ed clinically more susceptible by BCG + HKML. Clinical interpretations were supported by phenolic glycolipid-I (PGL-I) antigen levels in plasma. Highly significant blastogenic responses (to lepromin, Rees antigen, ML 10Kd protein & tuberculin) were seen in all 3 vaccinated RM groups post-vaccination (PV), post-boosting (PB), immediately post-inoculation (PI) with ML and 2 years PI. Similar responses to ML antigens occurred mostly in BCG + HKML groups of SMM PV and PB; suppression of some responses occurred in SMM approximately 2 years PI. Blood lymphocyte subsets with the following phenotypes significantly changed in vaccinated vs unvaccinated ML-challenged SMM and RM: CD2, CD4, CD8, CD16, CD20, CD4CD29 and CD4CD45. Patterns of changes in the subsets differed between the 2 species. Serum IgG vs IgM anti-PGL-I responses supported our prior observations that serum IgG anti-PGL-I responses favor protection while IgM responses correlate with susceptibility to leprosy. Lepromin skin testing confirmed successful long-term immunization of RM and failure to induce significant responsiveness in SMM by these immunizations. SMM immunized with BCG + LDHKML had significantly suppressed lepromin skin-test responses 33 months post-ML challenge. The immune response to vaccination with HKML and/or BCG is complex, multifaceted and dose-dependent. BCG protects individuals who are susceptible to either PB or MB leprosy; BCG + HKML protects PB-prone and heightens susceptibility in MB-prone individuals.

MICROBIOLOGY

MI01

IDENTIFICATION OF SPECIES-SPECIFIC ANTIGENIC DETERMINANTS OF M.LEPRAE IN CULTIVABLE MYCOBACTERIA

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At the present stage of leprology in some countries great efforts are aimed at searches for cultivable mycobacteria having close antigenic relationship with M.leprae so to use them as vaccine and diagnostic preparations in leprosy. With using immunoblotting we studied antigens of mycobacteria isolated from tissues of lepromatous leprosy patients (M.01, M.011) and cultivated in vitro on Shkolnikova's nutrient medium modified with perfluorine decaline as well as antigens of M.lufu, known as highly susceptible to dapsone. For identification following six clones of MAbs to M.leprae antigens obtained from WHO Bank were used: anti-12KDa mc8908-MLO6-A, anti-18 kDa mc8026-L5, anti-36 kDa mc5828-F47-9-36, and mc9215-IIIE9, mc5205-IIH9 and mc2404-IVD8 against three epitopes of 65kDa-protein. For reference, a sonicate of M.leprae isolated from nine-banded armadillos (WHO Bank) was used. The obtained results were following. In antigenic reference-preparation proteins of molecular weights 12, 18, 36 and 65 kDa (IIIE9) were identified. 36 kDA-protein was detected in M.01 and M.lufu, 12 kDa - in M.011 and M.lufu. MAb mc2404-IVD8 actively interacted with 65 kDa in M.01 and M.011. Presence of antigenic determinants specific for M.leprae in mycobacterial strains under study might suggest their antigenic relationship with M.leprae, hence, necessitating further investigation of their biological properties.

MI02

DETECTION OF MYCOBACTERIUM LEPRAE IN NASAL SECRETIONS OF HOUSEHOLD CONTACTS OF MULTIBACILLARY AND PAUCIBACILLARY PATIENTS

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It is generally accepted that one mode of transmission of *M. leprae* is via aerosols originating from infected individuals shedding *M. leprae* through the nose. Workers have reported either acid-fast bacilli (AFB) or PCR-positive nasal secretions from MB and PB patients. To better understand the risk of exposure to *M. leprae* from index cases we evaluated nasal carriage rates among household contacts (HHC) of MB and PB cases in Cebu, PI. Two hundred and forty-two HHC of both treated and untreated MB and PB index cases were enrolled in the study. Evidence of *M. leprae* in secretions by PCR was found in 8 of the 242 HHC (3.3%). Two of 8 of the contacts were positive from both nasal swabs and their serum tested positive for PGL-I antibody. All PCR-positive HHC were at least 18 years old and were contacts of either LL or BL index cases. PCR-positive contacts lived with their index cases between 2-21 years. Whereas 2 index cases of the positive HHC were untreated LL's, the remaining index cases had either finished WHO-MDT or were currently under treatment with the same or a combination of rifampin and ofloxacin. All contacts of PB disease tested negative by PCR. Further serial testing of nasal secretions from contacts is warranted in an attempt to distinguish a true carrier state from transient contamination.

MI03

USE OF PCR MEDIATED AMPLIFICATION OF MYCOBACTERIUM LEPRAE IN DIFFERENT TYPES OF CLINICAL SAMPLES AND SEROLOGY IN LEPROSY PATIENTS AND CONTACTS.

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The purpose of this study is to determine the usefulness of different clinical samples obtained from skin lesions with slit-skin smears before and after biopsy, ear lobes and nasal swabs together with serum samples in different stages of the disease and compare the results of each group with the less sensitive conventional methods.

Samples were obtained from 54 leprosy patientos (42 multibacillary and 12 paucibacillary) and 37 contacts (12 household contacts and 25 leprosarium staff members). The smear and histopathological stainings were done by the classical methods. The PCR primers used were S-13 and S-62 (Hartskeerl et al.1989) that amplify specifically the 530 bp fragment of the proline-rich (pra) gene of M.leprae. The serological assays include detection of antibodies to phenolic glycolipid I (PGL-I), LAM-B and protein antigens by enzyme-linked immunosorbent assays.

The results reveal that PCR was more sensitive in detecting M.leprae in biopsy and slit-skin smears specimens with no or low bacterial loads than the conventional microscopic examination and that the serological assys also correlated well with decrease of the bacterial index (BI) suggesting their usefulness for following leprosy patients responses to therapy.

MI04

A COMPARATIVE STUDY OF THE DETECTION OF Mycobacterium leprae BY PCR-BASED METHODS

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Two "freezing-thawing" methods were used to extract genomic DNA from M. leprae and compared. The primer combination of R1 & R2 and C6 & C7 was chosen for subsequent amplification by PCR. PCR reaction volume was reduced proportionally to 50, 20 and 10ul from the original volume of 100ul. Two PCR amplification methods (Woods and Cole, 1991; Jamil et al., 1994) were compared by using the purified M. leprae genomic DNA. Both methods were able to detect M. leprae in human skin punch biopsy samples with at least BI = 3.0 and MI = 0.2, microscopic density count = 2+, and viability count = 1/85. Both methods had also detected M. leprae DNA in human skin punch biopsy samples that were kept at -20°C for up to 7.5 years. Of 46 biopsy samples from leprosy patients, 45 were tested positive by the method of colorimetric OTN PCR. They had BI from 0.7 to 5.6 and MI from 0 to 24.3; and their homogenates had microscopic density counts from 0 to 5+ and viability counts of 0/100 to 9/91. Of 33 biopsy samples collected from household contacts (with suspicious skin lesions) of leprosy patients, 19 were positive by colorimetric OTN PCR. This study showed that the bacterial suspension from human skin punch biopsy could be used as an alternative material in colorimetric OTN PCR, if the skin biopsy sample was not available. Finally, no correlation was found between the colorimetric OTN PCR and IgM-phenolic glycolipid (PGL) ELISA.

MI05

IDENTIFICATION OF STRAIN VARIATION IN M.LEPRAE BY AMPLIFIED RIBOSOMAL DNA FINGER PRINTING.

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With the widespread use of MDT, there have been major changes in the epidemiology of leprosy. However, continued high incidence rates and persistent foci of disease point to the need of developing techniques for eliciting strain variation for studying the transmission of disease. Our earlier observations and data published by others suggested some strain differences within M.leprae. In this study different primer combinations targetting ribosomal gene region have been tested for eliciting these differences. Biopsies from different types of leprosy cases attending the OPD of our Institute belonging to different geographical locations were collected. Nucleic acids were extracted and fractionated by a physiochemical technique being used at our laboratory. Different stretches of rDNA gene region were amplified, restricted with different enzymes and probed with rRNA probes by our earlier published methods. One fragment encoding most part of 16S rRNA and spacer sequences has been finally selected as this showed maximum variation. Using this strategy different subgroups within M.leprae have been identified. Results indicate that amplified rDNA-RFLP analysis can be used as a tool for molecular epidemiology of the leprosy.

MI06

DETECTION OF MYCOBACTERIUM LEPRAE BY POLYMERASE CHAIN REACTION IN NASAL SPECIMENS OF LEPROSY PATIENTS AND HOUSEHOLD CONTACTS

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Several Hundred new cases of leprosy are diagnosed annually in the United States. The route of transmission is hypothesized to be by aerosolization of bacteria present in the nasal cavity. To investigate nasal carriage and transmission of M. leprae, nasal secretions were collected serially from patients, contacts, and controls. Samples were analyzed by standard microscopic technique to detect acid-fast bacilli. In addition, nasal samples were analyzed by a M. leprae specific PCR assay.

The test is based on the amplification of a 360-base-pair region of an 18-kDa protein gene of M. leprae. Use of a molecular assay with enhanced sensitivity and specificity will allow detection of M. leprae nasal carriage in individuals potentially with subclinical infection as well as patients with clinical disease. Preliminary results from 21 patients and 28 household contacts have been PCR negative.

To our knowledge, this is the first study that will provide data concerning carriage and transmission of leprosy in a non-endemic area.

MI07

A COMPREHENSIVE STUDY ON PCR FOR DETECTING INFECTION WITH M. LEPRAE

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Authors have conducted a comparative analysis of nested primer gene amplification assay (NPGAA), PCR basing on ML gene coded 65kd (ML-PCR) and PCR basing ML gene coded 16s rRNA (16s rRNA-PCR) for detecting infection with M. leprae (ML). In view of sensitivity, specificity, time consuming, substrate saving and practical value, ML-PCR was finally selected as first choice.

Considering ML-PCR was the best one of three PCRs established by us, it was futher used for studying on the optimum conditions and the factors influencing sensitivity of ML-PCR. The results indicated that 1) the sensitivity of ML-PCR was increased 100 folds when this PCR's denaturation and annealing temperature to be changed into 91 °C and 61 °C respectively, 2) the repeated freeze-thawing is the best method to release DNA from ML several tests used in our laboratory, 3) MI and BI were positively correlated with the detection rate of PCR (DRP), 4) the DRP was higher in slit-skin scrapings than those in skin lesion biopsies of leprosy, and 5) the DRP was decreased after chemotherapy in mice and nude mice.

The authors concluded that 1) although all the mentioned PCR tests were highly sensitive and specific, comprehensitive comparative analysis indicated that ML-PCR may be qualified as a representative method for detecting infection with ML, 2) although PCR technologies were advanced and their parameters basically were definited, it is still in need of studies on optimization, 3) DRP was influenced with many factors, for example, DNA amount releasing from bacilli, viability of bacilli, chemotherapy and modes of taking specimens from leprosy patients.

MI08

IMMUNOSTIMULATORY "CpG" MOTIFS IN THE DNA OF M. leprae

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It has recently been shown that the immune system is capable of responding to oligonucleotide motifs which are present in bacterial DNA. These motifs contain "CpG" dinucleotides at their core. Since mycobacterial DNA is GC rich one would predict that such motifs would be relatively common and hence that mycobacterial DNA should have powerful adjuvant properties. We have studied the immunostimulatory effects of synthetic oligonucleotides based on mycobacterial "CpG" motifs in the priming of the immune response.

Because dendritic cells are the principal antigen presenting cell in peripheral tissue, we have studied the response of primary or conditionally immortalized dendritic cells following exposure to synthetic oligonucleotides based on mycobacterial sequences. We have detected the specific induction of cytokine genes including IL-12, IL-6 and interferon alpha/beta after exposure to such DNA. One of these motifs is present in the repetitive sequence which is found in multiple copies in the M. leprae genome. We have also shown that co-immunisation of mice with purified mycobacterial proteins and immunostimulatory oligonucleotides induces a strongly TH1-dominated response to the protein.

These results emphasise that the powerful immunostimulatory properties of mycobacteria are not confined to the cell wall, but also extend to the mycobacterial genome. Such properties are likely to play a key role in the immunopathogenesis of leprosy.

MI09

PCR ASSAY FOR THE DETECTION AND IDENTIFICATION OF *M. leprae* IN PATIENTS IN THE UNITED STATES.

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Background: The differentiation of leprosy from other cutaneous granulomatous diseases is routinely based on characteristic histopathologic features and the demonstration of *M. leprae* by acid-fast staining. Increased ascertainment of other mycobacterial infections in the skin has made this task more difficult, but the distinction remains fundamental in selecting appropriate treatment.
Design: Experience with formalin-fixed & paraffin embedded tissues, frozen tissues, and tissue lysates referred for *M. leprae*-PCR during the past four years has been reviewed. PCR was performed using primers and probes previously developed (TPG & DLW) to amplify a 360 bp fragment of the gene for an 18 kD protein of *M. leprae*.
Results: Among 32 biopsies, PCR was positive in 8/16 diagnosed as leprosy, and in 0/13 diagnosed as non-leprosy by histopathologic criteria. PCR also identified *M. leprae* in 1/3 biopsies in which acid-fast organisms were seen but leprosy was not diagnosed histologically.
Conclusion: In a non-endemic population, the sensitivity and specificity of this technique recommend its use primarily to identify *M. leprae* when acid-fast organisms are discernable but atypical clinical or histopathologic features obscure the diagnosis.

MI10

THE PROTEOME OF *Mycobacterium leprae*.

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Definition of the genome of *M. leprae* has been matched by similar progress in defining the *in vivo* expressed proteins. The genome project identifies open reading frames, i.e., genes for potential proteins identified as such by comparison with genes in other organisms. However, the proteome project identifies actual protein end-products that govern the immunogenicity and pathogenesis of the organism and can provide antigens for assay development. The approach involves the separation of highly purified, armadillo-derived *M. leprae* into its subcellular components (cytosol, membrane, and cell wall). The proteins of these fractions are separated by 2-D PAGE and selected products excised for in gel digestion with site-specific proteases. The resulting peptides are resolved by reversed-phase HPLC, directly analyzed by electro-spray mass spectrometry, and the molecular ions of these peptides searched against existing databases, thus allowing for identification of the whole protein by way of matches to calculated molecular ions of theoretical peptide fragmentation patterns. To date, about 50 major expressed proteins have been identified, some previously recognized, for instance: 65 kDa/GroEL-2; 10 kDa/GroES; 28 kDa/Sod A; 18 kDa heat-shock protein; 18 kDa/ MMP II/bacterioferritin; 35 kDa/MMP II; antigens 85 A,B,C; L7/L12 ribosomal protein; CysA/thiosulfate sulfur transferase/rhodanase; AphC/alkyl hydroperoxide reductase; the protein elongation factor EF-Tu; a homolog of the MtrA response regulator; and a 34 kDa pseudolipoprotein. Recombinant forms of these are being generated to provide a new array of tools applicable for study of the immunopathogenesis of leprosy and for diagnosis. Supported by NIAID/DMID Contract NO1 AI 55262.

MI11

SECRETED PROTEINS OF *MYCOBACTERIUM LEPRAE*

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In mycobacteria secreted proteins represent a distinct group, probably of particular importance for immune responses following infection. Quantification of individual proteins in culture fluid and corresponding disrupted washed bacilli permits determination of a Localization index (LI) which is essential for identification of secreted proteins. LI is >5 for soluble secreted proteins and close to 0 for known cytoplasmic constituents like heat shock proteins. This procedure cannot be applied for *M. leprae* since secreted proteins are lost during isolation of bacilli from tissues. Occurrence of the secreted antigen 85 in *M.l.* was first shown by formation of corresponding antibodies in armadillos with systemic infection after inoculation with *M.l.* (Wiker, Int Arch Allergy 81:307, 1986). We have now compared DNA sequences of the cloned secreted proteins of *M.tuberculosis* with the available sequences of *M.l.* The close proximity between the *85a* and *mpt51* homologues also occurs in *M.l.*, the distance being 185 and 167 base pairs (bp) in *M.tb* and *M.l.* respectively. Additional sequences are available in *M.l.* corresponding to the *85b*, *85c*, *mpt32* (*apa*), and *erp* genes of *M.tb*. All contain typical signal sequences for secretion in *M.l.* In *M.tb*. the distance between *85b* and *mpt32* is 26,178 bp with *mpt32* on the complementary DNA strand. In *M.l.* the corresponding genes are located on the same cosmid, B38, with a similar distance of 23,032 base pairs, and again on different DNA strands. The 24.1 kDa lipoprotein of *M.tb*. also has a homologue in *M.l.* The genomic organisation of the genes coding for secreted proteins is thus very similar in *M.l.* and *M.tb*, the homology being higher for the mature polypeptide chains than for the corresponding signal peptides.

MI12

PREPARATION AND APPLICATION STUDY OF α 2 ANTIGEN OF MYCOBACTERIUM LEPRAE

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The α antigen gene is one of the dominant mycobacterial proteins that are secreated from the mycobacteria. One of the most interesting features of α antigen is antigenicity against T and B cells in patient infected with Mycobacteria. The authors have focused on α antigen as antigens for serodiagnosis of leprosy because of their antigenitity.

In the course of this study, the authors have constructed the genomic library of M. leprae Thai 53 strain, cloned a new α antigen gene with a plaque hybridization method using DNA fragment of M. leprae α 1 antigen as probe which was characterized in the previous study, the authors termed it as α 2 antigen gene. The α 2 antigen gene has been characterized by sequencing. From the results, α antigen of M.leprae have turned out to be members of gene family like M. bovis, BCG and M. tuberculosis. By comparing of the deduced amino acid sequence of α antigen with 85 complex antigen of other mycobacteria, the authors have found the homology as 74.3% - 85%.

MI13

PROSPECTS OF USING M.LUFU IN LEPROSY STUDIES

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Our previous investigations demonstrated a number of common biological properties in M.lufu, M.leprae and cultivable mycobacteria isolated from tissues of leprosy patients and designated as M.01 and M.011. The mentioned mycobacteria showed comparable indices of their sulphone susceptibility (MIC 1,1 and 4 mkg/ml, respectively). With using different methods, such as precipitation reactions, ELISA and tests on sensitized guinea pigs it was found out that M.leprae, M.lufu, M.01 and M.011 shared common antigens and had similar protein spectra. Intracutaneous injections of M.lufu, M.01 and M.011 in to mice 28 days before M.leprae inoculation resulted in a marked protective effect (the highest degree of protection (log10 average harvest in controls minus log10 average harvest in test groups) was 2,96 for M.lufu, 2,26 - for M.01 and 2,73 - for M.011. Blood samples from 158 subjects including leprosy patients along the whole spectrum of the disease, patients with other infectious diseases and healthy donors were investigated in ELISA and it was found out that preparations made of M.lufu and M.leprae had antigens interacting with specific antibodies from blood samples of leprosy patients suggesting antigenic similarity of M.lufu and M.leprae. A comparison between test-systems developed on the basis of antigens of M.lufu and M.leprae showed comparable

specificity (98,5% and 97,0%) and effectivity (98,6 and 97,2%) of both tests at 100% sensitivity. The results obtained appear to be rather interesting and promising for further development of diagnostic and vaccine preparations based on M.lufu.

MI14

Gene Vaccination for *M. leprae* hsp65 using plasmid DNA carrying Immunostimulatory Sequences.

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Recently it has been popular using DNA vaccines. The method is more easier to manufacture, provide prolonged antigen expression and when co delivered with costimulatory molecles, enhance the subsequent response to the DNA encoded antigen compared with conventional protein vaccines.

We constructed the recombinant DNA for expression of *M. leprae* hsp65 using pACB which carry two immunostimulatory DNA sequences located within ampicillin resistance gene. The BALB/cA mice vaccinated with the recombinant DNA were sacrificed for analysis of immune responses against *M. leprae* by splenocytes and macrophages cultivation. IFN-γ was produced from splenocytes culture of the vaccinated mice, and enhanced by co-cultivation with *M. leprae* lysate or hsp65 *in vitro*.

This result suggest that hsp65 is useful protein antigen against defense for *M. leprae* infection, and this method is a good technique for development of vaccines.

MI15

DEVISING A MORE PERFECT LEPROSY VACCINE

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The basic problems of immunity in LL and non-TT cases of leprosy are highlighted by defects in the cell mediated immunity, a progressive hyperbacillimea, a harmful persistent immune-complex state which actually protects the lepra bacilli, rather than killing them, a macrophage granuloma reflecting a surrender to the leprosy bacillus, and massive bacillary invasion of almost all tissues and organs. In theory and practice, experimental evidences show that immunological unresponsiveness of LL cases to leprosy bacillary antigens can be partly or wholly changed to a positive response by various permutations and combinations of BCG, other mycobacteria and leprosy bacillus, modulated by immunoboosters. Such responses may also be long lasting. The immunological failure reflected by lepromin anergy, immune complex and macrophage granuloma formation, may be corrected substantially, firstly by reducing the bacterial load using chemotherapy and subsequently various combinations of immunotherapy ; this will help achieving lepromin positivity and conversion of the cases by recruiting appropriate subsets of the T-cells. An in vitro grown killed culture of the leprosy bacillus will be the most suitable ingredient of all combination vaccines. Our studies show that these in vitro cultures (CAN bacteria) have same immunological specificity as the leprosy bacillus. Selective chemotherapy is a suitable adjunct based on in vitro sensitivity tests. As of today, all studies indicate that the CAN bacteria are in vitro cultivated forms of leprosy.

MI16

IN VITRO DETERMINATION OF ANTIMICROBIAL SUSCEPTIBILITY OF *MYCOBACTERIUM LEPRAE* BY THE MICROPLATE ALAMAR BLUE ASSAY

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The Alamar Blue reagent, an oxidation-reduction indicator, was used to assess antimicrobial activity of drugs against the non-cultivable *M. leprae* in axenic medium in 96-well plates. 10^7 *M. leprae*/well were exposed to antimicrobial agents for 1-2 weeks at 33C under ambient or microaerophilic conditions. Alamar Blue reagent was added and the reduced form of the dye was measured fluorometrically after 48 hours incubation and compared to that of drug-free cultures. Marked dose responses were observed to rifampin and clarithromycin, both effecting a significant reduction in fluorescence at 39 ng/ml. Dapsone was also active at this concentration while sparfloxacin, minocycline and ofloxacin showed significant inhibition at 62.5, 125 and 500 ng/ml respectively. Alamar Blue is an inexpensive, non-toxic dye which can be used to rapidly determine the viability and drug susceptibility of *M. leprae*.

MI17

A NEW RAPID METHOD TO MEASURE RIFAMPICIN RESISTANCE IN *MYCOBACTERIUM LEPRAE*

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Rifampicin is the key bactericidal drug in multi-drug therapy (MDT) for leprosy. Resistance to rifampicin is rare in leprosy and measurement of rifampicin resistance is dependent on the mouse foot pad assay which is rarely applicable to the detection of resistance in patients relapsing after MDT. The mutations of the *rpoB* gene associated with rifampicin resistance in *Mycobacterium leprae* have been identified and employed in a novel line probe assay. PCR amplification of the *rpoB* gene from skin biopsies from reactivated/ relapsing leprosy patients was followed by hybridisation to oligonucleotides complementary with the wild-type gene and with 5 of the point mutations which account for 70% of the recognised mutation in rifampicin resistant leprosy. Binding to oligonucleotides was visualised by binding streptavidin-alkaline-phosphatase to the biotin labelled PCR product and incubating with chromagen.
The application of this test to the detection of rifampicin resistance and preliminary results from relapse patients in India and Nepal will be presented.

MI18

THE DIHYDROPTEROATE SYNTHASE OF *MYCOBACTERIUM LEPRAE* AND DAPSONE RESISTANCE

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Dihydropteroate synthase (DHPS), encoded by *folP*, is a key enzyme in the folic acid biosynthesis pathway of bacteria which catalyzes the condensation of para-aminobenzoic acid (PABA) and 7,8-dihydro-6-hydroxymethylpterin-

pyrophosphate to form dihydropteroate. Sulfonamides are a class of drugs that act as substrate analogues competing with PABA and thereby, exerting their antimicrobial effect. Dapsone (DDS), a sulfonamide analogue, is an effective antileprosy drug; however, resistance to this drug is quite high in some regions of the world with the potential for undermining current control strategies for leprosy. The mechanism of DDS resistance in *Mycobacterium leprae* is unknown, however, it is thought to be associated with DHPS. We have previously cloned the putative *folP* from a DDS-susceptible strain of *M. leprae* into a DHPS temperature sensitive mutant of *E. coli* (MC4100ts3) and showed that expression of this gene product complemented the folate pathway at 42°C. Three possible mechanisms of DDS resistance directly associated with DHPS in *M. leprae* were investigated: 1) missense mutations in *folP* leading to an altered DHPS, 2) *folP* gene amplification leading to overexpression of DHPS and 3) mutations in promoter-like regions upstream of *folP* which may up regulate DHPS. Comparison of *folP* sequences from DDS-resistant and DDS-sensitive *M. leprae* strains showed no differences. Restriction fragment length polymorphism analysis using a *folP* probe and *Stu*I digests of chromosomal DNA from DDS-resistant and DDS-susceptible strains of *M. leprae* showed that one copy of the *folP* gene was present in all strains, indicating that gene amplification was not a mechanism for DDS resistance in *M. leprae*. Comparison of promoter-like regions upstream of *folP* from DDS-sensitive and resistant strains showed no sequence differences. Therefore, these data suggest that resistance of *M. leprae* to DDS is not directly associated with overexpression of *folP* based on gene amplification, altered promoter activity or an altered structural *folP* gene in the DDS-resistant strains analyzed in this study.

MI19

RIFAMPICIN RESISTANT *MYCOBACTERIUM LEPRAE* ISOLATED FROM JAPANESE PATIENTS AND MUTATION IN *rpoB* GENE

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Many rifampicin resistant *M.leprae* have been detected and threaten the effective leprosy control. Genetic method might be useful for the early detection of resistant bacilli, since the mutation in the *rpoB* gene confers the rifampicin resistance. Two rifampicin resistant *M.leprae* were isolated from Japanese patients and nucleotide sequence of *rpoB* gene was deduced to clarify the causative mutation in the gene. Usefulness of the simple method for the detection of mutation was also apprised.

The nude mice were inoculated with 8.0×10^5 or 1.0×10^4 bacilli into the foot fad and fed on diets containing with rifampicin in the concentration of 0.01gm per 100gm of diet from 2months after infection. Bacillary growth in the rifampicin treated mice was compared to that in the untreated mice by 55 weeks after infection. The susceptibility to drugs was examined by mouse foot pad method or Buddemeyer system. One isolate was resistant to rifampicin, KRM 1648 and DDS. The mutation in the codon for serine-531 which resulted in the substitution to leucine was detected as in most resistant cases so far reported. Markedly different banding pattern in PCR-SSCP was depicted between wild type and the resistant isolate. Heteroduplex analysis revealed the two bands in the heterogeneous mixture of 305bp PCR products and single band in the homogeneous one. Another isolate was considered to be low resistant to rifampicin, however confirmation is in progress. The codon for glycine at 524 was changed from GGC to GGT, but no change in amino acid, silent mutation, was shown. Other point mutations were revealed in other regions of the gene. It is concluded that some genetic methods are applicable for detecting mutation in the gene, which confers rifampicin resistant, but susceptibility for the rifampicin must be confirmed by other methods such as sequencing.

MI20

HOW *M.LEPRAE* DEFEATS A DEFENSE MECHANISM OF THE HOST

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Mycobacterium leprae possesses a highly active enzyme that rapidly hydrolyses lysophospholipids. Lysophospholipids generated by host tissues are extremely reactive molecules that can lyse cell membranes and kill pathogenic organisms. The cell membranes of both prokaryotes and eukaryotes are mainly made up of phospholipid bilayers. Phospholipases that degrade phospholipids are classified according to the site at which each enzyme acts on the substrate molecule. Phospholipase A_2 (PLA_2) comprises a diverse family of enzymes that cleave the *sn*-2 fatty acylester bond of glycophospholipids to yield a fatty acid and a lysophospholipid. PLA_2 has a central role in generating arachidonic acid that leads to the formation of thromboxanes and prostaglandins. We detected lysophospholipase in *M.leprae* by a sensitive radioisotopic method. Optimum activity was at 37°C and at pH 6.0. Temperatures above 70°C completely inactivated the enzyme. The compound AACOCF3, a trifluromethylketone analog of arachidonic acid, inhibited the activity. The inhibition appeared to be of the uncompetetive type. The K_m of the enzyme was 2.5×10^{-4}M, suggesting a fairly strong affinity for the substrate. Lysophospholipids have been shown to be microbicidal to invading organisms. Possession of lysophospholipase by *M.leprae* apparently is one of the mechanisms which enables the bacilli to survive and proliferate in the phagocytic cells of the host.

MI21

STRUCTURAL ANALYSIS OF THE HIGH MOLECULAR MASS PENICILLIN-BINDING PROTEIN 1 OF MYCOBACTERIUM LEPRAE

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Two high molecular mass multimodular penicillin-binding proteins (PBPs) of class A have been previously identified from the collection of ordered clones of Mycobacterium leprae, produced and characterized in Escherichia coli. PBP1 behaves as a PBP of low penicillin affinity. Deletion of the N-terminal amino acids suggested that a region between residues 39 and 81 probably functions as a membrane association site. Deletion of the C-terminal 164 amino acids did not affect membrane association. Deletion of the non-penicillin -binding (n-PB) module led to production of a truncated PBP localized in inclusion bodies. The PBP was solubilized with guanidine-HCl and refolded in a renaturation buffer. Refolding was confirmed by measuring intrinsic fluorescence of unfolded and refolded forms. The truncated PBP showed kinetic properties similar to the intact PBP. This is the first report of a class A PBP retaining penicillin-binding activity after removal of the n-PB module. This PBP appears to be a low-affinity penicillin target unique to mycobacteria; since a protein with amino acid sequence similarity has recently been identified in a cosmid of M. tuberculosis. The fact that it retains activity as a soluble protein carrying the PB module only, should facilitate future X-ray crystallographic studies on this target.

MI22

SOME BIOCHEMICAL FEATURES OF THE INTERACTION OF *MYCOBACTERIUM LEPRAE* WITH LAMININ. Marques MAM, Brennan PJ* and Pessolani MCV. Laboratório de Hanseníase, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. 21045. *Department of Microbiology, Colorado State University, Fort Collins, CO. USA. 80523

The neural tropism of *M. leprae* has been recently attributed to the specific binding of the leprosy bacillus to laminin 2 (LN2), a LN isoform preferentially distributed on the Schwann cell basement membrane (Rambukkana et al., Cell 88: 811-821,1997). However, the molecule on *M. leprae* surface responsible for laminin binding is still unknown. In order to characterize the laminin receptor of the leprosy bacillus, we investigated several biochemical features of *M. leprae*-laminin interaction. By using microtiter wells coated with the bacilli, we confirmed the capacity of soluble LN2 to bind to *M. leprae*. Enhanced binding capacity of laminin was

observed with purified cell wall of the bacilli, indicating a surface location for this receptor. Also, binding of LN2 to *M. leprae* was reduced by treatment of the bacterial cells with proteinase K or trypsin, suggesting that the binding to this basement membrane protein is mediated by bacterial surface proteins. Since phenolic glycolipid I (PGL-I) is abundantly present on the surface of *M leprae*, we also investigated a possible role of this molecule on the interaction of LN2 with the bacteria. Preincubation of LN2 with PGL-I resulted in significant enhancement of LN2 binding to the bacteria. These preliminary results suggest that LN2 binds to proteins located on the surface of *M. leprae* and that PGL-I enhances the binding of LN2 to these proteins. Three independent approaches are currently being undertaken in order to isolate the laminin receptor(s) from bacterial extracts: i) far western (gel-overlay), ii) laminin affinity chromatography, and iii) immunoprecipitation of laminin-receptor complexes.

Financial support: CNPq and NIH

MI23

STRUCTURAL CHARACTERIZATION OF THE CELL WALL OF *Mycobacterium leprae*.

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Mycobacterial cell wall polysaccharides include cell wall arabinogalactan (AG) and peptidoglycan (PG). AG is esterified at the distal ends with mycolates and covalently linked to a peptidoglycan layer at the reducing terminus. In our effort to address the structures of these molecules in *M. leprae*, walls of *M. leprae* were purified on a discontinuous sucrose density gradient. Purified cell wall cores were subsequently treated with mild base to remove bound mycolates and mild acid to release PG from the covalently linked AG. The mild acid-solubilized AG was fractionated using size exclusion column chromatography and analyzed using chemical derivatization and mass spectrometry.

We have also undertaken the formidable task of digestion of peptidoglycan (PG) of *M. leprae* , since mycobacterial PG are known to be notoriously resistant to lysozyme. The acid-released peptidoglycan is reduced and subjected to degradation using several methods (acid hydrolysis, mutanolysin digestion, trifluoromethane sulphonic acid treatment). We hope to assign the primary structure of *M. leprae* PG using an integrated approach of LC/ESMS (electrospray mass spectrometry). A comparative study is also carried out using *M. tuberculosis* cell wall in order to address subtle differences/modifications between the cell wall components of these two mycobacterial species.

We are also studying the non-covalent bound cell envelope polysaccharides of *M. leprae*, such as the secreted arabinomannans, glucans and lipoarabinomannans, by examining polysaccharides isolated from infected and non-infected armadillo livers.

(The research is supported by NIH, NIAID/DMID Contract NO1 AI 55262).

MI24

COMPARISON OF ASSESSMENT OF VIABILITY BY NORMAL MOUSE FOOT PAD, ATP MEASUREMENT AND PCR ASSAYS.

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Various techniques for the estimation of M.leprae viability have been described. Mouse foot pad (MFP) is one of the old and main techniques for this purpose. Besides MFP, bacilllary ATP measurements and PCR assay have been used in this study to determine the viability. In this investigation, an effort has been made to compare the usefulness of these techniques to assess the therapeutic effect of a newer drug regimen comprising of conventional drugs as well as newer drugs like ofloxacin & minocycline in smear positive MB cases in the beginning and after completion of one year of treatment. Biopsies were processed for viability assessment by normal mouse foot pad (Shepard et al), bacillary ATP measurement (Katoch et al) and PCR assay (Williams et al) by the techniques established at this Institute. Comparison of results by these techniques in untreated as well as treated patients suggests overall good usefulness of MFP & ATP measurement in assessing the effect of the MDT including this regimen. ATP assay has been found to be more sensitive in cases with low bacillary load. PCR assay appears to be suitable only for knowing the trends.

MI25

IDENTITY OF THE LEPROSY BACILLUS WITH CAN BACTERIA

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The CAN bacteria or chemoautotrophic nocardioform bacteria (CANb) had been reproducibly cultivated and propagated continuously from human, mouse footpad and armadillo leprosy tissues ; these had been repeatedly isolated. The CANb, like the leprosy bacillus (LB) are non-cultivable on any of the known/acceptable media, but grow only on chemically defined media containing $(NH_4)^{+2}$, liquid paraffin, urea etc. These have an identical mycolate profile, long generation time (Ca. 44 hr), nocardioform, weak acidfastness, identical mouse footpad pathogenicity, lepromin response(s) across the LL-TT spectrum and presence of PGL-I. Its possession of collagenase and gelatinase further identifies it with the LB. Its guanine auxotrophy possibly explains it s selective preference of the macrophage nucleus for scavenging it. Multiple molecular biological parameters applied on the CANb viz-a-viz the LB show these as two converging on identical points.

MI26

GRAM-POSITIVE COCCOID MICROORGANISMS CULTIVATED FROM THE HANSEN'S DISEASE PATIENT SPECIMENS, AND M. LEPRAE.

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Since Hansen, G.A., after observing the Hansen's disease (HD=Leprosy) bacillus in the lesions, made the first unsuccessful attempts to cultivate it outside the host, as reported by Terni (1950) in a long time back, a great number of research workers have claimed that they have succeeded while others even now are doubtful that it has been accomplished.

It is said that the microorganisms most commonly isolated and known as the results of pioneer research works of leprosy bacilli are included in following categories: (a) strains belonging to diphtheroid bacilli which were either non-acid-fast or only weakly acid-fast, (b) chromogenic acid-fast bacilli, (c) non-chromogenic acid-fast bacilli and (d) anaerobic bacilli or actinomyces.

Amongst such microorganisms, the Gram-positive coccoid organisms are of compelling interest as observed and examined by Delville (1973) and Chatterjee (1976). The organisms are easy to be stained with Gram's method and to be cultured on media. From the bacteriological observations of their properties, they may be seen at a glance as the so-called contaminants or various bacilli, though they show a cyto-morphological change in state from cocci to rod — from rod to cocci.

The present report is a result scrutinized the bacteriological observations of the Gram-positive coccoid microorganisms cultivated from the HD patient specimens and the bacteriological mutual relation to HD bacilli varied from Gram-positive to Gram-ghost or Gram-neutral and examined as the acid-fast bacilli stain red.

MI27

IN VITRO CULTIVATION of *Mycobacterium leprae* with INTERLEUKIN-2

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I reported that colony forming acid-fast organisms are isolated from armadillo spleen and nude mouse footpad infected with *M. leprae* and that these two organisms maintain their multiplication in 3% Ogawa media containing recombinant human interleukin-2 (IL-2).

Continued cultivation trial showed that these two organisms maintain their IL-2 dependency up to 15th and 16th generation, in case of armadillo and nude mouse originated *M. leprae*, respectively.

Next step of experiment was to identify these organisms with polymerase chain reaction (PCR) using *M. leprae* specific primers C & D and primers L1-L4 (nested PCR). These showed that the template of armadillo *M. leprae* DNA prepared at Colorado State University, USA, the template of nude mouse *M. leprae* prepared at National Institute for Leprosy Research, Japan, the 11th generation isolated from nude mouse at my laboratory, and the 10th generation isolated at my laboratory from armadillo spleen infected with *M. leprae*, all these 4 templates amplified the expected and the identical sizes of DNA fragments (372bp in case of primers C & D; 347bp with primers L1-L4).

Quantitative PCR showed that 10^3 fold multiplication of the 11th generation (isolated at my lab from a nude mouse footpad infected with *M. leprae*) for 2 months after the inoculation. The control culture tube without IL-2 showed 10^{-2} fold for the same time period.

Also, the *M. leprae* template prepared with the 10th generation culture isolate from armadillo spleen amplified a 390bp DNA fragment in a PCR using γ3 & γ10 primers specific for human IL-2 receptor γ chain gene. This PCR product was sequenced successfully and further studies such as cloning and the DNA hybridization are under investigation to identify the full IL-2 receptor gene sequence in *M. leprae*.

In conclusion, *M. leprae* is successfully cultivated in Ogawa media with IL-2.

MI28

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 thyroxine containing Dubos medium, have produced a visible colony on the surface of L.J. slant during 8-16 weeks of incubation at 37°C. The enhanced growth of this recalcitrant organism is due to stimulating effect of thyroxine as well as supply of readymade basic nutrients in the Dubos medium. Intradermal inoculation of 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 of M.leprae, may open out a new era in preparation of purified vaccine, production of specific monoclonal antibody and study of in-vitro drug sensitivity, and as such ensure rapid eradication of leprosy.

MI29

INHIBITION OF METABOLISM AND GROWTH OF *MYCOBACTERIUM LEPRAE* BY GAMMA-IRRADIATION

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Mycobacterium leprae is an extremely slow growing organism, having an in vivo doubling time of ~12.5 days. In vitro, it is uncultivable on artificial medium, but viability can be maintained, without multiplication, for a limited time. Traditionally, incubation at 100°C or autoclaving has been used to kill *M. leprae* for experimental use. Both of these methods cause extensive denaturation and damage to the bacilli. In this study, we evaluated gamma-irradiation (γ-rad) as a means to kill this slowly growing organism. Freshly harvested, viable athymic *nu/nu* mouse-derived *M. leprae* were exposed to varying doses (10^2 to 10^5 Rad) of γ-rad in a Sheppard Model 484 ^{60}Co irradiator. The cultivable, environmental mycobacterium, *M. lufu*, was used as a control. To assay metabolic activity, both species were inoculated into the BACTEC 460 system which measures the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$. Growth of *M. leprae* was evaluated using the proportional bactericidal mouse foot pad assay. Growth of *M. lufu* was evaluated by plating serial dilutions on 7H11 agar plates for enumeration of colony forming units (CFU). γ-rad of both *M. leprae* and *M. lufu* resulted in a dose-dependent inhibition of metabolic activity. γ-rad of up to 10^3 Rad had no effect on the oxidation of palmitic acid by either organism. With *M. leprae*, 10^4-10^5 Rad caused an intermediate inhibitory effect, whereas 10^6 Rad yielded almost total inhibition of metabolic activity. With *M. lufu*, in contrast, 10^5 Rad inhibited metabolic activity by 99%. However, culture of γ-rad *M. lufu* showed that while 10^5 Rad caused ≥2 log reduction in the number of CFU, ~ 10^4 CFU/ml survived. γ-rad of 10^6 Rad sterilized the culture of *M. lufu* as no CFU were recovered. The effect of γ-rad on growth of *M. leprae* is currently being evaluated. These data suggest that γ-rad may be an efficient way to kill *M. leprae* without causing extensive damage to the cell architecture, as opposed to boiling or autoclaving. Killing *M. leprae* by γ-rad may be preferable when comparing cellular responses to live versus dead bacilli in vitro and in vivo.

MI30

GENE FUSION EXPRESSION OF AN IMMUNOMODULATOR FROM *MYCOBACTERIUM LEPRAE*

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Gene fusion expression technology has come a long way from the days of insoluble lacZ fusion constructs that were useful only as antigens. Today's fusion systems not only provide high levels of gene expression, but also often produce soluble and correctly folded fusion proteins that can be conveniently purified and efficiently cleaved. Since mycobacterial transcription and translation signals are poorly functional in *E. coli*, it is necessary to put the required gene under a strong and inducible promoter system to achieve the production of desired amount of the product. If that expression system has a fusion partner, then, it provides high stability and solubility for the fusion protein with an opportunity to fold correctly instead of precipitating into inclusion bodies.

Delipidified Cell Component (DCC) of *M.leprae* has been shown to have immunomodulatory effect both *in vitro* and *in vivo*. By screening the λgt11 expression library of *M.leprae* with antibodies raised against DCC, various clones were selected. Out of these, one clone of 1.6 Kb length, expressing a protein of 25 kD showed functional properties similar to DCC.

This gene was cloned into the *E.coli* gene fusion expression system with a fusion to Maltose Binding Protein (MBP) which is 42 kD. The fusion protein expressed was 67 kD indicating that the gene was coding for 25 kD protein. The expression level was high (~15-20% of the total soluble protein) and the purification was achieved by the affinity chromotography using Amylose resin. Factor Xa protease was used for cleaving at the junction of the fusion and the MBP was removed by repeating the Amylose resin affinity chromotography. The biotin-labelled Factor Xa was also removed from the sample by the immobilized streptavidin.

This purified product, which was part of the 65 kD hsp, was found to have similar immunomodulatory effect as DCC. By the use of internal restriction sites, this gene was further spliced into three distinct pieces, and cloned and expressed in the similar way as explained above. The MBP fusion system helped to make these peptides in large amount and by subsequent experiments the immunomodulatory effect of them was proved. Part of the work was done at the Foundation for Medical Research, Mumbai, India.

MI31

MYCOLATES OF DIFFERENT LEPROSY-RELATED MYCOBACTERIA : A COMPARATIVE STUDY

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Mycolate profile is used for identification of Mycobacterium spp. In this study, the pattern of mycolic acids have been studied in chemoautotrophic nocardioform bacteria (CAN b) derived from infected tissues of rat, human leprosy and tissues of epizootic ulcerative syndrome (EUS) - infected fish which also yielded a leprosy bacillus like pathogen. Closeness of the mycolate profiles of these 3 mycobacteria with that of different mycobacteria have been studied to distinguish these from each other, if possible, as well as other mycobacteria.

In this study, mycolates have been isolated from all the test bacterial samples by alkaline hydrolysis followed subsequently by thin layer chromatography. Spots were visualised by iodine vapour method.

CAN b, derived from human lepromatous tissues, showed the same profile as that of M.leprae and M. gordonae, containing three types of mycolic acids, namely, α , α' & β . Subsequently, CAN b derived from rat leprosy tissues and from tissues of EUS-affected fish showed α , α' & ω bands for mycolic acids, suggesting possible close proximities of these last 2 with that of M.avium rather than with M.leprae. The taxonomic value is evaluated.

MI32

BETA-LACTAM TARGETS IN *Mycobacterium leprae*

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Multimodular high molecular mass penicillin-binding proteins (PBPs), involved in bacterial peptidoglycan biosynthesis, are lethal targets of beta-lactam antibiotics. Peptidoglycan is one of the main structural component of mycobacterial cell wall. Identification of the genes of two high molecular mass PBPs in an ordered cosmid library of *Mycobacterium leprae*, overexpression of these genes in *Escherichia coli* and characterization of the expressed proteins reveal that these genes encode two PBPs, PBP1 (M, 81 kDa) and PBP1* (M, 69 kDa). PBP1* is a high affinity PBP and is unstable at temperatures above 25°C (Lepage *et al.*, 1997, *J. Bacteriol.* 179, 4627-4630). PBP1 is thermostable and binds penicillins with low affinity (Basu *et al.*, 1996, *J. Bacteriol.* 178, 1707-1711). This is reminiscent of the situation in *E. coli* PBP, PBP1a and PBP1b. Both *M. leprae* PBP1 and PBP1* bear the nine motifs characteristic signature sequence of multimodular bacterial class A PBPs at the same order and with the same spacing but their enzymatic and biochemical properties are markedly different. Critical analysis reveal that *M. leprae* PBP1 contains an altered sequence in one of the domains of the penicillin-binding (PB) module. Similar altered sequence is also present in a low affinity PBP, PBP C of *E. coli*. This domain may be critical for penicillin sensitivity and the altered sequence may be related to the differences in the penicillin sensitivity and thermal stability of *M. leprae* PBP1 and *M. leprae* PBP1*. The mycobacterial PBPs are the counterparts of the multimolecular class A PBPs, key components of morphogenetic networks. These studies are the first steps towards understanding the penicillin targets in mycobacteria at the molecular level. This knowledge is essential for future predictive studies on the interaction of new generation beta-lactams with the beta-lactam targets, and for the rational use of beta-lactams in antimycobacterial chemotherapy.

MI33

IN VITRO GROWTH OF *MYCOBACTERIUM LEPRAE* IN STIRRED CELLS.

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We have presented earlier evidence on limited in vitro multiplication of *M. leprae* in DH medium. The growth has been extremely slow (about 20-30 fold increase in cell mass in 16-20 weeks), and terminated after 20-24 weeks. The growth was partly attributed to the presence of a growth factor that was demonstrated to be present in tissues of armadillos infected with *M. leprae*. The reason for the termination of growth was the accumulation of hydrogen peroxide in the growth medium during incubation. Addition of catalase in the DH medium did not alleviate the situation. Addition of ketonic acid, such as pyruvic acid, to neutralize hydrogen peroxide was also not helpful unless fresh pyruvic acid was supplemented every week. This resulted in extending the growth till 24 weeks with average growth of 55-65 fold. Still the growth was terminated after 24 weeks and subcultures were not possible.

Finally, the cultures were grown in Stirred Cells using 0.2µ membrane so that the fresh DH medium will be continuously supplemented at the same rate as the spent medium is removed from the chamber. This resulted in approximately 70 fold increase in cell yield in 24 weeks. The growth was enhanced further to approximately 100 fold by incorporating both catalase and pyruvic acid in the DH medium. Subcultures from these primary cultures in Stirred Cells could be achieved only when the growth factor was added to the DH medium along with catalase and pyruvic acid. However, the growth of subcultures in Stirred Cells could not be maintained beyond 10-12 weeks with maximum yield of 10-15 fold. Nevertheless, this is the first time we were successful in achieving subcultures of *M. leprae*. The studies are being continued further.

(Supported through the funding from German Leprosy Relief Association).

MI34

PRESENCE OF CALMODULIN-LIKE PROTEIN IN *M. LEPRAE* AND ITS IMPLICATIONS ON THE DEVELOPMENT OF NEW ANTILEPROSY DRUGS

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Calmodulin-like protein has been established as the primary receptor for calcium in eukaryotic as well as in prokaryotic cells. Calmodulin-calcium complex regulates a variety of enzymes including nucleotide phosphodiesterase. Recently, we have been able to show the presence of this protein in *M. leprae* harvested from armadillos infected earlier with human-derived *M. leprae*. Thus, studies were undertaken to evaluate the effects of calmodulin antagonists on the in vitro growth of *M. leprae* using the previously established DH medium.

Among the six phenothiazine-type calmodulin antagonists tested, trifluoperazine appeared to be the most potent in inhibiting the in vitro growth of *M. leprae*, with MIC of 10 µg/ml. Chlorpromazine, trifluopromazine and thioridazine were less active than trifluoperazine, with MIC of 20 µg/ml, while the other two, acetopromazine and fluphenazine were totally ineffective even at 80 µg/ml. These findings suggest that a methylpiperazinylpropyl group attached to the nitrogen at position 10 and trifluoromethyl group at second carbon confer the antileprosy activity to the phenothiazine molecule. Results from our preliminary studies based on the incorporation of ^{14}C-acetate, ^{3}H-thymidine and ^{14}C-glycine indicate that the effect of trifluoperazine is on the synthesis of lipids, DNA and protein of *M. leprae*.

(Supported through the funding from German Leprosy Relief Association).

MI35

MODELING ACTIVITY OF SINGLE DOSE COMBINATION REGIMENS BY SIMULATING HUMAN PHARMACOKINETICS IN MACROPHAGE CULTURES OF *MYCOBACTERIUM LEPRAE*

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Since, with the exception of rifampin, the pharmacokinetics of the potent anti-leprosy drugs are markedly different in mice and humans, it is impossible to accurately simulate the drug concentration/time exposure in the latter by using a mouse model. Attempting to match the human AUC in most cases necessitates using a dose which results in a much higher maximum serum concentration (Cmax) than is possible in humans. The mouse model becomes even less representative when single doses of drug combinations are being assessed. The human pharmacokinetics of various combination regimens can be simulated by using *M. leprae*-infected mouse peritoneal macrophages. Cultures, in 24-well plates, are initially exposed to the expected drug concentrations following the customary doses of the corresponding drugs. At intervals of 4 hours or less, media

is easily removed and replaced with fresh media containing drug combinations at concentrations which would be expected based on their respective serum half-lives. This process continues for several days until drug concentrations are at levels below the expected minimum inhibitory concentrations. After an additional week in macrophage culture without drugs, the macrophages are lysed and the viability of the released *M. leprae* are evaluated by radiorespirometry. Data will be presented on the relative inhibitory activities of combinations of rifampin, minocycline, ofloxacin, sparfloxacin and clarithromycin in addition to dapsone and clofazimine.

MI36

ELONGATION OF *MYCOBACTERIUM LEPRAE* IN MACROPHAGES CULTURED IN THE PRESENCE OF INTERLEUKIN-10

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Mycobacterium leprae, the causative agent of leprosy, is an obligate intracellular pathogen that prefers the mononuclear phagocyte as its host cell. Numerous efforts have been devoted to the in vitro cultivation of *M.leprae* since its discovery, yet the leprosy bacillus remains uncultivable. Difficulty in assessing the viability of *M.leprae* has also impeded leprosy research. The availability of a constant supply of a large number of highly viable *M.leprae* from nude mice allowed us, to adapt radiorespirometry as a measure of the metabolic activity of *M.leprae*, affording a rapid (2 weeks), quantitative method to assess the viability of the leprosy bacillus in vitro.

In the present study mouse macrophages were infected in vitro with *M.leprae* from nude mice and were cultured. Radiorespirometry data over 2 weeks showed *M.leprae* in macrophages at 31°C to be more metabolically active than at higher temperatures, such as 37°C. Moreover addition of IL-10 to the cultures clearly sustained *M.leprae* metabolism in macrophages for 8 weeks. The apparent increase in number of *M.leprae*/macrophage was likely an artifact of loss and lysis of macrophage and re-phagocytosis of released *M.leprae*. Noteworthy, however, was the elongation of individual bacilli after 4 week-culture in macrophages maintained in media with IL-10 observed under light microscopy. Transmission electron microscopy also confirmed elongation of *M.leprae* in mouse macrophages sectioned after 4 weeks under the ideal culture conditions described above. In addition armadillo macrophages cultured in vitro also supported metabolism as well as elongation.

MI37

GLOBAL EPIZOOTIC IN FISH BY A LEPROSY LIKE ACTINOMYCETE POSING A POTENTIAL HAZARD TO HUMAN HEALTH.

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Presence of actinomycetic organisms have been reported in different varieties of fish affected with epizootic ulcerative syndrome (EUS) in India and elsewhere since 1988. The same organisms were isolated repeatedly from lesions of dermis, subcutaneous tissues, muscles and internal organs. Acid-fast bacilli, bacillary clumps, globi, mycelia, free acid fast bacilli (AFB) and 'Coccoid' bodies were present in the muscle tissues of fish, somewhat resembling human and rat leprosy bacilli. The characteristic macrophage granuloma surrounding these actinomycetic mycelia appeared to be consistent and compatible with actinomycetic pathogenicity. These isolates possessed fundamental similarities to the human isolates of chemoautotrophic nocardioform (CAN) bacteria, not cultivable in any common or conventional type of media. These could, however, grow easily on media for chemoautotrophy, composed of simpliest sources of C&N. These have been compared with the human leprosy bacilli on the basis of their morphological, staining, metabolic, and enzymological characteristics, lipid profile and PGL I specificity. All the above tests showed close parallelism between the CAN bacteria isolated from fish, rat and human leprosy infection. Thus EUS has not only posed a severe economic problem but also a potential hazard to fish handlers, fish mongers and even possibly fish eaters.

MI38

STRICT CONSERVATION OF *MYCOBACTERIUM LEPRAE* DNA SEQUENCES

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Nucleic acid sequence-based differentiation of microorganisms at the genus, species and strain level has fostered new approaches for comparative taxonomy, provided insight on mechanisms of microbial pathogenesis and exposed powerful markers for epidemiologic studies of infectious diseases. This approach has been informative for some mycobacterial species (MAC) and strains (*M. tuberculosis*). Systematic attempts to define DNA polymorphisms in *M. leprae* have met with limited success. While minor, independent polymorphisms have been identified in a few strains of *M. leprae*, a universal model based on genomic differences of *M. leprae* strains has not been demonstrated. We have applied restriction-fragment length polymorphism (RFLP) analysis, using numerous restriction enzymes and probes, as well as direct sequencing analysis of the 18kDa gene and the 16-23S rDNA internal transcribed spacer (ITS) region to evaluate similarities and differences between eleven strains of *M. leprae*. *M. leprae* strains originated from geographically distinct regions of the world (Philippines, Vietnam, US, Thailand, India) and the DNA from each strain was purified from bacilli either expanded in armadillos, nude mice or obtained directly from lesions of patients. In all instances RFLP patterns and DNA sequence comparisons between individually isolated "strains" showed exquisite homologies at all DNA sites studied, supporting earlier findings suggesting minimal divergence in *M. leprae*.

MI39

DETERMINATION OF METABOLICALLY ACTIVE STATE OF SLOW GROWING MYCOBACTERIA.

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To determine the metabolically active state of various slow growing mycobacteria such as *Mycobacterium leprae, M. lepraemurium and BCG*, a rapid method was developed. *M. phlei* was used as a control. *M. leprae* bacilli were isolated from the nude mice foot pads while *M. lepraemurium* were recovered from C3H mice lepromata. *BCG* were grown on Sauton medium. *M. Phlei* were grown on Lowenstein-Jensen medium. Bacilli from various mycobacteria used in this study were purified by differential centrifugation and bacillary suspensions were prepared in 0.05 M phosphate buffer, pH 6.5. To determine the metabolically active state of mycobacteria two parameters namely oxygen uptake of bacillary suspensions and ATP content were used. By using this method, meaningful information concerning *M. leprae, M. lepraemurium* and *BCG* can be obtained in four hours while only one hour was required using *M. phlei*. The information gained could be very helpful for the *in vitro* cultivation trials as well as for the biochemical studies of slow growing *M. leprae* and *M. lepraemurium*. Bacillary suspensions which showed relatively higher rates of endogenous oxygen uptake and ATP concentrations were found to be in a better metabolically active state than suspensions exhibiting lower rates of oxygen

consumption and ATP content. Suspensions showing higher rates of respiration when used for *in vitro* cultivation trials showed relatively better multiplication, though limited, of *M. leprae* and the bacilli maintained their morphology very well for 16-20 weeks.

MI40

A METHOD OF STORING LEPROMAS

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Foot pad technique of M.leprae inoculation of mice (Shepard, 1960) permitted to study M.leprae survival in different environments. Through many years' experiments we succeeded in confirming a survival of M.leprae under the conditions when biopsies from untreated LL patients were put into 40% saline solution of glycerin (in refrigerator or at room temperature). In past this method was used for preserving viability of different organisms including M.lepraemurium (Marchoux, 1934; Chorine, 1934). At the moment of biopsy, then in three and subsequently in every six months M.leprae (10000) from lepromas were inoculated into 10 BALB/c mice by Shepard's technique. Inoculum and "harvests" were counted according to Shepard and McRae (1968). During the initial 12 - 24 months characteristics of M.leprae multiplication in mice foot pads (lag-phase, log-phase and plato phase) did not almost change in the most of cases. After 3-4 years of storing an amount of M.leprae increased more slowly. Preliminary results of these experiments were published in 1984. Maximal period during which it was succeeded to preserve M.leprae viability in 40% glycerin at room t was 12 years (longer experiments were not carried out). The results obtained could account for some unsolved questions of epidemiology and pathogenesis of leprosy. The method proposed is successfully used for transportation of infected tissues from leprosy patients without ice from any region of Russia to Leprosy Research Institute (Astrakhan) for studying. During a special study (Vishnevetsky & Juscenko, 1991) it was also proved that lepromas kept in 60-80% solution of glycerin at room temperature for 2-4 weeks remain suitable for enzyme and histochemical investigations.

MI41

DNA AMPLIFICATION FOR DETECTION OF LEPROSY AND ASSESSMENT OF EFFICACY OF LEPROSY CHEMOTHERAPY

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PCR for the detection of M.leprae was applied to fresh skin biopsies and skin slit smears from 122 untreated leprosy patients. The PCR positivity rates in biopsies were 95.6% in MB cases and 44.2% in PB cases. Following 1 month of treatment MB cases declined by 54.3% and PB cases by 61.8% of initial values. Six month values also declined from initial positivity rates to 50.3% and 53.8% of initial values in MB and PB, respectively. Larger declines in rate of positivity were seen for smear samples at 1 and 6 months in both MB and PB but overall PCR positivity rates were lower than biopsy rates for M.leprae.

MI42

CONFIRMATION OF DIAGNOSIS IN MONO LESION LEPROSY CASES BY GENE AMPLIFICATION TECHNIQUES

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With the successful use of WHO MDT there have been major changes in the profile of leprosy. Most of new emerging cases present with single lesions. It is difficult to diagnose these cases as the clinical features are vague in most of such cases and histopathology is of nonspecific type. Various gene amplification techniques developed during the last decade may help in confirming the presence of gene sequences in these lesions. In this study, DNA as well as rRNA targetting amplification techniques have been evaluated for their usefulness in the diagnosis of these early forms. Biopsies from these suspected early leprosy cases attending OPD of our Institute were collected. Nucleic acids were extracted and fractionated by a physiohemical technique being used at our laboratory. A DNA targetting (Williams et al) and a RT-PCR targetting a part of 16S rRNA of M.leprae (standardised at our laboratory) have been tried to detect the presence of M.leprae sequences. Ribosomal targetting gene amplification assay was found to be slightly more sensitive (65%) than DNA targetting (55%) method. Results indicate a good potential for gene amplification techniques for confirmation of diagnosis of early leprosy.

MI43

ON THE NERVE LESIONS CAUSED BY A LEPROMA-DERIVED AND CULTIVATED MYCOBACTERIUM HI-75 PRODUCED IN MICE

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The aim to make every experimental disease model has been the simulation of the pathognomonic lesion which characterize human disease by the simplest possible way. In leprosy, nerve lesions have been regarded as the one which characterize this disease. The present study was conducted to make the one modifying the methods to make experimental leprous lesions produced in nude and immunologically attenuated mice by Sasaki et al. by the inoculation of a leproma-derived and cultivated *mycobacterium HI-75* (HI-75) which was reported as *M. lepraе HI-75* by Skinsnes et al. including one of the present author (Matsuo E) in 1975 and was identified as *M. scrofulaceum* (MS) by Stanford et al. in 1977.

In this study 11 million in total of HI-75 mixed with hyaluronic acid were divided into two and injected into both upper lip of each female nude and SPF ddY(ddY) mice expecting the easier access of the bacilli to the sensory nerves and periodically examined the lesions in these portion histopathologically for a few months.

As the results, the lesions produced in nude and ddY were much different. In nude mice macrophages laden with abundant mycobacteria proliferated at the injection sites and in the limited cases and portions bacilli were seen in the endoneurium of tiny peripheral nerves. In ddY the abundant bacilli were often seen in the center of the granulomatous lesions which include the nerves with invaded AFB.

The questions arose from the results which should be solved to understand the pathologic mechanism of leprous neuropathy are firstly whether the mycobacteria other than the so-called *M. leprae* grow in nerves or not in certain conditions and secondly whether some of the mycobacteria might have the ability to transform into genetically different kind of those such as M.leprae or not.

MI44

LIMITED GROWTH OF *MYCOBACTERIUM LEPRAE* IN CELL-FREE LIQUID MEDIUM CONTAINING ADENOSINE

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No credible reproducible *in vitro* multiplication of *M. leprae*, either in a tissue culture or in a cell-free culture system has been reported to date, despite more than 120 years since the discovery of the bacilli by Hansen in 1873. Here we report the first evidence that the cells of the Thai-53 strain of *M.leprae* can multiply in a cell-free liquid system, when the cells are cultured in enriched Kirchner medium, at pH 6.8-7.0, containing adenosine, at 30 C. For cultivation, we employed two methods; inoculation of the bacillary suspension into the medium, and cultivation of slide glasses smeared with the suspension of *M.leprae* in the medium. The results obtained indicated that the number of the cells morphologically increased with increasing cultivation period. A 2 to 4-fold increase in the DNA content extracted from the cultured cells specifically amplified by the PCR method, was observed after 6-8 weeks of cultivation. Moreover, an approximately 2-fold increase in the intracellular ATP content was demonstrated after 2 weeks' cultivation, and an approximately 4-fold increase after 4 weeks' cultivation. However, the ATP content gradually decreased thereafter, and no further increase in the ATP content could be achieved. Either AMP, ADP or ATP could be substituted for adenosine obtaining the same results. From these results, it was evident that initiation of the growth of *M.leprae* in a cell-free system took place when the cells were cultured in the liquid medium containing adenosine. The limit in growth after 6 weeks' cultivation has remained difficult to clarify, and further studies are required.

MI45

THE ANTIMYCOBACTERIAL ACTION OF AMPICILLIN/SULBACTAM IN CELL-FREE AND INTRACELLULAR SYSTEMS

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We have demonstrated previously that ampicillin/sulbactam is bactericidal to drug-resistant *Mycobacterium leprae* multiplying in mice and *Mycobacterium tuberculosis* in vitro. Sporadic reports continue to appear of HD patients who relapse after undergoing multidrug therapy. Multidrug-resistant tuberculosis is becoming a world-wide problem now. A gene designated *mdr,* mediating multidrug resistance has been reported in bacteria. Recently, a bacterial protein LmrA that extrudes antibiotics from the cell was shown to be identical to the human multidrug efflux pump P-glycoprotein causing multidrug resistance in cancers. Drugs that kill multidrug-resistant microorganisms would be of use in controlling infections caused by them. ß-Lactam antibiotics are the most widely used antimicrobial agents. Since mycobacteria, including *M.leprae,* synthesize ß-lactamase, these drugs are inactive against them. Now, ß-lactam/ß-lactamase-inhibitors are being employed successfully to treat diseases caused by ß-lactamase-positive organisms. We screened the action of three ß-lactam/ß-lactamase-inhibitors [ampicillin/sulbactam (Unasyn®), amoxicillin/clavulanate (Augmentin®) and piperacillin/tazobactam (Zosyn®)] on four potentially-pathogenic (to humans or to animals) mycobacteria [*M.simiae, haemophilum, avium & microti*] in axenic cultures; the action of ampicillin/sulbactam was also tested against the mycobacteria phagocytized by macrophages. The mycobacteria were cultured axenically in 7H 9 medium with or without the drugs. The drugs suppressed the growth of the mycobacteria in the cultures; ampicillin/ sulbactam proved to be the most potent of the three drugs. In the acute phase of mycobacterial infections, the organisms multiply within macrophages. Peritoneal macrophages were harvested from BALB/c mice. The bacteria were exposed to monolayers of the cells in RPMI 10 medium. Unphagocytized bacilli were washed off. Three concentrations of ampicillin/sulbactam were tested. The drug killed 58-97% of the mycobacteria within the macrophages [assessed by CFU]. ß-Lactam/ß-lactamase-inhibitors, especially ampicillin/sulbactam might provide an alternative effective therapy against diseases caused by mycobacteria resistant to other drugs.

MI46

PROTEIN KINASE REGULATES PHAGOCYTOSIS OF *MYCOBACTERIUM LEPRAE* BY MACROPHAGES

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Pathogenic bacteria have developed a variety of mechanisms to invade their hosts. Many intracellular microorganisms interact with host cell receptor molecules to induce their own internalization. Bacteria exploit eukaryotic protein kinases as part of a strategy to enter mammalian cells by stimulating these enzymes at the receptor sites in the host cells. Protein kinase inhibitors prevent cell infection by blocking bacterial internalization. *M.leprae* was purified from the foot pads of experimentally-infected *nu/nu* BALB/c mice. Peritoneal macrophages were collected from BALB/c mice, using heparinized Hanks' Balanced Salt Solution; the cells were sedimented by centrifugation at 200xg for 10 min at 4°C. The macrophages were suspended in a small volume of RPMI 1640 medium containing 15% heated fetal bovine serum, 20 mM HEPES, 2 mg/ml $NaHCO_3$, 2 mM glutamine and 50 µg/ml gentamycin. The number of cells in the medium was adjusted to $4x10^6$ /ml. A round 13 mm coverslip was introduced into each well of a 24-well tissue culture plate; 0.5 ml of the cell suspension was added to each well. After 2h, nonadherent cells were washed off. The inhibitors tested (staurosporine, genistein and erbstatin) were dissolved in 0.25% DMSO. Medium with DMSO was added to one set of 3 wells. Each inhibitor solution (0.5 ml) was added to three wells each. After 60 min, the wells were washed with PBS and then with the medium; 0.5 ml medium was added to each well. In another group, the wells were replenished with medium containing inhibitor. *M.leprae* ($2x10^7$/20 µl) was added to each well. After 2 h at 37°C, the cover slips were washed in PBS, and stained by the Ziehl-Neelsen method. The coverslips were photographed under high power. The control macrophages and those exposed to genistein showed good internalization of *M.leprae*. Phagocytosis of the bacteria was suppressed in the cells exposed to staurosporine and erbstatin, even when the inhibitors had been removed after preincubation. Different inhibitors act on different types of protein kinases. Further work will elucidate this. The results suggest that protein kinase regulates phagocytosis of *M.leprae* by macrophages.

MI47

GENOMIC IDENTIFICATION OF THE LEPROMA-DERIVED, CULTIVABLE AND NERVE INVADING *MYCOBACTERIUM HI-75* BY THE COMPLETE DNA SEQUENCING OF 16S RIBOSOMAL RNA GENE

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The complete sequence of 16S ribosomal RNA (16S rRNA) gene was analyzed with the leproma-derived and cultivable *Mycobacterium HI-75* (*M.HI-75*) in order to obtain its taxonomic characteristics by the direct sequencing of the polymerase chain reaction (PCR) products. The mycobacterium examined in this study was first isolated by Skinsnes *et al.** in 1975 from a lepromatous type Hansen's disease patient and was maintained in Ogawa's medium enriched with glucuronic acid and N-acetyl-D-glucosamine in one of the authors' laboratory. DNA extracted from the cultured bacilli was amplified by the PCR using five sets of primers. Thus obtained product was sequenced by the use of biotinylated and DyeAmidite-667 conjugated primers. The detected sequences of 16S rRNA were compared with the gene data base issued from GenBank and the published data by Rogall *et al.* The results revealed that the sequence of *M.HI-75* was most similar to that reported for *M. scrofulaceum* (*MS*) with 5 bases (0.35%) differences in the sequenced 1493 bases of 16S rRNA gene. *M. leprae* (*ML*) differed from *M.HI-75* with 47 bases (3.3%). Stanford *et al.* identified *M.HI-75* as *MS* in 1976 from their biochemical characteristics. Sasaki and Hamit reported the nerve invasion and the growth of the inoculated *M.HI-75* either to the nude mice or the ^{131}I treated immunocompromised Swiss mice. The results of this study indicates that *M.HI-75* is one of the variant of *MS* possessing an ability to invade into peripheral nerve, claiming a nature of a pathogen in developing leproma-like lesions.

Ref) *Skinsnes OK, Matsuo E, Chang PHC, Andersson B. Int J lepr 1975;43:193-209

MI48

A METHOD OF CHANGING REVERSION OF IN VITRO MORPHOLOGY OF CAN BACTERIA TO IN VIVO FORMS OF LEPROSY BACILLUS

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Leprosy bacillus (LB) in vivo shows the preponderance of acid fast bacillary bodies. However, when cultivated in vitro, they show a reduction in typical lepra bacillary forms and a preponderance of mycelia, spores and granules. This has, for a long time, confused recognition of the in vitro cultivated chemoautotrophic nocardioform (CAN) bacteria as the counterpart of the LB. We report on selective in vitro cultivation conditions which help revert the CAN bacteria to in vivo morphology.

Gelatin minimal (GM) medium supplemented with sodium palmitate was further supplemented with 5% Dextrin, 2% Fructose, Actinomycin D at 50 µg/ml concentration and this medium was inoculated with 10^4- 10^6 CFU/ml of in vitro maintained CAN bacteria which was allowed to grow for 4-6 weeks. Morphology was studied every week by staining with acid fast stain, Gram stain and Giemsa stain. Control medium was GM without these supplements. The results showed unequivocal reversion of in vitro non-acid fast bacillary, mycelial and granular forms of CAN bacteria to typical acid fast bacilli seen in vivo in LL leproma. The role of individual factor by a process of elimination has been determined and evaluated.

MI49

OXIDATION OF ^{14}C-PALMITATE AS AN INDEX OF INFECTIVITY AND VIABILITY OF 'FAST' AND 'SLOW' GROWING M. LEPRAE ISOLATES.

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Though there are as yet no recognized strains of *M. leprae*, variants which exhibit 'fast' or 'slow' growth in the mouse foot pad have been described. The factors which underlie these variant traits are not yet understood. We compared the metabolic activity and infectivity of different preparations of *M. leprae* including known 'fast' and 'slow' growing isolates. Inocula were compared between freshly harvested *M. leprae* with tissue preparations and suspensions preserved at different temperatures and times. A number of good handling practices are noted. Metabolic activity was assessed as the relative ability of 10^7 *M. leprae* to oxidize ^{14}C-Palmitate in Middlebrook 7H12 (BacTec) incubated at 33C and was expressed as a growth index (GI). Infectivity was determined using the mouse foot pad technique. Armadillos were inoculated with some preparations. The metabolic activity of *M. leprae* varied markedly by its tissue of origin, means of preparation, duration of storage, and general character of the tissue from which it was derived. The GI of freshly harvested preparations were roughly bimodally correlated with infectivity. Preparations with low GI tended to show poor to no growth upon reinoculation into animals while those with high GI usually achieved good growth results. Above or below a threshold, the GI was not proportional to growth rate and was not associated with 'fast' or 'slow' growth characteristics of different isolates. Preparations held at 4-33C showed first an increase and then a gradual decline in GI over a 4 week period, but infectivity was lost rapidly and generally eliminated by 3 weeks. Preparations held at 4C for less than 7 days retained best infectivity. Metabolic activity was not detectable following a single freeze-thaw of tissue or bacillary suspension and was generally associated with a 99% or greater decline in infectivity. Growth results achievable after inoculation of armadillos and mice were comparable. Oxidation of ^{14}C-palmitate can be used effectively as a relative measure of the quality of *M. leprae* preparations. The lack of linear correlation between metabolic activity and 'fast' or 'slow' growth characteristic suggests that differential growth rates are inherent and may be useful markers to help identify strain variants.

MI50

CULTURE AND PATHOGENIC CHARACTERISTICS OF THE *MYCOBACTERIUM LEPRAE* ISOLATE "THAI-53" IN ARMADILLOS (*DASYPUS NOVEMCINCTUS*).

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Though no genetic variation has been detected in *M.leprae* from different patients, regions and animal hosts, some isolates can be distinguished by their differential growth rates the mouse foot pad. Thai-53-*M. leprae* is a well characterized "fast" growing isolate commonly propagated in athymic nude mice. To determine its pathogenic characteristics in another host we inoculated 2 groups of 10 armadillos with 1.9 X 10^7 bacilli and compared their time course of serological, hematological and histopathological events of their infection with those of other animals inoculated with bacilli passaged from humans to armadillos. Thai-53 showed high metabolic activity for oxidation of ^{14}C-palmitate. Consistent with its growth characteristics in the nude mouse, Thai-53 inoculated armadillos showed more a rapid progression of infection, developing fully disseminated leprosy within 8-12 months with all classical signs of the disease. Serological antibody profiles for infection with Thai-53 were low and inconsistent in comparison to that seen with other isolates and the bacilli did not commonly disseminate to distant somatic sites prior to the animals entering the terminal stages of disease. Necropsy examination showed that armadillos infected with Thai-53 had extensive neurological involvement. Interspecies transfer of bacilli do not effect their overt pathogenicity and similar culture characteristic can be observed in different hosts.

MI51

EFFECT OF UV RADIATION ON *MYCOBACTERIUM LEPRAE*.

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The relative importance in leprosy transmission of contaminated dust, droplets or fomites remain unclear. *Mycobacterium leprae* is an extremely slow growing obligate intracellular parasite which is yet to be cultivated on artificial media. Several environmental factors could limit its transmissibility and survival in nature including its tolerance to ultraviolet radiation (UV). In the laboratory, UV-C is generally recognized as a potent sterilizing aid but its effectiveness against *M. leprae* also has not been shown. We examined the influence of UV on the growth and metabolic activity of *M. leprae*. Temporary static cultures in 10x35mm polystyrene petri dishes with 500 ul 7H12 media containing $1x10^4$ *M. leprae* obtained from foot pads of nude mice were exposed to varying timed intervals of UV radiation generated from an artificial source. Exposure times ranged from 0-80 seconds and constituted doses totaling from 0-12.64x10^4 ERGS/cm^2. The bacilli were immediately resuspended and $1x10^7$ organisms from each exposure dose were inoculated into BacTec 12B vials to assess for metabolic effects. Another $1x10^4$ bacilli from each exposure were inoculated into the foot pads of Balb/c mice (MFP) to assess long term effects on cell division. The growth index achieved in BacTec cultures showed an immediate dose response related decline to a minimum of about 50% of the control activity after exposure to 6.3x10^4 ERGS/cm^2. MFP results assessing cell growth were similar and showed that doses of 3x10^4 ERGS/cm^2 resulted in a 90% killing and 6.3x10^4 ERGS/cm^2 killed 99%. *M. leprae* shows sensitivity to UV-C similar to *M. tuberculosis* and lacks mechanisms that might allow it to substantially avoid UV injury.

MI52

RAPID DETECTION OF RIFAMPIN RESISTANCE IN MYCOBACTERIAL PATHOGENS: EVALUATION OF PCR/UHG-RIF ASSAY

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Rifampin resistance in *Mycobacterium leprae* and *Mycobacterium tuberculosis* has been attributed to missense mutations within an 81 base pair fragment of *rpoB* (Rif region), encoding the *B*-subunit of the DNA-dependent RNA polymerase. Using this information, we have developed and characterized DNA-based assays using heminested PCR and universal heteroduplex generator (PCR/UHG-Rif) for the rapid detection and rifampin-susceptibility of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in clinical specimens. In a double-blind study, 655 sputum specimens were obtained from individuals suspected of having tuberculosis and analyzed for the presence of *M. tuberculosis* and rifampin-susceptibility using the PCR/UHG-Rif assay. Results of this study demonstrated that the PCR/UHG-Rif assay detected all smear-positive, culture-positive specimens containing *M. tuberculosis*. In addition, 50% of smear-negative, culture-positive specimens for *M. tuberculosis* were detected. This assay also correctly identified the rifampin susceptibility of *M. tuberculosis* from 98% of these specimens. All specimens containing only nontuberculous mycobacteria or no acid-fast bacilli were negative in the PCR/UHG-Rif assay. The presence of *M. tuberculosis* in culture-negative specimens from patients with previous culture-positive specimens but receiving anti-tuberculosis therapy when specimens were obtained for PCR/UHG-Rif testing was detected by the use of this assay. In addition, PCR amplification primers and conditions were modified to specifically amplify and detect the rifampin-susceptibility of *Mycobacterium leprae* in purified DNA samples and crude cell lysates of skin biopsy specimens from lepromatous leprosy patients. Even though, a small number of rifampin-resistant clinical specimens were available for analyses (no. = 4), the PCR/UHG-Rif assay correctly identified the rifampin susceptibility of these specimens. These assays require approximately six hours to run (post sample preparation) and should provide rapid tools for detection of the rifampin-resistant phenotype of these mycobacterial pathogens directly from clinical specimens.

MI53

PREPARATION AND APPLICATION STUDY ON a2 ANTIGEN OF MYCOBACTERIUM LEPRAE

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The a antigen gene is one of the dominant mycobacterial proteins those are secreted from the mycobacteria. In the course of our study, we have constructed the genomic libray of M. leprae Thai 53 strain, cloned a new a antigen gene with a plaque hybridization method using DNA fragment of M. leprae al antigen DNA as a probe and termed it as a2 antigen gene. The a2 antigen gene was characterized by sequencing. By comparing the deduced amino acid sequence of a antigen with 85 complex antigen of other mycobacteria, the homology of 74.3%-85% was found.

The over expression system of M.leprae a2 antigen gene in Escherichia coli was constructed. Recombinant a2 antigen was purified by amylose column chromatograph at the purity of more than 95%.More than 10mg of recombinant a2 antigen has been obtained from 200ml of liquid culture. Then we studied the serological activity of recombinant M. leprae a2 antigen using enzyme-linked immunosorbent assay (a2-ELISA) in sera from 100 leprosy cases and 50 normal persons, and determined the optimum conditions for a2-ELISA.The results indicated: the antibody titer to a2 antigen in leprosy patients was IgG>IgM>IgA>and there was same serological activity between a2-ELISA and ND-ELISA. These results suggested that the a2-ELISA may be useful for the serodiagnosis in leprosy.

MI54

A METHOD OF STORING LEPROMAS

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Foot pad technic of M.leprae inoculation of mice(Shepard, 1960) permitted to study M.leprae survival in different environments. Through many years' experiments we succeeded in confirming a survival of M.leprae under the conditions when biopsies from untreated LL patients were put into 40% saline solution of glycerin (in refrigerator or at room temperature). In past this method was used for preserving viability of different organisms including M.lepraemurium (Marchoux, 1934; Chorine, 1934). At the moment of biopsy, then in three and subsequently in every six months M.leprae (10^4) from lepromas were inoculated into 10 BALB/c mice by Shepard's technic. Inoculum and "harvests" were counted according to Shepard and McRae (1968). During the initial 12-24 months characteristics of M.leprae multiplication in mice foot pads (lag-phase, log-phase and plato phase) did not almost change in the most of cases. After 3-4 years of storing an amount of M.leprae increased more slowly. Preliminary results of these experiments were published in 1984. Maximal period during which it was succeeded to preserve M.leprae viability in 40% glycerin at room t^0 was 12 years (longer experiments were not carried out). The results obtained could account for some unsolved questions of epidemiology and pathogenesis of leprosy. The method proposed is successfully used for transportation of infected tissues from leprosy patients without ice from any region of Russia to Leprosy Research Institute (Astrakhan) for studying. During a special study (Vishnevetsky & Juscenko, 1991) it was also proved that lepromas kept in 60-80% solution of glycerin at room temperature for 2-4 weeks remain suitable for enzyme and histochemical investigations.

PATHOLOGY

PA01

HISTOPATHOLOGIC FINDINGS OF IRIDOCYCLITIS IN LEPROMATOUS LEPROSY

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Cataract is found to be a common complication in lepromatous patients belonging to the older age group. During cataract surgery an iridectomy is performed as a routine procedure. The iris specimens obtained from lepromatous patients undergoing cataract surgery are studied histopathologically using hemoloxylin eosin stain and acid fast stain. Iris atrophy with evidence of chronic inflammation was found in a majority of patients even in the absence of acid fast organisms. In one specimen active inflammation with macrophage granuloma infiltrating the iris tissue including constrictor muscles was noticed. Acid fast organisms were present in large numbers inside macrophages and in bundles of smooth muscle cells regulating the pupil. Nerve fibers were not detected. Details of these findings will be presented and their significance will be discussed.

PA02

In severe ENL reactions we found acute necrotizing and exsudative vasculitis in deep dermis and subcutis. These vasculitis follow severe acute inflammatory reactions in the neighboring tissues. We describe in 10 lepromatous leprosy patients, many years after discharge, episodes of one or few erythematous nodules in the limbs. Microscopical examina - tion revealed a exsudative and necrotizing segmentary vasculitis like Polyarteritis nodosa.
There are discrete inflammatory reaction in dermis and sub - Cutis and the clinical data, residual lepromatous infiltrate and Bacili in vessel's wall sugests a late ENL reaction

PA03

Theren't envolvement of Central nervous system, peripheral nervous systems, skin, muscles and lungs in secondary amyloidosis in leprosy. In these sites the blood capillaries shows tigh junctions between the endothelial cells and the transport is made by pinocitosis. Otherwise in sites where blood capillaries are fenestrate (kidneys, bowel, endocrine glands) or sinusoids (liver , spleen , bone marrow) the Amyloid deposition is ever present and frequently massive. The amyloidogenic SAA protein is produced in the liver, their molecular weight is 250.000 and is found in the blood as a apolipoproteic complex. This suggests that there are a relationship between the size of the precursor protein molecule and capillary pattern in the distribution of amyloid deposition in leprosy