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THIRTIETH U.S.–JAPAN TUBERCULOSIS RESEARCH CONFERENCE LEPROSY RESEARCH CONFERENCE AND TUBERCULOSIS/LEPROSY SYMPOSIUM

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OPENING REMARKS

On behalf of the U.S. Leprosy and Tuberculosis Panels, my colleagues here at Colorado State University, and the National Institutes of Health, it is my great privilege and pleasure to welcome members of the Japanese Leprosy and Tuberculosis Panels, led by Drs. Saito and Tsuyuguchi, and their guests and all guests to Fort Collins for the 30th Joint U.S.–Japan Conference on Tuberculosis and Leprosy. Like several of you here today, I have been associated with this Program for almost 20 years, so it is time to try to return the favor for all of the good times.

It is interesting to note that throughout the 30 years of the existence of the U.S.–Japan Cooperative Program, not one Panel or research area has been subtracted. In other words, throughout these years there has been the constant addition of new, important research topics. However, this tradition ends this year and at this meeting with the full amalgamation of the leprosy and tuberculosis panels. Leprosy is emerging as

one of the great success stories in infectious diseases, with a massive decrease in patient load worldwide and with plans afoot for the elimination of the disease as a major public health problem, and also with plans being formulated for its final eradication. Panel members—both leprosy and tuberculosis—and, indeed, delegates to the parent U.S.–Japan Cooperative Program and, also, most research workers are now satisfied that leprosy research will prosper within an amalgamated tuberculosis and leprosy panel. However, an important task of the Joint U.S. and Japanese Panels, both tuberculosis and leprosy, when we meet on Thursday will be to recommend to the U.S.–Japan Delegation a charter that will ensure the preservation of leprosy research for years to come.

At this meeting, we have an exceptional turnout of leprosy and tuberculosis researchers (154 in total, up from an original estimate of 60), and this has resulted in crowding here in this lecture hall and a shortage of hotel rooms, and we apologize. We have assembled an exciting roster of topics to discuss, such as, for instance, the tissue culture cultivation of *Mycobacterium leprae,* new approaches to the alleviation of nerve damage and to the diagnosis of leprosy, description of new antigens and new information on the genome of *M. leprae* and *M. tuberculosis,* exciting information on the physiology of *M. tuberculosis,* new data on the epidemiology of tuberculosis, on protective immunity and immunopathogenesis. Not only are there oral presentations, but we also have lined up about 40 posters. This is a concept—this idea of including posters—which we have tried previously at meetings in Seattle and in San Diego but, on this occasion, we take this one step further, with greater numbers and greater sophistication.

Of course, the beginning of a meeting is not the time to thank people and to acknowledge all the help that we have had. But just the act of getting you here required much effort, regardless of how the meeting turns out. So I would like to thank some of the people who have helped us get this far, in particular, Dr. Darrel Gwinn, who has orchestrated these meetings for 16 or 17 years and is responsible for much of the planning, particularly for the scientific content. Also, Gordon "Hap" Hazard of the CSU Conference Services Offices made many of the local arrangements. Marilyn Hein has helped tremendously, and Jack Hurdelbrink is responsible for the planning of much of the social activities and the planning for transportation, particularly of the Japanese guests. I should mention Ian Orme and the people in his lab, John Belisle and the people in his lab, Del Besra and his co-workers, Julie Inamine and her co-workers, Mike McNeil and his co-workers, Delphi Chatterjee, and my own co-workers who have participated in various aspects of this planning.

There are two very special features about the relationship between the United States and Japan. Number 1 is Hideo Nomo, the amazing rookie Japanese pitcher now with the Dodgers. Japanese guests, tell your fellow Japanese that we love him and thank you for sending him to the Western Division of the National League, so that we can see him play in Denver. The Number 2 feature in the relationship between our countries is the United States–Japan Cooperative Medical Sciences Program. The Cooperative Program was founded 30 years ago, in 1965, by President Lyndon Johnson of the United States and Prime Minister Sato of Japan. In April 1965, medical advisors from our two countries met in Tokyo and selected cholera, leprosy, parasitic diseases, tuberculosis, and viral diseases as the initial subjects for joint research. In subsequent years, malnutrition, immunology, hepatitis, and environmental mitogenesis and carcinogenesis and, recently, AIDS were added as Panel topics. Throughout these 30 years, some of the greatest scientists in the areas of tuberculosis and leprosy have been Panel members or participated in Panel meetings. Among leprosy researchers, figures such as Drs. Namba, Abe, Ito, Namamura, Ishiyura, Yoshi; and Drs. Shepard, Bloom, Bullock, Jacobson, Morrison, Binford, Hastings, Rea, Krahenbuhl, and Meyers have been Panel members or contributed to this Program, and among TB researchers, eminent scientists such as Drs. Yamamura, Kato, Shimao, Someya, Azuma; and Drs. Ribi, Chaparas, Wolinsky, Salvin, David, Collins, McInnis, Goren, Myrvick have been Panel members and contributors. Indeed, it is wonderful to wel-

come back many of these stalwarts of past leprosy and tuberculosis Panel meetings to this 30th meeting in Fort Collins. Accordingly those of us here today, as participants in this 30th meeting, are part of a long and auspicious Program and a wonderful tradition.

It is an adventure to have the U.S.–Japan meeting in the hinterland of America. So, look at this meeting as a kind of adventure and have a good time.

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

ABSTRACTS*

Adams, L. B., Gillis, T. P. and Krahenbuhl, J. L. Regulation by arachidonic acid metabolites of the immune response toward pathogenic mycobacteria.

The induction of enhanced local macrophage arachidonic acid (AA) metabolism by *Mycobacterium leprae* or its constituents in lepromatous (LL) leprosy granulomas could profoundly affect the function of the afflicted and surrounding cells and tissues, both T cells and macrophages, and yet have little influence on cells in the rest of the body. In our own model of *in vitro* perturbation of the macrophage function in experimental leprosy, enhanced PGE_2 production was antagonistic to the effects of IFN-gamma (IFN-γ) as measured by metabolism, Ia presentation and microbicidal and cytotoxic effector function of the macrophage. Infection of macrophages with large numbers of viable *M. leprae* results in a burst of PGE_2 production which, in turn, downregulates the ability of the infected macrophages to be activated to a microbicidal state by treatment with IFN-γ. Treatment of normal macrophages with PGE_2 mimics this effect and inclusion of the COX inhibitor, indomethacin, reverses the effects of *M. leprae* infection on the macrophages. Macrophages from the nu/nu mouse LL granulomas are similarly refractory to IFN-γ-activation and produce large amounts of PGE_2 in culture.

We have made numerous attempts to reconstitute *M. leprae*-infected nu/nu mice fed a diet of standard mouse chow with highly activated, T-cell-enriched immune cells in classic adoptive transfer experiments. The endpoint in these experiments (i.e., determination of the viability of *M. leprae* harvested from the foot pad) generally exhibited great variability with little demonstrable inhibition of the bacilli. In the *M. leprae*-infected nu/nu mouse, large amounts of PGE_2 produced locally in the foot pad could potentially downregulate the immune competence of an adoptively transferred, highly activated T-cell population, rendering it incapable of cytotoxic functions or of activating monocytes. Alternatively, this T-cell population could retain its helper functions and activate monocytes which, in turn, could be downregulated by the elevated PGE_2 levels as they migrate into the foot pad. We have shown previously that there is a substantial infiltration of monocytes into the *M. leprae*-infected foot pad, with 15%–20% of the cells newly arrived. Furthermore, the influx of monocytes into the foot pad nearly doubles upon administration of IFN-γ.

The seemingly contradictory data obtained in *M. tuberculosis*-infected mice (i.e., decreased resistance upon elimination of AA) is not easily explained. However, the numerous dissimilarities between these two models of infection, including the sites of infection, the toxicity of the organisms, the tremendous difference in growth rates between the two species of bacilli, the capability of AA metabolism in the host upon initial infection, and the environment in which the immune response develops, do not readily lend these models to comparison. Nevertheless, these data suggest that local macrophage AA metabolism, especially COX activity, induced by pathogenic mycobacteria or their constituents in the microenvironment of the granuloma can mod-

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

ulate the local immune response.—[Laboratory Research Branch, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA, U.S.A.]

Brennan, P. J., Donahue, S. A., Scott, D. K., Rivoire, B. L. and Walsh, G. P. A plan for preparation and application to humans of new leprosy skin-test antigens.

It is agreed by most of those contributing to the global leprosy elimination program that the most important contribution from current research endeavors will be "Tools to identify subclinical infection of sufficient sensitivity and specificity . . . to facilitate epidemiological monitoring of the disease in the community." Clearly serological and gene amplification approaches have not met the demanding requisites of such epidemiological tools in terms of specificity, sensitivity and ease of operation. We feel that the remaining hope lies in new skin-test antigens. The products from some years ago, Lepromin H, Dharmendra lepromin, and Lepromin A, and those obtained more recently from fractionated *Mycobacterium leprae,* Convit's SDA and Rees' MLSA, have their place in leprosy control but fail as universal diagnostic/epidemiological tools; SDA and MLSA meet the ideal for potency but fail in terms of sensitivity and specificity. Yet the quest for more useful reagents must lie in the continuing fractionation and identification of appropriate antigens of *M. leprae* and testing of products initially in guinea pigs and then in humans. The approach cannot be long term, since leprosy is declining, and must be directed to immediate human application and, hence, must involve manufacture under GMP/GLP conditions and IND applications through the FDA. Our approaches and the challenges are as follows:

FDA approval for Phase I trials has three major aspects to consider. The most important consideration is product safety. Therefore, skin-test antigens must be produced under Good Manufacturing Practices (GMP). The antigens must also satisfy the requirements of potency and stability.

We are in the process of setting up a GMP/GLP facility in our own department for purposes of producing skin-test antigens. In order to be in compliance with GLP conditions, an entire documentation system is the first essential ingredient. A network of standard operating procedures (SOPs) is used to validate every piece of equipment and to control every step of the processing to ensure that performance is consistent, and that all antigens satisfy exact standards of quality control. The other aspect of GMP is the installation of "clean room conditions" to ensure safety of the product. An environmentally controlled workspace suitable for the aseptic processing of the skin-test antigens has been installed and consists of a suitably constructed, properly functioning, and regularly certified Laminar Airflow Workbench (LAFW), which sweeps the workspace with HEPA-filtered air at a velocity of 90 feet per minute ± 20%. The air blower of the sterile head has to be operated without interruption in order to sweep the workspace continually. The air quality within the LAFW adjacent to critical sites has to meet a Class 100 (MCB-1) clean room specification during normal work activity.

The air entering the Buffer Room must be fresh and HEPA-filtered. The air in this room should meet the requirements for a Class 100,000 clean room. The inflowing air must go through 10 air changes per hour. The level of cleanliness of the air in the Buffer Room, in conjunction with the expertise of the operator, will be critical in maintaining the Class 100 (MCB-1) conditions within the LAFW.

Tasks carried out within the Buffer Room will be limited to those for which a controlled environment is necessary. Only the furniture, equipment, supplies, and other goods required for the tasks to be performed may be brought into this room, and they should first be cleaned and sanitized by designated SOPs. The Buffer Room itself contains cement walls, and floors which are smooth, impervious and free from cracks and crevices and non-shedding, thereby promoting cleanability and minimizing spaces in which microorganisms and other contaminants may accumulate. The shelves are stainless steel, smooth and non-shedding. All surfaces are resistant to damage by sanitizing agents. There are no sinks or functioning floor drains.

The Anteroom is available for the decontamination of supplies, equipment, and personnel before they enter the Buffer Room.

There is only one sink. Non-shedding towels are available near the entrance to the Buffer Room so that personnel can scrub their hands and arms before donning non-shedding, sterile, eyes-only hoods, shoe covers, and clean gowns as per designated SOPs. The door to the Buffer Room must remain automatically positively closed.

Validation of the "Clean Room" will begin with the monitoring of the air. This will be accomplished qualitatively via settling plates, which are petri plates containing sterile Soybean Casein Digest media which are exposed to the environment at the designated critical areas. Viable organisms that settle on the media surface will grow after suitable inoculation. Quantitative environmental monitoring will be carried out via the use of a Personnel Sampling Pump and Cyclone Filter Assembly, which will inform us of the number of particulates in the Skin Test Antigen Manufacturing Facility air. Evaluation of surfaces will be carried out via the Wipe Test, which entails the use of sterilized swabs which are immersed in a Soybean Casein Digest liquid culture media using aseptic techniques. Swabs are then rubbed over the test surface and placed in the medium. The number of colony forming units, per unit area, will be quantified using standard microbiological techniques as plate counting. This type of rigorous control of the microbiological contaminates assures the safety of the final product.

Manufacture of the first generation of skin-test antigens. We have already introduced two new reactions, modified versions of earlier antigens for the purpose of increasing specificity/sensitivity. First, we have modified the Rees-type MLSA so as to remove immunosuppressive, crossreactive components (LAM, LM, and PIMs, and other lipids). Secondly, in extensive *in vitro* immunological studies and guinea pig DTH testing with Drs. B. R. Bloom, V. Mehra, and R. L. Modlin, we have demonstrated that the cell-wall proteins of *M. leprae* (which were discarded in the Rees and Convit preparations) are powerful immunogens. Accordingly, these cell-wall-derived antigens are called MLCwA. Both antigens are presently awaiting FDA approval.

The procedure for the manufacture of these skin-test antigens begins with the purification of the bacteria from infected armadillo tissues using the Draper 3/77 protocol. This protocol involved homogenization using dipotassium EDTA as a metal chelator, followed by alkali treatment for the removal of pigment, and overnight collagenase treatment for the degradation of tissue. The partially purified bacteria are then applied onto a two-phase separation system involving polyethylene glycol and Dextran T500. The hydrophilic tissues partition to the Dextran layer and hydrophobic bacteria to the PEG phase. Purified bacteria are examined microscopically for residual tissue and pigment contaminants. Quality control measures for sterility involve the inoculation of Brain Heart Infusion Broth, Blood Agar Plates and Lowenstein-Jensen 11 slants. The concentration of the final product is established by measuring absorption at 540 nm.

Preparation of the MLSA-LAM starts with the Rees protocol, exactly. *M. leprae* bacilli are sonicated in PBS and centrifuged twice at 4°C. The supernatant is then centrifuged at 105,000 × *g*, and this supernatant (Rees MLSA) is adjusted to 1 mg/ml PBS, to which precondensed Triton X-114 is added to a final concentration of 4%. This mixture is rocked gently at 4°C for 30 min and then placed in a 37°C water bath for 10 min to condense the detergent. After a low-speed centrifugation at 2500 × *g* for 10 min, the two phases separate, and the aqueous layer is removed and re-extracted two additional times. Lipoglycans and other hydrophobic molecules will partition to the detergent layer. The aqueous phase is applied to an Extracti-gel D column to remove residual detergent. The product is the skin-test antigen called MLSA-LAM.

Formulation of the MLCwA begins with the 27,000 × *g* pellet. The cell pellet is subjected to extraction with 2% SDS in PBS while stirring at 56°C for 1 hr. The suspension is centrifuged and applied to an Extract-gel D column to remove SDS. The suspension is Triton X-114 extracted as described above. An additional step of safety, performed on both antigens, is a terminal sterilization of 121°C for 20 min.

These antigens are then subjected to a battery of quality control assays. These include: 1) protein profiles by 1D-SDS-PAGE stained with silver nitrate; 2) Western blotting with several monoclonal antibodies; 3)

protein estimation by BCA assay; 4) endotoxin quantitation using the Limulus amebocyte assay; and 6) sterility determination.

The potency of these antigens is tested on guinea pigs. The outbred Hartley strain is sensitized by injection with 200–500 μg of autoclaved *M. leprae* suspended in Freund's incomplete adjuvant at the base of the neck. After 6–8 weeks, the skin-test sites are injected to the dorsal surface. Measurements of induration using calipers, taking two measurements on both diagonals, are performed at 24 and 48 hr. The experiment was designed to provide data on the dose response and specificity of MLSA-LAM and MLCwA compared to the Rees antigen. Results demonstrate that MLSA-LAM and the Rees MLSA elicit equal DTH responses at a concentration of 0.1 μg. The dose response curve suggests that MLCwA causes a DTH response at the lowest concentration tested, 0.04 μg/ml); whereas the Rees and MLSA-LAM did not respond at this low concentration. The antigens must stimulate a DTH response at 0.1 μg to pass quality control.

Antigen stability studies have been performed with a "mock run" preparation (batch 9). Each antigen was diluted with PBS to a concentration of 100 μg of protein per ml of diluent. Each antigen was then filtered and terminally sterilized. Then each sample was placed at −70°C (for controls), 4°C, 37°C, or at 56°C.

At 45 days after the preparation was manufactured, the samples were analyzed in the guinea pig potency test as described earlier. The concentrations tested were 1.0 μg and 0.1 μg of protein. The results were very encouraging. There was no significant difference in potency, leaving us a comfortable margin of time to get the antigen into the field.

After completing the manufacturing of the antigens, we will proceed to the Phase I/Phase II clinical testing. This will be conducted under the direction of Dr. Gerald P. Walsh, Director, Leonard Wood Memorial Leprosy Research Center, Cebu, The Philippines, an endemic area for leprosy, and Stander, The Philippines, a non-endemic area for leprosy. Part of Phase I clinical testing will also be conducted here in Fort Collins, Colorado, U.S.A. The protocols have been approved by the responsible Review Boards.

Phase II/Phase III trials. Assuming a satisfactory outcome from Phase I trials, we are planning Phase II/III trials for sites in Rio de Janeiro through the Oswaldo Cruz Foundation (Dr. M. C. V. Pessolani); Anandaban, Nepal (through Drs. W. Britton, P. Roche, R. Weir, R. Hussain, and H. Dockrell); Madras, India (through Dr. M. D. Gupta) and Malawi (through Dr. P. Fine). It is not yet certain whether products produced in our own GMP/GLP facility or one designated by NIH/FDA will be utilized in Phase II trials.

Toward more specific, sensitive skin-test antigens. We still question whether the reductionist approach is the best way to a more specific, sensitive product. Certainly a remarkable array of individual proteins of *M. leprae* have been resolved, characterized, expressed in recombinant form and are available for testing. However, results to date in support of this approach are not encouraging. We will review this question. Rather, we favor fractionation of MLSA-LAM and MLCwA. Preliminary results in this respect will be presented.—[Colorado State University, Fort Collins, CO, U.S.A.; Leonard Wood Memorial Leprosy Research Center, Cebu City, The Philippines]

Cho, S.-N., Kim, J.-D., Cellona, R. V., Balagon, M. V. F., Villahermosa, L. G., Fajardo, T. T., Jr., Abalos, R. M., Walsh, G. P., Thole, J. and Brennan, P. J. Association of antibodies to *M. leprae* and nerve antigens with lepra reactions in leprosy patients.

In this study, we investigated whether or not antibodies to *Mycobacterium leprae* or nerve antigens are associated with reversal and erythema nodosum leprosum (ENL) reactions in leprosy patients following WHO-MDT. In the literature, anti-PGL-I IgM antibodies decreased sharply during ENL reaction, thus suggesting that the antibodies are implicated in the pathogenesis of the ENL reaction. This study also showed that about two thirds of ENL patients had such marked decrease in IgM or IgG antibodies to PGL-I before or during ENL reaction. Due to the lack of subsequent serum sam-

ples just after ENL reaction in this study, we could not support the previous report that anti-PGL-I antibodies reached back to the original level after the subsiding of each ENL episode.

In relation to reversal reaction, there is not much information about antibodies to *M. leprae* or nerve antigens in serial samples from leprosy patients with reversal reaction. Unexpectedly, a marked decrease in IgM or IgG antibodies to PGL-I also was noted before or during reaction in about 65% of leprosy patients with reversal reactions. In contrast, there was no significant difference in changes of IgG levels to LAM or 45-kDa antigen between patients with and without reversal reaction, although the antibody levels markedly fluctuated during the course of MDT. The results again support that anti-PGL-I antibodies may also be implicated in the pathogenesis of reversal reaction in some leprosy patients.

Although reversal reaction has been associated with nerve damage in leprosy, there was not much change in antineural antibodies in leprosy patients during the course of MDT except in the IgM antibodies to ceramide. Antibodies to ceramide tend to decline before reversal reaction in leprosy patients and increase again after the reversal reaction, but not in ENL patients and patients with no reaction. Therefore, IgM antibodies to ceramide seemed to be associated only with reversal reaction. The reason why there is no significant change in antibodies to other nerve antigens, such as ASM1, Galc, and NF-enriched protein, in leprosy patients during chemotherapy remains to be explained. In mangabey monkeys experimentally infected with *M. leprae,* antibodies to ceramide, AGM1, and GalC declined after chemotherapy was initiated and remained low afterward. Therefore, it may be postulated that antineural antibodies were lowered in general even before diagnosis and seemed to remain at low levels throughout the course of MDT. Of interest, anticeramide antibodies increased steadily after reversal reaction in some patients. It is not known yet whether or not these antibodies result from nerve damage by reversal reaction. It also may be reasonable to speculate that patients who have an increase in antibodies to ceramide after reversal reaction are inflicted with nerve damage during reversal reaction, resulting in disability. The assumption needs to be evaluated by a long-term follow up of these patients, including appropriate controls.

Although serum samples were not obtained as frequently as possible before, during or after reaction in study patients, this study gave a rough picture of the dynamics of antibodies to *M. leprae* and nerve antigens in relation to lepra reactions in leprosy patients following MDT. Not surprisingly, antibodies to PGL-I, both IgM and IgG classes, declined sharply before or during reversal reaction, as shown in ENL reaction, more frequently than in patients with no reaction. One explanation for this phenomenon is that chemotherapy stopped PGL-I antigen production, thus lowering circulating PGL-I antigen produced by only live *M. leprae.* The clearance of circulating PGL-I may result in the recovery of T-cell responses to *M. leprae* antigens which are suppressed by the antigen in some patients. This resurrected cell-mediated immunity may induce reversal reaction, while patients with no reaction had no ability to recover T-cell responses from anergy to *M. leprae* antigens. Further studies are required to understand reversal reaction and its relevance to nerve damage in leprosy.—[Department of Microbiology, Yonsei University College of Medicine, Seoul, Korea; Leonard Wood Memorial Center for Leprosy Research, Cebu, The Philippines; Department of Immunohaematology, University Hospital, Leiden, The Netherlands; Department of Microbiology, Colorado State University, Fort Collins, CO, U.S.A.]

Fukutomi, Y., Matsuoka, M. and Minagawa, F. Cultivation of *M. leprae* in macrophages.

Mycobacterium leprae is an obligate intracellular pathogen, the causative agent of Hansen's disease. The organism was discovered more than 100 years ago. Since then, a large number of attempts to cultivate the bacilli have been made. One of the approaches to cultivate the bacilli is to use host cells which the bacilli inhabit, achieving similar conditions as *in vivo.* While there have been a few reports on successful *in vitro*

cultivation of *M. leprae*, no satisfactory confirmation of these reports has been published.

The inability to assess the viability of *M. leprae* has impeded progress in leprosy research for a long time. Recently, radiorespirometric assays were reported in which the monitoring of the metabolic activity of *M. leprae* was applied to evaluate the viability. Until the 1970s a major obstacle in studying leprosy was the difficulty in obtaining fresh and viable *M. leprae*. Moreover employment of the organism from human specimens may induce the growth of contaminating mycobacteria. To avoid such a situation, we have been using established strains of *M. leprae* maintained in nude mouse colonies for culture experiments.

In the present study, macrophages were infected *in vitro* with *M. leprae* obtained from nude mice followed by incubation in the presence of the suppressive cytokines IL-10 and TGFβ. The metabolic activity of the bacilli was examined at the end of the incubation period by lysing the infected macrophages. Results showed a significant increase in the metabolic activity of *M. leprae* in macrophages incubated for 1 month at 31°C in the presence of IL-10. In parallel, elongation of the bacilli was observed under the microscope after acid-fast staining of the culture. Neither the control culture nor the TGFβ-treated culture showed these phenomena.

We believe that the incidence of elongation and enhanced metabolic activity reported here will provide us with clues leading to the actual multiplication of *M. leprae* in tissue culture systems.—[National Institute for Leprosy Research, Tokyo, Japan]

Gillis, T. P., Williams, D. L. and Frothingham, R. Sequence analysis of the 18-kDa gene and the 16S-23S rDNA ITS region from strains of *M. leprae*.

Systemic attempts to define DNA polymorphisms in *Mycobacterium leprae* have not met with much success. While independent polymorphisms have been identified, a model system for identifying *M. leprae* strains has not been developed. Our group and others have attempted to define *M. leprae* strain differences by RFLP analysis, using numerous restriction enzymes and probes to detect polymorphisms. Most of the probes were developed from single-copy genes or from an *M. leprae*-specific repetitive element (RLEP) not known to contain characteristics of an insertion sequence. To continue our investigation of potential DNA sequence polymorphisms in *M. leprae* we compared the DNA sequence of a highly conserved gene (18-kDa protein) and a noncoding sequence [16S-23S rDNA ITS (internal transcribed spacer) region] from five geographically distinct strains. We report here no differences in the DNA sequence between the strains examined, supporting earlier studies suggesting minimal strain divergence in *M. leprae*.

DNA from all strains of *M. leprae* produced the expected size fragments for both the 16S-23S ITS region (480 bp) and the 18-kDa gene (360 bp) when analyzed by agarose gel electrophoresis. Variations in sequences between individual isolates were not detected in either the ITS region or the partial sequence obtained from the 18-kDa protein gene of the seven isolates.

Earlier studies using ITS sequence comparisons for 35 reference strains of the *M. avium* complex showed clear divisions of strains into 12 sequevars, each consisting of a unique ITS sequence. Since this complex of organisms is generally thought to be composed of at least two species, some degree of differentiation was not surprising. However, when the analysis was applied only to the subgroup of *M. avium* strains, 4 sequevars were discerned. In addition, phylogenetic trees based on ITS sequences were comparable to phylogenetic trees of this group based on the more constant sequences found in the 16S rDNA. In contrast to the differentiation of strains of the *M. avium* complex, *M. tuberculosis* complex strains showed no differences in ITS sequences when 13 strains were examined. This appears to be the case for *M. leprae* as well, since the seven strains of *M. leprae* we analyzed gave identical ITS sequences. The absence of sequence variation in the 18-kDa gene from the *M. leprae* strains analyzed here further supports the notion that *M. leprae* has maintained minimal sequence variation over time. Sequence conservation could be a result of stringent requirements for survival as an obligate intracellular parasite with few opportunities for recombi-

national events with other species. Other mycobacterial species, such as *M. avium,* can survive intra- and extracellularly and, therefore, may have evolved a more flexible genome as a consequence of varied environmental pressures and interaction with other microbial species and their DNA.—[Laboratory Research Branch, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA; Infectious Diseases Section, Durham Veterans Affairs Medical Center, Durham, NC, U.S.A.]

Goto, M., Kitajima, S.-I. and Sato, E. Expression of a neurotrophic factor midkine in the spinal cord of leprosy.

Slowly progressive motor paralysis in cured leprosy is one of the serious problems in the clinical practice of leprosy. In order to evaluate the contribution of central nervous system lesions on the paralysis, we performed midkine immunohistochemistry, which reflects the site and degree of neuronal damage and the repair process. In 7 of 10 cured lepromatous leprosy spinal cords, the anterior horn motor neurons showed midkine immunohistochemistry, and its distribution was similar with anti-LAM immunostaining; 4 of 10 cured tuberculoid leprosy spinal cords showed midkine immunohistochemistry. This study suggests the novel possibility that spinal cord damage is one of the causes of quiet nerve paralysis.—[National Leprosarium Hoshizuka-Keiaien; Department of Pathology, Kagoshima University School of Medicine, Kagoshima, Japan]

Izumi, S., Hatta, M., Kawatsu, K. and Matsuoka, M. Seroepidemiological study of *M. leprae* infection in the inhabitants of endemic villages in South Sulawesi, Indonesia.

Despite the rapid decline in prevalence due to the increasing coverage of MDT, the incidence of leprosy has not declined in most endemic regions. This strange discrepancy between prevalence and incidence raises questions about the dogma that the source of infection of *Mycobacterium leprae* is mainly untreated multibacillary patients. Recently, we conducted an epidemiological survey in South Sulawesi, Indonesia, a well-known hyperendemic area of leprosy, to estimate the rate of *M. leprae* infection and to ascertain the possible nonhuman source(s) of the bacilli.

The seropositive rates of anti-PGL-I antibody in the general inhabitants were about 32% and 47% in males and females, respectively. The positive rates in household contacts were about 33%–47% in males and 68% in females. The antibody titer in household contacts was significantly higher than that of the general inhabitants. It was found that 43%–67% of young villagers were positive to PGL-I, suggesting that active transmission of the leprosy bacilli is still taking place in this area. On the other hand, 11 out of 341 (3.2%) of the general inhabitants and 2 out of 11 (15.4%) of the household contacts retained leprosy bacilli on the surface of their nasal mucosa.

These days we have increasing evidence to prove that general inhabitants in endemic regions are infected with the leprosy bacilli without direct contact with leprosy patients. We should pay more attention to the epidemiological significance of possible nonhuman sources of *M. leprae* in the environment.—[National Institute for Leprosy Research, Tokyo, Japan; Hasanuddin University, Faculty of Medicine, Ujung Pandang, Indonesia]

Matsuo, E., Komatsu, A., Maekawa, S., Sudik, H., Sumiishi, A., Inoue, T. and Skinsnes, O. K. On the serum antibody against beta-glucuronidase binding protein (BGBP) among normal individuals and leprosy patients.

Beta-glucuronidase binding protein (BGBP) is a molecule biosynthesized by several paracytic microorganisms, such as *Mycobacterium leprae, M. avium-intracellulare* and hepatitis B virus, to combine and utilize the host's enzymes and related metabolic machinery. In this study we examined whether leprosy patients, who should have been immunized strongly with the molecule synthesized by *M. leprae* in their bodies, have tried to cope with the paracytism by developing humoral antibody against BGBP.

Sera were obtained from 9 lepromatous leprosy patients and 55 healthy individuals. The anti-BGBP antisera were titrated by ELISA in which the BGBP antigen was pu-

rified from trypsin-digested *Pisum sativum,* which is cross-immunoreactive with that of *M. leprae.* The assay was the standard indirect method utilizing microtiter plates with solid-phase coating of the antigen and peroxidase-labeled antihuman=IgG antibody to detect human antibody reacting with BGBP.

Both groups showed the presence of anti-BGBP. However, the highest titer among leprosy patients was 12, 800 which was the same as that of healthy individuals. The results indicate that leprosy patients are not stimulated to develop higher titers of anti-BGBP antibody over normal individuals in spite of the enormous amount of antigen which should have been produced by *M. leprae* in their bodies.—[Department of Pathology, Kyorin University School of Medicine, Tokyo, Japan; Sun Yat-Sen University of Medical Sciences, Guangzhou, China]

Moreira, A. L., Tsenova-Berkova, L., Wang, J., Laochumroonvorapong, P., North, R. and Kaplan, G. Effect of thalidomide on the cellular immune response in experimental tuberculosis in mice.

Thalidomide (α-N-phthalimidoglutarimide) has been shown to selectively inhibit tumor necrosis factor-alpha (TNF-α) production by stimulated human monocytes *in vitro.* Treatment with thalidomide has also been shown to inhibit TNF-α production in leprosy patients with erythema nodosum leprosum (ENL) and in tuberculosis patients with and without HIV-1 co-infection. In this study we describe the effects of thalidomide on *Mycobacterium tuberculosis* Erdman infection in B6D2F1 mice. We demonstrate that a reduction, not abrogation, of TNF-α production is associated with an improvement in lung pathology with less inflammatory infiltration in the lung parenchyma and smaller, tighter granulomas, resulting in containment of the infection.

We have demonstrated that in this murine model of aerosol tuberculous infection TNF-α production is reduced by treatment with thalidomide. It is noteworthy that the integrity of granuloma formation was maintained in the thalidomide-treated animals, where TNF-α production was inhibited by only 40%–60%. This agrees with the observation that TNF-α is required for granuloma formation in mycobacterial infection. Thalidomide treatment was associated with the persistence of inflammation and the exacerbation of immunopathology. The persistence of apoptosis in the granulomas on day 28 in the thalidomide-treated mice may be indicative of more efficient cell turnover at the site. Apoptosis was associated with the reduction in colony forming units in the lungs. The importance of apoptosis in the control of mycobacterial infection *in vivo* warrants further investigation.—[The Rockefeller University New York, New York; The Trudeau Institute Inc., Saranac Lake, NY, U.S.A.]

Nakanaga, K., Nomaguchi, H. and Matsuoka, M. Mouse cell lines expressing *M. leprae* heat-shock protein 65-kDa gene: lymphoproliferative responses to the cell lines.

Among several protein antigens of *Mycobacterium leprae,* heat-shock protein 65 kDa (hsp65) has noticeable characteristics. Hsp 65 is a highly conserved protein throughout biology. It belongs to the family of groEL proteins. The amino acid sequence of *M. leprae* hsp65 shows homology of more than 90% with other mycobacterial hsp65s and shares 48% amino acid identity with the mammalian mitochondrial P1 or hsp60. Hsp65 was also reported to be a highly immunogenic protein of *M. leprae* in both B-cell and T-cell responses. Recently, many reports have described the relationships between some autoimmune diseases and hsp65; however the role of hsp65 in immunological disorders is unclear.

To analyze hsp65-related cellular immune responses, mouse cell lines showing stable expression of *M. leprae* hsp65 were thought to be an efficient tool for *in vitro* experiments. For that reason, the *M. leprae* hsp65 gene was inserted into the mammalian expression vector pMAMneo to construct pMAMneo65k-B. PMAMneo65k-B was introduced into the BALB/c-derived cell line A31 by the calcium phosphate precipitation method to establish hsp65-expressing cell lines. Almost a third of the pMAMneo65k-B introduced and G418 selected cell colonies showed transient expression of hsp65 by Western blotting. One

of the colonies which produced hsp65 even after several passages was shown to express mRNA hybridized with the hsp65 DNA probe.

The lymphoproliferative responses of *M. leprae*-infected or untreated mouse splenocytes to the hsp65-expressing cells were measured by ^{3}H-thymidine incorporation. A significant lymphoproliferative response against one of the hsp65-expressing cells was observed in the *M. leprae*-infected mouse splenocytes. No obvious response was observed to *M. leprae*-infected or naive mice spleen cells to the cell lines which were introduced with vector without the hsp65 gene. It was noteworthy that spleen cells of untreated mice also showed significant proliferation against one of the hsp65-expressing cells, but much less than that of infected mice.—[National Institute for Leprosy Research, Tokyo, Japan]

Nomaguchi, H., Ahsan, C. R., Yogi, Y., Matsuoka, M., Nakanaga, K., Fukutomi, Y. and Kawatsu, K. Immune responses of heat-shock protein 65 on *M. leprae*-inoculated mice.

In this study we tried to determine the effect of heat-shock protein 65 (hsp65) in bacterial progression and granuloma formation in mouse foot pads inoculated with *Mycobacterium leprae.* Cytokine stimulation by hsp65 and hsp60 in the splenocyte, *in vitro,* was also checked to evaluate the immunostimulatory properties of these proteins in mice infected with *M. leprae.*

Foot pad swelling in BALB/c mice inoculated with *M. leprae* was highly increased by challenge with hsp65/FIA.

By histological observation, in the case of hsp65/FIA challenge, the number and size of the granulomas and lymphocyte infiltration were greatly increased, together with an increased thickness of the foot pad. Some of the nonsolid (beaded form) bacilli were detected 1 month after *M. leprae* inoculation, even though the total number of bacilli seemed the same compared with the nonchallenged cases. However, the number of bacilli decreased slowly thereafter, with occasional granuloma containing epithelioid-like cells and a few bacilli still detectable after 1 year. On the other hand, many bacilli were detectable in the nonchallenged cases mentioned above. All of these data clearly suggest that hsp65 enhances the development of bactericidal granuloma in mice infected with *M. leprae* and, also, probably inhibits the invasion process of *M. leprae* into the nerve cells.

The lymphocyte proliferation (stimulation index, SI) was high in *M. leprae*-inoculated mice, and the SI observed with hsp65 and hsp60 stimulation was the same for both.

The culture supernatants of the splenocytes isolated from *M. leprae*-inoculated mice in the presence of hsp65 showed a high titer of gamma-interferon (IFN-γ) production, but no production in the presence of hsp60. These results show that the epitope for IFN-γ induction may be the hsp65-specific or hsp-specific-containing region. In addition, the amino acid homology between hsp65 and hsp60 is around 50%.

In summary, exogenous hsp65 enhanced the development of bactericidal granulomas in *M. leprae*-inoculated mice. IFN-γ stimulation by hsp65 *in vitro* also shows that hsp65 is a good immunostimulator for killing the bacilli.—[National Institute for Leprosy Research, Tokyo, Japan]

Pessolani, M. C. V., Marques, M. A. M., Smith, D. R. and Brennan, P. J. Mapping the *in vivo*-expressed proteins of *M. leprae.*

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 this obligate intracellular pathogen once considered unattainable. In this study, the analytical power of two-dimensional gel electrophoresis and procedures in amino acid microsequencing were combined to establish the N-terminal amino acid sequences of five 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* L5/L12 ribosomal protein homolog and another, a 34-kDa protein, as the product of the *M. leprae* cysA gene that codes for sulfate sulfurtransferase, a rhodanese-like enzyme probably involved in iron-sulfur cen-

ter formation and cysteine biosynthesis. Another protein of 23 kDa shows high homology to the *M. avium* Avi-3 antigen and to a *Corynebacterium diphtheriae* iron-repressible gene product. Further comparisons showed that *M. leprae* and *M. avium* Avi-3 proteins are members of a recently defined family of antioxidant proteins present from bacteria to humans called the AHPC/TSA family. Recognition of these proteins allows speculation on the mode of survival of this obligate pathogen in host cells and the molecular basis of its virulence.—[Setor de Hanseniase, Fundacao Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; Collaborative Research Inc., Waltham, MA; Department of Microbiology, Colorado State University, Fort Collins, CO, U.S.A.]

Saito, H., Tomioka, H. and Sato, K. *In vitro* and *in vivo* anti-*M. leprae* activity of benzoxazinorifamycin, KRM-1648, in combination with other antimicrobials and biological response modifiers.

We studied *in vitro* and *in vivo* anti-*Mycobacterium leprae* activity of benzoxazinorifamycin, KRM-1648 (KRM), in combination with dapsone (DDS), clofazimine (CFZ), and clarithromycin (CAM) (Exp. 1), or with some biological response modifiers (BRMs) including gamma-interferon (IFN-γ) and GM-CSF (Exp. 2). *In vitro* anti-*M. leprae* activities of test antimicrobials were measured by the BACTEC 460 TB system. Combinations of KRM (0.01 μg/ml) with DDS (5.0 μg/ml), CFZ (1.0 μg/ml), or CAM (2.0 μg/ml) caused slight increases in the efficacy at day 4, a 36%, 20%, 18% and 38% reduction in GI by KRM, DDS, CFZ, and CAM alone, versus a 54%, 51%, and 53% reduction by KRM+DDS, KRM+CFZ, and KRM+CAM, respectively. In experimental infections, BALB/c nude mice were infected subcutaneously with 1×10^6 of *M. leprae* Thai-53 into the foot pad. All drugs were given by gavage except for BRMs which were injected into the mice.

In Exp. 1, the above drugs were given daily, 6 times/week for 30 days from day 91 to day 120; thereafter followed by further 60-day administrations of each drug in the protocols as follows: KRM (0.2 mg) once/week; DDS (0.05 mg) daily 6 times/week; CFZ (0.05 mg) and CAM (0.2 mg) twice/week. Each drug alone exerted significant therapeutic efficacy, causing a 3.7-, 1.5-, 2.8-, and 2.1-log unit decrease, respectively, in the number of acid-fast bacilli (AFB) per foot pad. In this case, KRM exhibited a combined effect with DDS against *M. leprae* infection, causing further reduction in the number of AFB—a 4.6-log unit decrease. In contrast, such a significant combined effect was not observed for KRM with either CFZ or CAM.

In Exp. 2, KRM (0.2 mg), IFN-γ (10^4 units) and GM-CSF (100 ng) alone or in combination with KRM with the BRMs were given to mice for 90 days, from day 91 to day 180, in the protocols as follows: KRM once/month; BRMs once/week. KRM displayed potent therapeutic efficacy, causing a 3.2-log unit decrease. IFN-γ but not GM-CSF caused a slight reduction in the growth of the organisms, a 0.35-log unit decrease. KRM given in combination with IFN-γ but not with GM-CSF displayed weak combined efficacy, causing a further reduction in the number of AFB of 3.7-log units. Thus, the antileprosy activity of KRM seems to be potentiated by combination with immunotherapy using IFN-γ.—[National Institute for Leprosy Research, Tokyo; Department of Microbiology and Immunology, Shimane Medical University, Izumo, Japan]

Sampaio, E. P., Malta, A. M., Sarno, E. N. and Kaplan, G. Effect of rhuIFN-γ treatment in multibacillary leprosy patients.

It has long been known that interferon gamma (IFN-γ) can activate human monocytes *in vitro* and thereby stimulate killing of intracellular organisms. IFN-γ has thus been used in studies of patients with lepromatous leprosy in which there is a high bacillary load despite multidrug therapy (MDT). Our previous studies have described the response of patients with lepromatous leprosy to the intradermal administration of recombinant human IFN-γ (rhuIFN-γ). Intradermal injection of rhuIFN-γ for 6 days, as an adjunct to MDT, results in both a local reduction in the number of acid-fast bacilli (AFB) in the skin at the site of injection as well as a reduction in distal sites. However, when rhuIFN-γ was

administered for the 6-day treatment period and then monthly for 6–10 months, the cytokine was associated with the development of erythema nodosum leprosum (ENL) in 6 of 10 patients. Monocytes isolated from the blood of these patients secreted elevated levels of tumor necrosis factor-alpha (TNF-α) in response to stimulation *in vitro*. Thalidomide treatment of the patients during ENL resulted in a reduction of toxic symptoms and the elimination of the lesions of the reactional state. The addition of thalidomide to cultured monocytes from these patients resulted in suppression of TNF-α secretion *in vitro*. These studies suggested that ENL might be associated with enhanced TNF-α production by monocytes and/or accelerated bacterial clearance.

To understand the development of rhuIFN-γ-induced ENL and bacillary clearance, multibacillary (MB) leprosy patients were treated with rhuIFN-γ for 6 consecutive days and then monthly for 10 months. Two groups of patients were studied. In one group (Group 1) the patients were newly diagnosed and on MDT. These patients received rhuIFN-γ and a single 100-mg dose of oral thalidomide at the same time as each rhuIFN-γ injection. The second group (Group 2) of patients, BI-negative and BI-positive patients who had completed 2 years of MDT, received only rhuIFN-γ. Local and systemic responses, the frequency of ENL episodes and the rate of bacillary clearance were evaluated in each group.

When rhuIFN-γ was administered together with thalidomide to patients in Group 1, the mean reduction in the bacterial load was equivalent to the reduction observed with MDT therapy alone. Since IFN-γ combined with MDT previously had been shown to induce an accelerated clearance of bacteria, the present observation suggests that thalidomide prevents the IFN-γ induction of bacterial clearance. Thalidomide has been shown to inhibit the production of TNF-α. It may be that bacterial clearance depends in part on TNF-α production by monocytes activated by either endogenous or exogenous IFN-γ. TNF-α may render infected macrophages in the skin better capable of bacteriocidal activity.

In addition, thalidomide treatment was associated with a reduction in the occurrence of ENL in this group of patients. Previous studies have shown that when MB leprosy patients are treated with rhuIFN-γ and MDT, approximately 60% develop ENL during the first 6 months of the treatment period. In contrast, the addition of thalidomide to this regimen reduced the frequency of ENL episodes to 12% in the same time period. This lower frequency of ENL is comparable to the frequency observed in patients treated with MDT alone. The present results suggest a potential role for TNF-α in the induction of ENL.

In the second group of patients, who had already completed MDT before the start of this study, the presence or absence of *M. leprae* in the skin (BI) appeared to be associated with the frequency of development of ENL. In BI-negative patients, ENL did not occur during rhuIFN-γ treatment despite the appearance of systemic symptoms following rhuIFN-γ injections. However, these patients did not develop erythema or induration at the site of the IFN-γ injection. In BI-positive patients, the frequency of ENL was much higher as was the frequency of local erythema and induration. The local cutaneous response appeared to precede the development of ENL. In contrast, the mild systemic symptoms did not necessarily indicate development of ENL.

It is of interest to note that rhuIFN-γ treatment without concomitant MDT did not result in a reduction in the bacterial load in the skin of BI-positive patients. This finding suggests that IFN-γ does not, by itself, accelerate bacterial clearance. Treatment with rhuIFN-γ can lead to reduction in bacillary load only when administered as an adjunctive therapy together with MDT.—[Leprosy Unit, Oswaldo Cruz Foundation, Manguinhos, Rio de Janeiro, Brazil; Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY, U.S.A.]

Shannon, E. J. and Sandoval, F. Thalidomide is agonistic to the synthesis of IL-2 and it can be agonistic or antagonistic to the synthesis of TNF-α.

Thalidomide (α-phthalimidoglutarimide) is used investigationally in erythema nodosum leprosum (ENL), graft-versus-host disease after bone marrow transplantation, and a variety of dermatological diseases, es-

pecially aphthous ulcers of unknown origin in human immunodeficiency virus (HIV)-positive patients. The mechanism of action of thalidomide in arresting these inflammatory disorders is unknown. However, it has been suggested that modulation of the immune response plays an important role. Modulation of cytokines such as TNF-α and IL-2, which are known to promote inflammatory activity, could explain the antiinflammatory activity of thalidomide.

Thalidomide increases the amount of IL-2 in the supernatant of peripheral human mononuclear cells stimulated with mitogen or antigen. Cells exposed for 2 hr to 4.0 μg/ml of thalidomide and then stimulated for 16–18 hr with 20 μg/ml of concanavalin-A (ConA) showed significantly more IL-2 in the culture supernatant as detected in an IL-2 capture ELISA ($p < 0.001$). Cultures treated with thalidomide and stimulated with ConA produced 1531 $\pm$ 93 pg/ml of IL-2; whereas cells stimulated with ConA alone produced 1015 $\pm$ 61 pg/ml of IL-2. In similar experiments the pg/ml of IL-2 in the supernatants of the thalidomide-treated cells stimulated with 50 ng/ml of staphylococcal enterotoxin A (SEA) significantly increased (2170 $\pm$ 114 vs 1493 $\pm$ 20; $p < 0.0001$). The IL-2 in the supernatants of thalidomide-treated cells stimulated with 5.0 μg/ml of purified protein derivative of *Mycobacterium tuberculosis* (PPD) was also significantly increased (167 $\pm$ 18 vs 139 $\pm$ 14 pg/ml; $p < 0.02$).

The significant increase in IL-2 does not appear to be a reflection of thalidomide causing a decrease in membrane-anchored IL-2 receptors (IL-R) on activated IL-2R+ cells. Thalidomide did not decrease the ability of IL-R+ cells to express membrane-anchored IL-R. The effectiveness of thalidomide in enhancing IL-2 produced by cells stimulated with the superantigen SEA also indicates that the stimulatory effect of thalidomide on IL-2 production is independent of immunological "recognition" of SEA and MHC Class II HLA compatibility.

Depending on the type of cells treated with thalidomide and the antagonist used to provoke TNF-α, thalidomide can suppress or enhance the amount of TNF-α secreted by the cells. We reported that when cultures of human mononuclear cells from eight male donors enriched for adherent cells or cultures of the human monocyte cell line THP-1 are treated with 0.5, 1.0, 2.0 and 4.0 μg/ml of thalidomide and then stimulated with LPS, the thalidomide-treated cultures generally contain more TNF-α than the cultures stimulated with LPS. In cultures of the human monocyte cell line THP-1 or in cultures of human adherent cells from female donors, 4.0 μg/ml of the +/− or the +, or the − forms of thalidomide-enhanced LPS provoked synthesis of TNF-α. In contrast, when cultures of unfractionated human mononuclear cells are treated with thalidomide for 2 hr and then stimulated with LPS for 16–18 hr, thalidomide suppresses TNF-α production.

The suppressive effect of thalidomide on TNF-α produced by unfractionated mononuclear cells stimulated with LPS and its enhancing effect on IL-2 produced by mononuclear cells stimulated with ConA offer two bioassays to study structure-activity relationships of thalidomide. Considerable information is available from animal sources suggesting relationships between the structure of thalidomide and teratogenic effects. While there are differences depending on the animal species studied, basically the phthalimide moiety appears to be essential for teratogenicity.

The planar nature of phthalimide seems to be required for thalidomide to be antagonistic in the TNF-α assay and agonistic in the IL-2 assay. From modifications of thalidomide made by a) introducing electron withdrawing (NO_2) or electron donating (NH_2 or OH) groups on phthalimide and b) substitution of chemical groups for the glutarimide moiety of thalidomide, we have characterized and ranked the structural requirements for analogs of thalidomide to be active suppressants in the TNF-α assay. The modified molecule of thalidomide that is nontoxic in cultures and ranks above thalidomide in its ability to suppress TNF-α does not enhance the synthesis of IL-2 in the IL-2 bioassay.

The ability of thalidomide to be antagonistic or agonistic to the synthesis of TNF-α and its ability to apparently enhance the synthesis of IL-2 does not help to clarify the role of these cytokines in inflammatory reactions like ENL. Structure-activity relationships are being studied based on the ability of analogs of thalidomide to enhance

the production of TNF-α in cell lines stimulated with phorbol esters or to decrease the production of TNF-α in unfractionated human mononuclear cells stimulated with LPS (permitted personal communication, Dr. Kaplan). Regardless of the screening assay, hopefully an analog of thalidomide or another class of reagents will be found that overcomes the major disadvantages of thalidomide—teratogenicity and neuritis. Additional studies on the molecular mechanisms by which thalidomide enhances the synthesis of IL-2 *in vitro* and its use as an immune potentiating drug to facilitate the synthesis of IL-2 in IL-2-deficient patients are warranted.—[Laboratory Research Branch, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA, U.S.A.]

Sieling, P. A., Chatterjee, D., Porcelli, S. A., Prigozy, T. I., Mazzaccaro, R. J., Soriano, T., Bloom, B. R., Brenner, M. B., Kronenberg, M., Brennan, P. J. and Modlin, R. L. CD1-Restricted T-cell recognition of *M. leprae* lipoarabinomannan.

T cells activated in response to infection can generally be classified into distinct functional subsets corresponding to their surface expression of accessory molecules, CD4 and CD8. It is now apparent that T-cell populations which lack both CD4 and CD8, termed double negative (DN) T cells, are involved in the immune response to infectious disease. The analysis of human T-cell clones that responded to *Mycobacterium*-derived antigens demonstrated the ability of a unique antigen-presenting element, CD1, to present foreign antigen. In this study, $\alpha\beta$ DN T-cell lines from normal donors responded to mycobacteria in the contest of CD1b, in a manner independent of both classical Class I and Class II major histocompatibility complex (MHC) proteins.

The human cluster of differentiation 1 (CD1) gene family consists of a small number of genes which are structurally related to the Class I MHC genes and, thus, encode cell-surface glycoproteins which form heterodimers with β_2 microglobulin. Five CD1 genes—CD1a, b, c, d, and e—have been identified in humans. The recent discovery that particular combinations of cytokines can induce CD1 expression has facilitated our ability to study CD1-restricted T-cell responses.

Our goal was to determine if CD1-restricted $\alpha\beta$ DN T cells have a role in leprosy. We therefore attempted to derive $\alpha\beta$ DN T cells from leprosy lesions. From one such lesion, we found that $\alpha\beta$ DN T cells comprised 8% of $\alpha\beta$ T cells; whereas $< 1\%$ of $\alpha\beta$ T cells in the peripheral blood mononuclear cells of the same patient were double negative. We interpret these data to indicate a tenfold enrichment of $\alpha\beta$ DN T cells at the site of disease.

One DN T-cell line, LDN4, derived from a leprosy lesion proliferated in response to *M. leprae*. The *M. leprae*-induced proliferation of the LDN4 T-cell line was completely inhibited by anti-CD1b; whereas anti-CD1a and CD1c antibodies had no effect on the proliferation of *M. leprae*. Similarly, LDN4 cells lysed CD1b-transfected C1R cells in an antigen-specific manner. Therefore, CD1b restricts the response of *M. leprae*-responsive $\alpha\beta$ DN T cells from leprosy skin lesions.

In order to characterize antigens presented by CD1 molecules to T cells, we derived mycobacteria-reactive DN $\alpha\beta+$ T-cell lines from the skin lesion of a leprosy patient (line LDN4) and from the peripheral blood of a normal donor (line BDN2). These T cells were examined for their ability to respond to subcellular fractions of mycobacteria in the presence of CD1-expressing antigen-presenting cells (APCs).

In preparations in which the bacterial cytosolic proteins had been removed, substantial T-cell antigen activity for both cell lines was found associated with mycobacterial cell walls, specifically with a soluble fraction obtained after repeated extraction of cell walls with sodium dodecyl sulfate. This fraction contains several cell wall-associated proteins as well as the glycosylphosphatidylinositols (GPIs), lipoarabinomannan (LAM), lipomannan (LM), and phosphatidylinositol mannosides (PIMs). To confirm that the antigenic activity was due to such lipoglycans, highly purified LAM was tested and shown to maintain T-cell stimulatory activity. Protease treatment of LAM had little or no effect on the antigenic activity of LAM. Recognition of purified LAM from *M. leprae* was restricted by CD1b

for both cell lines because only CD1b-specific mononuclear antibodies blocked T-cell proliferation. These data demonstrated that a defined mycobacterial lipoglycan is presented to T cells in a CD1-restricted manner.

Our results indicated that LAM recognition required intracellular processing, as does peptide antigen on MHC. LAM-pulsed APCs could be glutaraldehyde-fixed and still retain antigen-presenting capacity. On the other hand, T-cell responses could not be detected if APCs were fixed prior to the addition of LAM. Furthermore, treatment of APCs with agents that prevent endosomal acidification, including the weak base chloroquine and concanamycin A, during the APC-pulsing period abrogated antigen presentation. These experiments demonstrate that presentation of LAM requires uptake and localization to an endosomal compartment, where LAM may be processed.

These results extend the spectrum of antigens presented by human CD1 molecules and recognized by T cells to include the lipoglycans, which thus potentially represent an abundant and diverse pool of microbial antigens. We speculate that CD1 molecules transport endosomally processed lipoglycan antigens of intracellular pathogens to the cell surface, thereby allowing T-cell recognition of this class of molecules which are central to the pathogenesis of infectious disease. Preliminary studies of the *in vitro* effector functions of the LAM-specific T-cell lines described here indicate that these cells mediate effector functions that correlate with protective T-cell responses *in vivo,* including lysis of antigen-sensitized macrophages and selective production of interferon gamma over IL-4. These findings, together with our recent observations that CD1 proteins are highly expressed in the lesions of patients with the resistant form of leprosy, support a role for CD1 in presentation of microbial lipoglycan antigens in host defense.—[Division of Dermatology, Department of Microbiology and Immunology, and Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA; Department of Microbiology, Colorado State University, Fort Collins, CO; Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, MA; Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, NY, U.S.A.]

Suzuki, Y., Ying, Y.-P., Katsukawa, C. and Makino, M. Application of recombinant 85 complex of *M. leprae* for the serodiagnosis of leprosy.

Sera from 38 lepromatous (L) leprosy patients, 53 clinically cured L (LC), 34 clinically cured tuberculoid (T) (TC), 7 borderline (B), 9 clinically cured B (BC), 11 tuberculosis (TB), 3 atypical mycobacterial infection (AM) and 21 healthy control subjects (N) were examined for antibody titers against recombinant *Mycobacterium leprae* 85A (rML85A), 85B (rML85B) and NTP-BSA that carries the sugar portion of PGL-I on bovine serum albumin. Reactivity of L sera against recombinant 85A was much higher than those of TC, TB, AM and N. Significant differences could be observed between the antibody titers of L and those of others. Similar observations were seen with the reactivity of various sera against rML85B and NTP-BSA. Some differences could be observed between them. One of the differences was that higher reactivities were observed on the titers against rML85B and rML85A than NTP-BSA. Another difference was that the number of sera which exhibited a lower titer than N was smaller in the case of rML85A and rML85B than in NTP-BSA. In addition, no false-positive sera were observed when 21 N sera were examined.

The rML85A and the rML85B established, overproduced and purified in our laboratory could be a powerful tool for the serodiagnosis of leprosy.—[Department of Pathology, Department of Microbiology, Osaka Prefectural Institute of Public Health, Osaka; National Sanatorium Oku-Komyoen, Oku-cho, Okayama, Japan]

Walker, L. L., King, C. H. and Shinnick, T. M. Continuous cultivation of *M. leprae* in primary cell cultures.

Mycobacterium leprae were grown to late log-phase ($\sim 1 \times 10^6$ bacilli/foot pad) in the foot pads of CFW mice (Charles River Laboratories, Portage, Michigan, U.S.A.) as originally described by Shepard. Foot pads were harvested and the tissue and eukary-

otic cells disrupted by homogenization with 3-mm glass beads in a Mickle apparatus. After allowing tissue clumps to settle out, the supernatants (i.e., isolated bacilli) were added to tissue-culture flasks containing primary cell cultures of murine spleen or lymph node cells prepared from uninfected CFW mice. The cultures were incubated at 30°C with 5% CO_2 and the medium was replaced with fresh Eagle's Modified MEM medium/5% fetal calf serum weekly. Periodically the cultures were assayed for the presence of acid-fast bacilli (AFB) by the method of Shepard and McRae, and cultures containing AFB were subcultured by harvesting the cell monolayer by scraping and using the resulting suspension to inoculate flasks containing fresh medium.

AFB have been observed in infected cell cultures maintained for more than 10 months, and electron-microscopic studies revealed that the bacilli were located within the eukaryotic cells. Observations made using phase-contrast microscopy of the viable monolayers indicated the presence of bacilli associated with tissue-culture cells, and acid-fast stains of the monolayers confirmed the presence of associated AFB. Some cells contained what appeared to be vacuoles containing AFB.

Bacilli were not uniformly distributed throughout the tissue-culture monolayers, but were found clustered in groups of cells within the monolayer. The precise nature of the infected cell has not yet been determined, but we are examining the infected cells by histological staining.

The identity of the AFB as *M. leprae* was confirmed by polymerase chain reaction amplification studies using two *M. leprae*-specific primer sets and a *Mycobacterium* genus-wide primer set. The *M. leprae*-specific primers confirmed the presence of *M. leprae* in most of the tissue-culture specimens that were also positive for AFB.

A preliminary analysis suggests that the number of bacilli per culture is increasing over time. For example 4×10^6 foot pad-grown AFB were inoculated into a primary spleen-cell culture and, after 4 months, 2×10^6 AFB were found associated with the cell monolayer. Portions of this culture containing 2×10^5 AFB were then used to inoculate fresh flasks. After 3 months, replicate subcultures were found to contain 4×10^5 to 1.2×10^6 AFB. Similarly the number of AFB in subcultures of these subcultures increased from 1×10^5 to 4×10^5 after 3 months. Thus, the bacilli appear to be increasing with a doubling time of 30–40 days.

Portions of the subcultures have been used to inoculate foot pads of CFW mice to assess the viability and virulence of the cultured bacilli. Additional studies are under way to compare the genotype and phenotype of the cultivated bacilli with authentic *M. leprae*. Overall, the preliminary studies are encouraging for the eventual development of a cell-culture system for the cultivation of *M. leprae* and for studies of the intracellular survival of replication of *M. leprae*.—[Division of Bacterial and Mycotic Disease, Centers for Disease Control and Prevention, Atlanta, GA, U.S.A.]

Yamada, T., Ohara, N., Matsumoto, S., Matsuo, T., Kitaura, H., Takano, M., Nishiyama, T., Yukitake, H., Miyazaki, R., Nomaguchi, H. and Matsuoka, M. Biochemical and immunological characterization of ribosomal fraction and culture filtrate from BCG.

Mice were immunized with 100 μ or 10 μg of ribosomal fraction isolated from BCG Tokyo. After 1 month, 5×10 *Mycobacterium leprae* were injected into foot pads of mice. After 30 weeks, the number of *M. leprae* were assessed. The numbers of acid-fast *M. leprae* were log 4.92 ± 0.43, log 4.86 ± 0.41, and log 5.59 ± 0.35 in the respective mouse groups immunized with 100 μg, 10 μg, and zero ribosomal fractions.

Biochemical and immunological characterization of individual components of the ribosomal fraction was initiated. DTH reaction, lymphocyte stimulation, and biochemical analysis of individual components were carried out. The results were presented at the conference.—[Nagasaki University, School of Dentistry, Nagasaki; National Institute for Leprosy Research, Tokyo, Japan]

CLOSING REMARKS

Ladies and Gentlemen:

In closing the 30th Joint U.S.-Japan Tuberculosis and Leprosy Conference, on behalf of the Japanese participants I would like to express my sincere thanks to Dr. Brennan, Chairman, U.S. Leprosy Panel; Dr. Ellner, Chairman, U.S. Tuberculosis Panel; and to Dr. Gwinn, Dr. Foulds, and Dr. Ginsberg for the nicely arranged, successful organization of the Conference over the last three days. A 14-hour air trip and an additional 2-hour drive from Osaka to Fort Collins was indeed troublesome and exhausting for me and probably for most of my Japanese colleagues. However, the warm heartfelt welcome we received from Dr. Brennan and his colleagues and the beautiful, wonderful scenery of Fort Collins surely compensated for it.

We have still many scientific problems left unresolved, both in leprosy and tuberculosis (TB) research. We are, in fact, facing an increasing number of patients with multidrug-resistant (MDR) TB; also the case in Japan, too. It is indeed quite miserable for a young man infected with and developing drug-resistant TB. Worldwide, on the other hand, there are still many, many leprosy patients. We should recognize the fact.

The merger of the Leprosy and Tuberculosis Panels into one single Panel which we have to be prepared for very soon may, in a sense, accelerate and concentrate our research on urgently to be solved first priority issues, such as better vaccine development for mycobacterial diseases, immunotherapy for MDR-TB, and intervention of HIV-TB. I believe some problems, particularly in immunological research, could be solved better by the close cooperation of researchers in leprosy and TB. At the Panel meeting yesterday, a new guideline for the combined TB and Leprosy Panel was discussed. Actually, we have started our new Panel, Tuberculosis and Leprosy.

We should accelerate our research on mycobacterial diseases in the coming year and meet together again with fruitful results next year in Nagasaki, Japan. Nagasaki is an old seaport town with a long history, the only port open abroad even during the ancient days of national isolation of Japan. Nagasaki is indeed a city with an enterprising, progressive spirit. The meeting will be organized by Professor Yamada, of Nagasaki University, who is now a member of the Japanese Leprosy Panel.

Finally, I would like once again to express my sincere appreciation to Dr. Brennan for the warm, warm hospitality we have received here in the past several days. Last but not least, I would also like to extend my many thanks to Dr. Gwinn for his decades-long, extraordinary contributions to the U.S.–Japan Cooperative Medical Science Program. Dr. Gwinn is retiring at the end of this year. Goodby, Dr. Gwinn, and goodby (sa-yo-na-ra) to every participant in this meeting until next year in Nagasaki, Japan.

—Izuo Tsuyuguchi, *Chairman*
Japanese Tuberculosis Panel