Schwann cells to participate in microbial recognition according to their expression of Toll-like receptor 2 (TLR2). In this paper, FACS analysis of a human Schwann cell line ST88-14 and immunohistochemistry of leprosy skin lesions demonstrate expression of TLR2 on the surface of human SC (double-fluorescence labeling showed colocalization of a Schwann cell marker, neural cell adhesion molecule (NCAM) and TLR2). Given that TLR2 mediates recognition of microbial lipopeptides, we engineered a synthetic lipopeptide comprising the first six amino acids of the putative *M. leprae* 19 kD antigen. Activation of the human Schwann cell line with the the *M. leprae* lipopeptide triggered an increase in the number of cells with condensed nuclei and evidence of DNA fragmentation, characteristics consistent with cell death. Hoescht stain and 7-AAD showed a 2 or 3 fold enhancement in the cell death when compared to the unstimulated cultures. The ability of *M. leprae* components to induce apoptosis of Schwann cells through Toll receptors might provide a mechanism for nerve damage in leprosy in the absence of inflammation.

MICROBIOLOGY & MOLECULAR BIOLOGY

PM & BM 1

A HISTOLOGICAL AND BACTERIOLOGICAL ASSESSMENT OF LEPROSY PATIENTS WITH < 5 LESION

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Aim: To study the histological and bacteriological features of leprosy patients with 5 or less than 5 lesions and relate it to clinical features.

Methods: 76 consecutive leprosy patients (M 57 F 19) who had 5 lesions were included in the study. Clinical features were recorded, slit skin smears and skin biopsies were done on all patients. A nerve biopsy was performed (radial cutaneous or Sural nerve) in 18 patients who had a clinically thickened cutaneous nerve.

Results: Out of the 76 patients, 28 patients had single skin lesions, 17 had 2 lesions, 13 had 3 lesions, 5 had 4 lesions and 2 had 5 lesions. The clinical diagnosis was TT leprosy in 4, BT in 68 and indeterminate in 4. Slit skin smears were positive in only 1 BT leprosy patient.

Histological examination revealed features of TT leprosy in 2 patients (2.6%), BT leprosy in 42 patients (55.3%), BL in 4 patients (5.3%), indeterminate leprosy in 16 (21%) and non-specific inflammation in 12(15.8%). Acid fast bacilli ranging from a bacterial index of granuloma (BIG) of 1+ to 4+ were present in 10 of the skin biopsies (13.2%). The cutaneous nerve biopsies in 16 of the 18 patients (88.8%) revealed features of BT leprosy consisting chiefly of lympho-epithelioid granuloma. 12 of these nerve (66.7%) revealed AFB in them with a BI ranging from 1+ to 4+.

Conclusion: The findings from the study indicate that the number of lesions does not determine the type or extent of the disease.

PM & BM 2

A HISTOPATHOLOGICAL STUDY OF TYPE II (ENL) REACTION IN LEPROSY

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Type II lepra reaction produces a defined clinical picture of painful and tender erythematous nodules which are described as 'Erythema Nodosum Leprosum'. The histology of these lesions have been variously described. The aim of this study was to document the different components that constitute a histological diagnosis of ENL and their consistency of occurrence in each lesion.

A detailed study was made of 22 skin biopsies from ENL lesions. A histological diagnosis of 'LL in ENL' was made in 11 biopsies (50%). The most consistent feature noticed in these 11 biopsies was the presence of foamy macrophage granulomas in a pale oedematous dermis. The oedema was more prominent in the upper dermis and was associated with dilated vascular channels. Neutrophilic infiltrate was a consistent finding in 9 biopsies and vasculitis in 8. Plasma cells were present in 5 and panniculitis was noticed only in 1 biopsy.

Acid-fast stain revealed predominantly beaded and granular bacilli in the macrophages, nerves, smooth muscle and in the sub epidermal zone. Bacilli were also seen in the endothelial cells in 2 biopsies and in the wall or lumen of the blood vessels in 2 biopsies.

In the remaining 11 biopsies although the patient was clinically diagnosed as LL in ENL the histology did

not reveal features that were sufficient to label as ENL. The findings in these biopsies were of 'lepromatous leprosy' with macrophage granulomas and acid fast bacilli. Oedema, vasculitis and neutrophilic infiltrate were absent in these lesions.

PM & BM 3

ALTERED PROTEIN PHOSPHORYLATION IN LEPROSY LYMPHOCYTES – A PRELIMINARY STUDY

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Protein phosphorylation is a post-translational modification that modulates the specific functions of various effector proteins and is a major biochemical mechanism by which cells integrate extracellular signals and respond to it. Recently, a detailed picture of protein kinases involved in the regulation of immune cells has been reported. cAMP dependent kinases, Calcium/Calmodulin dependent kinases and Protein Kinase C (PKC) have been shown to be involved in B and T cell responses to antigens. Leprosy is a disease in which varied types of immune responses to *M. leprae* are observed.

To understand the molecular basis of immune response, we carried out protein phosphorylation of lymphocytes from leprosy patients in the presence and absence of modulators - cAMP, cGMP and Phoshotidylinositol. A wide range of proteins were phosphorylated in lymphocytes of normal and leprosy patients. The modulators had a similar effect on both normal and leprosy lymphocyte phosphorylation patterns, except for the 20 and 29 kDa proteins which showed a decreased phosphorylation. The pattern and significance of the phosphorylation in leprosy lymphocytes is presented.

PM & BM 4

ARMADILLO-DERIVED *Mycobacterium leprae* PRODUCES A HEPARIN-BINDING HEMAGGLUTININ ADHESIN (HBHA)

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As described for several bacterial pathogens, *Mycobacterium tuberculosis* expresses a surface-exposed heparin-binding hemagglutinin adhesin (HBHA), which is specifically involved in epithelial adherence through interactions with heparan sulfate-containing proteoglycans. Recent data showed that the disruption of the *hbhA* gene impaired *M. tuberculsis* dissemination from the lungs after intranasal infection of mice, indicating that HBHA plays an important role in the pathogenesis of tuberculosis. The aim of this study is to investigate the role of the *M. leprae* HBHA homologue in leprosy. Indeed, the recent conclusion of the *M. leprae* genome revealed the presence of a *hbhA* gene coding for a protein of 199 amino acids and sharing 81.4 % identity with the *M. tuberculosis* homologue. To demonstrate the expression of this adhesin in armadillo-derived *M. leprae*, the bacilli were sonicated and subcellular fractions were isolated and analyzed by western blot developped with a panel of anti-*M. tuberculosis* HBHA antibodies. A reactive band with the expected apparent molecular weight was detected in the cell wall and soluble fractions, suggesting that the adhesin is present on the bacterial surface. As observed for *M. tuberculosis*, the HBHA expressed by *M. leprae* is also posttranslationally modified by methylations of the lysine residues present in the carboxy-terminal heparin-binding domain of the adhesin. Investigations are currently in progress to determine the role of HBHA in the interaction of *M. leprae* with Schwann cells.

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PM & BM 5

ASSOCIATION OF NRAMP1 GENE POLYMORPHISM WITH GENETC SUSCEPTIBILITY TO LEPROSY

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Mitsuda test is an intradermic lepromin test, which measure the specific immune response to heat-killed leprosy bacilli that have a high prognostic value, meaning susceptibility to the lepromatous form when negative. Linkage analyses have confirmed association of NRAMP1 gene with susceptibility to tuberculosis and leprosy. However, case-control studies could not found association of NRAMP alleles with leprosy status. This study aimed the association of the (GT)n repeats at the NRAMP1 gene promoter region with susceptibility to leprosy and also to the positive Mit-

suda test on high endemic Brazilian population assisted by Sanitary Dermatology/Leprosy Reference Center of Uberlândia, Federal University of Uberlândia (UFU). Leprosy patients (69) were diagnosed by WHO requirements, classified in sub-clinical forms, and submitted to the Mitsuda Test and BCG scar evaluation. Statistical analysis has clustered patients in paucibacillary-PB (36) and multibacillary-MB (33) forms. The control group consisted of 34 healthy non-consanguine household contacts of leprosy patients. Genotypes were obtained by the polymerase chain reaction (PCR), followed by detection through LIS-SSCP (14% PAGE, 49:1 acrylamide:bis, for 20h, 10V/cm, at room temperature). There were no significant differences among 2, 3 and 4 allele frequencies with Mitsuda test average and leprosy status. The allele 3 frequency (0.666) has shown a slight increase in MB patients compared to PB (0.611) and to the control group (0.573). The NRAMP1 gene may be associated to Leprosy resistance; however, our results do not agree with this affirmative, probably due to others factors, such as bacillus exposition, BCG status and genetic heterogeneity.

Support: FAPEMIG

PM & BM 6

ASSOCIATION OF VITAMIN D RECEPTOR GEN (VDR) *Taq1* POLYMORPHISM WITH SUSCEPTIBILITY TO LEPROSY

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Leprosy is a chronic disease caused by *Mycobacterium leprae*, with a wide spectrum of clinical manifestations. This spectrum of clinical/histological characteristics ranging from the polar paucibacillary (PB) form, which corresponds to tuberculoid leprosy (TT), exhibiting strong cellular immunity and a predominance of TH1-cytokine pattern, to the multibacillary (MB) form, which corresponds to lepromatose leprosy (LL) and a TH2-cytokine pattern. The mechanism of TH1/TH2 shift remains unclear but early studies of the leprosy treatment with medications containing vitamin D (VD) analogs are consistent with a possible immunomodulatory effect of VD on bacteriostasis. Also, the VDR gene polymorphism has been implicated with susceptibility to *M. malmoense*, *M. tuberculosis* and with clinical types of leprosy. This case-control study inquired the association of VDR with susceptibility to leprosy *per se* and also to leprosy types on high endemic Brazilian population assisted by Sanitary Dermatology/Leprosy Reference Center of Uberlândia, Federal University of Uberlândia (UFU). Leprosy patients (67) were diagnosed by WHO requirements and classified in sub-clinical forms as described by Ridley and Jopling (1966). Statistical analysis has clustered patients in paucibacillary (36) and multibacillary (31) forms. The control group consisted of 34 healthy non-consanguine household contacts of leprosy patients. The genotypes were obtained by the polymerase chain reaction (PCR), previously described by Roy et al (1999), and followed by *TaqI* restriction. Frequency distribution of *Taq1 T/t* polymorphism ($p = 0.5967$; $q = 0.4033$) for MB patients was significantly different from general population as detected by logistic regression. The MB group exhibits a lower frequency of "T" allele when compared to the control and PB groups, for which frequencies were: $T = 0.7352$; $t = 0.2648$ and $T = 0.7083$ and $t = 0.2917$, respectively. This study suggests that VDR polymorphism modulate susceptibility to leprosy development probably by affecting the TH1/TH2 differentiation of the host immune response.

Support: FAPEMIG

PM & BM 7

CHANGES IN THE PREVALENCE OF DAPSONE RESISTANT LEPROSY SINCE THE IMPLEMENTATION OF MDT

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Aim: To screen all new previously untreated multibacillary leprosy cases and relapses presenting at our leprosy referral hospital for dapsone resistance using MFP culture.

Methods: Skin biopsies were taken from all appropriate consenting patients presenting at Anandaban clinics. These were homogenised and injected into the hind footpads of Swiss Albino mice. Test drugs were included in mouse feed throughout the course of the experiment. When control group mice showed two logs of growth, experimental mice were sacrificed, and numbers of bacteria estimated.

Results: During the period 1987- 2000 a total of 348 samples were tested in our system. Twenty-three of 266 tested for primary dapsone resistance (0.09%) showed resistance at low dose (0.0001%, equivalent to 0.1mg/kg in humans); only one showed resistance at high dose (0.01%, ?10mg/kg). Levels of DDS resistance in patients treated with DDS monotherapy prior to MDT decreased over the period of monitoring. In 11 patients treated with MDT only, none had secondary dapsone resistance.

While there was evidence that some secondary dapsone resistant strains were resistant at high dose, only a single case of primary resistance to high dose dapsone was observed within our population.

Conclusion: Our studies indicate: i) that dapsone resistance has almost entirely disappeared as the remaining dapsone monotherapy patients have died or been treated with MDT, and ii) that secondary dapsone resistance does not develop in MDT regimens.

PM & BM 8

CHANGES IN VIABILITY OF INTERDERMAL *M. leprae* ASSOCIATED WITH THE HISTOPATHOLOGICAL RESPONSE OF SUSCEPTIBLE AND RESISTANT ARMADILLOS

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Leprosy manifests over a broad clinical and histopathological spectrum associated with Th1/Th2 immunological responses. Other than man, nine banded armadillos are the only hosts which develop the full clinical spectrum of leprosy. The CMI that they can manifest towards *M. leprae* has been indexed only with heat killed *M. leprae* (Lepromin) in a Mitsuda reaction. While the form of leprosy that a host may develop is generally consistent with the type of granuloma that they manifest with Lepromin, the Mitsuda reaction is known to be a poor indicator of susceptibility for leprosy. Several Mitsuda (-) armadillos resist experimental infection with *M. leprae*. To better understand the differences between granuloma formation and resistance, we examined the granulomas formed in the skin of armadillos in response to intradermal inoculation of highly viable *M. leprae* and to killed leprosy bacilli. We found that the granulomas formed in response to live *M. leprae* were significantly larger than those produced to *M. leprae* killed by heat, gamma irradiation or by freeze/thaw. Among Mitsuda(-) animals (n=20) granulomas involving viable bacilli ranged 2-12 times larger in size than those made to killed *M. leprae*, but their cellular composition was little changed and the bacillary number remained high. Mitsuda (+) animals showed similar enhancement with little qualitative difference in cellular composition. We used Radiorespirometry (RR) and conventional mouse foot pad technique (MFP) to examine the viability of *M. leprae* recovered from these intradermal inoculations. *M. leprae* viabilities fell markedly after initial inoculation but then stabilized. Bacilli recovered from *living-Mitsuda* reactions showed a broad range of viabilities and varied by the Ridley-Jopling classification of the animal (n=8). Highest *M. leprae* viabilities were found among multibacillary hosts and lowest among BT's. Over a six week period, intradermal *M. leprae* viabilities among most multibacillary animals tended to increase, while they decreased or remained very low among BT animals and other paucibacillary hosts. The pattern for intradermal *M. leprae* viability among leprosy resistant Mitsuda (-) animals (n=4) resembled that seen among BT hosts, with the higher initial viabilities waning over time. The histopathological response of these animals to Lepromin remained the same. The trends in viability seen for intradermal *M. leprae* generally correlated with the outcome of systemic infections. Within 15 months after intravenous challenge with 1×10^9

M. leprae, the LL-spectrum animals that had accommodated high intradermal *M. leprae* viabilities developed signs of fully disseminated disease, while the resistant Mitsuda(-) and paucibacillary spectrum animals remained free of leprosy. Actively metabolizing bacilli may produce antigens that are not present among killed bacillary preparations, and they secrete them to the host over a long period of time. Histopathology is likely too insensitive to reveal the full range of variable resistance across the leprosy spectrum. A better understanding of the *M. leprae* antigens involved in resistance to leprosy by armadillos, and the specific cytokine profile of their responses, would be useful in our efforts at *in vivo* propagation, and could significantly benefit our ability to identify disease susceptible and resistant individuals in human populations

PM & BM 9

DETECTION OF *M. leprae* AND ITS SUSCEPTIBILITY TO DAPSONE USING DNA HETERODUPLEX ANALYSIS

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Current recommended MDT for leprosy should control the spread of drug-resistant leprosy; however, dapsone resistance continues to be reported. Comprehensive estimates of dapsone-resistant leprosy are difficult to obtain due to the cumbersome nature of the conventional drug susceptibility testing methods using mouse foot pad inoculation, which requires at least 6 months to obtain results. Recently we have determined that dapsone-resistant strains contain mutations in codons 53 and 55 of the *folP1* gene encoding the dihydopteroate synthase, a key enzyme in the folate biosynthetic pathway, and used this information to design a, PCR-based heteroduplex assay for rapid detection of *M. leprae* and dapsone susceptibility from clinical specimens. PCR was used to amplify a 231-bp *folP1* fragment from crude cell lysates of biopsy homogenates. The PCR products were annealed to a universal heteroduplex generator and the resultant DNA duplexes were separated on a PAGE

mini-gel. This assay took 6 hrs to perform, correctly detected the presence of *M. leprae* from eight biopsy specimens and from 14 separate *M. leprae* strains harvested from either armadillos or mice. It addition, this assay demonstrated a 93% correlation with dapsone susceptibility results as determined by both DNA sequencing of *folP1* and mouse footpad susceptibility testing and was sensitive enough to detect 10^3 bacteria. Therefore these results demonstrate that a new tool has been developed for rapid detection of dapsone resistance. This tool should be useful for drug resistance surveillance in leprosy control programs when combined with similar molecular tests developed of other drug resistance markers

PM & BM 10

DETECTION OF mRNA CODING FOR PROTEASE ENZYMES IN *Mycobacterium leprae* ISOLATED FROM HUMAN BIOPSIES

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The knowledge about the mechanisms of *Mycobacterium leprae* pathogenesis and the virulence genes responsible for it is very limit. Bacterial proteases have been proposed as virulence factors in a variety of diseases, contributing in different ways to the establishment and maintenance of microorganisms in the host. In this work, we have investigated the *in vivo* expression by *M. leprae* of genes annotated as putative proteases in the genome of this pathogen (Cole et al., Nature 409: 1007-1001, 2001). Five out of 32 protease genes were initially selected for this study: ML0041, ML0176, ML2659, *gcp* gene (ML0379) and *clpC* gene (ML0235). These genes code for putative secreted proteases, or for proteases with homology to virulence factors of other microorganisms. *M. leprae* was purified from biopsies of lepromatous leprosy patients and total bacterial RNA was isolated by guanidine thiocyanate extraction. cDNA was synthesized in a reverse transcriptase reaction with random hexanucleotides. PCR reactions were conducted in the presence of protease specific primers designed for the amplification of the full lengh of the genes. Preliminary results indicate that *M. leprae* expresses the ML2659 gene, which shares homology with the serine protease *pepA*, a virulence factor of *Pseudomonas aeruginosa*. Additional experiments are under way to further characterize the expression of these genes and to investigate their protease activities.

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PM & BM 11

DICHOTOMY OF -238 AND -308 SINGLE NUCLEOTIDE POLYMORPHISMS IN TNF-α GENE: CLINICAL AND BACTERIOLOGICAL EVALUATION IN LEPROSY

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Tumor necrosis factor alpha (TNF-α) plays a key role in orchestrating the complex events involved in inflammation and immune response. The presence of single nucleotide polymorphisms (SNPs) within the promoter region of the TNF-α gene has been associated to a number of diseases. Since the genetic predisposition could be considered as one of the factors in the outcome of different clinical forms of leprosy, the aim of this paper was to investigate the occurrence of (G/A) polymorphisms at positions -238 and -308 within the TNF-α promoter and its possible association with degree of severity. By definition, multibacillary (MB) forms was considered severe and the paucibacillary (PB) form, mild. Besides, the bacteriological index (BI) was evaluated among genotyped MB patients in order to investigate the possible influence of each polymorphism on the levels of bacterial load. The results of this study, which included a total of 631 leprosy patients (MB= 401, PB= 230) suggest that the -238A allele was associated to the more severe clinical form of leprosy (MB), whereas, the -308A allele with the mild form (PB). These data are in compliance with the BI analyses of MB patients in that the bacterial load among the -308 carriers was lower while among -238 carriers it was increased.

PM & BM 12

DRUG RESISTANT *MycobacteriumlLeprae* FROM RELAPSE OR INTRACTABLE LEPROSY CASE

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Current strategy against leprosy is mainly based on multi drug treatment (MDT). On the other hand, some cases of the drug resistant *Mycobacterium leprae* were reported by deferent study groups. Recently, there have been advances in the elucidation of molecular events responsible for drug resistance in Mycobacteria. Molecular analysis technique takes place *in vivo* drug susceptibility test and enables to know correctly the distribution of resistant strains by

examining many samples. In this study, The DNA sequences of particular regions of *M. leprae,* which are responsible for resistance to daposon, rifampin, and fluoroquinolones were analyzed respectively. Samples are collected from Japanese relapsed or intractable cases, newly registered cases in Philippines and Indonesia. For the Japanese cases, 13 out of 16 samples analyzed *folP* gene, 9 out of 16 analyzed *rpoB,* 4 out of 8 analyzed *gyrA* indicated mutation at the position responsible for drug resistant. Results of the samples form Philippines were as follows, *folP*: 3 mutated/27 examined, *ropB*: 6 mutated/23 examined. Indonesian samples revealed as follows, *folP*: 2 mutated/27 examined, *ropB*: 5 mutated/23 examined. Two cases of Philippines regarded resistant daposon and rifampin. Frequent drug resistant cases in Japanese cases may attribute to irregular and/or monotherapy. We thank E. Nagao, K. Kinjoh, M. Namisato, M. Goto, A. Hosokawa, T. Yanagihashi, R. Nogami, A.T. Agdamag and I. Agsuni.

PM & BM 13

EVALUATION OF GENETIC VARIABILITY IN *Mycobacterium leprae* AND POSSIBLE APLICATION FOR DEVELOPMENT OF MOLECULAR TOOLS FOR STRAIN TYPING

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Despite a considerable reduction in registered leprosy cases over the last 15 years, the disease is still a major public health problem in several countries with no substantial decrease in case detection rate. Control programs could be improved by identification of the source of infection, better understanding of transmission and if relapse cases could be differentiated from re-infection.

Attempts to identify individual strains of *Mycobacterium leprae* has so-far been disappointing and development of fingerprinting technology is hampered by the lack of DNA polymorphism. Very recently however, one study demonstrated a considerable isolate-associated difference in the number of copies in a TTC repeat in a single locus.

We confirmed the difference in TTC copy number in this locus in *M. leprae* from different Brazilian leprosy patients using gel electrophoresis and automatic sequencing. After analyzing the *M. leprae* genome sequence for simple repeats, sets of primers for amplification of five more loci containing $(AT)_n$, $(TAC)_n$ or $(C)_n$-$(G)_n$ were developed. Our preliminary data, using skin biopsy samples from 3 different patients and agarose gel electrophoresis, demonstrated size variability in 3 of the 5 PCR systems so at the moment, 4 loci have been defined containing isolate-associated polymorphism. Considering the limited resolution of agarose, variability in the other 2 systems will be searched for on polyacrylamide gel and by sequencing. More samples are being collected, including biopsy and lymph samples from multi- and paucibacillary patients, in order to establish the degree of genetic variability in the different loci and look for a possible association between genetic composition of the bacilli using these markers and clinical and epidemiological characteristics of the patients.

PM & BM 14

FURTHER STUDIES ON THE HISTOLOGICAL CHANGES IN THE SKIN IN PRIMARY NEURITIC LEPROSY

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Primary neuritic leprosy presents with peripheral nerve damage and no evident skin patches. Clinical diagnosis has centred on demonstration of anaesthesia and nerve enlargement. Laboratory confirmation is based on a histological diagnosis of leprosy in the cutaneous nerve biopsy. We have previously shown that although the skin shows no visible patches there are histological evidences of the disease in the skin biopsy.

In the present study 24 PNL cases were subjected to a skin biopsy from the area of sensory change to look for any histological evidences of leprosy. 18 of the 24 biopsies (75%) showed changes specific to leprosy. The changes ranged from Indeterminate leprosy in 7 (29.2%), Indeterminate -> BT leprosy in 2 (8.3%), Indeterminate to BL leprosy in 3 (12.5%), BT in 5 (20.8%) and BL in 1 (4.2%). 6 of the biopsies revealed no significant lesion. The histological features suggest a schematic progression of the disease from non-specific changes to specific changes such as indeterminate leprosy and further progression to determined forms of BT and BL leprosy.

This gives PNL an important status as a stage in the development of full blown disease.

PM & BM 15

IMPROVED PROCEDURES FOR THE GENERATION OF THE RECOMBINANT ANTIGENS OF *M. leprae*

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To date, most developmental work for new leprosy diagnostic antigens has been conducted on native *M. leprae* products. Since the *M. leprae* genome sequence has become available, emphasis has shifted to individual proteins, notably those that are *M. leprae* specific. For the purpose of producing recombinant antigens, we have modified standard methods. The basic 3-step strategy is well established: PCR, cloning of the genes into an expression vector, and purification of six-histidine-tagged proteins using the standard immobilized metal affinity column (IMAC). However, in order to produce large quantities of soluble recombinant proteins, we have modified these methods. We use touchdown PCR to overcome high annealing temperatures for high GC-rich DNA. In purifying recombinant proteins, we modify the pH gradient buffer system using the knowledge of pI values of the 6-histidine tag and IMAC. In this way, several soluble recombinant proteins have been produced in milligram quantities per one liter of cultured cells. The hydrophobicity and pI values of the original proteins determine the solubility and quantity purified. Ten *M. leprae* recombinant antigens (ESAT-6; CFP-10; MMP-I; MMP-II; EF-Tu; Ag85B; and the Ag85B+ESAT-6, CFP-10+ESAT-6, 10kDa+ ESAT-6 fusion proteins) were purified by these methods and are under investigation (supported by a grant and contract from the NIAID, NIH).

PM & BM 16

INTRACELLULAR SIGNALS TRIGGERED DURING ASSOCIATION OF *Mycobacterium leprae* WITH SCHWANN CELLS

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Interaction of bacterial pathogens with their host cells triggers signal transduction pathways that, in turn, leads to a variety of cellular responses which ultimately favor their perpetuation in the host. Among these responses are those given rise to an extensive reorganization of the cytoskeleton, which results in morphological changes, and the secretion of cytokines into the medium. Although this interference in host cell metabolism by bacteria represents a central feature of their pathogenesis, these events are poorly understood in Schwann cells (SCs) infected with *Mycobacterium leprae*, the causative agent of leprosy. To gain a better understanding of *M. leprae*-SC interaction, the present study investigates the signal transduction events triggered during the interaction of *M. leprae* with SCs. The assays consiste of pre-treating or not ST88-14 cells – a human Schwann cell line – with specific kinase inhibitors, followed by incubation with fluorescein-labeled bacteria and analysis of bacterial association via fluorescence microscopy. The use of tyrphostin AG126, bisindolylmaleimide I and wortmannin which, respectively, inhibit tyrosine kinase (TK), protein kinase C (PKC) and phosphatidylinositol 3- kinase (PI 3-K) produced association inhibition suggesting that TK, PKC and PI 3-K are activated during the interaction of the leprosy bacillus with SC. Currently these preliminary results are being confirmed and the involvement of other transduction elements are being investigated by the use of specific inhibitors.

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INTRACELLULAR TRAFFICKING OF *Mycobacteriuml leprae* IN SCHWANN CELLS

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The effects of *M. leprae* invasion on the physiology and metabolism of Schwann cells and its relation to the progressive and irreversible degenerative process of peripheral nerves are poorly understood. *M. leprae* almost exclusively infects macrophages and Schwann cells. The fate of other pathogenic mycobacteria, once inside macrophages, has been the object of many recent studies. The exact molecular events leading to the *M. tuberculosis* phagosome-lysosome fusion inhibition, initially identified by Armstrong and Hart (*J Exp Med* 134:713-740, 1971), are not yet completely understood, though great progress has been made in the delineation of the molecules involved in uptake of *M. tuberculosis* and its interference with fundamental trafficking processes in host cells. Earlier studies, using bone-marrow derived macrophages, have shown that, like other pathogenic mycobacteria, *M. leprae* resides in non-acidified phagosomes (Frehel and Rastogi, *Infect Immun* 55: 2916-2921, 1987).

We have used a human Schwannoma cell line (ST 8814) as an *in vitro* model for *M. leprae* infection. Tissue culture cells were incubated with fluores-

cently labeled live and heat-killed *M. leprae* (kindly provided by J. Krahenbuhl, Louisiana State Univ., Baton Rouge, Louisiana, USA). Our data demonstrate that the cells avidly take up both live and dead *M. leprae*. Further, in preliminary experiments using fluorescent markers of lysosomal compartments, we show that live *M. leprae* do not collocalize with acidified vesicles inside Schwann cells, whereas dead bacilli do. This is the first demonstration of the utility of the ST 8814 cell line to study the trafficking of *M. leprae in vitro*. Studies are under way to further characterize the intracellular fate of *M. leprae* in Schwann cells.

This work received financial support from WHO/TDR and the Brazilian Ministry of Health

PM & BM 18

LEPROSY TRANSMISSION AND MUCOSAL IMMUNE RESPONSE: DO SEASONS PLAY ANY ROLE?

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Environmental association with the incidence of leprosy is not yet well understood. Reports from the literature indicate association of the rainfall and proximity of water sources to the inhabitants with the incidence. This can be an important aspect in the transmission of leprosy, especially in reference to the reports that suggest the increased viability of *M. leprae* outside human body under moist and shaded conditions. The objective of the present analysis was to look at the presence of *M. leprae* on the nasal mucosa in general population in different seasons using the data obtained from the study that was designed to look at the transmission and the development of mucosal immunity. Individuals from three villages were screened. Polymerase Chain Reaction (PCR) was used to detect presence of *M. leprae* DNA on the nasal mucosa and mucosal immunity was tested by measuring the salivary *M. leprae* reactive IgA antibodies (sML-IgA) using ELISA. PCR positivity was seen to be highest during the monsoon season. The PCR positivity was seen in 2.5% (36 out of 1464), 1% (19 out of 1824) and 4% (68 out of 1701) subjects during winter, summer and monsoon seasons respectively. Both children and adults show peak of positivity in the monsoon suggesting an increased exposure to *M. leprae* in monsoon. The percentage of non-exposed subjects i.e. subjects negative for PCR and sML-IgA is highest in summer (37.9%) and lowest in monsoon (27.4%). Seasonal effect and dynamic nature of the exposure needs to be looked more closely with shorter duration follow-ups to understand the mechanism of transmission and factors affecting it, which in turn can help us to design intervention strategies to interrupt the transmission.

PM & BM 19

MAST CELL SUBSETS AND NEUROPEPTIDES IN LEPROSY REACTIONS

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The immunohistochemical identification of neuropeptides (calcitonin gene-related peptide, vasoactive intestinal polypeptide, substance P, -melanocyte stimulating hormone and -melanocyte stimulating hormone) quantification of mast cells and their subsets (tryptase/chymase-immunoreactive mast cells = TCMC and tryptase-immunoreactive mast cells = TMC) were determined in biopsies of six patients with leprosy reactions (three patients with type I reaction and three with type II). Biopsies were compared with those taken from the same body site in the remission stage of the same patient. We found a relative increase of TMC in the inflammatory infiltrate of the reactional biopsies compared to the post-reactional biopsy. Also, the total number of mast cells and the TMC/TCMC ratio in the inflammatory infiltrate was significantly higher than in the intervening dermis of the biopsies of both periods. No significant difference was found regarding neuroptide expression in the reactional and post-reactional biopsies. The relative increase of TMC in the reactional infiltrates could implicate this mast cell subset in the reported increase of the immune response in leprosy reactions

PM & BM 20

MORPHOLOGICAL EVALUATION OF NERVE BIOPSIES FROM PURE NEURITIC FORMS OF LEPROSY USING TOLUIDINE BLUE-STAINED SEMITHIN SECTIONS. CORRELATION WITH THE RESULTS OF POLYMERASE CHAIN REACTION

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Pure neuritic leprosy is difficult to diagnose if acid-fast baccilli is neither detected in nerve biopsy sections nor in skin smears. Nerve biopsies of seventeen patients with neuritic form of leprosy were submitted to the routine histopathology (H-E and Wade staining) and also to semi-thin (0,5 m) sectioning, toluidine blue staining and were observed under optical microscopy. A small piece of the nerve biopsy was submitted to polymerase chain reaction (PCR) for the detection of *M. leprae* DNA. The morphological findings of the biopsies were: inflammatory infiltrate (9), fibrosis (8, six of them with concomitant inflammatory process), myelinated fiber loss (13, large or small fibers), demyelination (3), active axonal degeneration: (2), remyelination (7), axonal regeneration (4), endoneurial angiogenesis and multilayering of capillary wall (5), acid-fast baccilli positivity (5). Eleven biopsies were PCR-positive, (6 of them were AFB-negative in Wade staining and one of them exhibited normal histological appearance). The most predominant findings for leprosy neuritic form were perineurijm and endoneurium inflammatory infiltrate, nerve fiber loss (small and large fibers) and fibrosis. PCR contributed decisively in 6 cases for the diagnosis

PM & BM 21

MULTIPLE ENDOTHELIAL MEMBRANE PROTEINS BIND *M. leprae*

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Morphologic evidence has suggested that endothelial cells (EC) may be the gateway through which *M. leprae* enter peripheral nerve. Studies in vitro have demonstrated that uptake of *M. leprae* by EC is time- and dose-related. Experiments have therefore been undertaken to identify the EC membrane proteins capable of binding *M. leprae*.

Cytoplasmic membranes from 12×10^6 EC grown in vitro were solubilized and their proteins conjugated to biotin. *M. leprae* (2×10^9) were allowed to bind these biotinylated proteins for 4 hr at 4° C. The bacterial pellet was washed to remove unbound proteins; bound proteins were separated by SDS-PAGE and electro-transferred to PVDF membranes. Biotinylated EC proteins were visualized by staining with an avidin-alkaline phosphatase conjugate.

Biotinylated EC proteins bound to *M. leprae* were separated into several distinct bands, 7 of which have been consistently identified in 8 different experiments. In these preliminary experiments, the smaller molecules (29, 32, 47, and 54 kDa) have yielded discrete single bands on 8% and 10% gels; the larger molecules have appeared more diffuse, with bands at 59-63, 125-130, and 175-185 kDa.

These studies suggest that EC are capable of binding *M. leprae* using multiple surface proteins. Although these probably include proteins already used by other cell types to *M. leprae*, they may also include binding proteins unique to EC.

PM & BM 22

NEURAL PREDILECTION, MOLECULAR MIMICRY AND NERVE DAMAGE- COMPUTATIONAL COMPARISIONS BETWEEN *M. leprae* BINDING PROTEINS AND THE *M. leprae* GENOME

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Mycobacterium leprae, the causative organism of leprosy is known to target and infect Schwann cells of the human peripheral nerve and trigger host-mediated immune reactions and destruction of myelin membranes leading to nerve damage. Antigenic mimicry is a mechanism adopted by *M. leprae* to evade the efficient human immune system. Various receptor mediated mechanisms such as the laminin 2-α dystroglycan/β integrin bridge, the fibronectin (FN)-β integrin bridge and the myelin P0 glycoprotein are known to play a role in the binding and invasion of Schwann cells by *M. leprae*. Computational comparison of the *M. leprae* proteins and the human peripheral nerve - *M. leprae* binding proteins has revealed sequence similarities. Laminin had a homology to 60 kDa Chaperonin 1 and heat shock protein (P values 0.55 and 0.94 respectively).

Fasta searches of fibronectin and Blastp searches of myelin P0 revealed a homology to the secreted P60 family protein. The significance of secreted proteins as antigenic determinants is of importance because in tuberculoid leprosy nerve pathogenesis is observed even in the absence of *M. leprae*.

The secreted P60 family protein has sequence similarities to the immunoglobin domains of myelin P0, which have significance in protein-protein and protein-ligand interactions. FSSP studies showed that many of the structural neighbours of myelin P0 were antigenic determinants and/or immunogens, which could have implications in understanding nerve damage.

These sequence similarities need to be further analyzed by extending this bioinformatic knowledge to wet experimentation to recognize potential drug targets and peptides to counteract leprosy.

PM & BM 23

OBSERVATION OF ACID FAST BACILLI BY MERGE TECHNIQUE OF DIFFERENTIAL INTERFERENCE CONTRAST AND POLARIZED MICROSCOPES

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Approximately 400 autopsy cases of leprosy were done during past 50 years by our group. There are still many unsolved problems in the pathological field of the acid fast bacillary infections.

We have studied the polarization of *M. leprae* under the polarized microscope up to a thousand magnification in these six years and trying to extend this study to the other mycobacterial infections such as tuberculosis and MOTT (Mycobacterium Other Than Tuberculosis). The polymorphonuclear leukocytes respond to these mycobacterium in the first phase of infection, then later phagocytic histiocytes take place of the role of responder. Polarization of mycobacterium was not observed in the polymorphonuclear leukocytes and monocyts in early stage when acid fast bacillary stains clearly. On the contrary, after the acid fast bacillary stain become negative in the late stage of treatment, polarized particles similarly looking to mycobacterium come appear in the cytoplasma of phagocytic histiocytes. Later on, the polarized mycobacteria are seen in the surrounding collagenous connective tissue. We will try to investigate these polarized particles using marge technique of differential interference contrast.

These sequence similarities need to be further analyzed by extending this bioinformatic knowledge to wet experimentation to recognize potential drug targets and peptides to counteract leprosy.

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PCR DETECTION OF *Mycobacterium leprae* IN NASAL MUCOSA FROM LEPROSY PATIENTS

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Leprosy characteristics of long incubation period and wide spectrum of clinical manifestations prevent a fast and reliable diagnosis, especially in the initial forms. Discoveries have indicated an airway transmission, in which the nose plays a central role. Polymerase Chain Reaction (PCR) has been used to amplify *Mycobacterium leprae* DNA, allowing detection of low amounts of bacillus, and the spreading and transmission mechanisms of leprosy. The objective was to standardize PCR and RT-PCR to detect, respectively, DNA and RNA of *Mycobacterium leprae* in nasal mucosa biopsies of leprosy patients and to correlate it with histopathology and patient's clinical form. A preliminary test of Untreated (6), in treatment (1), and treated leprosy patients (1) and their contacts (2) from the Centro Colaborador Estadual em Hanseníase/UFU was done. PCR primers amplify 372 bases-pairs of a repetitive sequence of the bacillus DNA. RT-PCR was standardized using Ready-to-Go RT-PCR beads. Amplicons were detected in 1.5% agarose gels. PCR was positive in three samples, two BT patients with bacilloscopic index (BI) 0 and normal histopathology, and a BL with BI 4. RT-PCR was positive in two LL untreated patients and it was negative in 5 samples: two LL, one treated and another after one month of treatment, one TT, and two contacts of multibacillary patients. The results demonstrated that PCR identifies *Mycobacterium leprae* in leprosy patients' nasal mucosa and RT-PCR showed the viability of the bacillus, attesting treatment effectiveness. Larger sampling of patients and their contacts is being processed aiming to identify factors related to transmission, subclinical infection, and healthy bearers, looking for target-groups for new prevention strategies.

Support: FAPEMIG/UFU

PM & BM 25

PREDICTION AND EXPRESSION ANALYSIS OF *Mycobacterium leprae* SECRETED PROTEINS

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Secreted proteins represent a distinct group of proteins with respect to their structure and function and their contribution to virulence. Secreted proteins are of particular importance for vaccine development because they are often immunogenic and have the potential to be recognized early in infection. Annotation of the completed *M. leprae* genome has provided new information related to proteins constituting *M. leprae*'s hypothetical proteome. Because *M. leprae* cannot be grown *in vitro*, novel approaches are needed to determine which proteins are expressed

during infection and whether these proteins are related to pathogenesis. The objective of this study was to identify proteins from the *M. leprae* genome database that had high predictive values for secretion and to determine whether they were transcribed during infection. Our strategy was to 1) select known and predicted secreted proteins from *M. tuberculosis* and search for homologs in *M. leprae*, 2) select proteins from the *M. leprae* annotation with high predictive values for secretion and 3) study their expression by probing cDNA libraries prepared from nude-mouse derived *M. leprae* mRNA. Signal P was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences, and TMHMM was used to predict the location and orientation of transmembrane helices in protein sequences. The analysis of 200 *M. leprae* sequences with Signal P yielded 32 potentially secreted proteins. These sequences were analyzed with TMHMM resulting in 24 sequences with high probability of encoding secreted proteins and 8 sequences likely to be transmembrane proteins. While the analysis suggests a relatively low number of potentially secreted proteins, it correlates with the fact that these algorithms detect only those proteins secreted via the general sec-dependent export pathway and because *M. leprae* has only 1,600 potential genes in its chromosome. Expression analysis indicated that a number of known and unknown secreted proteins were expressed in *M. leprae* during infection while others were not detected in the same cDNA library. PREDEP and TEPITOPE algorithms were used to predict MHC class I and class II binding motifs, respectively, in an attempt to prioritize the secreted proteins for vaccine development.

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PROFILE OF DRUG RESISTANT *M. leprae* FROM A LABORATORY IN SOUTH INDIA IN THE PAST DECADE

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Mouse footpad inoculation data from skin specimens of patients belonging to the control area of S.L.R and T.C. and elsewhere between the years 1988 to 1998 were analyzed. Suspensions of 10^4 *M. leprae* prepared from skin biopsies of each of these patients were inoculated into both foot-pads of thirty-three inbred CBA strain mice. Dapsone feeds were prepared with concentrations of 0.01%,0.001%,0001%, Clofazamine with 0.01%, and 0.0001% and Rifampicin 0.01%, and 0.003%. On each of these drug concentrated feeds three inoculated mice were fed. Twelve animals were used as controls.

Out of the total 265 biopsies tested, 216 were sensitive to all of the concentrations of dapsone, rifampicin and clofazamine. Biopsies from 49 patients (19%) showed resistance to varying concentrations of dapsone, rifampicin and clofazamine. Out of the 122 biopsy samples received from patients belonging to the leprosy control area, 21(17%) showed drug resistant strains. 9(7%) of these exhibited primary drug resistant strains and 12 (10%) secondary resistant strains.

Though it took more than a decade to report dapsone resistant strains, secondary resistance to other drugs have been reported in a shorter period of time. There appears to be a gradual emergence of primary, low degree resistant *M. leprae* strains to clofazamine and rifampicin, in addition to dapsone, during the last decade, among newly diagnosed patients. Continuing drug sensitivity evaluations with clearly defined indications and careful follow up of patients is becoming crucial because of the frequent changes in the therapeutic regimens that have taken place in the past decade.

PM & BM 27

PURE NEURITIC LEPROSY: IMPORTANCE OF THE POLYMERASE CHAIN REACTION (PCR) IN THE DIAGNOSIS

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Introduction: Leprosy is a disease of nerves, skin and other tissues. The clinical diagnosis may be definied with, minimally, two of these three principal signs: hypopigmentation and/or skin, infiltration, nerve thickening and/or sensitive alterations, and the presençe of Mycobacterium leprae in the skin or nerve. Leprosy represents in developing countries a real and severe problem of public health. According to WHO (1998), there are around 11 or 12 million leprosy patients in the world, being 105 thousand in Brazil that occupies the first place in Latin America. The pure neuritic leprosy (PNL), condition with one ou more nerves compromised, without skin lesion, has a prevelence of 3,9% to 8,2% per 1000 patients. In these cases, even with acurate investigation, the diagnosis es difficult. In the last years the use of PCR for *M. leprae* detection and identification in nerve biopsies has been an alternative for the differential diagnosis of PNL.

Objectives:

1) To identify general aspectos of the patients;

2) To study the diagnostic importance of PCR.

Material and methods: Fifty-eight patients with clinical suspicion of PNL were studied. They were patients from the Service of Neuromuscular Diseases of the Clinical Hospital of UFPR and Dona Libania Health Center (a reference unit for leprosy in the state of Ceará) and eventually other units. All cases were submitted to a pre-determined protocol including anmnesis, dermato-neurological examination, laboratorial routine, bacilloscopia research, electroneuromiography, histopatology and PCR of the selected nerves. The nerves were biopsied and divided in two fragments: one put into eppendorf tube for PCR and another fixed in gum adagrath both frozen in liquid nitrogen. For histopatogical study 4 and 8 slices were done, stained in HE, Gomori and Ziehl and analyzed. From the sample for PCR, DNA was extracted and the sequence of DNA M-lepraespecifc was amplified with primers ML 1 and ML 2, according to Woods and Cole (1989). After it was visualized with plates of agarose gel, compared with a positive control for M.leprae and negative for another non-leprosy neuropathy.

Results: From 58 patients, 41 (70.7%) were males and 17 (29.3%) females. The evolution of the disease ranged from 2 months to 8 years (mean of 1.9 year). The age varied from 15 to 77 years (mean 42.1 years). The patients were classified according to Ridley and Jopling (1966) in to Borderline-Tuberculoid BT 40 cases (69%) and Tuberculoid polar TT 18 cases (31%). The patterns of neuropathy: multiple motor-sensitive 36 cases (62.1%), multiple sensitive 7 cases (12.0%), motor-sensitive mononeuritis 11 cases (19.0%), sensitive mononeuritis 4 cases (6.9%). The main nerves involved was ulnar, common peroneal, tibial posterior, superficial peroneal and sural. The nerve sural was bipsied in 38 cases (65.1%). The Acid-fast-bacilli (AFB) was positive in the nerves in 20 cases (34.4%) of BT in none of TT. The PCR was positive in the nerves of 29 patients (50%). From theses the PCR was positive in 14 cases (48.2%), AFB negative, from whick 12 cases (85.8%) BT and 2 cases (14.2%) TT.

Conclusion: PCR is useful diagnostic method in pure neural leprosy and allow to confirm diagnoses in AFB negative cases in nerve.

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RELATIONSHIP BETWEEN INFECTION AND GENETIC SUSCEPTIBILITY MARKERS

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Some point mutations at the promoter region of the TNF-α and IL10 genes may have an influence on the production of TNFα and IL10 cytokines and consequently to the susceptibility to leprosy infection. TNFα mediates host defense by stimulating effector mechanisms that kill mycobacteria and by promoting granuloma formation. Nevertheless, a high concentration of TNFα can cause immunopathology, including direct damage to myelin and oligodendrocytes, which can lead to deformities. Therefore, TNF may be a valuable prognostic marker that reflects inflammatory activity in leprosy. IL10 may also play a role in leprosy in inhibiting inflammatory and cell-mediated immune responses. Interestingly, an increased TNFα/IL-10 ratio seems to restrain mycobacterial invasion and replication early in infection and could be associated with protection against leprosy.

The presence of IgM antibodies to Phenolic Glycolipid-I in serum is a marker for *M.leprae* infection and could be related to the TNF and IL10 polymorphism genotype. We studied the correlation between mutations at TNF-238, TNF-308, IL10-819, IL10-1082 and IL10-2849 and PGL-I levels in 224 contacts of leprosy patients. Correlation between IL10 point mutations and PGL-I were not observed. Mutation at TNF-308 seems to be associated with a lower positivity rate for PGL-I ELISA and therefore possibly to protection against the development of severe forms of leprosy.

PM & BM 29

ROLE OF Zn AND Cu IN DNA DAMAGE OBSERVED IN LYMPHOCYTES OF LEPROSY PATIENTS MEASURED USING THE COMET ASSAY

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Accumulating reports indicate the involvement of almost every organ in leprosy. Massive infection of *M. leprae* affects the homeostasis of vitamins and minerals, which participate in various known and unknown biochemical reactions, occur in the body. This derangement of the balance of vitamins and trace elements observed in leprosy patients may be an outcome of the disease process as such or an effect of the therapeutic agents given to them. In leprosy, the serum Zn level progressively decreased from TT to LL. Cu is reported to increase in serum of the leprosy

patients. Zn and Cu are important minerals that involve in several biochemical process of the body. Zn is a vital component of at least twenty enzymes and required for RNA and DNA synthesis. Adhesive Zn tapes used in the treatment of leprosy patients have been shown to promote healing of the wounds. Our previous studies on leprosy patients indicated higher levels of DNA damage in lymphocytes. Since Zn and Cu are essential for the maintenance of genetic stability, to find out the role of these elements in DNA damage in leprosy, we carried out an in vitro study on lymphocytes of leprosy patients. Lymphocytes isolated from leprosy patients and healthy individuals were exposed to various concentrations Zn and/or Cu and DNA damage was measured using the alkaline single cell gel electrophoresis.

PM & BM 30

STUDY OF THE RELATION AMONG NEW CASES OF LEPROSY AND POSITIVY IN THE BACTERIOSCOPY IN THE STATE OF RIO GRANDE DO NORTE – BRAZIL

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Leprosy is an infectious and contagious disease caused by Mycobacterium leprae and is transmited by the upper respiratory tract and skin. There are 04 types: indeterminate, tuberculoid, dimorph and virchowian. The bacillus prefers the skin and nerves. The most peculiar clinical findings are related to the peripherical involvement. The diagnosis is based on clinical and laboratorial findings. The direct baciloscopy is the best method of diagnosis, in the public health services the purpose of this study was to establish the percentage of new patients who had a positive baciloscopy. Records were acquired from patients who were seen in GISELDA TRIGUEIRO HOSPITAL.

GISELDA TRIGUEIRO HOSPITAL is a reference in infectious and contagious diseases in Natal – RN. The baciloscopies were done at the Central Laboratory since January 1996 until March 2002. A total of 435 cases were diagnosed; 221 (50,8%) had a positive baciloscopy: 30,3% Mycobacterium leprae dimorph and 20,5% M. Leprae virchowian. This research shows that more than half of new diagnosed patients in the period of the study were classified as multibacilar, showing the failure of the health services in the procecious diagnosis of this diseases

PM & BM 31

STUDY ON THE APPLICATION OF PCR ON THE LEPROSY DIAGNOSIS

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[Abstract] For understanding the possibility of detecting the *M. leprae*, we use the technique of polymerase chain reaction (PCR) with 16Sr RNA primer to test more than 20 strains different mycobacteria including mycobacteria leprase. 72 leprosy cases diagnosed by classical methods, such as clinical demonstration, bacteriological and histopathological examination, and 45 health volunteers from leprosy endemic area were also tested by this method for comparison. The result shows that among the 22 strain mycobacteria, mycobacteria leprase has unique positive reaction. There is no cross-reaction found among those mycobacteria. Among the 72 leprosy cases, diagnosed by classical method, 55 cases were positive in bacteriological examination, 59 cases show positive results by PCR test with 16 Sr RNA as primer. Statistic analysis show there is no difference in those two-laboratory tests. (x^2=0.38, $p>0.05$) All of the 45 health volunteers from leprosy endemic area show negative result. We think polymerase chain reaction with primer of 16SrRNA has prosperous future in detecting leprosy.

PM & BM 32

THE EXPRESSION OF NGFR AND PGP IN LEPROSY REACTIONAL CUTANEOUS LESIONS: NERVE FIBER STATUS USING IMMUNOHISTOCHEMISTRY

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The effects of reactional episodes on the cutaneous nerve fibers of leprosy patients was assessed in six patients (three with reversal reactions and three with erythema nodosum leprosum). Cryosections of cutaneous biopsy of reactional lesions taken in the episode and in the remission period were immunostained with anti-NGFr and anti-PGP 9.5 (indirect immunofluorescence) and counted with a fluorescent microscope. Wilcoxon, ManWhithey U and ANOVA tests were applied. We found no significant statistical difference in the number of NGFr- and PGP 9.5-pos-

itive fibers between the reactional and post-reactional groups. A significant difference was detected between the number of NGFr and PGP 9.5-stained fibers inside of the reactional group of biopsy cryosections; this difference could be due to the distinct aspects of the same nerve fibers displayed when stained with anti-NGFr and with anti-PGP 9.5 (NGFr-positive branches looked larger and so interpreted as containing more fibers, also some NGFr-positive fibers were PGP 9.5-negative). No differences in the number of stained fibers among the distinct cutaneous regions examined (epidermis + upper dermis, mid and deep dermis) was detected. This study shows also that nerve fibers should be evaluated with immunohistochemistry using markers for both Schwann cell and axons

PM & BM 33

THE USE OF IMMUNO STAINING TECHNIQUES TO ENHANCE THE DIAGNOSIS IN DOUBTFUL SKIN LESIONS OF LEPROSY

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Clinical and bacteriological examinations are generally adequate for making a diagnosis of leprosy. However a few cases present with 'doubtful' skin lesions and they usually remain on observation until a definitive diagnosis can be made.

The present study is of 15 such patients who presented to our centre and who were not confirmed as leprosy on clinical criteria. 6 mm skin biopsies were taken from the doubtful skin lesion. These were routinely processed and 5 micron thick section were cut and stained with Haematoxylin and Eosin stain to study the morphology and modified Fite Faraco stain to identify acid fast bacilli.

Parallel sections were immunostained with S100 to identify nerve involvement and BCG antibodies to identify and localise mycobacterial antigen in the sections.

The significance of these immuno stains in comparison to the routine staining techniques in enhancing the sensitivity for a definitive diagnosis of leprosy are presented and discussed.

PM & BM 34

VIABLE *M. leprae* AS A RESEARCH RESOURCE: EFFECTS OF PURIFICATION WITH NaOH AND STAINING WITH PKH DYES ON VIABILITY

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Mycobacterium leprae is a slow growing uncultivable organism, with an in vivo doubling time of ~13 days. Properly passaged in the athymic (nu/nu) mouse foot pad (MFP) fresh viable *M. leprae* can be maintained in vitro for a limited time. We have described conditions for producing and maintaining viable bacilli in axenic medium and cell culture. In brief, the bacilli do not tolerate freezing and are rapidly killed at 37°C, preferring temperatures <33°C. The present report describes ongoing efforts to improve the quality of viable *M. leprae* as a research reagent and the use of a vital fluorescent stain to track viable bacilli in vivo in mice and intracellularly in cell culture.

Because of the urgency of harvesting viable bacilli rapidly the bacilli are "contaminated" with mouse foot pad tissue but can be purified by treatment with NaOH. We describe here the effects of treatment with a range of NaOH concentrations (0.1M to 0.9M) on *M. leprae* viability.

Studies were also carried out to determine the effects of labeling viable *M. leprae* with highly aliphatic tracker dyes containing fluorochrome head groups which are retained in lipid bilayers of eukaryote cells and some prokaryotes because of their inherent insolubility in aqueous media. Two tracker dyes, PHK26 (red) and PKH65 (green) were employed to label *M. leprae* and yielded bacilli clearly flourescent extracellularly and intracellularly in cultured mouse macrophages. Viability of stained *M. leprae* was not affected as determined by radiorespirometry and growth in the MFP.

These findings complement our laboratory's goal of characterizing fresh, abundant nu/nu mouse derived *M. leprae* as a research resource and will offer an important tool to investigators interested in the intracellular interaction of the live (or dead) leprosy bacillus with various host cells, subcellular components and organelles.

PM & BM 35

VIABLE *M. leprae* AS A RESEARCH RESOURCE: EVALUATION OF FLUORESCENT STAINING FOR LIVE AND DEAD *M. leprae*

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Our laboratory is concerned with the routine production and characterization of freshly harvested viable *M. leprae* from the infected foot pads (FP) of athymic nu/nu mice. An ideal passage schedule for weekly harvests of *M. leprae* was defined for our own use and for shipment to qualified investigators. Our standard test for viability, in vitro radiorespirometry (RR) closely correlated with growth of *M. leprae* in the mouse FP. In addition we determined the highly detrimental effects on viability of freezing and incubation at 37°C. Storage at 4°C is ideal and the ideal temperature for experimentation is 26°C to 33°C. The minimum lethal dose of U.V. or gamma irradiation and the minor effects on viability of purification of *M. leprae* suspensions using NaOH treatment was shown. Finally, we have shown that *M. leprae* can be stained with highly aliphatic red or green fluorescent tracker dyes without affecting their viability in vitro or in vivo.

RR was a breakthrough in comparing the viability of one suspension of *M. leprae* from another. RR allows more elaborate experimental design and is certainly superior to the tedious, expensive mouse FP assay. But RR data must accumulate, usually for 7 days, to assess viability. We are currently testing a novel, two-color fluorescence assay to determine if a reliable, quantitative, direct count viability assay is applicable to *M. leprae*. Using the *Molecular Probes BacLight Bacterial Viability Kit®*, two nucleic acid stains are employed in combination, a green fluorescent stain and a red fluorescent propidium iodide (PI) stain. PI penetrates if the cell membrane is damaged and reduces the green fluorescent stain to reveal dead (red) bacteria. A variety of experimental conditions are being employed to quantitate this "viability stain," including: killing extra cellular *M. leprae* with heat, fixatives, freeze-thawing and incubation at non-permissive temperatures. Kinetic studies are being employed to measure the effects of microbicidal leprosy drugs such as rifampin, ofloxacin and minocycline. Finally, these drug studies are being explored on intracellular *M. leprae* in normal and interferon gamma-activated macrophages

OPERATIONAL ASPECTS OF ELIMINATION

POA 1

A ESTRATÉGIA SAÚDE DA FAMÍLIA NO PROCESSO DE ELIMINAÇÃO DA HANSENÍASE NO MUNICÍPIO DE SOBRAL

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Um dos problemas que impedem o controle da Hanseníase é a dificuldade de acesso das pessoas atingidas a serviços que efetivamente realizem o manejo de casos. Em Sobral, município de 153.000 habitantes da zona norte do Ceará, a partir de 1997, iniciou-se a implantação do Programa de Saúde da Família, tendo sido implantadas um total de 35 equipes em 25 Unidades. Até 1999, os portadores de hanseníase eram atendidos em uma única Unidade de Saúde, referência inclusive para vinte e cinco municípios da região norte do Estado. A partir de então iniciou-se um processo de descentralização do atendimento de casos que compreendeu a capacitação dos profissionais de saúde da família: agentes de saúde, auxiliares de enfermagem, médicos e enfermeiros de família. Houve ainda uma intensa mobilização social que contou com o envolvimento de escolas, clubes de serviço, rádios, lideranças comunitárias e espirituais, como as rezadeiras e curandeiros. Esse processo levou a efetiva descentralização de todas as ações de controle da doença para todos os Centros de Saúde da Família, com aumento do Coeficiente de detecção de casos novos de 7:10.000 habitantes em 1998 para 10,9: 10.000 em 2001. A taxa de abandono foi reduzida de 19,9% em 1998 para 2,6% em 2000.

POA 2

A IMPORTÂNCIA DA SUPERVISÃO TÉCNICA ESTADUAL NAS AÇÕES DE CONTROLE DA HANSENÍASE DOS MUNICÍPIOS DE PEQUENO PORTE

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Os pequenos municípios enfrentam dificuldades comuns na administração da saúde pública. As mudanças políticas locais e a constante inversão de prioridades a cada novo governo têm como conseqüência a desestruturação de alguns serviços e programas de saúde. Aliado a isto está a falta de profissionais qualificados, a baixa remuneração e profissionais sobrecarregados assumindo inúmeras funções.

Neste contexto, a manutenção dos serviços de hanseníase, assim como de outros programas de saúde, requer um esforço contínuo de todos os profissionais envolvidos.