THE ARMADILLO (DASYPUS NOVEMCINCTUS) HAS BEEN ACCEPTED AS THE CHOICE ANIMAL FOR LEPROSY RESEARCH. IN ORDER TO STUDY THE POSSIBILITY OF ESTABLISHING THE ARMADILLO AS A MODEL FOR NEURAL LEPROSY INVOLVEMENT EXPERIMENTALLY, THIS REPORT DESCRIBES THE NERVE CONDUCTION STUDY TECHNIQUE IN THIS ANIMAL, PROVIDED THE LITERATURE ON THIS ISSUE IS SCARCE.

WE EXAMINED THE TIBIAL NERVE OF BOTH SIDES OF 10 ANIMALS FROM THE ARMADILLO FARM OF THE INSTITUTO LAURO SOUZA LIMA. THE TECHNIQUE PERFORMED WAS NERVE CONDUCTION STUDY, THE COMPOUND MUSCLE ACTION POTENTIAL WAS MADE FROM THE PLANTAR MUSCLES IN THE FOOT PAD OF THE LOWER LIMBS. THE STIMULATION SITES WERE DISTALLY, BELLOW THE ANKLE, AND PROXIMALLY JUST CLOSE TO THE KNEE IN THE MEDIAL ASPECT OF THE LIMB. THE DISTANCE BETWEEN THESE TWO POINTS WERE MEASURED WITH A TAPE MEASURE AND THE TEMPERATURE WAS MEASURED BY MEANS OF AN DIGITAL SKIN THERMOMETER WHICH ELECTRODES WERE PLACE HALF WAY OF THE ABOVE MENTIONED POINTS, IN BOOTH SIDES.

WE CONCLUDED THAT THE MOTOR NERVE CONDUCTION STUDY IN THE ARMADILLO IS A FEASIBLE AND EASY TECHNIQUE TO BE PERFORMED IN A STANDART LABORATORY AND COULD BE OF UTMOST IMPORTANCE TO BE USED IN EXPERIMENTAL LEPROSY NEURAL INVOLVEMENT. OUR DATA WITH STATISTICS STUDIES WILL BE PRESENTED.

EX34

THE ARMADILLO AS A MODEL FOR LEPROSY

Eleanor E. Storrs, Florida Institute of Technology, Melbourne FL U.S.A.

Leprosy is unique among human diseases in that the bacillus causing it does not grow in artificial culture media, and until 1971 would not produce disseminated disease in experimental animals. Research was at a standstill. Since then, leprosy bacilli grown in armadillos have been used to produce lepromin-A, a reagent used to predict the course of disease; and PGL-1, a reagent used for its diagnosis. Armadillo-derived vaccines for prevention of leprosy are under test on 470,000 people throughout the world. The biochemistry and genome of the leprosy bacillus, once complete mysteries, are slowly unraveling. As an animal model, the armadillo has led to a better understanding of the pathology, immunology and transmission of disease. The armadillo provides the ultimate answer to people who would like to ban use of animals in medical research. Without this model there would be no research on diagnostic reagents or vaccines. Leprosy would still linger in the shadows of medieval medicine.

EX35

LEPROSY IN WILD ARMADILLOS

Eleanor E. Storrs,
Florida Institute of Technology, Melbourne FL

Until the coming of AIDS, leprosy was the most feared of infectious diseases because the Bible linked it with corruption of both spirit and body. It was a punishment by God for transgression. Most physicians do not think that Biblical leprosy was the disease we know today, but these ancient fears lingered into modern times. In 1975, just four years after the discovery of the armadillo as an animal model, we found that some wild armadillos are naturally infected with leprosy. This was a remarkable coincidence that caused great consternation in the lay and scientific press. Since then, other workers have confirmed that leprosy occurs in many wild armadillos. A few years later, a mangabey monkey housed in our animal colonies at Gulf South Research Institute was found to have leprosy. Within a few years, leprosy was downgraded from its ancient status as a Biblical curse to just another disease common to humans and animals. This discovery opened up a vast natural laboratory for studies of transmission of leprosy in wild animal populations.

IMMUNOLOGY

IM1

THE 65 KDa PROTEIN OF MYOBACTERIUM HABANA AND ITS PUTATIVE ROLE IN IMMUNITY AGAINST M. TUBERCULOSIS.

N.B. SINGH
Division of Microbiology,
Central Drug Research Institute, Lucknow, India.

Mycobacterium habana (M. simiae serovar-1) an atypical mycobacterium has protective efficacy against M. tuberculosis $H_{37}Rv$ and M. leprae infections in mouse. It generates cell mediated immune responses and shares several immunodominant proteins with these mycobacteria.

The 65 KDa protein of this mycobacterium has been isolated in pure form by isotachophoresis. The isolated protein was run on SDS-PAGE gel, alongwith molecular weight marker, electro-transferred on nitrocellulose membrane and probed with two monoclonal antibodies (mab) IIC8 and IIH9. Both the mabs have identified a single band discrete protein at the same molecular mass. The yield from single dose of (1.5 mg weight = $6.27X10^8$ = 63.3 ug protein) M. habana vaccine is 3 ug. This dose has provided significant degree of protection in mice. The leucocytes/lymphocytes obtained from vaccinated animals and patients of T.B. & Leprosy had stoppage of migration and had shown strong lymphoproliferative response under antigenic influence. Strong CMI responses have been generated by this protein in animal against homo and hetrologous antigens.

IM2

A 25kDa PORTION OF 65kDa PROTEIN OF MYCOBACTERIUM LEPRAE HAS IMMUNO PROTECTIVE PROPERTIES.

P.R. Mahadevan & Asha Kulkarni
The Foundation for Medical Research
Bombay 400 018, India.

Rabbit antibodies to delipidified cell components (DCC) of M.leprae were used to screen the λgtll library of M.leprae genes and reactive colonies were picked out. One such colony had 1.6kb insert DNA and was expressing a 65kDa protein. This protein was identified in immunoblot using antibodies to DCC. This protein was not reactive with M.leprae 65kDa specific IIIE9 Mab. The DNA sequence showed that the insert started from 1.15kb portion of the classical 65kDa protein gene (Mehra et al,1986). This protein was reactive to another monoclonal antibody to DCC of M.leprae, but this monoclonal had no reactivity to 65kDa hsp of M.leprae. The DNA sequence and the antibody reactivity indicated this protein as a second 65kDa protein of M.leprae. The pUC19 lysate containing this 65kDa had good immunoreactivity listed below. However this 1.6kb insert on recloning in a modified pET vector expressed in BL21 De3 E.coli, a 25kDa protein. This was because of the restricted open reading frame available. This protein has reactivity with specific Mab IIIE9. The protein both in the crude lysate and as partially purified protein

showed (a) immunoprotectivity in vaccinated mice against M.leprae. (b) activation of peritoneal macrophage of vaccinated mice, to kill M.leprae in vitro. (c) ability to induce lymphocyte proliferation in PBMC of lepromatous leprosy patients with release of IL2 and IFN and lastly activation of the macrophages of lepromatous leprosy patients to phagocytose and kill M.leprae.

IM3

CLONING AND CHARACTERISATION OF A 42-kDA SERINE-RICH ANTIGEN FROM MYCOBACTERIUM LEPRAE.

F. Vega-López**, L.A.Brooks, H.M.Dockrell, K.S.E.De Smet, J.K.Thompson, R.Hussain*, and N.G.Stoker.

Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, UK. *Department of Microbiology, Aga Khan University, Pakistan. **Department of Dermatology, National Medical Centre, Mexican Institute of Social Security, Mexico.

In order to identify and characterise protein antigens from Mycobacterium leprae which are relevant in the immunology of the disease, a screening strategy of a λgt11 library was carried out using pooled sera from lepromatous leprosy patients. Three positive plaques were identified which contained an identical 1.7kbp insert coding for an immunoreactive 145-kDa β -galactosidase fusion protein. The 1.7kbp insert was subcloned into the lacZ gene in pUR290 and sequence analysis of the end adjacent to lacZ revealed an ORF with no significant homology to sequences already reported. In order to isolate the gene for this protein, the 1.7kbp insert was used to screen an M.leprae cosmid library by hybridisation. Five overlapping cosmid clones were identified, and an M.leprae 1.8kbp HindIII fragment was subcloned from one of these to perform sequence analysis. A 1227bp ORF was found to code for a 408 amino acid protein with a predicted mass of 42,466-Da. The hydrophilic domain in the centre of this protein contains a high proportion of serine residues, and the hydrophobic amino terminal showed some homology to a 51-kDa hypothetical antigen of M.tuberculosis.

It was found that sera from multibacillary and paucibacillary patients (78 & 68% of cases respectively), had IgG antibodies directed against this molecule, whereas endemic control sera did not recognise a similar band in immunoblotting studies. We also demonstrated that this major M.leprae antigen carries cross reactive determinants since 26% of patients with active pulmonary tuberculosis had antibodies recognising this 42,466-Da protein.

IM4

HEAT SHOCK PROTEINS IN LEPROSY REVERSAL REACTIONS

Diana Lockwood[1], Douglas Young[2], Jo Colston[3], John Stanley[4], and Saroj Young[1]

1. Dept of Clinical Sciences, London School of Hygiene and Tropical Medicine, London WC1E 7HT.
2. MRC Tuberculosis Unit, Royal Postgraduate Medical School, Du Cane Rd, London W12 0HS
3. National Institute for Medical Research, The Ridgeway, Mill Hill, London
4. Dhoolpet Leprosy Research Unit, Karwan, Hyderabad, India

Heat shock proteins are synthesised by cells in response to cellular stresses. They have a wide distribution and have important roles in the immune, inflammatory and auto-immune responses. The immunodominant 70kDa M leprae protein has been shown to belong to the heat shock protein family 70. We have examined skin and nerve biopsies from reactional patients to determine whether heat shock proteins play a role in these acute inflammatory episodes.

39 skin biopsies and 10 nerve biopsies have been stained for constitutive and inducible heat shock proteins. Positive staining for HSP 70 was seen in macrophages in leprosy lesions. In reversal reactions there is a statistically significant, specific increase in the number of HSP 70 positive cells in both skin and nerve. It is possible that the elevated expression of host HSP70 in leprosy patients already primed to recognise *M leprae* 70 Kda results in the development of local auto-immunity with exacerbation of damage to nerve and skin.

IM5

CROSS-REACTIVE RECOGNITION OF HUMAN T CELL EPITOPES IN THE M.LEPRAE 18KDA ANTIGEN.

Beatrice Menz, Neil Stoker and Hazel Dockrell.

Molecular Immunology Unit, Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

The M.leprae 18kDa antigen was one of the first recombinant leprosy antigens to become available. It is strongly recognised by the T cells of healthy leprosy contacts who are presumed to have protective immunity (Dockrell et al. Infect. Immun. 57:1979(1989)), which might make it a candidate leprosy vaccine. However BCG-vaccinated donors, not previously exposed to leprosy, can also respond to this protein. We have used a panel of M.leprae 18kDa reactive human T cell clones, and a series of 15mer overlapping peptides which span the 18kDA sequence, to map the epitopes BCG vaccinated donors recognise in the M.leprae 18kDa antigen. One clone recognised the region amino acids 38-50, previously shown to be recognised by M.leprae-specific human T cell clones isolated from donors vaccinated with a killed M.leprae vaccine (Oftung et al, J.Immunol. 144: 1478(1990)). The region between amino acids 38 and 50 cannot therefore be considered to be M.leprae specific.Two further clones recognised the region 21-35, not previously described as a human T cell epitope. This region does not show striking homology with the M.tuberculosis 16kDa antigen (previously described as 14kDa (Verbon et al, J.Bact. 174:1352 (1992)).

IM6

A NOVEL PROTEIN ANTIGEN SECRETED BY *M.LEPRAE*: THE HOMOLOGUE OF *M.TUBERCULOSIS* MPT32

BRIGITTE WIELES[1], MIRANDA VAN AGTERVELD[1], ANNEKE JANSON[1], JOSEPHINE CLARK-CURTISS[2], TOBIAS RINKE DE WIT[3], MORTEN HARBOE[4] AND JELLE THOLE[1]

Department of Immunohaematology and Bloodband, Leiden University Hospital, PO box 9600 RC Leiden, The Netherlands[1]; Department of Biology and Molecular Microbiology, Washington University, St. Louis, Mo. 63130, USA[2]; Armauer Hansen Research Institute (AHRI), PO box 1005, Addis Ababa, Ethiopia[3] and Institute of Immunology and Rheumatology, University of Oslo, N-0172 Oslo 1 Norway[4]

Recent studies have indicated that secreted proteins are major targets in the immune response to mycobacteria, including *M.leprae*. To identify potential secreted *M.leprae* antigens we tested polyclonal rabbit antisera specific for culture filtrate proteins of *M.tuberculosis* against a panel of novel recombinant *M.leprae* antigens that were recently identified by leprosy patient sera. As expected secreted antigen 85 complex proteins specified by clones L7 and L44 were recognized by the rabbit antisera, but in addition three other clones designated L14, L24 and L2 were identified to express polypeptides that were recognized. Consecutive use of monospecific sera specific for distinct secreted proteins of *M.tuberculosis* indicated that L14 expressed an antigen similar to MPT32, a 41 kilodalton (kDa) secreted protein of *M.tuberculosis*. Sequence analysis of a cosmid clone homologous to L14 revealed the presence of an open reading frame (ORF) predicting an *M.leprae* protein of 287 amino acids. This ORF consists of a potential signal sequence of 39 amino acids followed by a mature protein of 248 amino acids. The 20 N-terminal amino acids of the mature protein show extensive homology with the N-terminal sequence of MPT32. We conclude from these findings that clone L14 expresses a homologue of MPT32, and is likely to be a secreted *M.leprae* protein. No significant homology was found with sequences in EMBL/Genbank databases. The C-terminal 230 amino acids expressed by L14 are extensively recognized by antibodies and T-cells from leprosy patients and healthy contacts. Together with the reported strong immune response to the secreted antigen 85 complex, these findings further indicate a major role for secreted antigens in the immune response to *M.leprae*.

IM7

NEW LEPROSY SKIN TEST ANTIGENS

Becky Rivoire, Julie McCormick, and Patrick J. Brennan

Department of Microbiology, Colorado State University
Fort Collins, Colorado 80523 U.S.A.

The dramatic reduction in the prevalence of global leprosy does not necessarily correspond to a fall in the incidence of disease. One of the most pressing needs of leprosy control programs is epidemiologically acceptable assays to measure incidence, reservoirs of infection, and transmission of disease. Yet our

options are limited. Serology has failed us. Gene amplification protocols may not be applicable. To address this immediate need, three new leprosy skin test antigens were developed: SP-, soluble proteins of M. leprae; SolPCW, soluble proteins extracted from the cell wall of M. leprae; and rMCP-I, recombinant major cytosolic protein of M. leprae of Mr 10.8 kDa. The SP- antigen is similar to the skin test antigens of Rees (STA, Lepromin, MLSA) and of Convit (STA, SPA, SA) except that cross-reactive "suppressive" carbohydrates and lipids were removed. SolPCW is composed of the SDS-extracted cell wall proteins which are known to be highly immunogenic. By separating cell wall proteins from the underlying peptidoglycan complex, it is thought that the undesirable feature, characteristic of lepromin A, of sensitization to a subsequent test, will be eliminated. The recombinant MCP-I protein is identical to its native counterpart and stimulates the proliferation of peripheral blood T-cells better than other native and recombinant products tested. Preliminary testing of each of these new skin test antigens in sensitized guinea pigs resulted in a strong DTH response. Based on these results, an investigational new drug (IND) application has been submitted to the Federal Drug Administration. Upon approval, phase 1 testing in humans will be conducted by Dr. G.P. Walsh in Cebu, Philippines. Work supported by NIH, NIAID Contract NO1 AI-05074.

IM8

B-CELL EPITOPES OF HSP65 IN THE AUTOIMMUNE DISEASES

H. Nomaguchi, K. Chaicumpar, M. Matsuoka, K. Nakanaga, F. Minagawa, and S. Yokota

Nat. Ins. For Lepr. Research
4-2-1, Aoba-cho Higashimurayama-shi
Tokyo 189 JAPAN

We describe B-cell epitopes of HSP65 of Kawasaki diseases (KD), Rheumatoid arthritis (RA) and leprosy patients, and will discuss the role of HSP65 in autoimmunity.

The sera from KD patients in convalescent but not acute phase cross-reacted with HSP65 of M. leprae. To determine whether the endogenous and/or exogenous 65kD protein are activated for B-cell epitopes in Kawasaki diseases, two kinds of chemically synthesized peptides were used. One is the epitope for MAb-IIIE9 for exogenous 65kD, and another is the corresponding site of a human homolog to the mycobacterial 65kD protein, P1 protein in human mitocondria, for endogenous epitope. The convalescent sera but not acute phase sera of KD reacted with both of these epitopes for endogenous and exogenous proteins. On the other hand, sera from mice immunized withM. leprae lysate or purified 65KD reacted with IIIE9 -epitope, but did not react with the P1 epitope.

In leprosy, 20% of lepromatous leprosy patients and 29% of tuberculoid leprosy patients show a significantly higher titer to HSP65 of M. leprae compared to the healthy controls. Since about 30% were sero-positive to RA factor in leprosy, the titer of sera from leprosy patients to HSP65 may be correlated to the RA factor. Sero-positivity to HSP65 was 20% in the group of RA positive sera, and 13% in RA negative sera.

IM9

RECOGNITION OF 21- AND 14- KILODALTONS ANTIGENS OF MYCOBACTERIUM ICRC BY ANTI-MYCOBACTERIUM LEPRAE ANTIBODIES

Shubhada Chiplunkar, Jyoti Kudalkar, Sudha Gangal and Madhav Deo

Immunology Division, Cancer Research Institute, Bombay, India

ICRC, a cultivable mycobacterium isolated from human lepromata is undergoing clinical trials as an anti-leprosy vaccine in India. Antigens of ICRC share cross-reactive epitopes with antigens of Mycobacterium leprae. Radioimmunoprecipitation of 125-I labelled ICRC antigens with sera from lepromatous leprosy patients, borderline lepromatous leprosy patients, borderline tuberculoid leprosy patients, tuberculoid leprosy patients, healthy contacts, tuberculosis patients and healthy individuals, demonstrated that 21- kD antigen of ICRC was exclusively precipitated by sera from all lepromatous leprosy patients and those undergoing erythema nodosum leprosum reaction. The 14-kD antigen of ICRC was identified by sera from a few lepromatous leprosy patients (5 of 26) and all the contacts. However, using M.leprae antigens, it was not possible to distinguish between reactivities of sera from leprosy patients across the clinical spectrum. It was observed that polyclonal anti-ICRC and anti-M.leprae antibodies also showed predominant reactivity to 21-kD protein of ICRC. Furthermore, M.leprae-specific monoclonal antibody WML06 showed reactivity to 21- and 14-kD proteins of ICRC. Studies are in progress to map the relevant epitopes on the 21- and 14- kD antigens of ICRC showing reactivity with patients' sera and anti-M.leprae polyclonal and monoclonal antibodies.

IM10

T CELL RESPONSES TO SYNTHETIC PEPTIDES IN HUMAN LEPROSY

Anwar Murtaza, Gopal Ramu*, N.P. Shankar Narayanan*, Radhey Shyam Misra#, H.Krishna Prasad & Indira Nath.

Department of Biotechnology, All India Institute of Medical Sciences; Department of Dermatology, Safdarjung Hospital#, New Delhi; VHS Leprosy Project, Shakti Nagar, Tamil Nadu , India.

12- 24mer peptides (kindly provided by M.E.Pattarayo, Institute de Immunologia, Bogota, Columbia and M.J. Colston, NIMR, Mill Hill, London, UK) were synthesised based on the sequence of the immunodominant protein LSR2, reported by us from the lambda gt 11 library of *M.leprae* (Laal et al PNAS, 88, 1054-58, 1991). They were screened in lymphoproliferation assay using peripheral blood of leprosy patients from three geographic regions comprising of Tamil Nadu (South India), New Delhi (North India), and Bogota (Colombia).

The pattern of recognition by T cells varied in different clinical types of leprosy as well as in different ethnic populations. Interestingly, peptide *CGAAIREWARRNGHVSTRGRIGC* was recognised by 60% of BL/LL patients who showed unresponsiveness to the total recombinant protein.

This peptide was recognised by all patients in Type I and II reactions. Studies using overlapping peptides indicated a preferential recognition of RGR and REW motifs.

PCR based mRNA cytokine profile of LSR2 and peptide stimulated lymphocytes of lepromatous and tuberculoid individuals showed discriminatory signals for IL-2, IFN-γ, GM-CSF, IL-4, IL-6 and IL-10.

IM11

DEMONSTRATION OF MYCOBACTERIAL ANTIGENS IN THE SKIN SMEARS OF TUBERCULOID PATIENTS OF LEPROSY

U. SENGUPTA, OM PARKASH AND B.K. GIRDHAR

CENTRAL JALMA INSTITUTE FOR LEPROSY,
AGRA-282001, India.

Although many specific serological assays were developed for diagnosis of leprosy but none of these assays is able to detect more than 60 per cent of established cases of tuberculoid leprosy. It was noted that at any early stage of the disease the levels of both antibody (Ab) and antigen (Ag) were not above the level of the background level present in the endemic population. Hence Ag/Ab levels of many tuberculoid leprosy patients fell below the cut off point. The present study was, therefore, carried out to find out the level of Ag/Ab in in situ situations in the lesions. Using a cross reactive anti-BCG antibody about 80 per cent of tuberculoid leprosy cases could be identified in dot-blot immunoassay from routine slit and skin smear samples. It was interesting to note that many of these samples were positive for the presence of local antibodies also. The results will be presented in detail and discussed.

IM12

A 15-KILODALTON ANTIGEN OF *MYCOBACTERIUM LEPRAE* THAT IS RECOGNIZED BY BOTH HUMORAL AND CELLULAR IMMUNE SYSTEMS IN LEPROSY PATIENTS

Shlomo Sela[1], J.E.R. Thole[2], T.H.M. Ottenhoff[2] and Josephine E. Clark-Curtiss[1,3], Departments of Biology[1] and of Molecular Microbiology[3], Washington University, St. Louis, MO 63130, and Department of Immunohaemotology and Blood Bank[2], University Hospital, Leiden, The Netherlands.

A colony immunoblot technique was used to screen the *Mycobacterium leprae* cosmid library with pooled sera from lepromatous (LL) leprosy patients. Four of the 100 clones that produced immunologically reactive proteins were found to specify a 15 kDa antigen (A15) that reacted strongly with LL patients' sera on a Western blot. This 15 kDa antigen also reacted with pooled sera from tuberculoid leprosy patients from the U.S. and Brazil. Each of these clones contained a common 1.2 kb *Pst*I fragment. Nucleotide sequence analysis of the 1.2 kb fragment revealed the presence of three open reading frames (ORFs), only one of which (ORF II) contains sufficient genetic information to code for A15. Sequences homologous to the A15 gene were also detected in chromosomal DNA from *Mycobacterium avium, Mycobacterium bovis* BCG, and *M. tuberculosis*. One of the γt11::*M. leprae* clones (L8) previously identified by us expresses a β-galactosidase fusion protein with 89 amino acids from the C terminus of A15. This fusion protein was clearly recognized by *M. tuberculosis*-stimulated T cells from both LL and BT leprosy patients.

IM13

ANTIBODY RESPONSE OF PATIENTS WITH BORDERLINE LEPROMATOUS AND BORDERLINE TUBERCULOID LEPROSY TO MYCOBACTERIAL 29/33 KDa DOUBLET AND 65 KDa SINGLET ANTIGENS.

Jan D. Burggraaf, Anura Rambukkana, William R.Faber, S. Yong and Pranab K.Das.

Departments of Dermatology and Pathology, Academic Medical Center, University of Amsterdam, The Netherlands.

Using immunoblot assays (ImBA) and enzyme-linked immunosorbent assays (ELISA) for antibodies to mycobacterial immuno cross reactive antigenic components (Im-CRAC) we have already shown that sera of patients with lepromatous (L) and tuberculoid (T) leprosy reacts in significant manner to 29/33 (KDa) doublet and 65 KDa mycobacterial Im CRAC respectively (J Clin Microbiol 1990; 28: 379-382).
Furthermore, we proposed that measurement of antibodies to these antigens by an ELISA will be useful for distinguishing two polar types of leprosy. In this report we have extended our previous studies on a serological survey in which both EILISA and ImBA have been extensively used for diagnosis of different stages of leprosy, particularly those of borderline groups (borderline lepromatous (BL) and borderline tuberculoid (BT). The patient groups consisted of 32 LL, 26 BL, 22 BT and 37 TT patients. By making use of both serological methods we have been able to discriminate the four types of leprosy from each other with an average of sensitivities: 85 - 95 % and specificities: 80 - 95 %. We hypothesize that this presently described serology may be useful for diagnosis and follow-up.

IM14

IMMUNOGENICITY AND PROTECTION STUDIES WITH RECOMBINANT VACCINIA AND BCG EACH EXPRESSING THE 18kD ANTIGEN OF *Mycobacterium leprae*.

K.W. Baumgart[1], A. Radford[2], & W.J. Britton[1].
Centenary Institute of Cancer Medicine and Cell Biology, the University of Sydney, NSW, [2]Division of Animal Health, CSIRO Australia at Parkville, VIC

The 18kD antigen is relatively specific to the leprosy bacillus and no homologue has been found in *M.bovis* BCG. T lymphocytes, which are essential for the control of mycobacterial infection in humans and mice, recognise a number of epitopes within this antigen following infection with *M.leprae* or immunisation with *M.leprae* sonicate. Whether immune responses to this particular antigen contribute to the control of *M.leprae* infection is uncertain. Since vaccination with recombinant vectors (rV) may enhance T cell responses to co-expressed antigens compared to adjuvant-based vaccination, rV using vaccinia virus (VV) and *M.bovis* BCG were prepared. The gene for the 18kD protein was inserted into the thymidine kinase region for the VV constructs under control of both the early and late promoter. The gene for the 18kD protein was inserted into BCG constructs under the control of either the 18kD promoter or the 65kD (heat shock) promoter. Different mouse strains were injected with viable VV (10^6 PFU) or BCG constructs (10^6 CFU) by various routes, and sera as well as lymphocytes from regional lymphoid tissue collected. IgM and IgG (including IgG1 and IgG2a) antibody responses to the 18kD antigen were detected by ELISA from all strains of mice, although the vaccinia constructs induced the highest specific antibody titres. T cell proliferative responses to the 18kD antigen, were maximal in B6-H2k mice after subcutaneous immunisation with either construct at 2 weeks. Recombinant BCG stimulated delayed type hypersensitivity responses to soluble 18kD antigen in guinea pigs. Prior vaccination with VV co-expressing the 18kD antigen conferred partial protection to BCG-18kD, as measured by a reduction in the number of CFUs of BCG in the spleens of mice challenged with recombinant BCG. This model system permits the comparison of the protective efficacy of recombinant viral and BCG vectors encoding the same *M.leprae* protein.

IM15

LYMPHOCYTE PROLIFERATION AND CYTOKINE SECRETION IN RESPONSE TO PURIFIED MYCOBACTERIAL HSP AND TO Ag85 DURING INFECTION WITH *MYCOBACTERIUM LEPRAE*.

P. Launois*, M.N. Niang*, P. Vandenbussche°, J-L. Sarthou*, J. Millan* and K. Huygen°. Institut Pasteur de Dakar*, SENEGAL and Instituut Pasteur van Brabant°, Brussels, BELGIUM.

Peripheral blood leucocytes from 9 paucibacillary and 12 multibacillary leprosy patients, 18 healthy controls and 34 healthy leprosy contacts were stimulated with three mycobacterial heat shock proteins with respective m.w. of 70, 65 and 18 kDa and with the secreted 30-32 kDa protein, also called antigen 85. Ag85 was found to be the most powerful T cell antigen (as measured by lymphoproliferation and IFN-γ secretion), eliciting a response in 9/9 paucibacillary patients, in 10/10 lepromin positive controls and in 25/25 lepromin positive contacts. The three hsp were less active T cell stimuli. The 70 kDa hsp elicited responses in only 4/9 paucibacillary patients, in 8/10 lepromin positive controls and in 15/25 lepromin positive contacts. The 65 kDa hsp stimulated T cells of 8/9 paucibacillary patients, of 8/10 lepromin positive contacts and of 20/25 lepromin positive contacts. The 18 kDa hsp finally, elicited T cell responses in 7/9 paucibacillary patients, in 4/10 lepromin positive controls and in only 1/25 lepromin positive contacts. T cell reactivity of lepromin negative controls (n=8), lepromin negative contacts (n=9) and of multibacillary leprosy patients were low to all the antigens tested.

These data confirm our previous findings on the immunodominant character of Ag85 during *M. leprae* infection and suggest that this antigen is indeed a potentially protective T cell immunogen.

Secretion of the monokine IL-6 was also examined in this study. Elevated IL-6 levels were found, in response to all the antigens tested, in PBMC culture supernatants from paucibacillary and especially multibacillary leprosy patients. In lepromin negative healthy contacts, the 70 kDa hsp was the only antigen capable of inducing significant IL-6 production. In lepromin positive healthy contacts finally, Ag85, the 65 kDa and especially the 70 kDa hsp induced substantial IL-6 titers. The 18 kDa hsp did not induce any IL-6 in these healthy lepromin positive contacts.

IM16

LEVELS OF IL-6 AND TNF RECEPTORS IN ENL.

Sampaio EP, Nery JAC, Selmaj K', Sarno EN. Leprosy Department, Oswaldo Cruz Foundation, Av. Brasil, 4365 - Manguinhos, 21.045-900, Rio de Janeiro, Brazil; 'Neurology Department, Lodz University, Poland.

ENL is an acute inflammatory complication of leprosy disease. it has been demonstrated that ENL patients present high TNFα levels in the serum and that the amount of TNF seems to correlate with the intensity of their clinical symptoms. Although high TNFα levels are associated to the fatal outcome in other syndromes, elevated TNF in ENL coexists with the benign course of the disease. Increased levels of other inflammatory cytokines in the serum and the presence of inhibitors seem to modulate the toxic systemic effects of TNFα in organs and tissues of the body. In this study, we investigated the levels of IL-6 and soluble TNF receptors by specific Elisa in the serum of 13 ENL patients previously found to be positive for TNFα. Nine patients were found to be positive for IL-6 in the sera with a mean ± SEM of 587 ± 286 pg/ml, ranging from 0 to 4,880 pg/ml. Although

IL-6 is also overproduced during the reactional state, IL-6 values were inversely related to those of TNFα. The amount of TNF-Rα (the 75 KD protein) was found to be elevated in all patients tested which correlated with the high TNF levels present in the circulation. The present data suggest that both presence of TNF-R and lack of simultaneous high levels of IL-6 in the circulation, during ENL, can justify at least in part the benign course of reactions in leprosy.

Supported by grants from TDR-WHO.

IM17

ASSOCIATION OF NK(CD16+) CELLS AND CYTOKINES WITH M.LEPRAE-SPECIFIC RESPONSES IN TYPE 1 REACTIONS

Danuza A. Esquenazi, Andre L. Moreira, Jose A.C. Nery, Jorge L. Salgado, Euzenir N. Sarno and Geraldo M.B. Pereira. Leprosy Unit, FIOCRUZ and State University of Rio de Janeiro, Rio de Janeiro, Brazil.

During Type 1 reaction(TR1), a leprosy manifestation more frequent on borderline leprosy patients, and associated with neuritis, there is emergence of antigen-specific immune response to M.leprae, in previously unresponsive individuals. Enhancement of natural killer function was also observed during the course of TR1(PJ Converse & G Bjune, 1986). To further evaluate the participation of antigen-specific immunity and innate responses in TR1, we analyzed 18 patients(10 BL, 4 BB, and 4 LL; 12 male and 6 female). The 18 patients were previously negative for lepromin skin test and turned positive during TR1. The relative proportion of CD16+ on peripheral blood mononuclear cells(PBLs) had a 3x increase during TR1(13.0±0.4%, n=7) when compared with values post TR1(4.6±0.5%, n=10). T-lymphocyte *in vitro* proliferation to *M. leprae*, and production of IFN-γ were also present during TR1 but returned to absence of response after the end of the reaction(50.0±12.0 U/ml during TR1, n=5; and 3.5±3.2 U/ml after TR1, n=3). In the course of TR1, serum levels of IL-6 were higher than on normal volunteers(NV) and BT leprosy patients(340.0±58.5 pg/ml, TR1, n=5; and 79.5±20.1 pg/ml, BT+NV, n=5) but TNF-α serum levels were similar to BT and NV(10.6±0.4 pg/ml vs. 34.36±19.0 pg/ml. These observations are consistent with an enhancement of NK function during TR1 as a consequence of increment in NK cell number in the PBLs. It remains to be determined if the appearance of *M. leprae*-specific immune response during TR1, is a consequence of the transient increase in the level of NK activity, or if the specific immune response drives the innate functions during the reactional episode. Supported by grants from TDR-WHO and CNPq

IM18

PROBLEMS IN DETECTION OF SECRETORY ANTIGENS OF INTRACELLULAR MYCOBACTERIA IN MACROPHAGE AND SCHWANN CELL TISSUE CULTURE

Nerges Mistry, Kamal Sethna, Morten Harboe* & Noshir Antia

The Foundation for Medical Research,84-A, R.G. Thadani Marg, Worli, Bombay-400018, India and *IGRI,Fr.Qvamsgt.1, 0172 Oslo-1, Norway.

Identification of mycobacterial (ML and H37 Rv) antigens in supernatants of infected growth promoting host cells viz. Schwann cells (Sc) and mφs constitutes the first step in the development of an immunodiagnostic test that can be used ultimately to monitor efficacy of treatment in mycobacterial diseases such as tuberculosis and leprosy.

The well characterized BCG 85 antigen complex was used as a model detection indicator in a capture ELISA & 2-D electrophoresis for testing of supernatants derived from ML/H37 Rv infected murine Sc/mφs respectively. A significant problem was the establishment of BCG 85 as a true marker for viability as opposed to its release from lysed bacteria. This was attempted by co-detection of exclusively cytoplasmic antigens in the culture system. Approaches to enhance sensitivity of detection included physical concentration, minimization of serum content in the medium and use of protease inhibitors.

Future purposes for these observations will be outlined.

IM19

CYTOKINE PATTERNS IN LEPROSY

Padmini Salgame and Barry R. Bloom
Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, N.Y. USA

Previous evidence from our laboratory indicated that T-suppressor (Ts) cells may be one mechanism of maintaining peripheral tolerance to *M.leprae* in lepromatous leprosy. To understand the cellular basis of Ts cell mediated suppression, we studied the lymphokine profile of the Ts clones and compared it with mycobacteria and tetanus toxoid reactive $CD4^+$ clones and allo-reactive $CD8^+$ cytotoxic clones. All of the mycobacteria reactive $CD4^+$ clones generated from tuberculoid lesions or healthy contacts produced γ-IFN and IL-2 but little or no IL-4 and IL-5, similar to the pattern of lymphokines characteristic of murine Th1 cells. These cells were designated "Type 1 cells". The $CD4^+$ tetanus toxoid clones produced little γ-IFN and high levels of IL-4, IL-5 and IL-10, a pattern similar to murine Th2 cells and were designated as "Type 2" cells. The clones making IL-4 also had helper activity for B-cells. The $CD8^+$ clones from lepromatous patients produced predominantly IL-4 but little or no IL-6, IL-5, IL-10 and γ-IFN. In contrast the $CD8^+$ cytotoxic clones secreted γ-IFN, IL-6 and IL-10 and made no detectable levels of IL-4 and IL-5. Although IL-4 and IL-5 production are highly associated in $CD4^+$ cells, IL-5 was not secreted by the IL-4 producing $CD8^+$ Ts cells. Our data suggest that the human $CD8^+$ population can also be divided into two subsets: Type 1 $CD8^+$ cytotoxic cells and Type 2 Ts cells. IL-4 production by the Ts cells was a necessary condition for suppression in this system because anti-IL-4 antibody was able to block the suppressor activity. Further the effect of IL-4 was at the level of IL-2 gene transcription of the Type 1 cells. Our findings suggest an explanation for the classical immunological dichotomy between the development of humoral immune responses and those of cell-mediated immunity. The observations of Type 1 and Type 2 T-cell functions in many infectious diseases can in part be explained by the action of IL-4, probably in conjunction with other cytokines, not only to enhance antibody formation but also to depress cell-mediated immunity required for protection.

IM20

CYTOKINES IN IMMUNOPATHOLOGY OF LEPRA REACTION

Shreemanta K. PARIDA, Christian VESIN, Pierre-François PIGUET, Pascal LAUNOIS, Rama MUKHERJEE and Georges E. GRAU

Dept. of Pathology, University of Geneva, Geneva, Switzerland, Microbiology Division, National Institute of Immunology, New Delhi, India. & Cellular Immunology Unit, Institute Pasteur, Dakar, Senegal

Cytokines are involved in the immunopathological complications of several infectious conditions, but their role in leprosy has yet to be clearly defined. We have studied serum TNFα, IL-1β and IL-6 levels in 220 leprosy patients across the spectrum of the disease (lepromatous, borderline and tuberculoid types) and during lepra reaction. Dramatically elevated cytokine levels were observed: levels as high as 8,000 pg/ml of TNFα (382±176), 5,000 pg/ml of IL-1β (373±183) and 3,500 pg/ml of IL-6 (223±104) in lepromatous leprosy patients and also in patients during reaction, while cytokines remained within normal range in most of the patients with tuberculoid leprosy and in all clinically healthy individuals. At study entry, there was a significant correlation between serum levels of these cytokines. All these patients were followed up for 1-2 years. It was observed that 73% of patients having raised serum TNFα level >100 pg/ml and all patients with IL-1β level >200 pg/ml at admission time point manifest with severe episodes of lepra reaction following 2-4 months period in comparison to about 10% of patients having <100 pg/ml of TNFα and <200 pg/ml of IL-1β suggesting potential prognostic implications of these cytokines in predicting the onset of reaction, thereby helping to identify patients at risk. In the skin lesions of the patients, immunohistochemistry and *in situ* hybridization revealed elevated expression of TNFα in the epidermal layers and in granuloma areas implying the direct role of these cytokines in the immunopathology of leprosy. Localisation studies of different cytokines in the nerves of patients with reaction will be discussed.

IM21

DETERMINATION OF SUBCELLULAR LOCATION OF THE IMMUNOPROTECTIVE MOIETIES IN MYCOBACTERIUM HABANA

Vinita Chaturvedi[1], Sudhir Sinha[2] and N.B. Singh[1]

Divisions of [1]Microbiology and [2]Membrane Biology, Central Drug Research Institute, Lucknow, India.

M. habana, a nonpathogenic, cultivable mycobacterium offers protection against experimental infection with M. leprae and M. tuberculosis. Present study aims at subcellular localization of the protective moieties in this candidate vaccine.

The cell wall, cell membrane and cytosol fractions were prepared by sonication and differential centrifugati on of the bacilli grown in liquid shake cultures. Groups of mice were immunised with these fractions (dosages adjusted in relation to the dose of integral vaccine) in addition to the integral vaccine (killed M. habana) and placebo, and subsequently challenged with M. tuberculosis H_{37} Rv. The animals were examined for survival and associated parameters.

Preliminary results indicate that the protective moieties most probably are located in the cell membrane. Data on morphological, biochemical and immunological characterisation of M. habana membrane will also be presented.

IM22

M.leprae 10kD HEAT SHOCK PROTEIN IS A MAJOR T CELL ANTIGEN

Vijay Mehra, Robert Modlin**, J. Convit***, and Barry Bloom##. Department of Microbiology and Immunology, and the ##Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461, ***Institute of Biomedicine, Venezuela; **UCLA School of Medicine, Los Angeles, California.

The development of reagents for prevention, control and early diagnosis of leprosy depends on identification of antigens relevant for eliciting T cell responsiveness. Several approaches to identify immuno-reactive determinants of mycobacteria have resulted in identification and characterization of many proteins that elicit T cell reactivity in small numbers of immunized donors. Our previous studies on the analysis of M. leprae antigens using T cell Western blots indicated that most M.leprae reactive T cell lines developed from lepromin positive individuals recognized an antigen of 7-10kD mol. wt. The gene encoding this antigen was isolated from lambda gtll library and sequenced. The deduced amino acid sequence of M.leprae 10kD protein was found to have 44% identity with hsp 10 (GroES) of E.coli. As native 10kD protein was found to be highly immunoreactive in inducing T cell proliferation in M.leprae immunized individuals, and DTH responses in guinea-pigs, we expressed it in E.coli using pMAL-c expression vector, to produce the recombinant 10kD protein in large quantitites for further evaluation. The immunological studies using recombinant protein show significant lymphoproliferation in vitro of PBL from leprosy contacts and TT patients to 10kD antigen. The magnitude of T cell proliferation to 10kD protein was similar to that with whole M. leprae throughout the spectrum of leprosy. Limiting-dilution analysis indicated that one third of M.leprae reactive T cell precursors responded to 10kD antigen. It evoked greater lymphoproliferation than other purified antigens tested. T cell lines derived from Mitsuda reaction showed marked proliferation to this antigen. Further, purified recombinant 10kD antigen elicited strong delayed hypersensitivity recactions in guinea-pigs sensitized to M.leprae. Strong T cell responses to M.leprae 10kD protein suggest a role for this protein in protection against leprosy.

IM23

STUDIES ON POTENTIAL USES OF MLPA IN LEPROSY

Wu Qinxue Li Xinyu Wei Wanhui Shen Jianping Ye Ganyun

Institute of Dermatology, CAMS, Nanjing, China

Gelatin Particle Agglutination Test (GPAT or MLPA, MA) and NT-P-BSA-ELISA (NT-ELISA, NE) are two methods for detecting infection with M.leprae. In order to evaluate them, we conducted systematically comparison of MA with NE.Samples:Leprosy patients 158(LL 58,BL 55,BT 20,TT 20), Household contacts(HC) 155, random population(RP) 149, normal controls(NC) 40.

Results:1.MA at 1:32 serial dilution(MA 1:32),positivity rate(PR) was 73%, MA 1:16—91%;PR of NE1(OD=0.10) was 98%,PR of NE2 (OD=0.20)—91%. The individual agreement (IA) were more than 90% between MA 1:16 and NE,while the IA were more than 70% between MA 1:32 and NE. In multibacillary patients(MB), the IA were 96—100% between MA 1:16 and NE,and 83—96% between MA 1:32 and NE. Quantitative data support above results.These results suggested that NE could not be replaced with MA, and MA is suitable to detect MB.

2. Comparison of sera with dried sera and dried blood on filter paper: results indicated that: (1) the best results of them were those in sera in MA and NE, and in MA, the defferences were no significances between sera and dried blood (X^2 = 3.2, P>0.05), and the identical results were obtained in dried blood which were reconstituted by means of calculating sera content of dried blood. If the reconstitution does not consider the sera content of dried blood, the PR and IA were all to be decreased (<10%).

3. Comparison of U-bottom plate with V-bottom plate: the differences of results were of no significance between U-bottom and V-bottom plate (X^2=0, P>0.99, IA =93.3%). It is easier to judge the results using V-bottom plate.

IM24

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST MYCOBACTERIUM LEPRAE

Wu Qinxue Li Xinyu Yin Yueping Wei Wanhui Ye Ganyun

Institute of Dermatology, CAMS, Nanjing, China

A series of hybridoma cell lines,which secrete monoclonal antibodies(McAbs),were produced by means of fusion between mouse myeloma cells SP2/0 and spleen cells from BALB/C mice immunized with whole M.leprae plus unique phenolic glycolipid I(PGL-I) of M.leprae and M.leprae sonicates supernatant fluid (MLSS) as immunogen.Primary identification indicated that H2 cell line can secrete McAb against the epitope of PGL-I; IIIE10 cell line can secrete McAb against PGL-I and MLSS and (5)24D6C8 cell line only against whole M.leprae. The uses of these McAbs in serodiagnosis of leprosy, identification of M.leprae, analysis and purification of M.leprae antigens, and key problems in technology for producing McAbs against M.leprae were also discussed.

IM25

REACTIVITY OF LEPROSY AND CONTROL SERA TO CRUDE AND RECOMBINANT MYCOBACTERIUM LEPRAE ANTIGENS.

F.Vega-López, O.Rodriguez*, E.Castro*, O.Flores*, E.Macotela R.Hussain **, G.Moraila#, J.L.Ayala#, H.M.Dockrell##, N.G. Stoker##.

Departments of Dermatology, National Medical Centre, IMSS, *Dermatological Centre, SSA, #Health Centre, SSA, Culiacan, Mexico; **Microbiology. Aga Khan University, Pakistan; ##Clinical Sciences, London School of Hygiene and Tropical Medicine, UK.

Immunoblotting studies were carried out in order to identify reactivity patterns of sera from leprosy patients and controls to crude and recombinant M.leprae antigens. Four armadillo-derived M.leprae sonic extracts and a 145-kDa M.leprae β-gal fusion protein were fractionated by SDS-PAGE and blotted onto nitrocellulose filters. Sera from leprosy patients across the disease spectrum, patients with active pulmonary tuberculosis, and healthy endemic controls were used to probe antigenic strips. The leprosy patients were from Mexico and Pakistan, whereas the patients with tuberculosis and endemic controls were from Mexico.

It was found that a total of 89 out of 116 (77%) individual leprosy sera contained IgG antibodies directed to antigens in the sonicates (90% of 75 multibacillary and 54% of 41 paucibacillary patients). Antigenic bands of 65, 33,

18, and 15-kDa were the most frequently identified. In particular, 83% of patients with a history of Erythema Nodosum Leprosum (ENL) had antibodies direct to the 33-kDa protein; a similar band was recognised by 16% of tuberculosis patients but not by sera from endemic controls.

A 42-kDa M.leprae recombinant antigen partially expressed as a 145-kDa β-gal fusion protein was recognised by 75% of 76 individual leprosy sera. Analysis of recognition patterns by different patient groups revealed that 78% multibacillary and 68% paucibacillary cases had antibodies directed to this molecule. The recombinant antigen was recognised by 94% of patients with a history of ENL, 26% of tuberculosis patients, and none of the control sera. Statistical analysis of recognition patterns among patients and controls suggested that the 42-kDa recombinant antigen has potential as a prognostic marker of leprosy reactions.

IM26

M.LEPRAE ANTIGENS CAN BE RECOGNISED BY BOTH TH1-LIKE AND TH2-LIKE HUMAN T CELLS

Anne MacFarlane and Hazel Dockrell

Molecular Immunology Unit, Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

Recent studies have suggested that tuberculoid leprosy may represent a Th1 form of the disease, while T cells with the properties of Th2 T cells predominate in lepromatous leprosy. A panel of human T cell clones have been obtained from BCG vaccinated donors who respond to M.leprae sonicate and to the M.leprae recombinant 18kDa antigen. Cloning was performed in the presence of antigen, IL-2 and autologous irradiated peripheral blood mononuclear cells as antigen presenting cells. Clones were selected by the ability to incorporate tritiated thymidine in a dose dependent fashion to the antigen used for cloning. Supernatants from antigen-stimulated cultures of clones were screened for the secretion of interferon-g by sandwich ELISA. On the basis of proliferation and interferon-g production, the clones could be separated into three groups. The majority of the clones showed a positive correlation between interferon-g production and proliferation resembling Th1 T cells. A smaller group showed much higher levels of interferon-g production relative to proliferation. A few clones gave proliferation without detectable interferon-g production. These clones, one of which recognised the 18KD antigen, produced IL-4, detected by ELISA. All the clones were CD4⁺ CD8⁻, αß⁺ and δ⁻. Thus even in donors with predominant Th1 T cell responses, a minority of Th-2-like T cells responsive to mycobacterial antigens are present, in the absence of any clear ongoing Th-2 response to allergens or worms. We are now investigating the role of other cytokines in the development of Th1 and Th2 CD4⁺ T cell response to M.leprae antigens.

IM27

MODULATION OF MHC CLASS-II ANTIGEN EXPRESSION ON ANTIGEN PRESENTING CELLS OF LEPROSY PATIENTS BY *MYCOBACTERIUM LEPRAE*.

Siva Sai SR Krovvidi, Anushree Gupta, Radhey Shyam Misra*. Prakash S Bisen**, H.Krishna Prasad.

*Department of Biotechnology, All-India Institute of Medical Sciences; New Delhi, * Department of Dermatology, Safdarjung Hospital, New Delhi-110 029; ** Department of Microbiology, Barkatullah University, Bhopal-462 026, India.*

MHC antigens play an important role in antigen presentation to T cells. Down regulation of MHC class-II antigen by any pathogen would therefore hamper the full expression of T cell functions in the diseased host. The present study was undertaken to address whether aberrations in modulation of MHC class-II antigens on monocytes could explain the antigen specific T-cell anergy seen in lepromatous leprosy patients. Flow cytometric analysis using two colour fluorescence was used to analyse the expression of MHC class II antigens (HLA-DR, -DQ, -DP) and CD 14 as a marker for monocytes. MHC class-II antigen expression was induced with time in healthy individuals, tuberculoid (TT/BT) and lepromatous (BL/LL) leprosy patients. This was independent of antigenic stimuli. The expression of HLA-DR was 6-8 fold more than that of HLA-DQ and HLA-DP. Down regulation of class-II antigen expression was observed at 12 and 24 hours with live and heat killed *M.leprae*. However, this down regulation was not specific as it was also observed with live *M.tuberculosis* H37Ra.

IM28

IgG ACTIVITY TOWARDS PURIFIED HEAT SHOCK PROTEINS AND ANTIGEN 85 IN LEPROSY PATIENTS AND THEIR CONTACTS

P. Launois[1], M. NDiaye-Niang[1], J.L. Sarthou[1], T. Lalu[2], A. Drowart[3], J.P. Van Vooren[3], J. Millan[2] and K. Huygen[4]

1 - Institut Pasteur, Dakar, Sénégal
2 - Institut de Léprologie Appliquée de Dakar, Sénégal
3 - Hôpital Erasme, ULB Brussels, Belgium
4 - Institut Pasteur du Brabant, Brussels, Belgium

Sera from 9 paucibacillary and 12 multibacillary leprosy patients, 18 healthy controls and 34 healthy leprosy patients contacts were analysed towards three heat-shock-proteins with M.W. of 70, 65 and 18 kDa and towards the secreted antigen 85 in a dot blot assay.

High reactivity to the 70 kDa molecules was observed in all groups of subjects. Indeed, 10/12 of multibacillary patients, 7/9 of paucibacillary patients, 20/25 Mitsuda positive contacts and 6/9 of Mitsuda negative contacts recognized the 70 kDa protein.

IgG activity towards the hsp 65 was higher in patients with positive Mitsuda reaction -i-e paucibacillary patients and lepromin positive contacts - than in patients with negative Mitsuda reaction.

None of the serum samples tested showed binding to the 18 kDa molecules.

12/12 of multibacillary patients, 2/9 of paucibacillary patients, 7/25 Mitsuda positive contacts and 3/9 of Mitsuda negative contacts were positive with antigen 85.

In conclusion, multibacillary leprosy patients recognized preferentially the 70 kDa molecules and the antigen 85 and sera from paucibacillary patients exhibited reactivity to the 65 and 70 kDa molecules.

IM29

RECONOCIMIENTO DE ANTIGENOS DE *MYCOBACTERIUM LEPRAE* EN PACIENTES HANSENIANOS

Carlos Santaella, Elsa Rada y Jacinto Convit.

Instituto de Biomedicina, Caracas, Venezuela.

Las células mononucleares (linfocitos T) de pacientes con lepra tuberculoide, de sus contactos sanos y voluntarios no relacionados dieron una buena respuesta al estímulo con un extracto soluble de Mycobacterium leprae. En estudios preliminares realizados en el laboratorio, se aportaron evidencias experimentales, mediante T-cell blotting, de una respuesta positiva de células mononucleares de sangre periférica proveniente de dichos pacientes y familiares contactos Mitsuda positivos frente a antígenos proteicos separados por SDS-PAGE. Se encontró actividad en las fracciones con intervalos de peso molecular de 45-29, 22-18 y 14 kDa, principalmente. Estos hallazgos se han relacionado con ensayos posteriores de la respuesta humoral (inmunoblotting), utilizando sueros policlonales de 10 pacientes con lepra lepromatosa(LL). Se detectó actividad frente a algunos de estos mismos antígenos (30, 16 y 14 kDa) despues de la absorción de dichos sueros con M. bovis, evidenciándose en consecuencia una alta especificidad con respecto a M. leprae.

IM30

SEROLOGICAL RESPONSE AGAINST CROSS REACTIVE CELL-WALL ANTIGENS (65 kD PROTEIN & LAM-B) IN LEPROSY.

Elangeswaran N, Senthilkumar S, Menaka K, Jayasheela M, and Rao, P.S.

Central Leprosy Teaching and Research Institute, Chengalpattu, Tamil Nadu - India.

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Two hundred leprosy patients and one hundred and fifty six endemic controls were screened for IgG antibody response against 65 kDa proteins of M.leprae, M.bovis BCG, M.fortitum

M.lepraemurium and LAM-B by ELISA with the objective of discrimination of antibody response between leprosy patients and endemic controls against these antigens. The following observations were noticed:-

In cases: The lepromatous leprosy patients had significantly higher antibody level against M.leprae, M. bovis BCG and LAM-B and significantly low antibody level 65 kDa of M.fortitum. The other types of leprosy(Indeterminate,Borderline tuberculoid and Tuberculoid, Borderline leprosy) there is a significantly higher antibody response against 65 kDa of M.fortitum and M.lepraemurium with low antibody response against LAM-B but the Borderline lepromatous type alone showed higher antibody response against LAM-B.

In controls: The household contacts of treated LL(Adult and children) had significant antibody response against 65 kDa of M.bovis BCG and significantly very high response against LAM-B was noticed in adult contacts only. The occupational contacts elicited significantly higher response against all the antigens used in this study. This study of antibody response against cell wall antigens may help us to discriminate the leprosy patients with the endemic controls along with the subtyping of the subjects included in this study.

IM31

DETECTION OF 65 KILODALTON HEAT SHOCK PROTEIN FROM SERA OF LEPROSY PATIENTS - MYCOBACTERIAL OR AUTO ANTIGEN ORIGIN.

Elangeswaran,N., Senthilkumar,S., Menaka, K., Jayasheela, M., Rao, P.S.

Central Leprosy Teaching and Research Institute, Chengalpattu-603001, Tamil Nadu - India.

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Sera from 167 leprosy cases of 65 kilodalton heatshock protein antigen by Reverse Passive Heamagglutination(RPHA). The antigen positivity rates varied from 72-100 % along the clinical spectrum of leprosy. The same sera were also examined for the presence of IgG antibody to recombinant 65 kDa of M.leprae by ELISA. 72.6% of the patients found to be positive for antibody to 65 kDa protein in leprosy patients. There was no significant difference in the percentage of 65 kDa antigen positivity between the patients under treatment and patients released from treatment. The 65 kDa antigen detection by RPHA found to be more sensitive than the antibody detection by ELISA, and having a higher predictive value for the diagnosis of leprosy. The role played by this Heatshock 65 kDa protein and auto-immune response in leprosy will be discussed.

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IM32

MAJOR ANTIGENS OF *Mycobacterium habana* RECOGNIZED BY SERA OF PATIENTS WITH LEPROMATOUS LEPROSY

Magdalena Vilchis*, José Esparza*, Fausto Quesada-Pascual**, Sergio Estrada-Parra**, Alfonso Islas** and Iris Estrada**. **Centro de Invest. Inmunol. Dermatol, U. de Guadalajara, Inst. Dermatol., Guadalajara, Jalisco. *Depto. de Inmunología, Escuela Nal. de C. Biológicas, IPN. México, D.F. 11340

Although *Mycobacterium leprae* infection of mice is self-limiting, it is possible to vaccinate mice such that they are protected against footpad infection. Intradermal immunization with killed *M.leprae* is the most effective means of vaccination. Of the cultivable mycobacteria which have been tested in this system, only *M.bovis* BCG has been found to give consistent cross-protection. Nevertheless, recently it was shown that *M.habana* may protect in a similar way to *M.leprae*, conferring 100% protection. Since immunization of mice with *M.habana* results in protection against infection with *M.leprae*, we have investigated, using a serological approach, the cross-reactive antigens shared by *M.habana* and *M.leprae*. By definition, the *in vivo* antibody (Ab) response to a given protein, where the response is preferentially of the IgG class, is an assay for Th cells. In this work we describe two *M.habana* proteins, with molecular masses of 30 and 28kDa. These doublet was recognized by all patients with lepromatous leprosy when their sera was diluted 1:100 and used in a Western blot analysis. The doublet was only recognized by IgG Ab and not by IgM. Neither the sera from tuberculosis patients nor from healthy people showed any antibodies against the doublet, when tested in a similar system. These proteins must have some cross-reactive epitopes with the *M.leprae* protein homologous, and since they are only recognized by IgG Abs we suggest they may play a role in protection. We also identified a similar doublet in *M.leprae*, BCG and *M.tuberculosis* with our LL sera. It is possible that the *M.habana* doublet is the simile of a doublet (33/29 KD) previously described in BCG and *M.tuberculosis*. The possibility of using a molecular genetic approach to investigate the role of these proteins in protective immunity is raised.

*Becarios de COFFA
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IM33

ANTIGENIC SIMILARITY MAY BE RESPONSIBLE FOR IMMUNE REACTIVITY IN LEPROSY.

Roël A.M. Chin-A-Lien*, Arend H.J. Kolk**, Theo W. van den Akker*, Bob Tank*, Theodoor van Joost*, Bernard Naafs*.
*Department of Dermato-Venereology, Dijkzigt Hospital, Erasmus University, Rotterdam, The Netherlands
**N.H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands

Antigenic similarity indicates that host and parasite share antigenic determinants that react with the same antibody or evoke cell-mediated immunity. It has been suggested that on one hand, such a similarity may fool the immune system and enable the parasite to avoid detection and destruction. This may occur in lepromatous leprosy. On the other hand, antigenic similarity may induce a state of enhanced immunity which is not only directed at parasites but also at the tissue of the host. Even when the parasite has been eliminated, damage to the tissue of the host may continue. This may occur in tuberculoid leprosy and during a reversal reaction.
For the human host, using an immunoperoxidase technique we demonstrated that skin and nerve had antigenic determinants that were in common with M.leprae. This was confirmed using Western Blot technique. It is interesting to note that macrophages also were able to express antigenic determinants that were similar to those expressed by M.leprae.
It has been shown that these antigenic determinants were often associated with heat shock proteins.

IM34

AN IMMUNODOMINANT .30KDa ANTIGEN(S) OF A CANDIDATE ANTI-LEPROSY VACCINE, *Mycobacterium w* SHARES T AND B CELL DETERMINANTS WITH *M.leprae & M.tuberculosis*.

Anjali Yadava and Rama Mukherjee

Microbiology Division, National Institute of Immunology, Shaheed Jeet Singh Marg, New Delhi 110 067. INDIA.

Peripheral T cell repertoire of *M.w* vaccinated leprosy patients was analysed using fractionated antigens of *Mycobacterium w*. Responses of unimmunised lepromatous patients, tuberculoid leprosy patients and healthy contacts (HC) were also analysed. All the subjects, except the unimmunised LL, recognized a number of low molecular weight antigens of *M.w in vitro*. One of these antigens, having a molecular weight of 30KDa, was recognized by a majority of the vaccinated subjects as well as the tuberculoid patients and HC. This antigen(s) mimicked *M.leprae* in the sense that the unimmunised LL showed a good antibody response to this antigen(s) but failed to show a T cell response, while the immunized LL, TT and HC showed a T as well as a B cell response to this antigen(s). Further studies on this antigen(s) using polyclonal antibodies against it revealed that it is associated with the cell surface. Immunofluorescence and Western blot studies demonstrate that it has homologues present in *M.leprae* as well as *M.tuberculosis*. DTH studies carried out in guinea

pigs immunized with this antigen(s) show that this immunodominant antigen(s) of *M.w* shares T as well as B cell determinants with *M.leprae* and *M.tubercuosis*.

IM35

IMMUNOCHEMICAL CHARACTERISATION OF 22 KD CYTOSOLIC PROTEIN OF MYCOBACTERIUM HABANA:A CANDIDE VACCINE

Sudhir Sinha[1] Deepa Bisht[1], Vinita Chaturvedi[2] and N.B. Singh[2]

Divisions [1]Membrane Biology & [2]Microbiology, Central Drug Research Institute, Lucknow, India.

The known adverse effects of certain constituents of integral mycobacterial vaccines have reemphasized the need for subunit vaccines based on immunodominant protein antigens. Present study aims at characterization of the major cytosolic protein antigen of M. habana, a candidate leprosy vaccine.

Cytosol was obtained as the supernatant (at 140,000 gx1 h) of the sonicate of logarithmic growth of M. habana. Pattern of cytosolic protein was analysed by SDS-PAGE. The major protein (Mr ~22 kd) was identified which almost exclusively got precip-itated at 80 to 95% ammonium sulfate concentration. Purity of the isolated protein was checked by silver staining, HPLC and isoelectric focussing. Initial immunological characterization was done by immunoblotting using homologous and heterologous polyclonal antibodies, LTT and DTH.

IM36

PGL-I LIKE ANTIGEN IN RENAL CARCINOMA

Masanao Makino, Yue Ping Yin, and Yasuhiko Suzuki

Osaka Prefectural Institute of Public Health, Osaka 537, Japan

Many methods for sero-diagnosis of leprosy have been established by using *M. leprae* specific phenolic glycolipid I(PGL-I) as an antigen. Gelatine agglutination tests by Izumi et al. and micro hemoagglutinin method by Minagawa et al. are two of most popular methods and used widely. By using Izumi's gelatin particle agglutinination test, we have examined sera from gravid women and obtained the conclusion that the sera from gravid women of gestation nine month especially had the anti PGL-I antibodies and would loose them after the labors. These results strongly suggest not only the relationship between anti-PGL-I antibody and fetus, but also existence of a new enbrionic antigen. In extensive studies, we examined sera from patients suffering from some kinds of cancer. We will discuss about the relationship between PGL-I like antigen and renal carcinoma.

RESULTS AND DISCUSSIONS

1) Anti-PGL-I antibody titer in serum: 200 times diluted sera from patients of renal carcinoma, L-type leprosy, and healthy control were examined with reactivity to sugar portion of PGL-I conjugated BSA as antigen by ELISA. Sera from 40 patients have been examined and about 70% of them showed high anti-PGL-I antibody titer. It was surprising that there existed a serum which is containing as high titer as those of L-type leprosy patient.

2) PGL-I like antigen in urine: PGL-I like antigen in urine was examined by dot blot detection. Urine from patient of renal carcinoma and healthy control have been serialy diluted with phosphate buffered saline and dotted on Nytran 13N nylon membrane filter. PGL-I like antigen was detected by anti-PGL-I monoclonal antibody SF1 and alkarine phosphatase conjugated anti-mouse immunoglobulins. Out of 10 tested urine from patients, PGL-I like antigen have been detected from 7 patients whereas all of 8 healthy controls were negative.

Wheather PGL-I like antigen might be usefull for the early diagnosis of renal carcinoma or not must be examined more.

IM37

IGG HUMORAL RESPONSE AGAINST THE ANTIGEN 85 COMPLEX HOMOLOGS IN LEPROSY

A Drowart(1), K Huygen(2), P Launois(3) JC Yernault(1) and JP Van Vooren(1)

1. Hôpital Erasme, ULB, Brussels, Belgium;
2. Instituut Pasteur van Brabant, Belgium;
3. Institut Pasteur de Dakar, Senegal.

Antigen 85 complex is the major protein component present in M. bovis BCG culture filtrate (CF) and consists of a family of 3 components 85A, 85B and 85C. Combining isoelectric focusing and Western blot analysis, we have identified different antigenically related proteins in CF from other mycobacteria (M.tuberculosis, M. kansasii, M. avium, M. gordonae and M. fortuitum) using monoclonal antibodies directed against the antigen 85 complex of M. bovis BCG. IgG antibodies directed against the antigen 85 cross reactive homologs from the 6 species were investigated in sera from 20 patients with multibacillary leprosy (BL/LL), from 20 patients with paucibacillary leprosy (BT/TT) and from 15 healthy leprosy contact subjects.

All the antigen 85 homologs identified with the monoclonal antibodies in these CF were recognized by the multibacillary leprosy patients sera but not by the paucibacillary leprosy patients sera nor by the healthy subjects sera.

The similarity in the recognition patterns of these different 85 homologs suggests that the epitopes inducing a significant humoral response in multibacillary leprosy are common to the 85 antigenically related proteins of all mycobacterial species.

IM38

MODULATION OF CITOTOXICITY AGAINST M.LEPRAE BY CYTOKINES IN LEPROSY PATIENTS.

María Sasiain, Susana Fink, Silvia de la Barrera, Fernando Minnucci, Raúl Valdez, Marta Finiasz and Luis Baliña. IIHema. Academia Nacional de Medicina and Hospitales San Martín and Argerich, Buenos Aires, Argentina.

We studied the lack of cytotoxicity induced by M.leprae in multibacillary patients (MB), to determine whether it was due to a deficiency of activating factors. Peripheral blood mononuclear cells (PBMC) ($2x10^6$cell/ml) were cultured for 7 days in tissue culture medium (RPMI-FCS), in the presence or absence of whole M.leprae ($1.8x10^7$ bac/ml), and then used as effector cells (E) (19 MB(6 BL,1 BB,12 LL), 6 paucibacillary (PB) (2 BT, 4 TT) and 7 BCG vaccinated healthy individuals(N) Adherent cells were cultured in RPMI-FCS for 6 days, pulsed overnight with M.leprae and used as target cells(T). IL-2(50U/ml); IL-6(10U/ml) and IL 4(20U/ml) were added at the beginning of the PBMC incubation period, and IFN-γ(100U/ml) 18hr.before perfoming the cytotoxicity assay. ^{51}Cr release was measured after 4 hr incubation of E + radio-labelled T (E/T=40/1). Results were expresed as % cytotoxicity (Mean±SEM). Basal: MB:16±2, PB:33±4, N:29±2; +IL-2: MB:16±1, PB:33±4, N:28±1; +IFN-γ: MB:20±1, PB:43±4, N:36±2; +IL-6: MB:21±2, PB:38±5, N:33±2; +IL-6+IL-2: MB:26±2, PB:40±3, N:40±1; +IL-6 +IFN-γ: MB:26±3, PB:49±4, N:44±2; +IL-4: MB:2±1, PB:19±3, N:15±1; +IL-4+IFN-γ: MB:5±2, PB:24±4, N:26±2. Statistical differences were found for IFN-γ and IL-6 alone ($p<.05$) or when IL-2+IL-6 or IFN-γ +IL-6 ($p<.02$) were added to MB, PB and N. Addition of IL-4 diminished the cytotoxicity ($p<.05$) and IFN-γ antagonized the effect of IL-4 in a dose dependent fashion. Cytokines would be involved in the regulation of cytotoxicity against M.leprae.

IM39

RECEPTOR SELECTIVE ENKEPHALINS AS EFFECTIVE IMMUNOMODULATORS IN LEPROSY

Shibnath Mazumdar, M.M. Dhar*, Radhey Shyam Misra** and Indira Nath

*Department of Biotechnology, All India Institute of Medical Sciences, New Delhi; *CDRI, Lucknow, **Department of Dermatology, Safdarjung Hospital, New Delhi, India.*

Enkephalins influence neuroendocrine and immune systems through receptors present on the cells. Of the four opiod receptors identified, δ receptor has immunostimulatory and μ receptor has immuno suppressive effects. Using Met enkephalins which binds to both receptors and δ selective (DPDPE), and μ selective (TOPA) peptides, *in vitro* immunomodulation was undertaken on lymphocytes derived from leprosy patients and healthy contacts.

Antigen specific lymphoproliferation and numbers of rosette forming T cells were significantly ($p < 0.05$) enhanced on *in vitro* treatment with Met enkephalins in both tuberculoid and lepromatous patients. This was further increased ($p < 0.001$) in the presence of the δ selective DPDPE. In contrast, treatment with μ selective TOPA inhibited lymphoproliferation substantially ($p < 0.01$) and rosette formation to a lesser extent.

These results indicate that 1) receptor selective enkephalin peptides have greater immunomodulatory effect than the total compound 2) DPDPE may have applications as immunoenhancing compound in lepromatous leprosy and 3) TOPA may be useful as an immunosuppressant agent in reactional leprosy.

IM40

SUPPRESSION OF HUMAN MONOCYTE CYTOKINE RELEASE BY PHENOLIC GLYCOLIPID I (PGL-1) OF *Mycobacterium leprae*.

Celio L. Silva, Lucia H. Faccioli, and Norma T. Foss

School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.

Defective macrophage activation is a prominent feature of lepromatous leprosy that depends on highly localized conditions occurring within macrophage-rich granulomas that contain numerous bacilli. It has been suggested that PGL-I play a role as protectors of resident **M. leprae** within phagosomes of phagocytic cells. Here we have measured monocyte activation by cytokine release in response to LPS in the presence or absence of PGL-1.

Peripheral blood mononuclear cells (PBMC) from healthy individuals were incubated in the presence of medium only or in the presence or absence of PGL-1 and LPS. After incubation for 24 h, culture medium was aspirated and TNF-alpha, IL-1 and IL-6 concentrations determined by using ELISA Kits.

Over a concentration range of 0.1-10 ug/ml PGL-1,no significant stimulation to produce IL-1, IL-6 or TNF was observed in cultured monocytes when compared to that observed for monocyte stimulated with LPS. The results were similar to that found for PBMC in the presence of medium alone. In contrast, a significantly increased ($p < 0.005$) levels of suppression of cytokine release was observed by the addition of PGL-1 (1 ug/ml) within the LPS-stimulated PBMC cultures. Thus, the percent suppression ranged from 35-74% for IL-1, 42-68% for TNF and 38-71% for IL-6 release (median of the experiment repeated 5 times).

These results might have a profound implication in the host response to **M. leprae**, once to our knowledge that acquired populations of specifically sensitized T cells and activated macrophages interact by means of cytokines to give rise to a state of protective immunity to this class of human pathogen.

IM41

EFFECTS OF GLUCOCORTICOIDS, DAPSONE AND THALIDOMIDE ON INTERLEUKIN-1 PRODUCTION

Wook Lew, Jung Bock Lee, and Joon Lew*
Department of Dermatology, Yonsei University College of Medicine,
*Lew Institute of Biomedical Research, Seoul, Korea

Interleukin-1(IL-1) is a cytokine which has multiple biological effects on the immunity and inflammation.

Dapsone(DDS) has been used as an anti-leprosy drug for a long time. It has also been an effective treatment for neutrophilic dermatosis like dermatitis herpetiformis and erythema elevatum diutinum. Thalidomide was developed as a hypnotic and sedative since the 1950's, but use was discontinued due to teratogenicity. Recently, it has been reintroduced for limited use in the treatment of erythema nodosum leprosum. But, the mechanisms of the anti-inflammatory effect of these drugs are still controversial. On the other hand, glucocorticoids have been shown to suppress production of IL-1, which was used as a positive control in this experiment.

Therefore, we studied the effect of DDS, thalidomide on the IL-1β mRNA production in U937 cells measured by Northern blot method and IL-1 production in the supernatant of human adherent peripheral blood mononuclear cells which was measured by thymocyte comitogenic assay, in order to determine whether the anti-inflammatory activity derives from the suppression of IL-1. We found the following effects on IL-1 production after treatment with DDS, thalidomide and several glucocorticoids.

1. IL-1β mRNA production of U937 cells and IL-1 production of human adherent mononuclear leukocytes were not suppressed by either the treatment with DDS(1ug/ml, 10ug/ml, 100ug/ml) or thalidomide(1ug/ml, 10ug/ml).
2. IL-1β mRNA production of U937 cells was well suppressed by 10^{-7}M glucocorticoids(prednisolone, prednicarbate, dexamethasone acetate), of which dexamethasone acetate was the strongest, followed by prednicarbate and prednisolone.

In conclusion, neither DDS nor thalidomide suppress IL-1β mRNA production or IL-1 production. Therefore, their anti-inflammatory mechanism may be different from glucocorticoids.

IM42

COMPARATIVE EVALUATION OF ANTIBODIES IN THE SERUM AND URINE OF LEPROSY PATIENTS THROUGH DIVERSIFIED MYCOBACTERIAL ANTIGENS

H.P. Gupta, Shalini Bhatnagar and N.B. Singh
Division of Microbiology,
Central Drug Research Institute,
Lucknow (U.P.) INDIA.

Approximately 1.5 billion people are globally exposed to the risk of contracting leprosy and the problem is expected to be exaggerated due to relentless prevalence of HIV incidence and as a resultant shift in the disease spectrum due to AIDS. Case detection and treatment are the principle methods currently used for control of leprosy but the former is inadequate in detecting early leprosy and contact cases. This study has been done with a view to increase the sensitivity of the assay and to replace the scarcely available M. leprae antigens.

We present here our latest efforts to detect M. leprae antibodies through Conventional ELISA test by using different antigens, namely PGL1, sonicates of M. leprae and M. habana, arabinomannan and 65 KDa protein derived from M. habana. A simultaneous detection of antibodies have been made from two different sample sources - the serum and urine of leprosy patients. The level of detection of M. leprae antibodies from both type of samples through antigens of M. habana (antileprosy vaccine candidate) showed superiority over M. leprae antigens. Antibodies levels were more in the serum than urine. Smear negative doubtful paucibacillary cases were also detectable and confirmed serologically for initiation of treatment. Significance of these findings has been discussed.

IM43

IgM SERUM ANTIBODIES TO PGL-I BY ELISA IN HEALTHY SUBJECTS AND LEPROSY PATIENTS

Meng Meibai Deng Yunshan Wu Xingzhong Sun Chuanzhen Ma Jiaju
Wang Zhenbao Zhang Xibao Li Yunwen Sun Xianguang

China Leprosy Control and Research Center, Guangzhou, China

Sera from 969 healthy subjects and 974 leprosy patients were tested for anti-PGL-I antibody (APGL-I) by ELISA with NT-P-BSA.

The results and discussion showed that,1)The mean OD($\overline{OD}$)and positivity rate of healthy females (0.090 and 6.9%)were significantly higher than those of healthy male(0.068 and 2.8%)(P>0.01), and there was a similar tendency between sex groups of leprosy patients. The female false positivity rate would be increased if calculated by overall positive criteria, thus the authors suggested that the positive criteria of $\overline{OD}$ of males and females must be calculated separately; 2) The levels of APGL-I were of skew distribution in all groups except the active MB patient group, suggesting it was not suitable to calculate cut-off point of $\overline{OD}$ according to $\overline{OD}$+2SD based on normal distribution. Therefore, the comparison of $\overline{OD}$ among various populations is more reliable than that of positivity; 3) From the epidemiological point of view, this detection can not reflect slight difference when leprosy prevalence is reduced to a lower level, but may reflect epidemiological dynamics in some focus with relatively higher incidence; 4) $\overline{OD}$ and positivity rate were still significantly higher in inactive MB and PB patients than those in healthy subjects. It remains to be proved what are their clinical and epidemiological significance and if t hey can serve as an applicable parameter in detecting relapses; 5)In order to have a good quality control of the detection at different times and in different laboratories, the procedures and reagents used, especially the control pool sera for correcting results, must be standarized.

IM44

LIPOARABINOMANNAN (LAM) BASED ENZYME-LINKED IMMUNOSORBENT ASSAY IN SERODIAGNOSIS OF LEPROSY

Sanjay Gandhi, M.D.[1], Shereen Wahab, M.D.[1], Fazal Raheman, M.D.[2]

[1] [2] Government Medical College, Nagpur, INDIA
[2]DynaGen, Inc., 99, Erie Street, Cambridge, Massachusetts 02139, USA

To determine whether antibodies to mycobacterial cell wall carbohydrate would be valuable in serodiagnosis of leprosy, serum IgG antibodies to lipoarabinomannan (LAM) antigen were assayed, 40 leprosy patients, their household contacts which comprised of 31 individuals and 86 controls which included 46 apparently healthy individuals and 40 new-born babies, were recruited for this study. The serum samples from the study subjects were tested for anti-LAM IgG antibodies using an enzyme-linked innunosorbent assay. When results among leprosy cases were compared with control group and considering clinical diagnosis as gold standard, sensitivity and specificity of LAM based ELISA was 72.5% and 90.7% respectively. When ELISA results were compared with skin smear for AFB, the difference was significant by McNemar test for changes. A positive linear correlation was observed between Bacillary index and IgG anti-LAM antibody levels. The relevance of these findings to the serodiagnosis of leprosy and its importance in household contacts of leprosy patients is discussed.

IM45

THE ANTIBODY RESPONSES TO VARIOUS ANTIGENS OF M. Leprae IN BORDERLINE LEPROSY WITH OR IN REACTION

Mrs. Ratna Sudha R, & Dr. Stanley J.N.A.*

School of Biotechnology, J.N.T. University, Masab Tank, Hyderabad, A.P. 500 028, INDIA,

*Dhoolpet Leprosy Research Centre
Balram Seth Galli, Karwan, Hyderabad - 500 006, INDIA.

The serological activities to various M. Leprae specific antigens (18KD, 65KD, WML, PGL, ND-O-BAS & NT-O-BSA) were assessed by ELISA against 365 sera from 95 patients classified Clinico Pathologically as BT(25), BTRR(17), BTRR-RFT(6), BT-RFT(9), BL(10), BLRR(10), BL-RR-RFT(5), BL-RFT(4), BTRR-RFT-RR(9) & 36 healthy volunteers. The sera from 71 patients were collected periodically at monthly invervals for 6-9 months during treatment and rest were collected only once.

A similar pattern of reduction of Antibody responses in both BT & BTRR groups suggests that steroids do not influence the antibody responses in the doses of 30-40mg of Prednisalone per day in both BT with nerve damage and BTRR patients.

On analysis of both the IgM & IgG response patterns during treatment to various antigens, it is quite likely that the IgM responses to any of these antigens may not play any significant role during reversal reactions, however by monitoring the IgG responses to WML alone it would be possible to differentiate early reaction from active disease alone and also from relapse, so that appropriate treatment can be given to the patients. The implications to clinicians of these results will be discussed in detail.

IM46

ANTIBODIES TO CEREBROSIDE-SULPHATE IN LEPROSY.

Paul R Wheeler, KPWJ McAdam, R Hussain#, and John G Raynes

Molecular Immunology Unit, Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. #Professor of Microbiology at the Aga Khan University, Karachi, Pakistan.

A study was set up to investigate whether antibodies against cerebroside sulphate (sulphatide) may have pathological consequences in leprosy. Sulphatide is found on the surface of human cells including NK and notably Schwann cells. It was found that sera from leprosy patients contain antibodies to sulphatide and that anti-sulphatide IgM was higher in sera from lepromatous patients than tuberculoid patients (levels were
in 24/24 and 5/16, respectively, outside 95% confidence limits of levels in control sera). Anti-sulphatide IgG was also present in sera from lepromatous patients.

Lack of variation ($< \pm 20\%$ from mean values) of both anti-sulphatide IgM and IgG during ENL was noted; however, in 3 patients where sera were taken before ENL commenced, anti-sulphatide IgM fell 1.5 to 3 -fold at the onset of the reaction. Thus anti-sulphatide antibodies are present in leprosy but their role in the pathogenesis of nerve damage remains to be clarified.

IM47

AUTOANTIBODIES TO NEURAL ANTIGENS IN LEPROSY

Nalini Vemuri, Leila M. Viera and Rama Mukherjee

National Institute of Immunology,Shahid Jeet Singh Marg,New Delhi-110067,India.

Neural lipids, namely galactocerebroside(Galc) and gangliosides(Gg) have been implicated in demyelinating diseases. In order to asses their role in leprosy, the humoral immune response to these lipid antigens as well as to ceramide, sulpholipids and human Myelin Basic Protein (MBP) was quantitated by microtitre enzyme linked immunosorbent assay. Sera from 219 leprosy patients, 18 neuritic patients and 43 normal healthy controls were screened. High titers of IgM antibodies directed to total nerve lipid (TNL), Galc and ceramide were present in patients across the spectrum while the antibodies to sulpholipids and gangliosides were present in low titers. Varying titers of IgG class of antibodies directed to MBP were detected in all categories of leprosy patients. No anti- lipid/ protein antibodies were detected in normals. Anti- TNL and anti-Galc antibodies were highest in TT patients with clinical evidence of nerve damage. A statistically significant positive corelation was observed between anti-TNL and anti-Galc antibodies in TT and neuritic patients. However anti-MBP antibodies were significantly high in LL-BL patients with evident nerve damage.These observations suggest that the neural pathology in these two forms of disease may be different.

IM48

COMPARISON OF A GELATIN PARTICLE AGGLUTINATION TEST AND ELISA FOR DETECTION OF ANTIBODY TO M.LEPRAE

Lishi Qian, James T. Douglas, and Gertrude P. Chan
Department of Microbiology, University of Hawaii at Manoa Honolulu, Hawaii
Research Institute for Tropical Medicine, Alabang, Philippines

Two rapid diagnostic methods, Enzyme-linked Immunosorbant Assay(ELISA) and the Gelatin Particle Agglutination Test (GPAT), have been developed recently for epidemiological monitoring of the efficacy of chemotherapy and post-treatment follow-up of patients. Both these tests make use of semisynthetic analoques of the phenolic-glycolipid-I, the specific antigen of M.leprae, but differ in methodology. This report examined the two tests with 1059 sera collected at an endemic area in Manila, Philippines. ELISA and GPAT were compared in terms of the seropositivity, the concordant rates, the sensitivity and specificity.
The GPAT was found to be much simpler and easier to perform and generally more sensitive but less specific compared with ELISA. The sensitivity of GPAT in MB patients (83.04%) group was much higher than those in PB patients (41.86%). The concordant rates, ranged from 87% in normal populations and 57.89% in household contacts. Chi-square test of homogeneity indicated that the concordant rate between ELISA and GPAT in normal populations was significantly higher than that in patients and contacts populations. The concordant rate of MB untreated decreased after being treated by multi-drug therapy six months or longer. The seropositivity of GPAT was higher than that of ELISA in all study populations except the household contacts. The seropositivity of both GPAT and ELISA in MB patients were higher than the seropositivity of these two tests in PB patients. In conclusion, GPAT was found to be a sensitive but less specific test compared with ELISA. However, it is easy to perform and inexpensive. Therefore, it is considered as a screening test for detection of the M.leprae infection, especially of the multibacillary type.

IM49

A PROGNOSTIC VALUE OF SEROLOGICAL ASSAYS IN REGRESSED LEPROSY

O.Degtiarev, M.Dyachina
Leprosy Research Institute, Astrakhan, Russia

610 outpatients with different types of leprosy being under specific therapy over 10 years were examined with using enzyme immunoassay and counter electrophoresis. Native antigen from ultrasound-desrupted M.leprae (USD -ML) and a semi-synthetic analogue of PGL-1 (ND -O-BSA) (provided by WHO Bank) were used.Among the patients with inactive leprosy (BI=0) 182 cases (29,8%) were invariably seropositive for 1-3 years of the observational period. It was found out that the group above included the patients with chronic specific polyneurites, visceral and eye pathologies (hepatitis, orcho-epididimitis, iridocyclitis, uveitis, ètc). Only in 15% of the patients from this group antibody titers to ND-O-BSA were significantly higher as compared with the titers of antibodies to USD-ML.In other cases the ratios were inverse indicating the necessity of serological testing of regressed leprosy patients with either antigens. 26 patients relapsed, 80% out of them being seropositive 1 - 3 years before the occurrence of relapse. 23 (88,4%) cases out of them had high levels of antibodies to both antigens that is characteristic to active leprosy. In three paucibacillary patients anti-M.leprae antibodies were detected neither before nor at the moment of relapse. The investigation showed a value of serological assays in patients with regressing leprosy for assessment of the effectiveness of chemotherapy and early prognosis of relapses.

IM50

THE INFLUENCE OF BCG VACCINATION AND CLOSE CONTACT WITH LEPROSY PATIENTS ON THE RESULTS OF SKIN TESTS WITH 4 NEW TUBERCULINS

O.Bottasso[1], H.Williams[2], L.Cannon[2], N.Ingledew[2], V.Merlin[3] A.Dalla Fontana[3], J.Morini[1], and J.Stanford[2]
Division Inmunologia[1] Facultad de Ciencias Medicas de Rosario , Programa Provincial de Lepra[3] , Argentina; School of Pathology, University College and Middlesex School of Medicine, London, England[2]

Our study in Santa Fe Province of Argentina investigated the results of skin tests with Tuberculin, Leprosin A, Scrofulin and Vaccin on 672 young adults residing in a leprosy endemic area. Of them, 350 were in-house contacts, usually relatives, of MB patients, 87 were similar contacts of PB patients, and 235 were members of the leprosy service frequently exposed to patients. Within these groups distribution by sex, BCG scar and length of exposure were similar, but the leprosy workers tended to be the oldest group. The results were analysed individually for each reagent, and by responder categories (Category 1, +ve to all 4 reagents; category 2, -ve to all 4 reagents; category 3, +ve to some but not all reagents). Age made no difference to categorization, but the presence of a BCG scar significantly increased categories 1 and 3 at the expense of 2 ($p < 0.03$). A similar finding was recorded in leprosy workers when compared to MB and PB contacts ($p < 0.01$). BCG scars were also associated with increased positivity to Leprosin A ($p < 0.02$). These results emphasize the roles played by BCG vaccination and close contact with leprosy patients on the cellular immune response to mycobacterial antigens.

IM51

BCG VACCINATION AS A PROTECTION AGAINST LEPROSY. Maria Fernanda Sardella Alvim, Nadia Duppre, José Augusto Nery, Ana Maria Malta, Maria Lucia Penna e Euzenir Nunes Sarno. * Leprosy Department Fundação Oswaldo Cruz, Av. Brasil, 4365 - Manguinhos, 21.045-900 - Rio de Janeiro - RJ. ** Department of Epidemiology State University of Rio de Janeiro.

The role of BCG vaccination in protection against leprosy disease is not definitively clarified. Randomized controlled trials in four different populations demonstrated a range of efficacy against leprosy from 20% in Burma to 80% in Uganda. In Brazil BCG vaccination has been widely used since 1976 as a prophilatic measure against tuberculosis applied at birth. The official rules of the leprosy control program recommended since 1990, two BCG doses with a one year interval in contacts of multibacillary leprosy patients. The impact of this measures in leprosy control must be clarified in the future.In This study, 503 contacts of multibacillary patients with age 2 - 20 years were included to the trial with different periods of follow-up. These 55 leprosy cases and 448 controls were matched for age, close-association of index case, and BCG scar. Considering as control group those vaccinated, the unvaccinated contacts have a risk, estimated through the odds ratio, 4.3690 times greater than the control group. In the group without BCG scar, persons over 10 year old had an increased risk of developing leprosy (odds ratio = 9.0909). When compared to those under 10 years (odds ratio = 2.6556). Household contacts had to risk of contracting of disease 2,02 times greater than non-household. Among children under 10 years of age with BCG scar, 11 (50%) developed the Infantum Nodular Leprosy (Tuberculoid lesion). From the 55 new cases of leprosy multibacillary forms were diagnosed in 62% without BCG scar and 38% with BCG scar. This study strongly suggest a considerable value of BCG vaccination on protection of leprosy disease and protective efficacy against the more serious forms of leprosy

Supported by grants from TDR-WHO

IM52

INDUCTION OF LEPROMIN POSITIVITY BY A CANDIDATE ANTI-LEPROSY VACCINE MYCOBACTERIUM w AND ITS IMMUNO——PROPHYLACTIC EFFECT IN LEPROMIN NEGATIVE HEALTHY CONTACTS OF MULTIBACILLARY LEPROSY PATIENTS

*H K Kar *A K Sharma, **R S Misra,@S A Zaheer, çA Mukherjee,@R Mukherjee, @K R Beena, @H Kaur, @S K Nair and @G P Talwar

* Deptt.of Skin, STD ad Leprosy, Dr.RML Hospital, New Delhi-110 001.
** Dept. of Skin, STD and Leprosy, Safdarjung Hospital, New Delhi-110 029
ç Institute of pathology, ICMR,New Delhi-110 029.
@ National Institute of Immunology,New Delhi-110067.

In a hospital based study, 362 household contacts of multibacillary leprosy patients under MDT were screened for evidence of leprosy and 54 (14.9%) were found to be having leprosy. The remaining 308 apparently healthy contacts were lepromin tested and 109 (35.4%) were observed to be negative to Mitsuda lepromin. M.w vaccine was administered intradermally to 95 of these 109 lepromin negative contacts. Sixty eight of them could be retested for lepromin A reactivity. Fifty six (82.35%) manifested lepromin conversion. The twelve subjects who did not show lepromin conversion, received a second dose of the vaccine, and eleven subsequently became lepromin positive. The overall lepromin conversion rate was thus 98.5% (67 out of 68). Follow-up of these contacts upto a period of 5 years did not demonstrate reversion of lepromin positivity back to negativity status. Among original lepromin positive contacts, so for four cases of Paucibacillary leprosy have been detected, but none from vacine induced lepromin converted contacts.

IM53

M.LEPRAE-RESPONSIVE T LYMPHOCYTES AND BLOOD MONOCYTES IN A RARE REACTIONAL FORM OF LEPROSY, THE LUCIO'S PHENOMENON.

Geraldo M.B. Pereira, André L. Moreira, Damma A. Esquenazi, José A.C. Nery, A. de M. Machado and Eumenio N. Sarno. Leprosy Unit, FIOCRUZ and State University of Rio de Janeiro, Rio de Janeiro, Brazil.

The Lucio's phenomenon(LP), a leprosy reaction with systemic manifestations, characterized by necrosis of the dermal capillaries and epidermal areas, was investigated in 3 polar lepromatous(LL) patients(LP1, LP2, LP3; male, 65 to 70 yr. old, 0 to 12 months of treatment), treated with thalidomide(LP1,LP3) or thalidomide plus glucocorticoid(LP2). Before treatment, patients had increased serum TNF-α(LP2), IL-6(LP1, LP2, LP3), and their peripheral blood mononuclearleukocytes(PBL) generated spontaneous TNF activity in vitro, as well as very high levels of oxidative metabolism intermediates(chemiluminescence; LP2,LP3). FACS analysis of PBL demonstrated absence or reduction of γδ+, CD8+ and CD14+ cells. On day 7, the lesions were healing. The parameters above with the exception of IL-6 serum levels were returned to normal or diminishing; and surprisingly the PBL(LP2,LP3) proliferated in vitro in response to *M. leprae*, but without IFN-γ production. The specific inhibition of TNF-α by thalidomide, and these observations support the hypothesis of a major function for TNF-α in LP, and suggest a role for *M. leprae*-specific T lymphocyte response in this leprosy reaction. Supported by grants from TDR-WHO and CNPq

IM54

LYMPHOCYTE SUBPOPULATIONS AND THEIR FUNCTIONAL PROPERTIES IN LEPROMATOUS LEPROSY

L. Saroyants, A.Juscenko, L.Alekseyev
Leprosy Research Institute, Astrakhan, Russia

Lymphocytes from LL patients were studied for their proliferative responses to PHA, ConA, PWM, and specific M.leprae antigen (lepronin). Besides,the functional activity of ConA-induced T suppressors and T cell subsets with CD2+, CD4+, CD8+ phenotypes were studied.It was shown that in active leprosy patients lymphoproliferation to mitogens, function of nonspecific T-suppressors and the relative percentage of CD2+ cells were significantly low as compared with cured patients and healthy donors ($p<0,01$). In long treated patients lymphoproliferation was also low, though at less degree than in untreated patients ($p<0,05$). LTT to lepronin was low in both groups of the patients. In leprosy patients irrespective of their disease status relative contents of CD4+ cells was lower while CD8+ content was higher as compared with normal values. CD4+/CD8+ ratio in both patient groups was significantly lower than in the control group. Increased level of CD8+ cells with simultaneous decrease in the functional activity of non-specific T-suppressors in leprosy patients seems to be a consequence of compensatory mobilization of suppressor/cytotoxic cells into circulation to replenish their functional deficiency. Thus, in LL patients there is a definite interrelationship between immune aberrations and their disease status. Prolonged specific therapy results only in partial recovery of disturbed functional activi*y and changed contents of lymphocyte subpopulations.

IM55

IMMUNOGENETIC ASPECTS OF SUSCEPTIBILITY TO LEPROSY

L.Saroyants, A.Juscenko, L.Alekseyev
Leprosy Research Institute, Astrakhan, Russia

According to literary data, an association between HLA antigens of the major histocompatibility complex and leprosy in different populations was extensively studied, but the patients belonging to Russian nationality were assessed only for HLA class I antigens. The investigation presented was aimed at studying a distribution of HLA class 1 and 2 antigens in lepromatous leprosy. 125 Russian patients who were admitted to the clinical department of our Institute were examined. For control 120 unrelated healthy individuals of the same ethnic group were randomly selected. HLA typing was carried out as described by Terasaki. It was stated that the frequency of HLA-DR3, DR2 and B7 antigens was significantly higher for LL patients than in the control group, whereas DR5 was absent. After the correction for the number of antigens tested statistically significant differences remained only for HLA B7 ($Pc=0,049$) and DR3 antigens ($Pc=0,00014$). The highest relative risk was to HLA-DR3 antigen ($RR=10,22$). Attributive risk, characterizing a strength of association with the disease, was also the highest for HLA-DR3 - carriers ($\sigma = 0,67$). Thus, based on these results it was concluded that HLA-B7 antigens (possibly, haplotype B7-DR2) and HLA-DR3 might be the markers of susceptibility to lepromatous leprosy in Russians that should be borne in mind when identifying risk groups among leprosy contacts.

IM56

ROLES OF LYMPHOCYTE SUBPOPULATIONS IN ERYTHEMA NODOSUM LEPROSUM AND ACUTE ANTERIOR UVEITIS

Erkut Bahçeci, Tülay Çakıner, Tevfik Akoğlu, Türkan Saylan

Istanbul Leprosy Hospital, Istanbul Leprosy Research Center, Istanbul, Turkey.

One of the many sistemic complications of leprosy is a reactional state known as erythema nodosum leprosum (ENL). During ENL some patients develop acute anterior uveitis (AAU). It is still unknown why some patients develope AAU during ENL and others do not.

Considering that changes in lymphocyte subpopulations might be important in pathogenesis of ENL and AAU, we determined proportions of lymphocyte subpopulations, namely B cells (CD19+), T cells (CD3+), NK cells (CD56+), helper/inducer cells (CD4+), suppressor/cytotoxic cells (CD8+), CD4+ CD29+, CD4+ CD45RA+ and CD8+ CD11B+ cells, by flowcytometry. The study group consisted of patients with AAU, ENL, post reactional patients and patients who never suffered from these reactions.

Proportions of CD4+ cells and CD4+ CD29+ cells were significantly higher in AAU and ENL patients than post reactional and non reactional groups. In AAU patients CD4+ CD45RA+ cells were also high.

CD4/CD8 ratio was significantly high in ENL patients compared to non reactional group.

CD56+ (NK) cells were significantly lower in AAU and ENL group than other groups.

The importance of these results will be discussed.

IM57

IN VITRO LABELLING AND FUNCTION OF GRANULOMA MACROPHAGES FROM LEPROMATOUS LEPROSY

Gue-Tae Chae, Gun-Yeon Na, Nan-Hee Kim, Yong-Ma Ha, Hae-Young Choi

Chronic Diseases Laboratory, Department of Pathology, Catholic University Medical College, Seoul 137-701, Korea

Granuloma is an immunologic and pathologic unit of human lepromatous leprosy (LL), which is chiefly composed of macrophages (MACs) packed with *M. leprae* and lymphocytes. Until now little is known about the influx and turnover of these granuloma MACs in human LL, except high influx of bone marrow-derived MACs in experimental LL granuloma of nu/nu foot pad. We have been conducting autoradiography studies to evaluate influx of bone-marrow-derived MACs in Ll granuloma using [3H]TdR pulse *in vitro*. MACs are isolated by enzyme digestion and plated on LUX coverslips 1 x 10^6/ml with RPMI-10% AB serum, MACs with AFB bacilli and more than ten sliver grains on nucleus are counted to score Labelling Index (LI). LI are calculated labeled nucleus with AFB per on-thousand nucleus. In spite of major roles of suppressor lymphocytes in unresponsiveness of LL as reported, defective activation of LL MACs to exogenous gamma interferon (IFN-γ) is likely due to any cytokines or mediators such as prostaglandin E_2 (PGE_2) which inhibit gamma interferon in the microenvironment of LL granuloma. To find any difference following IFN-γ, BCG, IL-2 treatment *in vitro*, we have examined levels of PGE_2 production by radioimmunoassay and Toxoplasmacidal effect of MACs from LL granuloma.

IM58

KILLING OF MYCOBACTERIA-INFECTED MACROPHAGES BY LAK CELLS FROM LEPROSY PATIENTS

Shubhada Chiplunkar, Madhu Deshmukh, Jyoti Kudalkar, * Prabhakar Samson, Madhav Deo and Sudha Gangal

Immunology Division, Cancer Research Institute, Bombay, * Richardson Leprosy Hospital, Miraj, India

Host resistance to bacteria is multifaceted with both specific and non-specific immune mechanisms playing important roles. In the present investigations, we have assessed the ability of lymphokine-activated killer (LAK) cells generated from lepromatous leprosy (LL) patients, tuberculoid leprosy (TT) patients and healthy individuals, to lyse targets (macrophages and T-24, bladder carcinoma cell line) infected with mycobacteria (Mycobacterium leprae/Mycobacterium ICRC). We observed that LAK cells generated from LL patients could preferentially lyse M.leprae or ICRC-pulsed macrophages and T-24 cells, compared to non-pulsed targets. However, LAK cells from TT patients failed to distinguish between non-pulsed and mycobacteria-pulsed target cells. The specificity of lysis of mycobacteria-pulsed targets by LAK cells was confirmed in a cold target competition assay. Furthermore, we have studied the killing of mycobacteria by LAK cells. ICRC bacilli incubated with LAK cells or bacilli obtained from infected macrophages incubated with LAK cells showed a significant reduction in the number of colonies of bacilli after plating on soft agar. Thus, our studies demonstrate that LAK cells may play a significant role in killing of intracellular bacteria and may serve as an immunotherapeutic modality in the treatment of leprosy.

IM59

MODULATION OF MONOCYTES/MACROPHAGES OF LEPROSY PATIENTS BY TUFTSIN FOR BIOCHEMICAL AND IMMUNOGENIC FUNCTIONS.

Sangeeta Khare, L.K. Bhutani & D.N. Rao.
Deptt.of Biochemistry & Deptt.of Dermatovenereology
All India Institute of Medical Sciences
New Delhi 110029, India.

Mycobacterium leprae is an obligate intracellular pathogen that is ingested by and proliferate within the cells of monocytes/macrophages (Mφ). Intracellular pathogens may escape killing mechanism either by inhibiting production of reactive oxygen intermediates (ROI) or by neutralizing these intermediates. We have investigated the effect of macrophage stimulant "tuftsin" on immunogenic functions of monocytes/Mo derived from leprosy patients as a function of in vitro culture age. Since we have earlier observed an aberrant phagocytic and microbicidal response in Lepromatous patients towards tuftsin pulsing, the present study was undertaken to correlate the microbicidal functions (by measuring O_2^- & H_2O_2) and maturation profile (by measuring adenosine deaminase activity) of monocytes/Mφ. Further the signals involve (by measuring $[Ca^{++}]$) in triggering the Mo membrane by tuftsin and tuftsin receptor expression (by radio receptor assay) on these Mφ were studied in detail. ROI production and $[Ca^{++}]$ release towards tuftsin pulsing showed a progressive increase with increasing in vitro culture age till day 3, than tapered off in older cultures of normal and BT/TT Mφ. BL/LL cultures were unable to undergo tuftsin mediated ROI production and $[Ca^{++}]$ release after day 1. ADA activity was found to be maximum in the early cultures of BL/LL Mφ. These results indicate that BL/LL Mφ has a differential maturation profile and there may be specific enzyme(s) defect associated with ROI production. From the study it can be also concluded that there is an altered signalling during Mφ activation and finally these defect may lie at the tuftsin receptor expression.

IM60

IS NERVE DAMAGE IN LEPROSY AN AUTO-IMMUNE PHENOMENON INVOLVING ANTI-PERIPHERAL NERVE ANTIBODIES

Prabha Desikan, Om Parkash and Pratibha Narang

M.G.Institute of Medical Sciences,Sevagram, Wardha- 442 102, Maharashtra, India.

The role of anti-peripheral nerve antibodies directed against the peripheral nervous system in leprosy patients in the pathogenesis of nerve damage is, till date, inconclusive and controversial. A study was therefore taken up to detect such antibodies in the sera of one hundred leprosy patients belonging to the entire spectrum of the disease, along with sera from normal individuals as controls. Using antigen derived from normal human nerve,

9% showed demonstrable levels of antineural antibodies of the IgG type and 11% of the IgM type. However, with antigen derived from nerve of a cured, bacteriologically negative leprosy patient, on testing twenty out of the above hundred sera, 40% tested positive for antineural antibody of the IgM type, and none tested positive for the IgG type. There was no correlation found in the present study between the presence of the antibodies and of neuropathy or of occurrence of active neuritis. There was also no correlation with the type or duration of the disease. The findings will be discussed.

IM61

ANTIBODIES TO A NERVE LIPID EXTRACT (NLE) IN SERA OF LEPROSY PATIENTS.

Rekha Vartak*, Damayanti Shah, Shubhada Dandekar**, Sharad Naik*, and Evelyn Sequeira*.**

***Acworth Leprosy Hospital Society for Research, Rehabilitation and Education in Leprosy, Wadala, Bombay - 400 031, India.**
****Radiation Medicine Centre, BARC, Parel, Bombay - 400 012, India.**

Soluble serum factors have long been suspected to play an important role in the neuro-pathogenesis of leprosy. Humoral immune response to a Nerve Lipid Extract (NLE) was evaluated in patients of leprosy (n=131), patients with neuropathies other than leprosy (n=23) and normal healthy volunteers (n=51) using microtitre plate ELISA test. High titres of IgM class of anti-neural-antibodies directed to NLE were detected in 21% (29/131) sera of leprosy patients. The reactivity was minimal with normal healthy volunteers as only 2 out of 51 (4%) showed detectable anti-neural antibody titres. Interestingly, none of the sera from patients of neuropathies other than leprosy showed raised anti-neural antibody titres. All the twenty nine sera showing raised anti-neural antibody titres belonged to borderline or lepromatous type of leprosy conferring 30% positivity to the group whereas none of the 34 patients of BT/TT/P type of leprosy showed raised anti-neural antibody titres.

It also appears from this study that the anti-neural antibodies develop at a later stage of multidrug therapy as the majority of untreated LL patients(14/16) did not show reactivity against NLE. This suggests a possible role for cytoplasmic antigens of M.Leprae released during the period of treatment.

IM62

PREVALENCE OF ANTI-NEURAL ANTIBODIES AMONG LEPROSY PATIENTS

Sang-Nae Cho,[1] Roland V. Cellona,[2] Rodolfo M. Abalos,[2] Tranquilino T. Fajardo, Jr.,[2] Laarni G. Villahermosa,[2] Gerald P. Walsh,[2] and Joo-Deuk Kim[1]

Department of Microbiology, Yonsei University College of Medicine, Seoul, Korea,[1] and Leonard Wood Memorial Center for Leprosy Research, Cebu, Philippines[2]

Nerve damage is a major clinical manifestation in leprosy. As an effort to elucidate the pathogenic mechanisms of nerve damage in leprosy, this study was initiated to determine whether or not there is any association between anti-neural antibodies and nerve damage in leprosy. Lipid and glycolipid antigens including ceramide and galactocerebrodide (GC) were prepared, and the prevalence of antibodies to the antigens and asialo-GM$_1$ (AGM$_1$) was determined among leprosy patients and controls. The major immunoglobulin class to the nerve lipid antigens was IgM;therefore, only IgM class to the antigens was analyzed. Of 291 Korean controls who had no recent history of having traumatic injuries, 43 (14.8%) had elevated antibodies to ceramide, 54 (18.6%) to AGM$_1$, and 24 (8.2%) to GC, respectively, and 83 (28.5%) to at least one of the three antigens. In contrast, among 170 Philippine controls, 52 (33%) were seroreactive to ceramide, 38 (22.4%) to AGM$_1$ and 18 (10.6%) to GC, respectively, and 81 (47.6%) to at least one antigen, indicating that controls in Philippines have significantly greater opportunity to expose to neural antigens than in Korea. Of 105 untreated leprosy patients from Philippines, 57 (54.3%) were seropositive to ceramide, 40 (38.1%) to AGM$_1$, and 42 (40.0%) to GC, respectively, and 129 (58.9%) to at least one antigen, indicating that the prevalence of anti-neural antibodies in leprosy patients was significantly higher than controls. In addition, the prevalence of anti-neural antibodies was correlated with the extensiveness of anesthesia and nerve enlargement. The results suggest that anti-neural antibodies are closely associated with neurologic damage following *Mycobacterium leprae* infection.

IM63

MYCOBACTERIUM LEPRAE LAM AND PGL-I ANTIBODIES CROSS-REACT WITH HIV-1 ANTIBODIES IN SERA FROM HIV-1-SERONEGATIVE LEPROSY PATIENTS AND THEIR CONTACTS.

Oscar Kashala[1,2], Ilunga Mbayo[3], Richard Marlink[4], and Max Essex[4]
[1]Cambridge Biotech Corp, Worcester MA, USA; [2]Dept of Pathology and Oncology, Kinshasa University Hospital, University of Kinshasa, Kinshasa, Zaire [3]Ministry of Public Health, Kinshasa, Zaire [4]Dept of Cancer Biology, Harvard School of Public Health, Boston MA, USA

Fifty seven leprosy patients (LP), 39 leprosy contacts (LC), and 500 pregnant women (PGW) were evaluated for the presence of antibodies to human immunodeficiency virus type 1 (HIV-1), human T cell lymphotropic virus type 1 (HTLV-1), and type 2 (HTLV-2). Antibodies to mycobacterium leprae phenolic glycolipid 1 (PGL-I), and lipoarabinomannan (LAM) were analyzed to assess the association between leprosy and human retroviral infections. A low prevalence of HIV-1 infection occurred among LP (3.5%), LC (0%), and PGW (3.6%). LP and LC had a significantly higher prevalence of HTLV-1 infection compared with PGW (8.7% and 12.8% vs 0%, respectively). Sera from LP and LC were often false-positive by ELISA (64.7% and 23%, respectively), and indeterminate by Western Blot for HIV-1 (83.6% and 64.1%, respectively). In LP sera, both LAM IgM, and PGL-I IgM antibodies cross-reacted significantly with anti-HIV-1 Pol (p31, p61) and Gag antibodies (p24), respectively. Mycobacterial cell-wall antigens may share common epitopes with HIV. Caution should therefore be exercised when interpreting HIV-1 ELISA and western blot data from regions where leprosy or other mycobacterial diseases such as tuberculosis are endemic.

IM64

SEROPROFILE OF HBV, HIV and HTLV INFECTIONS IN ZAIRIAN LEPROSY PATIENTS AND THEIR CONTACTS.

N. ILUNGA 1, L.O. KASHALA 2,3, M.R. KALENGAYI 2

Leprosy Hopital de la Rive (1) Kinshasa,Zaire, Dept of Pathology, Kinshasa University Hospital Zaire (2) and Dept of Cancer Biology, Harvard School of Public Health, Boston, USA.

Objective : Preliminary study to determine the seroprevalence of HBV,HIV and HTLV infections in Zairian Leprosy patients and their contacts

Materiel & Methods : Patients with lepromatous (LL) and tuberculoid (TT) lepra and their contacts were serologically examined for HBV (HBS, AntiHBS seroconversion, AntiHBS titer, pre-S1 and Pre-S2), HIV (antiHIV), HTLV (anti HTLV1 and antiHTLV2) and for Antibodies to M.leprae specific antigens PGL-1 and LAM-B.

Results. HBs prevalence was significantly higher in LL (20%) than in TT (6,6%) patients and contacts. Anti HBs seroncoversion was significantly low in the 3 groups. Anti HBs was significantly higher in TT (4572,40 +/- 5234,61 UI/ml) than in both LL patients and contacts.Pre-S1 and Pre-S2 prevalence rates were higher in TT (26,6% and 30%) than in LL (12% and 20%).
HIV1 seroprevalence was 3,5% in leprosy patients, 0% in their contacts while it is known to be 3-8% in the general population. Unlike in this last group, high rates of HIV false + in Elisa (63,6%) and W-B indeterminate patterns (30,7%) were seen in the leprosy patients.
HTLV1 antibodies were detected in 8,7% of leprosy patients and in 12,8% of their contacts. No HTLV2 antibodies were seen in any group.
Antibodies to M leprae specific antigens were seen in 43.8 (antiPGL-1) and 42,1% (antiLAM-B) of patients.

Implications and Perspectives :
It is worth to extend such a study to the nuserous leprosomia in the country in order to asses the impact of these virus infections on the pathogenesis and the course of Leprosy. Furthermore one must be precautious in the interpretation of the HIV tests in people with leprosy.

IM65

ASSOCIATION OF HLA-A_1-B_1-C, AND DR ANTIGENS WITH LEPROSY

S.K. Ghei[1], U. Sengupta[1], S.Kailash[2], N.G. Sekaran[2], K.S. Sudhakar[3], K.V. Desikan[4], Kondala Rao[3], C.C. Shepard[5] and T. Shinick[5].

[1] Central JALMA Institute For Leprosy, Agra, [2]All India Institute of Medical Sciences, New Delhi, [4] LEPRA (India), Hyderabad,[3] GREVALTES, Visakhapatnam and [5]CDC, Atlanta, USA.

The human major histocompatibility system (HLA) has been known to be associated with a variety of diseases. Various groups of workers have searched for HLA antigens and their associations with leprosy. However, except for the association of DR2 with tuberculoid type of leprosy, no other association could be related to any of the types of leprosy. As no strong association could be observed with single locus antigens, it was decided to find out if the combination of genes (haplotypes) have got any association with the types of leprosy. The present study was undertaken in families from an endemic area, Visakhapatnam (A.P.) In all 2009 individuals from 408 families were taken for the study. The normal healthy sibs were taken as controls. None of the HLA-A_1-B_1-C and DR antigen showed any significant correlation with the disease types except for BB group only. In this group HLA-A10 specificity showed a significant association (P=0.008) even after correction of the P value. Certain haplotypes also showed some significant associations which will be presented and discussed.

IM66

IN VITRO TEST SYSTEMS TO DETERMINE SUSCEPTIBILITY TO LEPROMATOUS LEPROSY.

P.R.Mahadevan & Prema Robinson
The Foundation for Medical Research
Bombay 400 018, India.

In vitro systems to demonstrate the level of superoxide produced by macrophages on exposure to live M.leprae and ability to kill the phagocytosed M.leprae, showed that lepromatous leprosy patients, before, during and after treatment had poor positive response in the two parameters. The normally leprosy resistant individuals in an endemic city like Bombay, showed these two potentialities in their macrophages. This has ben consistently found to be true in subjects analysed 30 patients 30 normal healthy. Extending this observation to families it was found that the index patient had the defects and the defects were shown by one or more of the children even though the male or female partner of the index patient was normal. Such progeny showing the defects in the macrophage were seen as either healthy or exhibited symptoms of leprosy infection. The complete analysis of the observations showed that in the normal population monitoring of macrophage interaction with live M.leprae by the in vitro systems could lead to indication of susceptibility. This along with lepromin negativity in skin test should help narrowing the susceptible population.

IM67

STUDIES OF LYMPHOCYTE PHYSIOLOGY-A STUDY OF MARKER ENZYMES OF METABOLIC PATHWAYS

C.S. SURI BABU, K.B. KANNAN, V.M. KATOCH, & V.P. BHARADWAJ

Biochemistry Deartment, Central JALMA Institute for Leprosy, Tajganj, AGRA-282001 (India).

Infection with Mycobacterium leprae, the causative organism of leprosy is the result of defect in CMI. The abnormality has been correlated with defects both in number and functions of T-Lymphocytes. The changes that occur in the physiology of lymphocytes might be one of the reasons for their depressed functions, specially in the effector limb. We have studied a number of enzymes to elucidate different metabolic pathways like Arginase, Iso-enzymes of LDH and Aldolase, SOD and Peroxidase, Alkaline and Acid Phosphatases, beta-D-Glucuronidase, Adenosine deaminase and Amino Acyl t-RNA Synthetases besides rate of translation by labelled amino-acids, Lymphocyte Arginase showed an increase of activity in LL (5.48 units/mg), BB and BT (2.54 units/mg), TT (1.81 units/mg) in comparison to that in healthy controls (0.87 units/mg). Adenosine deaminase shows a reversal of this trend as in LL (265 units/mg), TT (375 units/mg), which is common in immunoproliferative disorders. Activities of all other enzymes also varied throughout the spectrum of the disease, which is well correlated with LTT and LMIT. Enzyme induction studies have been carried out to rectify the defects in lymphocytes which showed increasing response to different antigens including M. leprae.

IM68

OBEERVATION ON SUBCUTANEOUS IMMUNNE CELLS IN SITU IN LEPROUS NONREATIVE SLIN LESIONS

Zhu Simin Zhao Guohua Wu Huixi Olaf K. Skinsnes

Department of Pathology, Sun Yat-Sen University of Medical Sciences, Guangzhou, China

David M.Scollard Peter Chang

Department of Pathology, Leahi Hospital, Hawaii University, U.S.A

This study attempts to evaluate changes in T-cell subsets, macrophages and natural-killer cells by using Leu-2a, Leu-3a, Mac718, Interleukin-2-receptors (IL-2R) and HLA-DR antingen in situ in leprous nonactive skin lesions of 24 leprosy patients and healthy normal controls(LL13, BL3, BB4, BT2,TT2, normal control 5).Cells were obtained from fluid aspirated from suction-induced blisters directly over nonreactive skin lesions. Significant differences were observed between LL/BL, TT/BT and normal control by HLADR, Leu-3a, Leu-2a. The LL/BL group had lower positive rate than the TT/BT group and normal controls. The T-helper/supressor ratio (Leu-3a+/Leu-2a+) was 0.39 in LL/BL and 2.39 in TT/BT. This indicated that even macrophages containing phagocytosed M. leprae could not react with T-helper cells. Macrophages due to HLA-DR antigen absence, and also T-helper cells are decreased in LL. The cell-mediated immunity defect is caused by lymphokines absence. IL-2R is also decreased in LL/BL. On the other hand, Leu-2a positive cells (supressor cells)were increased in LL/BL.They can supress cell-mediated immunity. The results can be interpretated as part of the reason for lymphokines absence in lepromatous leprosy. No significant difference was observed between LL/BL and TT/BT in Leu-7(NK cells). This indicates that perhaps NK cells are not importnant in cell-mediated immunity in leprosy. Macrophages had higher levels in LL/BL than TT/BT from nonactive skin blister. This is inadequately understood as yet.

IM69

BORDERLINE TUBERCULOID HANSENIASIS: PARAMETERS OF IMUNOLOGICAL ACTIVITY.

Pimentel, M. I. F.; Sarno, E. N.; Sampaio, E. and Moreira, A. L.

Oswaldo Cruz Foundation, Hanseniasis Department Rio de Janeiro

Forty-eight patients with Borderline Tuberculoid Hanseniasis, according to Ridley & Jopling classification system, were studied in order to determine their capacity in circunscribing the disease in the skin (number of cutaneous lesions) and in the peripheral nerves (number of afected nerves). These clinical features were compared to each other, and to an humoral immunity test (ELISA test for PGL-I, phenolic glycolipid spe-

cific from Mycobacterium leprae) and to celular immunity tests "in vivo" (cutaneous Mitsuda test) and "in vitro" (lymphocyte proliferation stimulated by M. leprae and gama-interferon detection in lymphocyte culture stimulated by M. leprae supernatant). The celular immunity test that best related to the capacity of the patients in limiting their disease, with reference to the skin, was the gama-interferon detection test. BT form of Hanseniasis seems to be of systemic nature, though with tendency to circunscribe the manifestations in the skin and/or in the peripheral nerves, one feature not necessarily being accompanied by the other.

IM70

A POTENTIAL MARKER FOR THE PREDICTION OF ENL REACTIONS.

Satish Singh, N.P. Shankar, Narayanan*, Peter. J. Jenner$, Gopal Ramu , Radhey Shyam Misra#, H. Krishna Prasad, M. Joseph Colston$ & Indira Nath.

Department of Biotechnology, All India institute of Medical Sciences; Department of Dermatology, Safdarjung Hospital#, New Delhi; VHS Leprosy Project, Shakthi Nagar, TN, India; National Institute for Medical Research. Mill Hill, London, UK$.*

A double blind study was conducted to identify a serological marker for the prediction of Erythema nodosum leprosum (ENL) reactions which occur in lepromatous patients. 538 sera samples {ENL,stable LL, LL with history of reactions (H/O REACTION), BT-TT,Familial contacts (FC), Non contacts (NC), Pulmonary Tuberculosis (TB)} obtained from endemic and non endemic areas of India were screened in an ELISA using Recombinant *M.leprae* antigen LSR, that was previously reported by us and 16 overlapping peptides(15-19 mer) based on its sequence. Significantly > 90% of the ENL and H/O reaction patients showed seroreactivity to the LSR antigen in comparison to only 68% of the stable LL patients. In contrast none of the NC individuals were seropositive to this antigen. On screening with the peptides,greater than 92% of the ENL patients recognised peptides 2 (GVTYEIDLTNKNAA), 3 (IDLTNKNAAKLRGD) and 13 (REWARRNGHNVSDRGRI) as compared to < 43% of the stable LL patients. The degree of seroreactivity based on the Optical Density (OD) values showed highly significant differences between stable and reactional patients (P value <0.01 to 0.001) for peptides 2,3,13 & LSR. Peptide 13 was found to be cross reactive with sera from TB patients (18%). It is proposed that peptides 2 and 3 are diagnostic markers for active ENL. They may also be useful for identifying lepromatous patients with a high risk of developing ENL.

IM71

HUMAN LEPROSY LESIONS IN SITU USING SUCTION-INDUCED BLISTERS: CELL CHANGES WITH IGM ANTIBODY TO PGL-1 AND INTERLEUKIN-2 RECEPTOR IN CLINICAL SUBGROUPS OF ERYTHEMA NODOSUM LEPROSUM.

Lertlakana Bhoopat†, David M Scollard‡, Choti Theetranont†, Siri Chiewchanvit†, David L Nelson§, Utaiwan Utaipat*

†Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
‡John A Burns School of Medicine, University of Hawaii, Honolulu, Hawaii.
§National Cancer Institute, National Institutes of Health, Bethesda, Maryland.
*Research Institute for Health Sciences, Chiang Mai University, Chiang Mai Thailand.

To examine the immunopathogenesis of type 2 erythema nodosum leprosum (ENL) reactions in leprosy, we studied cellular and soluble immunologic components of skin lesions in 57 patients with reactions (19 acute ENL and 38 chronic ENL), 61 active patients without reactions, and 33 control patients whose leprosy had been treated and cured. Cells, IgM antibody to PGL-1 and Tac peptide levels were obtained from fluid aspirated from blisters induced by suction directly over representative skin lesions. During ENL reactions: a) the lesions in chronic ENL showed a decreased number of $CD8^+$ (T-suppressor) cells and increased helper/suppressor ratio as compared to those in acute ENL and non-reactional leprosy; b) Tac peptide and IgM antibody to PGL-1 levels were elevated in the chronic ENL lesions; c) and systemic administration of corticosteroids appeared to cause a reduction in the intralesional $CD4^+$ cell population and IgM antibody to PGL-1 but did not change $CD8^+$ cell population and the levels of Tac peptide in the lesions. The elevated levels of Tac peptide were localized in the skin lesions while increased levels of IgM anti-PGL-1 seemed to be filtered from the peripheral blood. We conclude that spontaneous lymphocyte activation in situ, primarily of decreased $CD8^+$ and relatively increased $CD4^+$ cells, are important features of chronic, recurrent ENL reactions and may be an intermittent or cyclic phenomenon during the reaction. Understanding the mechanisms of these spontaneous changes in immunity in leprosy will enlarge our knowledge of reactions and of the underlying determinants of delayed type hypersensitivity and cell-mediated immunity in leprosy, which in turn will allow us to realize the potential for artificially manipulating these responses as proposed with vaccines or immunotherapy.

IM72

LEPROSY IN WOMEN: CLINICAL AND IMMUNOLOGICAL ASPECTS

Marian Ulrich, Manuel Zúñiga, Celsa Sampson, Maria E. Pinardi and Jacinto Convit

Instituto de Biomedicina, Caracas, Venezuela

Renewed interest has been shown in the impact of tropical diseases in women. One of the basic areas of interest in the analysis of sex and gender differences is related to the relative contribution of biological and cultural factors. Disease characteristics and immunological reactivity in leprosy in Venezuela suggest a more effective immune response to *Mycobacterium leprae* in both healthy women and female patients. Among 64,559 contacts of patients, 48-hr skin test reactivity was higher to *M. leprae* soluble extract (MLSE) in females; the antibody response to PGL-I was significantly higher in females at every age level. Clinical leprosy of all forms was more frequent in males. The highest frequency in females occurred in the 15-24 year age group; the peak in males was 20 to 30 years later. A retrospective family study in Venezuela demonstrated a significantly higher incidence of leprosy in male offspring when the mother was infected, but multibacillary incidence was low in this group, suggesting maternal immunomodulation. Skin tests with PPD and MLSE were larger in females throughout a 5-yr follow-up after vaccination. Taken together, all of these data suggest a stronger immunological response to *M. leprae* in females which appears to reflect true physiological differences.

IM73

THE INFLUENCE OF THALIDOMIDE ON THE HISTOLOGICAL MANIFESTATIONS OF ENL. Miranda A, Sampaio EP, Maypel GT, Sarno EN. Leprosy Department, Oswaldo Cruz Foundation, Av. Brasil, 4365, Manguinhos, 21.045-900, Rio de Janeiro, Brazil.

Leprosy patients with various forms of the histological spectrum have peculiar reactional states, which are associated with changes in immunological reactivity. Biopsies from six multibacillary leprosy patients were collected at the time of developing ENL, during thalidomide therapy, or after thalidomide treatment was discontinued. In contrast to the lepromatous leprosy lesions, the ENL lesions demonstrated local changes identified as parameters of immune activation in situ. The overlying thickened epidermis exhibited pronounced keratinocyte ICAM-1 expression, increased number of Langerhan's cells and contained lymphocytes (mainly CD8+ cells) expressing the ICAM-1 ligand, LFA-1. HLA-DR staining was always present in ENL lesions from 50 to 100% of epidermal cells. The lesions showed recruitment of CD4+ cells into the dermal infiltrate. TNFa-positive cells in the dermis were increased in number as compared to the non reactional biopsies. Surprisingly biopsies taken from patients under different periods of thalidomide treatment showed complete remission of those manifestations. Treatment with thalidomide dramatically reduces the positivity in situ for ICAM-1, HLA-DR and TNFa protein in the tissue. Within 7 days of treatment, a decreased number of neutrophils and T cells in the dermis was noted. The histological changes seen in ENL biopsies, as well as the expression of activation molecules in in situ provide evidence for a cellular immune activation in this type of leprosy reaction.

Supported by grants from TDR-WHO.

IM74

LIPOARABINOMANNAN (LAM) - A POSSIBLE IMMUNOREGULATORY MOLECULE OF M.LEPRAE INFECTED SCHWANN CELLS?

Nerges Mistry, Vanaja Shetty, Varsha Shetty & Noshir Antia

The Foundation for Medical Research, 84-A, R.G. Thadani Marg, Worli, Bombay-400018, India.

Despite bacterial killing by drugs, persistence of Mycobacterial antigen (LAM) in tissues may result in prolonged immunosuppression in and/or progressive damage to peripheral nerves. It is therefore important to define the nature of the immunoregulatory effects of LAM, if any, on the Schwann cell of peripheral nerves. Murine dissociated Schwann cells in tissue culture were tested for their ability to induce lymphoproliferation in the presence of purified LAM from H37Ra. This was studied in isolation and in conjunction with accessory cells viz. the endothelials and the fibroblasts. Simultaneously the functional competence of these tissue culture sensitized cells was examined in assays for M.leprae cytotoxicity, cytokine release, induction of nerve damage and granuloma formation.

Observations indicate LAM as a potent immunoregulatory antigen in all cell types albeit in different conditions. The findings indicate a novel mechanism for the precipitation of lepra reactions in peripheral nerves in leprosy.

IM75

SENSITIZATION TO MYCOBACTERIA IN TWO AREAS IN ZIMBABWE WITH DIFFERENT DISTRIBUTION OF LEPROSY TYPE AND LEPROSY INCIDENCE: ELISA.

De Lange, W.E.*, Gwanzura, L.*, Mous, H.V.H.*, Mason, P.R.*, Naafs, B*.
*Dept. of Medical Microbiology
University of Zimbabwe, Harare
Zimbabwe
*Dept. of Dermato-Venerology
Erasmus University, Rotterdam
The Netherlands.

Antibody titers against eight mycobacterial antigenic preparations were determined using an Enzyme Linked Immunosorbent Assay (ELISA) in sera from individuals from two different areas in Zimbabwe. In the two areas that were selected, Chipinge (C) and Nenyunka (N), significant difference in the ratio of Paucibacillary and Multibacillary leprosy (C: 1:7, N: 6:1) and in the incidence of leprosy were observed. Leprosy patients (C: 13, N:24), their contacts (C:26, N:31), secondary school pupils (C:52, N:48) and healthy non-contact adults (C:10, N:10) were tested. The results of this study showed that sera of leprosy patients had higher antibody titers against all antigens than healthy controls from the same area. Significant differences in antibody titers in the sera of the leprosy patients from both areas were also observed for five antigenic preparations. These findings support the concept that humoral antibodies may not have any direct influence on the immunity against M. leprae and the pathogenesis of leprosy.

IM76

SENSITIZATION TO MYCOBACTERIA IN TWO AREAS IN ZIMBABWE WITH DIFFERENT DISTRIBUTION OF LEPROSY TYPE AND LEPROSY INCIDENCE: SKIN TESTS.

Mous, H.V.H.*, Mason, P.R.*, De Lange, W.E.*, Gwanzura, L.*, Naafs, B*.
*Dept. of Medical Microbiology
University of Zimbabwe, Harare
Zimbabwe
*Dept. of Dermato-Venerology
Erasmus University, Rotterdam
The Netherlands.

Delayed type hypersensitivity (DTH) skin tests with eight mycobacterial antigenic preparations were performed to evaluate the relationship between cell-mediated immune response (CMI) and the type of leprosy in two different areas in Zimbabwe. In the two areas that were selected, Chipinge (C) and Nenyunka (N), significant difference in the ratio of Paucibacillary and Multibacillary leprosy (C: 1:7, N: 6:1) and in the incidence of leprosy were observed. Leprosy patients (C: 13, N:24), their contacts (C:26, N:31), secondary school pupils (C:52, N:48) and healthy non-contact adults (C:10, N:10) were tested.
Significant differences between the two areas and differences between leprosy patients and their contacts and between leprosy patients and controls were observed. These findings support the concept that sensitization by environmental microorganisms (mycobacteria) may influence the incidence and the pathogenesis of leprosy.

IM77

LEVELS AND COMPOSITION OF CIRCULATING IMMUNE COMPLEXES IN PATIENTS WITH ERYTHEMA NODOSUM LEPROSUM AND ACUTE ANTERIOR UVEITIS

Tülay Çakıner, Erkut Bahçeci, Tevfik Akoğlu, Turkan Saylan

Istanbul Leprosy Hospital, Istanbul Leprosy Research Center, Istanbul, Turkey.

Acute Anterior Uveitis (AAU) is one of the ocular manifestations of type II reactions which leads to serious disabilities. Exact mechanism of development of AAU is still unknown. In the hope to determine the significance of specific factors manifested in AAU we determined the levels of circulating immune complexes (CIC), IgA, IgM, IgG, C4, C3c and CRP in patients with ENL, AAU, post reactional patients and patients who never suffered from these reactions.

There were no significant difference between levels of CIC of these groups. However, when reactional and post reactional levels of individual patients were compared, we found significant decrease in levels of CIC after reaction.

Considering these results we analysed composition of CIC by immunoblotting.

The results of these experiments will be discussed.

IM78

AN IMMUNOGENETIC STUDY OF DIFFERENTIAL MANIFESTATIONS OF LEPROSY IN NORTH INDIA

Rajni Rani,*@ M. A. Fernandez-Vina,@ S.A. Zaheer, K.R. Beena,* Peter Stastny@

*National Institute of Immunology, New Delhi-110067, India.
@ Southwestern Medical Center, Dallas, Texas-75235, USA

Leprosy is a heterogeneous disease which presents in the form of multibacillary lepromatous (LL) type at one pole of the spectrum and paucibacillary tuberculoid (TT) type at the other end. To study the role of host factors in determining the immune response and the differential manifestations of the disease, the class II HLA alleles were studied by a PCR-oligotyping technique. DRB1, DRB3, DRB5, DQA1, DQB1 and DPB1 alleles were studied in 93 patients and 47 normal controls. DRB1*1501 and DRBI*1502 account for 83.1% of the multibacillary patients and 57.1% of the TT patients compared to 21.27% in controls. The much stronger association of DBR1*1501 and 1502 with the multibacillary form suggests a possible role of these alleles in the differential immune response to the M. leprae antigens. DQB1*0601 and DQA1*0103 was found significantly more often

than in controls throughout the leprosy spectrum. On the other hand, DQB1*0201 and DQA1*0201 were decreased in the LL patients as compared to TT patients and controls, suggesting a possible protective effect of these alleles against multibacillary leprosy.

IM79

GLUCOCORTICOIDES AND REGULATORY PEPTIDES IN LEPROSY PATHOGENESIS

V.Naumov, L.Saroyants, E.Balybin
Leprosy Research Institute, Astrakhan, Russia

In LL patients the production of hydrocortisone and regulatory peptides (ACTH and beta-endorphine) was studied. Immunity status was assessed by lymphocyte blast transformation and by activity of non-specific T-suppressors. Mononuclear cell reactivity to glucocorticoides and interleukin-1 (IL-1) was assessed. It was found out that in active leprosy hydrocortisone levels were increased while glucocorticoide reserve was decreased. ACTH levels were in normal range in active leprosy and significantly increased when the disease regressed. Concentrations of beta-endorphine in leprosy patients were below the norm. A positive correlation between hydrocortisone levels and lymphocyte resistance to glucocorticoides and T-cell suppressing activity was found out. Lymphocytes from the patients with leprosy reaction responded to IL-1 while they were unresponsive to it in the patients with no leprosy reactions. The results suggest that marked neuro-endocrine disturbances in leprosy might be at hypothalamus-hypophysial and adrenal levels. Proliferative and regulatory potential of lymphocytes is dependent on the state of glucocorticoides production. These findings as well as the peculiarities of lymphocyte response to IL-1 in leprosy reaction represent the interrelationships between immune and neuroendocrine systems and might be used for prognosis of the course of leprosy and optimization of pathogenetic therapy with glucocorticoides and immune modulators.

IM80

EFECTO DEL IONOFORO A23187 Y 12,13-FORBOL MIRIACETATO SOBRE LA PROLIFERACION DE LINFOCITOS T DE PACIENTES CON LEPRA LEPROMATOSA.

Fernando Alfaro, Gabriela Ramírez, Amado González, Martín Arce, Alfonso Islas, Roberto Morales, Mary Fafutis.
Centro de Investigación en Inmunología y Dermatología. Universidad de Guadalajara/Instituto Dermatológico de Guadalajara (SSBS) Guadalajara, Jalisco MEXICO.

Los linfocitos T (LT) de pacientes con lepra lepromatosa (LL) son deficientes en proliferar adecuadamente ante estímulos antigénicos y/o mitogénicos (Bloom, Hastings), así como en la producción de IL-2 (Islas, Moghaghepour, Harege woin), por otro lado se ha demostrado que el receptor para IL-2 (IL-2R) está intacto (Fafutis). Cuando los eventos antes mencionados se llevan a cabo adecuadamente, el ciclo celular de LT humanos progresa. En LT de individuos sanos existen evidencias de que el Ca++ y la PKC son mensajeros secundarios importantes en la biosíntesis de IL-2, pero sólo PKC es necesaria para la expresión de RIL-2 (Mills). El ionóforo A23187 es una molécula que se disuelve en la bicapa lipídica de la membrana celular incrementando la permeabilidad al Ca++ (Alberts) y se le ha considerado un agente mitogénico de LT, así como cofactor de 12,13-forbol miriacetato (PMA) para activar PKC (Truneh, Akerman). En este trabajo el ionóforo A23187 y PMA se adicionaron a LT de sangre periférica para determinar su efecto sobre la proliferación y compararlo con el de fitohemaglutinina (PHA) en el cultivo celular.
Los resultados obtenidos sugieren que los LT de la mayoría de los pacientes con LL forman dos grandes grupos: los que requieren la presencia de ionóforo A23187 (incremento de Ca++) y los que requieren de PMA (activación de PKC) para mejorar su respuesta linfoproliferativa.

IM81

SERUM TRANSFERRIN (Tf) CONCENTRATION AND Tf RECEPTORS (R) ON THE SURFACE OF T LYMPHOCYTES IN LEPROMATOUS LEPROSY (LL) PATIENTS.

Mary Fafutis, Fernando Alfaro, Amado González, *Luis Santos coy, José Barba-Rubio, Roberto Morales, Alfonso Islas.
Centro de Investigación en Inmunología y Dermatología. Universidad de Guadalajara/Instituto Dermatológico de Guadalajara (SSBS). *Unidad de Patología Clínica. Guadalajara, Jalisco MEXICO.

The iron transport via systemic circulation is assured by Tf a serum globulin which binds iron and conveys it to high-affinity Tf-R on cell surfaces. It has been shown that the complex Tf/Tf-R plays an important role in the progression of the antigen and/or mitogen, that leads to the expression of interleukin-2 (IL-2) and its receptor. We have reported (Int.J.Leprosy 55:566. 1987,58;126. 1990) that the lymphocytes from LL patients have a deficient biosynthesis of IL-2, but express their IL-2 receptor. The present study concerns the serum concentration of Tf measured by radial immunodiffusion and the expression of Tf-R by flow cytometric analysis in PHA stimulated T lymphocytes from 25 LL patients.
In this work we found that T lymphocytes from LL patients under PHA stimulation, present a diminished percent of TfR positive cells and the serum Tf levels are not significattly different from those of normal subjets.

IM82

RECOMBINANT IL-2 IN UNTREATED AND TREATED LEPROMATOUS LEPROSY PATIENTS

L.G.Villahermosa, R.M.Abalos, T.T.Fajardo,Jr.
E.C.dela Cruz and G.P.Walsh

Leonard Wood Memorial Center for Leprosy Research
Cebu, Philippines

Interleukin 2 (IL-2), is a 15-kilodalton single chain protein produced by thymus-derived lymphocytes and is believed to stimulate T-cell proliferation after antigenic stimulation.

Ten lepromatous leprosy patients: 5 untreated, 3 currently receiving WnO-MDT regimen and 2 post-lepromatous patients were injected with interleukin 2 at specified dosage levels and intervals and administered as single and multiple injections.

In all IL-2 injected sites, early recruitment of cells: neutrophilic granulocytes, lymphocytes and mononuclear cells were noted to appear as early as 24 hours and which was more pronounced among the treated lepromatous patients as compared to the new untreated lepromatous patients. The migratory cells in the dermis noted after injection of IL-2 were identified as T-lymphocytes by surface phenotyping. There was an increase in both CD4 and CD8 cells and ratio of CD4 to CD8 was 2 to 3 times greater than in uninjected control sites.

These results suggest IL-2 may enhance cellular immune response among lepromatous leprosy patients. However, its clinical utility as a supplement to chemotherapy can only be determined by long term clinical studies in lepromatous leprosy.

IM83

TUMOR NECROSIS FACTOR (TNF) IN LEPROSY PATIENTS WITH TYPE II REACTION

Mario Gómez**, Santiago Partida*, Luis Favila*+, Sigifredo Pedraza*, Sergio Estrada-Parra*+, Iris Estrada*+ & Amado Saúl Cano**.*Depto. de Inmunología, Escuela Nal. de C. Biológicas, IPN. México, D.F.**Servicio de Dermatología, Hosp. General de México, S.S. México, D.F.

The broad spectrum of host responses of leprosy patients to antigens from *Mycobacterium leprae* provides a model for investigating the role of cytokines in the pathogenesis of the reactional state designed as "type II reaction", which includes ENL and Lucio's phenomenon. Of particular interest is TNF, a cytokine which may have both antimycobacterial and immunopathological effects. The production of one of the two types of TNF molecules, TNF-α, has been shown to be selectively inhibited by thalidomide, an anti-inflammatory drug, which is used to cure type II reactions. To evaluate the potential role of TNF in type II reactions and the effect of thalidomide upon the levels of TNF *in vivo*, we measured the levels of this cytokine in the sera of ten patients with type II reaction with no thalidomide treatment. After a first blood sample was taken, patients were put on thalidomide (100 mg/day). One month later, a second blood sample was taken from patients which had clinically recovered from the reaction (no clinical symptoms, one month on thalidomide). TNF levels in serum samples were measured by a bioassay with L929 cells. Levels of TNF in the first sample (reaction and no thalidomide) were high (x=235.7 U/ml). In the second sample (no reaction, thalidomide for 1 month), levels were similar (x=214.2 U/ml) to those of the first sample (not statistically significant). However, when four months later, a third blood sample from the same patients was obtained (no reaction, thalidomide for 4 months), levels of TNF were significantly lower (x=92.48 U/ml), compared with those obtained in the first and second samples ($p \leq 0.01$), and similar to those of a control group consisting of LL patients with no clinical history of reactions. Immunopathological damage in type II reaction can be trigger by immune complexes, TNF may also be responsible for some part of this pathology, a notion which could be supported by the fact that thalidomide accelerates the recovery from reaction. However, according to our results no correlation was observed between clinical recovery and serum levels of TNF. Since we used a bioassay, the TNF we measured was active, and no pathological conditions were observed (second sample), this suggest that type II reaction is accompanied by the production of TNF inhibitors.

*Becarios de COFFA.

The complex symptoms observed in lepromatous leprosy patients with reactive episodes of the erytema nodosum leprosum (ENL) type, is associated with different serum components actively participating in the acute inflammatory reaction. Among them are the tumor necrosis factor (TNF)and the acute-phase protein C-reactive protein (CRP). TNF and CRP were found at significantly more elevated concentrations in the serum of patients with ENL, with a positive correlation of about 95% when compared with patients with nonreactive LL or TT forms of the didiase or with control individuals. Furthermore, in another series of experiments, CRP had a specific and significant suppressive action on Con A-induced lymphoproliferation in culture from patients and controls, the reduction being more marked(75%) in patients with ENL. By extrapolation from its known actions, production of TNF may have a number of potential consequences for the immunobiology of ENL. Thus, TNF may cause direct injury to compromissed cells, facilitating mononuclear cell activation and production of cytokines such as IL-1 and IL-6, and upregulating hepatocyte expression of CRP. Both CRP and TNF in high serum concentration have the ability to enhance the acute inflammatory process in ENL; favoring increased macrophage activation and phagocytosis; contributing to the elimination of damaged cells and bacilli; as well as in the reduction of T suppressor cells, with a consequent improvement in the immunologic response of ENL patients.

IM84

CYTOKINE mRNA EXPRESSION IN LEPROMATOUS LEPROSY AND THE REACTIONAL STATES IN LEPROSY.

Sampaio EP, Sarno EN, Marques MAM, Persechini PM, Felipe APL, Kaplan G. Leprosy Dept., Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; Laboratory of Electrophysiology & Immunology, Federal University of Rio de Janeiro; Laboratory of Cellular Physiology & Immunology, Rockefeller University, New York.

Lepromatous leprosy patients expression of monocyte, NK and T cell cytokine mRNA was evaluated. Ten active LL/BL patients were compared to 5 normal controls and 3 patients who had completed therapy and did not have active disease. In addition 6 lepromatous patients in erythema nodosum leprosum (ENL) and 4 in reversal reaction (RR) were evaluated. Cytokines were evaluated directly after isolation from the blood, and in the absence of in vitro stimulation. Cytokine mRNA was not expressed in the PBMC of normal healthy individuals. However, mRNA for leukocyte cytokines was expressed in LL/BL patients. The majority of patients' PBMC constitutively express mRNA for TNFα and IL-8. The IL-2 receptor chain p55 mRNA expressed on NK and T cells and perforin mRNA expressed in cytotoxic cells were also observed. None of these patients demonstrated IL-2, IFNγ or GM-CSF mRNA. Lepromatous patients who were analyzed after having completed the therapy were negative for cytokine messages. A clear difference between ENL and RR patients was observed. All reactional patients expressed in addition to TNFα, IL-8, p55 and perforin, IL-1, IL-6 and GM-CSF mRNA. IFNγ mRNA was expressed only by patients with RR, not with ENL. Expression of IFNγ mRNA was associated with the release of IFNγ following in vitro stimulation of the PBMCs with M. leprae (mean IFNγ levels ± SD = 342 ± 146 pg/ml). Lepromatous patients without RR were unresponsive to the antigen, and cytokines such as IL-2 and IFNγ were not detected in these cultures (mean IFNγ levels ± SD = 11 ± 1.4 pg/ml).

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IM85

Correlation between TNF production, increase of CRP level and suppression of T lymphocyte during ENL reaction

Norma T. Foss, and Célio L. Silva
School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.

IM86

ANALYSIS OF CYTOKINE PRODUCTION BY MYCOBACTERIUM REACTIVE T CELLS: FAILURE TO EXPLAIN *MYCOBACTERIUM LEPRAE* SPECIFIC NONRESPONSIVENESS OF PERIPHERAL BLOOD T CELLS FROM LEPROMATOUS LEPROSY PATIENTS

TUNA MUTIS*, E.M. KRAAKMAN*, Y.E. CORNELISSE*, J. B. A. G. HAANEN*, H.SPITS**, R. R. P. DE VRIES*, AND T.H.M. OTTENHOFF*
*Department of Immunohaematology and Blood Bank, Building 1, E3-Q, University Hospital Leiden, The Netherlands. **DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California

Recent analyses of antimycobacterial T cell clones indicate that mycobacteria preferentially induce helper T cells that produce high levels of IFN-γ and no or little IL-4 in *M.leprae* resistant TT patients and healthy subjects, whereas in one study *M.leprae* induced Ts clones from LL patients showed a reciprocal cytokine secretion profile and mediated their suppressive activity via the release of high levels of IL-4. We have evaluated these findings in peripheral blood T cells from a larger panel of TT and LL patients as well as healthy individuals. Mycobacterium reactive T cell lines generated from the PBMNC of these individuals were tested for cytokine secretion and proliferative capacity in response to *M.leprae*, *M.tuberculosis* and various individual mycobacterial antigens. The lepromatous pole of the leprosy spectrum was additionally investigated by analyzing the cytokine secretion profile of *M.leprae* induced (suppressor) T cell clones as well as primary ex-vivo PBMNC. Our results show that mycobacteria preferentially induce Th1 like cells across the whole leprosy spectrum. Although some T cells from lepromatous leprosy patients secrete IFN-γ as well as IL-4 and /or IL-10 neither IL-4 or IL-10 seem to play a pivotal role in the *M.leprae* specific T cell unresponsiveness observed in the peripheral blood of lepromatous leprosy patients.

IM87

RESPONSES OF CYTOKINE TREATED HUMAN MONOCYTE-DERIVED MACROPHAGES TO CHALLENGE WITH *MYCOBACTERIUM LEPRAE*

L.B. Adams and J.L. Krahenbuhl

Gillis W. Long Hansen's Disease Center, Baton Rouge, LA

An interesting dilemma in the study of anti-microbial effector mechanisms is the dichotomy between the ability of murine and human macrophages (MΦ) to inhibit, in vitro, an intracellular infection with mycobacteriae. While IFN-γ will prime murine MΦ to kill or restrict mycobacterial growth, primarily via an L-arginine-dependent pathway, this lymphokine will either have no effect on, or may actually augment, the growth of *M. tuberculosis* and *M. avium* in human MΦ. In the current study, human MΦ were evaluated for their ability to inhibit the metabolic activity of *M. leprae*. Peripheral blood was collected from nine different donors. The monocyte-derived MΦ were stimulated, for 24 hr, with IFN-γ alone, IFN-γ +

indomethacin, IFN-γ + LPS, IFN-γ + TNF-α, LPS alone or TNF-α alone. The MΦ were infected with 2 x 10^7 *M. leprae* per well for 4-5 hr and the culture fluid analyzed for TNF-α and PGE_2. The infected monolayers were reincubated, in the presence of the stimulants, for an additional 48 hr. The MΦ were lysed and the viability of the released *M. leprae* evaluated by measuring the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ in a modified Buddemeyer assay. As a control for antimicrobial activity, similarly treated MΦ were assessed for the ability to kill the intracellular protozoan, *Toxoplasma gondii*. Regardless of the stimulants employed, the human MΦ were incapable of consistently inhibiting the metabolism of *M. leprae*. In fact, in many cases the level of ^{14}C-palmitic acid oxidation was augmented in the bacilli recovered from IFN-γ-primed MΦ. In contrast, these same MΦ were quite capable of killing *T. gondii* if activated with IFN-γ + LPS. Interestingly, even though unable to inhibit *M. leprae*, infection of the MΦ with the bacilli induced TNF-α production in untreated MΦ, and these levels were greatly amplified if the MΦ were pretreated with IFN-γ alone, IFN-γ + indomethacin, or IFN-γ + TNF-α. Pretreatment with IFN-γ + LPS, TNF-α alone or LPS alone, however, reduced *M. leprae*-induced TNF-α production. In addition, pretreatment with IFN-γ + indomethacin or IFN-γ + LPS augmented *M. leprae*-induced PGE_2 production by MΦ. In summary, human monocyte-derived MΦ could not be activated in vitro with the stimulants employed to inhibit the metabolism of *M. leprae*. In contrast, great differences in TNF-α and PGE_2 synthesis were induced by challenge with the bacilli. This demonstrates that human MΦ are responsive to *M. leprae* and that these responses can be manipulated with cytokine treatment, even though they cannot, as yet, be activated to inhibit the bacilli in vitro.

IM88

POST-VACCINATION SENSITIZATION WITH ICRC VACCINE

M.D. Gupte, R.S. Vallishayee, and D.S. Aanatharaman

CJIL Field Unit (Indian Council of Medical Research), 271 Nehru Bazaar, Avadi, Madras 600 054, India.

ICRC is one of the anti-leprosy vaccines tested in the multi-arm Leprosy Vaccine Trial being conducted by our Unit in Tamil Nadu, South India. A study was conducted in 368 individuals, from one village and a nearby school in Chingleput district, to obtain information on local reaction after ICRC vaccination and post-vaccination sensitization.

Each individual received either ICRC vaccine, in a dose of 10^9 bacilli, or normal saline by random allocation. They were tested and read for Rees' soluble skin test antigen (MLSA) and Lepromin-A tests 12 weeks after vaccination. Character and size of local response, at the vaccination site, were recorded.

Healing of vaccination lesions was uneventful. No vaccine related complications were observed. The mean size of lesion was 10 mm.

The mean sizes of post-vaccination sensitization to Rees' MLSA, Lepromin-A (early) and Lepromin-A (late) in the vaccine group were significantly larger than that in the normal saline group clearly demonstrating the ability of the vaccine to induce sensitization. The sensitizing effect attributable to vaccine was of the order of 3.5 mm, 1.7 mm and 2.2 mm respectively.

Thus ICRC vaccine was acceptable to the population and showed apparent potential for immunoprophylactic efficacy.

IM89

PROTECTION OF MICE BY VACCINATION WITH PURIFIED AND RECOMBINANT MAJOR *M. leprae* PROTEINS AND POLYPEPTIDES

Robert H. Gelber, Shirley Hunter, V. J. Mehra, Nahid Mohagheghpour, Patricia Siu, Lydia P. Murray, Mabel Tsang, and Patrick J. Brennan.

Medical Research Institute of San Francisco, CA, Colorado State University, Fort Collins, CO, and Albert Einstein Medical School, Bronx, NY, USA.

Groups of 10 mice were vaccinated intradermally in the right flank with in Freund's incomplete adjuvant (FIA, negative control), 10^7 killed *M. leprae* (positive control), and 10 μg of a number of purified and recombinant *M. leprae* proteins plus a single synthesized polypeptide diluted in FIA; 1 month later these mice were challenged in the right hind footpad with 5,000 mouse-derived viable *M. leprae*, and protection was assessed both at the peak of *M. leprae* multiplication of the mice vaccinated with FIA alone and 3 months subsequently utilizing the rank sum test and the Wilcoxon distribution. In these studies killed *M. leprae* was found generally, but not always, to be protective. The following proteins afforded no consistently significant mouse protection: a recombinant 35 kD *M. leprae* protein, a purified 16-17 kD *"M. leprae* protein", a recombinant 18 kD *M. leprae* protein (Watson), a purified 22 kD *M. leprae* protein, and a synthesized 27 amino acid N-terminal peptide of the 10 kD *M. leprae* protein. On the other hand, vaccination with the following *M. leprae* proteins resulted in consistent and significant protection: a purified 10 kD protein, a recombinant 10 kD protein (Mehra), a recombinant 65 kD protein (van Embden), and a purified 28 kD protein. It was noteworthy in these studies that when mice were vaccinated with each of the proteins found to be protective there was induction of significant splenic T cell responses *in vitro* to sonicated *M. leprae* (stimulation indices ≥ 4). These studies suggest which *M. leprae* protein epitopes are important to protective immunity and hence those which would best be included in a future-generation leprosy vaccine.

IM90

BCG VACCINATION PROTECTS AGAINST LEPROSY IN VENEZUELA

Jacinto Convit, Peter G. Smith, Marian Ulrich, Celsa Sampson, Manuel Zúñiga and Victor García

Instituto de Biomedicina, Caracas, Venezuela

The protective effect of BCG vaccination in the control of leprosy has been widely variable in trials carried out in different areas of the world. In Venezuela, repeated BCG vaccination of contacts of leprosy patients has been one of the components of the leprosy control program. Using the case-control approach, we have carried out a retrospective study of the efficacy of repeated BCG vaccination in reducing the occurrence of leprosy. The clinical examination of 63,878 contacts during the intake phase of a large vaccine trial revealed 91 previously undetected cases of leprosy. There appeared to be an inverse relation between the number of BCG scars and the prevalence of leprosy (no scar, crude rate 2.87/1000; 1 to 5 or more scars, 1.31 to 0.45/1000). In addition, multi-bacillary LL and BL cases were found only in the group with no BCG scar (18 cases) or one scar (3 cases). There was no evidence that the protective effect of BCG was different among household or non-household contacts nor that it was age-related. Both specific responses to shared antigens and non-specific activation of immunological mechanisms by BCG may be involved in the apparent BCG-induced protection observed in this study.

IM91

ANNUAL IMMUNOTHERAPY IN TREATED LEPROMATOUS LEPROSY WITH 3 DIFFERENT BCG-BASED VACCINES - A 6 YEAR ASSESSMENT.

Michael F R Waters, Elaine Filley, and John L Stanford.

Hospital for Tropical Diseases, London, and School of Pathology, UCMMS, London.

44 treated LL (37) and BL (7) patients were investigated clinically, bacteriologically and histologically and allocated to one of three annual vaccination regimens, either BCG alone (9 patients) or BCG plus 6 x 10^8 dead M leprae (18 patients) or BCG plus 1 x 10^9 dead M vaccae (17 patients). Patients were assessed by means of the rate of fall in the BI (in those still skin smear positive), by annual lymphocyte transformation tests and by skin testing with 5 different mycobacterial antigens,

including standard lepromin and sonicated M leprae (Rees skin-test antigen).

Although it was expected that lepromin conversion would constitute a major assessment, some very longstanding patients were found to be weakly lepromin positive on admission to the trial (Waters and Ridley, 1990); therefore lepromin conversion has be be interpreted with caution.

Results obtained with the various parameters over a 3 to 6 year follow-up will be described, and the value of immunotherapy discussed.

IM92

FIELD TRIALS WITH AN ANTI-LEPROSY VACCINE MYCOBACTERIUMw.

R.Malia K.G.Sarathchandra, R.Mukherjee, G.Prakash and G.P. Talwar.

National Institute of Immunology ,New Delhi-110067

A double blind field trial was started with an anti-leprosy vaccine, Mycobacterium w, as an immunotherapeutic and immunoprophylactic agent in a highly endemic region of Kanpur Dehat District in the Northern Indian State of Uttar Pradesh.
The study population, estimated to be around 400,000, has been divided into 4 groups, group I consists of multibacillary (MB) patients receiving a placebo (1/8th the dose of tetanus toxoid) and their healthy household contacts the vaccine, group II consists of MB patients receiving the vaccine and their contacts placebo while in groups III & IV the MB patients and their contacts receive placebo and vaccine respectively.
A total of eight vaccine doses are given at 3 monthly intervals together with multi-drug therapy to the MB patients,while the immunoprophylactic schedule consists of 2 doses at 6 months interval. Analysis regarding the immunotherapeutic effect of the vaccine is being assessed on the basis of clinical improvement, bacterial index, lepromin status & histopathology all of which are regularly monitored.
Upto the 31st of December 1992, about 350,000 people have been surveyed and 4045 patients have been resistered of which 1050 are multibacillary cases. A total of 21732 eligible contacts have been screened for disease of which 18131 have received the initial dose of vaccine /placebo showing a complaince rate of 83.4%. Booster dose for immunoprophylaxis has been given to 11533 healthy contacts. The vaccine has been well tolerated and there has not been any incidence of systemic reactions to the vaccine /placebo in any of the study groups.

IM93

T CELL RECEPTOR USAGE IN BLOOD AND SKIN LESIONS IN LEPROSY.

Caroline Cross[1], Maggie Hackett[2], Sebastian Lucas[2], Rabia Hussain[1], and Hazel Dockrell[1].

[1] Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK, [2] Department of Histopathology, University College & Middlesex School of Medicine and [1] Microbiology Department, Age Khan University Medical Centre, P.O Box 3500, Stadium Road, Karachi 74800, Pakistan.

Previous studies have shown a 100 fold enrichment of M.leprae reactive T cells in skin lesions compared to the peripheral blood in tuberculoid leprosy. We have used a panel of anti T cell receptor antibodies to quantitate the usage of various Vα and Vß genes in leprosy. In peripheral blood, positive cells were quantitated by FACScan, while immunochemistry was used to stain lesional T cells. T cells expressing Vα2, Vß2, Vß3, Vß5.1, Vß5.2 + 5.3, Vß6, Vß8 and Vß12 were analysed. There was no evidence for deletion of any family of T cell receptor genes in the leprosy patients. Comparing blood and lesional T cells, leprosy skin lesions contained more Vß2 and Vß5.1 positive T cells then the blood, although there was no connection with the clinical status of the patient. These results show that Vß2 and Vß5.1 positive T cells are attracted to or preferentially expanded in, leprosy skin lesions - perhaps stimulated by a leprosy superantigen.

IM94

Human T cell clones recognise mycobacterial specific and shared epitopes on *Mycobacterium leprae* 70kD protein.

Adams E, Britton W J*, & Basten A.
Centenary Institute of Cancer Medicine & Cell Biology and *Department of Medicine, University of Sydney. 2006. Australia

The 70kD protein of *Mycobacterium leprae* (hsp70) stimulates both cellular and humoral immune responses in leprosy patients and their contacts. We have previously demonstrated that the C-terminal region of the protein including the *M.leprae* specific C-terminal 70 residues is the major target for antibody responses of leprosy patients. Using peripheral blood lymphocytes from known responders to the protein and short synthetic peptides of 12 amino acids, we were able to identify two T cell epitopes in sequences 380-396 and 418-433. In order to characterise the fine specificity of T cell epitopes we screened a panel of human T cell clones generated against *M.leprae* sonicate with *M.leprae* hsp70 and synthetic peptides Two epitopes contain sequences specific to *M.leprae* and *M.tuberculosis*. One (71-90, restricted by HLA-DR3 or 4) spans a region containing a deletion restricted to the mycobacterial sequences. The second mycobacterial specific epitope (241-260) was DR7 restricted. A further two epitopes towards the C-terminal end show partial homology with human hsp70. The minimal epitope length of these to be 414-427 and 471-486. The key residues which determine the antigenicity of the mycobacterial peptides are being determined with sequential replacement of amino acids. The *M.leprae* hsp70 T cell clones produced IFN-γ and TNF-β, but one also released IL-4 in comparable amounts. Functionally each clone was cytolytic against autologous EBV targets pulsed with *M.leprae* sonicate, hsp70 or the specific peptide. Analysis of other CD4+ *M.leprae* reactive T cell clones confirmed that some recognising the *M.leprae* 65 and 18 kD proteins were also cytolytic, but others with undefined specificity were not cytolytic. Therefore there is a spectrum in cytolytic activity in anti-*M.leprae* CD4+ T cell clones.

In summary, the defined T cell epitopes are present in regions distant from major human antibody determinants of *M.leprae* hsp70.

IM95

FACS ANALYSIS OF CD4+ T-CELL SUBSETS IN REACTING AND NON-REACTING LEPROSY SKIN LESIONS.

U. Utaipat[1], D. Scollard[2], K. Pattanapanyasat[3], L. Bhoopat[1], and C. Theetranont[1].

[1]RIHES, Chiang Mai University, Chiang Mai, Thailand; [2]GWL Hansen's Disease Center, Carville, LA., USA; [3]AFRIMS, Bangkok, Thailand.

Increases in the number and percentage of CD4+ T-cells have been noted in lesions of leprosy patients with Type I (RR) and in some Type II (ENL) reactions. We therefore asked whether the so-called helper-inducer (HI) or suppressor-inducer (SI) subsets of CD4+ cells were preferentially increased in skin lesions in these reactions.

Cells were obtained in suspension from blisters induced by gentle suction directly over reacting or non-reacting lesions, 48 hours after induction. Control cells were obtained simultaneously from peripheral blood and, when possible, from blisters induced on non-lesional skin. A total cell count was followed by simultaneous labelling with FITC- and PE-conjugated Mabs Leu3 and Leu8, anti-γ,δ and other markers. Flow cytometry was performed using a FACScan, and results analyzed using Lysis software.

Twenty samples from active lesions from 14 patients were examined. Pairing of BB and BT lesions with and without RR indicated a definite increase in the Leu3+, 8- (HI) subset in RR lesions (without corticosteroid treatment). Although no consistent effect was observed in ENL, all ENL lesions studied were in patients receiving prednisolone. Cell subsets in non-lesional skin were generally similar to those in clinical lesions, but all cutaneous subsets were significantly different from those in the peripheral blood.

These results indicate that precise quantitation of T-cell subsets by multiple labelling and flow cytometry is possible with cells obtained from lesions using suction-induced blisters, and suggest that the previously observed increase in CD4+ cells in Type I reactions is accompanied by an increase in the Leu3+, 8- subset.

IM96

MYCOBACTERIUM LEPRAE INFECTION TRIGGERS SYNTHESIS OF STRESS INDUCIBLE hsp 70 IN SCHWANN CELLS AND ANTI hsp 70 ANTIBODIES IN SERA.

YASMIN MISTRY,[1] DOUGLAS B. YOUNG,[2] AND RAMA MUKHERJEE[1*]

National Institute of Immunology, Shahid Jeet Singh Marg, New Delhi-110067, and Medical Research Council Tuberculosis and Related Infections Unit, Hammersmith Hospital, London W12 OHS, United Kingdom.

Murine and monkey Schwann cells were exposed to elevated temperatures and the induction of heat shock protein synthesis was monitored. Synthesis of the stress-inducible 70-kDa heat shock protein (hsp 70) was detected in both murine and primate Schwann cells by metabolic labelling and by immunoblotting with a specific monoclonal antibody. Infection with Mycobacterium leprae caused induction of hsp 70 synthesis in Schwann cells which was detected within 24 hours and persisted upto one week post-infection. hsp 70 was purified from the Schwannoma cells and antibody response to it in leprosy was studied using Western blot technique. These antibodies were directed to both the constitutive and inducible members of hsp 70 family, as ascertained after 1D and 2D PAGE and Western blot of the purified protein. The presence of high levels of antibodies to self proteins suggests their possible role in nerve damage observed in leprosy.

IM97

THALIDOMIDE DOES NOT AFFECT SELECTED IMMUNOMODULATING SURFACE RECEPTOR MOLECULES ON CELLS WITH IMMUNE POTENTIAL.

[1]E.J. Shannon, [2]McClean K., [2]Howe, R.C., [1]Hastings, R.C.

[1]Laboratory Research Branch, GWL HD Center at LSU, P.O. Box 27072, Baton Rouge, LA. and [2]Armauer Hansen Research Institute, Addis Ababa, Ethiopia.

The uncertainties concerning the pathogenesis of ENL are underscored by the unknown mechanism of thalidomide's therapeutic effect in this condition.

Previous reports have described an increase in cells expressing CD4 molecules and an increase in Ia (HLA-DR) molecules on keratinocytes in reactive skin lesions of patient experiencing ENL.

As these manifestations are associated with parameters of CMI and were confined to the acute phase of ENL, it has been suggested that ENL is the consequence of a transient activation of the CMI cascade. If ENL is the consequence of an activated CMI response, an alteration of the existing or the density of immunomodulating surface receptor molecules would be expected.

In the present study, thalidomide did not alter the expression of immunoregulatory molecules such as CD4, CD8 and CD5 on lymphocytes from four healthy male donors; nor did it influence the expression of the interferon-γ induced cell surface molecules HLA-DR, HLA-DP, CR1, CD 14, CR2, N-CAM-1, FcR-γ, and CR3 on THP-1 monocytes.

IM98

SOLUBLE M.LEPRAE ANTIGEN SKIN TESTING AND LEPROMIN POSITIVITY IN CHILDREN OF MOTHERS WITH LEPROSY AND HEALTHY CONTROLS STUDIED PROSPECTIVELY FROM BIRTH TO PUBERTY

ME Duncan, T Miko, R Howe, A Demissie, S Menzel, R Melsom, D Frommel

Department of Medical Microbiology, University of Edinburgh, UK; Armauer Hansen Research Institute, Addis Ababa, Ethiopia.

149 children (K) of mothers with leprosy (MB and PB) and healthy mothers (NL) living in the same environment (80MBK, 40PBK, 29NLK) were studied from birth up to 2 years of age (Phase 1). Reassessments were made at age 3-4 years (Phase 2): 89 children (49MBK, 25PBK, 15NLK); at age 7-8 years (Phase 3): 86 children (48MBK, 23PBK, 15NLK); and at puberty aged 12-15 years (phase 4): 99 study children with an additional cohort of 79 healthy children (55MBK, 31PBK, 95NLK). Skin testing with soluble *M.leprae* antigen in Phases 1,2 and 3 readings at 48-72 hours (± = negative): Phase 1:- MBK: 1/47 (2%), PBK: 3/21 (14%), NLK: 1/11 (9%); Phase 2:- MBK: 16/37 (43%), PBK: 11/23 (48%), NLK: 8/14 (57%); Phase 3:- MBK: 10/43 (23%), PBK 11/22 (50%), NLK: 4/14 (33%). Phase 4 Mitsuda lepromin testing Fernandez readings: MBK: 38/55 (69%), PBK: 26/32 (81%), NLK: 78/95 (82%); Mitsuda readings (± (2mm) = negative): MBK:46/52 (88%), PBK: 28/29 (97%), NLK: 78/86 (91%). Lepromin reaction (Mitsuda) ++/+++ was seen in 35/52 (63%) MBK, 21/29 (72%) PBK and 54/86 (63%) NLK. Punch biopsies of Mitsuda reactions from 41 children (MBK23, PBK11, NLK7) all showed BT type/pattern of Mitsuda reaction. Stimulation index (SI) in LTT against intact *M.leprae* was: MBK 23.0±25.3 (n=54), PBK 28.0±34.9 (n=25), NLK 7.2±10.7 (n=80). SI › 4.0 *vs.* intact *M.leprae* was: MBK 27.8±25.7, PBK 28.0±34.9, NKL 14.1±13.3; numbers of SI ›4: MBK 44/54 (81/5%), PBK 24/25 (96%), NLK 35/80 (43.3%). Rank correlation test (Kendall) SI *vs.M.leprae* and size of Mitsuda gave r= 0.19, ($p<0.001$).

IM99

IMMUNOBIOLOGY OF MELANOCYTES IN RELATION TO HYPOPIGMENTATION IN LEPROSY.

Caroline Le Poole*, Tuna Mutis**, René M.J.G.J.van den Wijngaard*, Wiete Westerhof*, Tom Ottenhof**, René R.P.de Vries** and Pranab K.Das*.

Departments of Dermatology and Pathology*, Academic Medical Center, Univ.Amsterdam, and Department of Immunohaematology**, Univ.Leiden, Leiden.

Hypopigmentation is a feature of all forms of leprosy and predilicts for neuronal involvement. However, it is strikingly more common in paucibacillary tuberculoid type of the disease. Although there is no strong correlation between cellular infiltrate and hypopigmentation in leprosy, it is assumed that destruction of melanocytes (MC: which originate from neural crest like Schwan cells) is a consequence of local T-cell mediated immune response. The potential importance of MC in the local immune response of human skin is being studied in our laboratory. Results of such studies have been recently reported by us (Arch.-Dermatol.Res.in press, 1993; Exptl.Cell Res.in press, 1993; Exptl.Dermatol. 1 p.95, 1992). Using immunohistological methods, we could demonstrate that MC can express MHC class I/II molecules, ICAM-1 and cytokines e.g. IL-1, IL-2 and IL-6. Interestingly, by using latex beads and applying confocal microscopy/electronmicroscopy and FACS analysis we could show that MC are capable of phagocytosis. These results are suggestive of an antigen processing and presenting ability for melanocytes. Indeed we could demonstrate that cultured human MC can process intact HSP-65 as well as whole M.leprae and can present processed antigenic peptides to CD4+ cytotoxic and proliferative Th1-like T-cell clones in a HLA-restricted manner. These T-cell clones were obtained from lesional skin biopsy material from tuberculoid leprosy patients. Such functions of MC can be involved in the pathogenesis of depigmentation in tuberculoid leprosy and can be extrapolated to the immunologic damage of these cells as "bystander targets" to some T-cell clones in the lesional infiltrates.

C.Le Poole and R.M.J.G.J.v.d.Wijngaard are recipients of subsidy from Stiefel.

IM100

NATURAL KILLERS IN LEPROMATOUS LEPROSY

L.Saroyants, A.Juscenko, L.Alekseyev
Leprosy Research Institute, Astrakhan, Russia

The present work is aimed at studying functional activity of natural killers (NK) in view of the distribution of HLA class I antigens in LL patients belonging to the Russian nationality. Cytotoxical activity of NK in leprosy patients and in healthy subjects was determined by their response to H3-uridine-labelled cells of myeloleucotic line K-562. HLA-typing by class I antigens was performed by standard microlymphocytotoxicity test.In active

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

MICROBIOLOGY

MI1

A MOLECULAR ANALYSIS OF MYCOBACTERIAL ANTIGENS WHICH STIMULATE γδ T CELLS

J.Sanchez-Garcia, P.J.Jenner and M.J.Colston

National Institute for Medical Research, London, UK.

Most T lymphocytes in human peripheral blood (hpb) express the αβ T cell receptor (TCR). T cells expressing the γδ TCR account for less than 10% of CD3+ hpb T cells. Several microorganisms, including mycobacteria, have been shown to produce a marked in vitro expansion of γδ T cells.

The nature of the γδ stimulatory molecule(s) is controversial. In this study we have used a variety of fractionation methods to identify these molecules, and to charactarise the γδ T cell response.

We find that virtually all individuals tested show a stimulation of Vγ9 γδ T cells when hpb are incubated in the presence of low molecular weight (<5kDa) fractions of mycobacteria, and that there are at least five low molecular weight molecules, all very close in molecular nature, involved in this stimulation.

The γδ T cell response to these molecules has been further charactarised in terms of the lymphokine profile, the involvement of the TCR, and the requirement for antigen processing.

MI2

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

Cristina Pessolani, Aimee Stanley, and Patrick J. Brennan

Department of Microbiology, Colorado State University
Fort Collins, Colorado 80523 U.S.A.

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

(Work supported by NIH, NIAID Contract NO1 AI-05074.)

MI3

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

T.P. Gillis, E.V. Tan, D.L. Williams, L.G. Villahermosa, M.V.F. Balagon and G.P. Walsh.

G.W. Long Hansen's Disease Center, Baton Rouge, LA, Leonard Wood Memorial, Cebu City, Philippines.

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

MI4

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

Adalberto R. Santos, José T. Góes, Philip Suffys and Wim Degrave

Leprosy unit , Oswaldo Cruz Foundation, Av. Brasil , 4365, Rio de Janeiro, RJ 21045-900 Brasil

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