

THIRTY-SEVENTH U.S.–JAPAN
TUBERCULOSIS-LEPROSY
RESEARCH CONFERENCE

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THIRTY-SEVENTH U.S.-JAPAN TUBERCULOSIS-LEPROSY RESEARCH CONFERENCE

The 37th Research Conference on Tuberculosis and Leprosy of the U.S.-Japan Cooperative Medical Sciences Program was held at Kyoto University Hall, Kyoto University, Kyoto, Japan from August 21–23, 2002. Dr. Masao Mitsuyama and his staff from Kyoto University, Graduate School of Medicine, Kyoto, Japan, organized the meeting. Panel members in attendance were Drs. Masao Mitsuyama

(Japan Panel Chairman), Kazuo Kobayashi, Kiyoshi Takatsu, Masamichi Goto, Hatsumi Taniguchi, Cliff Barry, III, Tom Gillis, David McMurray, and Philip Hopewell (US Panel Chairman). The abstracts of oral presentations in the area of leprosy research are presented below. Abstracts of presentations on tuberculosis research will appear in *Tubercle and Lung Disease*.

ABSTRACTS

Linda B. Adams, L., Ray, N. A., and Krahenbuhl, J. An *in vitro* model for the dynamic leprosy granuloma. Fate of *Mycobacterium leprae* from infected target macrophages after interaction with normal and activated effector macrophages.

The leprosy granuloma is a dynamic entity requiring a steady influx of cells for its maintenance. We have developed an *in vitro* model to study the turnover of macrophages in a leprosy lesion. Murine target macrophages, consisting of foot pad granuloma macrophages or macrophages infected *in vitro* with viable *M. leprae*, were challenged with normal or activated (ACT) effector macrophages. The bacilli were then recovered and assessed for viability. *M. leprae* recovered from target macrophages incubated alone or with normal effector macrophages possessed high metabolic activity. In contrast, bacilli recovered from target macrophages challenged with ACT macrophages exhibited a markedly decreased metabolic activity. An effector to target ratio of 10:1 was optimal for maximum *M. leprae* inhibition. In addition, the inhibitory effects exerted by the ACT effector macrophages were dependent on the production of reactive nitrogen products. Thus, the state of the macrophages infiltrating the granuloma may markedly affect the viability of *M. leprae* residing in macrophages in the leprosy lesion.

Yasuo Fukutomi, Y., Toratani, S., Kimura, H., and Matsuoka, M. Requirement of cyclooxygenases in induction of IL-10 production in *M. leprae*-stimulated macrophages.

Leprosy is a chronic infectious disease caused by an obligate intracellular pathogen, *Mycobacterium leprae*, and shows characteristic clinical manifestations. Cytokines produced by immune cells play an important role to form the clinical lesions. We have focused our research on the role of macrophages to study the mechanisms of cytokine production, and found that both PGE2 and cytokines, such as TNF- α , IL-10,

and IL-1 were induced in macrophages by phagocytosis of *M. leprae*. Newly synthesized cyclooxygenase (COX)-2 protein was also observed in macrophages. COX-1 protein levels were unchanged by *M. leprae* phagocytosis. Selective inhibition of COX-2 activity, but not COX-1 activity, was followed by significant decrease of PGE2 and IL-10. PGE2 receptor antagonist also suppressed IL-10, whereas TNF production was not affected. Enhanced accumulation of cAMP was observed in macrophages by *M. leprae* phagocytosis and PGE2 receptor antagonist markedly reduced cAMP accumulation. Collectively these results suggest that COX-2 expression in *M. leprae*-stimulated macrophages induce PGE2 followed by stimulation of its receptor and production of IL-10 through the stimulation of adenylate cyclase and accumulation of cAMP in the cells. In addition, steroids, which are used in leprosy to treat patients during reactions, suppressed TNF production, although enhanced IL-10 production was observed when lower doses were used. It is possible that steroids stimulate IL-10 production by stimulating adenylate cyclase. IL-10 is known as a cytokine synthesis inhibitory factor, therefore, it is conceivable that steroids suppress reactions (especially type 2) in leprosy by suppressing the production of inflammatory cytokines through an enhancing effect on IL-10 production.

Gillis, T. and Robbins, N. Enumeration of *M. leprae* using taqman-based PCR.

Quantification of *M. leprae* requires microscopic counting of bacilli relying on special staining and counting methods yielding results with limited specificity and sensitivity. We developed and tested a real time PCR assay for quantifying *M. leprae* DNA in tissue samples. Primers and probes were identified in the *M. leprae*-specific RLEP region of the genome and tested for sensitivity and specificity in the TaqMan format. The assay was specific for *M. leprae* and was able to detect 3 fg of purified *M. leprae* DNA or 300 bacteria in infected tissues.

The RLEP TaqMan PCR was used to assess the results of a protection study and yielded comparable results to conventional direct counting of bacilli.

Kitajima, S., Goto, M., Nomoto, M., and Taki, C. Cured tuberculoid leprosy in patients live longer than lepromatous patients in Japanese sanatoria.

Leprosy patients lack specific cellular immunity against *Mycobacterium leprae*, but other immunological functions are thought to be preserved. However, in Hoshizuka-Keiaien located in south Kyushu between 1982–2000, we found that cured lepromatous leprosy patients, who completely lack *M. leprae* immunity, die about 5 years earlier than cured tuberculoid patients who partly lack *M. leprae* immunity. This trend was also observed in autopsy records of two other leprosy sanatoria in Japan. We do not know whether immunological mechanisms are related to this particular phenomenon.

Krutzik, S., Ochoa, M. T., Sieling, P., Ng, Y. W., Legaspi, A., Liu, P. T., Cole, S. T., Godowski, P., Maeda, Y., Sarno, E. N., Norgard, M. V., Brennan, P. J., Rea, T. H., and Modlin, R. L. Activation and regulation of toll-like receptors 2 and 1 in human leprosy.

The expression and activation of Toll-like receptors (TLRs) was investigated in leprosy, a spectral disease in which clinical manifestations correlate with the type of immune response to the pathogen, *Mycobacterium leprae*. *M. leprae* was found to activate through TLR2 homodimers and TLR2/TLR1 heterodimers, known to mediate the response to microbial lipoproteins. Scanning of the *M. leprae* genome revealed 31 putative lipoproteins. Synthetic lipopeptides representing the 19-kDa and 33-kDa lipoproteins were found to activate monocytes and dendritic cells in a TLR2 dependent manner. The ability of the 19-kDa lipopeptide to activate cells was enhanced by the type 1 cytokines IFN- γ , GM-CSF, IL-12 and IL-18 and inhibited by the type 2 cytokines IL-4 and IL-10. Furthermore, the type 1 cytokines IFN- γ and GM-CSF en-

hanced TLR1 expression in monocytes and dendritic cells, respectively, whereas the type 2 cytokine IL-4 downregulated TLR2 expression. In patients with leprosy, both TLR2 and TLR1 were more strongly expressed on monocytes and dendritic cells in the lesions from tuberculoid patients, characterized by localized infection and type 1 cytokines than lesions from lepromatous patients characterized by disseminated infection and type 2 cytokines. These data provide evidence that the regulated expression and activation of TLRs at the site of disease contributes to human host defense against microbial pathogens.

Makino, M. and Maeda, Y. Dendritic cell-mediated production of IL-12 and IFN- γ by *Mycobacterium leprae*-derived cell membrane

The antigenicity of *M. leprae*-derived cell membrane fraction was examined using human dendritic cells (DCs). Immature DCs internalized the cell membrane expressed Ags, which reacted to leprosy patient's sera, and up-regulated the expression of MHC class II and CD86 Ags. The Ag-pulsed DCs induced significantly higher proliferation of autologous CD4⁺ and CD8⁺ T cells and higher IFN- γ production by the T cells than those pulsed with equivalent doses of *M. leprae*-derived cytosol fraction or whole live *M. leprae*. The CD40 signaling on the DCs enhanced IFN- γ production, and induced perforin production by CD8⁺ T cells. T cells from tuberculoid leprosy patients produced marked IFN- γ by stimulation with membrane pulsed DCs. Furthermore, the cell membrane was more efficient in the IL-12 p70 production from DCs than the cytosol fraction. These results suggest that *M. leprae* cell membrane has antigenic molecules that might be useful as the vaccinating agents against leprosy.

Matsuoka, M., Kashiwabara, Y. Ozaki, M., and Maeda, S. Distribution of the drug resistant *Myobacterium leprae* in Japan and Southeast Asian countries.

Distribution of *Mycobacterium leprae* resistant to dapsone, rifampin and quinolones

was investigated by the detection of mutations conferring drug resistance. Sequences of the *folp1*, *rpoB* and *gyrA* gene were analyzed for the isolates from Japanese, Indonesian and Philippine leprosy patients. Isolates originated in Japanese relapsed cases harbored dapsone, rifampin and quinolone resistant bacilli with the ratio of 78%, 64% and 36% respectively. Two isolates were regarded as multidrug resistant to three drugs. The prevalence of the dapsone resistant bacilli obtained from newly registered cases in Southeast Asian countries ranged between 4.5% and 11%. The proportion of rifampin-resistant cases ranged between 0% and 20%. Bacilli with resistance to both dapsone and rifampin were detected in two new cases out of 28 Philippine samples and one new case out of 30 Indonesian materials. The results suggested an increase of multidrug-resistant *M. leprae* among intractable cases and also in new cases.

Ohyama, H., Yamada, H., Takeuchi, K., Matsuoka, T., and Matsushita, S. A study on the polymorphism of IL-12R gene in patients with leprosy.

The susceptibility to leprosy was partially explained by the IFN- γ productivity from activated T cells in the presence of IL-12 in our previous study. The objective of this study is to assess the effect of IL-12R polymorphism on the IFN- γ productivity from activated T cells. The SNPs on IL-12RB1 were determined to compare the allele frequencies between patients and healthy donors, using PCR-RFLP method. The polymorphism of IL-12RB2 was analyzed by using direct sequencing technique. The results are as follows: 1) The variants of SNPs on IL-12RB1, which were suggested to be associated with a susceptibility to *Mycobacterium* infection, were not frequently detected in the Japanese patients. However, the SNP in the intronic region upstream exon 10 was detected only in the patients. 2) Several coding SNPs on IL-12RB2 were detected in the subjects in this study. However, the effect of these SNPs on the IFN- γ productivity from activated T cells in the presence of IL-12 was not apparent.

Scollard, D. M., McCormick, G. T., and Gillis, T. P. Multiple Endothelial Membrane Proteins Bind *M. leprae*

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

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

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

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

Shannon, E. J., and Sandoval, F. Thalidomide's ability to enhance correlates of T-cell activation *in vitro* is dependent on the stimulus.

Thalidomide is a drug that is being used in the treatment of several diseases like erythema nodosum leprosum (ENL). Despite studies conducted for over a quarter of a century, thalidomide's mechanism of action in arresting ENL is still unknown.

Published reports on thalidomide's effect on lymphocytes, especially mitogen-induced synthesis of cytokines like IL-2 and

mitogen-induced proliferation are contradictory. Some of these directly contradictory observations may stem from the nature of the stimulating agent or the cells that were stimulated. The purpose of this study was to determine if thalidomide influenced correlates of T-cell activation when peripheral blood mononuclear cells (PBMC) were stimulated with mitogens and to partially characterize the immune-phenotype of the responding cell.

PBMC from healthy individuals were incubated in the presence or absence of thalidomide and then stimulated with Staphylococcal enterotoxin A (SEA), anti-CD3, Con-A, or PHA. After 18 hr the culture medium was sampled for IL-2. At the end of 72 hr the culture medium was sampled for IFN- γ , and the cells harvested to assess cellular incorporation of [H^3]-thymidine during the last 24 hr of the 72 hr incubation period. IL-2 and IFN- γ were assessed by antigen capture ELISA. The incorporation of [H^3]-thymidine was assessed by liquid scintillation.

Regardless of the mitogen used to stimulate the PBMC, the thalidomide-treated-group produced significantly more IL-2 as compared to the untreated group. The PBMC treated with thalidomide and stimulated with anti-CD3 or with Con-A produced more IFN- γ than the cells in the untreated group. In the presence of thalidomide, the PBMC stimulated with SEA or PHA were suppressed in their ability to incorporate [H^3]-thymidine; whereas, thalidomide enhanced the incorporation of [H^3]-thymidine when the PBMC were stimulated with anti-CD3.

To partially characterize the immune-phenotype of the responding mitogen stimulated cells in the cultures of PBMC, the PBMC were sorted into CD3+, CD4+ T-cells and CD3+, CD8+ T-cells. The PBMC were sorted into CD4+ or CD8+ cells by negative selection using micro beads conjugated with anti-human CD8 or anti-human CD4. The cells that eluded the magnetized column were stained by a three-color direct immunofluorescence stain and labeled as +CD4+ or +CD8+ cells. When SEA or anti-CD3 were used to stimulate thalidomide-treated PBMC or +CD4+ or +CD8+ cells, the PBMC responded best in the synthesis of IL-2 and incorporated more [H^3]-thymi-

dine than the +CD4+ which responded far better than the CD8+ cells.

Among mitogen stimulated PBMC, thalidomide acts synergistically with the mitogen to stimulate the production of IL-2. The particular T-cell subset targeted by thalidomide is the CD4+ lymphocyte. The type of response depends on the nature of the stimulus.

John S. Spencer, Hee Jin Kim, M., and Marques, M. Antigenic specificity of the *Mycobacterium leprae* homologue of CFP-10.

The recent completion of the sequencing of the genomes of *M. tuberculosis* and *M. leprae* provides the opportunity to identify leprosy-specific antigens. An analogous approach applied to *M. bovis* BCG allowed the identification of deleted genes and the development of antigens that can distinguish between *M. tuberculosis* infection and vaccination with BCG. Among those antigens which have shown promise are two low-molecular weight *M. tuberculosis* culture filtrate proteins, ESAT-6 (esat-6) and CFP-10 (lhp), both encoded by genes in the RD1 region, a genetic segment that has been deleted from all strains of BCG. The operon containing these two genes, and a cluster of surrounding genes which are postulated to be involved in the processing and transport of these signal peptide-lacking proteins, has been duplicated five times within the *M. tuberculosis* genome. These duplications are conserved in the genomes of other mycobacteria, including virulent *M. bovis* strains, *M. avium*, *M. leprae*, and the avirulent *M. smegmatis*. In *M. leprae*, which has experienced a massive downsizing of its genome through deletion and decay, only three members of the ESAT-6 family (ML0049, ML2531, and ML0363), and only two members of the CFP-10 family (ML0050 and ML2532) remain as functional genes; the other members have either been deleted or have been converted to non-functional pseudogenes. Because the *M. leprae* ESAT-6 (ML0049) and CFP-10 (ML0050) have only 36% and 40% amino acid identity, respectively, to their homologs in *M. tuberculosis* (Rv3875 and Rv3874), we decided to analyze the im-

munologic cross-reactivity of these proteins in mice by characterizing the B and T cell epitopes recognized. We had previously reported this analysis of the ESAT-6 homologs, and found that the dominant B and T cell epitopes recognized in H-2^d haplotype (BALB/c) strain mice for the *M. tuberculosis* and *M. leprae* proteins were in different regions. Using algorithms that predicted B cell epitopes based on hydrophilicity and surface exposure and other algorithms that predicted T cell epitopes based on their amphipathic nature and structural motifs, there was good correlation between the predicted dominant epitopes and those that we observed. In addition, polyclonal antisera against the two forms of ESAT-6 did not cross-react at the level of the whole protein or with any of the heterologous peptides. We have since performed a similar immunological analysis of cross-reactivity with the CFP-10 homologs, and found that polyclonal antiserum raised against ML0050 did not cross-react with the *M. tuberculosis* homolog, and vice versa. At the cellular level, only one T cell hybridoma raised against either *M. leprae* or *M. tuberculosis* CFP-10 exhibited a cross-reactive response against the N-terminal heterologous CFP-10 15-mer peptide, a region that has the highest level of identity (67%) between the two proteins. Nevertheless, the remaining peptide epitopes recognized by T cell hybridomas specific for each protein did not cross-react with heterologous peptides, suggesting the possibility of developing peptides that can be used to differentiate infection caused by these two related microorganisms. Although there have been recent reports that *M. leprae* ESAT-6 is more widely recognized as cross-reactive in *M. tuberculosis* infected patients and even in healthy endemic control individuals with no known prior exposure to leprosy or tuberculosis disease, there remains the possibility that synthetic ESAT-6 peptides may be able to discriminate between these two diseases. We are currently in the process of analyzing antibody and T cell immune responses against members of the ESAT-6 and CFP-10 family of proteins and other unique proteins discovered in the analysis of the *M. leprae* genome.

Truman, R. and Gillis, T. Analysis of TTC VNTR of *M. leprae* as a tool for distinguishing variants of laboratory strains.

Genotyping has practical application in outbreak investigations and variant classification of microorganisms. Until the sequence of the genome was completed, however, remarkably little variability had been noted among *M. leprae*. Recently, a few loci for allelic diversity have been identified, including small insertion sequences and tandem repeating elements. At least one of these, the TTC triplet occurring in a non-coding region of the putative sugar transporter pseudogene of *M. leprae*, also has been found to occur at variable copy numbers in different clinical isolates. The utility of a single VNTR for epidemiological genotyping is quite low and will require further examination to establish its frequency and range in populations. To better understand the suitability of this and other VNTR markers in differentiating variant strains of *M. leprae*, we examined a battery of 12 *M. leprae* isolates derived from leprosy patients in different regions of the United States, Brazil, Mexico, and the Philippines, as well as from wild nine-banded armadillos and the Sooty Mangaby Monkey. The stability of the TTC VNTR was compared among the individual isolates as well as to those from bacilli obtained on subsequent passage in nude mice and armadillos. Copy numbers for the TTC repeat ranged from 10–15 among the isolates tested. No regional clustering was noted and all of the U.S. isolates showed a wide number of different repeats. Strains derived from different wild animals also were not identical. Greatest variability was seen over long term passage with the Thai-53 strain which has been maintained continuously in nude mice for many years. Thai-53 TTC copy number varied markedly over 8 passage intervals. However, the TTC VNTR genotype of most individual strains remained relatively constant for isolates passaged outside man for fewer than 12 generations. In addition, the TTC VNTR genotype of these strains tended to remain constant when passaged through an alternate animal host, experimentally infected nine-banded armadillos. These data demonstrate and expand the utility of our nude

mouse and armadillo colonies, affording an opportunity to examine the stability of certain genotypes through potentially hundreds of passages with both nude mice and armadillos. Even though the TTC VNTR occurs in a non-coding region of the *M. leprae* chromosome, its apparent stability among most short term passaged isolates suggests that it has utility for differentiating laboratory strains of *M. leprae*, and may be useful in assessing drift amongst isolates carried in long term culture. Future studies with these and other allelic markers will likely evolve suitable variant typing schemes for *M. leprae* that will aid in assessing specimens harboring mixed strains, determining transmission patterns among endemic populations and provide a means to standardize reference strains of this organism.

Williams, D., Truman, R., Bishai, W., and Gillis, T. Defining a Partial Transcriptome of *Mycobacterium leprae*

The genome of *Mycobacterium leprae* has been completely sequenced and annotated. Approximately, 1604 open reading frames, encoding potentially functional proteins, and 1104 inactivated genes (pseudogenes) have been identified. However, the minimum gene set required for intracellular growth and survival (transcriptome) has not yet been defined. To address this, we have recently developed a protocol for purification of large quantities of total RNA from highly viable, nude mouse-derived *M. leprae* using reciprocal shaking in TRIzol® and extraction and precipitation of RNA using standard methodology. RNA was obtained from two geographically distinct strains of *M. leprae* (T-53 and 4089) and as a prelude to evaluating global gene expression using an *M. leprae* cDNA array, the expression of approximately 5% of the potential transcriptome was analyzed using RT-PCR. To accomplish this, cDNA was produced using 1 µg of RNA from each strain, random hexamers (Clontech) and reverse-transcription (RT). Gene transcripts of interest were amplified from cDNA using PCR with primer sets flanking gene fragments of sev-

eral potentially functional families of *M. leprae*. PCRs were initially characterized using DNA from *M. leprae* T-53. Then the cDNA from both strains was amplified and DNA sequence of the resultant PCR fragments was obtained. Results of RT-PCR experiments demonstrated that genes encoding a variety of enzymes were transcribed in both strains. These include enzymes involved with, folic acid synthesis, iron utilization, cofactor biosynthesis, gluconeogenesis, degradation of phosphorous compounds, degradation of DNA, detoxification, synthesis of mycolic acids, modification and maturation of ribosomes, synthesis of RNA, glycolysis, glyoxylate bypass, and genes containing secretion motifs or encoding stress proteins, and several genes with unknown functions. These data have provided us with the first insight into the transcriptome of *M. leprae* and further demonstrated the homogeneity of this species. However, not all genes were expressed in both of these strains. Comparative analysis of gene expression these strains will be discussed in greater detail. In addition to RT-PCR analysis, the expression of a larger number of genes is presently being analyzed by DNA microarray analysis using the Operon Technologies set of 4100 seventy-mers printed in duplicate on glass slides and representing the open reading frames of the *M. tuberculosis* H37Rv genome. To perform this analysis, cDNA is being produced from 5 µg total RNA from T-53 and 4089 and labeled with either Cy3 or Cy5 fluorochromes using RT. The labeled cDNAs will be hybridized to the slides, the slides will be washed and scanned using an Axon Scanner. The intensities of the two dyes at each spot will be quantified using the GenePix software package. It is anticipated that this analysis will help to identify a larger set of functional genes in *M. leprae* which will potentially help us to understand the minimal requirements for growth and replication of this pathogen. This information may lead to the identification of new drug targets, skin test antigens and to identify factors that allow this pathogen to evade the immune system and destroy peripheral nerves.