

TWENTY-NINTH JOINT LEPROSY RESEARCH CONFERENCE

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ABSTRACTS*

Fiss, E. H. and W. R. Jacobs, Jr. Identification of genes involved in the ferric exochelin biosynthetic pathway for the sequestration of iron in mycobacteria.

As a first step in the characterization of iron uptake in mycobacteria, a genetic approach was taken. Molecular biology techniques were utilized to complement the mutation in an exochelin-deficient mutant of *Mycobacterium smegmatis* using DNA libraries from *M. smegmatis*, *M. leprae* and BCG. Subcloning was used to identify the complementing gene. The sequence of the proposed gene, *fxbA*, required exochelin biosynthesis has been analyzed. In addition, two additional genes are reported, genes which share homology with the *Escherichia coli* iron permease genes.—[Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, N.Y., U.S.A.]

Fukutomi, Y., Matsuoka, M., Bramhne, H. G. and Minagawa, F. Analysis of induction of anti-*M. leprae* activity in macrophages.

One of the major roles of macrophages in the immune system is to kill intracellular parasites. The killing activity closely cor-

relates with the activation of macrophages, and this process is commonly induced by cytokines. In the present study, fresh *Mycobacterium leprae* were obtained from the foot pads of nude mice and, by means of radiorespirometric assay (the Buddemeyer system), the expression of anti-*M. leprae* activity of macrophages was analyzed.

Interferon-gamma (IFN- γ) pretreatment followed by infection with *M. leprae* induced the macrophage to exhibit potent anti-*M. leprae* activity after 1 or 2 days. *M. leprae* harvested from IFN- γ -pretreated macrophages exhibited very low oxidation metabolism of palmitic acid (less than 1/10) compared to the bacteria harvested from normal macrophages. In contrast, treatment of a 1-week culture of *M. leprae*-infected macrophages with IFN- γ alone could not induce the activation, while the co-presence of tumor necrosis factor (TNF) did induce the activation. Previously, we reported on TNF production by *M. leprae*-phagocytosed macrophages. TNF, therefore, is a key cytokine which induces the activation of IFN- γ -treated macrophages.

In addition, interleukin 4 (IL-4) and IL-6, which mainly regulate the B-cell response, neither enhanced nor suppressed IFN- γ -mediated induction of the anti-*M. leprae* response of macrophages. On the other hand, T-cell growth factor- β (TCF β) suppressed the activation to some extent.

* 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.

These results indicate that IFN- γ and TNF definitely are involved in the induction of the anti-*M. leprae* response of macrophages, although other cytokines such as IL-4 and IL-6 exhibit less direct effects on macrophage activation.—[National Institute for Leprosy Research, Tokyo, Japan]

Gelber, R. H., Murray, L. P., Siu, P., Tsang, M. and Rea, T. H. Efficacy of minocycline 100 mg twice daily and an initial single 200-mg dose in the therapy of lepromatous leprosy.

Ten previously untreated lepromatous leprosy patients were admitted into a clinical trial to evaluate their responses to an initial single 200-mg dose of minocycline (8 patients) followed subsequently by minocycline 100 mg twice daily for 3 months in all patients. These studies were initiated to determine whether twice-daily therapy had advantages over what had been found previously with once-daily therapy, and whether single doses of minocycline were sufficiently effective so as to be of use in once-monthly regimens such as is recommended for rifampin. Patients were carefully monitored for clinical response, the occurrence of reactional states, drug side effects and toxicities, and the clearance of viable *Mycobacterium leprae* from sequential skin biopsies. These evaluations occurred 1 week after the single dose and 2 weeks, 1 month, 2 months, and 3 months thereafter.

Trial patients experienced a uniform and remarkable rapid clinical response to minocycline therapy. Five of the eight patients who received a single, initial 200-mg dose, when evaluated 1 week later, were improved clinically, three profoundly so. By 1 month of therapy all patients were significantly improved and by 3 months' therapy all patients had 80% or more resolution of skin nodularity and dermal infiltration. Four patients experienced vertigo: in two this was severe, occurring after the initial 200-mg dose and mitigating against further minocycline therapy; in two patients the vertigo was mild, evanescent, and did not require interruption of therapy. Two other patients developed minocycline-related hyperpigmentation which resolved after the completion of therapy, and one patient had diarrhea during the first week of treatment

that abated spontaneously. Sequential hemograms and blood chemistries remained within normal limits in all trial patients. Of note, only a single patient developed erythema nodosum leprosum (ENL) during the trial, this being mild but becoming very severe only when minocycline was discontinued and while on therapy with dapsone and rifampin. Perhaps the known antiinflammatory effects of minocycline (Antimicrob. Agents Chemother. 36, p. 277, 1992) could account for both the unusually rapid clinical response and the relative absence of ENL.

M. leprae (5000) from sequential skin biopsies were inoculated into both hind feet of groups of mice. *M. leprae* were considered to be viable if growth to 10^5 /foot pad occurred either in pools of four feet (two mice) obtained both 8 and 12 months after infection or in any individual foot pads (generally 10) harvested 12 months after infection. All pretreatment skin biopsies were found to contain viable *M. leprae* which were uniformly sensitive to dapsone, clofazimine, rifampin, and a low level of minocycline (0.01% in diet). In none of the eight patients who received a single, initial 200-mg dose of minocycline were viable *M. leprae* totally eliminated. However, the proportion of viable *M. leprae* was reduced in 6 of these 8 patients. By 1 and 2 months of therapy the number of patients harboring viable bacilli was convincingly reduced, and by 3 months none of the treated patients harbored detectable viable *M. leprae*.

This clinical trial, as with our first clinical trial (Br. Med. J. 304, p. 91, 1992) and that of others (J. Infect. Dis. 168, p. 188, 1993), confirms that minocycline is highly effective in the therapy of lepromatous leprosy. The clinical response found in trial patients was, in our experience, unique and consistently rapid. The clearance of viable *M. leprae* found in this study and our previous one at 100-mg daily were remarkably similar, being superior to dapsone and clofazimine but not rifampin. Because of the increased evidence of side effects found herein and the increased difficulty of maintaining compliance with twice-daily therapy, we currently recommend that minocycline be administered 100-mg daily for the therapy of leprosy patients. Because of its proven safety on chronic administration and perhaps its additional advantage of providing an im-

portant antiinflammatory effect, minocycline appears the most promising of the newer agents for application to the therapy of patients.—[San Francisco Regional Hansen's Disease Program, San Francisco, CA.; University of Southern California School of Medicine, Los Angeles, CA, U.S.A.]

Gillis, T. P., Tan, E. V., Williams, D. L., Balagon, M. F., Cellona, R. V. and Walsh, G. P. Detection of *M. leprae* in nasal secretions of household contacts of multibacillary patients.

Recent reports have focused attention on the rates of nasal carriage of *Mycobacterium leprae* in various segments of populations with endemic leprosy. We, as well as others, have reported the rate of nasal carriage of *M. leprae* in untreated patients with multibacillary (MB) and paucibacillary (PB) disease and have found that between 50% and 70% of MB cases produced nasal secretions containing *M. leprae* detectable by polymerase chain reaction (PCR). Not unexpectedly, rates of nasal carriage of *M. leprae* in PB disease were extremely low, mirroring the overall bacilli load evident in lesions. With this in mind, we tested nasal secretions from 242 contacts of 133 index cases for the presence of *M. leprae*. All contacts of PB index cases tested negative for the presence of *M. leprae*. In contrast, 3.3% of the household contacts of MB index cases tested positive by PCR. A rate of 7.9% for contacts of MB patients and 1.9% for PB patients was reported by Pattyn, *et al.* While the two rates reported by Pattyn, *et al.* were different, a statistically significant difference could not be shown, suggesting that our PB household contact population may not have been large enough to detect positive contacts in this group, should they exist.

Klatser, *et al.* reported a 2.4% rate of positivity in contacts of leprosy patients in two villages in Indonesia. While this rate of positivity in nasal secretions is similar to our results, the disease classification of the index cases in the two studies were much different. In the Indonesian study 42 contacts of 11 index cases were studied with 60% of the index cases being classified as PB leprosy. In our study in The Philippines all positive nasal secretions came from contacts associated with a MB index case. While

our results support the idea that a higher rate of positive contacts is found in MB households, further study is necessary to determine the relationship between nasal carriage, infection, and the spread of *M. leprae*.—[Laboratory Research Branch, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA, U.S.A.; Leonard Wood Memorial Center for Leprosy Research, Cebu City, The Philippines]

Goto, M. and Matsuoka, M. Ultrastructural immunohistochemistry of *M. leprae* in dermal nerves; comparison of human leprosy and nude mouse.

A high-resolution, ultrastructural, immunohistochemistry profile for *Mycobacterium leprae* using convention Epon-embedded ultrathin sections, anti-BCG antibody, and colloidal gold was established. By this technique, dermal nerves from five cases of fresh lepromatous (LL) or borderline lepromatous (BL) leprosy and a *M. leprae*-inoculated nude mouse were examined. In 3 of the 5 cases, most of the BCG-positive *M. leprae* were localized in the cytoplasm of unmyelinated Schwann cells, some of them in macrophages, and only rarely in the myelinated Schwann cells of one LL case. *M. leprae* in macrophages were surrounded and digested by lysosomes, while *M. leprae* in both forms of Schwann cells were not related to lysosomes. In the nude mouse, the major host cells were macrophages, but distinct BCG-positive *M. leprae* were detected in a few unmyelinated and myelinated Schwann cells. These results confirmed our previous idea that unmyelinated Schwann cells are the major host cell of *M. leprae* and, furthermore, demonstrated that lack of lysosome digestion in Schwann cells seems to be an important mechanism for escaping from host immunity.—[National Leprosarium Hoshizuka-Keiaien, Kagoshima; National Institute for Leprosy Research, Tokyo, Japan]

Izumi, S., Cao, Y.-H., Kawatsu, K. and Bharadwaj, V. P. Comparative study on the utility of various serological tests for prediction of high-risk household contacts to develop leprosy; a case-control study.

In 1991, we demonstrated that considerable numbers of the inhabitants in the endemic villages in South Sulawesi, Indonesia, had been infected with leprosy bacilli without direct contact with leprosy patients. Klatser, *et al.*, using nose swab and polymerase chain reaction (PCR), demonstrated that about 8% of the general inhabitants in the same villages carry *Mycobacterium leprae* on the surface of their nasal mucosa. These results suggest that the strong infectivity of leprosy bacilli and the wide distribution of *M. leprae* in the environment play an important role in the continual high incidence of leprosy in the area. The application of chemoprophylaxis to those infected inhabitants who are at high risk of developing leprosy will become important for leprosy control programs in the near future.

Recently, we developed a new ELISA technique using partially delipidified whole *M. leprae* and *M. tuberculosis* absorbed serum. Its use in predicting high-risk groups was tested by a case-control study. It was found that leprosy cases had significantly higher IgG antibodies to *M. leprae* compared to the controls. The odds ratio was 9.8. The new technique was found to have a significantly higher ability to predict a risk group than PGL-I- or LAM-based serodiagnosis.—[National Institute for Leprosy Research, Tokyo, Japan; Central JALMA Institute for Leprosy, Agra, India]

Kashiwabara, Y., Nakamura, M., Kusunose, E., Ichihara, K., Kusunose, M. and Asano, A. Cloning of the gene encoding superoxide dismutase of *M. lepraemurium*.

The gene encoding superoxide dismutase (SOD) has been cloned from *Mycobacterium lepraemurium* by screening a genomic library constructed in λ gt11 with a polyclonal antibody toward the purified SOD of *M. lepraemurium*. The open reading frame contained 621 nucleotides and encoded 207 amino acid residues. The deduced amino acid sequence of the SOD of *M. lepraemurium* revealed a close similarity to those of *M. leprae* and *M. tuberculosis*; 93% and 87% identity, respectively. It also showed a similarity to manganese-containing SODs from other microbes and mitochondria of

eukaryotes. The results from Southern hybridization suggested that *M. lepraemurium* has one copy of the Mn-SOD gene in its genome. Moreover the drastic changes in the content of SOD in *M. lepraemurium* grown *in vitro* and *in vivo* suggest that some regulatory mechanism of gene expression may operate to control the production of SOD.—[National Institute for Leprosy Research, Tokyo; Institute for Protein Research, Osaka University, Osaka; Toneyama Institute for Tuberculosis Research, Osaka City University Medical School, Osaka; Department of Industry, Fukuyama University, Fukuyama, Japan]

Kimura, T., Yahara, O., Izumi, S., Goto, M. and Inoue, K. Perineurial involvement of leprosy neuropathy.

Leprosy is characterized by skin lesions and neuropathy. We previously reported that the perineurium lost continuity and the continuities of the tight junctional complexes and the basal lamina disappeared completely in tuberculoid leprosy. Thus this perineurial involvement was pathognomonic of tuberculoid leprosy. We evaluated the perineurial changes of 9 cases of tuberculoid and lepromatous leprosy.

Patients with tuberculoid leprosy were apparently divided into two groups pathologically, a mild group and a severe group. In the mild group, the distance between the perineurium and the endoneurium was widened, which was indicative of the presence of subperineurial edema. The perineurium partially lost the basal lamina and showed splitting of the basal lamina occasionally. In the severe group, the perineurium was thickened and infiltrated with lymphocytes and plasma cells. Occasionally the severely infiltrated perineurium lost its continuity. Neither myelinated nor unmyelinated fibers presented in the endoneurium. The perineurium lost continuity and the continuities of the tight junctional complexes and the basal lamina disappeared completely.

Patients with lepromatous leprosy had *Mycobacterium leprae* in the endoneurium, the epineurium and the perineurium. The perineurium was mildly thickened with preserved continuity. There was a subperineurial edema. Some cases had decreased myelinated and unmyelinated fibers in the

endoneurium; others had normal myelinated fibers. The basal lamina of the perineurium partially disappeared. There were no abnormalities of the basal lamina in either the Schwann cells or the vascular endothelial cells. It is apparent that the perineurium constitutes a diffusion barrier to various externally applied substances. We think that these changes suggest that dysfunctions of the blood-nerve barrier might play a central role in the neuropathy of both tuberculoid and lepromatous leprosy.— [First Department of Internal Medicine, Asahikawa Medical College; National Institute for Leprosy Research; National Leprosarium Hoshizuka Keiaien; Department of Neurology, Yokohama Rosai Hospital, Japan]

Laochumroonvorapong, P., Molly, A., Moreira, A. L., Sarno, E. N. and Kaplan, G. An *in vitro* model of resistance in leprosy: apoptosis, but not necrosis of mycobacteria-infected monocytes is coupled with killing of intracellular mycobacteria.

We have demonstrated by light and electron microscopy that tuberculoid leprosy lesions contain epithelioid and multinucleated giant cells, surrounded by a mantle of lymphocytes, predominantly CD4+. A significant proportion of the mononuclear macrophages in these lesions appear injured or dead. The foci of cell death are interspersed with large numbers of viable epithelial cells and lymphocytes, suggesting a "high turnover" with the continuous death and disintegration of older cells and the influx of new mononuclear cells from the circulation. Cell death is not observed in lepromatous lesions. Thus, the number and integrity of intracellular bacilli appears inversely related to the turnover of macrophages and the intensity of the T-cell response.

Cells die by one of two known mechanisms: necrosis or apoptosis. Necrosis, often referred to as accidental cell death, is induced when the plasma membrane of a cell is irreversibly damaged. Hydrogen peroxide (H_2O_2) produced by mononuclear phagocytes during the phagocytic event kills cells by destroying the integrity of their membranes, leading to osmotic dysregulation and necrosis. Apoptosis, or pro-

grammed cell death, is distinguished from necrosis on the basis of biochemical and morphological criteria. Unlike necrosis, apoptosis is associated with rapid and profound changes in nuclear organization, and can be accompanied by fragmentation of the chromatin in the dying cell. Apoptosis has been shown to be induced by variety of mediators, including glucocorticoids and ATP^{4-} .

In order to understand the dynamics of the granulomatous response described above, we have developed an *in vitro* model of human monocyte mycobacterial infections to investigate whether apoptotic or necrotic host cell death is associated with a reduction in mycobacteria viability. Cultured monocytes are infected with the attenuated mycobacterium *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), and allowed to support several rounds of intracellular replication. Chronically infected cells are induced to undergo apoptosis or necrosis, and the effect on the viability of intracellular mycobacteria is investigated using a colony forming unit assay.

Our results indicated that freshly explanted monocytes could restrict the replication of BCG, but that as monocytes differentiated into macrophages, they lost the small capacity they initially had to inhibit mycobacterial replication. H_2O_2 and ATP^{4-} killed cultured monocytes in a dose-dependent fashion while GTP^{4-} had no effect on cell viability. The dose-response curves of parallel assays with infected and uninfected cells were identical, suggesting that the long-term presence of replicating bacilli neither predisposed toward, nor protected against, either necrosis or apoptosis. Pretreatment with interferon-gamma ($IFN-\gamma$) did not alter the dose-response curve or the kinetics of cell death. More importantly, ATP^{4-} -induced apoptosis, but not H_2O_2 -induced necrotic cell death, was coupled with killing of BCG. No killing of bacilli was detected if cultures were lysed before ATP^{4-} treatment, indicating that bacillary killing was coupled with the apoptotic event. Morphological studies of ATP^{4-} -treated cells revealed alterations in morphology, including shrinkage of cytoplasm and condensation of nuclear chromatin. When free ends of DNA were enzymatically detected and fluorescently labeled, pyknotic nuclei induced by

ATP⁴⁻ treatment were intensely fluorescent, suggesting that the chromatin inside had been fragmented. Fragmentation of DNA into discrete, nucleosome-sized fragments was detected by agarose gel electrophoresis.

Currently, skin biopsies from lesions of various forms of leprosy (borderline lepromatous, mid-borderline, borderline tuberculoid, tuberculoid and erythema nodosum leprosum) are being analyzed for evidence of apoptosis *in vivo*.—[Department of Cellular Physiology and Immunology, The Rockefeller University, New York, NY, U.S.A.; Hanseniasis Section, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil]

Matsuoka, M., Bormate, A. B. and Nak-anaga, K. Diversity among isolates of *M. leprae* detected by polymerase chain reaction with arbitrary primers.

The identification or differentiation of individual isolates of *Mycobacterium leprae* would be useful for epidemiological studies and in the analysis of infection mechanisms. No genotypic diversity within *M. leprae* isolates was exhibited by restriction fragment-length polymorphism (RFLP) analysis so far. We set about developing an improved method to subtype *M. leprae* isolates. The arbitrarily primed polymerase chain reaction (AP-PCR) method is able to distinguish among isolates of other bacteria belonging to a single RFLP class. The feasibility of applying this method to discriminate between *M. leprae* isolates was examined.

M. leprae isolated from eight different patients and further passaged through nude mice were used. Bacterial suspensions were prepared by differential centrifugation. DNA was extracted by freezing and heating or by mechanical disruption in liquid nitrogen, followed by phenol extraction. Various arbitrary primers consisting of 6 to 13 nucleotides were prepared and used as single primers in PCR amplification.

Among the 10-mer primers tested, two primers with a G + C content of 50% yielded many fragments, ranging between approximately 2k bps and 100 bps. The banding patterns were affected by various factors, such as primer sequence, template concentration, MgCl concentration, annealing temperature, and purity of sample. Under

optimal conditions, reproducible results were obtained with highly purified samples. Diversity in banding patterns between the different isolates were demonstrated. It is considered that this reflects genetic diversity among the *M. leprae* isolates. No diversity in the banding patterns were noted among samples prepared from different generations and batches belonging to one original isolate. The results suggest that *M. leprae* is maintained without any selection or considerable sequence divergence during passage in mouse foot pads up to the 11th passage. The polymorphism in the banding patterns revealed by AP-PCR of *M. leprae* shows that this method can be used as a tool in studying the epidemiology of leprosy.—[National Institute for Leprosy Research, Tokyo, Japan]

Nakanaga, K., Nomaguchi, H. and Matsuoka, M. Establishment of mouse cell lines expressing *M. leprae* genes.

As one of the approaches to analyze *Mycobacterium leprae* gene function, we tried to establish mouse cell lines expressing *M. leprae* genes. The mouse cell line BALB/3T3 clone A31-1-(A31 cell) and the gene of the *M. leprae* heat-shock protein 65 kDa (hsp65) were chosen as the model system.

The source of the *M. leprae* hsp65 gene for this experiment was the plasmid pUC-N5 (pUC-N5 is constructed from pUC8 into which the gene of the *M. leprae* hsp65 was subcloned as a 3.6-kb Eco RI fragment of λ gt11). The full length of the hsp65 gene or a fragment of it was inserted into the mammalian higher expressing vector pMAMneo (pMAMneo has the mouse mammary tumor virus' long terminal repeat (LTR) as the promoter upstream of the multicloning site; it also have the SV40 promoter allowing expression of the G418 resistant gene).

These three different constructions of mammalian expression vectors were introduced individually into the A31 cells by the calcium phosphate precipitation method. The gene product protein was detected by Western blotting using the anti-hsp65 monoclonal antibodies B2C, A5B, and 3A as the first reaction antibody. The second reaction antibody was the antimouse IgG conjugated with alkaline phosphatase. The positive signal was visualized by the blue-

purple color reaction with BCIP/NBT as the substrate reagent.

Two different constructions of the vectors which contain no promoter sequence nor nonsense sequences of the hsp65 gene were able to express the hsp65 when they were introduced into A31 cells. These cells were cloned by limiting dilution and one of the clones, 146-2-9, was shown to express the hsp65 at a much higher rate. These mouse cell lines that express the hsp65 may be useful in analyzing cell-mediated immunity generated during mycobacterial infections.—[National Institute for Leprosy Research, Tokyo, Japan]

Nomaguchi, H., Ahsan, C. R., Yogi, Y., Matsuo, M., Fukutomi, Y. and Kawatsu, K. The 65-kDa heat-shock protein enhances bactericidal granuloma formation in mice infected with *M. leprae*.

Leprosy is a chronic granulomatous disease involving both the mononuclear phagocyte and *Mycobacterium leprae*. Resistance to the *M. leprae* organisms in the host is mediated by cellular immune responses involving macrophages and sensitized T cell, and is associated with the formation of granulomas. The heat-shock proteins (hsp) are evolutionarily highly conserved polypeptides that are produced under a variety of stressed conditions to preserve cellular functions and are highly immunogenic protein antigens.

Foot pad swelling in the mouse was caused by challenge with hsp65/Freund's incomplete adjuvant (FIA). The foot pad enlarged significantly and remained unchanged during the whole experimental period. Histological examination revealed that many lymphocytes infiltrated the lesion site and fluid infiltration also appeared after 1 month's observation after the challenge with hsp65. One year after the *M. leprae* inoculation, the mice challenged with hsp65 were found to possess no bacilli in their granulomas or neural cells. However, the non-challenged mice were found to possess bacilli in granuloma cells and neural cells.

All of these data suggest that hsp65 enhances the development of a bactericidal granuloma in mice infected with *M. leprae* and also probably inhibits the invasion process of *M. leprae* into the neural cells in

mice.—[National Institute for Leprosy Research, Tokyo, Japan]

Pessolani, M. C. V., Smith, D. R., Rivoire, B., McCormick, J., Hefta, S. T., Cole, S. T. and Brennan, P. J. Developments from the *M. leprae* genome project: discovery, purification, characterization, gene sequence and significance of a *M. leprae* bacterioferritin.

Present developments were due, firstly, to our technical ability to isolate, even from scarce tissue-derived *Mycobacterium leprae*, sufficient amounts of this major protein to obtain partial amino acid sequence, to establish other relationships to known bacterioferritins (Bfrs). Oligonucleotides based on the amino acid sequences and polymerase chain reaction (PCR) allowed amplification of a fragment accounting for approximately 70% of the gene. Recognition of the entire gene encoding MMP-II was possible through the new *M. leprae* genome project. At present the *M. leprae* chromosome is represented by four contigs (blocks of contiguous DNA sequences) of overlapping cosmids and, by summing their sizes, one can estimate the chromosome to have a size of about 2.8 megabases. The sequence of one cosmid has been described in detail and the sequences of 26 more (18 sequences in Genbank), or about one third of the *M. leprae* chromosome, have been determined. Over 300 *M. leprae* genes have been identified and, in many cases, precise functions can be attributed on the basis of extensive sequence homology. However, the present work, in that it defines a protein that corresponds to a gene identified in this fashion, extends the implications of the research beyond the level of comparisons with known gene sequences. Clearly the derived amino acid sequence from the MMP-II gene showed significant homology with Bfrs already described in the literature, and it also shared 100% identity with the first 42 amino acids of antigen D, a Bfr isolated from *M. paratuberculosis*. Bfrs are haem proteins, and the optical spectrum of reduced MMP-II showed α , β , and Soret bands at 557, 523, and 422 nm, respectively, demonstrating the presence of protoporphyrin IX as a prosthetic group. Recently, a bismethionine axial ligation of haem has been demonstrated

in Bfr from *Pseudomonas aeruginosa* and, based on this information, alternative models involving two conserved methionine residues have been postulated as providing the haem ligands in other Bfrs. With the inclusion of the *M. leprae* Bfr, it can be seen that only the methionine at position one remains conserved among the sequence of all Bfrs.—[Department of Microbiology, Colorado State University, Fort Collins, CO; Collaborative Research, Inc., Waltham, MA.; Beckman Institute of the City of Hope, Duarte, CA, U.S.A.; Institut Pasteur, Paris, France]

Saito, H., Tomioka, H. and Sato, K. *In vivo* antileprosy activity of benzoxazinorifamycin KRM-1648 in combination with other microbials.

Previously, we found that the new benzoxazinorifamycin derivative KRM-1648 (KRM) displayed excellent *in vivo* therapeutic efficacy against *Mycobacterium leprae* infection induced in athymic nude mice. In addition, KRM showed combined therapeutic efficacy with dapsone (DDS) and clofazimine (CFZ) when these drugs were given to mice once daily for 50 days from 31 to 80 days after infection. In this study we evaluated the therapeutic efficacy of KRM in combination with DDS, CFZ, or ofloxacin (OFLX) in the case where KRM was given with long intermission. Moreover, the therapeutic effect of KRM in combination with a new macrolide, CAM, was examined. BALB/c nude mice were infected subcutaneously with 1×10^6 of *M. leprae* Thai-53 into the left hind foot pad. Test drugs were emulsified in gum arabic-Tween 80 solution and given to mice by gavage (Experiment 1). KRM, CAM or their combination, was administered daily six times per week from day 31 to 80. After 360 days, the mice were killed and the number of acid-fast bacilli in the left hind foot pad was measured. KRM and CAM given at doses of 0.001 mg and 1.0 mg/mouse, respectively, exhibited significant antileprosy activity, causing a 1.1- and a 1.5-log-unit decrease in the number of leprosy bacilli per foot pad, respectively. However, there was no significant increase in the therapeutic efficacy when KRM was given to mice in combination with CAM (Experiment 2). KRM was

administered once per week and CFZ, DDS, or OFLX were given twice per week from day 91 to 180. KRM, CFZ, DDS, and OFLX given at the dose of 0.001, 0.05, 0.02, and 3.0 mg/mouse, respectively, exerted significant antileprosy activity, causing a 2.5-, 1.9-, 1.3, and 1.5-log-unit decrease in the number of leprosy bacilli per foot pad, respectively. In this case, KRM exhibited a significant combined effect with either CFZ or DDS against *M. leprae* infection, causing a further reduction in the number of leprosy bacilli per foot pad, i.e., a 3.9- and a 4.3-log-unit decrease, respectively. In contrast, such a combined effect was not observed for KRM + OFLX. Therefore, the *in vivo* anti-*M. leprae* activity of KRM can be enhanced when combined with DDS or CFZ, when compared to the efficacy of each drug alone, even if KRM was given with long intermission.—[National Institute for Leprosy Research, Tokyo; Department of Microbiology and Immunology, Shimane Medical University, Izumo, Japan]

Sampaio, E. P., Pimentel, M. I. F., Moreira, A. L., Kaplan, G. and Sarno, E. N. Effect of HIV-1 infection on the immune status of leprosy patients.

Our present study suggests a possible dissociation of immune status from the histology of the lesions in leprosy patients. Our observations demonstrate that HIV-1 infection and even very progressive AIDS are not associated with a significant modification of the appearance of the cutaneous lesions of *Mycobacterium leprae* infection. The cellular composition and phenotype appear unaffected by HIV-1 infection. However, the *in vitro* immunoresponsiveness of the cells in the circulation of borderline tuberculoid (BT) HIV-1-positive compared to HIV-1-negative leprosy patients is impaired. Cells obtained from our BT patients do not proliferate in response to mycobacterial antigens and the mitogen PHA. Cytokine mRNA is hyperexpressed in the peripheral blood mononuclear cells of these patients. Also, their ability to extravasate in response to the administration of soluble *M. leprae* antigens into the skin is abrogated. This dichotomy is as yet unexplained. Nor do we understand why *M. leprae* infection appears to be affected differ-

ently by HIV-1 co-infection than do other mycobacterial infections.

Although the numbers of patients are small, our results suggest that HIV-1 infection may be activating subclinical leprosy infection since five of the patients first diagnosed with HIV-1 infection later or simultaneously developed BT skin lesions. This is in accordance with earlier studies carried out in Haiti and Zambia, in which HIV-1-infected BT patients developed new lesions after the initiation of multidrug therapy, suggesting that co-infection accelerates the development of leprosy.—[Leprosy Sector, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; The Rockefeller University, New York, NY, U.S.A.]

Sieling, P. A., Barnes, P. F. and Modlin, R. L. IL-12 in mycobacterial infection.

The data indicate a role for interleukin 12 (IL-12) in the generation of cell-mediated immunity in both leprosy and tuberculosis (TB). First, IL-12 was evident at the site of human leprosy and TB infection and appeared to correlate with strong expression of the type 1 cytokine IFN- γ . Second, T-cell responses to mycobacteria were IL-12 dependent, abrogated by the addition of neutralizing IL-12 antibodies and enhanced by the addition of rIL-12. Third, IL-12 augmented T-cell responses in both leprosy and TB patients who manifested weak T-cell proliferation and did so by enhancing the CD4⁺ type 1 responses. These data indicate a central role for IL-12 in the cell-mediated immune response to mycobacteria.—[Division of Dermatology, University of California at Los Angeles, Los Angeles, CA; Department of Medicine, University of Southern California, Los Angeles, CA, U.S.A.]

Suzuki, Y., Yin, Y.-P., Katsukawa, C. and Makino, M. Overproduction of recombinant 85 complex of *M. leprae* in *E. coli* and their application for serodiagnosis of leprosy.

The 85 complex is one of the dominant mycobacterial proteins that are thought to be secreted from mycobacteria. These proteins have been studied independently and designated by various names— α -antigen,

MPB59, P32 and antigen 6—by various investigators. One of the most interesting features of the 85 complex gene is that these genes are members of a gene family. Both the BCG and tuberculosis 85 complex have been reported to consist of three structurally related components designated as A, B, and C. Another striking feature is the antigenicity of the 85 antigen complex against T and B cells in patients with mycobacterial infections.

We have constructed the genomic library of *Mycobacterium leprae* Thai-53 strain, cloned, and characterized two kinds of genes coding the 85 complex. From the results, the 85 complex genes of *M. leprae* have turned out to be similar to the gene family of *M. bovis* BCG and *M. tuberculosis*. From a comparison of these with 85 complex homologs of other mycobacteria, we found the possible *M. leprae*-specific epitopes in the two genes.

We have also constructed an overexpression system of the 85 complex of *M. leprae*. Polymerase chain reaction (PCR) amplified DNA fragments coding for either 85A or 85B antigen were inserted downstream of the maltose binding protein gene of the *Escherichia coli* expression vector pMALc-RI which can express MBP-*M. leprae* 85 complex fusion protein. Cells were grown to mid-log phase, and the expression of MBP-*M. leprae* 85 complex fusion proteins were induced by adding IPTG to the final concentration of 2 mM. Fusion proteins in the sonic extracts were absorbed to amylose resin followed by elution with 10 mM maltose. Both 85A and 85B recombinant fusion proteins were purified to a purity level of more than 95%. In addition, more than 10 mg of fusion proteins were obtained from a 200-ml culture.

The reactivities of lepromatous and tuberculoid patient sera to recombinant *M. leprae* 85A, 85B, and *M. tuberculosis* 85 complex were examined by an absorption ELISA. Antibodies reactive to the *M. tuberculosis* 85 complex in the sera were absorbed by incubating with various concentrations of the *M. tuberculosis* 85 complex. Reactivity of lepromatous patient sera against recombinant 85A was much higher than that against recombinant 85B or *M. tuberculosis* 85 complex. The reactivity against recombinant 85B of lepromatous

patient sera was reduced to on fifth when absorbed by *M. tuberculosis* 85 complex at the concentration of 320 ng/ml; whereas the reactivity against recombinant 85A still showed high reactivity. Reactivity of tuberculoid patient sera against recombinant 85A was also higher than that against recombinant 85B or *M. tuberculosis* 85 complex. Some reactivity against 85A remained after absorption by *M. tuberculosis* 85 complex at the concentration of 320 mg/ml; whereas little reactivity was observed against recombinant 85B or *M. tuberculosis* 85 complex.

The recombinant 85A antigen of *M. leprae* overproduced in this study may become a powerful tool for the serodiagnosis of lepromatous and tuberculoid leprosy.—[Departments of Pathology and Microbiology, Osaka Prefectural Institute of Public Health, Osaka; National Sanatorium Oku-Komyoen, Okayama, Japan]

Williams, D. L. and Gillis, T. P. Diagnosis of rifampin resistance in pathogenic mycobacteria by molecular techniques.

In 120 *Mycobacterium tuberculosis* isolates a 305-bp fragment of the *rpoB* gene was sequenced. Sixteen mutations were

identified in 110 rifampin-resistant isolates, and no mutations were observed in 10 rifampin-susceptible strains. Similar mutations were also observed in *M. leprae*, *M. africanum*, and *M. avium*. Direct mutation detection was accomplished using a polymerase chain reaction/heteroduplex formation (PCR/HDF) assay. The 305-bp PCR products from *M. tuberculosis* isolates were mixed with the 305-bp PCR product from a rifampin-susceptible strain (wild type). The DNAs were denatured at 94°C for 5 min and then allowed to anneal slowly. Loading dye was added and the samples were electrophoresed at 700 V on 1 × mutation detection enhancement gel in TBE buffer. DNA bands were observed by ethidium bromide staining and UV transillumination. In all rifampin-resistant strains, two or three bands were observed. However, only a single band of 305 bp was observed when susceptible strains were analyzed by PCR/HDF analysis. PCR/HDF analysis was able to detect the rifampin phenotype of *M. tuberculosis* directly from sputum specimens of 20 tuberculosis patients within 24 hours.—[Laboratory Research Branch, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA, U.S.A.]

CLOSING REMARKS

I would like to close on a note of optimism on the future of the U.S.–Japan Cooperative Tuberculosis and Leprosy Programs. Yesterday, Dr. Shimao, Overall Chairman of the Japanese Delegation, mentioned the 29-year history of the U.S.–Japan Cooperative Medical Science Program and its importance. Indeed, it is an extremely important program encompassing most of the major areas of biomedical research within our respective countries, and it is a program that is supported by some of the highest scientific, administrative and political authorities among our respective peoples.

As we leave here, it is important that we realize this point. It is a wonderful privilege, a great professional honor, to be invited to present our research and to serve, within

the context of the U.S.–Japan Cooperative Medical Sciences Program, to be able to come to this truly beautiful setting, to work for a few days with such gifted, wonderful people and to partake of such exceptional hospitality.

At this meeting, we have lived up to such high expectations. Never before have we invited, from among both Japanese and U.S. participants, so many new inductees into these research areas, and the presentations were uniformly exceptional, covering clinical, epidemiologic, immunologic, and molecular aspects of the two diseases. In the two Japanese Panels, we have met a new generation of enlightened leadership. Dr. Tsuyuguchi has assumed Chairmanship of the Tuberculosis Panel for the first time. Dr. Saito is into only his second year, and he is

the new Director General of the National Institute of Leprosy Research. In fact, Dr. Saito is the third Chairman of the Japanese Leprosy Panel with whom I have served, reminding me that my time also has come.

In closing, I would also like to offer a note of caution about the future of leprosy research, especially within the U.S. context. The World Health Organization has done an enormous service to mankind in helping develop and implement multiple drug therapy and, as a result, it now is a very realistic possibility that leprosy within a few short years will show even more substantial reduction. Yet WHO may be severely damaging the very cause it espouses by stridently naming deadlines and speaking in terms of leprosy elimination. On page 6 of WHO's own document, the latest *TDR News*, it is mentioned in the one article that there are about 2½ million people worldwide with leprosy, and yet 600,000 new cases are being discovered every year. There is an apparent dichotomy in these figures.

But we ourselves, as American scientists, are showing the same type of ambivalence. On the one hand, we are trying to impress on Dr. Shimao, Dr. Carpenter, other delegates and the National Institutes of Health (NIH) that this Leprosy Panel should remain separate. Yet several laboratories in the U.S., long dedicated to leprosy research, no longer work in this area; leprosy research is now concentrated in two to three laboratories; good abstracts for participation in this meeting and other important meetings are not forthcoming; important Panel members are not attending meetings; materials are not being exchanged; cooperative interactions in leprosy research are dying. There

clearly is a crisis in leprosy research; there is a brain-drain, and it is of our own doing. The fear now is not that the U.S.-Japan Delegation nor NIH will terminate the leprosy component of this Program, but that we ourselves will do so at a time when the great scientific challenges, as we heard over the past four days, still remain. So I exhort you and those whom you can influence to be imaginative, interactive and cooperative, and re-dedicate yourselves to leprosy research, so that when the Delegation makes its decision, it at least can say that there are vibrant leprosy research programs underway in the U.S. and thereby the delegates' decisions can be based on other factors. Thus, let me offer a suggestion. The NIH, thanks to the efforts of Dr. Gwinn, has just issued a new RFP for leprosy research. Let some of us (Carville, CDC, Rockefeller) combine in responding to this initiative under the banner of the U.S.-Japan Program.

Anyway, let's not end on a negative note. We are so grateful to Dr. Kuzo for creating such a memorable event in such a wonderful setting; we are so grateful to the two Japanese Panels for such an exceptional scientific program; we are grateful to Dr. Shimao and Dr. Someya for their attendance and their support; we are grateful to Dr. Darrel Gwinn for his sterling public service in the cause of this program over many years; and we are grateful to all of you, especially the newer generation of Japanese and American scientists, for your research and your company. Welcome, next year, to the foothills of the Colorado Rockies, sunny Fort Collins.

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