected from 10^9 armadillo derived M. leprae with a viability of <0.1%. Three out of 6 M. leprae infected nude mouse footpad mRNA preparations give strong PCR bands which disappear after heat killing the bacilli, indicating that mRNA levels reflect viability. Correlation of mRNA levels with viability assessed in the nude mouse footpad after in vitro exposure of M. leprae bacilli to different drug regimens is underway. This technique combines the sensitivity of PCR based detection systems with the measurement of mRNA as a rapid and highly sensitive assay for the viability of M. leprae.

MI27

CULTIVATION OF MYCOBACTERIUM LEPRAE IN ARTIFICIAL CULTURE MEDIUM

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A novel procedure in the cultivation of M.leprae in combined Dubos-Lowenstein-Jensen medium after addition of thyroxine sodium is being reported. This has been found to be successful as the organisms, after multiplying vigorously in the thyroxine containing Dubos medium, have produced a visible colony on the surface of Lowenstein-Jensen medium during 8-16 wks of incubation at 37°C. The enhanced growth of this recalcitrant organism is due to stimulating effect of thyroxine as well as supply of ready-made basic nutrients in the synthetic Dubos medium. Intradermal inoculation of the bacterial suspension from the subculture into the foot pad of cortisone treated swiss mouse revealed an early appearance of specific histological lesion of leprosy with infiltration of nerve fibres by lepra cells.

The methodology, described here for in-vitro cultivation, may open out a new era in the preparation of purified vaccine, the study of in-vitro drug sensitivity, and as such ensure rapid eradication of leprosy.

MI28

EFFECTS OF PALMITATE ON THE GROWTH OF *MYCOBACTERIUM LEPRAE* UNDER DIFFERENT GASEOUS ENVIRONMENTS.

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Low oxygen tension has often been considered important for the growth of Mycobacterium leprae. Palmitate has been suggested as the oxidizable substrate for the growth of leprosy bacilli. Effects of palmitate on the growth of M. leprae under various gaseous environments were investigated. No multiplication of bacilli was observed in liquid or solid medium without palmitate when incubated under various gas mixtures or air. However, when palmitate was included in the media six to ten fold increase in the number of bacilli was obtained between 12 to 20 weeks of incubation under gas mixtures containing 2.5% O_2 and 5 or 10% CO_2 as well as under air. The use of different gas mixtures is tedious, laborious and time consuming. Since the cultures incubated under air gave the same cell yield as obtained when incubated under optimal gas mixtures, air alone can be used for the in vitro cultivation trials of M. leprae when palmitate is included in the culture media.

MI29

IN VITRO CULTIVATION OF *Mycobacterium leprae* WITH CYTOKINES

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Ever since its discovery, numerous investigators have tried to cultivate *M. leprae in vitro*, but in vain. However, this organism maintains its multiplication *in vivo*, with all the requirements for its growth.

It was hypothesized that the lack of nutritional factors may not have been the problem, instead, certain cytokines alone or in combination, may be the essential growth factor(s) for this organism. As a first step to test this hypothesis, the tissue homogenate of armadillo spleen infected with *M. leprae* was inoculated into Ogawa media with interleukin-2, interferon-α, or -γ, alone or in combination. Incubation temperature was 36°C, without CO_2. Gross and microscopic examination revealed the following results.

1. Round, oily, and white colonies were observed on the surface of the liquid in all the tubes with both *M. leprae* and interleukin-2, 3-6 months after the initiation of the cultivation. Above colonies could be observed 1-2 months earlier in the test tubes with *M. leprae*, interleukin-2, and interferon-γ. But no colonies could be seen in all the test tubes without *M. leprae* or interleukin-2.

2. The size of the colonies grew with time and they were the compact and solid collections of acid fast organisms under a microscope.

3. This experiment has been repeated 2 times, with the same results.

These data indicate the possibility that *M. leprae* can interact with interleukin-2, interferon-γ, and probably some other cytokines, which would turn out to be the essential growth factor(s) for this organism. Subcultivation, identification, and animal studies are under investigation. Furthermore, the effect of various cytokines on the growth of other microorganisms, including uncultivable ones, is also under investigation.

MI30

A COMPARISON OF DNA/RNA, MYCOLATES, PGL-I, ANTIGENICITY, ENZYMES, MORPHOLOGY AND STAINING CHARACTERISTICS OF THE LEPROSY BACILLUS WITH THE LEPROSY DERIVED CHEMOAUTOTROPHIC NOCARDIOFORM BACTERIA(IN VITRO).

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The chemoautotrophic nocardioform (CAN) bacteria had been isolated as single organisms from 90 different LL cases so far, as well as from the mouse footpads (MFP) and armadillo tissues experimentally infected with M.leprae. These CAN bacteria and M. leprae have been compared on the basis of their morphological, staining, metabolic and enzymological characteristics, and found to be extremely similar. Both the organisms also exhibited closely similar or identical patterns of lipid profiles, anergy and 'Mitsuda' responses to a large number of LL and TT cases respectively, PGL-I specificity, as well as, for DNA characteristics and resistances to γ - and UV - radiations. All were DOPA oxidase positive and lost acid-fastness due to pyridine extraction. The comparative study and an evaluation of all the above characters reveal an extreme closeness of the CAN bacteria to the leprosy bacillus to a point beyond which it may not be possible to distinguish them from each other any further.

MI31

DETECTION OF PGL-I IN THE CHEMOAUTOTROPHIC NOCARDIOFORM (CAN) BACTERIA

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PGL-I is a unique M.leprae antigen, detecting which may lead to identification of M.leprae. We have investigated the presence of PGL-I in the in vitro CAN-cultures derived from human, mouse-footpad and armadillo tissues infected with leprosy bacillus.

For this purpose, antisera were produced in rabbits against the 4 CAN bacterial suspensions. Microtitre gelatin particle agglutination test (Serodia M.leprae Fujirebio, Japan) had been used to detect and assay anti-PGL-I antibodies, if any, in such antisera. Use of synthetic antigens seemed to exclude possible artifacts.

The gelatin particle agglutination tests showed high titre anti-PGL-I antibodies to be present in

the immune sera. Biological controls, human serum (LL) and normal rabbit serum controls were used to exclude false positivity, and decide on reliability of the tests. The animals responded best by producing antibody when injected by the intramuscular route. Most of the animals initially developed immunologic paralysis towards the antigens, but showed gradually rising antibody titres.

In the light of other tests reported previously, e.g. chemoautotrophism, lipid profile, DNA(36K) specificity etc. the present test becomes significant with respect to the true identity of the CAN bacteria, viz-a-viz M.leprae. The close parallelism observed between lepromin and the CAN-AGs is thus explainable.

MI32

TOWARDS DEVELOPING A NEW SCHEME FOR RAPID CHARACTERIZATION OF MYCOBACTERIA WITH SPECIAL REFERENCE TO M.LEPRAE

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Difficult to grow and non-cultivable mycobacteria provide new challenges for the taxonomists, epidemiologists and immunologists all of whom have related interest in defining the degree of relatedness of mycobacteria. Data based on M.leprae gene probes reported so far supports the concept that M.leprae is a highly homogenous species in nature. However this may be because of the fact that only a small fraction of the genome could be examined by this type of analysis. Protein electrophorogram and multi-locus enzyme electrophoresis has advantage that large number of protein enzymes encoded by a sizeable proportion of mycobacterial genome can readily be compared. We have adapted and applied these techniques using nine enzymes and protein electrophorograms to 23 species of mycobacteria, many of which are related to M.leprae in one way or other so as to develop strategies to characterize strains of M.leprae or 'alleged isolates'. Our findings suggest that the approach can be helpful as adjunct to studies of rRNA genes and other molecules in strains of pathogenic mycobacteria specially M.leprae. Such investigations may possibly help to establish "Epigenetic Finger Prints". As a result, a simple and rapid identification scheme has been evolved which has potentiality to be used for several difficult to grow mycobacteria, armadillo derived mycobacteria, mycobacteria isolated from leprosy lesions as well as 'alleged isolates' of M.leprae.

MI33

ELECTRON MICROSCOPIC STUDY OF M.LEPRAE PASSED ON LABORATORY ANIMALS

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Ultrastructure of M.leprae (ML) taken from LL patients (original strain) and passed on laboratory animals (nine-banded armadillos and mice) were studied. ML of the 1st passage were characterized, as compared with the original strain, by a reduced microcapsule, thickened cell wall and condensed cytoplasmatic matrix with poorly differentiated ribosomes and nucleoide, that is typical for surviving bacterial forms. ML of the 2nd passage (mouse-to-mouse and armadillo-to-mouse) were identical and had fragmented microcapsule, normal cell wall with closely adjacent cytoplasmatic membrane and rare inclusions ("homogeneous bodies") in their cytoplasm. These strains differed from the 1st passage by the abundance of dividing cells, pronounced nucleoide and more developed mesosomes. For ML of the 3d passage the presence of numerous "homogeneous bodies" in their cytoplasm as well as the increased number of volutin granules were peculiar features suggesting ML adaptation to a new environment. In subsequent passages (4th-8th) no further changes in ML ultra-structure were observed. It was concluded that the changes in ultrastructure of ML passed from man to laboratory animals (within three passages) might be accounted for mycobacterial adaptation to the environmental conditions in a new host. These data should be taken into account when studying taxonomical properties of the adapted strains of M.leprae and in bio-technological developments as well.

MI34

PHAGOCYTIC MYELOPEROXIDASE AS A KILLING FACTOR FOR M.LEPRAE

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It is well known that functional activity of phagocytes depends on the state of their enzymes, in particular the activity of macro-phage myeloperoxidase. The present work is devoted to electron cytochemical study of myeloperoxidase (MPO) activity in active LL patients. Various activity and different lo-calization of MPO in macrophages correlated with the degree of completeness of M.leprae phagocytosis. In macrophages with a large number of peroxidase-active mitochondria alongside with the presence of MPO in phagosome membranes and electron-transparent zone around myco-bacterial cells intensive lysis of M.leprae was observed. On the contrary, in macrophages with low level of MPO M.leprae appeared mainly intact. Observation of MPO-activity in macrophages of leprosy granulomas in long-treated BL and LL patients showed that with low phagocytic activity of MPO the disease improved slowly and such patients were at risk of relapses. With a high level of phagocytic activity of MPO leprosy patients demonstrated a rapid improvement of their lesions and did not relapse throughout the observational period (up to 14 years). The results obtained were assumed as a basis for the development of our method for identification of patients at risk of leprosy relapses and could be used in improving experimental leprosy models available.

MI35

BIOLOGICAL PROPERTIES OF M.LEPRAE PASSED ON LABORATORY ANIMALS

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The alterations in some biological properties of the original strains (OS) of M.leprae, isolated from leprosy patients and passed (PS) to mice, rats and armadillos were studied. M.leprae from mouse and rat foot pads of the 3d-5th passages and M.leprae from armadillo spleen of the 1st passage were studied for the spectra of fatty acids (FA), antigenic structure and DOPA-oxidase activity with using gas-liquid chromatography, enzyme immunoassay and electron cytochemistry. It was shown that in FA spectrum in OS-M.leprae C22:0 > C24:0 ratio predominated. Being passed M.leprae showed FA spectrum similar to that in M.lepraemurium and M.avium, i.e. C24:0 >C26:0 ratio. M.leprae strains from the 1st passage to armadillo retained C22:0 > C24:0 ratio. DOPA-oxidase was present both in OS- and in PS-M.leprae while M.lepraemurium and cultivable mycobacteria had no such enzyme. With using a set of monoclonal antibodies (WHO Bank) protein epitopes 12, 18 and 65 kDa were demonstrated in the antigenic structure of OS-M.leprae while PGL-1, unique for OS-M.leprae and M.leprae from

the single passage on armadillos, was not discovered. The data obtained should be taken into account in taxonomical studies on M.leprae passed on laboratory animals, and when developing novel experimental leprosy models,specific diagnosticums and antileprosy vaccines.

MI36

OVEREXPRESSION OF *MYCOBACTERIUM LEPRAE* ANTIGENS IN *ESCHERICHIA COLI*

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Three genes encoding *Mycobacterium leprae* antigens selected with monoclonal antibodies were cloned in *Escherichia coli*. Nucleotide sequencing revealed that these genes are capable of encoding proteins of apparent molecular weights of 16, 26 and 45 kDa. The functions of these proteins in *M.leprae* are as yet unknown. However, the 45 kDa protein shows homology to a number of integral membrane proteins involved in the transport of compounds over the cellular membrane. We tried to overexpress these proteins in *E.coli* as native proteins using the T7 system. We found no overexpression despite efficient transcription, indicating that the lack of protein expression is due to inefficient translation. We were capable of overexpressing the proteins in *E.coli* as fusion proteins using a variety of expression systems (i.e. pUC8-2, pNGS21, pGEX1-3 and pVW500).In the present paper we will discuss the characterization, overexpression and purification of these proteins and their use in serology.

MI37

OVEREXPRESSION AND SEROREACTIVITY OF 15 KDA ANTIGEN OF *MYCOBACTERIUM LEPRAE*

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By screening lambda gt11 *Mycobacterium leprae* genomic DNA library with leprosy patient's sera, we isolated 16 clones of strong and weak reactivities. Sequence determination of these clones revealed that clone R9 has part of the gene coding for 15 kDa *M. leprae* antigen. The 4 kb insert DNA from this clone was sub cloned into pMal-c expression vector and expressed in *E.coli* HB101 as maltose binding fusion protein. The affinity purified fusion protein showed strong reactivity with leprosy patient's sera in western blot analysis. An ELISA developed using this protein showed 81.8%, 47.1%, 41.7%, 10% and 20% seropositivity to untreated LL, BL, BB, BT, and TT sera at 1:300 dilution. No significant reductions in the seropositivity rates were noticed on sera from treated patients as they showed 68.6%, 50%, 13%, and 25% to LL, BL, BT, and TT, respectively. This protein detected antibodies in contacts of leprosy patients as well as tuberculosis patients in which the seropositivity were 15.8% and 7.2%, respectively. This protein was also found to react with serum samples obtained from mice immunized with *M.* leprae.

MI38

CHARACTERIZATION OF M.LEPRAE STRAINS BY RFLP ANALYSIS OF AMPLIFIED rDNA

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Restriction Fragment Length Polymorphism (RFLP) analysis has emerged as a powerful tool to characterize various prokaryotic and eukaryotic organisms. These studies can not be easily done on M.leprae as sufficient amount of organisms can be isolated only from highly bacillated leprosy types. Earlier studies on strains grown in experimental animals showed very little divergence among such strains. In this study, nucleic acids from biopsies from leprosy patients across the spectrum belonging to different geographical locations in India, were extracted by a modified technique standardised at this laboratory. By using different sets of primers targetting variable regions towards the ends and flanking regions of ribosomal RNA genes, rDNA fragments were amplified. These were restricted with different restricted endonucleases and hybridized with ribosomal RNA probes by the techniques reported by us earlier. The origin of amplified rDNA was confirmed by using a set of oligonucleotide probes targetting specific sequences on rRNA genes of M.leprae. The combination of patterns obtained after restriction with different restriction enzymes revealed interesting findings. Overall, strong resemblence among the different strains was observed. However, some divergencies have also been observed. The relevance of these findings is being investigated further at sequence level and in larger number of strains. Amplified rRNA restriction analysis appears to be promising for rapid identification and characterizing of M.leprae directly from the lesions in leprosy cases.

MI39

DEVELOPMENT OF IMPROVED TECHNIQUES FOR EXTRACTION OF NUCLEIC ACIDS FROM LEPROSY LESIONS

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During the last 3-4 years, several probes and gene amplification techniques for detection/amplification of nucleic acid sequences of M.leprae have been developed. For the optimum application of these methods, the extraction of nucleic acids from leprosy tissue is an important step. Techniques reported for extraction of nucleic acids from tissue include physical as well as chemical approaches. In this study, different techniques for lysis such as freeze--thawing, freeze-boiling, Proteinase K treatments for 1 to 16 hrs and a new modified lysozyme/SDS+Proteinase K method (followed by stepwise purification wherever necessary) based on optimised concentrations and duration have been compared and evaluated in the clinical specimens across the spectrum. The nucleic acids extracted were processed for detection of rRNA by oligonucleotide probes and for gene amplification by primers targetting 18kd, 36kd genes and reverse transcription-amplification of 16S rRNA sequences. All the extraction procedures appeared to be adequate for biopsies from multibacillary cases. However the freeze-thawing and freeze-boiling methods were found to be less efficient for biopsies from smear negative paucibacillary cases. The modified procedure standardised in this study is reasonably fast and appeared to be very efficient for all type of cases. The technique needs to be tried in a larger number of specimens.

MI40

STUDIES OF α ANTIGEN GENES IN *M. LEPRAE*

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Sero-diagnosis for leprosy has been performed mainly by two methods. One is agglutination test using gelatine particles coated with phenolic-glyco-lipid I(PGL- I). The other is ELISA method using bacterial cellular protein(s) as antigen(s). Among these methods cross reactivity with another *Mycobacteria* was one of the majour problems. To establish a new reliable method, antigen with high specificity is very important.

In this study, we planed the construction of recombinant antigen which contains *M. leprae* specific epitope(s). And we performed

molecular cloning and analysis of *M. leprae* α antigen gene family and constructed the overproduction system of *M. leprae* α antigen.

RESULTS AND DISCUSSIONS

1) We have cloned and characterized *Mycobacterium leprae* α antigen gene family. We obtained 2 kind of α antigen gene by screening 10000 plaques of *M. leprae* genomic library. Homology between *M. leprae* α antigen was lower than those between *M. leprae* α1 and α antigen of *M. bovis* BCG or 85 complex of *M. tuberculosis*.

2) Recombinant α1 antigen of *M. leprae* has been constructed and purified by amirose resin affinity chromatography. More than 20 mg of recombinant protein was obtained from 250 ml liquid culture.

3) Antibody titer against recombinant α antigen in the serum of leprosy patient was much higher than healthy control. Fusion protein produced in this study could be used as a new specific antigen for sero-diagnosis of lepromatous leprosy.

MI41

THE ABNORMAL STRUCTURE OF MYCOBACTERIAL recA GENES

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Mycobacteria are intracellular pathogens which are exposed to DNA-damaging agents as part of the host's defence against infection. In most bacteria the response to such agents involves the coordinated expression of over 20 genes through a common regulatory mechanism involving the RecA protein. We have previously reported the cloning and characterisation of the recA gene of M.tuberculosis. This gene is very unusual in that it contains a protein splicing element which is removed post-tranlationally.
We have now characterised the M.leprae recA and found that it too contains a protein splicing element. However the M.leprae and M.tuberculosis spliced sequences are inserted at different positions within the gene, are unrelated in sequence and differ in size, suggesting that their insertion into recA has occurred independently. Southern blotting with protein splicing element-encoded DNA indicates other mycobacteria do not possess these elements, suggesting that there has been positive selection for the abnormal recAs seen in M.tuberculosis and M.leprae.

MI42

BIOCHEMICAL CHARACTERIZATION OF TWO NEW MAJOR PROTEINS OF MYCOBACTERIUM LEPRAE

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Proteins synthesized in significant amounts by in vivo grown Mycobacterium leprae presumably play a key role in the host-parasite interface. In order to complete the definition of the major proteins present in armadillo-derived M. leprae, two polypeptides originally described as major membrane protein I (MMPI) and major membrane protein II (MMPII), with Mr of 35 kDa and 22 kDa, respectively (Hunter et al. 1990. J. Biol. Chem. 265: 14065-14068), were purified and subjected to amino acid sequencing. The sequencing strategy involved digestion of the polypeptides with different endoproteinases and purification of the generated peptides on a C18 reverse phase column. Peptides were then subjected to automated Edman degradation and their sequences were confirmed by fast atom bombardment mass spectrometry. Eleven peptides were isolated and sequenced from MMPI and eight peptides from MMPII, accounting for approximately 40 % of the total amino acid content of both proteins. The generation of the complete amino acid sequence of both proteins is in progress. In order to proceed with the immunological characterization of these proteins through use of their recombinant equivalents, the genes coding for MMPI and MMPII are under investigation (in conjunction with Drs. Brigitte Gicquel and Nathalie Winter). The C-terminus region of the gene coding for the 35 kDa protein has been sequenced. Fragments containing the N-terminus and internal region of the gene were identified using oligonucleotide probes derived from peptide sequence, and are presently being sequenced. A region from the 22 kDa gene coding for 50% of the protein was amplified from M. leprae DNA by using oligonucleotide primers derived from the amino acid sequence. We are currently sequencing this fragment and using it as a probe to clone the entire gene. Thus, the major cellular proteins of the leprosy bacillus are close to complete definition. (Work supported by NIH, NIAID Contract NO1 AI-05074.)

MI43

STRUCTURAL DEFINITION OF LIPOARABINOMANNAN: ITS BIOLOGICAL SIGNIFICANCE IN HOST-PARASITE INTERACTION

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All mycobacterial species are endowed with two dominant, highly complex polysaccharides, the mycolylarabinogalactan-peptidoglycan (mAGP) complex and lipoarabinomannan (LAM). Whereas the structure of mAGP is highly conserved, that of LAM varies among mycobacterial species. For instance, in a rapidly growing strain of Mycobacterium, the terminal arabinan ends are occupied with branched hexaarabinofuranosyl and linear tetraarabinofuranosyl arrangements, giving a product now designated as AraLAM. However, the same arrangements are capped extensively with mannose-containing oligosaccharides in strains of Mycobacterium tuberculosis, a product called ManLAM. Most importantly, LAM from Mycobacterium leprae demonstrates a hybrid structure, in that it shares the "naked" arabinofuranosyl arrangements of AraLAM as well as some of the mannose capping typical of ManLAM, and thus it is termed LepLAM.

Work by many collaborators has implicated LAM in a wide spectrum of immunoregulatory functions, such as inhibition of IFN-γ-mediated activation of macrophages, the scavenging of potentially cytotoxic oxygen-free radicals, inhibition of protein kinase C activity and evocation of a large array of those cytokines characteristically associated with macrophages. Thus, it appears that LAM may mediate production of macrophage-derived cytokines which, in turn, may evoke many of the clinical manifestations of leprosy. The relationship of structural features of LAM to these biological properties will be discussed. (This work has been supported by NIH/NIAID Contract No. AI-05074.)

MI44

SUBCELLULAR FRACTIONATION OF MYCOBACTERIUM LEPRAE AND THE SEARCH FOR NEW PROTEINS

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The availability of adequate quantities of armadillo-derived Mycobacterium leprae has allowed the complete characterization of many of its major constituents. However the subcellular location of these is largely a matter of speculation. Hitherto, subcellular fractionation of mycobacteria never did yield a subcellular fraction totally free of the components of another, possibly because sonication resulted in organelle fragmentation. To minimize wastage of M. leprae, pilot studies were conducted on M. smegmatis leading to the conclusion that microbead disruption and sucrose density centrifugation results in less cross contamination of subcellular fractions as determined by assays for membrane specific enzymes (NADH dehydrogenase and lactic dehydrogenase) and 2-D SDS-PAGE of proteins and carbohydrates. Application of the procedure to M. leprae resulted in two cell wall fractions of different specific gravity, cell membrane and cytosolic fractions. SDS-PAGE and Western blot analysis of the fractions revealed significant differences in terms of the various proteins and carbohydrates. The 10 kDa protein was significant only in the cytosolic fraction. The 71 kDa and 18 kDa HSPs and the 28 kDa (SOD) protein were seen in the cell wall and cytosolic fractions but were totally absent in the membrane. The 65 kDa HSP was mostly membrane-associated. LAM was predominant in the cytosolic and membrane fractions with only faint traces in the cell wall fractions. The goal of this work is to identify the major subcellular constituents (proteins, lipids and carbohydrates) of the various compartments of M. leprae towards a better understanding of its physiology and pathogenesis. (Work supported by NIH, NIAID Contract NO1 AI-05074.)

MI45

MYCOBACTERIUM LEPRAE METABOLISM: IN VITRO UTILIZATION OF GLUCOSE, URIDINE 5'-DIPHOSPHOGLUCOSE AND GLUTAMATE

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As a non-cultivable, intracellular parasite, a direct relationship between Mycobacterium leprae growth and specific cell nutrients has not been established. When maintained in vitro with a suitable axenic medium, limited metabolic patterns have been observed in M. leprae by measuring the utilization of radioactive substrates. In previous studies we observed the incorporation of ^{14}C-palmitate into the complex liquids of M. leprae. However, we were unable to detect any incorporation of ^{14}C-acetate into lipid. These results indicate that M. leprae does not synthesize lipids through the de novo, acetyl-CoA pathway, but must depend principally upon host metabolites.

We have recently shown that M. leprae does not readily utilize exogenous ^{14}C-glucose, as measured by the formation of $^{14}C\text{-}CO_2$. These results suggest that the glycolytic and hexose monophosphate pathways may not be functioning in M. leprae. However, M. leprae must synthesize various bacterial polysaccharides such as those present in complex glycolipids. As an alternate source of glucose, we incubated M. leprae in the presence of uridine 5'-^{14}C-diphosphoglucose (UDPG). The results revealed that significant amounts of $^{14}C\text{-}CO_2$ were released in the presence of labelled UDPG.

Organisms which do not glycolyze glucose, may use other pathways as energy sources, e.g. glutamate oxidation. When ^{14}C-glutamate was incubated in the presence of M. leprae, oxidation of glutamate was observed. Oxidation appeared to be enhanced by the presence of pyruvate, thus, indicating possible transaminase activity.

This study suggests that M. leprae has the potential to use host-derived UDPG as substrate for polysaccharide synthesis. Utilizing a high energy nucleotide would probably be metabolically advantageous for the organism. Further studies may also reveal any involvement of the Krebs cycle with glutamic acid oxidation in M. leprae.

MI46

RECENT RESULTS ON *IN VIVO* DRUG EFFICACIES FROM MASS ANALYSIS OF INDIVIDUAL *M.LEPRAE* ORGANISMS

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Laser microprobe mass analysis (LAMMA) of a limited number of individual *M.leprae* organisms allows the determination of the physiological state (viability) of a bacterial population and its changes upon the influence of drugs from the measurements of intrabacterial Na^+,K^+-ratios and the evaluation of mass fingerprint spectra. One of the applications of the method is *in vivo* therapy control. For this, *M.leprae* are isolated for mass analysis from patients' skin biopsies taken at different times during treatment. In principle, the results can be obtained already a few days after arrival of the biopsy specimens. Limitations of the method arise from the fact that not every biopsy contains sufficient numbers of bacteria and from difficulties in connection with the isolation procedure. For cultivable bacterial species a limiting value of the Na^+,K^+-ratio could be determined up to which the bacteria are viable ("limiting value"). Assuming that this limiting value is valid also for non-cultivable species, the degree of correspondence between intrabacterial cation ratios of *M.lepraemurium* treated *in vivo* with various drugs and the ability of the organisms to multiply in mice was examined. A linear relationship between the proportion of viable organisms, calculated from the Na^+,K^+-ratio and that calculated from the ID_{50} was found, suggesting that from measurements of the Na^+,K^+-ratio the effects of drugs can be predicted. The method is particularly useful for the early detection of drug resistance. Surprising results on the influence of certain drug regimens on the phagocytosis of *M.leprae* will be presented and discussed with respect to effectiveness and clearance.

MI47

THE EFFECTS OF RIFAMPICIN AND CIPROFLOXACIN ON MYCOLIC ACID LEVELS IN HUMAN TISSUE DERIVED M.LEPRAE AND ITS CORRELATION WITH VIABILITY

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Rifampicin is a potent anti-mycobacterial drug whereas Ciprofloxacin is a broad spectrum drug also posses anti-mycobacterial activity. The effect of these agents on mycolic acids was investigated and correlated with their killing mechanism(s) in M.leprae. Purified bacilli were incubated in a modified Dubos medium containing these drugs over 14 days. Biopsies without drug served as controls. Mycolates and ATP were analysed by HP-TLC and ATP photometry respectively. The results showed drastic reduction in the level of mycolates which was found directly proportional to decline in ATP level at 5 mcg/ml of Ciprofloxacin and 3 mcg/ml of Rifampicin, where the cells have poorly synthesized methoxy mycolate and other mycolate components. Though the ATP contents were markedly decreased at 5 mcg/ml of Rifampicin, mycolic acid contents were found to remain intact. The biosynthetic as well as catabolic processes are affected by Rifampicin at very high concentrations leading to preservation of cell wall skeleton. In case of Ciprofloxacin, catabolic process seems to be less affected thus leading to continuing degradation process. These observations have therapeutic implications.

MI48

INACTIVATION OF INTERLEUKIN-2 BY THE CULTURE ISOLATE FROM *Mycobacterium leprae* IN OGAWA MEDIA WITH CYTOKINES

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Round, oily, and white colonies were isolated in Ogawa media with interleukin-2 (IL-2) and interferon-γ, after the inoculation of the tissue homogenate of armadillo spleen infected with *M. leprae*. The size of the colonies grew with time and they were the compact and solid collections of acid fast organisms under a microscope (presented in a separate paper at this meeting).

To test whether or not the acid fast organisms forming these colonies inactivate IL-2, the microorganisms from a colony mentioned above were incubated with IL-2 in RPMI 1640 media, and the IL-2 activities were assayed on days 0, 5, 10, & 20 by measuring the CTLL-2 proliferation. Autoclaved microorganisms were used as control.

The results are:

1. The same levels of IL-2 activity were observed on days 0 & 5 in the media with autoclaved or nonautoclaved micororganisms.

2. The IL-2 activity in the media with the autoclaved was 4-8 times higher than that in the ones with the nonautoclaved on days 10 & 20.

These data strongly suggest the possibility that *M. leprae* may interact with and inactivate IL-2, which is essential for the activation of normal defense mechanisms. The immune defects associated with Hansen's disease may be explained partly based on this finding. Receptor assay, subcultivation, identification, and animal studies are under investigation. Furthermore, the effect of various cytokines on the growth of other microorganisms, including uncultivable ones, is also under investigation.

MI49

IN VITRO CULTIVATION OF MYCOBACTERIUM LEPRAE — EXISTENCE OF A GROWTH FACTOR.

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Inability of M. leprae to grow in culture medium in vitro has been a bottleneck in leprosy research. We had reported earlier about the limited growth of M. leprae in DH medium and our inability to achieve subcultures. One reason for this is the accumulation of oxygen radicals in the growth medium and the other is existence of a possible growth factor.

In our study, normal growth of M. leprae in DH medium was obtained when inocula were from livers and spleens of infected armadillos. However, M. leprae harvested from the foot pads of nude mice failed to multiply in the same medium. Even when the inocula were from armadillo lymph nodes or from human biopsy, the growth was much slower. Furthermore, using inocula from livers and spleens of armadillos, gradual decrease in inoculum size resulted in proportionally slower multiplication.

When the DH medium was supplemented with irradiated M. leprae from livers and spleens of armadillos, nude mouse-derived M. leprae exhibited growth in DH medium similar to that obtained with armadillo-derived M. leprae. Similar results were also obtained with cell-free extracts of non-irradiated M. leprae. All these findings point to the possibility of the existence of a growth factor in armadillo-derived M. leprae.

MI50

ANERGY AND MITSUDA RESPONSES TOWARDS CHEMOAUTOTROPHIC NOCARDIOFORM ANTIGENS RUN PARALLEL TO LEPROMIN ACROSS THE LEPROSY SPECTRUM

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Chemoautotrophic nocardioform (CAN) bacteria had been repeatedly isolated from infectious human, mouse-footpad and armadillo leprosy tissues which had been found to share similar/same metabolic, physiological, enzymological, 36K DNA, lipid profile, pathogenicity and other specificities with Mycobacterium leprae. For further studies on their homology with leprosy bacillus, anergy or contrarily, Mitsuda-type responses towards 4 of these CAN - AGs and a control lepromin were tested on 93 LL, TT and borderline cases of leprosy, categorised clinically and bacteriologically. Final results were obtained for 73 cases. The antigens injected per patient varied from a maximum of 5 to a minimum of 2. The suitability standard of the control lepromin was verified first in 4 TT cases where it produced nodules (+++) $\geqslant$10 mm diameter. Complete anergy to CAN - AGs was seen in 92/92 instances tested on 24 LL cases, while the anergy was weakly modified or unmodified in 3 other LL cases which had been vaccinated before. Concurrent studies with the same antigens tested on 33 TT cases showed clearcut, dose-dependent, Mitsuda-type late responses in 80/81 instances which included 3 cases where the control lepromin was omitted. The CAN bacteria, therefore, despite their origin from different unrelated human, mouse footpad and armadillo tissues appeared to be identical with each other and also with the leprosy bacillus, on the basis of these and other parameters. Phenolic glycolipid-I could be demonstrated among these.

OPHTHALMOLOGY

OP1

A LONGITUDINAL FOLLOW-UP STUDY OF EYE IN 649 LEPROSY PATIENTS

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Periodic examination of eyes were done for leprosy patients attending this centre. The condition of eye in 649 patients for a period ranging from 3 to 20 years (mean 8.29) are discussed. Throughout this period, 458 (70.79%) eyes were normal while the rest 191 (29.21%) were affected and majority recovered with treatment. In tuberculoid and borderline patients, lagophthalmos was the only complication. In lepromatous patients, who had monotherapy, except for fleeting scleritis/ iridocyclitis no complications occurred. In lepromatous patients of short duration on MDT, complications were few and subsided with treatment. In lepromatous patients of long duration and in M.B. relapses on MDT, eye complications were more and in some scleritis/ iridocyclitis lasted for 4-6 years. Even in those who were normal at the beginning of treatment, some developed scleritis/irido-cyclitis after 3-5 years. Blindness was mostly due to non-leprosy causes like cataract and corneal ulcer. Lagophthalmos and corneal hyposthesia were the only causes of blindness in tuberculoid and borderline cases. In lepro-matous patients on treatment, blindness occurred only in those with severe pre-existing lesions. Steroid induced cataract led to blind-ness in a few. Early detection of disease, management of reactions and periodic eye examination prevent eye complications.

OP2

CONSENSUAL OPHTALMOTONIC REACTION IN LEPROSY PATIENTS

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The cosensual ophtalmotonic reaction (TOR) describes the phenomenon whereby alterations of the intraocular pressure in one eye is accompanied by a corresponding pressure change in the contralateral eye. It has been postulated that the COR is mediated via a nervous reflex mechanism. In this study the COR was determined in normals and in the leprosy patients with and without ocular involvement. The theory and the potential usage of the COR in the eye clinics will be discussed.

OP3

OCULAR PROBLEMS IN CASES RELEASED FROM TREATMENT

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