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TWENTY-EIGHTH JOINT LEPROSY RESEARCH CONFERENCE

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OPENING REMARKS

Colleagues:

Over the past number of years, we have had a tradition in this Panel of reviewing aspects of leprosy that affect our research mission as members and participants in the Joint U.S.–Japan Leprosy Program. This exercise is particularly important this year, since our program is now being reviewed by the Joint U.S.–Japan Delegation.

We are all very aware of the vastly improved quantitative figures for global leprosy, and the ambitious goal of the World Health Organization (WHO) to raise $420 million over the next 6-year period in order to purchase drugs, train health workers and organize a world-wide campaign to achieve the elimination of leprosy is highly praiseworthy. However, the impression that leprosy is already eliminated has been sown already even though it is still a serious problem in several major global areas. There clearly has been a massive reduction in funds for leprosy research by WHO and other agencies and an exodus of researchers from the field. This dual problem of the exit of key researchers and waning interest in leprosy presents those of us with responsibilities for leprosy research with special problems. Firstly, we really do not know the full extent of global leprosy. Peter Smith, the noted leprosy epidemiologist in reflecting on the revised estimates of global leprosy, advised "considerable caution," noting that these figures relate to estimated prevalence and do not necessarily correspond to a fall in incidence. To quote Dr. Smith in a recent issue of *Leprosy Review* [(1992) 62, 317–318], "The WHO Leprosy Elimination goal requires that there must be a marked fall in incidence of leprosy. It is hoped that high coverage and prompt treatment of prevalent and new cases of leprosy with multidrug therapy will rapidly render (patients) non-infectious to others and thus prevent the secondary cases that they would otherwise have caused. While this seems a reasonable expectation, the direct evidence that such therapy reduces the incidence of leprosy in a population is scanty and more time will have to pass before a definitive assessment can be made of the impact of multidrug therapy on transmission."

Added to this ignorance of ours of the true extent of leprosy is the fact that there are at least 2–3 million individuals, with many more to come, who no longer require chemotherapy and, therefore, are not counted but who, nevertheless, have or will de-

velop deformities because our knowledge of the pathogenesis of leprosy and how to treat it is still in its infancy. So, we, who are the guardians of research on leprosy, have to be concerned that these two major research areas, namely, development of the tools to measure incidence and understanding the immunopathogenesis of leprosy, may be compromised.

On a different note, we have to think about ways in which to continue to foster some recent extraordinary developments in fundamental leprosy research. I am referring, firstly, to the excitement and enormous potential of the *M. leprae* genome project. As you know, first results from international cooperative efforts on sequencing of the *M. leprae* genome have appeared. Due to the effort of Dr. Stewart Cole, the *M. leprae* chromosome is now represented in a collection of about 140 clones organized into four contigs and, already, the sequence of five cosmids has been published and, due to the work of Dr. Douglas Smith of Collaborative Research, another nine are well advanced, another four are nearing completion and five more are being assembled. In fact, about 50% of the *M. leprae* genome has been sequenced. The enormous potential of this visionary effort on the parts of Drs. Cole and Smith and the responsible funding agencies, the National Center for Human Genome Research of NIH and the WHO/TDR and PVD programs, is already bearing fruit with the identification of the genes encoding new possible virulence factors, as you will hear from Dr. Pessolani, and also in the recognition of the genes that are probably responsible for the synthesis of many of the secondary gene products of *M. leprae.* However, excitement in these developments is tempered by the likelihood that the National Center for Human Genome Research and WHO will not continue to support sequencing of the *M. leprae* genome, and, accordingly, we will have to use the weight of our prestigious joint program to encourage these efforts.

There have also been exceptional developments in our understanding of the immunopathogenesis of leprosy, and I am referring, in particular, to the work from the laboratories of Dr. Gilla Kaplan and Dr. Robert Modlin, which you will also hear about. Dr. Modlin has now identified the immunological hallmarks of a relatively healthy immune response in leprosy, and he has recognized the roles of IL-4 and IL-10 in the inhibition and suppression of cell-mediated immunity and the onset of lepromatous leprosy and, of course, you will hear about Dr. Kaplan's headline research on the effects of tumor necrosis factor in erythema nodosum leprosum and its modulation by thalidomide.

Other stirring research called to mind is the development of polymerase chain reaction as a genuine, highly reproducible, sensitive tool for leprosy diagnosis or confirmation, and its application to nasal secretions and slit-skin smears in a practical, beneficial way, as we will hear from Dr. Sugita and Dr. Gillis, and also the elegant molecular definition of the basis of rifampin resistance in *M. leprae* from Dr. Diana Williams, which, thank heavens, is apparently not a problem in clinical leprosy.

Accordingly, we Panel members are the gate keepers of outstanding research that in its fundamental significance almost transcends the status of the disease itself. But, in addition, we are cautious about the disease, feeling that information on true incidence is crucial before the book is closed on leprosy and also are convinced that we must work toward understanding and rectifying the problem of leprosy pathology. In addition, we are conscious of the lessons from tuberculosis: there must be a cadre of researchers and key materials in existence should the disease resurrect its ugly head. Nevertheless, in light of the encouraging state of leprosy as a disease, it is now proving difficult to justify the continuing existence of a separate U.S.–Japan Leprosy Program within the U.S.–Japan Cooperative Medical Sciences Program. And yet, realizing our concerns, it is imperative that a leprosy component continue to exist within the body of the Medical Sciences Program. Accordingly, I propose to you that we continue to evolve toward a single U.S.–Japan Tuberculosis and Leprosy Panel with equal representation from active tuberculosis and leprosy researchers. The measured integration with tuberculosis over the past few years has proved beneficial and, I believe, that within this arrangement, we can continue to fulfill our mission as the sentinels of leprosy research.

In welcoming you, Dr. Saito, as the Chairman of the Japanese Leprosy Panel, and your other Panel members (Dr. Izumi, Dr. Yamada, Dr. Goto and Dr. Matsuoka) and your guests, and in welcoming the other U.S. Panel members and our guests, I look forward to discussing these thoughts with you and arriving at consensus recommendations and workplan which we can then present to the delegates on Thursday.

While the prospects of defining new directions for ourselves in the next few days is a joyous one, this is also a sad meeting in that the recent passing of one of our own, Dr. Zanvil Cohn, is fresh in our minds. Zan Cohn was a truly good, warm and generous man and a great scientist. He came on this Panel at a time when we needed help and guidance and wisdom and experience, and he provided all of these. We were enlightened by his contributions and enjoyed his company at many meetings here, in Japan, and as recently as the U.S.–Japan meeting 2 years ago in Seattle. As joint Panels, our sympathy goes to his wife, children and his close friend and colleague, Dr. Gilla Kaplan, a member of our Panel.

Thank you very much.

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

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ABSTRACTS*

Azuma, I., Suzuki, Y., Sakamoto, Y., Yin, Y. P., and Makino, M. PGL-I-Like antigen in renal cell carcinoma.

Many methods for the serodiagnosis of leprosy have been established by using *Mycobacterium leprae*-specific phenolic glycolipid-I (PGL-I) as an antigen. Gelatin agglutination tests and the micro-hemagglutination method are two of the most common methods widely used in Japan for the diagnosis of leprosy. By the gelatin particle agglutination test, we have examined sera from gravid women and obtained the results that sera from gravid women of 9-month's gestation especially had high anti-PGL-I antibodies and lost them immediately after labor. These results strongly suggest not only the relationship between anti-PGL-I antibody and the fetus, but also the possibility of the existence of a new embryonic antigen. In extensive studies, we examined sera from patients suffering from some kinds of cancer. One of the strongest relationships existed between PGL-I-like antigen and renal cell carcinoma. Twenty-nine out of 40 renal cell carcinoma patients carried anti-PGL-I antibody in their sera. A small number of sera from pelvic tumor patients or renal cyst patients also have been tested and very low anti-PGL-I antibody titers were obtained.

PGL-I-like antigen in urine was examined by dot-blot detection. Urine from patients of renal cell carcinoma and healthy controls was tested by anti-PGL-I monoclonal antibody SF1. In 28 out of 39 tested urines from patients, PGL-I-like antigen has been detected; 12 out of 15 healthy controls tested negative. The combination of detection of anti-PBL-I antibody titer in the sera and the PGL-I-like antibody in urine brings us excellent results. Whether PGL-I-like antigen might be useful for the early diagnosis of renal carcinoma or not must be elucidated further.—[Departments of Microbiology and Pathology, Osaka Prefectural Institute of Public Health, Osaka; National Institute for Leprosy Research, Tokyo, Japan]

Franzblau, S. G., Chan, G. P., Ignacio, B. G., Chavez, V. E., Livelo, J. B., Jimenez, C. L., Parrilla, M. L. R., Calvo, R. F., Williams, D. L., and Gillis, T. P. Clinical trial of fusidic acid in leprosy.

Clinical response (graded according to erythema, diffuse infiltration, and size/ele-

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

vation of nodules and plaques) at the end of 8 weeks of treatment with fusidic acid was judged moderate in 6 patients and marked in 3 patients. No reversal reactions were noted. Bacilli recovered from skin biopsies showed a mean decrease in radiorespirometric activity of 84%, 96%, and 99.5% after 2, 4, and 6 weeks of treatment, respectively. Serum phenolic glycolipid-I titers showed a time-dependent decrease in all patients. No significant difference in response was noted between patients receiving 500 mg or 750 mg. Mouse foot pad infectivity and polymerase chain reaction results are given.

This clinical trial was based primarily on the impressive *in vitro* activity of fusidic acid against *Mycobacterium leprae* in the BACTEC system together with existing documentation of human pharmacokinetics, safety and efficacy in other infections. This may thus be the first drug since the 1960s to go to clinical trial in leprosy without prior demonstration of activity in the mouse foot pad system. Based on results obtained thus far, fusidic acid appears promising as an antileprosy agent. Fucidin is routinely used in combination with rifampin in treating other infections and thus it would be expected to combine well in a multidrug regimen for leprosy. Finally, a number of laboratory studies indicate an immunosuppressive activity as well which may suggest the potential for simultaneous suppression of reversal reaction.—[GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, Louisiana, U.S.A.; Research Institute for Tropical Medicine, Metro Manila, The Philippines]

Gillis, T. P., Tan, E. V., Williams, D. L., Villahermosa, L. G., Balagon, M. V. F., and Walsh, G. P. Evaluation of polymerase chain reaction for detecting *M. leprae* in skin scrapings and nasal secretions from leprosy patients.

We have shown that polymerase chain reaction (PCR) technology for *Mycobacterium leprae* detection can be transferred successfully to a reference laboratory in a leprosy-endemic country. In addition, we have shown that the PCR test can be developed to detect the presence of *M. leprae* DNA in the nasal secretions and slit-skin smears of both multibacillary (MB) and paucibacillary (PB) leprosy patients. Results indicated that in both MB and PB disease slit-skin scrapings were superior to nasal secretions for detecting *M. leprae* DNA in routine samples obtained by relatively noninvasive sampling techniques. These techniques should allow further analysis of patients during therapy as well as for studying signs of early disease in contacts of patients in an attempt to better define the early events associated with *M. leprae* infection.—[GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, Louisiana, U.S.A.; Leonard Wood Memorial Center for Leprosy Research, Cebu City, The Philippines]

Goto, M., Izumi, S., and Kimura, T. Early nerve lesions of leprosy; immunohistochemistry and ultrastructure in fresh cases in India.

In order to clarify the entry of *Mycobacterium leprae* and early lesions into peripheral nerves, we have examined skin (N = 11) and nerve (N = 3) biopsies of fresh leprosy cases in India by light- and electron-microscopic immunohistochemistry using anti-BCG, anti-LAM-B, and anti-PGL-I antibodies.

(A) *M. leprae* antigens were localized mainly in normal-looking nerve bundles more than extraneural foamy macrophages in about half of the fresh BL/LL skin biopsies. (B) Perineural lymphocytic cuffing around the normal *M. leprae*-free nerve bundles was common in BT skin biopsies. (C) Waxy degeneration of nerve bundles was rare in fresh BL/LL skin biopsies. (D) A radial cutaneous nerve taken from a fresh BL case with clinically mild nerve damage showed an almost normal myelinated nerve population without inflammation. Careful comparison of conventional electron-micrograph and ultrastructural immunohistochemistry revealed the presence of *M. leprae* antigens only in the bacillary and degenerated structures of unmyelinated Schwann cell cytoplasm and macrophages, but not in the bacilli-like structures of myelinated Schwann cells or axons. The small cutaneous nerve described in (A) also showed the identical findings.

This study suggests that the unmyelinated Schwann cell is the initial target of *M. leprae*

infection, and sensory nerve tropism of *M. leprae* may be due partly to the distribution of unmyelinated Schwann cells. If our interpretation is correct, a considerable number of the pure neuritic leprosy cases in endemic areas should be classified as multibacillary leprosy and a more sophisticated therapeutic protocol, which can prevent intraneural reaction, should be planned for these patients.—[National Leprosarium Hoshizuka-Keiaien, Kagoshima; National Institute for Leprosy Research, Tokyo; Asahikawa Medical College, Japan]

Izumi, S., Kawatsu, K., and Maeda, Y. Development of immunological tools for diagnosis of leprosy in an early stage.

In the leprosy-endemic countries, leprosy is diagnosed by clinical examination and detection of leprosy bacilli by slit-skin smear. These techniques, however, are not enough to make a precise diagnosis of leprosy in an early stage. When a paramedical worker or doctor in a peripheral health center cannot make a final diagnosis, the patients usually are referred to a local hospital with better laboratory facilities, and the hospital doctors make a conclusive diagnosis. The presently available techniques, however, are not sufficient to make a reliable diagnosis of leprosy in an early stage. To overcome this, we have developed new techniques of immunohistopathology, a new axon staining method, and serological tools useful under the field conditions of leprosy-endemic countries. The applicability of our diagnostic techniques was tested by collaboration with leprosy hospitals in Pakistan, Indonesia, and Brazil. It was found that a better conclusive diagnosis can be reached when we fully use the data from these new techniques.—[National Institute for Leprosy Research, Tokyo, Japan]

Kaplan, G., Sampaio, E. P., and Sarno, E. N. The influence of thalidomide on the clinical and immunological manifestation of erythema nodosum leprosum.

In this report we show that circulating levels of tumor necrosis factor-alpha (TNF-α) are often associated with either systemic erythema nodosum leprosum (ENL) or more circumscribed inflammatory foci. The intensity of the inflammatory state of ENL is positively correlated with the highest levels of TNF-α. It is of interest that in some BL patients with subtle inflammatory lesions and in the absence of full-blown systemic toxicity TNF-α levels are elevated. In contrast, the majority of less-reactive, polar LL patients failed to show high TNF-α levels prior to multidrug therapy (MDT). The indolent inflammatory state of BL patients, in which cytokine levels are chronically elevated, may be of some concern for future damage to peripheral nerves.

It is also clear that some of the patients demonstrating only skin lesions of ENL do not have elevated levels of TNF-α in the serum. One suspects that in these cases cytokine production is occurring at the local tissue level but, for as yet unknown reasons, TNF-α is not being released into the circulation. The presence of soluble TNF-α receptors may serve to reduce the immunoreactive concentration of the cytokine in the serum. These and other modulatory factors will be examined in the future in this cohort of patients.

The effect of thalidomide in reducing the serum levels of TNF-α, ameliorating the local and systemic manifestations of ENL and inhibiting the egress of inflammatory cells into the lesions has been documented in a convincing fashion. Thalidomide in the *in vitro* environment is a selective inhibitor of TNF-α production. Similarly, in ENL patients, who are no doubt generating a spectrum of lymphokines and cytokines, it is the prompt reduction of TNF-α levels which can be correlated with clinical improvement. With an overall suppression of inflammation, the levels of other cytokines would eventually also reach basal levels.

The prompt beneficial effect obtained with thalidomide, steroids and cyclosporine A highlight the central role of the host inflammatory mediators in this syndrome. The importance of interferon-gamma (IFN-γ) in the regulation of cytokine production is suggested by the appearance of ENL in patients after intradermal injections of IFN-γ. We have already demonstrated the IFN-γ priming of blood monocytes for enhanced agonist-induced TNF-α release both *in vitro* and *in vivo*. Thus, IFN-γ in the sera of ENL patients could lead to the priming of patient monocytes, resulting in enhanced TNF-α

production and the induction of ENL. Since most of these patients demonstrate *Mycobacterium leprae* T-cell anergy, the source of IFN-γ in the serum is probably not *M. leprae*-specific activated TCR$\alpha\beta$ cells but rather natural killer or TCR$\gamma\delta$ cells. The role of these cell types in ENL is also under investigation.—[Laboratory of Cellular Physiology and Immunology, Rockefeller University, New York, New York, U.S.A.; Leprosy Unit, Oswaldo Cruz Foundation, Manguinhos, Rio de Janeiro, R.J., Brazil]

Makino, M., Yin, Y. P., Katsukawa, C., Kim, K. H., and Suzuki, Y. Studies of the alpha antigen gene family in *M. leprae.*

The alpha (α) antigen (or 85 complex) is one of the dominant mycobacterial proteins thought to be secreted from the bacteria. Both BCG and tuberculosis 85 complex have been reported to consist of three structurally related components designated as A, B, and C. But only one gene of the α antigen in *Mycobacterium leprae* has been cloned and reported.

By screening 10,000 plaques of the *M. leprae* Thai 53 strain genomic library, we obtained nine clones that hybridized with the digoxigenin-labeled probe-α-1. By physical mapping, they could be distinguished into two kinds of α antigen gene. We termed the gene encoded by one group as α1 gene; the other as α2 gene. The nucleotide sequence of the α1 and α2 genes of *M. leprae* were compared with homologs of other mycobacteria. Homology, at the nucleotide level, between *M. leprae* α1 and α2 antigens (73%) was lower than those between *M. leprae* α1 and α antigens of *M. bovis* BCG (81%) or 85B antigen of *M. tuberculosis* (78%). The same observation could be seen at the amino-acid level. We do not know what phylogenetic meanings these results have.

We also found many regions to be potentially *M. leprae*-specific epitopes on both the α1 and α2 antigens. The question of whether these regions would be *M. leprae*-specific epitopes or not must be elucidated further.—[Departments of Microbiology and Pathology, Osaka Prefectural Institute of Public Health, Osaka, Japan; Department of Microbiology, Kohsin Medical College, Pusan, Korea]

Matsuo, E., Komatsu, A., Maekawa, S., Matsushita, A., Sumiishi, A., Sasaki, N., and Skinsnes, O. K. On the common immunogenicity of pisum sativum derived fraction to the receptor for beta-glucuronidase (BGR) of a *Mycobacterium,* HI-75.

Our previous studies suggested that *Mycobacterium leprae* grow in peripheral nerves and lepra cells because they metabolize hyaluronic acid and use its component for their growth with the aid of the host β-glucuronidase combined to its receptor (BGR). The possibility of the growth disturbance of *M. leprae* by the elimination of the BGR function by immunological means also was suggested. In order to prove that by experiment, however, sufficient BGR or its alternative is required.

In this study, therefore, we first examined some properties of BGR to help in developing purification methods. Secondly, the pisum sativum extract (PSE) was examined as a possible alternative immunogen to BGR from a strain of *Mycobacterium,* HI-75, originating from a leproma and maintained by cultivation. As a result, the preparatory electrophoresis separated BGR as an isolated peak. Molecular sieving of BGR with marker proteins was not successful in isolating BGR and, therefore, was useless for the determination of its molecular weight. However, the molecular weight was determined to be 11,990 by liquid chromatomass spectrometry. Immunization of affinity-separated PSE produced anti-BGR antisera in all three rabbits and titrated up to 25,600× at the end of 2 months. The results indicate: a) preparatory electrophoresis can allow good separation of BGR unlike molecular sieving and b) pisum sativum provides an alternative source of BGR.—[Department of Pathology, Kyorin University School of Medicine, Tokyo; Tohoku Shinseien, Miyagi, Japan; Sun Yat-Sen University of Medical Sciences, Guangzhou, China]

Matsuoka, M., and Dayanghirang, J. A. Dissociation between tolerance to *M. leprae* antigen and bacillary multiplication in mice.

Susceptibility against *Mycobacterium leprae* was examined in mice with depressed

delayed-type hypersensitivity (DTH) reactions which were generated by intravenous (i.v.) injection with heat-killed (HK) *M. leprae* according to the early studies of Shepard, *et al.* C57BL/6 mice were injected i.v. with 1.0×10^7 or 1.0×10^9 HK *M. leprae* prior to foot pad infection with the bacilli, and the effects on the bacillary growth in the foot pads and subsequent host reactions were investigated. Foot pad swelling at the inoculated site, based on cellular reaction, was depressed by i.v. injection with 1.0×10^7 HK *M. leprae* in the mice infected with 1.0×10^4 or 1.0×10^7 bacilli. The effects of the treatment on other host reactions, DTH, and enlargement of popliteal lymph nodes were vague with this dose. No enhancement of the bacillary growth in the foot pad was indicated. Marked suppression of host reactions were induced by i.v. administration of 1.0×10^9 HK *M. leprae.* Foot pad swelling, DTH, and weight of lymph nodes were 0.21 or 0.28 mm, 0.16 or 0.19 mm, and 14.8 or 27.6 mm, respectively, at 20 weeks postinfection in mice without treatment and infected with 1.0×10^7 bacilli. These host reactions were decreased to a negligible level by the i.v. injection of HK bacilli. Despite the definite deficiency of the host reaction considered as the parameter of protective immunity, no prominent bacillary growth was exhibited in these immunologically deficient mice. Small lymph nodes at 65 weeks postinfection in the mice treated with i.v. injection suggested long-lasting suppression of the host reaction by the treatment, although the bacillary numbers recovered from the foot pads were the same as those of nontreated mice.

The results confirm the early study of suppressed DTH by i.v. administration, and indicate dissociation between protective immunity and host reactions as a parameter of immunity. The existence of a different subpopulation of T cells was suspected to be the possible mechanism of the phenomenon.—[National Institute for Leprosy Research, Tokyo, Japan; Dr. Jose N. Rodriguez Memorial Hospital, Caloocan, The Philippines]

Pessolani, M. C. V., Suzuki, A. E., and Brennan, P. J. Further molecular definition of the leprosy bacillus; integration of the results of protein mapping and genome sequencing.

The results of two major research endeavors, sequencing of the *Mycobacterium leprae* genome and characterization of its major antigens, are now merging to produce the type of information on the composition of the bacillus once considered unattainable. In this study, the analytical power of two-dimensional gel electrophoresis and established procedures in amino-acid microsequencing were combined to establish the N-terminal amino-acid sequences of six new proteins produced by *in vivo*-grown *M. leprae.* Chromosome sequences emerging from the *M. leprae* genome project allowed the identification of one of these, a 15-kDa cytosolic protein, as the *M. leprae* L7/L12 ribosomal protein homolog. Likewise, chromosome sequence allowed the full characterization of a 22-kDa protein as the *M. leprae* bacterioferritin equivalent (unpublished data supplied by Dr. S. T. Cole, Pasteur Institut, Paris, France). A new 23-kDa cytosolic protein also was identified as the *M. leprae* homolog of Avi-3, a protein originally defined as *M. avium* specific. This report is but one example of the sheer excitement and enormous potential of the *M. leprae* genome project with the capability to help explain many of the great puzzles of leprosy, such as the nature of the antigens expressed *in situ,* incapability of *in vitro* growth, tissue and cellular tropism, host persistence, and propensity for nerve damage.—[Department of Microbiology, Colorado State University, Fort Collins, Colorado U.S.A.]

Saito, H., Tomioka, H., Sato, K., and Hidaka, T. Therapeutic efficacy of benzoxazinorifamycin, KRM-1648, in combination with other antimicrobials against *M. leprae* infection induced in nude mice.

Multidrug therapy consisting of rifampin (RMP), clofazimine (CFZ), and diaminodiphenylsulfone (DDS, dapsone) is considered to be the most effective treatment for patients of leprosy. However, the development of new protocols which enable more rapid therapy for leprosy patients that contain other types of antileprosy drugs is con-

sidered desirable. The new benzoxazinorifamycin derivative, KRM-1648 (Kaneka Corporation, Hyogo, Japan), has excellent *in vitro* and *in vivo* antimycobacterial activities, and is much more potent than RMP. In this study, *in vivo* anti-*Mycobacterium leprae* activity of KRM-1648 was evaluated in combination with DDS, CFZ, and clarithromycin (CAM).

When drug susceptibility testing was done using the BACTEC 460 TB system, KRM (0.01 μg/ml), CAM (0.02 μg/ml), CFZ (0.5 μg/ml), and DDS (2.0 μg/ml) alone exhibited significant anti-*M. leprae* activity showing 80%, 40%, 53% and 41% reduction in GI values on day 18, respectively. Combinations of KRM with either CFZ, DDS or both caused a slight increase in the efficacy as compared to KRM alone on days 11, 18, and 27. However, KRM + CAM showed no combined effect.

For an experimental infection test, 1×10^6 of *M. leprae* Thai-53 were injected subcutaneously into the left hindfoot pad of female BALB/c nude mice. Test drugs emulsified in 0.1 ml of 2.5% gum arabic-0.1% Tween 80 solution were given by gavage, once daily 6 times per week, from day 31 to 80. When the number of acid-fast bacilli (AFB) in the left hindfoot pad was counted 360 days after infection, the following results were obtained. *In vivo* anti-*M. leprae* activity of KRM (0.001 mg/mouse) was intensified by combined use of either DDS (0.2 mg/mouse) or CFZ (0.1 mg/mouse) when compared to the efficacy of each drug alone. The combination of KRM + DDS + CFZ exhibited the most potent efficacy. In contrast to this, a significant combined effect was not noted between KRM (0.001 mg/mouse) and CAM (1.0 mg/mouse), although they alone exhibited proper therapeutic efficacy in these doses and protocol.

This study revealed that *in vivo* anti-*M. leprae* activity of KRM can be enhanced when combined with other agents, such as DDS and CFZ, compared to the efficacy of each drug alone. This indicates the usefulness of KRM in multidrug regimens for the clinical control of bacilliferous leprosy patients. Since KRM possesses remarkably more potent antileprosy activity *in vivo* than RMP, KRM seems to be preferable to RMP in multidrug regimens for clinical control of leprosy patients, given that it has a similar toxicity, pharmacokinetics and probable cost compared to RMP.—[Shimane Medical University, Izumo; Biochemical Research Laboratories, Kaneka Corporation, Takasago, Japan]

Shannon, E. J., Sandoval, F., McClean, K., and Howe, R. C. Thalidomide does not affect selected immunomodulating cell surface receptor molecules on cells with immune potential nor does it affect synthesis of selected cytokines from these cells.

The uncertainties concerning the pathogenesis of erythema nodosum leprosum (ENL) are underscored by the unknown mechanism of thalidomide's (Thd) therapeutic effect in ENL. *In vivo* it is known that patients experiencing ENL have: an increase in responsiveness to dinitrochlorobenzene; an increase in the percent of CD4+ cells in blood and in reactive skin lesions, and an increase in HLA-DR molecules on keratinocytes in reactive skin lesions. In *in vitro* assays lymphocytes from patients with ENL have an increase in responsiveness to mitogens and respond to *Mycobacterium leprae* antigens in culture. Since these manifestations are associated with parameters of cell-mediated immunity (CMI) and are confined to the acute phase of the reaction, it has been suggested that ENL is a consequence of an activation of the CMI cascade.

We postulated that a reduction in the number of CD4+ cells detected by fluorescent microscopy in ENL patients treated with Thd could be related to the ability of Thd to: modify CD4 molecules and prevent binding of fluorescein-conjugated anti-CD4 monoclonal antibodies (mAB); or to significantly reduce the density of CD4 molecules on the surface of CD4+ cells.

Thd did not modify CD4 molecules on the surface of mononuclear cells nor did it reduce the density of CD4 molecules on the membrane of mononuclear cells. Thd did not influence the expression of recombinant human interferon gamma (rhIFN-γ)-induced cell surface HLA-DR molecules on THP-1 monocytes.

The synthesis of interleukin-1 (IL-1) by *Escherichia coli* lipopolysaccharide (LPS)-stimulated monocytes derived from ten

healthy males was detected by the concanavalin-A (ConA)-mouse-thymocyte-culture assay and an IL-1β capture ELISA. In comparison to 10^{-3} M antiinflammatory drugs such as prednisone, prednisolone, and dexamethasone, Thd at 10^{-3} M did not inhibit the synthesis of IL-1 or IL-1β.

The synthesis of IL-2 was induced in mononuclear cells in culture with 4 or 0.4 or 0.04 μg/ml of ConA (optimum, midrange, and minimum stimulatory concentrations, respectively). The addition of Thd at 10, 1, and 0.1 μg/ml at the initiation of the 3-day ConA-stimulated cultures did not alter the ability of the cells to incorporate ^{3}H-thymidine. As determined by the ability to support the growth of the IL-2-dependent cell line CTLL-2, there was no reduction in fluid-phase IL-2 at its maximum detection level after 24 hr in the culture supernatant.

The synthesis of tumor necrosis factor (TNF) has been shown to be inhibited by Thd. Employing a similar assay system, we occasionally have observed Thd to cause marginal inhibitory effects; however, in general, we observed enhancing effects on the synthesis of TNF-alpha (TNF-α).

The mechanism(s) by which thalidomide attenuates ENL is not thoroughly understood. Its broad clinical usefulness as a sedative, analgesic, immunosuppressant and antiinflammatory drug mandates continued studies.—[GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, Louisiana, U.S.A.; Armauer Hansen Research Institute, Addis Ababa, Ethiopia]

Sieling, P. A., Mehrali, M., Porcelli, S., Brenner, M. B., Rea, T. H., and Modlin, R. L. CD1b restricts the response of $\alpha\beta$+, CD4−, CD8− T cells to *M. leprae*.

Several populations of T cells have been shown to function in cell-mediated immunity (CMI) to infection. CD4 T cells produce interferon-gamma (IFN-γ) and increase the antibactericidal activity of macrophages. CD8 cytotoxic T cells lyse infected cells, leaving the pathogen unprotected from ingestion by activated macrophages. $\gamma\delta$ T cells, found in increased numbers in some infectious disease lesions, appear to act early in CMI. We examined CD4−, CD8−, $\alpha\beta$+ T cells, at the site of human infectious disease, using leprosy as a model. Our data indicate that antigen-specific CD1-restricted DN T cells play a role in the immune response to infection.

Two DN T-cell lines derived from peripheral blood mononuclear cells (PBMC) were found to be *Mycobacterium leprae*-specific and CD1b-restricted. *M. tuberculosis*-reactive DN T cells previously have been shown to be CD1b restricted as well. However, the biological significance of these DN T cells has not been determined. CD4−, CD8−, $\alpha\beta$+ T cells were identified in the lesion of a leprosy patient, suggesting that DN T cells function in CMI to *M. leprae* infection.

CD1a, b, and c expressions were induced by GM-CSF on monocytes of both tuberculoid and lepromatous leprosy patients as well as normal donors. These CD1+ monocytes were effective antigen-presenting cells (APC) for DN T cells in a nonMHC-restricted manner. CD1 is normally expressed on Langerhans' cells (LC) and other interdigitating cells of tissue. LC and CD1+ dendritic cells have been shown to present antigen to CD4+ T cells. Therefore, LC and dendritic cells may be populations of APC in skin that can present antigen to both CD4+ and DN T cells.

In order to assess the biological role for CD1 in human infectious disease, the levels of CD1 in leprosy lesions were determined. CD1 was detected at higher levels in tuberculoid lesions than in lepromatous lesions. The present data, however, also demonstrate that monocytes of both tuberculoid and lepromatous patients can be induced by GM-CSF to express CD1. Tuberculoid lesions express higher levels of GM-CSF than lepromatous lesions and, thus, the necessary stimulus for CD1 expression is present in tuberculoid but not in lepromatous lesions. The increased level of CD1 in tuberculoid lesions may, therefore, influence the immune response to *M. leprae* infection by providing more effective APC for *M. leprae*-reactive DN T cells.

Our preliminary data indicate that the functional role of $\alpha\beta$, CD4−, CD8− T cells may be the local release of GM-CSF and IFN-γ. Production of GM-CSF and IFN-γ by DN cells at the site of infection would induce the expression of CD1 and MHC class II, respectively, thereby promoting

T-cell activity against *M. leprae* by CD4−, CD8−, $\alpha\beta$+ T cells and CD4+ T cells at the site of disease. In conclusion, the identification of DN T cells in the active lesions of leprosy patients indicates that this population of T cells functions in CMI against a human pathogen.—[U.C.L.A. School of Medicine, Los Angeles, California; Harvard Medical School, Boston, Massachusetts; U.S.C. School of Medicine, Los Angeles, California, U.S.A.]

Sugita, Y., Koseki, M., Murakami, K., Narita, M., Kim, S., Ishii, N., and Nakajima, H. A simple diagnostic system for leprosy using polymerase chain reaction.

A simple diagnostic system for leprosy has been developed. This system consists of DNA extraction, polymerase chain reaction (PCR), and the detection of probe hybridization on an ultrafiltration membrane. DNA for PCR analysis was obtained from a needle used for skin-smear examination. A set of primers [5′-TACCGACATTTCCGCGATAAAGTCGGCA-3′,5′-CGTCAACACATCGTCAGTAGA-3′] were derived from the gene encoding heat-shock protein (hsp) 70 in *Mycobacterium leprae* DNA. The PCR primers were selected from the parts showing low amino-acid sequence homology among the hsp 70 family. A 157-bp DNA was amplified from clinical samples, and sequencing analysis revealed identity with the published data for the *M. leprae* hsp 70 gene. The detection of hybridization between the amplified DNA and the biotin-labeled oligonucleotide probe [5′-biotin-GGGCAGGCGATCTATGA-3′] was carried out on an ultrafiltration membrane using an enzymatic color reaction. This diagnostic system does not require skin biopsy or conventional Southern blot analysis. In the future, similar improvements might be employed for the PCR diagnosis of leprosy, and an early diagnosis will be done even in leprosy-endemic areas where special equipment and experienced technicians are unavailable.—[National Sanatorium Tama-Zenshoen, Tokyo; Department of Dermatology, Yokohama City University School of Medicine, Yokohama, Japan]

Tantimavanich, S., Nagai, S., Nomaguchi, H., Kinomoto, M., Ohara, N., and Yamada, T. Immunological properties of ribosomal proteins from *M. bovis* BCG.

Although some possible roles of ribosomal vaccines have been postulated, no data have been presented about immunogens in ribosomes. In this work, some proteins were identified as important immunogens in ribosomal preparations. The 65-kDa heat-shock protein (HSP) is tightly associated with ribosomes. The 12-kDa protein analogous to HSP GroES is loosely associated with ribosomes. The 16-kDa ribosomal protein in the 50S subunit induced a strong delayed-type hypersensitivity reaction. The 17-kDa protein revealed a sequence (AXTLP VQDXP) quite similar to that of the α-crystallin family of low-molecular-weight HSP. These immunogens may be either tightly or loosely associated with ribosomal RNA which possesses a complicated stem structure and potent adjuvant properties.—[Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand; Toneyama Institute for Tuberculosis Research, Osaka City University Medical School, Toyonaka; National Institute for Leprosy Research, Tokyo; Department of Bacterial and Blood Products, National Institute of Health, Gakuen, Tokyo; Nagasaki University School of Dentistry, Nagasaki, Japan]

Truman, R. W., Kumaresan, J. A., and Alexander, S. A. Geographical information systems for selecting cohorts in analysis of the origins and risks of leprosy among wild armadillos.

A leprosy-like disease was first reported among wild armadillos in 1975. Since that time the etiologic agent has been confirmed to be *Mycobacterium leprae,* and armadillos are now viewed as highly endemic natural hosts of leprosy. How armadillos became infected with *M. leprae* and what possible risks infected armadillos present to man have been unclear. Our studies address those issues. We surveyed for leprosy among armadillos at locations across the south-central U.S.A. and elsewhere. By the end of the field studies, we had sampled nearly 1400 armadillos and evaluated their population characteristics in many of the sites. Like others before us, we found leprosy to be

widely distributed among armadillos in the state of Louisiana. Excluding those sites where sampling was less significant, antibody prevalence rates ranged from 8%–24%. Armadillos were most plentiful in low-lying areas. Their density declined with movement inland from the coast and comparison of prevalence rates between these habitats showed marginally significant differences. In Texas, we found an antibody prevalence rate of 17.5% among the armadillos taken near Corpus Christi, and the rate was similar to what we observed in similar habitats in Louisiana. But no serologically positive animals were found among the 69 armadillos taken from outside Lawton, Oklahoma. Others had previously found 1/61 histopathologically positive armadillos near Palestine, Texas (approximately 125 miles southeast of Lawton), and the histopathological prevalence rate for armadillos along the Texas Gulf Coast had been estimated at 4.5%. Armadillos expanded their range into Oklahoma around the same time that they populated northern Louisiana, and the significant differences in antibody prevalence rates between these locales suggest that environmental factors largely influence the distribution of the infection. The literature contains reports totaling approximately 27/853 histopathologically positive armadillos in Texas. Among these, 24/465 infected animals were found along the coast and only 3/388 infected armadillos have been found more than 30 miles inland. Somewhat similarly, we and others have found no evidence of leprosy among armadillos sampled in Florida, and note a significantly reduced rate of disease among armadillos in the state of Mississippi (Woodville, 5%). Other than these cases reported here only 2 other infected armadillos have been found among the 224 armadillos examined in Mississippi.—[GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, Louisiana, U.S.A.]

Williams, D. L., Waguespack, C., Gillis, T. P., Eisenach, K., Crawford, J., and Portaels, F. Characterization of rifampin-resistant mutations in pathogenic mycobacteria.

The development of a rapid DNA diagnostic assay for the specific detection of rifampin-resistant tuberculosis and leprosy could facilitate the effective therapy for those individuals harboring rifampin-resistant (Rif-r) mycobacteria in a shorter time than by the use of conventional drug-susceptibility testing and radiorespirometric methods. To develop such an assay requires knowledge of the molecular basis of rifampin resistance in pathogenic mycobacteria. The majority of mutations which result in the Rif-f phenotype in prokaryotes have been mapped to a 250 bp (Rif) region of the rpoB gene encoding the β-subunit of the DNA-dependent RNA polymerase. Recently, the entire rpoB gene of *Mycobacterium leprae* and part of the rpoB gene in *M. tuberculosis* have been sequenced, and several Rif-r mutations have been identified in both species. We have developed a rapid DNA sequencing protocol to study rifampin resistance in pathogenic mycobacteria. Using the *M. tuberculosis* rpoB sequence, we have synthesized PCR primers which amplify a 305 bp fragment encompassing the 250 bp Rif region in mycobacteria. Using the amplimers as sequencing primers and a direct DNA sequencing protocol, we have determined the nucleic acid sequence and deduced the amino-acid sequence of this region in several rifampin-susceptible (Rif-s) and Rif-r strains of *M. leprae* and *M. tuberculosis.* In addition, we also have sequenced this region in Rif-s and Rif-r strains of *M. avium,* a common pathogen of AIDS patients. Subsequently, we have identified Rif-r mutations in all strains analyzed.

In all Rif-r strains analyzed point mutations were observed within a 48 bp region of the 305 bp fragment of the rpoB gene. All mutations were found in bases encoding three amino acids: Ser531 (50%), His526 (37.5%), and Asp516 (12.5%). In *M. leprae* and *M. tuberculosis* mutants, C-T transitions occurred in the Ser531 codon, changing a serine residue to a leucine. In the *M. avium* mutant, a C-G transition occurred in the Ser531 codon, resulting in a change from a serine residue to a tryptophan. Other mutations found in *M. tuberculosis* were located in the Asp516 codon, changing the aspartic acid residue to either a valine or glycine, and in the His526, changing the histidine residue to either a tyrosine or aspartic acid. Most of these mutations have been reported previously in Rif-r *M. leprae* and

M. tuberculosis strains obtained from other sources. In addition the Asp516 mutation, which resulted in a change of the aspartic acid residue to glycine (A-G), has not been reported previously.

Comparisons of secondary structure predictions for deduced amino-acid sequences from Rif-r and Rif-s rpoB polypeptides using the Chou-Fasman and Hopp-Woods algorithms showed that the serine-to-leucine substitution profoundly increased the hydrophobicity of this region, possibly contributing to the antibiotic resistance observed in this strain. A point mutation in the same codon in *Escherichia coli* changes the serine residue to a phenylalanine in the Rif-r mutant strain. This mutation has been characterized as the genetic basis of rifampin resistance in this mutant. All other Rif-r mutations described in this report also appear to change the hydrophilicity of this region.—[GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, Louisiana; Veterans Affairs Medical Hospital, Little Rock, Arkansas; Centers for Disease Control, Atlanta, Georgia, U.S.A.; Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium]

Wolucka, B. A., de Hoffman, E., Chojnacki, T., Kalbe, L., Cocito, C., McNeil, M. R., and Brennan, P. J. Identification of the carrier lipid for arabinan biosynthesis in mycobacteria.

We have little comprehension of the biogenesis of mycobacterial cell walls, and yet they are the site of action of many antituberculosis drugs and the cause of much of the pathology associated with mycobacteria. A family of glycosylated polyprenylphosphates was isolated from *Mycobacterium smegmatis* which contained arabinose, ribose, mannose, and glucose. The isoprenoid nature of the lipid component was established by ^{1}H-NMR. FAB-MS demonstrated the predominance of C50 decaprenyl-P with much smaller amounts of the C35 octahydroheptaprenyl-P. Decaprenyl-P-D-arabinose was isolated from this family and identified by ^{1}H-NMR, FAB-MS and DEI-MS analysis of the phospholipid portion, and GC-MS of the sugar. The *cis/trans* geometry of the mycobacterial decaprenol was established as di-*trans*, poly-*cis* prenol. The identification of decarpenyl-P-arabinose, the obvious transmembrane carrier of cell-wall D-arabinase, provides the first clue to the biosynthesis of the arabinan component of cell-wall arabinogalactan and lipoarabinomannan. Ethambutol, a powerful antituberculosis drug known to inhibit arabinan biosynthesis, results in the accumulation of decaprenyl-P-arabinose, indicating that its site of action is in the transfer of arabinose to unknown acceptors in cell-wall synthesis.—[Microbiology and Genetics Unit, Institute of Cell Pathology, University of Louvain Medical School, Brussels, Belgium; Department of Microbiology, Colorado State University, Fort Collins, Colorado, U.S.A.; Mass Spectrometry Unity, University of Louvain, Louvain-la-Neuve, Belgium; Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland]