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TWENTY-SEVENTH JOINT LEPROSY RESEARCH CONFERENCE

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Hotel Shinjiko
Matsue, Shimane Prefecture, Japan
4–7 August 1992

OPENING REMARKS

Good morning, Ladies and Gentlemen:

It is a great pleasure for me to declare the opening of the Twenty-Seventh U.S.–Japan Joint Research Conference on Leprosy and Tuberculosis. I wish to express my gratitude to my colleagues who came from the United States, and also from various parts of Japan, for participation in this Conference. I would like to express my thanks to Dr. Brennan, Chairman of the U.S. Leprosy Panel; Dr. Ellner, Chairman of the U.S. Tuberculosis Panel; Dr. Azuma, Chairman of the Japan Tuberculosis Panel; and the members of the U.S. and Japan Leprosy and Tuberculosis Panels who helped and supported me to arrange this joint conference.

In these several years, research in the field of mycobacteria is rapidly progressing. This is due to the introduction of modern molecular biology and genetic engineering to the bacteriology and immunology of mycobacteria. There are many excellent papers on these matters which will be presented at this Conference. I believe these studies on mycobacteria will offer us the knowledge for the development of vaccines for leprosy and tuberculosis. Some investigators will speak of recent advances in diagnosis, epidemiology, pathology and chemotherapy of leprosy and tuberculosis—important themes for improvement of the clinical control of these diseases.

Matsue is one of the most beautiful cities in Japan, facing Shinji-ko Lake. From Shinji-ko Lake, we receive abundant fish and shellfish, some of which will be served at the garden party at Hotel Shinjiko on 5 August. The Matsue area is called the Izumo district; "Izumo" means the appearance of many huge and small clouds. In the west of Matsue, there is the Izumo-Taisha Shrine, one of the oldest shrines in Japan. It is traditionally said that all the gods in Japan will get together at the Izumo-Taisha Shrine every November. Therefore, in this district November is called "Kami-Ari-Zuki" (the month when the gods are staying here); in other districts of Japan, November is called "Kanna-Zuki" (the month when the gods are absent). It is also said that the medical sciences originated from Izumo, since a Japanese myth tells of Okuninushi-No-Mikoto, one of the gods who cured an injured white rabbit.

Izumo City, located about 30 km west of Matsue and 650 km west of Tokyo, is situated on the coast of the Sea of Japan. The city is one of the homes of Japanese myths

Participants of the U.S.–Japan Joint Research Conference on Leprosy and Tuberculosis, Hotel Shinjiko, Matsue, Japan, 5 August 1992.

and legends, and the whole area is rich in beautiful scenery. Shimane Medical University is found in the southern part of Izumo City and has a quiet atmosphere ideal for academic pursuits.

I sincerely hope that this joint Conference will be successful, and that you will enjoy your stay in Matsue and Izumo. Thank you.

Hajime Saito, *Chairman*
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TWENTY-SEVENTH JOINT LEPROSY RESEARCH CONFERENCE
ABSTRACTS*

Franzblau, S. G., Gillis, T. P., Williams, D. L. and Chan, G. P. Leprosy clinical trials of sparfloxacin and clarithromycin.

Patients receiving sparfloxacin showed improvement in the appearance of their lesions after only 2 weeks of treatment. At 4 weeks of treatment, clinical improvement was approximately 50%, increasing to 70%–95% by 8–12 weeks of treatment. There was a concurrent decline in phenolic glycolipid-I (PGL-I) antigen during treatment. Eight of nine patients had a 3+ titer before treatment and half of these were rated +/− at the end of 8 weeks' treatment. No patient had a titer > 1+ after 12 weeks of treatment. Bacilli taken from the 2-week post-treatment biopsies showed a reduction in radiorespirometric activity of 75%–95% compared to pretreatment activity. After 4 weeks of treatment there was no detectable radiorespirometric activity, suggesting rapid bactericidal activity of sparfloxacin.

Patients receiving clarithromycin had a similar clinical response as those receiving sparfloxacin. Although the initial response was minimal (probably due to a 6-day interval of no treatment), by 4 weeks considerable resolution of the lesions was apparent with further improvement throughout the 12 weeks of observation. PGL-I antigen titers of all 9 patients showed a time-dependent decrease. Radiorespirometric activity of bacilli harvested 1 week following a single day of 1500 mg BID clarithromycin showed a reduction of 52%–79%. Bacilli recovered after an additional 2 weeks of 1000 mg daily clarithromycin failed to demonstrate any activity.

Both sparfloxacin and clarithromycin were well tolerated, with only minor side effects noted during the first day (clarithromycin) or first week (sparfloxacin). A mild reversal reaction was noted in one patient in the sparfloxacin group.

Data currently available suggest that clarithromycin is rapidly bactericidal for *Mycobacterium leprae* in man. In addition, the results obtained with daily 200 mg sparfloxacin appear to be comparable with those found previously in trials of 400 mg ofloxacin. This suggests that sparfloxacin may eventually be the quinolone of choice in leprosy due to, if nothing else, cost considerations. Subsequent harvesting of mouse foot pads will allow correlations to be made between the newer, rapid methodologies and the traditional mouse foot pad assay. [GWL Hansen's Disease Center Laboratory Research Branch at Baton Rouge, Louisiana, U.S.A.; Research Institute for Tropical Medicine, Metro Manila, The Philippines]

Gelber, R. H., Azouaou, N., Abel, K., Brennan, P. J., Rivoire, B., Murray, L. P., Siu, P., Tsang, M., Sasaki, D. T., Locksley, R. M., Mehra, V. J. and Mohagheghpour, N. Protection of mice from *M. leprae* infection by T cells and protein vaccines.

Unlike human lepromatous leprosy, the murine infection with *Mycobacterium leprae* is localized and self-limiting. We used SCID mice to examine the ability of adaptively transferred T cells to confer protection against *M. leprae* infection. SCID mice have an autosomal recessive mutation that prevents the formation of functional B and T cells. We found that SCID mice infected with *M. leprae* developed a significantly more profound foot pad infection that BALB/c mice ($p < 0.05$). A T-cell line producing IFNγ upon stimulation by leprosy bacilli was generated from splenic lymphocytes of an *M. leprae*-immunized BALB/c mouse. Transfer of these *M. leprae*-immune T cells resulted in a significant reduction in the number of *M. leprae* found in the footpad infection of SCID mice ($p < 0.03$). The yield of *M. leprae* in the foot pads of SCID mice reconstituted with the *M. leprae*-immune cells was also significantly lower than that found in normal BALB/c mice ($p \leq 0.05$), and at levels we found previously only

* Many of these abstracts were not provided by the authors but are a synopsis prepared by the Chairman. The abstracts are printed here in alphabetical order by last name of first author.

in BALB/c mice that had been effectively immunized. It is further noteworthy that SCID mice treated with *M. leprae*-immune T cells developed fewer *M. leprae* than those mice reconstituted with *M. leprae* murine nonimmune T cells ($p < 0.03$). Although the numbers of *M. leprae* found in mice receiving *M. leprae* nonimmune T cells were less than in SCID mice that received no T cells, this difference did not reach the level of statistical significance. Flow cytometric analysis of spleen and peripheral blood cells confirmed the reconstitution with T cells. *In vitro* lymphokine production and the proliferation of spleen cells from the reconstituted mice established that the donor cells had maintained their functional activity for the duration of the study (275 days). Thus, adoptively transferred T cells home effectively and control *M. leprae* infection in SCID mice.

Previously we found that intradermal vaccination with killed *M. leprae* in various cell-wall preparations of *M. leprae* prevented the growth of *M. leprae* when foot pads were infected 1 month subsequently. More recently, we have found that a detergent extract of one of these cell-wall preparations, "soluble proteins," consistently provided long-term protection with viable *M. leprae* challenge at 6, 9, and 12 months after vaccination, time intervals when killed *M. leprae* and more purified cell walls are no longer protective. These protection studies provide the first *in vivo* data that mycobacterial vaccines devoid of lipids and carbohydrates, which have been found by others to be immunosuppressant *in vitro*, are superior vaccines to whole mycobacteria and, by extension to man, that vaccine efficacy of BCG for mycobacterial diseases can be surpassed by mycobacterial proteins alone.

Because "soluble proteins" do not migrate on gels, 10 separate density-gradient subfractions were prepared from superose 12 columns in an effort to determine which proteins therein are the protective epitopes. Vaccination of mice with approximately 1 μg of several, but not all, of these fractions afforded protection against a subsequent viable *M. leprae* challenge. These results, together with end-group analysis, suggest that the protective epitopes of the "soluble proteins" are contained in the 10-kDa, 1–3-kDa, and high molecular weight proteins.

In order to define the activity of specific proteins involved in vaccine protection, groups of mice were vaccinated intradermally with 10 μg of a variety of chemically purified and bioengineered major *M. leprae* somatic proteins. The following proteins offered no consistently significant mouse protection: recombinant 35-kDa *M. leprae* protein, chemically purified 16–17-kDa "*M. leprae* protein," the *M. leprae* 18-kDa protein of Watson, the purified 22-kDa *M. leprae* protein, and the synthesized 27 amino-acid N-terminal peptide of the 10-kDa *M. leprae* protein. On the other hand, the following proteins afforded significant protection: the purified 10-kDa *M. leprae* protein, the recombinant 10-kDa *M. leprae* protein (Mehra), the recombinant 65-kDa *M. leprae* protein (Van Embden), and the purified 28-kDa *M. leprae* protein. Vaccination with newer preparations derived from 10^7 *M. leprae* of the three major *M. leprae* subcellular protein fractions, cell wall, cell membrane, and cytoplasmic, all afforded significant mouse protection. In general, there was a correlation between vaccine protection and the induction of splenic lymphocyte recognition of sonicated whole *M. leprae* (stimulation index ≥ 3), but this was not always the case.

These two separate studies provide the basis for unique experimental opportunities to address several important issues: a) the relative role in the protective response provided by both murine and human (across the leprosy spectrum) T cells and subsets; b) the ability that various *M. leprae* subunit, protein, and peptide vaccines provide to induce T-cell lines, clones, and cytokine production profiles that result in molecularly specific and salutary protective responses. Our approach to these future studies is outlined. [Medical Research Institute, San Francisco, California; GWL Hansen's Disease Center, Carville, Louisiana; Systemix, Palo Alto, California; Colorado State University, Fort Collins, Colorado; Albert Einstein School of Medicine, Bronx, New York; University of California, San Francisco, California, U.S.A.]

Gillis, T. P., Williams, D. L. and Chan, G. Comparison of PCR with different clinical forms of leprosy and with multibacillary patients on MDT.

Early diagnosis and treatment of leprosy constitute the major public health strategy for controlling leprosy. Improved methods for detecting *Mycobacterium leprae* in known and suspected cases of leprosy could identify stages of infection preceding clinical disease and possibly elucidate preclinical manifestations of infection which lead to overt disease or self-healing. We describe here the application of a highly specific and sensitive polymerase chain reaction (PCR) test for detecting *M. leprae* DNA in skin biopsies from patients exhibiting various clinical forms of the disease and from patients with multibacillary disease receiving multidrug therapy (MDT) for leprosy.

Newly diagnosed multibacillary (MB) and paucibacillary (PB) leprosy patients were recruited for this study by Dr. Gertrude Chan in Metro Manila, The Philippines. Two, 4-mm punch biopsies were taken at the border of an active lesion from each patient. One biopsy was fixed in neutral formalin for histopathological evaluation and confirmation of disease status. The other biopsy was lyophilized and shipped to the GWL Hansen's Disease Center for PCR testing. All samples were coded by Dr. Chan prior to shipping, and the code was broken after PCR results and histopathologic data were obtained.

Initial screening of samples by PCR was done on a 10-μl aliquot taken from the biopsy homogenate. Samples were frozen and thawed three times and then subjected to 45 cycles of amplification by PCR using *M. leprae*-specific primers as described earlier (D. L. Williams, *et al.*: J. Infect. Dis. 162: 193–200, 1990). Agarose gel electrophoresis was used to analyze the PCR product, and the samples were scored positive or negative based on the predicted 360 bp product. Samples which tested negative by gel electrophoresis, following the freeze/thaw method, were subjected to a more intense nucleic acid extraction following enzymatic digestion of the original biopsy homogenate. Resultant PCR products from these samples were analyzed by slot-blot hybridization using an *M. leprae*-specific, P-32-labeled DNA probe as described earlier (D. L. Williams, *et al.* Mol. Cell. Probes, 1992, in press).

A sample preparation of the longitudinal study of MB patients receiving MDT consisted of analyzing a specified number of *M. leprae* using the freeze/thaw method as described above with the following modifications. One-hundred-thousand *M. leprae* in a specific volume derived from each homogenate were recovered by centrifugation and washed two times prior to resuspension in 100 μl of buffer. This suspension was frozen and thawed three times and 10 μl were removed for PCR. PCR products were analyzed by slot-blot hybridization using a P-32-labeled, 212 bp DNA probe (*M. leprae*-specific). After hybridization and washing, a specified area (equivalent to the slot created on the membrane by the slot-blot manifold) of the blot was removed and radioactivity was quantified by scintillation counting.

The general histologic features of the MB cases followed characteristic patterns, with the LL cases showing predominantly macrophage granulomas packed with bacilli and occasionally lymphocytes scattered throughout the lesions. The bacterial indices (BI) ranged from 3+ to 6+ and the morphologic indices (MI) were between 2% and 4%. Since the PCR test results for these samples were reported as positive, weakly positive, or negative, and because all MB patients gave strongly positive PCR results, correlation with the above histopathologic parameters was impossible. The percent of biopsy involved by the granuloma was also scored but, again, no direct correlation between this parameter and PCR reactivity was observed.

Biopsies from BT patients showed characteristic histology with epithelioid cell granuloma, lymphocytes, and occasional giant cells in skin lesions. The PCR results in BT cases were generally weaker when no acid-fast bacilli were seen in the lesions. For example, BT patients with BIs of 1+ to 4+ all tested positive by PCR, while 55% (6/11) of BT patients with BIs = 0 tested negative by PCR. Since as many BI-negative BT cases tested positive by PCR, these results suggest that the ability to detect a positive result by PCR in PB leprosy may be a reflection of the age and/or "immunologic status" of the granuloma. In other words, disease which is improving (destruction of bacteria) may be recognized as PCR-negative, while stationary or progressive disease may test as PCR-positive.

The sensitivity (the incidence of true positive results obtained when a test is used for patients known to have leprosy) of PCR for detecting *M. leprae* in leprosy patients, regardless of disease status, was 92%. Sensitivity was even higher in MB disease and TT disease. However, in the case of TT leprosy not enough patients were sampled to make the statistic meaningful. A lesser certainty of positivity was seen in the PB group, which gave a sensitivity of 80%. The decrease in sensitivity in PB leprosy was the result of 6 BT cases testing negatively (false-negatives).

Specificity (a measure of the ability of a test to give a negative result in the absence of leprosy) of the PCR test was 100%. This indicates that the test is based on a highly stringent reaction (i.e., amplification of a specific DNA segment directed by specific primers) and that false-positives are not likely to interfere in the interpretation of the test results.

The efficiency (the ability of a test to give a positive result on positives and a negative result on negatives) was calculated for PCR and shown to be 93%. Finally, the predictive value for a positive test result was 100%, while the predictive value for a negative test was relatively low at 60%, reflecting the relatively high rate of false-negatives in the BT group of patients.

The most important inferences obtained from this analysis of test accuracy for the *M. leprae* PCR was: a) the high degree of sensitivity of PCR for detecting the leprosy bacillus in all forms of leprosy studied (absent indeterminate disease), particularly MB disease; and b) the test's high degree of specificity, demonstrating no false-positives in testing uninfected skin biopsies from The Philippines. The "normal" skin biopsies, which tested uniformly negative by PCR, were screened for PCR inhibitors and found to lack any such activity. More importantly, these biopsies were taken from patients suspected of having either indeterminate or TT disease and, in each case, a histopathologic evaluation did not confirm the clinical diagnosis.

Based on preliminary findings (D. L. Williams, *et al.* Mol. Cell. Probes, 1992, in press), we set up a prospective, longitudinal study on MB patients over the first few months of treatment with MDT and gathered quantitative PCR data on *M. leprae* isolated from serial biopsies.

From the data obtained we conclude that somewhere between 1 and 3 months, following the initiation of MDT, leprosy bacilli in the skin reach a point of disintegration, such that their DNA no longer support primer-directed amplification of the 18-kDa gene. Since the BIs of these patients' biopsies are not significantly reduced during the early months of treatment, the observed change in PCR during the early phase of treatment could be exploited as a means of monitoring short-term drug trials of new antibiotics or combinations of the same. The apparent delay in detecting the effect of drugs on *M. leprae* by PCR as compared with other methods, such as metabolic activity or viability of *M. leprae* in the mouse foot pad, must reflect host clearance mechanisms, including enzymatic activity and cellular turnover in the lepromatous granuloma. Even though PCR detects the effects of MDT somewhat later than other methods, the number of bacteria required for PCR testing is minimal and, therefore, may be appropriate for some types of patient monitoring. In addition, *M. leprae* found in other parts of the body (e.g., nasal mucosa) may be a better site for the analysis of immediate effects of drugs, and PCR may be the only test capable of providing such analysis based on its exquisite sensitivity and specificity. [GLW Hansen's Disease Center, Laboratory Research Branch at Baton Rouge, Louisiana, U.S.A.; Research Institute for Tropical Medicine, Albang, Muntinlupa, Metro Manila, The Philippines]

Goto, M., Izumi, S., Nobuhara, Y. and Sato, E. Central nervous system lesions in leprosy—immunohistochemical study using anti-PGL-I antibody.

Despite the advance of chemotherapy, deformities evoked by nerve damage are still a great problem in the management of leprosy. In the 1990 U.S.-Japan meeting, we demonstrated a sensitive immunohistochemical method to identify phenolic glycolipid-I (PGL-I) in Formalin-fixed skin tissue using an anti-PGL monoclonal antibody. Using this method, we have exam-

ined the peripheral nerves of the lower extremities, spinal cord and brain stem of clinically cured (slit-skin smear negative for > 10 years), leprosy autopsy cases [lepromatous (L; N = 6), tuberculoid (T; N = 6)] in which ordinary Fite's acid-fast staining did not reveal *Mycobacterium leprae*. Positive staining was observed as follows: a) peripheral nerves: L = 6/6, T = 1/6. b) dorsal root ganglia and posterior spinal roots: L = 6/6, T = 2/6. c) anterior roots: L = 0/6, T = 0/6. d) spinal cord: L = 6/6, T = 2/6, observed in posterior horn cytoplasm and anterior horn neurons. e) medulla oblongata: L = 6/6, T = 2/6, observed mainly in ambiguous, facial, hypoglossal, cuneate and gracile nuclei. These findings indicate that *M. leprae*-specific antigen remains in the central sensory and motor nerves as well as in peripheral sensory nerves long after the clinical cure, especially in lepromatous patients, which suggests the role of motor neurons in the pathogenesis of quiet nerve paralysis. [National Leprosarium Hoshizuka-keiaien, Kagoshima; Department of Pathology, Kagoshima University School of Medicine, Kagoshima; National Institute for Leprosy Research, Tokyo, Japan]

Izumi, S., Maeda, Y., van Beers, S., Mdjid, B. and Kawatsu, K. Distribution of anti-PGL-I and anti-LAM-B antibodies among inhabitants of leprosy-endemic villages in South Sulawesi, Indonesia.

Infectivity and the mode of transmission of the leprosy bacillus are among the most important subjects to be studied in the context of the epidemiology of leprosy and control of the disease. Recent development of serological tools based on the *Mycobacterium leprae*-specific phenolic glycolipid-I (PGL-I), such as the gelatin particle agglutination (MLPA) or indirect ELISA, made it possible to detect at least some individuals infected with *M. leprae*. In July 1991, we conducted a seroepidemiological survey of inhabitants in South Sulawesi, Indonesia, where leprosy is highly endemic. It was found that many villagers were infected with the leprosy bacillus regardless of whether or not they had a known history of household contact.

One of the important observations from the present study was the recognition of higher seropositive rates in the younger generation. This fact suggests that infection with the leprosy bacillus is still common in South Sulawesi. Although the majority of seropositive individuals may not develop leprosy, a certain percent of them will certainly develop clinical disease in the future. Leprosy control programs in endemic countries should take this fact into account. We should also pay more attention to the high prevalence of infection with the leprosy bacillus in the inhabitants of endemic areas in developing strategies for worldwide leprosy control and subsequent global elimination of the disease. [National Institute for Leprosy Research, Tokyo, Japan; Hasanuddin University, Ujung Pandang, South Sulawesi, Indonesia]

Kimura, T., Hashimoto, K., Izumi, S. and Inoue, K. Perineurial involvement in tuberculoid leprous neuropathy.

Both light-microscopic and electron-microscopic studies were applied to the question of perineurial changes in nerves from two patients with tuberculoid leprosy. Microscopically, the perineurium of the patients had thickened with infiltration of lymphocytes, occasionally losing its continuity. Most strikingly, the basal lamina in the perineurial cells disappeared entirely; whereas those of Schwann cells and endothelial cells of vessels showed a normal appearance when examined by electron microscopy. This type of appearance may be specific for the tuberculoid type of leprosy and may play an important role in the pathogenesis of leprous neuropathy. [Department of Neurology, National Sanatorium Dokohu Hospital, Hokkaido; National Institute for Leprosy Research, Tokyo; Department of Neurology, Institute of Brain Research, University of Tokyo, Tokyo, Japan]

Kaplan, G. Role of TNFα in the pathogenesis of leprosy and tuberculosis.

Thalidomide (α-N-pthalimidoglutarimide) has a long pharmacological history, having been employed as a sedative and as an antiinflammatory agent. Currently, it is

used for the therapy of erythema nodosum leprosum (ENL, or type 2 reaction), an acute inflammatory state occurring in lepromatous leprosy and characterized by severe systemic symptoms, including fever, painful cutaneous lesions, arthritis, glomerulonephritis, and the presence of circulating immune complexes. In this serious complication of leprosy, thalidomide has a prompt and dramatic effect, decreasing inflammation and enhancing patient well-being. The fever, weight loss and general debility of ENL, as in other forms of acute and chronic disease, may be associated with the production of macrophage-derived cytokines. Recently, serum levels of TNFα and IL-β were found to be markedly elevated in some ENL patients. In addition, we noted that TNFα levels were reduced following treatment of patients in ENL with thalidomide. This prompted a detailed examination of the effects of thalidomide on cytokine production by human monocytes in a number of mycobacterial disease states.

In a recent publication (E. P. Sampaio, *et al.* J. Exp. Med. 173:699–703, 1991), our *in vitro* data showed that thalidomide selectively inhibits the production of human monocyte TNFα triggered by lipopolysaccharides (LPS) and other agonists in culture, with 40% inhibition occurring at the clinically achievable concentration of 1 μg/ml. Thalidomide does not affect general protein synthesis, and the amount of total proteins observed on SDS-PAGE are not influenced. Most significantly, IL-1β, IL-6, IL-8, and granulocyte/macrophage colony-stimulating factor (GM-CSF) produced by monocytes remain unaltered. It follows that if the compound also selectively inhibits TNFα production *in vivo* it could be used as a) a tool for determining the role of TNFα in the immunopathology of a variety of infections and inflammatory conditions, and b) modulating TNFα-induced toxic effects in various clinical settings.

In several previous studies we have described the response of patients with lepromatous leprosy to the administration of rIFNγ. This T-cell- and natural killer (NK)-cell-derived lymphokine is a major activator of monocyte/macrophage antimicrobial activity. Lepromatous patients are anergic to antigens of *Mycobacterium leprae,* resulting in their inability to produce IFNγ in response to the bacillus. Therefore, IFNγ replacement therapy seemed warranted in these patients.

In a recent study, 10 patients with lepromatous leprosy were injected intradermally with rIFNγ (total of 1.2 mg) administered monthly for 11 months or every 2 weeks for 5 months. Following lymphokine injection, mild systemic symptoms were noted. An enhanced reduction in the mean bacterial index (BI) was detected in patients during treatment. Increased respiratory burst activity by monocytes was detected 4 hr after one 30 rIFNγ injection. Systemic ENL was observed in 6 patients undergoing therapy. Most patients with ENL (64%) demonstrated high serum TNFα levels (mean $\pm$ S.E.M. 2000 $\pm$ 1178 pg/ml), and exhibited elevated levels of monocyte-cytokine release from monocytes after *in vitro* response to a variety of agonists, relative to lepromatous leprosy patients (twofold) and normal controls (sixfold). Thalidomide therapy of patients in ENL (300 mg/day) selectively reduced TNFα release from monocytes by 50%–80% after stimulation with LPS and mycobacterial agonists. A similar reduction in TNFα release was obtained by exposure of the cells to 10 μg/ml thalidomide *in vitro.*

A possible explanation for these results involves rIFNγ priming of the patient's monocytes for enhanced TNFα release. Primed monocytes might then be triggered by mycobacterial agonists, possibly complexed with immunoglobulin, found in high concentrations in the circulation as a result of enhanced rIFNγ-induced bacterial killing. Thus, abnormally high levels of TNFα, and possibly other cytokines, could be released into the circulation, bringing about the clinical symptoms and toxicities of ENL. Accordingly, thalidomide may well be effective at controlling ENL precisely because it reduces TNFα levels released by monocytes and possibly other leukocytes.

We have also found that mononuclear blood cells from tuberculosis patients release enhanced amounts of TNFα *in vitro* in response to mycobacterial agonists, and that these cells express TNFα mRNA *in vivo.* The high TNFα levels are associated with high monocyte numbers in the blood, IFNγ production in response to *M. tuberculosis*

antigens, and *M. tuberculosis* agonist-induced TNFα release. *In vitro* TNFα production by agonist-stimulated monocytes from tuberculosis patients is inhibited by thalidomide. We have initiated clinical trials in which thalidomide is administered to tuberculosis patients who have typical TNFα-mediated toxicities of fever, weight loss, vascular leakage, and cellular extravasation and necrotizing lesions. The early results of these studies are discussed. [Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York, U.S.A.]

Makino, M. and Suzuki, Y. Molecular cloning of the α-antigen gene from *M. leprae* and its application to serodiagnosis of leprosy.

In this study, we report the construction of a new recombinant α(-85 complex)-antigen which contains *Mycobacterium leprae*-specific epitope(s). Through the use of polymerase chain reaction (PCR) with ML1 and ML2 primers that share sequences common to three mycobacteria, a 0.8 kilobase pair (kbp) fragment was amplified. This fragment was cloned into pUC18 and subcloned into M13mp18. The DNA sequence was determined by the dideoxy terminator method. According to the determined nucleotide sequence, primers ML3 and ML4 for secondary anchored PCR were designed. After secondary PCR using primers ML3 or ML4 and M13RV, 0.5 kbp and 1.2 kbp fragments were amplified from reactions using *Eco*R I and *Xba* I ligation products, respectively. From the nucleotide sequence of the amplified 0.8 kbp fragment, the cloned *M. leprae* α-antigen gene proved to be a homolog of the 85B protein of several mycobacteria, such as *M. bovis* (BCG) and *M. kansasii*. By comparing the results of the deduced amino-acid sequence of the *M. leprae* α-antigen with those of the 85B protein of other mycobacteria and plotting the flexibility of the *M. leprae* α-antigen, two candidates of *M. leprae*-specific epitopes were revealed.

The PCR products amplified by MLR1 and MLR2 were inserted into *Eco*R I + *Hin*d III digests of pMALc-RI and transformed into *Escherichia coli* TB1. Recombinant antigen was purified by Amirose resin affinity chromatography. According to ELISA, using the recombinant α-antigen, the antibody titer in the sera of leprosy patients proved to be much higher than that of healthy controls. [Osaka Prefectural Institute for Public Health, Osaka, Japan]

Matsuo, E., Komatsu, A., Takatsu, A., Sumiishi, A., Sasaki, N. and Skinsnes, O. K. On the cross-immunoreactivity of the receptor for beta-glucuronidase (BGR) of *M. leprae* with the extracts of beans.

We regard the receptor for beta-glucuronidase (BGR) of *Mycobacterium leprae* as a fundamental part of the system by which the bacillus is able to grow in the host cell and to borrow its metabolic machinery. Therefore, we believe that immunological interference with BGR will be harmful to the microorganism. Our previous study showed that *Mycobacterium* HI-75, originally separated from a leproma and a cause of neuritis in nude mice, is a good source of extracted BGR. Nevertheless, we are still interested in more ready sources of BGR. It is present not only in *M. leprae* but also in *M. avium-intracellulare* and also in hepatitis-B-infected cells, suggesting a wide distribution of BGR in nature. In this present study, we sought the presence of BGR in beans, namely, soybean, Azuki-grain, black soybean, peanut, French kidney bean, jackbean and concanavalin A (ConA). All of these, with the exception of ConA, were extracted by the method developed for BGR of *Mycobacterium* HI-75. The resulting fractions and ConA were examined for cross-immunoreactivity with BGR from *Mycobacterium* HI-75 by dot ELISA utilizing anti-BGR rabbit globulin. Two types of anti-ConA from Sigma and Dako were also utilized as the reference sera to look for common immunoreactivity among BGR, soybean and jackbean extracts, and ConA. All of the extracts and ConA appeared to be immunologically stained although not strongly, except ConA with anti-BGR. Anti-ConA from Sigma and not that from Dako appeared to stain weakly the extracts of soybean, jackbean and BGR. The results showed that substances crossreactive with BGR seem to be present in beans. Whether

immunization with these alternative sources of BGR could produce antibodies reactive to BGR extracted from *Mycobacterium* HI-75 and, therefore, reactive to *M. leprae* should be revealed by future experiments. ConA seemed to share cross-immunoreactivity with BGR, and was previously reported to combine with B-Gase. However, it does not seem to be a good alternative for BGR since anti-ConA either reacts with BGR weakly or not at all. [Department of Pathology, Kyorin University School of Medicine, Tokyo; National Leprosarium Tohoku Shinseien, Miyagi, Japan. Department of Pathology, Sun Yat-Sen University of Medical Sciences, Guangzhou, People's Republic of China]

Matsuoka, M., Kohsaka, K. and Dayanghirang, A. J. Characteristics of *M. leprae* Thai-53 strain.

Mycobacterium leprae Thai-53 strain has been used widely for many experiments since this strain grows excellently in the foot pads of nude mice. This strain was isolated from an untreated lepromatous leprosy (LL) case in Thailand in 1982, and has been maintained by serial passage in nude mice foot pads. Properties of this strain were investigated not only by the conventional methods but also by techniques recently available. Bacillary multiplication of the strain in the foot pads of nude mice was greater than that of another strain which was isolated in Japan, but the bacillary growth in the foot pads of conventional mice was similar for both strains and showed a plateau as usual. No bacillary growth was observed on the Ogawa egg-yolk medium or 1% Ogawa medium after 10 weeks of cultivation. Acid-fastness was lost by treatment with pyridine. The DOPA oxidation test was applied on filter paper and clear color development was exhibited by the bacillary suspension containing the Thai-53 strain. Dot ELISA was also applied using the *M. leprae*-specific monoclonal antibodies which recognize specific epitopes on the 65-kDa, 38-kDa, 18-kDa, and 12-kDa proteins. Color development by IIIE9 was a little weak, but clear positive results were obtained with the other antibodies. Polymerase chain reactions using four kinds of primers for the previously deduced sequence (65 kDa, 36 kDa, 18 kDa and repetitive sequence) revealed that products of the desired length were amplified from the template DNA prepared from the Thai-53 strain. The results showed that all properties of the Thai-53 strain were identical with previously reported characteristics of *M. leprae*. [National Institute for Leprosy Research, Tokyo, Japan]

Molloy, A., Smith, K. D. and Kaplan, G. Cell-mediated immunity to mycobacteria: the effect of cytolysis on parasite viability.

Macrophage activation by secreted cytokines has been shown to be important in the control of many macrophage parasites but no lymphokines have yet been demonstrated to inhibit pathogenic mycobacteria. We were interested in investigating the role of another potentially important arm of the cell-mediated-immune response, cell-mediated cytotoxicity. We were interested in the role of cell-mediated cytotoxicity in both the protection against and the pathology of mycobacterial infections. We have studied the effect of cytolysis *in vitro* on BCG viability. Although lymphokine-activated killer (LAK) cells preferentially kill infected monocytes, they fail to directly inhibit intracellular BCG. However, LAK-mediated killing may have a role in the focus of infection in that necrosis and turnover of infected cells may be important in initiating and perpetuating a protective granulomatous response. Apoptosis induced by a soluble mediator directly resulted in a reduction of bacillary viability. T cells have been shown to induce apoptosis in target cells and such cytotoxic T lymphocytes may directly inhibit mycobacteria. Experiments are underway in the laboratory to discover how host-cell suicide and BCG death are coupled, and also to develop T-cell clones from recovering tuberculosis patients. Clones that are lytic for infected monocytes will be identified, their mechanism of killing defined, and their effect of intracellular BCG viability studied. [Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York, U.S.A.)

Nomaguchi, H., Chaicumpar, K., Matsuoka, M., Kohsaka, K., Nakanaga, K., Mi-

nagawa, F. and Yokota, S. Characterization of the B-cell epitope on hsp65 in the immunized mice and autoimmune patients.

We describe B-cell epitopes of heat-shock protein 65 (hsp65) of Kawasaki diseases (KD), rheumatoid arthritis (RA), and leprosy patients, and discuss the role of hsp65 in autoimmunity. The sera from KD patients in the convalescent but not the acute phase crossreacted with hsp65 of *Mycobacterium leprae.* To determine whether the endogenous and/or exogenous 65-kDa protein is activated for B-cell epitopes in KD, two kinds of chemically synthesized peptides were used. One is the epitope for the monoclonal antibody IIIE9 for exogenous 65 kDa, and another is the corresponding site of a human homolog to the mycobacterial 65-kDa protein, P1 protein in human mitochondria, for the endogenous epitope. The convalescent sera but not the acute-phase sera of KD reacted with both of these epitopes for the endogenous and exogenous proteins. On the other hand, sera from mice immunized with *M. leprae* lysate or purified 65 kDa reacted with the 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 seropositive to RA factor in leprosy, the titer of sera from leprosy patients to hsp65 may be correlated to the RA factor. Seropositivity to hsp65 was 20% in the group of RA-positive sera and 13% in RA-negative sera. Future work will address the nature of the B-cell epitopes of hsp65, exogenous and/or endogenous, in individual leprosy patients. The results from these future studies should provide us with useful clues as to the bases of the phenomenon of autoimmunity in leprosy. [National Institute for Leprosy Research, Tokyo; Yokohama City School of Medicine, Yokohama, Japan]

Pessolani, M. C. V. and Brennan, P. J. *Mycobacterium leprae* express extracellular homologs of the antigen 85 complex.

The antigen 85 complex, consisting of at least three closely related proteins (A, B, C) of 30–32 kDa, is an immunodominant set of secreted proteins appearing in the culture medium of *Mycobacterium tuberculosis* and other cultivable mycobacterial species. Their prominence in *M. leprae,* the one obligate intracellular pathogen of the genus, has been assumed based on immunological evidence and proof of the existence of the genome. We have confirmed *in situ* expression of this family by analyzing *M. leprae* subfractions by Western blotting using monospecific rabbit sera raised against the individual *M. bovis* BCG 85A, B, and C components. A crossreactive band of apparent molecular mass of 30 kDa was detected in extracts of whole bacteria and in soluble fractions prepared from tissue of *M. leprae*-infected animals. Studies of the subcellular distribution of these proteins indicated that they are secreted by the pathogen. The stronger reactivity of the *M. leprae* 85 complex with the anti-BCG 85B serum, as well as two-dimensional electrophoresis, established that the B component was the predominant member of the complex in *M. leprae.* The fibronectin-binding capacity of the *M. leprae* and the BCG 85 complex was reinvestigated, using new approaches, and is questioned. Nevertheless, the results with the native proteins reinforce the belief that the 85 complex is one of the dominant protein immunogens of the leprosy bacillus. [Department of Microbiology, Colorado State University, Fort Collins, Colorado, U.S.A.]

Saito, H., Tomioka, H. and Sato, K. *In vitro* and *in vivo* antileprosy activities of benzoxazinorifamycin (KRM-1648) and clarithromycin.

We previously reported that a newly synthesized rifamycin derivative (KRM-1648) and some new quinolones have a potent therapeutic efficacy against *Mycobacterium leprae* infection induced in athymic nude mice. Furthermore, when *in vitro* antimicrobial activities of various agents against *M. leprae* were examined using the BACTEC 460 TB System, close correlation was found between *in vitro* and *in vivo* anti-*M. leprae* activities of a given test agent, including dapsone, clofazimine, rifamycins, and quinolones. In this study, we further investigated *in vitro* anti-*M. leprae* activities of these agents by the BACTEC system using NaOH-treated *M. leprae* as an inocu-

lum. Moreover, the *in vivo* antileprosy activities of KRM-1648 and clarithromycin (CAM), a new macrolide, were examined in detail.

Drug susceptibility testing using the BACTEC 460 TB System was performed according to Franzblau (Antimicrob. Agents Chemother., 33, 2115–2117, 1989), with some modifications. Briefly, *M. leprae* Thai-53 were harvested from infected foot pads of BALB/c nude mice by homogenizing with a glass homogenizer in RPMI medium, treated with 2% NaOH-treatment, followed by subsequent washing with the medium by centrifugation, and suspended in 7H9 medium. BACTEC 12B medium (4 ml) with or without the test drug was inoculated with 0.1 ml of the *M. leprae* suspension (10^8/ml). The vials were then incubated at 33°C without agitating for up to 4 weeks. The cumulative Growth Index (GI) value was read on days 4, 11, 18, and 27. At the concentration of 0.5 μg/ml, KRM-1648 caused a very marked reduction in the GI value of the organisms (65%–84%), followed by rifabutin (57%–74%), clofazimine (24%–84%), CAM (41%–71%), and sparfloxacin (18%–30%). At the concentration of 2 μg/ml, the degree of reduction in GI due to the test agents was of the order: clofazimine (78%–97%), KRM-1648 (67%–87%), rifabutin (55%–77%), CAM (37%–71%), rifampin (44%–66%), sparfloxacin (34%–51%), minocycline (26%–35%). Ofloxacin and dapsone caused a slight decrease in the GI value, but the other agents were not as efficient. These results are essentially the same as those obtained by using ampicillin and amphotericin B-treated *M. leprae*, prepared by the method of Franzblau, as an inoculum.

In order to study the antileprosy activity of KRM-1648 and CAM, athymic nude mice infected with 10^6 *M. leprae* in the hind foot pad were given the test drug, emulsified in 0.1 ml of 2.5% gum arabic–0.1% Tween 80 solution by gavage, once daily, six times per week, from day 31 to day 80 after inoculation. KRM-1648—even at 0.001 mg/mouse—caused a significant decrease in the number of leprosy bacilli recovered from the infected foot pad on day 360 (ca 0.6-log decrease). KRM-1648 at 0.005 and 0.01 mg/mouse caused a 1.0- and 2.2-log decrease, respectively. CAM at 0.3 mg/mouse caused a significant decrease in the number of leprosy bacilli in the infected foot pad on day 360 (ca 1.1-log decrease). CAM at 1.0 and 3.0 mg/mouse caused a 1.7- and 1.5-log decrease, respectively. Thus, these agents exhibited a dose-dependent antileprosy action. They also inhibited the foot pad swelling due to infection in a dose-dependent manner.

The present results indicate that measurement of the *in vitro* anti-*M. leprae* activity of various antimicrobials is possible by using NaOH-treated *M. leprae* instead of ampicillin-and amphotericin B-treated organisms. Moreover, it was confirmed that there is a good correlation between the *in vitro* activity of a given agent and its therapeutic efficacy against *M. leprae* infection, as previously observed. [Department of Microbiology and Immunology, Shimane Medical University, Izumo, Japan]

Salgame, P. and Bloom, B. R. Human T-cell subsets in leprosy.

One of the major interests of immunology today is lymphokine production by T-cell subsets and their crossregulation. Several laboratories over the last few years have reported the presence of two distinct nonoverlapping subsets of murine CD4+ T cells. TH_1 cells make IFN-γ, IL-2, and lymphotoxin; TH_2 cells make predominantly IL-4, IL-5, and IL-10. Recently we and others have found that human T-cell clones exhibiting a similar Type 1 (TH_1) and Type 2 (TH_2) phenotype exist in tissues and peripheral blood lymphocytes of strongly lepromin-positive patients and contacts. *Mycobacterium leprae*-specific CD4+ clones exhibited a TH_1 phenotype. Of the 9 tetanus toxoid-reactive clones tested 8 clones made IL-4 and IL-5. Using a panel of HLA-B7-reactive CD8+ cytotoxic clones and CD8+ *M. leprae*-specific T_S clones, we observed a similar dichotomy within the CD8+ population. The cytotoxic CD8+ clones made a Type 1 lymphokine response and the T_S clones produced lymphokines similar to the Type 2 subset, i.e., predominantly IL-4. IFN-γ and IL-4 production by *M. leprae*-reactive CD4+ and CD8+ clones was further confirmed by ELISPOT assays. These

results suggest that intracellular pathogens may be particularly effective at stimulating differentiation toward Type 1 and Type 2 subsets and that the lymphokines made by T cells play a key regulatory role in the development and expression of immune effector mechanisms.

What are the factors that determine activation of the different subsets? Antigen-presenting cells (APC) displaying different second signals, accessory molecules and cytokine production may be important. In preliminary experiments we observed that Type 1 cells neither make IL-2 nor proliferate to immobilized anti-CD3 antibody, unless provided with a costimulatory signal from APC. However, Type 2 cells proliferated well to anti-CD3 receptor antibodies in the absence of APC, suggesting different APC requirements by these two subsets. Type 2 clones also proliferate to both IL-2 and IL-4 unlike Type 1 clones that respond only to IL-2. We are further analyzing the APC requirements of the four T-cell subsets, especially *M. leprae*-reactive CD4-T_H and CD8-T_S clones. This may provide insight into the inverse correlation between antibody and cell-mediated-immune responses to *M. leprae* antigens observed in leprosy patients. [Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York, U.S.A.]

Sieling, P. A., Abrams, J. S., Yamamura, M., Rea, T. H. and Modlin, R. L. Interleukin-10 production in response to *M. leprae*: evidence for an immunosuppressive role in leprosy.

The disease spectrum of leprosy provides an opportunity to correlate clinical manifestations with immune responses to a pathogen. Tuberculoid leprosy patients mount strong cellular immune responses to *Mycobacterium leprae* that limit the growth of the pathogen. Lepromatous leprosy patients, however, are specifically unresponsive to the pathogen, which results in permissive infection. Cytokines are known to be pivotal regulators of such cell-mediated-immune (CMI) responses. Earlier studies in our laboratory demonstrated that tuberculoid leprosy lesions contain cytokines that promote CMI responses, particularly IFN-γ (analogous to the cytokines produced by TH_1 lymphocytes). In contrast, lepromatous leprosy lesions contain cytokines that augment humoral responses and suppress CMI responses (analogous to the cytokines produced by TH_2 lymphocytes). In a parallel study, CD4 and CD8 populations that produce analogous cytokine patterns were identified. Since interleukin-10 (IL-10) is prominent in lepromatous lesions, the role of this cytokine in immune unresponsiveness was investigated. In the present study, we found that: a) *M. leprae* induced IL-10 release from peripheral blood mononuclear cells (PBMC); b) monocytes/macrophages produce the *M. leprae*-induced IL-10; c) IL-10 inhibits PBMC proliferation to *M. leprae*; d) *M. leprae*-induced IL-10 inhibits monokine release.

The manner in which IL-10 functions to suppress immune responses in leprosy is analogous to the mechanism(s) in which it induces immune suppression in murine models of infection. Anti-IL-10 caused augmentation of antigen- and mitogen-driven TH_1 responses in spleen cells of schistosome-infected mice. Secondly, IL-10 mRNA was detected in spleens of *Toxoplasma cruzi*-infected mice and IL-10 blocked the ability of IFN-γ to inhibit intracellular replication of *T. cruzi* in mouse peritoneal macrophages. Furthermore, exogenous IL-10, added to murine macrophages, blocks TNF-α. The studies indicate that IL-10 acts through the macrophage to inhibit T-cell-mediated immunity to infection. Similarly, the present study shows that IL-10 inhibited monokines that lead to functional T-cell responses and bacterial killing (e.g., TNF-α and GM-CSF).

Most evaluations of IL-10 production have focused on the TH_2 phenotype. Our investigation, however, indicates that the monocyte/macrophage population is the primary course of IL-10 after mycobacterial exposure. An earlier study by Malefyt, *et al.* (J. Exp. Med. 174:1209–1220, 1991) demonstrated that lipopolysaccharide induced IL-10 production by human monocytes which inhibited cytokine synthesis by monocytes (most notably TNF-α and GM-CSF). Monocyte involvement in suppressed immune responses to *M. leprae* has been suggested for some time. Monocytes were

required for *M. leprae*-induced suppression of proliferative responses to mitogen. The present study, viewed in the context of our earlier studies of cytokine expression in leprosy lesions, indicates that by inducing IL-10 release from monocytes, *M. leprae* drives the immune system to a more TH_2-like response. Other factors must influence the response to *M. leprae,* since not all patients progress to the lepromatous pole of the leprosy spectrum, but IL-10 appears to play a pivotal role in perpetuating the immunosuppressed state. [Division of Dermatology; Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California; Department of Immunology, DNAX Research Institute, Palo Alto, California; Section of Dermatology, USC School of Medicine, Los Angeles, California, U.S.A.]

Wang, X.-H., Ohmen, J. D., Uyemura, K., Rea, T. H., Kronenberg, M. and Modlin, R. L. Limited T-cell receptor repertoire in the delayed-type hypersensitivity response to *M. leprae.*

The spectrum of leprosy is not static but, rather, dynamic, with alterations in the clinical presentation associated with rapid fluctuations in the level of cell-mediated immunity. Of particular interest are reversal reactions, which are generally known to be naturally occurring, delayed-type hypersensitivity (DTH) responses to *Mycobacterium leprae* associated with the clearance of bacilli from lesions. Patients with reversal reactions are generally upgrading, moving from the lepromatous region of the spectrum toward the tuberculoid region. Reversal reaction lesions are readily accessible to immunologic study and are characterized by an influx of CD4+ T cells and by the production of type-1-like cytokines, including IL-2 and IFNγ. There is a concomitant decrease of type-2-like cytokines, including IL-4, IL-5 and IL-10.

T cells recognize antigen in the context of the major histocompatibility complex (MHC) through a receptor which, in most instances, consists of two polypeptide chains termed α and β. Recent advances in molecular immunology have led to the characterization of the genes which encode T-cell receptors (TCRs). Each chain has a constant (C) region which is relatively invariant between cells. The ability of T cells to recognize a wide diversity in antigens is conferred by the choice of variable (V) and joining (J) segments, as well as nucleotide (N) and diversity (D) segments at the V-J junction. The usage of these gene segments vary from cell to cell. The identification of the germline sequences of these TCR segments has been exploited in determining the diversity of the T-cell repertoire in immune responses. If few variable genes account for a large part of the TCR repertoire, the immune response is thought to be directed against a small set of antigens. If many variable genes are used, the response is thought to be directed against a complex array of proteins. The genomic management of β-chain genes include at least 20 variable genes, 2 diversity genes, 13 junction genes, and 2 constant genes. After rearrangement and splicing, a single V-J-C complex is formed. We designed oligonucleotide primers for each Vβ and a single consensus Cβ for polymerase chain reaction (PCR).

Examination of the TCR β repertoire in reversal reactions in leprosy was undertaken to provide clues as to the nature and diversity of the set of antigens recognized by T cells mediating DTH in skin. Because resistance to *M. leprae* infection is associated with specific MHC class II alleles, we explored the influence of MHC class II alleles in shaping the TCR repertoire at the site of infection. To assess TCR V gene usage, we first isolated RNA from skin lesions and blood, synthesized cDNA, and performed PCR. PCR product is electrophoresed, transferred to nylon membrane, and hybridized with a radioactive Cβ probe internal to the Cβ PCR primer. The contribution of each Vβ family to the total TCR repertoire is assessed by radioanalysis.

To identify whether specific Vβ subpopulations accumulate at the site of disease activity in reversal reactions, PCR was utilized to determine Vβ gene usage within lesions and blood from three individuals. Simultaneous measurement of TCR repertoires in lesions compared to blood minimized some of the inaccuracies in absolute quantification of each particular Vβ family related to possible differences in primer efficiency in the PCR. Distinct compartmentalization of the immune response was ev-

ident in the overrepresentation of specific Vβ families in lesions relative to blood. For example, in Patient A, Vβ1, Vβ6 and Vβ7 encoded TCRs were preferentially used by T cells in lesions compared to blood by at least twofold. In Patient B we identified Vβ6, Vβ13, and Vβ19 gene families as frequently utilized in lesions. In the lesions from Patient C we found relative overrepresentation of T cells using Vβ6, Vβ12, and Vβ14 gene families. These data indicate that specific TCR Vβ populations accumulate at the site of disease activity in reversal reaction lesions. Note that Vβ6 was increased in the lesions relative to the blood in all three patients.

To determine whether TCR Vβ usage was consistent throughout the lesion, we measured the TCR Vβ repertoire in four adjacent regions of a single lesion. Several Vβ families were overrepresented about two- to sevenfold throughout the lesion compared to the blood. For example, Vβ13 and Vβ19 were similarly overrepresented throughout the different areas studied. Some Vβ gene families (for example, Vβ6) were overrepresented in some but not all regions of the biopsy specimen. Several Vβ families, including Vβ2, Vβ11, Vβ12, and Vβ18, were underrepresented by at least twofold throughout the lesion compared to the blood of this patient. Finally, the majority of Vβ families were equally represented throughout the lesion compared to the blood of the same patient. In summary, TCR Vβ gene families were identified which were generally overrepresented, focally overrepresented, or underrepresented throughout the specimen. Note again that Vβ6 increased during the reaction in both patients.

The dynamic changes in the TCR Vβ repertoire associated with the DTH response were studied in two patients from whom specimens were obtained before the onset of and during upgrading of reversal reactions. Striking changes in the TCR Vβ repertoire were observed in these DTH reactions. Several Vβ gene families were more than twofold increased in reversal reaction compared to pre-reversal reaction lesions. In Patient D, the onset of reversal reaction was associated with an influx of T cells expressing Vβ3, Vβ6, Vβ8, Vβ13.1, Vβ19, and Vβ20 gene families. In Patient E, T cells expressing TCRs belonging to the Vβ6, Vβ8, and Vβ12 families were more prominent in the reversal reaction lesion. These data indicate that the onset of DTH against *M. leprae* in reversal reactions is associated with the increase of specific T-cell populations locally.

Examination of the repertoire of seven patients indicated that T cells using the Vβ6, Vβ12, Vβ14, and Vβ19 encoded T-cell receptors were strikingly overrepresented in the lesions of patients as compared to blood and pre-DTH lesions from the same individuals. Furthermore, the data indicate a possible association between the predominant expression of a Vβ gene segment in lesions and the MHC class II haplotype of the individual. Vβ6 was prominent in the lesions of four patients who were DR15, a marker of resistance in leprosy infection. In two DR9 individual, Vβ6, Vβ12, and Vβ14 were overrepresented and in two DR13 individuals, Vβ14 and Vβ19 were overrepresented in skin lesions.

Sequence analysis of the Vβ6 TCRs from a reversal reaction lesion showed that: a) Vβ6.1 was used by 12/21 clones; b) Jβ2.7 was selected by 14/21 clones. There was conservation of a glutamine followed by a tyrosine encoded by different Jβ gene segments; c) in the V-J junction there was a conserved leucine encoded by the Vβ germline gene segment. There was a conserved glycine encoded by both Dβ and N segments; d) the occurrence of repeat sequences indicates local oligoclonal expansion. Preliminary sequence data in a second patient yielded similar results. [Division of Dermatology; Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California; Section of Dermatology, USC School of Medicine, Los Angeles, California, U.S.A.]

Yoon, K.-H., Cho, S.-N., Abalos, R. M., Cellona, R. V., Fajardo, T. T., Jr., Walsh, G. P., Lee, M.-K. and Kim, J.-D. Detection of *M. leprae* in biopsy specimens from leprosy patients by polymerase chain reaction.

Biopsy specimens were obtained from 95 leprosy patients before and during the course of multidrug therapy (MDT) and examined by polymerase chain reaction (PCR) using the primers amplifying 372 bp DNA of the

repetitive sequence of the *groE-L* gene of *Mycobacterium leprae*. The PCR results were then compared with bacterial indices (BI) of slit-skin smears and biopsy specimens. The intensity of the DNA bands was in general correlated with the acid-fast bacilli (AFB) numbers and even one organism gave a PCR positive result. Five 5-μm sections from each frozen tissue were pooled and processed for DNA preparation. PCR was positive in 6 (60.0%) of 10 patients with a BI of 0 at six sites and in 21 (77.8%) of 27 biopsy specimens with a BI of 0 determined in the paraffin sections from the same biopsy sample. PCR also gave positive results in 83 (97.6%) of 85 biopsy samples with a BI of 2–6. The intensity of the 372 bp DNA bands was well correlated with the BI of biopsy specimens and decreased little after 2 years' MDT in many other patients. This study showed that the PCR tool using primers targeting the repetitive sequence of the *groE-L* gene was highly sensitive and specific in detecting *M. leprae* in biopsy specimens from leprosy. Further modification of PCR protocols is, however, needed to see any significant change in the PCR results following chemotherapy. [Department of Microbiology, Yonsei University College of Medicine, Seoul, Korea; Leonard Wood Memorial Center for Leprosy Research, Cebu, The Philippines]

CLOSING REMARKS
(prepared but not delivered)

Highlights in Leprosy Research during 1991–1992 under the U.S.–Japan Cooperative Program and Other NIAID Programs

A major development that concerns U.S.- and Japan-sponsored and other international programs is the downward trend in global estimates of number of leprosy cases. While leprosy is still a major public health problem in most countries of Africa, Asia, and Latin America, the situation has vastly improved, at least in quantitative terms, over that of a decade ago. The revised global estimate for 1991 is 5,511,000 cases of leprosy as compared to the 10–12 million cases estimated earlier (Anon., Weekly Epidemiological Record, World Health Organization, Geneva, 67: 163–170, 1992). The number of registered cases of leprosy is now estimated at 3,087, 788, and it is further estimated that the cumulative multiple drug coverage of these is about 70%. Thus, about one half of the total estimated number of cases are having or have had multiple drug therapy.

Of implication also to many of the programs sponsored by the U.S.–Japan Cooperative Program was the disappointment of the WHO-sponsored leprosy vaccine trial in Venezuela; although results are only initial, no improvement in protection was provided by the live BCG/killed *Mycobacterium leprae* vaccine over and above BCG alone (Convit, *et al.*, Lancet 339: 446–450, 1992). However, the efficacy of the BCG vaccine alone against leprosy in a trial in northern Malawi has been further corroborated (Ponnighaus, *et al.*, Lancet 339: 636–639, 1992).

Despite the apparent success of multidrug therapy, it is estimated that there are at least 2–3 million persons worldwide with significant deformities, including those cured of disease. Considerable basic research progress has been made by U.S.–Japan-sponsored investigators in the immunological definition of immunopathogenesis in leprosy and in differentiating it from protective immunity, and in counteracting some deformities in leprosy. For instance, high production of IL-2 and γ-IFN auger a strong cell-mediated-immune response, self-healing, and resistance to *M. leprae*, whereas the predominance of IL-4, IL-5, and IL-10 are indicative of immunological unresponsiveness, uncontrolled proliferation of *M. leprae* and, in essence, lepromatous leprosy (Modlin, *et al.*, Science 254: 277–279, 1991; Salgame, *et al.*, Science 254: 279–282, 1991). Moreover, in erythema nodosum leprosum (ENL) reactions, the lepromatous profile of cytokines is maintained, but there is a se-

lective increase in IL-6, IL-8, and IL-10, and also TNF, which may be of prognostic value. Novel strategies including the application of recombinant granulocyte/macrophage colony stimulating factor and other cytokines (Kaplan, *et al.*, J. Exp. Med. 175: 1717–1728, 1992) and thalidomide therapy to alleviate systemic leprosy reactions through reducing of TNF-α production (Sampaio, *et al.*, J. Exp. Med. 175: 1729–1737, 1992) represent important milestones and, throughout the course of the meeting, we heard of these and associated developments.

To date, five major *M. leprae* protein antigens have been sequenced and cloned and are available to the research community; 70 kDa (Dnak); 65 kDa (GroEL); 28 kDa (SodA); 18 kDa (small hsp); 10 kDa (GroES). Other proteins which have been recognized and are under examination with a view to production are: 36 kDa (Pro-rich); 35 kDa (membrane); 28–30 kDa (antigen 85 complex); 28 kDa (Fe-regulation); 22 kDa; 15 kDa; and 12 kDa (Mehra, *et al.*, J. Exp. Med. 175: 275–284, 1992; Young, *et al.*, Mol. Microbiol. 62: 133–145, 1992). Thus, apparently all of the antigens necessary for a sub-unit vaccine against leprosy have been identified. In addition, the expression of many of these antigens in live recombinant BCG (Stover, *et al.*, Nature 35: 456–460, 1991) to present the prospects of a live recombinant vaccine, is a major development.

Over 70 genes and markers of the *M. leprae* genome have been mapped and characterized, and a canonical set has been identified for genomic sequence analysis which is now underway. This is unpublished work but has work implications, since it will allow a comparison of the genome of *M. leprae* and *M. tuberculosis,* and perhaps explain why *M. leprae* cannot be grown *in vitro.*

The molecular mechanism of phagocytosis of *M. leprae* is nearing definition with recognition of a three-component reactor-ligand-acceptor molecular system composed of complement receptors (CR1, CR3, and CR4) and complement component C3 and phenolic glycolipid (Schlesinger and Horowitz, J. Exp. Med. 174: 1031–1038, 1991).

Most of the secondary gene products of *M. leprae* have now been defined. Chatterjee, *et al.* (J. Biol. Chem. 267: 6234–6239, 1992; Infect. Immun. 60: 1249–1253, 1992) made the important observation that LAM (lipoarabinomannan) from *M. leprae* was capped with mannose residues and that this phenomenon had a profound effect on TNF secretion and, thus, on the entire cascade leading to pathogenesis in leprosy.

New chemotherapy regimens, described by Dr. Saito and Dr. Franzblau involving quinolones, minocycline, and clarithromycin, have been described.

In summary, the last 1–2 years, as reflected at this meeting, have seen enormous progress in containing the disease, defining protective immunity and immunopathogenesis, and counteracting reactions/nerve damage. Euphoria with fundamental developments was offset by disappointment with the outcome of the initial results from the vaccine trial.

The final point which I would like to make is that in these times of considerable debate on the status of global leprosy, especially *vis-a-vis* tuberculosis, and the contributions of basic research to the disease, we should be resolute and continue to identify leprosy as an important disease entity and then try to maintain the research thrust. And, specifically, our joint programs will come under review next year.

I propose to you that we do not follow the path of other international organizations and amalgamate leprosy and tuberculosis into a mycobacteriology sobriquet. Instead, we should request the retention of the two separate panels in this very successful half-amalgamated form for at least another 5 years, until such time as we can better assess the trends in prevalence and incidence of global leprosy.

—Patrick J. Brennan, *Chairman*
U.S. Leprosy Panel