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TWENTY-SIXTH JOINT LEPROSY RESEARCH CONFERENCE

Meany Tower Hotel
Seattle, Washington, U.S.A.
6–9 August 1991

U.S.–Japan Cooperative Medical Science Program

U.S. Leprosy Panel

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PROGRAM
JOINT U.S.–JAPAN TUBERCULOSIS AND LEPROSY SYMPOSIUM

8 August, Thursday

Morning Session

Co-Chairpersons: Dr. Hajime Saito
Dr. Thomas Shinnick

Lymphokines and Cytokines: Leprosy

9:00 a.m. Tumor Necrosis Factor in Experimental Leprosy: Yasuo Fukutomi, Linda Adams and James Krahenbuhl

9:20 a.m. Macrophages Infected with *Mycobacterium leprae* or Treated with Mycobacterial Constituents Release Factors that Modulate Afferent and Efferent Macrophage Function: Yasuo Fukutomi, Linda Adams and James Krahenbuhl

9:40 a.m. Defining Protective Immune Responses to Infectious Pathogens: Lymphokine and Cytokine Profiles in Leprosy Lesions: Masahiro Yamamura, Koichi Uyemura, Thomas H. Rea and Robert L. Modlin

10:00a.m. Coffee Break

10:50a.m. Lymphokine Profile of Functionally Distinct *M. leprae* Reactive CD4+ T-Helper and CD8+ T-Suppressor Clones: P. Salgame, J. Abrams, C. Clayberger, S. Lyu, R. Modlin and B. R. Bloom

11:00a.m. The Role of TNF alpha in Pathogenesis of Mycobacterial Infections. Mechanism of Modulation by Thalidomide: Gilla Kaplan and Elizabeth P. Sampaio

Afternoon Session

Co-Chairpersons: Dr. Yasuo Mizuguchi
Dr. Josephine Clark-Curtiss

3:30 p.m. Joint Meeting of U.S.–Japanese Tuberculosis and Leprosy Panel Members

PROGRAM
TWENTY-SIXTH JOINT LEPROSY CONFERENCE

9 August, Friday

Opening Remarks

8:30 a.m. Thoughts on Future Research Directions under the United States–Japan Cooperative Medical Science Program: P. J. Brennan, Chairman, U.S. Leprosy Panel

Morning Session

Co-Chairpersons: Dr. Tatsuo Mori
Dr. Patrick Brennan

Structure and Antigenicity

9:00 a.m. Molecular Characterization of Immunologically Reactive Proteins of *Mycobacterium leprae*: Shlomo Sela, J. E. R. Thole, T. H. M. Ottenhoff and J. E. Clark-Curtiss

9:20 a.m. Identification, Characterization and Expression of the Major Proteins of *Mycobacterium leprae* of Molecular Mass 14 kDa and 35 kDa: Becky Rivoire, Cynthia M. Bozic, Ellen Dysen, Vijay Mehra, Barry R. Bloom, Patrick J. Brennan and Shirley W. Hunter

9:40 a.m. On the Pathologic Significance of the Receptor for Beta-Glucuronidase (BGR) of *Mycobacterium leprae,* Especially in the Relationship to Its Other Antigen: Eiichi Matsuo, Akio Komatsu, Mizue Murakami, Miyuki Terada, Norisuke Sasaki and Olaf K. Skinsnes

10:00 a.m. Coffee Break

10:50 a.m. Application of Monoclonal Antibodies to Differentiate the Morphological Forms of *Mycobacterium leprae* Hawaiian Strain: N. Elangeswaran, Toshiyuki Saito, Masanori Matsuoka, Yoshiki Sakamoto and Tatsuo Mori

Chemotherapy of Leprosy

11:10 a.m. Bactericidal Antibiotics of Three Different Classes Emerge to Treat Leprosy: Recent Developments from the Laboratory to the Patient: R. H. Gelber, L. Murrary, P. Siu and M. Tsang

11:30 a.m. Anti-Leprosy Activity of Ofloxacin in Combination with Rifampin and Diaminodiphenylsulfone in Mice and *In Vitro* Activities of New Quinolones and Rifamycin Derivatives: Hajime Saito, Haryaki Tomioka and Katsumasa Sato

Afternoon Session

Co-Chairpersons: Dr. Takeshi Yamada
Dr. James Krahenbuhl

1:30 p.m. Evaluation of PCR Analysis for Monitoring Antileprosy Chemotherapy in Lepromatous Leprosy Patients: D. L. Williams, T. P. Gillis, S. Franzblau and R. Hastings

Immunology/Serology/Animal Models in Leprosy

1:50 p.m. Host and Bacterial Molecules Mediating Phagocytosis of *Mycobacterium leprae* by Human Mononuclear Phagocytes: Larry S. Schlesinger and Marcus A. Horwitz

2:10 p.m. The T-Cell Receptor Repertoire in Mycobacterial Infection: Robert L. Modlin, Koichi Uyemura, Jeffrey Ohmen, Xiao-Hong Wang, Thomas H. Rea and Peter F. Barnes

2:30 p.m. Coffee Break

3:00 p.m. Distribution of Anti-Lam-B Antibodies in Leprosy Patients and Household Contracts: Shinzo Izumi, Yumi Maeda, Kunio Kawatsu, M. Dali Amiruddin, Kentaro Hatano, Mauro F. Mendes and Agha M. Choudhury

3:20 p.m. Susceptibility of Severe Combined Immunodeficient (SCID) Mice to *Mycobacterium leprae*: The Multiplication of *M. leprae* Inoculated into Both Hind Feet at an Early Stage: Yasuko Yogi, Kazunari Nakamura, Tuyoshi Inouye, Konio Kawazu, Yoshiko Kashiwabara, Yoshiki Sakamoto, Shinzo Izumi, Muneo Saito, Kyoji Hioki and Tathuji Nomura

OPENING REMARKS

Thoughts on Future Research Directions Under the United States–Japan Cooperative Leprosy Program

Since its inception over 25 years ago, the U.S.–Japan Cooperative Leprosy Program under the aegis of the U.S.–Japan Cooperative Medical Science Program has devoted itself to the application of fundamental research to the control of global leprosy. Traditionally, it has concentrated on the dissection and definition of the immune response in leprosy, on the dissection and definition of the antigenic components of the leprosy bacillus and, with that, the cloning, sequencing and production of the major antigens of *Mycobacterium leprae* and, more recently, the creation of attenuated strains encoding the major *M. leprae* antigens in alternative hosts. In the arena of diagnostics, participants in the Program have concentrated on serology, both antigen and antibody detection, on the generation of a new order of skin test reagents and, more recently, on the application of gene amplification techniques for diagnostic purposes. In the arena of the pathogenesis of leprosy, participants in the Program have dwelt largely on cell and cytokine definition within leprosy lesions and the implementation of immunotherapy regimens. In addition, the definition of bacterial inducers of pathogenesis and the identification of genes and gene products involved in pathogenesis have been goals of the Program. Participants in the Program have worked with others in formulating and implementing the present-day multiple drug therapeutic regimens, and in designing and implementing the present-day three major vaccine trials. Participants in the Program, based on emerging information on the genome of *M. leprae,* are also collaborating with others in mapping the entire genome. Many of these endeavors are spearheaded by other programs, such as those sponsored by the WHO/TDR Special Program.

Progress in basic research in leprosy during this period under the U.S.–Japan Cooperative Program and like programs has been exceptional. For instance, nine major immunoreactive proteins have been cloned, sequenced, and are now available in quantity either in the native or in recombinant form. With this fundamental work has come the realization that several of the predominant immunogenic protein antigens of *M. leprae* share sequence homologies with pro-

teins throughout living kingdoms and may be involved in self-mimicry and auto-immunity which may have a bearing on such important events as nerve damage and reactional states in leprosy. The major carbohydrate-containing antigens of *M. leprae* have been isolated, structures elucidated, made available in quantity, and implicated in a range of host-pathogen events. A deeper understanding of the cellular immune response in leprosy has emerged, namely, how different classes of T-cell subsets contribute differently to protection and pathology in leprosy; how cytotoxic and helper T cells collaborate in certain responses; how different phenotypes of human helper T cells may explain the dichotomy between antibody production and T-cell proliferation in leprosy; how different classes of *M. leprae* antigens may activate different functional classes of T cells, including those of the gamma-delta receptor phenotype; how different cytokines evoked by different subsets of T cells, in turn activated by different classes of antigens, may contribute to protective immunity or to immunopathology. Innovative immunotherapeutic interventions, based on this information, are being implemented. In addition, during this period, counteracting the disappointment in the applicability of serological tests based on individual *M. leprae* antigens, have come new developments, new biological tools, and new techniques of potential use in disease control. These include DNA and RNA probes and a new generation of synthetic, recombinant, and native antigens of possible use as skin-test reagents. Gene amplification techniques now show promise in assaying the viability of the bacillus, in testing for resistance to drugs, and in providing measures of infection incidence and prevalence rates of disease and of the occurrence of bacteria in different body tissues and in the environment.

Throughout all of this time, the rationale for this surge of fundamental research was the perception of worldwide leprosy as an intractable disease and the belief that fundamental developments could aid in its control and ultimate eradication. However, there is now increasing evidence that the global leprosy of today has vastly changed and, consequently, the refocusing of basic research to address this new scenario may be called for. We are now hearing that the numbers of registered leprosy patients worldwide has been reduced from 5.4 million in 1987 to 4.9 million in 1988 to 3.9 million in 1990, and that the total number of cases in 1990 is 30% lower than those in 1985. In addition, a recent examination of the results of the implementation of multiple drug therapy (MDT) shows that in September 1990, 55.7% of total registered leprosy cases are on MDT, and already 1.2 million patients have completed the drug regimen. And, in view of these encouraging results, experts now indicate that it is not unrealistic to expect a reduction of the leprosy caseload by as much as 60% to 80% in the next 5 to 10 years, at least in countries with effective programs. Indeed, a goal was set at the recent World Health Assembly for the "elimination" of leprosy as a public health problem (i.e., a prevalence of less than 1 per 10,000 population) by the year 2000.

Nevertheless, it is imperative that participants in this Program should ask themselves whether such goals are feasible. Will, for instance, these anticipated major reductions in prevalence not be offset by problems in drug implementation in certain areas of the world, and will the incidence of new disease arising from infections caught years earlier not come to the fore some years from now? Is there a need to be concerned about the old bug-a-boos of resistance and relapse? Clearly, there is the belief abroad that leprosy is rapidly on the wane, and this view is already having profound effects on funding for research in leprosy and on the future of research-based programs. Thus, it is imperative that participants in the prestigious U.S.–Japan Cooperative Leprosy Program begin to determine what is perception and what is realism and plan accordingly. The future of this Program will come under scrutiny 2 years from now, and it is essential that facts, not perceptions, be the determinants.

Depending on the outcome of this assessment, by we ourselves the embodiment of the program, we must then ask ourselves whether there is still the need for vaccine development in light of the apparent excellent prospects for appreciable control

through MDT. The epidemiologists among us tell us that the real test for the epidemiological impact of MDT must be seen in terms of decline of the incidence rate, and these epidemiologists expect that the current drug regimen in conjunction with efficient implementation will bring about such an effect. On the other hand, some epidemiologists also argue that there has been no overwhelming evidence for a decline in the incidence rate of leprosy observed in the 5 or more years of multiple drug programs. Regardless, epidemiologists and field-control researchers also argue that a low caseload after MDT implementation will generate a demand for the integration of a vertical leprosy program into the general health services with the need for more early detection and management of patients with leprosy. Thus, a program such as the U.S.–Japan Cooperative Leprosy Program has an important role to play in the context of this scenario. Leprosy control programs will need sensitive and specific methods for leprosy diagnosis; there is a continuing need for methods for monitoring trends in leprosy disease and transmission of infection. And, most of all, the epidemiologists argue, there is still a genuine need for a primary preventative measure, viz., an effective prophylactic vaccine against leprosy.

Indeed, paramount to these considerations is the realization that preliminary results from the Venezuela vaccine trial initiated in 1984 by Dr. Convit will become known in September 1991, shortly after the upcoming Joint meeting. You will recall that this trial involved immunization of leprosy contact populations with either live BCG or live BCG plus heat-killed *M. leprae.* Assuming a positive outcome with either combination and assuming that we convince ourselves of the need for continuing vaccine development, the U.S.–Japan Cooperative Leprosy Program should be in a position to contribute hugely to the goal of a second-generation vaccine. For instance, from this Program has emerged the concept that not only macrophage activation but also T-cell lysis of target cells are major contributors to protective immunity. The concept that class-II-restricted T cells and perhaps natural-killer cells are implicated in protective immunity has also been advocated by participants in this Program. In addition, the role of γ-interferon in macrophage activation, the role of IL-2 in recruitment of cytolytic CD4 and CD8 cells, and the role of T-helper cells in the DTH response, which itself is regarded as a harbinger of protective immunity, were all advanced by participants in this Program. Thus, the Program has helped enunciate key immunological markers of protective immunity which could in the future become the yardsticks of the effectiveness of any new vaccine regimen. In addition, in terms of the actual protective antigen, participants in this Program have helped in the definition and production of such proteins as the 70 kDa, 65 kDa, 18 kDa, 36 kDa, 35 kDa, the several 28 kDa products, the 10-kDa protein, and the several smaller molecular weight products, most of which conform to at least some of the specifications of inducers of protective immunity, at least in *in vitro* and in whole animal studies. In addition participants in the Program have developed strategies for the upgrading of the cellular immune response in leprosy through the administration of recombinant IL-2 or γ-interferon.

Thus, where the U.S.–Japan Cooperative Leprosy Program might best contribute to the more stringent control of worldwide leprosy leading to its ultimate eradication could be in the practical implementation of second-generation vaccines or other immunological interventions. Such a joint venture could involve, firstly, the identification of suitable implementation sites in leprosy-endemic areas, perhaps within the geographical encompass of Japan, such as in areas of The Philippines, Thailand, Myanmar or Indonesia, and, secondly, the creation of immunological laboratories in these areas capable of measuring some of the key immunological parameters associated with protective immunity in response to immunizations with some of the key recombinant proteins already identified. A role for U.S. participants could be in the provision of the necessary recombinant and native proteins and the conducting of the necessary toxicity studies and in the seeking of the necessary regulatory permission. In addition, a group of joint immunologists (U.S. and Japanese), in conjunction with others from other international programs, could

convene in order to identify what would be the key harbingers of protective immunity to be measured in such trials. One would envisage such immunological trials presaging another future, large-scale protection study based on subunits or recombinant versions of *M. leprae.* In the meantime, the long-time, traditional efforts of participants in the U.S.–Japan Leprosy Program should continue in areas such as developing new generations of diagnostic procedures and in implementing a new order of immunotherapeutic intervention, as well as in the identification of new chemotherapy regimens and the recognition within the pathogen itself of new sites for tailored chemotherapy.

Thus, the challenge for participants at the upcoming Joint Leprosy forum is to analyze available data on the status of present-day leprosy and, accordingly, to formulate a revamped research strategy aimed at the elimination of leprosy, a long-time aspiration of this outstanding Program, and in accord with the worthy goal of the World Health Assembly.

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

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TWENTY-SIXTH JOINT LEPROSY RESEARCH CONFERENCE

ABSTRACTS*

He, H., Oka, S., Kashima, K., Ide, M., Yamamura, Y., Maekura, R., Kanetsuna, S., Izumi, S. and Yano, I. Production and characterization of antibody against cord factor (trehalose 6,6′-dimycolate) in mice and its application to rapid serodiagnosis of human mycobacteriosis including tuberculosis and leprosy.**

Mycobacterial infections, including tuberculosis and leprosy, have long been known to be important in human beings and other animals. The incidence of mycobacteriosis has been decreasing since World War II but it is still a problem in developing countries, and there is also current interest in mycobacteriosis associated with the acquired immunodeficiency syndrome (AIDS). However, the diagnosis of mycobacterial infections is still based on finding bacilli in the secretions of patients, and serodiagnosis has not been successful. Cord factor (trehalose 6,6′-dimycolate) is a cell-wall glycolipid found widely among *Mycobacterium, Nocardia* and related *Actinomycetes.* It is toxic and may be related to mycobacterial virulence. An antibody against cord factor has been produced experimentally, but an antibody against cord factor was not found in tuberculosis patients. We investigated antibody production against cord factor and related mycolyl glycolipids in the mouse and rabbit with several intravenous injections of cord factor in water/oil/water micelles, and found high titers of antibody in the sera. Here, we found a high titer of antibody against cord factor in the sera of patients infected with *Mycobacterium tuberculosis* or nontuberculous mycobacteria; the titer tended to be especially high in patients excreting acid-fast bacilli in the sputum. Also, in the case of sera from leprosy patients, IgG antibodies against cord factor were observed. To the best of our knowledge, this is the first report that an ELISA with cord factor as the antigen is useful in the serodiagnosis of general mycobacterial infections. [Department of Bacteriology, Osaka City University Medical School, Osaka; National Sanatorium Toneyama Hospital, Toyonaka; National Sanatorium Aoshima Seishoyen and National Institute for Leprosy Research, Tokyo, Japan]

Suzuki, Y., Mineta, T., Kobayashi, K., Ohara, N., Yukitake, H. and Yamada, T. The progress of the study of the ribosomes of BCG vaccine.***

From the point of view of developing a BCG vaccine to protect against leprosy, a detailed study on the molecular biology and immunology of the BCG vaccine is required. In such a study, we reproduced earlier work and further addressed the ribosomes of BCG. The important conclusions are summarized as follows: 1) Ribosomes for *Mycobacterium bovis* BCG could not translate the *Escherichia coli* phage f2 RNA in a cell-free system. 2) *M. bovis* BCG contained only a minimum set of ribosomal RNA (rRNA) genes. 3) The 16S rRNA gene was cloned and sequenced. It contained

* Many of these abstracts were not provided by the authors but are a synopsis prepared by the Chairman.

** From Tuberculosis Research Conference, Tuesday, August 6, 1991, morning session.

*** From Joint Tuberculosis and Leprosy Symposium, Wednesday, August 7, 1991, morning session.

Shine Dalgano sequence identical to *E. coli*. For comparison, a 16S rRNA gene of *Streptomyces lividans* was cloned and sequenced. *M. bovis* BCG had 89% homology with *S. lividans*; whereas it had 75% homology with *E. coli*. 4) The structure and function of the promoter were studied. The initiation site of transcription was estimated by the primer extension method. Seven signals were seen at −265, −187, and at five other positions. On inspection of the sequence, TTGACT (−215 to −220) and TATTAG (−197 to −202), which are similar to *E. coli* promoter "consensus" sequences (−35 to −10 regions), were found. These sequences are probably the promoter for the initiation at −187. The other initiation site (−265) contained *Bacillus subtilis* "consensus" like −10 sequence TATGCT, although the defined −35 region was absent. RNA was synthesized with *E. coli* RNA polymerase. *E. coli* RNA polymerase recognized the promoter located at about 200 bp upstream from 5′-end of 16S rRNA gene. That this promoter was also active in the *in vivo* system of *E. coli* was shown in the Maxi-cell strain of *E. coli* N17-9. The signal at −265 can probably proceed only with *M. bovis* BCG enzyme. Whether the signals at the other four positions in the primer extension system indicate the initiation or processing products remains to be elucidated. 5) *M. bovis* BCG contains a low number of ribosomes, a part of which was confirmed by biochemical and morphological methods. [Research Institute for Microbial Diseases, Osaka University, Yamada-kami; School of Dentistry, Nagasaki University, Sakamoto-machi 7-1, Nagasaki, Japan]

Mehra, V. L., Modlin, R. L., Wu-Hunter, S., Brennan, P. J., Bajardi, A. C., Alland, D. and Bloom, B. R. Characterization of cell-wall associated 10-kDa heat-shock protein of *M. leprae* which is a major T-cell antigen.***

We have previously demonstrated that cell-wall antigens are as potent as intact bacilli in inducing lymphoproliferation of T cells from immunized individuals, and in eliciting delayed-type hypersensitivity (DTH) reaction in guinea pigs and humans sensitized to *Mycobacterium leprae*. This immunological reactivity was destroyed by proteolysis, indicating that cell-wall protein(s) is a major contributor to cell-mediated reactivity of *M. leprae*. The immunoreactive protein determinants associated with the purified cell walls were identified by analyzing *M. leprae* proteins separated on SDS-PAGE using cell-wall reactive, T-cell lines/clones developed from tuberculoid leprosy patients and lepromin skin-test-positive individuals. The major T-cell-stimulating, cell-wall-associated proteins were of the 7-10-kDa protein, 16-18-kDa molecular weight ranges. In order to characterize these further, the 7-10-kDa protein was electroeluted and resolved on two-dimensional PAGE. The two-dimension spot was directly subjected to protein microsequencing to obtain the N-terminal sequence of the first 20 amino acid residues, which was found to be identical to BCG-a protein, except for residues 4, 15 and 17.

We have isolated the gene encoding *M. leprae* 10-kDa protein from the λgt11 library of *M. leprae* and determined its nucleotide sequence. The deduced amino acid sequence of this protein bears 90% identity with BCG-a protein of *M. tuberculosis* (10–12 kDa) which has been reported to have a 44% identity with the heat-shock protein GroES (hsp 10) of *Escherichia coli*. Further, we have produced the recombinant 10 kDa of *M. leprae* in *E. coli* as a fusion protein with maltose-binding protein (MBP), using the expression vector pMAL-c. The MBP-10 kDa is cleaved with the specific protease factor Xa at the four amino acid recognition sequence, present just before the N-terminal of the recombinant protein, to obtain recombinant 10-kDa protein.

Preliminary studies to determine the reactivity of lymphocytes from the leprosy patients to *M. leprae*, purified cell wall, and 10-kDa antigen show significant lymphoproliferative response of peripheral blood lymphocytes and cell-wall-reactive clones, obtained from *M. leprae*-immunized individuals and tuberculoid leprosy patients, to the native and recombinant 10-kDa antigen. The reactivity is greater than any other purified antigen tested. In addition, using the T-cell clones reactive to the 10-kDa antigen of *M. leprae* and/or *M. tuberculosis*,

*** From Joint Tuberculosis and Leprosy Symposium, Wednesday, August 7, 1991, afternoon session.

we have identified one *M. leprae*-specific epitope located at the N-terminal region. [Albert Einstein College of Medicine, Bronx, New York; Colorado State University, Fort Collins, Colorado; University of California, Los Angeles, California, U.S.A.]

Makino, M. and Suzuki, Y. Studies on alpha-antigen gene of *Mycobacterium leprae*: molecular biological studies of *M. leprae*-specific epitopes of alpha-antigen.***

The *Mycobacterium leprae* gene encoding the immunoreactive protein (the alpha-antigen) was amplified using polymerase chain reaction (PCR) and cloned. The primers for the PCR were designed by comparing three alpha-antigen genes of other species. The entire nucleotide sequence of the amplified alpha-antigen gene was determined by the dideoxi-termination method. The deduced amino acid sequence of the alpha-antigen of *M. leprae* showed 77%, 79%, and 70% homology with those of *M. bovis* BCG, *M. kansasii,* and *M. tuberculosis,* respectively. In addition, two possible *M. leprae*-specific epitopes were identified by comparison of deduced amino acid sequences of the four alpha-antigen genes. [Osaka Prefectural Institute of Public Health, 3-69 Nakamichi, 1-Chome, Higashinari-ku, Osaka, Japan]

Sathish, M. and Shinnick, T. M. Identification of genes involved in the resistance of mycobacteria to killing by macrophages.***

This study demonstrates that mycobacteria possess genes and gene products that actively resist the bactericidal activities of macrophages, and describes an effective strategy to isolate and identify such genes and gene products. In particular, we describe the identification of three different genetic elements from *Mycobacterium leprae* which appear to be involved in resistance to killing by macrophages. This study used *Escherichia coli* as a host cell for the initial isolation of the mycobacterial genetic elements which confer increased survival onto the bacterial host. However, since not all mycobacterial genes are efficiently expressed in *E. coli,* mycobacterial genomic libraries are being constructed in *E. coli*-mycobacterium shuttle vectors, and the enrichment process and screening will be repeated with recombinant DNA libraries in *M. smegmatis.*

One limitation of this approach is that it can effectively identify only genes which impart increased survival as a result of the direct effect of a gene product on resistance to killing by the macrophage bactericidal activities. This may include previously identified mycobacterial proteins, such as superoxide dismutase and catalase. Other genes involved in the ability of mycobacteria to survive and multiply intracellularly may not be isolated by this approach. Such genes may include those a) required for scavenging essential metabolites (e.g., mycobactin); b) involved in synthesis of components required for growth (e.g., thymidine, aromatic amino acids); c) which modulate (upregulate or downregulate) other genes or operons; or d) which are part of a multi-protein complex or pathway (enzymes required for cell-wall biosynthesis). Nonetheless, the approach described in this study will identify an important class of genes and gene products, and may provide invaluable insights into the survival of mycobacteria within macrophages and identify potential new targets for combatting these important pathogens. [Department of Microbiology and Immunology, Emory University, Atlanta; Division of Bacterial and Mycotic Diseases, Centers for Disease Control, Atlanta, Georgia, U.S.A.]

Fukutomi, Y., Adams, L. and Krahenbuhl, J. Tumor necrosis factor in experimental leprosy.

These studies show that exposure of normal macrophages to *Mycobacterium leprae* or mycobacterial constituents provides a potent stimulation of tumor necrosis factor (TNF) production. The use of Polymyxin B to neutralize lipopolysaccharide (LPS)-induced TNF production, together with the precautions used to prepare the bacilli and/or purify mycobacterial constituents, rules out the conclusion that observations such as these are attributable to contamination of the reagents with LPS. The role of local TNF production in host resistance to lep-

*** From Joint Tuberculosis and Leprosy Symposium, Wednesday, August 7, 1991, afternoon session.

rosy remains open to speculation. Compelling evidence exists that local TNF elaboration is important in the formation of the epithelioid granuloma and control of BCG growth in mice. Whether a similar role for TNF can be demonstrated in mice infected with *M. leprae* is currently under investigation. Even in the nu/nu mouse model for a lepromatous lesion, the continuous elaboration of low levels of TNF may have a profound local effect on the maintenance of the lesion by virtue of the potent effects that TNF may have on blood vessel endothelial cells and diapedesis of macrophages into the lesion. We present our findings regarding the ability of TNF-containing supernatant media to markedly upregulate the macrophage effector function in the absence of interferon-gamma. [Immunology Research Department, Laboratory Research Branch, GWL Hansen's Disease Center, Carville, Louisiana, U.S.A.]

Fukutomi, Y., Adams, L. and Krahenbuhl, J. Macrophages infected with *Mycobacterium leprae* or treated with mycobacterial constituents release factors that modulate afferent and efferent macrophage function.

In our previous studies we have decribed defective macrophage (MAC) function in experimental leprosy where a heavy burden of intracellular *Mycobacterium leprae* or treatment with *M. leprae* or *M. tuberculosis* lipoarabinomannan (LAM) renders MAC unresponsive to the ability of interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) to enhance MAC afferent and efferent effector function. This effect required 3–5 days of infection or a relatively high dose of LAM. The present studies are fundamentally different since we examined the striking ability of LAM and *M. leprae* to modulate MAC function in only a few hours. More importantly, although the 5-hr supernatant induced by *M. leprae,* LAM, or LPS downregulated Ia induction, these supernatant media actually enhanced MAC efferent effector function rather than render the MAC refractory to IFN-γ induced activation, as described previously.

The ability of *M. leprae* and LAM to induce activating supernatant is a property shared with LPS yet is fundamentally distinct since Polymyxin B blocked both tumor necrosis factor (TNF) induction and production of activating supernatant by LPS but not *M. leprae* or LAM. Once produced, the activity of the supernatant was not abrogated by Polymyxin B, regardless of the source. In exploring the identity of the active factor(s) in these supernatants, indomethacin or exogenous PGE_2 failed to alter the results, suggesting MAC-derived arachidonic acid metabolites were not involved. We examined the roles of a variety of cytokines using two approaches: addition of the cytokines to test media and depletion of cytokines from supernatant with neutralizing antibody. Interestingly, only neutralization of TNF activity with specific antibody abrogated the ability of supernatant to activate MAC as measured by enhanced microbicidal capacity for *Toxoplasma* or *M. leprae,* enhanced production of NO_2, and enhanced tumoricidal activity. However, the role of TNF remains very unclear since in each of these assays for MAC activation TNF alone was ineffective in activating the MAC. It is possible that TNF does play a key role in the supernatant activation described, but as a potent co-factor to an as yet unknown product of short-term MAC stimulation. The relevance of these findings is also unclear. Possibly we have described a mechanism whereby MAC exposed to *M. leprae* or mycobacterial products are able to secrete factor(s) that markedly modulate the function of nearby MAC in the absence of T-cell-derived lymphokines. The possible role of this phenomenon in the local microenvironment of the tuberculoid or lepromatous granuloma is worthy of further investigation. [Immunology Research Department, Laboratory Research Branch, GWL Hansen's Disease Center, Carville, Louisiana, U.S.A.]

Yamamura, M., Uyemura, K., Rea, T. H. and Modlin, R. L. Defining protective immune responses to infectious pathogens: lymphokine and cytokine profiles in leprosy lesions.

The present observations in human leprosy lesions clearly reveal two distinct patterns of cytokine production that correlate with the known levels of cell-mediated immunity (CMI) against infection. Th1-like

lymphokine mRNAs were abundant in tuberculoid lesions which are basically self-healing and characterized by resistance to growth of *Mycobacterium leprae*. In marked contrast, Th2-like lymphokine mRNAs were abundant in lepromatous lesions correlating with the immunologic unresponsiveness to *M. leprae*. The abundance of IL-2 IFN-γ, lymphotoxin, and GM-CSF in tuberculoid lesions is likely to contribute to the resistant state of immunity in these patients. IFN-γ is well known to enhance production of reactive oxygen and nitrogen intermediates by macrophages and to stimulate them to kill or restrict the growth of intracellular pathogens. IFN-γ also augments expression of HLA-DR and ICAM-1 which could facilitate T cell-accessory cell interaction. IL-2 may contribute to the host defense by inducing the clonal expansion of immune-activated, lymphokine-producing T cells and augments the production of IFN-γ. In fact, cutaneous administration of either IL-2 or IFN-γ to lepromatous patients results in some clearance of bacilli from lesions. As well, lymphotoxin, GM-CSF, IL-1β, TNFα and IL-6 can contribute to resistance. Although it is unclear that cytokines act individually and additively, more likely it is the matrix of lymphokines and cytokines locally that determines the ultimate biological response at the locus of infection.

In contrast to the set of cytokine mRNAs present in tuberculoid lesions that might be involved in CMI and inflammation, those found to be increased in lepromatous lesions might be expected to contribute to the immune unresponsiveness and failure of macrophage activation in these individuals. IL-4 was increased in lepromatous lesions compared with tuberculoid lesions, and is known to inhibit IFN-γ production as well as IFN-γ-mediated production of reactive oxygen intermediates, such as hydrogen peroxide, by macrophages. Additionally, IL-4 has been reported to inhibit IL-2 activation of T cells through IL-2R expression and to inhibit production of TNFα and IL-1β by macrophages. Elegant studies of murine immune responses to leishmania infection in BALB/c mice have demonstrated that T cells capable of adoptively transferring resistance to infection produce predominantly IL-2 and IFN-γ *in vitro*; whereas those that induce exacerbation and more rapid lesion formation produce primarily IL-4 and IL-5. Treatment of susceptible mice with anti-IL-4 antibodies resulted in increased IFN-γ production with a concomitant increase in the host resistance to these parasites. Similarly, in murine schistosomiasis induction of Th2 responses is accompanied by a downregulation of Th1 cytokine production. The IL-4 and IL-5 in lepromatous lesions could lead to similar enhancement of bacterial growth and even contribute to the elevated levels of anti-*M. leprae* immunoglobulin found in lepromatous patients. The presence of IL-10 in lepromatous lesions, another cytokine that can downregulate lymphokine production of CD4 T cells, in the relative absence of IFN-γ or IL-2, suggests a possible role for this cytokine in the specific immunological unresponsiveness to *M. leprae* antigens.

Two different episodes of significant inflammation were studied: reversal reaction and erythema nodosum leprosum (ENL), as well as the Mitsuda reaction, a measure of delayed-type hypersensitivity in these patients. In the reversal reaction and the Mitsuda reaction, IL-2, LT, GM-CSF, and IFN-γ mRNAs were strongly expressed. In contrast, in ENL, IL-4, IL-5, and IL-10 mRNAs were prominent. Interestingly, although most monocyte cytokines were similarly expressed in these reactions, IL-6 and IL-8 were more abundant in ENL as compared to reversal reaction. In the reaction of leprosy, reversal reactions and Mitsuda reactions appear to be Th1-type responses. IL-2, lymphotoxin, GM-CSF, IFN-γ were prominent in reversal reaction as well as in the Mitsuda reaction. On the other hand, IL-4, IL-5, and IL-10 appear to characterize the lesion of ENL, suggesting that these are Th2 responses. [Division of Dermatology; Department of Microbiology and Immunology, UCLA School of Medicine; Section of Dermatology, USC School of Medicine, Los Angeles, California, U.S.A.]

Salgame, P., Abrams, J., Clayberger, C., Lyu, S., Modlin, R. and Bloom, B. R. Lymphokine profile of functionally distinct *M. leprae*-reactive CD4+ T-helper and CD8+ T-suppressor clones.

Murine T-helper cells have recently been classified into two distinct groups based on

their lymphokine secretion patterns. Upon stimulation TH1 cells produce IFN-γ and IL-2. The TH2 cells make predominantly IL-4 and IL-5. Mouse IL-4 promotes switch of lipopolysacharride (LPS)-stimulated B cells to the expression of IgG1 and IgE; whereas IFN-γ inhibits the production of IgG3, IgG1, IgG2b, and IgE. The biological significance of the dichotomy in the CD4+ T cells is well illustrated in murine leishmaniasis, where protection is mediated by antigens inducing a TH1 response and the exacerbation of the disease is induced by TH2 cells making IL-4. Thus far, however, a comparable clear-cut dichotomy between IL-4- and IFN-γ-producing T cells has not been reported in humans.

The spectrum of clinical manifestations in leprosy correlate with cell-mediated immune responses to *Mycobacterium leprae* antigens, and ranges from the highly reactive tuberculoid form to the susceptible lepromatous form. We have established in our laboratory a panel of *M. leprae*-reactive CD8+ T-suppressor clones from lepromatous leprosy patients and CD4+ T-helper clones from lepromin-positive healthy contacts. In order to understand the cellular basis of T-cell-mediated protection and suppression, we studied the lymphokine profile of a panel of functionally distinct CD4 and CD8 clones. CD4+ tetanus toxoid responsive clones and CD8+ alloreactive cytotoxic clones were also included in the panel for comparison. All of the clones were stimulated with anti-CD3 antibody, and the supernatants were harvested at 18 hr and assayed for IFN-γ, IL-4, IL-5, and GM-CSF. IFN-γ was analyzed using the ELISA kit from Amgen Biological; IL-4 and GM-CSF were measured by using ELISA kits from Genzyme; IL-5 levels were assayed at DNAX Inc.

M. leprae-reactive CD4+ clones and alloreactive CD8+ cytotoxic clones made IFN-γ but not IL-4 or IL-5. The CD4+ tetanus toxoid specific clones, however, made IL-4, IL-5 but not IFN-γ. The CD8+ suppressor clones produced IL-4 but made none or very little IFN-γ. All of the clones analyzed secreted GM-CSF. All of the CD4 clones made IL-2 and either IFN-γ or IL-4 and IL-5. Based on the above lymphokine profiles, we have demonstrated consistent subtypes in CD8+ populations in humans comparable to the TH1 and TH2 phenotype in the mouse. The production of IL-4 by T-suppressor cells and not by cytotoxic cells enables us to distinguish the functionally different CD8+ populations for the first time based on markers other than function. There is growing evidence that IFN-γ and IL-4 mutually inhibit their respective functions. The role of IL-4 made by T-suppressor cells in immune suppression is being investigated. [Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York; Department of Immunology, DNAX Research Center, Palo Alto, California; Department of Cardiovascular Surgery, Stanford University Medical Center, Palo Alto, California; Department of Medicine, UCLA Medical School, Los Angeles, California, U.S.A.]

Kaplan, G. and Sampaio, E. P. Role of TNFα in the pathogenesis of mycobacterial infections; mechanism of modulation by thalidomide.

Leprosy patients often develop reactional states (acute episodes of immunologic and inflammatory responses) which may be associated with changes in immunological reactivity. Reactions are classified into two types according to their clinical and histological manifestations: Type 1 or reversal reaction (RR) and type 2 or erythema nodosum leprosum (ENL). These reactional states constitute serious complications of leprosy which require prompt diagnosis and therapy. Cytokines released from macrophages and lymphocytes may play an important role in these phenomena. Indeed, recent findings have suggested the association of high serum levels of IL-1 and tumor necrosis factor α (TNFα) with the presence of ENL and reversal reactions. Therefore, during *in vivo* clinical trials with recombinant lymphokines, reactional manifestations constitute a potential deleterious effect of immunomodulation in leprosy patients. Consequently, it is important to understand the mechanisms underlying these reactional episodes.

Three patients injected with 100 μg IFN-γ every month developed ENL (disseminated subcutaneous nodules) following the first, fourth or seventh monthly high dose rIFN-γ injection. One patient who developed re-

peated systemic symptoms was treated with thalidomide (100 mg/day for 21 days) and IFN-γ injections were discontinued after the fourth dose. All of the patients who received IFN-γ twice monthly developed leprosy reactions: 2 had multiple ENL skin lesions without other systemic symptoms (not treated with thalidomide), 1 had RR (treated with prednisone), and another developed RR followed by severe ENL (treated with thalidomide and prednisone). A correlation between a large area of induration (> 15 mm) in response to IFN-γ and the development of ENL was noted in 5 of 6 patients. A single ENL skin lesion was observed following the multiple low doses of rIFN-γ (six 30 μg injections) in 4 of the 6 patients who later developed ENL. No enhancement in serum levels of TNFα was observed. However, higher TNFα levels were induced in monocytes of patients after IFN-γ injection compared to pre-injection levels.

Five lepromatous patients with untreated ENL were examined for the ability of their peripheral blood mononuclear cells (PBMC) and monocytes to release TNFα *in vitro*. Cells from normal individuals, thalidomide-treated ENL patients, and lepromatous patients without ENL were also analyzed. Purified monocytes or PBMC were stimulated with lipopolysaccharide (LPS) or different mycobacterial products, and after 18 hr of culture supernatants were recovered and assayed for TNFα levels. After *in vitro* stimulation of PBMC with LPS or BCG, a higher TNFα response was detected in untreated reactional patients as compared to normal individuals or ENL patients treated with thalidomide (100 mg/day for 1–2 weeks). When purified monocytes were analyzed, patients with untreated ENL showed enhanced TNFα response after BCG stimulation. In addition, monocytes from patients with untreated ENL and lepromatous leprosy showed increased TNFα levels in response to stimulation with MDP as compared to thalidomide-treated ENL patients and normal controls. No spontaneous release of TNFα was noted in any of the groups studied.

Thalidomide, the drug of choice for the treatment of ENL, selectively inhibits the production of human monocyte TFN triggered by LPS and other agonists in culture; 40% inhibition occurs at the clinically achievable dose of the drug of 1 μg/ml. In contrast, the amount of total protein and individual proteins labeled with [^{35}S]methionine and expressed on SDS-PAGE are not influenced. The amounts of interleukin 1β, IL-6, and granulocyte/macrophage colony-stimulating factor produced by monocytes remain unaltered. [Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York, U.S.A.]

Sela, S., Thole, J. E. R., Ottenhoff, T. H. M. and Clark-Curtiss, J. E. Molecular characterization of immunologically reactive proteins of *Mycobacterium leprae.*

Immunological screening of the pHC79::*Mycobacterium leprae* cosmid library and the pYA626::*M. leprae* plasmid library with pooled sera from lepromatous leprosy patients has resulted in the identification of approximately 100 cosmid clones and two plasmid clones that specify a protein or proteins that react with antibodies in the patients' sera. Several cosmid clones and one plasmid clone possess a common 1.2 kb *Pst*I DNA fragment that carries a gene encoding a 15-kDa protein that reacts with antibodies in pooled sera from lepromatous leprosy patients and sera from tuberculoid leprosy patients. Subclones and λgt11 clones possessing sequences encoding 10-kDa of the 15-kDa antigen fused to *lacZ* have been shown to elicit proliferative responses in T cells from leprosy patients and household contacts. The gene specifying the 15-kDa protein does not share homology with any sequence in GenBank other than the sequences from λgt11::*M. leprae* clone LSR2, which codes for 10 kDa of the protein. The 15-kDa antigen appears to be a good candidate for further study to determine whether or not it can elicit a protective immune response against *M. leprae.* [Departments of Biology and Molecular Microbiology, Washington University, St. Louis, Missouri, U.S.A.; Department of Immunohaematology and Blood Bank, University Hospital, Leiden, The Netherlands]

Rivoire, B., Bozik, C. M., Duysen, E., Mehra, V., Bloom, B. R., Brennan, P. J. and Wu-Hunter, S. Identification, characterization, and expression of the major

proteins of *Mycobacterium leprae* of molecular mass 14 kDa and 35 kDa.

From the array of major somatic proteins of *Mycobacterium leprae* previously identified, two proteins of molecular mass 14 kDa (MCP-I) and 35 kDa (MMP-I) were chosen for further characterization and expression as recombinant proteins. The full amino acid sequence of MCP-I was established by a combination of Edman chemistry and FAB/MS. It differed from the GroES analog/BCG, a protein of *M. tuberculosis*/BCG, only in the nature of ten of its amino acids. Specific oligonucleotide primers were prepared for polymerase chain reaction (PCR) amplification of the MCP-I gene which was then cloned into *Escherichia coli* with a pTRP expression vector. Expressed recombinant MCP-I reacted at the same molecular weight as the native MCP-I in Western blots with specific monoclonal antibodies. The 35-kDa protein (MMP-I) was not amenable to N-group analysis. The nucleotide sequence of MMP-I was determined from a clone selected from the *M. leprae* λgt11 library of a specific anti-MMP-I monoclonal antibody. The clone contains 40% of the gene encoding the C-terminus of MMP-I, according to the molecular size of the β-galactosidase fusion protein produced by these clones. [Department of Microbiology, Colorado State University, Fort Collins, Colorado; Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, U.S.A.]

Matsuo, E., Komatsu, A., Murakami, M., Terada, M., Sasaki, N. and Skinsnes, O. K. On the pathologic significance of the receptor for Beta-glucuronidase (BGR) of *Mycobacterium leprae* in relationship to its other antigen.

Mycobacterium leprae seems to combine the enzyme beta-glucuronidase (B-Gase) of the host to its receptor (BGR) to release and utilize glucuronic acid (GA) from hyaluronic acid which is abundant in lepra cells and the endoneurium, wherein are located the sites of parasitism itself. On the other hand, a mycobacterium HI-75, originally separated from leproma and which has been regarded as *M. scrofulaceum,* grew remarkably in Ogawa's medium with only the supplementation of the medium with GA with or without N-acetylglucosamine. In this study, we tried further to confirm the cross immunoreactivity of the BGR of HI-75 and *M. leprae,* its distribution among mycobacteria and other microorganisms, the possibility of the inhibition of the BGR-B-Gase combination by antibody against BGR and the identification of HI-75. The question of whether or not *M. leprae* and HI-75 are similar was determined by an absorption test of positive control serum in SERODIA-Leprae, a *M. leprae*-specific antibody detection kit with HI-75 and its fractions.

First of all a method for the immunohistologic staining of BGR in leproma was developed. By this method BGR was shown to be present not only in *M. leprae* but also apparently in other intracellularly grown microorganisms, such as *M. intracellulare* and hepatitis B virus. The results suggest that BGR is a necessary component for some intracellular parasites, including *M. leprae.*

Secondly, the present study did not demonstrate the inhibition of the BGR-B-Gase combination by anti-BGR antibody. However, BGR might still become a potent immunogen for vaccination. The reason is that once the immune reaction takes place *in vivo* it might cause complement fixation or cellular reaction that completely damages the activity of BGR in microorganisms which may be essential for them to borrow the metabolic machinery.

Thirdly, among the fractions arising from HI-75, BGR absorbed some anti-PGL antibody, further indicating a relationship between the two. The reason why cardiolipin and lecithin are required for this absorption is unknown. [Department of Pathology, Kyorin University School of Medicine, Tokyo; Tohoku Shinseien, Miyagi, Japan; Sun Yat-Sen University of Medical Sciences, Guangzhou, China]

Elangeswaran, N., Saito, T., Matsuoka, M., Sakamoto, Y. and Mori, T. Application of monoclonal antibodies to differentiate the morphological forms of *Mycobacterium lepraemurium* Hawaiian strain.

Monoclonal antibodies (MOAbs) are useful tools for the identification and characterization of microbial and other antigenic

determinants. Also, the elucidation of the antigenic structure and taxonomy of an infectious pathogen has been greatly advanced with the aid of MOAbs. In order to identify the genes encoding the 65-kDa protein of *Mycobacterium lepraemurium,* we have developed a λgt11-based genomic library of *M. lepraemurium* (smooth form). To screen the library, two MOAbs to the 65-kDa protein of *M. lepraemurium* were generated. During characterization of the MOAbs it was noted that there were different reactivities with the MOAbs by ELISA. Reactivity of the MOAbs was carried out to compare our MOAbs with the established Lm.6 MOAb against the 65-kDa protein of *M. lepraemurium* (rough form) which reacted with the rough form more intensively than with the smooth form. To confirm this fact, the major protein fractions of *M. lepraemurium* (smooth) were purified according to Hunter, *et al.* and three types of 65-kDa protein antigens, i.e., cell-wall, membrane, and soluble 65-kDa proteins, were obtained from the respective fractions. We confirmed the different reactivity pattern of MOAbs against the purified 65-kDa proteins of *M. lepraemurium* by both ELISA and one- and two-dimensional SDS-PAGE immunoblotting. We identified the DNA fragments encoding the cell-wall and soluble 65-kDa proteins cloned in the λgt11 vector, and further sequencing work is in progress. Also, we have generated the MOAbs against purified 65-kDa proteins of the soluble and membrane-bound fractions of *M. lepraemurium.* The details of these studies are presented. [National Institute for Leprosy Research, Higashimurayama, Tokyo, Japan]

Gelber, R. H., Murray, L., Siu, P., Tsang, M., Iranmanesh, A. and Rea, T. H. Bactericidal antibiotics of three different classes emerge to treat leprosy: recent developments from the laboratory to the patient.

Previously, we described (J. Inf. Dis., *156,* 236, 1987) that minocycline was bactericidal for *Mycobacterium leprae* in mice at levels easily obtained in man. We can now confirm that minocycline consistently inhibits seven different *M. leprae* isolates, including one partially and one fully dapsone resistant. Furthermore, we have found that minocycline is consistently active in mice treated 3 days and 1 day weekly, and partially active even 1 day monthly. In the first clinical trial of minocycline in eight patients with lepromatous leprosy (100 mg daily for 3 months) we found it consistently and very rapidly effective clinically and without significant toxicity. We can now report on our complete results of the clearance of viable *M. leprae* from the skin of patients in that trial as assessed by serial skin biopsies 1 week, 1 month, 2 months, and 3 months after the initiation of treatment. *M. leprae* viability in these studies was determined by two means: pools of four foot pads and 10 individual foot pads, which resulted in the clearance of all viable *M. leprae* in 2 and 3 months, respectively. This rate of clearance of viable acid-fast bacilli (AFB), though slower than that found previously for rifampin, was faster than for either dapsone or clofazimine. Because minocycline has been used throughout the world for over 30 years and has been demonstrated to be safe on chronic administration, it will certainly be an important addition to the therapy of leprosy.

Previously, Franzblau, *et al.* (Antimicrob. Agents Chemother., *32,* 1758, 1988) found that the new macrolide, clarithromycin, was active against *M. leprae in vitro* and when fed mice at 0.01%. Newer macrolides offer the advantage over clarithromycin itself by virtue of better gastrointestinal absorption, tissue and macrophage accumulation, and enhanced spectrum of activity. In our studies we found in mice that while erythromycin (0.06% in diet) and azithromycin (0.1%) were inactive, both clarithromycin and roxithromycin (0.1%) were, in two separate studies, bactericidal at levels easily obtained in man: a) roxithromycin and clarithromycin, respectively, delayed multiplication of *M. leprae* for 5 and 11 months after drug discontinuation; b) roxithromycin and clarithromycin, respectively, were found to be 82% ± 13% and 96% ± 2% bactericidal.

Previously, Grosset, *et al.* found that pefloxacin and ofloxacin inhibit the growth of *M. leprae* in mice (Int. J. Lepr., *55,* 70, 1987; Int. J. Lepr., *56,* 259, 1988), and in a clinical trial cleared *M. leprae* from the skin in lepromatous patients within 2 months (Int. J.

Lepr., *58*, 281, 1990). In the past 2 years, newer quinolones that are more active against gram-positive bacilli and quinolone-resistant bacteria have been developed. We compared the activity of some of these (sparfloxacin, temafloxacin, lomafloxacin, WIN 57273, and PD 124816) with pefloxacin and ofloxacin in groups of mice treated 5 times weekly by gavage with doses of 50, 150, and 300 mg/kg by the kinetic technique of Shepard (treatment day 60–150 after foot pad infection). Additionally, sparfloxacin was tested at 15 mg/kg and 30 mg/kg. We judged drugs bacteriostatic if at the end of therapy, the number of AFB were less than untreated controls but multiplication commenced immediately thereafter, "partially bactericidal" if multiplication was further delayed, and "fully bactericidal" if *M. leprae* did not grow even 9 months after therapy was completed. Pefloxacin and lomafloxacin were found inactive and bacteriostatic, respectively, at 50 mg/kg, bacteriostatic at 150 mg/kg, and partially bactericidal at 300 mg/kg. PD 124816 was bacteriostatic at 50–150 mg/kg and fully bactericidal at 300 mg/kg. Ofloxacin was bacteriostatic at 50 mg/kg and fully bactericidal at 150–300 mg/kg. WIN 57273 was partially bactericidal at 50 mg/kg and fully bactericidal at 150–300 mg/kg. Temafloxacin was fully bactericidal at all three doses tested, while sparfloxacin was fully bactericidal at these doses, as well as 15 mg/kg and 30 mg/kg. These results suggest that certain of the newer quinolones, especially sparfloxacin and temafloxacin, are even more active than pefloxacin and ofloxacin against *M. leprae*-infected mice. [Kuzell Institute, San Francisco, California; GWL Hansen's Disease Center, Carville, Louisiana; University of Southern California, Los Angeles, California, U.S.A.]

Saito, H., Tomioka, H. and Sato, K. Antileprosy activity of ofloxacin in combination with rifampin and diaminodiphenylsulfone in mice and *in vitro* activities of new quinolones and rifamycin derivatives.

The therapeutic activity of ofloxacin (OFLX) (3 mg/mouse/injection) or rifampin (RFP) (0.01 mg/mouse/injection) alone or in combination was studied. RFP alone at this dose caused a 2.0-log decrease in the number of leprosy bacilli recovered from the infected mouse foot pad on day 365 after infection as compared to that of solute control mice. OFLX alone also caused a 1.6-log decrease in the value. Combined use of OFLX with RFP gave a 3.2-log decrease in the number of bacilli as compared to that of control mice. Thus, there was a clear combined effect between OFLX and RFP. These findings were confirmed in terms of the foot pad thickness of *Mycobacterium leprae*-infected animals. Infection-induced foot pad swelling was almost completely inhibited by RFP treatment with or without OFLX. OFLX alone caused a 1.5-mm decrease in foot pad swelling compared to the control value.

The antileprosy activity of OFLX (3 mg/mouse/injection) or dapsone (DDS) (0.2 mg/mouse/injection) alone or in combination was also studied. DDS alone given at this dose caused a 0.8-log decrease in the number of organisms recovered from the infected foot pads of host mice 365 days after infection as compared to control mice. In this experiment, OFLX alone caused a 0.9-log decrease in the value. In the case of combination use of OFLX with DDS, a 1.98-log decrease in the number of recovered *M. leprae* was found compared to control animals. Thus, the therapeutic efficacy of DDS was much improved by the combined use of OFLX. Foot pad swelling due to *M. leprae* infection was markedly decreased by administration of either DDS or OFLX alone; 1.6-mm and 1.4-mm decrease, respectively. A somewhat larger decrease in the foot pad swelling (1.8-mm decrease) was observed in mice given OFLX in combination with DDS.

Of the quinolones, sparfloxacin (SPFX) added to the BACTEC 12B medium at concentrations of 0.5 or 2.0 μg/ml caused a marked reduction in the GI value of *M. leprae* (61%–94% decrease). This effect was obvious in the readings during days 5–11 and days 12–18. Other test quinolones, such as pipemidic acid, piromidic acid, and enoxacin, slightly reduced the GI value at both concentrations (0–20% inhibition). Norfloxacin (NFLX), ciprofloxacin (CPFX), fleroxacin (FLRX), and OFLX at the concentration of 2 μg/ml caused considerable reduction of the GI value (37%–65% de-

crease). Although the GI values measured in this experiment were considerably lower than those reported by Franzblau, presumably due to low metabolic activity of the organisms, SPFX seems to have an excellent anti-*M. leprae* activity when compared with other quinolones. Intermediate *in vitro* activity of OFLX and FLRX and the lack of the activity in other quinolones, including CPFX, are fairly consistent with our previous findings that OFLX and FLRX had a significant *in vivo* antileprosy activity, but none of the latter quinolones exhibited such efficacy.

Excellent *in vitro* anti-*M. leprae* activities of rifamycin derivatives, such as RFP, rifabutin, and a newly synthesized benzoxazinorifamycin (KRM-1648), were observed. These agents at concentrations of 0.5 μg/ml and 2 μg/ml caused a marked reduction in the GI value (60%–97% decrease). In particular, KRM-1648, which has been evidenced by us to possess an excellent antimycobacterial activity, exhibited the strongest activity (77%–97% inhibition of GI value) among test rifamycins. The *in vivo* antileprosy activity of KRM-1648 is now under study in our laboratory. [Department of Microbiology and Immunology, Shimane Medical University, Izumo, Japan]

Williams, D. L., Gillis, T. P., Franzblau, S. G. and Hastings, R. C. Evaluation of PCR analysis in monitoring antileprosy chemotherapy in lepromatous leprosy patients.

The purpose of this research was to determine the potential of using polymerase chain reaction (PCR) analysis to monitor antileprosy chemotherapy directly from skin biopsies of leprosy patients. Homogenates of skin biopsies (4 mm) from lepromatous leprosy patients with no chemotherapy or chemotherapy up to 20 years were analyzed for the presence of *Mycobacterium leprae* using PCR and previously defined DNA primers which specifically amplify a 360 bp target sequence of *M. leprae* DNA. Agarose gel analysis of PCR products from 20 untreated patients showed equivalent quantities of PCR products when 3×10^4 *M. leprae* were analyzed. Similar analysis of 20 treated leprosy patients showed that most patients with more than 2 months of chemotherapy prior to biopsy had greatly diminished, or the absence of, PCR products. Three patients having 14 to 20 years of chemotherapy but harboring either dapsone- or rifampin-resistant *M. leprae* as determined by the mouse foot pad drug assay gave quantities of PCR product equivalent to untreated patients. PCR products of two patients clinically suspected of relapse due to intermittent drug treatment regimens were also equivalent to PCR products from untreated patients. We also compared the viability of *M. leprae* after treatment with a variety of antileprosy drugs with corresponding PCR results, in both *in vitro* (BACTEC) and *in vivo* (nude mice) systems. We have evidence supporting the relationship between the loss of viability, due to antibacterial therapy, and the diminution of PCR reactivity in the *in vivo* model. Therefore, it is anticipated that the *M. leprae* PCR assay may be useful in Hansen's disease patient monitoring. [Laboratory Research Branch, GWL Hansen's Disease Center, Carville, Louisiana, U.S.A.]

Schlesinger, L. S. and Horwitz, M. A. Host and bacterial molecules mediating phagocytosis of *Mycobacterium leprae* by human mononuclear phagocytes.

We have been examining the molecular basis for phagocytosis of *Mycobacterium leprae* by human mononuclear phagocytes. We have determined previously that *M. leprae* invasion of human monocytes is mediated by complement receptors CR1 and CR3 on the surface of the monocyte and fragments of complement component C3 fixed to the bacterium. We now report our studies on a) phagocytosis of *M. leprae* by human monocyte-derived macrophages (MDM), b) the influence of interferon-gamma (IFN-γ) activation on complement receptor function and *M. leprae* phagocytosis, c) the identity of C3 acceptor molecules on the *M. leprae* surface, and d) the role of natural antibody in C3 fixation.

To study MDM receptors mediating *M. leprae* uptake, we cultured human peripheral blood monocytes for 5 days, incubated the MDM with armadillo-derived *M. leprae,* and enumerated MDM-associated bacteria by fluorescence microscopy. Compared with monocytes, MDM adherence of

M. leprae was markedly increased (> six-fold). MDM adherence of *M. leprae* was highly serum-dependent and significantly decreased by heat inactivation of serum, demonstrating the importance of heat-labile serum opsonins. Electron microscopy studies revealed that all MDM-associated bacteria were intracellular. Soluble monoclonal antibodies (MOAbs) against MDM complement receptors but not MOAbs against other MDM surface antigens markedly inhibited adherence and phagocytosis of *M. leprae* in the presence of nonimmune serum. A combination of MOAbs against CR1 and CR3 significantly inhibited *M. leprae* adherence; however, a combination of MOAbs against these complement receptors and CR4, a complement receptor that is markedly upregulated on MDM, was required to obtain maximal inhibition of *M. leprae* adherence (nearly 1 log).

MDM activation by IFN-γ resulted in a dose-dependent reduction in *M. leprae* adherence (83% ± 2%) and phagocytosis (88%), assessed by phase contrast and electron microscopy, respectively. Paralleling this, complement receptor function, assessed by measuring adherence of E-C3b and E-C3bi, was also strikingly decreased (69% ± 4% and 70% ± 4%, respectively); whereas Fc receptor function, assessed by measuring adherence of E-IgG, was enhanced. By flow cytometry, IFN-γ activation resulted in a significant decrease in MDM surface expression of CR1 but not CR3 or CR4.

To evaluate the capacity of surface molecules of *M. leprae* to serve as acceptor molecules for fragments of complement component C3, we developed an ELISA to measure C3 fixed to purified *M. leprae* surface carbohydrates. Phenolic glycolipid-I (PGL-I) readily fixed C3 in a dose-dependent and serum concentration-dependent fashion. In contrast, lipoarabinomannan, lipomannan, and arabinogalactan did not fix appreciable amounts of C3. C3 fixation to PGL-I was classical pathway-dependent and required the presence of both the terminal trisaccharide and the lipid portion of this molecule. PGL-I bound to polystyrene microspheres specifically fixed C3 in nonimmune serum, and PGL-I and C3 mediated ingestion of the polystyrene microspheres by human monocytes, as assessed by electron microscopy.

To assess the role of antibody in nonimmune serum in C3 fixation to *M. leprae*, we used an ELISA to study the influence of antibody on C3 fixation and Clq binding to *M. leprae* and PGL-I. C3 fixation to *M. leprae* was strictly antibody-dependent. *M. leprae* did not fix C3 in agammaglobulinemic serum unless pure IgG or IgM or heat-inactivated nonimmune serum (as a source of antibody) was added. C3 fixation required the antigen-binding portion of the antibody molecule since equivalent amounts of pure Fc fragments of immunoglobulin did not mediate C3 fixation to *M. leprae* in agammaglobulinemic serum. At low concentrations of nonimmune serum, C3 fixation to *M. leprae* occurred by both the classical and alternative pathways. Consistent with a role for the classical pathway, C3 fixation to *M. leprae* was enhanced by Clq. C3 fixation in Clq-depleted serum was increased twofold by the addition of pure Clq. IgG, IgM, and Clq were readily detected on the surface of *M. leprae*.

Consistent with the finding that C3 fixation to PGL-I is classical pathway-dependent, Clq bound to PGL-I in a serum concentration-dependent manner. Clq binding to PGL-I was strictly antibody-dependent. Pure Clq bound to PGL-I only after the addition of pure IgG or IgM, or heat-inactivated nonimmune serum.

These studies demonstrate that a) MDM exhibit an enhanced capacity to ingest *M. leprae*; b) MDM complement receptors CR1, CR3, and CR4 mediate phagocytosis of *M. leprae*; c) IFN-γ-activated MDM exhibit decreased *M. leprae* phagocytosis and complement receptor activity; d) PGL-I of *M. leprae* selectively fixes C3, and PGL-I and C3 mediate monocyte ingestion of polystyrene microspheres; and e) natural antibody mediates C3 fixation to *M. leprae* and Clq binding to PGL-I. Thus, complement receptors on mononuclear phagocytes, fragments of complement component C3, and PGL-I on the outermost surface of *M. leprae* form a complete receptor-ligand-acceptor molecule system for mediating phagocytosis of *M. leprae*. This three-component phagocytic system is dependent upon natural antibody for fixation of C3 to PGL-I,

and it is down-regulated by IFN-γ via the effect of this lymphokine on complement receptor function. [Division of Infectious Diseases, Department of Medicine, UCLA School of Medicine, Los Angeles, California, U.S.A.]

Modlin, R. L., Uyemura, K., Ohmen, J., Wang, X.-H., Rea, T. H. and Barnes, P. F. The T-cell receptor repertoire in mycobacterial infection.

The diversity of gamma/delta ($\gamma\delta$) T-cell-receptor repertoire within lepromin skin tests was investigated to provide clues as to the set of antigens recognized by these cells. Immunohistologic staining of frozen sections with monoclonal antibodies directed against Vδ encoded determinants revealed that within the dermal granulomas, Vδ1- and Vδ2-bearing cells accounted for the majority of infiltrating $\gamma\delta$ cells. The Vδ2:Vδ1 ratio was approximately 2:1 in the skin lesions as compared to 9:1 in the blood of the same individuals. Additional distinct microanatomic patterns for Vδ1- and Vδ2-bearing cells were identified: the dermal infiltrate containing both populations, but the epidermal $\gamma\delta$ T cells preferentially expressing the Vδ1 chain. The localization of Vδ1+ cells to epidermis in these mycobacterial lesions suggests either homing, retention, and/or *in situ* expansion of Vδ1+ cells during the immunopathologic reaction. Further analysis of Vδ and Jδ gene segment usage was accomplished by polymerase chain reaction (PCR) amplification of DNA extracted from lesions. These studies revealed that both Vδ1 and Vδ2 gene segments rearrange with the Jδ1 gene segment. The proportion of Vδ1-bearing cells appears to be much greater than in normal skin. Similar to normal skin, the Jδ1 gene segment is preferentially used by these $\gamma\delta$ T cells.

The junctional diversity of $\gamma\delta$ T cells in lepromin skin tests was determined by cloning and sequencing PCR-amplified products. Strikingly, in each of the three lepromin skin tests subjected to nucleotide sequencing analysis, the majority of Vδ1-Jδ1 and Vδ2-Jδ1 junctional sequences were found to be identical, but distinct for each patient. This was clearly different from peripheral blood of these same individuals, which exhibited extensive diversity. The junctional sequences of $\gamma\delta$ cells in lesions were not found in the respective individual's peripheral blood, indicating that the lesional sequences do not represent an already existing clonal expansion in the peripheral repertoire. It is noteworthy that in each spatially separated region of the biopsy specimen, a limited number of junctional nucleotide sequences were represented multiple times, although the predominant sequence differed from site to site. Since there was conservation in the predicted amino acid sequence in various areas of the biopsy and among individuals, we hypothesize that the $\gamma\delta$ T-cell expansion in lesions is selected by a limited set of antigens within the tissue microenvironment. The microheterogeneity implies that a very small number of $\gamma\delta$ clones initiate the oligoclonal expansion and that their progeny do not disperse homogeneously throughout the lesion. The study of leprosy lesions indicates that the clonal selection by foreign antigen occurs in skin, from among a genetically diverse, resident skin population. [Division of Dermatology and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles; Section of Dermatology and Department of Medicine, USC School of Medicine, Los Angeles, California, U.S.A.]

Izumi, S., Maeda, Y., Kawatsu, K., Amiruddin, M. D., Hatano, K., Mendes, M. F. and Choudhury, A. M. Distribution of anti-LAM antibodies in leprosy patients and household contacts.

Based on the observations of 367 noncontact sera from a nonendemic area (Japan), we calculated the 95 percentile value for cut-off. The calculated cut-offs were 0.250 OD and 0.500 OD for IgG and IgM, respectively. The antibody titer and positive rates were higher in the multibacillary forms of leprosy and low in the paucibacillary form of the disease. The distribution of anti-LAM antibodies in 99 household contacts from an endemic area (South Sulawesi) were also determined. There were no statistically significant differences between contacts of the multibacillary and paucibacillary patients. The distribution of anti-LAM antibody in noncontacts from an

nonendemic area (Japan), noncontacts, tuberculosis patients, and pregnant women from an endemic area (South Sulawesi) were also determined. It was found that noncontacts from an endemic area show higher positivity in anti-LAM antibodies compared to noncontacts from nonendemic areas. The positivity in the antibody in tuberculosis patients was significantly higher compared to noncontacts from the same area, but the normal pregnancy sera showed no differences in the positivity in anti-LAM antibody.

The concordance rates between anti-LAM antibody and anti-PGL-I antibody in leprosy patients and household contacts were about 70%. To know the quantitative correlation between antibodies to PGL-I and LAM-B, the simple correlation coefficient was calculated by using 226 positive sera from leprosy patients and contacts. The calculated coefficient was 0.260, showing a statistically significant but weak positive correlation. [National Institute for Leprosy Research, Tokyo, Japan; Hasanuddin University, South Sulawesi, Indonesia; Christian Leprosy Center, Chittagong, Bangladesh; Sociedade Filantropica Humanitas, Sao Jeronimo da Serra, Brazil]

Yogi, Y., Nakamura, K., Inouye, T., Kawazu, K., Kashiwabara, Y., Sakamoto, Y., Izumi, S., Saito, M., Hioki, K. and Nomura, T. Susceptibility of severe combined immunodeficient (SCID) mice to *Mycobacterium leprae*: multiplication of *M. leprae* inoculated into both hind feet at an early stage.

The severe combined immunodeficient (SCID) mouse which has lymphopenia is severely deficient in both T- and B-cell immunity. In the present study, we determined whether the susceptibility to *Mycobacterium leprae* of SCID mice is significant due to its severe immunodeficiency characteristics and if it can be used as an animal model for the study of leprosy.

Employing 20 SCID (CB-17SCID;*scid/scid*) mice, with CB-17 (+/+) and BALB/cAJc1 mice as controls, inoculation of *M. leprae* derived from the foot passage of nude mice was made into both hind feet at a dose of 5.8×10^6 bacilli per foot. Following inoculation of *M. leprae,* the animals were sacrificed at varying periods of 3, 5, 7, and 8 months after inoculation. The right hind foot was used to count the bacilli; the left, for histopathological study. Mouse serum was processed for PAGE and ELISA. SCID mice were maintained in a vinyl isolator under SPF conditions. CB-17 and BALB/cAJc1 mice were housed in a conventional animal room.

Almost all of the SCID mice showed a slight enlargement of the injected foot at day 170 postinoculation; control CB-17 and BALB/cAJc1 mice did not. The bacillary counts of the SCID mice increased to over the inoculum count at approximately 3 months postinoculation. At 8 months postinoculation, the bacillary counts were over 10^9 per swollen foot. Subsequently, histopathological findings of the infected foot of SCID mice 5 months postinoculation indicated the presence of a number of bacilli around blood vessels in subcutaneous tissue and intermuscular layer on Fite-Faraco staining. At that time, there was systemic infection in the SCID mice and the bacilli extended to neighboring parts, such as popliteal lymph nodes. At 8 months postinoculation, multiplication of *M. leprae* was observed all over the inoculated foot. The presence of *M. leprae* inside the bone marrow and the nerve tissues was also noticed. Further multiplication of *M. leprae* in the draining lymph nodes was also observed at 8 months postinoculation.

In addition to the above findings at this stage, the multiplication of *M. leprae* was observed, though small in quantity, all over the body, such as the iliac or lumbar nodes, the liver, spleen, lung, coccygeus and, in male SCID mice, the scrotum and the epididymis. Interestingly, SCID mice lack detectable immunoglobulins, but *M. leprae*-inoculated SCID mice showed a low serum immunoglobulin production. For identification, normal mice were inoculated with the acid-fast bacilli collected asceptically from the SCID mice 7 months postinoculation. Cultivation on egg-yolk medium was done, but the results were negative. The presence of phenolic glyoclipid-I was demonstrated at the site of multiplication of the bacilli by immunohistopathological staining.

To summarize the results of early experimental findings, SCID mice were found to

have an extremely high susceptibility to *M. leprae*. In contrast to the usual findings of nude mice, the progress of the infection observed in SCID mice is considerably different, showing rapid multiplication at the inoculation site as well as the systemic spread of infections. [National Institute for Leprosy Research, Tokyo; National Defense Medical College, Tokorozawa; Central Institute for Experimental Animals, Kawasaki, Japan]

CLOSING REMARKS

Ladies and Gentlemen,

I wish to thank all of you for a most pleasant and successful meeting. It is the first time in which we have had a two-day joint panel symposium. It was very successful, and we would like to follow this format next year in Tokyo.

Dr. Brennan presented some opinions about the future of the U.S.–Japan Cooperative Leprosy Program. I would like to add a few points. It is certain that the number of leprosy cases will decrease year by year throughout the world through the implementation of multidrug therapy (MDT). However, it is less certain that chemotherapy alone can achieve elimination of the disease. My colleague, Dr. Shimao, a member of a WHO Committee, agrees that the caseload is decreasing. It was due to a belief in the efficacy of MDT that led the Sasakawa Memorial Health Foundation to support chemotherapy implementation in favor of vaccine development. Actually, about 10 years ago, I was very much impressed with the evidence that rifampin could kill all leprosy bacilli in lepromatous patients through only 2 days of administration, and I recommended rifampin treatment.

If one considers that there are about 4 million lepromatous leprosy patients in the world today, and that any one patient may contain 10–100 grams of *M. leprae* with one resistant bacillus in 10^7, then one worries that while MDT may be successful in the 20th century, MDT resistance may occur in the 21st century. In view of the remarkable progress in the molecular biology of *M. leprae* and the possibility that we may be close to an effective vaccine, I advocate continuing molecular studies, efforts to grow the organism, and the study of the question of immunological anergy in patients.

At the closing of this Conference, on behalf of the Japanese participants, I would like to say a great thanks to Dr. Gwinn, Dr. Brennan, Dr. Ellner, and the other organizers of the 26th U.S.–Japan Leprosy Research Conference. We shall meet again in Tokyo at the next Research Conference.

Thank you.

—Tatsuo Mori, *Chairman*
Japanese Leprosy Panel