Free Papers - Immunology and Molecular Biology

Role of 18 kDa Heat Shock Protein in Mycobacterium leprae Infection of Macrophages

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Introduction: M. leprae, the causative agent of leprosy, is an obligate intracellular pathogen. In order to understand the pathogen's immunopathological mechanism and to develop effective vaccine candidates, a large number of immunodominant antigens have been identified and characterized. Our studies are aimed towards understanding the role of M. leprae Hsp18, a major secreted antigen, in infection. Methodology: The 18kDa gene along with a 168 bp upstream promoter region was PCR amplified from the armadillo derived M. leprae genomic DNA, cloned into pSET152 vector and transformed into E. coli. The expression of this 18kDa protein was analyzed by western blot analysis. Further, the expression of this protein in E. coli under microaerobic growth conditions and its localization was also examined. The hsp18 gene with its promoter was integrated into the genome of the non-pathogenic M. smegmatis, mc2155 and the integration was confirmed by a plasmid rescue strategy. The survival of M. smegmatis, with and without the integrated copy of the hsp18 gene, in the macrophages is also under study. The survival of M. smegmatis at different time points after infection, ranging from 3-120 hrs, was assessed. Results: The Hsp18 protein is expressed in E. coli and slight enhancement of expression was seen when grown in microaerobic conditions. This protein localizes to the periplasm of E. coli. The plasmid rescue experiments revealed that the pSET152 vector could integrate in at least 3 different sites in the M. smegmatis genome. The hsp18 gene does not seem to confer survival advantage during the early hours of infection. Data pertaining to the role of Hsp18 in the survival of the pathogen during prolonged period of macrophage infection will be presented. Conclusions: The Hsp18 protein is expressed in E. coli and gets localized to periplasm. The integrated hsp18 gene with its promoter does not confer survival advantage to M. smegmatis during early hours of infection in macrophages. Keywords: M. leprae, Hsp18, integration of pSET152, M. smegmatis, macrophage.

IFN - γ and TNF -α Gene Polymorphisms in Leprosy

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Introduction: The main aim of the study was to identify pro-inflammatory cytokine gene differences in tuberculosis and lepromatous types of leprosy. The specific objectives were to study Single Nucleotide Polymorphisms (SNPs) in the genes of IFN-γ downstream region (+874 A/T) and TNF-α promoter region (-308 G/A) across the leprosy spectrum. Methodology: Amplification Refractory Mutations System (ARMS) - PCR was performed to detect SNPs in 71 healthy controls and 102 leprosy patients to in the above genes. The amplified products, 273 bp (TNF-α), 262 bp (IFN-γ) and 450 bp internal control was viewed on 2% agarose gel stained with ethidium bromide. Results and Conclusion: No significant association of TNF-α promoter region (-308 G/A) polymorphism was observed with leprosy. There was a significant association of high producer genotype (TT) of IFN-γ gene with tuberculosis leprosy and low producer genotype (AA) with lepromatous leprosy. Keywords: leprosy, ARMS PCR, TNF-α, IFN-γ, Polymorphism.

Metalloproteinases and Pure Neural Leprosy (PNL) Nerve Fibrosis

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Metalloproteinase expression is one of the regulating mechanisms in inflammatory extracellular matrix remodeling. We studied this expression in leprosy nerve fibrosis in PNL biopsies. Groups studied: G1 - normal-looking biopsies; G2 - PNL + inflammatory infiltrate; G3 - PNL + fibrosis; and G4 - PNL + inflammatory infiltrate + fibrosis. Results: m-RNA expression: G2 showed higher MMP2 and MMP9 expressions than did G1 and G4. In contrast, MMP9 expression was lower in G3. TIMP1 expression was highest in G2, followed by G3 and G4. Immunohistochemical expression: G1 showed MMP2, MMP9, and TIMP1 expression in Schwann cells and axons while G2 showed MMPs and TIMP1 expression in mononuclear inflammatory cells (macrophages and fibroblasts); G3 had TIMP-1 expression in a few residual inflammatory cells. G4 expressed MMPs in SCs and axons and TIMP1 in inflammatory cells. These results suggest that the mutually opposing activities of MMPs and TIMP1 contribute to the final configuration of the fibrotic extracellular matrix in leprosy-affected nerves.

[89]
Lucio's Leprosy, Lucio's Phenomenon and the Antiphospholipid Antibody Syndrome

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Introduction: Lucio's phenomenon (LP) occurs in patients with Lucio's leprosy (LuL). For some authors the cutaneous lesions and its histopathology show an underlying thrombotic/occlusive condition, but for others, it is a leukocytoclastic vasculitis. The similarity between the cutaneous manifestations of LP and Antiphospholipid syndrome (APS), took us to investigate the relationship between these two pathological conditions. Objectives: to establish the clinical, laboratorial and histopathological characterization of LuL and LP; to make a correlation between the latter and APS. Material and Methods: seven patients with LuL and LP were included; they were submitted to clinical evaluation; detection of antiphospholipid antibodies (aCL), verifying the 2-GP1 in six of them. The dependency to the coagulation cofactor histopathological exams of cutaneous biopsies of the skin were stained by hematoxylin-eosin (HE) and Fite-Faraco. Results: all the patients presented the features of classical LuL and LP, without signs of lepromatous reaction. One patient presented physical incapacity grade 1 and six, grade 2. Seven patients presented one clinical criterium and five, one laboratorial criterium for APS. 2-GP1 was positive in six patients. Fourth the test of aCL dependency on presented risk factors for thrombo sis. The histopathology showed thrombi in the vessel walls, the extensive fibrosis and changes of the vessel wall, besides the specific features or Lucio's Leprosy. It was not found leukocytoclastic vasculitis. Conclusions: the clinical pattern of LuL observed in the patients of this study is identical to that described by Lucio and Latapi. The study suggest that LP could be considered APS secondary to LuL. The treatment with multidrug therapy for multibacillary patients (MDT-MB) was successful in the patients studied.

Modulation on the Expression of Chemotactic Factors in Reactional Leprosy Lesions Following Treatment with Thalidomide

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During the ENL patients show augmented pro-inflammatory cytokine levels in skin lesions and treatment with thalidomide leads to remission. In order to evaluate the expression of chemotactic factors by real-time RT-PCR, skin biopsies were collected from reactional leprosy patients lesions at the onset of ENL (n=8), and one week after thalidomide treatment (n=8). The expression of IL-8, IL-10, FOX3, CCL2, CXCL10 and CCR5 was detected during ENL. After treatment with thalidomide (300 mg/day), it was found a reduction on IL-8, CCL2 and CXCL10 mRNA and sustained/augmented expression of IL-10, FOX3 and CCR5 at the lesion sites. The reduction of chemotactic CCL2 and IL-8 may lead to diminished migration of inflammatory cells into the tissue. Moreover, the sustained expression of IL-10, FOX3 and of CCR5 during treatment may well correlate with the local suppression of the immune response and reduced bacterial load at the lesion site. Evaluation of the expression of chemotactic factors may contribute to a better understanding of pathology progression during reactions and may reveal targets for therapeutic interventions. Keywords: Thalidomide.

Correlation Between A New Single Nucleotide Polymorphism in the Toll-like Receptor 1 Gene and Leprosy

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The TLR signaling pathway is required for protection against infectious diseases, but excessive signaling may lead to allergies and autoimmune diseases. Recently, a 1602S single nucleotide polymorphism (SNP) in the TLR1 gene was associated with abnormal trafficking of the TLR receptor to the cell surface resulting in a reduced response of blood monocytes to bacterial agonists. Remarkably, the 602S allele was associated with a decreased incidence of leprosy in a Turkish study population, suggesting that M. leprae subverts the TLR system as a mechanism of immune evasion. Here, we describe a study into the distribution of a previous unpublished SNP in the TLR1 gene among a study population consisting of 843 leprosy patients, 107 controls and 544 people from the general population. The geographical location of the patients had no effect on allele frequency, but the allele frequency of the patient and contact populations differed significantly from the general population. In the patient population homozygous allele combinations were more frequently observed than expected (P<0.0001). We will discuss the implications of the occurrence of this TLR1 SNP for leprosy.
Identification and Characterization of IFN-γ and TNF-α from Nine-Banded Armadillos (Dasypus novemcinctus)

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Nine-banded armadillos (Dasypus novemcinctus) are natural hosts of Mycobacterium leprae and they exhibit the full clinical spectrum of leprosy. Unfortunately, suitable immunological reagents have not been available to allow us to study disease pathogenesis in these animals. Recently, the whole genomic sequence of armadillos was published which is aiding development of a wide array of new armadillo specific immunological reagents. We report here identification and cloning of two prominent armadillo Th1 cytokines: interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). IFN-γ cDNA was amplified from ConA stimulated PBMC and TNF-α cDNA was amplified from LPS stimulated macrophages. Both sets of primers were designed using recently available genomic contigs. These cDNAs were subcloned into pET vectors and overexpressed in E.coli. Biological assays confirmed functionality of both recombinant products. DnTNF-α lysed PK15, L929 and WEHI cell lines and was inhibited by polyclonal antibodies prepared against synthetic peptid epitopes of the proteins. DnIFN-γ induced Indoleamine 2,3-dioxygenase activity (IDO) and killing of intracellular toxoplasma by armadillo macrophages. Similar to human macrophages DnIFN-γ activation did not induce killing of intracellular mycobacteria or discernable bursts of nitric oxide. Generation of immunological reagents such as these will further elucidate the pathogenesis of leprosy.

Relationships Between Antibodies to Mycobacterium leprae PGL-I, LAM and Recombinant Protein Antigens in Lepromatous and Tuberculoid Leprosy Individuals

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The current serodiagnostic test for individuals with leprosy is based on antibody responses to the M. leprae-specific phenolic glycolipid-I (PGL-I). To further understand the relationship of antibody responses to disease state, we have examined the reactivity patterns of 50 lepromatous and 20 tuberculoid sera to PGL-I, lipoaerabinomannan (LAM) and six recombinant M. leprae proteins. The response to Ag85 was consistently high in both patient groups, while responses to CFP-10 and GroES showed the most variability; from strongly positive to completely negative. The tuberculoid patient sera showed overall lower responses to all of the recombinant proteins, particularly in the case of GroES, where none in this group showed any reactivity to this protein. Only 25% of these same patients showed a weak response to CFP 10, while 76% of lepromatous patients showed a positive reaction to this protein. This analysis has given a clearer understanding of some of the differences in the responses, both between individuals at opposite ends of the disease spectrum, as well as illustrating the heterogeneity of antibody responses towards protein, carbohydrate, and glycolipid antigens within a group. We will attempt to determine if some of these response patterns can be correlated with a particular disease state or outcome. Key words: diagnostics, serology, recombinant proteins, LAM.

Mycobacterium leprae Induces Apoptosis and TNF Secretion via NF-kB in Monocytes

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Introduction and Objectives: Leprosy is a chronic infectious disease characterized by a broad spectrum of clinical features depending on the host's immune response to Mycobacterium leprae. In this work we have studied the role of the ubiquitin - proteasome pathway and NF-kB in M. leprae-induced apoptosis and cytokine secretion. Methods and Results: Flow cytometric analysis of cultures stained with Annexin V and propidium iodide showed that inhibition of proteasome and NF-kB by MG132 or SN50 respectively, significantly reduced monocytes apoptosis induced by M. leprae. The evaluation of cytokine levels in the culture supernatants by ELISA showed that both proteasome and NF-kB inhibitors significantly reduced the M. leprae-induced TNF secretion. Also, we observed that M. leprae increases p65 levels in nucleus and that NF-kB pathway inhibitors reverses this effect. Conclusion: Our data are consistent with the notion that M. leprae is able to affect the levels of ubiquitin-conjugates in monocytes and that proteasome function and NF-kB plays a role in apoptosis and modulation of TNF levels induced by this pathogen.
Potential Plasma Markers of Leprosy Reactions

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We assessed potential plasma markers of Type 1 Reactions (T1R) and Type 2 Reactions (T2R) in a cross-sectional study of untreated leprosy patients at the Reference Center for Diagnosis and Treatment, Goiania, central Brazil. Patients were classified by dermatological and histological methods, biocapsular index (BI) and histopathology, and plasma samples were frozen. Patients with T1R (n = 10) or T2R (n = 10) at diagnosis were matched for age, sex, and leprosy type with patients without reaction ('controls', n = 19). Cytokines were assayed using a pre-mixed human cytokine 27-plex panel (BioRad, USA). Exploratory data analysis and Mann Whitney tests were applied. Patients were mainly young adult males. Patients with T1R had BT leprosy (BI range = 0-1.5); those with T2R had LL leprosy (median BI = 3.25). Compared to matched controls, T1R patients had significantly greater IP-10 (p = 0.004) and IL-6 (p = 0.013). Compared to matched controls, T2R patients had significantly greater IL-7 (p = 0.039) and PDGF-bb (p = 0.041). No significant differences were found between reaction patients and their matched controls for other cytokines, including TNFα or IFNγ. Conclusions: IP-10/CXCL10, a chemokine known to attract Th1 cells to DTH sites, may be a potential plasma marker for Type 1 leprosy reactions. Funding: CNPq: Brazilian Research Council. Key Words: Immunopathology, leprosy type 1 and type 2 reactions.

A Study on Relationship of Host Protein and M. leprae Cell Walls in the Failure of Phagolysosome Process

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Introduction: The failure of phagolysosome process could be identified due to the expression profile of molecules: Rab5, Rab7 and TACO from host macrophage, and Lep-LAM and PGL-1 from M. leprae. The purpose of this study is to clarify the involvement of these molecules in relation to 16SrRNA M. leprae as a marker of viability. Methodology: Forty seven skin biopsy specimen from new untreated leprosy patients were examined for 16SrRNA M. leprae using Real Time PCR. The expression of Rab5, Rab7, TACO, Lep-LAM and PGL-1 was assessed by immunohistochemistry technique. Results: A significant difference (p < 0.05) on the expression profile of Rab5, Rab7, Lep-LAM and PGL-1 were found between positive and negative 16SrRNA groups. A significant correlation (p = 0.05) between the score of Rab5, Rab7, Lep-LAM and PGL-1 and the score of 16SrRNA were also found. Conclusions: These molecules have an important role in the failure of phagolysosome process in M. leprae infection. These result could be used for early diagnostic device and follow up treatment. Key words: host protein, M. leprae, phagolysosome.

Clofazimine-Induced Cell Death in Macrophages

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Clofazimine has been used in the treatment of leprosy at first for its antibacterial action against M. leprae. Later, it was also found to possess an anti-inflammatory action which makes it a very useful drug in the treatment of acute reactions, including ENL, neuritis, iritis etc. But the mechanisms are not fully understood yet. In the present study we found that the drug possessed cell death-inducing activity in macrophages. Mouse peritoneal macrophages were cultured in vitro in the presence of clofazimine for 24 hr. The cells exhibited marked decrease in metabolic activity by biochemical analysis and showed shrink in cell size under microscopic observation. Giemsa stain of the cells demonstrated nuclear condensation and fragmentation. The cell death-inducing activity of clofazimine was also observed in human peripheral blood monocyte-derived macrophages and human monocyte leukemia cell line, THP-1 cells. Moreover, human macrophages cultured in the presence of clofazimine were positively stained with annexin V prior to their death. Clofazimine-treated THP-1 cells also showed annexin V staining followed by demonstration of fragmented DNA. Enhanced caspase activity was observed in cell lysates of the clofazimine-treated cells. Collectively, these results suggest the apoptosis-inducing activity of clofazimine. There shall be a close relationship between apoptosis-inducing activity and anti-inflammatory property of the drug.
Inhibition of the Multiplication of *Mycobacterium leprae* by Vaccination with a Recombinant BCG that Secretes Major Membrane Protein-II of *M. leprae*

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To improve the potency of BCG as a vaccine against leprosy, we constructed recombinant BCG (BCG-SM) secreting Major Membrane Protein-II (MMP-II), which we identified as one of the immunodominant Ag of *Mycobacterium leprae* capable of activating T cells through TLR2. BCG-SM secreted MMP-II and the secreted protein stimulated human monocyte-derived dendritic cells (DC) to produce IL-12p70. DC infected with BCG-SM expressed the MMP-II, and more efficiently activated both naïve and memory type CD4+ T cells to produce IFN-γ than those infected with vector control BCG (BCG-pMV). However, naïve CD8+ T cells were significantly activated only by the stimulation with BCG-SM infected DC. The naïve CD8+ T cells were cross-primed with the secreted MMP-II, since the activation of the T cells was dependent on the expression of MHC class I, CD86 and MMP-II antigens. Further, BCG-SM efficiently produced perforin-producing T cells. Moreover, the MMP-II specific memory T cells were efficiently produced in mice inoculated with BCG-SM. A single intradermal injection of BCG-SM could confer better protective immunity than that of BCG-pMV against challenge of *M. leprae* in the mouse footpad. Taken together, these results indicate that BCG capable of secreting the MMP-II is more potent as a vaccine against leprosy. **Keywords**: Vaccine, Major Membrane Protein-II, BCG.

Toward Rational Design of a Defined Sub-Unit Vaccine for Leprosy

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We have studied the immune response of leprosy patients to identify immunogenic antigens, with the goal of rational development of a defined sub-unit vaccine to prevent leprosy. We screened a panel of *M. leprae* recombinant proteins for T cell recall responses in simple whole blood assay, using blood from leprosy patients and controls (household contacts, tuberculosis and healthy endemic controls). Upon 24 hour incubation, we found several antigens that specifically elicited IFN-γ secretion from paucibacillary (PB) patient blood, and surprisingly, IL-5 secretion from multibacillary (MB) patient blood. Based on these leprosy patient T cell responses, recombinant antigen ML0276 was tested in vaccine studies of experimentally infected mice. Vaccination promoted a strong Th1 cell response, and immunized mice did not develop the local inflammation that was observed in control mice. Furthermore, when cells from immunized mice were injected into recipient mice that were subsequently infected, transfer of ML0276 responsive cells provided protection. These results identify candidate antigens for a leprosy vaccine. **Sponsorship**: American Leprosy Mission. **Keywords**: T cells, vaccine.

Selection of Antibigens for a Rapid and Objective Point-of-care Leprosy Diagnosis

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Leprosy diagnosis is currently based on clinical signs and the development of diagnostic tests remains a research priority. To identify and evaluate *M. leprae* proteins with diagnostic potential, we studied antibody reactivity using patient sera from well characterized, untreated multibacillary (MB) and paucibacillary (PB) leprosy patients and endemic controls. New *M. leprae* recombinant protein antigens were first screened by protein array. Antigens that provided the most sensitive and specific responses in protein array were then validated by ELISA. Upon confirmation of protein array results in ELISA, we performed multiantigenic print immunoassay (MAPIA) to determine antigens that were suitable for use in a rapid test format. The chimeric fusion protein, LID-1, provided the best discriminatory diagnosis of leprosy by MAPIA. When imprinted into an advanced lateral-flow technology (the Dual Path Platform (DPP™) Leprosy antibody detection assay) that can be interpreted by a digital reader, LID-1 was capable of providing an objective and quantifiable leprosy diagnosis within 15 minutes. Our findings have contributed to the development of a cheap, prototype leprosy diagnostic kit with promising discriminatory power in a highly leprosy endemic regions. **Keywords**: diagnosis, serology. **Sponsorship**: American Leprosy Mission.
**Suitability of Slit Skin Smears for Molecular Detection of Strain Variation and Drug Resistance in Leprosy**

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**Introduction**: Since *Mycobacterium leprae* is not cultivable, strain typing by methods such as Variable Number of Tandem Repeats (VNTR) analysis would help us to correlate genotypes with level of incidence, clinical state of disease, re-infection and/or relapse. The aim of the present study was to assess the suitability of slit skin smears for strain variation and drug resistance.

**Methodology**: DNA was extracted from slit skin smears of bacteriological Index (BI) 1 to 5+. Using multiplex PCR 15 VNTR loci and 4 drug resistance determining regions in genes - *rpoB* (rifampicin), *folP1* (Dapsone), *gyrA* and *gyr B* (Fluoroquinolones) were studied. GeneScan analysis detected the number of repeats for VNTRs and sequencing for the DRDR mutations. **Results**: Most conserved locus in the isolates of this study was microsatellite 27-3 while considerable variation was seen at locus (TTC)21. A codon 55 mutation in *folP1* was detected in one patient who presented with clinical relapse and BI of 2+. **Conclusion**: *M. leprae* DNA could be amplified for detection of strain variations and drug resistance from slit skin smears showing BI as low as 1+. Multiplex PCR minimized the amount of DNA required to undertake both screening for strain variations and resistance to multiple drugs. **Key Words**: Leprosy, VNTR, Multiplex PCR, drug resistance, Rifampicin, Dapsone, Fluoroquinolones.

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**Strain Typing of *Mycobacterium leprae* Thai Clinical Isolates by Multiple Locus Variable Number Tandem Repeat Analysis**

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Multiple Locus Variable Number-Tandem Repeat (VNTR) Analysis (MLVA) has been proposed as a means of strain typing for tracking leprosy transmission. Many tandem repeats have been reported to be polymorphic with potential as genetic markers to differentiate strains of *Mycobacterium leprae*. However, the characteristics of polymorphism can vary depending on the population studied. We therefore measured the copy numbers of simple sequence repeats known as microsatellites and minisatellites, in *M. leprae* in Thai leprosy patients. MLVA at 10 loci was applied using total DNA extracts from skin biopsies. The number of alleles per locus ranged from 2-24 providing adequate strain discrimination. Microsatellite loci (TTC)21, (AT)17, (GTA)9, (AC)8a, (AC)9 are highly diverse. The minisatellites 6-7, 21-3, 23-3 and 27-5 exhibited a limited number of alleles while the *rpoB* locus was non-polymorphic. The combined use of nine VNTR loci revealed genetic diversity among 93 clinical isolates. The closely matched VNTR profiles found in household members of multibacilli families suggests infection through a common source. This MLVA method is thus useful for investigating of leprosy transmission in Thailand and targeted surveillance in defined communities.

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**Molecular Strain Typing for Microepidemiology of Leprosy in Cebu, Philippines**

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Nearly 400 new cases of leprosy are detected every year within the island of Cebu. Therefore, a study was initiated to understand the transmission patterns of leprosy within this geographically isolated and stable population using modern molecular methods. Seventy untreated, newly diagnosed leprosy patients with bacteriological index >2, and their household contacts residing in Cebu for at least 6 months were recruited. Interestingly, five of these enrollees were linked by blood; 3 siblings in one family, and a mother and son pair in another family. After epidemiological and clinical evaluation, biopsy and slit skin scrapings (SSS) from at least 3 active sites were taken from the patients and contacts showing signs of leprosy. The specimens are currently being processed for strain typing of *M. leprae* on the basis of genetic variability at multiple loci arising from variable number of tandem repeats (VNTR). To date, results indicate that SSS from a single site provides DNA of sufficient quality and quantity for strain typing at more than 13 loci by PCR and sequencing methodologies. The VNTR allelic diversity detected within this population is adequate for strain discrimination. Furthermore, preliminary results show identical *M. leprae* VNTR profiles from multiple SSS sites of a single patient. These attributes support the use of VNTR loci for short range transmission studies. The spatial and temporal distribution of strains and patients will be presented.

[94]
O-85
Implications of High Level Pseudogene Transcription in Mycobacterium leprae

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Transcriptional analysis of pseudogenes using an M. leprae genomic array and RT-PCR demonstrated that >40% of M. leprae pseudogenes were transcribed. This is the highest rate of pseudogene transcription documented to date. In silico analysis revealed that 22% of transcribed pseudogenes were within gene clusters or downstream of expressed ORFs. RT-PCR revealed that some of these pseudogenes appeared to be transcribed as polycistronic mRNAs. The paucity of stem-loop structures for transcript termination between upstream ORFs and pseudogenes and the presence of promoters upstream of some pseudogenes suggested mechanisms contributing this high rate of transcription. Since translation of large numbers of pseudogene transcripts could impact M. leprae's energy consumption without benefit to its survival, bioinformatics tools were applied to identify mechanisms of transcriptional 'silencing' of transcribed pseudogenes. The majority of these pseudogenes (73%) lacked translational start codons, strong Shine-Dalgarno sequences (SD), had multiple 'in-frame' stop codons and possessed high Ka/Ks ratios indicating lack of functionality. Very few transcribed pseudogenes possessed their own putative promoter, start codon, and strong SD, demonstrating their potential to express its protein product. Taken together, these data suggest that even though a large number of pseudogenes are transcribed in M. leprae, most are phenotypically 'silent'.

O-86
Development of a Rapid Molecular Viability Assay for M. leprae

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As a result of the inability to cultivate M. leprae on axenic media, the viability of a preparation, including that from biopsy materials, is often unknown for clinical or experimental purposes. Therefore, to address this we developed a 'real-time' RT-PCR viability assay for M. leprae based on the sodA gene transcript, a labile mRNA and using the previously developed RLEP DNA 'real-time' PCR TaqMan assay to normalize template for bacterial numbers. FastPrep/Trizol technology was used to purify both RNA and DNA from the same sample. When DNA and (c)DNA from rifampin-treated M. leprae were tested in this assay, results demonstrated that a significant decrease in viability could be seen in as little as 48 hr post treatment. Currently this assay is being evaluated to determine its sensitivity and ability to define the viability of M. leprae from the skin biopsies of leprosy patients pre- and post- MDT. It is anticipated that this assay will be useful as an additional molecular tool to assess the viability of M. leprae for experimental research and to assessment of the response to chemotherapeutic intervention as well as presence of persisters in relapse cases.

O-87
Cloning and Characterization of a Hypothetical Efflux Pump Gene in Mycobacterium tuberculosis

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Introduction: Multidrug Resistant Tuberculosis (MDR-TB) has dramatically emerged as a biggest challenge for the chemotherapy of tuberculosis and has been grown into a more deadly form Extensive Drug Resistant Tuberculosis (XDR-TB). Besides accumulation of mutations in the drug targets, multidrug efflux pumps have been reported to be one of the most important mechanisms of drug resistance. Genome sequencing of Mycobacterium tuberculosis has identified a number of open reading frames encoding proteins with hypothetical drug efflux function. Methodology: We have cloned and characterized one of these efflux pump genes (paten applied) in M. tuberculosis with the help of an E. coli-Mycobacterium shuttle vector. The amino acid sequence of this efflux pump is conserved among other mycobacterial species and distant homologues of the gene in M. leprae encode for putative transmembrane drug efflux proteins. The over-expression of the gene in the clones has been confirmed by reverse-transcription PCR as well as Real-Time RT-PCR. Results: The over expression of the gene in clones resulted in an increase in minimum inhibitory concentration of ethambutol and izoniazid, when studied by resazurin microtiter assay plate method. In the presence of efflux pump inhibitors CCCP and verapamil this increase in minimum inhibitory concentration of these drugs has reverted back. Conclusion: The gene studied possibly has important role in conferring resistance to ethambutol and izoniazid in M. tuberculosis. Keywords: Efflux pump, M. tuberculosis, M. leprae.
Detection of Genotoxic risks in the Offsprings of Leprosy Patients who Underwent ‘MDT’

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Leprosy, a chronic infectious disease caused by Mycobacterium leprae. Leprosy is considered to be a specific public health problem, owing to the permanent disabilities it causes as well as its social consequence such as discrimination and stigma. The different clinical presentations of the disease are determined by the quality of the host immune response. WHO recommends a protocol of multidrug therapy (MDT) effectively controls the disease, hence contributing to the global elimination program. Of the various mechanisms that influence the pathogenesis of the leprosy, oxidative stress caused by reactive oxygen species (ROS) is important. Besides the pathogenesis of the leprosy, the drugs used in MDT are also generating ROS which cause deleterious effect. There is a growing evidence suggesting that oxidative stress is believed to be associated with a host of a major disease processes. Despite the worldwide incidence and reported evidence of possible genotoxicity of components of MDT no comprehensive cytogenetic investigation has been conducted on leprosy patients and on their offspring. So this study aimed to determine the genotoxic risk in leprosy patients who are under MDT (group I) and the offspring of the leprosy patients who underwent MDT (group II). Single Cell Gel electrophoresis is a sensitive technique to detect both the DNA damage and the repair. Therefore the comet assay was used to evaluate the DNA damage and the repair in the peripheral blood lymphocytes (PBL) of the above said subjects and compared with control. The result of this study clearly indicated a significant increase in the incidence of endogenous DNA damage, DNA-DNA cross links, DNA-protein cross link in (group I) but not in (group II). The repair capacity of the PBL of group II significantly high when compare to group I. However it is lesser than the control. It is also observed that none of the confounding factors has influence on the basal endogenous DNA damage. Hence this study concludes that the DNA damage generated during the MDT is repairable and supplementation of certain antioxidants is recommended to protect the DNA damage.

Real Time PCR Based Approach for Monitoring the Effectiveness of Chemotherapy of Leprosy

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Introduction: Mycobacterium leprae, the causative agent of leprosy is uncultivable and hence development of new diagnostic tools, which does not depend on bacterial culture is needed for monitoring the effectiveness of chemotherapy and early detection of M. leprae. Methodology: Real Time PCR based assay was used for absolute quantification of the copy number of hsp18 DNA and mRNA from leprosy patients. Paraffin embedded biopsy samples were optimised for the preparation of DNA and RNA. Results: The assay was specific, sensitive and reproducible. A reduction in hsp18 DNA and mRNA was found during chemotherapy. Complete disappearance of hsp18 mRNA was observed in biopsies from patients treated for two years of Multi Drug Therapy (MDT). But considerable amount of DNA could be detected even after two years of MDT. In addition we also examined the level of pathogen specific DNA and RNA in reactional cases. Conclusions: Our results confirm the correlation of MDT treatment and decline in the gene expression level indicating the usefulness of this simpler and safer approach to monitor chemotherapy of leprosy. However, significant amount of mRNA and DNA of hsp18 could be detected in reactional cases and this finding raises a critical issue in the treatment of reactional cases. Key words: Leprosy, Paraffin-embedded tissues, Real time PCR, Multi Drug Therapy.
Distribution of *Mycobacterium leprae* genotypes in Uttar Pardesh, India

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**Introduction**: Although genomic analytical strategies have made a substantial impact on epidemiological studies of many infectious diseases, leprosy caused by the obligate intracellular organism *M. leprae*, has not benefited to the same extent. A primary reason is the inability to cultivate *M. leprae* and analyze whole genomic DNA directly from clinical samples, particularly for the paucibacillary form of the disease. The object of this study was to analyze the strain diversity among *M. leprae* strains from UP by targeting ten Short Tandem Repeats (STRs) as molecular markers; we measured the copy numbers of simple sequence repeats or minisatellites, in *M. leprae* from patients of Uttar Pradesh including Ghatampur area. **Methodology**: Thirty specimens including 14 biopsies and 16 slit scrapings were collected from leprosy patients across the spectrum, who attended the OPD of JALMA Agra, and from 9 villages of Model Rural Health Research Unit at Ghatampur, Kanpur. These biopsies were homogenized and DNA was extracted. STRs loci were amplified by using the primers and conditions earlier published by Groathouse et al (2004). Among these ten STRs primers and assays for three STRs were developed and standardized at JALMA. Differences in the mobility were observed in the gel electrophoresis. STRs showed good amplification in slit scrapings from PB specimens also. The copy numbers were also determined by sequence analysis. **Results**: Diversity was observed in a cross-sectional survey of strains from these 30 patients, but similar fingerprinting profiles found from the cases of nearby locations indicate a possible common transmission source. **Conclusion**: STRs as molecular markers appear to be useful for studying molecular epidemiology of leprosy in this region. **Key words**: STRs, *M. leprae*.

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*M. leprae* Genes Expressed in Human Host as Potential Drug Target(s)

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**Introduction**: Differential expression of genes encoding enzymes particularly concerning metabolic pathways of mycobacteria, could be important for mining new targets to design new drugs. A gene essential for the survival, over expressed during disease with low homology with any host protein could be selected as a drug target. **Methodology**: To study active transcription of *M. leprae* genes during the active infection in the host, a partial DNA Chip (Indian Patent application No- 2012/DEL/2006 and 884/DEL/2007) for selected genes (genes encoding metabolic checkpoints and genes hypothetically related to virulence) of *M. leprae* has been indigenously developed at the institute and active transcription of 11 (6-metabolism and 5-virulence) genes of *M. leprae* were detected. Active transcription was validated and relatively quantified by Real Time RT-PCR across the disease spectrum by using standard curve generated by using 16SrRNA gene. **Results**: Among these genes *sucA*, *pyrG*, *accA3*, *purN*, *mmiA1* and *purB* related to metabolism of *M. leprae* were found to be expressed across the disease spectrum. Protein sequence as well as 3D structural homology analysis showed presence of bacterial specific region in active sites of enzymatic proteins suitable for drug interaction. **Conclusion**: *sucA*, *pyrG* and *accA3* are reported as essential genes for in-vitro survival of *M. tuberculosis* also, thus supposed to be more promising potential both as anti leprosy, anti TB and other broad anti-mycobacterial as well as anti-bacterial therapeutic targets due to common bacterial metabolism and high protein similarity. **Keywords**: *M. leprae*, Metabolism, DNA Chip, Drug target.
Signaling Events Involving CDK5 and MAP Kinases Play a Role in Cytoskeletal Protein Abnormalities and Nerve Damage in Leprosy

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Introduction: Our studies demonstrated that hypophosphorylation of neurofilament (NF) proteins in both human and mouse lepromatous nerves precede atrophic changes in the axons resulting in paranodal demyelination. Studies in the mouse model indicated the regulation of CDK5 and other kinases in the leprosy process.

Methodology: In order to further decipher the cause-effect relationship, effects of foot pad inoculation of whole M. leprae, M. leprae antigens viz. ML4A and PGL-1 and host factors viz. NGF, and TGF-β on the sciatic nerve NF phosphorylation state was studied using Western Immuno blot analysis. Secondly, NFs are substrates for phosphokinases (PK) such as cyclin dependent kinase 5 (CDK5) and extracellular signal regulated kinase 1/2 (ERK1/2) MAP kinase. Levels of these enzymes in the sciatic nerve homogenates (triton x-100 soluble fraction) were estimated using a radiometric method and γ32p labeled ATP. Results: M. leprae as well as its antigens induced similar NF changes. Significant increase in PK activity with histone with both M. leprae and PGL-1 where as with anti-NGF and TGF-β there was a decrease. In case of M. leprae, PK activity in presence of inhibitors for specific kinases showed 1) Lack of inhibition of PK activity corresponding to CDK5 and ERK 1/2, b) Expression of stress associated kinases viz. p38 MAPK and C-jun N terminal kinase (JNK) and c) Activity for Phospho Kinase C. Conclusions: Down regulation of CDK5 and ERK1/2 leading to down regulation of NF phosphorylation could lead to atrophic changes in the axons of lepromatous nerves. Presence of p38 MAPK and JNK activities indicate a stress response, where as PKC activity indicates regenerative inputs. We therefore propose that perturbation of MAP kinase signaling by M. leprae and its components might be playing an important role in the initiation of a cascade leading to axonal cytoskeletal protein abnormalities and the de- and regenerative responses in leprosy nerves. Key words: Leprosy, nerve damage, axonal atrophy, neurofilaments, phosphorylation, CDK5, MAP kinase. Funding: DST, Govt. of India.

Evaluation of Genotoxicity of Thalidomide on Bone Marrow Cells of Male Wistar / Nin rats

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Despite of its history as teratogen thalidomide serves as a drug of choice for many inflammatory diseases. In leprosy this drug is used for the management of leprosy reactions. The immuno modulatory action by which the drug attenuates the leprosy reactions is still not fully understood. The present study is aimed to study the genotoxicity of thalidomide in male Wistar/NIN rats. Wistar/NIN rats were obtained from the National Infrastructure Facilities for Laboratory Animal Sciences at NIN Hyderabad, they were bred and maintained in the laboratory animal facilities of CLT& RI. Male Wistar/NIN rats was taken for the study. Thalidomide was obtained from the licensed leprologist of CLT&RI for drug incorporated diet preparations. Haematological and Biochemical parameters pertaining to Genotoxicity are also performed. Bone marrow is dissected and the cells extracted from the bone marrow are stained with Giemsa stain for the presence of micronuclei. Population of micro nucleated cells is enumerated in control, and experimental rats. We noticed statistically increased (P<0.05) scores of micro nuclei population in experimental rats fed with higher dosage of thalidomide indicating the genotoxicity when compared to animals fed with thalidomide equal to human dosage. There was no significant alterations in the haematological and biochemical parameters in therapeutic dose fed animals. Hence it is inferred the thalidomide currently administered for therapeutic management/ maintenance dose for leprosy patients is safe. This is evidenced by the supporting haematological and biochemical parameters. Key words: Leprosy, thalidomide, genotoxicology, experimental study.
**O-94**

**A New Mycobacterium Species Causing Diffuse Lepromatous Leprosy**

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Leprosy Research Support – Post Genome Era” (http://www.cvmbs.colostate.edu/mip/leprosy/index.html) Diffuse lepromatous leprosy (DLL) with Lucio’s phenomenon was originally described over 150 years ago in Mexico by Lucio and Alvarez. It is characterized by a distinctive necrotizing skin reaction in non-nodular leprosy, and affects individuals primarily in the Sinaloa and Jalisco provinces of Mexico, Costa Rica, and countries in the Caribbean. From an individual who was diagnosed with DLL at a clinic in Phoenix, AZ, mycobacteria were purified from skin lesions, lung, and lymph nodes, and DNA was isolated for genetic typing. The antibody titer to the M. leprae-specific PGL-1 was very high (50% endpoint, 1:1,500), although reactivity to only 3 out of 6 M. leprae recombinant proteins indicated an unusual pattern for a lepromatous patient. Sequence analysis of genes from two individuals with DLL revealed that the 16S RNA gene diverged by 2.1% and contained a unique sequence, while five other genes mismatched by 6-14% when compared to that of the TN strain of M. leprae. Phylogenetically, using evolutionary trees for a number of sequences, it appears that this mycobacterium evolved from a common ancestor with M. leprae that had branched off from other mycobacteria. These findings may have implications for the research, diagnosis and treatment of DLL.

**Key words:** diffuse lepromatous leprosy, Lucio’s phenomenon, 16S rRNA.

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**O-95**

**Disease Associated Bio-Markers in Leprosy: Are We Any Closer?**

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The spectral pathology of leprosy involves a complex interaction between M. leprae and the host immune system which is reflected in the accumulation of infiltrating cells and their products like soluble cytokines, activation molecules and antibodies in tissues and circulation. Profiling these parameters can help to identify valuable biomarkers for early detection and intervention in leprosy and reactions. The present report summarizes an assessment of an extensive panel of potential biomarkers within serum and tissues in patient populations from Indonesia, Brazil, the Philippines and the Netherlands. Although several potential markers such as the cytokines IFN-γ, the macrophage activation product neopterin and the soluble IL-6R were TNF-α found to be associated with reactions, the study raised critical questions regarding the pitfalls of present approaches in developing simple and reliable tests for early diagnosis of leprosy under field conditions.

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**O-96**

**The Use of Amplification Procedures in the Histological Diagnosis of Paucibacillary Leprosy**

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The histopathology of paucibacillary leprosy, particularly in its early stages, is often non-specific and acid fast bacilli are hard to demonstrate which places severe restraints on the histopathological diagnosis and confirmation of leprosy. Augmentation of histopathological diagnosis can be achieved by the demonstration of panthion specific antigens or nucleic acid sequences using the procedures of immunohistochemistry and in situ hybridization in situ PCR respectively. The present study examines the role of two amplification procedures in detecting nucleic acid sequences specific to M. leprae. The study was performed on 40 histological sections obtained from biopsies of patients belonging to the paucibacillary group (IId and BT leprosy), which on routine histopathological examination showed nonspecific features and were additionally, negative for acid fast bacilli. Two amplification procedures to amplify pathogen specific nucleic acid sequences were performed namely, in situ hybridization incorporating the nonenzymatic biotinyl-tyramide amplification system, and the enzymatic in situ PCR procedure. In both the procedures digoxigenin was used as the label and chromogenic detection done using alkaline phosphatase NBT/BCIP system. Positive signals were observed in 26 of the 40 (65 %) specimens studied using biotinyl-tyramide system and in 31 of the 40 (77.5 %) specimens using the direct in situ PCR procedure. The study demonstrates the potential role of two nucleic acid amplification procedures in the diagnosis of bacteriologically negative paucibacillary leprosy which can be performed on routinely processed paraffin sections.

**Key Words:** amplification methods, histology, diagnosis, paucibacillary leprosy.
Detection of Gene Mutations Related with Drug Resistance in Mycobacterium leprae from Leprosy Patients in Korea

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Identification of the presence and drug resistance of *Mycobacterium leprae* is key to the diagnosis and treatment of leprosy in non-endemic country like Korea. This study aims screen mutations on the drug target DNA such as *folP*, *rpoB*, *gyr*, and 23S rRNA. Sequences of those genes were analyzed for the 104 bacterial index positive cases out of 171 leprosy patients in Korea using touchdown PCR, single stranded conformational polymorphism. Twenty (19.2%) cases have shown the mutations in *folP* gene of dapsone-resistant *M. leprae* in which three (2.89%) cases were mutations in two genes, *folP* and *rpoB*, of multidrugs resistant strains to dapsone and rifampicin, and two (1.92%) cases in *folP* and *gyr* genes of resistance to dapsone and ofloxacin, respectively. Besides double mutation for *folP* gene was one case (0.96%) and for *rpoB* gene one case, respectively. There was no mutant isolates in 23S rRNA gene against clarithromycin. This result should leads to a better understanding of the status of multidrug resistant leprosy in Korea and may assist in the rapid screening of drug resistant *M. leprae* and the choice of the appropriate treatment regimens. **Keywords**: Leprosy; Drug-resistance; Multidrug therapy; *Mycobacterium leprae*; Mutation

Development and Evaluation of a Novel Multiple Primers-PCR Amplification Refractory-Mutation System for the Rapid Detection of Mutation Conferring Rifampicin Resistance in rpoB Gene of Mycobacterium leprae

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There is an urgent need for a simple method which can detect rifampicin resistance in clinical isolates. Here we develop and propose a Multiple primers-PCR Amplification Refractory-Mutation System (MARS), a simple, reliable, and economic method for clinical specimens that allows the rapid detection of mutation in the nucleotides of the codon for Ser425 to Leu, Met, Phe of the *M. leprae* *rpoB* gene associated with rifampicin resistance. The approach involves a multiple primers PCR in which both mutant specific and normal sets of primers are included in reaction. The mutant specific primer is complementary to the corresponding sequence of the wild-type gene except for one additional deliberate mismatch near the 3'-OH terminus. The assay was successfully evaluated using a panel of plasmids and *M. leprae* reference strains carrying the wild type or known *rpoB* mutations. It was applied to *M. leprae* DNA extracts from skin biopsies taken from patients and all were found to be wild type for Ser 425.

**Key words**: gene mutation, resistance gene, rifampicin resistance.

Detection of Mutations In RpoB and FolP 1 Gene of M. leprae Using Molecular Methods

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Molecular tools for rapid detection of drug resistance in *M. leprae* have been well established. A study was undertaken to determine the drug susceptibility of *M. leprae*, using Mouse Foot Pad (MFP) assay and molecular methods. 32 smear positive leprosy patients with features of Relapse / Irregular treatment / New cases were included in the study. Skin biopsy was done and the samples were processed for MFP assay and PCR assays were carried out to amplify 305bp of *rpoB* gene and 388bp of *fol P1* gene. PCR products were further purified and processed for direct sequencing and for non radioactive Single-strand conformation polymorphism (SSCP) analysis. MFP assay showed significant growth in 23 out of 31 samples processed (74%). 8 isolates were dapsone resistant and one isolate showed combined resistance against dapsone, rifampicin and clofazimine. Amplification of *rpoB* and *fol P1* genes was successful in all the 32(100%) samples. Among the *fol P1* products sequenced, 6 isolates showed mutations at 53(or) 55 amino acid positions and these isolates showed high (or) intermediate level resistance to dapsone in MFP assay. Among the *rpoB* products sequenced, mutation was detected in one isolate consistently at 433 amino acid position, which was resistant to rifampicin by MFP assay. However reverse sequencing ruled out any mutation. SSCP analysis showed results consistent with that of direct sequencing. Thus molecular methods are found to have potential scope for rapid detection of mutations against anti-leprosy drugs.
Expression of mce1A gene of Mycobacterium leprae in Escherichia Coli
Inhibits Host Cell Growth

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Introduction: Mammalian cell entry protein (Mce1A) helps in the survival as well as efficient entry of mycobacteria into the macrophages. Mycobacterium leprae genome has only one functional mce cluster, which may be essential for survival and possibly determines the virulence. Methodology: PCR amplified mce1A from leprosy biopsy was cloned into the expression vector pQE31 and transformed into E. coli M15 (pREP4). Histidine tag at the N-terminal end allowed purification as well as detection using NiNTA metal affinity column and anti-His antibody respectively. Protein was also identified using mass spectrometry. Overlap extension PCR was done to introduce mutations. Results: Using anti-His antibodies, Mce1A was detected as a 50.3 kDa protein. Viable counts showed that cells carrying mce1A failed to divide after IPTG induction. To examine the effect of rare codons a truncated protein (deleting 179 amino acids at N-terminal end) was constructed. This protein was overexpressed as inclusion body (27 kDa) and these cells were unaffected after IPTG induction. The cells having a mutated construct, with only one rare codon deleted, were also unaffected after IPTG induction. Conclusion: Removal of a rare codon eliminated the cessation of cell division which was due to possible ribosomatic stalling during translation. For over expression of Mce1A removal of entire rare codons from N-terminal end may be required. Key words: Mce1A protein, overexpression, rare codons, mutation, Mycobacterium leprae.

Immunogenetic Profile of Leprosy Patients of Karakalpak Nationality

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Karakalpak focus of leprosy was active in Central Asian region where leprosy karakalpak patients took the first place. The aim of investigation – to study distribution of alleles genes HLA II class among leprosy Karakalpak patients. 40 leprosy karakalpak patients and 76 controls were typed for HLA genes DRB1, DQA1, DQB1 by using PCR-MSSP. The frequencies of alleles HLA-DRB1-15, DQA1-0102, DQB1-0502, DQB1-0602, and of genotypes 15-0102-0602 and 15-0103-0601 were significantly increased in the patients as compared with the control subjects. On the other hand, the occurrence of HLA DRB1-08, DQB1-0401 and of genotype 09-0301-0303 was significantly lesser in the patients. Alleles DRB1-16, DQA1-0401 and genotypes 16-0102-0502 and 15-0102-0602 were met only in leprosy patients, genotype 08-0401-0401 only in healthy persons. Our results suggest that HLA-class II genes confer susceptibility to or protection from leprosy to the Karakalpak. This study along with our earlier studies on HLA association in leprosy suggests that HLA-DRB1-15 alleles may be universal marker of susceptibility to leprosy. Key words: leprosy, susceptibility, alleles, genotypes, karakalpaks.
Immunohistochemical Analysis of Skin & Nerve in MB Leprosy Patients in the INIFIR Cohort Study

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**Background & Objective:** Tissue expression of TNF-α, TGF-β and iNOS in 298 skin biopsies at first diagnosis and 68 nerve biopsies at reaction were studied in the INIFIR Cohort. In addition, biopsies were stained with H&E stain, modified Feito Feraco stain, CD68 cell marker and S100 to observe morphological changes in the tissues. The objective of the study was to characterize the cellular infiltrate and identify any association of these cell and cytokine markers with nerve function impairment in leprosy.

**Results & Discussion:** The cytokines TNF-α, TGF-β and the enzyme iNOS were detected in 79, 94 and 78 percent of skin biopsies respectively. The levels of staining for these molecules were similar across the spectrum. The presence of TNF-α, TGF-β and iNOS were significantly higher in the reactional group and may be involved in nerve damage. The presence of these molecules in non-reactional tissues suggests their involvement in the disease pathology as such. 68 nerve biopsies taken at the time of the event were available for analysis. CD68, TNF-α, TGF-β and iNOS staining were detectable in 88, 38 and 28 percent of the biopsies respectively. S100 staining was higher in group that showed no significant lesion histologically than in indeterminate group. 45% of BT nerves in TIR stained for S100 had a staining pattern reflecting extreme nerve destruction. **Conclusion:** For the first time the association of TNF-α, iNOS and TGF-β with RR has been clearly demonstrated. The three molecules detected in immunohistochemistry showed a significant association with the presence of skin reaction and nerve damage.

**Keywords:** Immunohistochemistry, Cytokines, Leprosy, Nerve Function Impairment.

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Comparative Tissue TNF-α and Anticeramide Antibody in MB Leprosy Patients in the INIFIR Cohort Study

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**Introduction:** Serum TNF-α protein and anticeramide antibodies were measured in 303 leprosy patients at diagnosis and every month for 12 months in the INIFIR cohort. These molecules were tested as potential markers for reversal reaction and nerve function impairment (NFI). **Methodology:** TNF-α and anticeramide antibodies were measured using Sandwich & Indirect ELISA techniques respectively. Serum TNF-α levels were also compared with immunohistochemical presence of TNF-α in skin and nerve biopsy in 147 patients in the cohort. **Results:** There was an association of TNF-α with new sensory and motor Nerve function impairment. Comparative analysis of TNF-α revealed the presence of TNF-α in only one of the tissues (e., plasma, skin or nerve (35%, 5% and 3% respectively). When concomitant expression of TNF-α in only two tissues was analyzed it was detected in 20% in plasma and skin; 10% in plasma & nerve; and 3% in skin and nerve. TNF-α was absent from all three tissues in 9% of the patients and present in all three tissues in 15% of the patients. **Conclusion:** TNF-α and anticeramide antibodies may be associated with the pathological processes of leprosy and in the acute immunological phase of the disease. The results also showed that TNF-α expression in the plasma, skin and nerve are differentially regulated by the extent of individual tissue inflammatory processes.

**Keywords:** TNF-α, Anticeramide antibodies, Leprosy, Neuropathy.
The Development and Manufacture of Novel Skin Test Antigens for Use as Early in vivo Diagnostics for Leprosy

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Current diagnostic methods for leprosy are laborious, lack sensitivity, and require trained clinicians to decipher results. Due to the need for improved early diagnostics, two novel leprosy antigens have already been produced. Presently, these antigens, composed of M. leprae soluble antigens (MLSA) and M. leprae cell wall-associated protein (MLCwa), are under investigation in a large phase II clinical trial in Nepal. Furthermore, new diagnostic candidates, such as the M. leprae membrane antigen (MLMA), have been developed and are ready for production. Development and manufacturing of this material is the responsibility of the PDM Core in collaboration with the NIH Leprosy Contract at Colorado State University. Clinical grade antigen is manufactured under current Good Manufacturing Practices (cGMP), which comply with US Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) guidelines for quality of product permitted in human clinical trials. Recent quality control assay development and process standardization allowed for product characterization and improved evaluation of sterility, safety, purity, potency, and identity of the final product. Thus, the PDM Core is poised for initiation of subsequent manufacturing campaigns, which consist of M. leprae whole cell purification, cellular subtraction, and refinement, eliciting the next generation of skin test antigen candidates.

Genetic Variability in Mycobacterium leprae on TTC Repeats by Polyacrylamide Gel Electrophoresis (PAGE) in Nepal

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Strain differentiation of Mycobacterium leprae would be of great value for several epidemiological investigations. From the M. leprae genome sequence database, TTC DNA repeats were identified. Primer sets designed to amplify the region flanking TTC repeats revealed PCR products of different sizes, indicating that the number of repeats at each locus may be variable among M. leprae strains. Upon analysis of the TTC repeat region in each of the M. leprae strains by PAGE showed a variation of 3 to 30 repeats. In the M. leprae strains of 117 patients, M. leprae with 13 and 15 TTC repeats were the most common, and this was followed by strains with 9, 14 and 17 repeats. This study thus indicates that there are variable numbers of TTC repeats in a non-coding region of M. leprae, and that the TTC region may be useful for strain differentiation for epidemiological investigations of leprosy. Key words: polyacrylamide gel electrophoresis (PAGE) in mycobacteria.

A Common Polymorphism in TLR1 is Associated with Impaired Signaling in Response to Mycobacteria and Protection from Reversal Reaction in Leprosy

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Introduction: The host factors that regulate leprosy susceptibility are poorly understood. We recently identified a common variant in the transmembrane domain of Toll-like receptor 1 (TLR1), T1805G (1602S) that regulates the immune response to tricycated lipopeptide, a synthetic ligand for TLR1. We investigated whether TLR1-deficiency is associated with susceptibility to leprosy and reversal reaction, an immune reaction characterized by TH1 T cell responses. Methods: Allele 1805G was associated with markedly impaired TLR1-mediated NF-kB signaling in response to stimulation with heat-killed extracts of M. leprae and M. tuberculosis in transfected HEK293 cells. We genotyped SNP T1805G in a case-control study of 938 Nepalese leprosy patients. Results: 11805G was not associated with susceptibility to leprosy type, although GG individuals showed an increased risk of lepromatous vs. tuberculoid leprosy (OR 5.12, 95% CI 0.64-41.17, P=0.087). However, the 1805G allele was associated with protection from reversal reaction (OR 0.51, CI 0.30-0.88, P=0.013). The GG/GT genotypes were also associated with a lower likelihood of reversal reaction when compared to TT individuals (OR 0.55, CI 0.32-0.97, P=0.036). Conclusion: Our findings suggest that TLR1-deficiency may impact clinical outcomes such as reaction. Key words: TLR1 polymorphism, leprosy.
**Definition of *M. leprae* Native Fractions Towards the Discovery of New Diagnostic Tools**

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Native subcellular fractions of *Mycobacterium leprae* have been extensively evaluated with state of the art techniques to define the proteome, identify immunogenic components, decipher surface proteins, and probe for secreted proteins. The sum of this work provides a substantial collection of unique antigens that may be ideal candidates for developing new diagnostic tools for assessing pre-clinical *M. leprae* infection. The purpose of this work is to evaluate the distinctive features of these antigens and/or subcellular fractions and to test them in a comparable diagnostic platform. Methods used to elucidate and define the cytosol, membrane and cell wall of *M. leprae* native fractions consists of extraction, phase separation, chemical manipulation, two-dimensional gel electrophoresis, proteolytic cleavage, mass spectrometry, and comparative genomic and proteomic analysis. The top candidate identified to-date is a membrane fraction exposed to oxidation, reduction, hydrolysis and butanol extraction to remove immunomodulatory lipoglycans. *In vivo* guinea pig testing suggests a highly potent and specific antigen, while *in vitro* testing reveals clarification of the two major membrane proteins, MMP-I (ML0841) Mr 35 kDa, and MMP-II (ML2038) Mr 22 kDa, bacteriophage. This candidate is being further evaluated for development as a skin test antigen, while examination of remaining antigens continues. **Key words**: native, protein, diagnostic.

**Extraction of Mycobacterial DNA and RNA From Soil**

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*Mycobacterium leprae* is a chronic pathogen causing leprosy. Very little is known about the transmission of the disease and reservoir for *Mycobacterium leprae*. For effective elimination and control of this disease, better understanding of epidemiology is required. Protocols for rapid detection of this organism from various different samples (clinical as well as environmental) are required. This study was designed to assess suitability of DNA and RNA extraction protocols for mycobacteria from soil, so that it can be used to determine the presence or absence of *Mycobacterium leprae* in the environment. We compared different cell lysis protocols for effective extraction of DNA and removal of PCR inhibitors from the soil samples. The DNA and RNA extraction method was standardised using soil seeded with serial dilutions of non-pathogenic mycobacteria like *M. smegmatis* and *M. phlei* followed by PCR and RT-PCR using primers for 16S rRNA gene specific for general mycobacteria. We found that lysis of cells with bead beating in the presence of ethanol effectively lysed cells releasing DNA as well as RNA. Further, use of Sepharose 4B columns removed humic acid compounds that inhibit PCR reaction. RNA could be selectively extracted using acid phenol: chloroform. Further RNAse free DNAse treatment gave pure RNA preparation that can be used for RT-PCR work.

**Key words**: extraction of mycobacterial DNA, extraction of mycobacterial RNA.

**Molecular Epidemiological Analysis of *M. leprae* Infection and Transmission of Leprosy**

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Epidemiology of *M. leprae* infection and consequent prevalence of leprosy is one of the most important research subjects in leprosy. However, until recently, we could not conduct scientific analysis due to lack of knowledge and technique. In 2000, Shin et al reported a genetic diversity of leprosy bacillus named “TTC repeat” which makes it possible to analyze *M. leprae* infection in detail. Since the end of 1990s, we are conducting molecular epidemiological survey at leprosy endemic areas in Indonesia to know the situation of *M. leprae* infection in inhabitant of endemic villages. The methods we are using are comparative analysis of the distribution of *M. leprae* genotypes in skin lesions, living environment and daily life water. **Followings are the results obtained so far**: 1. Considerable numbers of inhabitants are carrying leprosy bacillus on the surface of their nasal cavity. 2. TTC genotypes of *M. leprae* detected in a family are often different. 3. It is not rare that household cases have different genotypes; suggesting they are infected by the different sources. 4. The distribution of TTC genotypes is same in living environment and skin lesion; suggesting that patients are infected with bacilli in the living environment. **Key words**: Molecular epidemiology, genotyping of *M. leprae*, non-human sources.
Study of 18 Referral Multibacillary Relapse Cases in Leprosy Received During the Period 2004 -2007

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Objective and Methods : MDT was introduced in the year 1982 because of the emergence of resistance to DDS monotherapy. To evaluate the impact of MDT it is important to document and study the relapse cases as and when they appear. Eighteen referral multibacillary relapse cases received during 2004-2007 were studied in detail. History, body charting and treatment details were recorded. Slit skin smears and a lesional biopsy was also done in all. The lesional biopsy obtained was divided into 2 parts, one part was fixed and processed for histopathology and the second part was homogenized into bacterial load/gm wt was determined. The homogenate thus obtained was appropriately diluted and inoculated into the hind foot pads of normal S/W mice, where viability and drug sensitivity testing was done. Findings : Treatment details: Of the 18 cases, 8 (44 %) were treated MB- MDT for 12 months and 7 (38 %) with MB- MDT till smear negativity (2 – 4 years) and 3 cases had between 5 – 11 years of DDS monotherapy. All the 18 cases had an average BI of > 4+ in the slit skin smear on relapse. The average duration between cessation of treatment and appearance of new lesions (DCTR) was > 10 years in MDT treated cases and ~ 25 years in DDS monotherapy. We also found the release from treatment and all had an average BI of > 4+. Detection of resistance to Rifampicin (0.03 gm %) in 2/6 cases in Mouse footpad test is of concern. Keywords: Leprosy, Multibacillary Relapse, Rifampicin resistance, MFP test.

Application of New Serological Test for Leprosy in Vietnam

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Since early detection and rapid medication is important for leprosy control, serodagnosis is very useful to detect Mycobacterium leprae infection. We have established a new serodiagnostic test using major membrane protein-II (MMP-II) as an antigen for detecting leprosy infection. So, in this study, we conducted serological survey of leprosy patients, their contacts, healthy individuals living in the central part of Vietnam. Although Vietnam attained the WHO’s leprosy elimination target of less than 1 per 10,000 population in 1995, the number of newly detected cases in the year 2006 was still 666. We measured anti-MMP-II IgG antibody level and anti-PGL-I IgM antibody level in sera from 224 leprosy patients, their contacts and 211 healthy controls by enzyme linked immuno-sorbent assay (ELISA). The percent positivity by MMP-II ELISA was 85% for multi-bacillary leprosy (MB). For pauci-bacillary leprosy (PB) where cell-mediated immunity predominates, 47.0% showed positive results. Although, the positive rate of PGL-I ELISA were almost similar in both MB and PB to those of MMP-II ELISA. These results suggest that MMP-II antibody detection would facilitate diagnosis of leprosy. Keywords: Serodiagnosis, Major Membrane Protein-II, PGL-I.

Evaluation of the ML Flow Test as a Point-of-Care Serology Test

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We evaluated the ML Flow test as an additional, serological, tool for the classification of new leprosy patients in Brazil, Nepal and Nigeria. 2632 leprosy patients were classified using three methods: (1) counting the number of skin lesions, (2) slit skin smear examination, (3) serology using the ML Flow test. In Brazil and Nepal about 1/3 of the patients were MB against 1/5 in Nepal. Seropositivity was 63% in Nigeria, 51% in Brazil and 36% in Nepal. ML Flow test results and smears were negative in the vast majority of PB patients and in 16% of Brazilian and 38% of Nepali MB patients. Testing all PB patients with the ML Flow test to prevent under-treatment would increase the MB group by 18%, 11% and 46% for Brazil, Nepal and Nigeria. Using the ML Flow test as the sole criterion for classification would result in an increase of 11% and 44% of patients requiring MB treatment in Brazil and Nigeria and a decrease of 4% for Nepal. The ML Flow test could be used to strengthen classification, reduce the risk of under-treatment and minimize the need for slit skin smears.
Relapse Associated with Resistance in Leprosy Patients: A Prospective Study from 2003 to 2007

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In Brazil MDT was effectively implemented in 1991 and contributed to reduce leprosy prevalence, nevertheless, relapses have occurred and its causes are not commonly investigated: treatment failures, persistence of bacilli or drug resistance. **Objective**: to verify the occurrence of resistance bacilli in leprosy patients who relapsed at least 5 years after treatment (DDS monotherapy, DNDS, MDT). **Patients and methods**: multibacillary patients presented with new lesions, diagnosed clinically, attended by spontaneous demand. A skin biopsy was collected from the lesion for mouse foot-pad inoculation according to Shepard’s protocol. A total of 52 patients were evaluated. **Results**: Among relapse cases: 21 (40.3%) were sensitive to dapson and rifampicin, 06 (11.5%) were resistant to dapson (02 DDS monotherapy, 04 MDT), 02 (3.8%) resistant to rifampicin (MDT) and 23 (44.2%) were inconclusive; multiple resistance was not verified. **Conclusion**: The finding of resistance samples show the need of follow-up of patients to validate treatment schemes. Surveillance of drug resistance cases using appropriate approaches are needed, especially in endemic countries such as Brazil. **Key-words**: leprosy, relapse, drug resistance, dapson, rifampicin.

Recombinant BCG Expressing *Mycobacterium leprae* or *Mycobacterium tuberculosis* Ag85b Induce Protection Against *M. leprae* Challenge Comparable or Superior to BCG

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**Background**: Renewed interest in improving BCG as a vaccine for tuberculosis and leprosy have produced new vaccine constructs including recombinant BCG (rBCG). rBCG expressing *Mycobacterium tuberculosis* (MT) Ag85B (rBCG30MT) has been shown to provide enhanced protection against MT challenge in guinea pigs compared with parental BCG. **Methodology**: Here, we tested the efficacy of rBCG vaccines expressing either the *Mycobacterium leprae* (ML) or the MT form of Ag85B in the mouse model of leprosy. Methods rBCG30MT carrying plasmid pSMT3 encoding the MT Ag85B gene and rBCG30ML carrying plasmid pNBV1 encoding the ML Ag85B gene were constructed using BCG Tice. BALB/c mice (n = 15/group) were sham-immunized or immunized with 1x107 viable rBCG30MT, rBCG30ML, or BCG (Tice), or with heat-killed ML. Mice were challenged 2.5 months later in each hind foot pad with 5x103 viable ML, and 210 days after challenge, the number of ML/foot pad was enumerated. Splenocytes and lymph node cells from vaccinated (n=3) and control (n=3) mice were tested for sensitization to purified protein derivative (PPD) by lymphocyte transformation (LT). **Results**: All vaccinated groups showed sensitization to PPD by LT. Splenocytes from rBCG30ML–immunized mice [stimulation index (SI) = 14.5] and lymph node cells from rBCG30MT–immunized mice (SI = 67) showed the strongest LT responses. All vaccines provided substantial protection in the mouse foot pad assay (P < 0.0006, Mann-Whitney test). In the first experiment, both rBCG30MT and rBCG30ML gave protection superior to BCG and the difference between rBCG30MT and BCG was statistically significant (P = 0.04) as was the difference between the two rBCG30 groups combined and BCG (P = 0.04). In the second experiment, all BCG strains gave protection at the limit of detection. **Conclusions**: Both rBCG30 strains protect against leprosy in the mouse model, and in the one experiment in which an efficacy comparison was feasible, the efficacy of rBCG30MT was significantly greater than BCG. These data suggest that rBCG30MT may offer cross-protection against leprosy in humans that is comparable or superior to BCG.