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ABSTRACTS

TWENTY-THIRD JOINT LEPROSY RESEARCH CONFERENCE

Nara University
Nara, Japan
27 and 29 July 1988

U.S.–Japan Cooperative Medical Science Program

OPENING REMARKS

Ladies and Gentlemen:

It is a great pleasure to open the Twenty-Third Joint Conference on Leprosy Research today. We are particularly grateful to the United States scientists who have traveled far distances in order to participate in this conference.

The preceding Chairman, Dr. Abe, retired this April, and I have followed him as Chairman of the Japanese Leprosy Panel. I hope for your support in this responsibility.

Since the International Leprosy Congress will be held in September, this meeting is being held in midsummer. This season is usually the hottest in Japan, but this year the rainy season is not yet over and we are enjoying very cool weather.

One of the meeting members, Dr. Fujiwara, is from Nara University, and made a favorable offer to us to use this place for our meeting. The U.S. members agreed, and we chose to hold the meeting in Nara University. This university moved to a new campus in February. We are very obliged to Dr. Fujiwara for making the local arrangements for this meeting. I would like to deeply thank the staff of Nara University who have enthusiastically assisted in preparing for our meeting. The Silk Road Exposition is now open in Nara, and there are the historical and famous Daibutsu (the colossal bronze image of Buddha) and the Todai Temple. Please enjoy the old city of Nara in your free time.

These joint conferences have continued for 23 years, and over these years many good research works have been reported here and then sent out into the world. I wish to declare open the Twenty-Third Joint Conference on Leprosy Research.

Thank you.

—Tatsuo Mori, *Chairman*
Japanese Leprosy Panel

PROGRAM
TWENTY-THIRD JOINT LEPROSY CONFERENCE

27 July, Wednesday

Opening Remarks: Dr. Tatsuo Mori, Chairman, Japanese Leprosy Panel

Session I

Co-Chairmen: Dr. Robert Modlin
Dr. Kenji Kohsaka

Tsutsumi, S. and Gidoh, M. On search for new antileprous compounds and their enhancement with immunostimulants in experimental leprosy

Franzblau, S. G., O'Sullivan, J. F. and Hastings, R. C. *In vitro* activity of selected phenazines, fluoroquinolones, oxazolidinones, and macrolides against *Mycobacterium leprae*; identification of potential antileprosy agents

Osawa, N. and Akiyama, T. Effect of protamine and rifampin liposomes on *M. leprae* in murine macrophages

Kohsaka, K. and Ito, T. Effect of ofloxacin and minocycline on experimental leprosy

Session II

Co-Chairmen: Dr. Josephine Clark-Curtiss
Dr. Hajime Saito

Jacobs, W. R., Jr., Snapper, S. B., Lugosi, L. and Bloom, B. R. Development of genetic systems for the mycobacteria

Clark-Curtiss, J. A species-specific DNA probe as a candidate for diagnosis of leprosy

Mohagheghpour, N., Munn, M. W., Gelber, R. H. and Engleman, E. G. Identification of a major immunostimulating protein from *M. leprae*

Hirata, T. and Chyugun, M. Cellular peripheral parts of the "so-called" leprosy bacilli in comparison with avian tuberculous mycobacterium *in vitro* and/or *in vivo*

Matsuo, E., Sasaki, N. and Skinsnes, O. K. *In vitro* binding of beta-glucuronidase to a mycobacterium HI-75

29 JULY, FRIDAY

Session III

Co-Chairmen: Dr. James Krahenbuhl
Dr. Shinzo Izumi

Quismorio, F. P., Jr. and Rea, T. H. Serum antiphospholipid antibodies in leprosy

Izumi, S., Fujiwara, T., Ikeda, M., Nishimura, Y. and Sugiyama, K. Clinical and

seroepidemiological application of *M. leprae*-specific gelatin particle agglutination test (MLPA) for leprosy

Mehra, V., Bloom, B. R., Mandich, D., Hunter, S., Brennan, P. J., Torigian, V., Rea, T. H. and Modlin, R. L. Immunological significance of *Mycobacterium leprae* cell walls

Hunter, S. W., McNeil, M., Stewart, G. and Brennan, P. J. Isolation and characterization of large-molecular-size and hydropholic protein antigens from the cell walls of *Mycobacterium leprae*

Session IV

Co-Chairmen: Dr. Thomas H. Rea
Dr. Kazunari Nakamura

Krahenbuhl, J. L., Sibley, L. D. and Chae, G. T. Induction of macrophage responsiveness to interferon-gamma by *M. leprae*

Fukutomi, Y., Inui, S., and Onozaki, K. Macrophage activation after phagocytosis of *M. lepraemurium* and *M. avium in vitro*

Kaplan, G. and Cohn, Z. A. Role of lymphokines in the regulation of cell-mediated immunity in leprosy

Nakamura, K. and Yogi, Y. Experimental inoculation with leprosy bacillus in various hybrid nude mice (continued): Results of CD-I (ICR) hybrid nude mice

JOINT U.S.–JAPAN TUBERCULOSIS AND LEPROSY SYMPOSIUM
Nara University
Nara, Japan

28 July, Thursday

Session I

Co-Chairmen: Dr. Patrick J. Brennan
Dr. Tatsuo Mori

Young, R. A. Mycobacterial genes and antigens

Nomaguchi, H., Matsuoka, M., Nakata, A. and Ito, T. Purification of 65-kilodalton protein of *Mycobacterium leprae* in *Escherichia coli*

Session II

Co-Chairmen: Dr. Frank M. Collins
Dr. Ichiro Azuma

Barnes, P. F., Modlin, R. L. and Rea, T. H. A comparative study of the immunohistology of leprosy and tuberculosis

Izaki, S., Isozaki, Y., Tokairin, S., Segawa, I., Tanji, O., Hsu, P. S., Hibino, T. and Kon, S. Proteolytic and anti-proteolytic regulation in granulomatous tissue reaction

PANEL MEMBERS
U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

U.S. Leprosy Panel

Brennan, Patrick J. (*Chairman*), Professor, Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, U.S.A.

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Rea, Thomas H., Department of Health Sciences, Los Angeles County/University of Southern California Medical Center, 1200 North State Street, Room 8441, Los Angeles, California 90033, U.S.A.

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Mori, Tatsuo (*Chairman*), Director, National Institute for Leprosy Research, Higashimurayama-shi, Tokyo 189, Japan

Izumi, Shinzo, Chief, Laboratory of Pathology, National Institute for Leprosy Research, Higashimurayama-shi, Tokyo 189, Japan

Kohsaka, Kenji, Research Assistant, Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Yamadakami, Suita, Osaka, Japan

Nakamura, Kazunari, Director, First Research Department, National Institute for Leprosy Research, Higashimurayama-shi, Tokyo 189, Japan

Saito, Hajime, Professor, Department of Microbiology and Immunology, Shimane Medical University, Izumo-shi, Shimane, Japan

ABSTRACTS

Tsutsumi, S. and Gidoh, M. On search for new antileprous compounds and their enhancement with immunostimulants in experimental leprosy.

Using Hansen's bacilli (HB) collected from the foot pads (fps) of BALB/c (nu/nu) mice (kindly supplied by Kohsaka of Osaka University), the growth inhibitory effects of several novel compounds and the enhancement of a few immunostimulants to the bacteriostatic effect of dapsone (DDS) were examined.

The continuous dose of a quinolone compound, enoxacin, or a strong *in vitro* antimycobacterial compound, 2, 2'-dithiobis(N-(4-butylphenyl-1-yl) benzamide) (I, Okachi, *et al.*) to BALB/c (nu/nu) female mice could not inhibit the growth of HB even though dosed for 1 year at 0.02% in the diet.

Since the bioavailabilities of quinolones are known to be lower in mice than in humans, we examined the concentration of enoxacin in sera collected by carotid puncture from BALB/c (nu/nu) mice which were occasionally taking 0.02% of this drug in their diet. This concentration was found to be up to 30-50% of maximum concentration in humans under usual regimens.

All of an antimalarial pyridyl thiosemicarbazone possessing 3-azabicyclo (3.2.2) nonane ring (II, Klayman, *et al.*), an *in vitro* antimycobacterial compound, 2-adamantyl benzimidazole (III, Kuzmierkiewicz, *et al.*) and 2-mercapto-3-hydrazinoquinoxaline (IV, Tsutsumi, *et al.*) more or less delayed the start of the growth phase when com-

pounds II and III (10 mg/kg) were given to BALB/c (nu/nu) female mice once every day through a mouse catheter or compound IV was given 3 times weekly by the intramuscular route into both hind feet from the lateral malleolus toward knee articulations (5 mg/kg/foot) for 3 months (3rd–5th months). Among them, the effect of compound II was most distinct, but less than the effect by half that dosage of DDS. Nevertheless, the growth of HB in the DDS group became comparable to that in the untreated group at the end of the 10th month.

When DDS was given continuously over 1 year at 0.001% or 0.005% in the diet, the growth of HB was markedly inhibited even in BALB/c (nu/nu) mice. When given during the 3rd-5th months alone, the inhibitory effect seemed to be lower in BALB/c (nu/nu) mice than in a hybrid strain of nude mice named Jcl:AF (nu/nu), established in Japan by the Clea Company by the hybridization of male FNS/N (nu/nu) with female IAI (+/+) of an ICR strain in which resistance to infections is higher than in BALB/c (nu/nu) mice.

Before examining the enhancement by immunostimulants using semi-immunodeficient Jcl:AF (nu/+) mice, Jcl:AF (nu/nu) female mice were used. The counts of acid-fast bacilli (AFB) detected in fps after intraperitoneally injected muramyl dipeptide (MDP, 100 μg/mouse) or a water-soluble lipoidal amine, CP-46665 (Pfizer Inc., 0.6 mg/kg) once weekly (9th–32nd weeks) during and following DDS administration (0.005% in diet, 9th–20th weeks) were significantly lower than those found in the group given DDS alone. The growth of HB in the untreated group was comparable to that in BALB/c (nu/nu) female mice. A significant increase in foot pad swelling was observed by microvolumetry in each of the untreated mice. But the swelling was insignificant among the treated animals.

The growth of HB was examined in the fps of Oseanian DA rats which have a high response to adjuvant-induced arthritis (AIA). However, AFB counts after inoculation with 10^8 HB and fed for 485 days were at the 10^6 level. On the other hand, AFB counts in fps of Fischer 344 nude rats inoculated with 10^7 HB reached the 10^8 level after 529 days. The development of an animal model which can induce erythema nodosum leprosum (ENL)-like symptoms with the growth or acute destruction of HB is indispensible. For this purpose, the development of a strain of rodent animals which has high responses to both the growth of HB and arthritogenicity is presumed to be needed.

Along with these findings, a pharmacokinetic problem of the dosing method, such as the intake through catheter or from diet, in connection with the long-activity of drugs is discussed, as well as that of the immunostimulative therapy using semi-immunodeficient mice.—[National Institute for Leprosy Research, Tokyo, Japan]

Franzblau, S. G., O'Sullivan, J. F. and Hastings, R. C. *In vitro* activity of selected phenazines, fluoroquinolones, oxazolidinones and macrolides against *Mycobacterium leprae:* identification of potential antileprosy agents.

Selected phenazines, fluoroquinolones, oxazolidinones and macrolides were evaluated *in vitro* against nude mouse-derived *Mycobacterium leprae* by a semi-automated radiorespirometric technique involving oxidation of [1-^{14}C] palmitic acid to $^{14}CO_2$. Among the 13 phenazines tested, activity in ascending order was observed in compounds containing: no chlorine atoms, a monochlorinated phenazine nucleus, and chlorines in the *para* positions of both the anilino and phenyl rings. The most active compounds contained a 2,2,6,6 tetramethylpiperidine substitution at the imino nitrogen. Most of these chlorinated phenazines were considerably more active *in vitro* than clofazimine (B663). Among fluoroquinolones, ciprofloxacin and pefloxacin were active at $\geq$1.25–5 μg/ml while norfloxacin was active only at 20 μg/ml. The oxazolidinones, a new class of antimicrobials, were active at $\geq$0.125–0.5 μg/ml. All macrolides except azithromycin were fairly active. Clarithromycin (TE-031) was most active *in vitro,* was detectable in the serum of mice receiving the drug in the feed at 0.01% (w/w), and was strongly bactericidal in a modified, kinetic, mouse foot pad test.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.; Health Research Board Laboratories, Trinity College, Dublin 2, Ireland]

Osawa, N. and Akiyama, T. Effect of protamine and rifampin liposomes on *M. leprae* in murine macrophages.

There are similar defense mechanisms in leprosy, tuberculosis, and salmonellosis. In *in vivo* and *in vitro* infections, these bacteria grow in phagocytic cells. In the present study, we tested the effect of antibiotic liposomes or other antibiotics on *Mycobacterium leprae* or other bacteria in murine and human macrophages. Mice of the CBA and DDN strains, 8–10 weeks old of both sexes weighing 25–30 g, were used for *M. leprae* and salmonella infections. *M. leprae* T-53 and *Salmonella enteritidis* 116-54 were also used. *M. leprae* were obtained by nude mice inoculation. The peritoneal macrophages were cultured in a TD-15 culture chamber with four cover glasses (9 × 12 mm). The culture medium consisted of 20% horse serum and 80% Waymouth solution. At appropriate time intervals after phagocytosis, the cover glasses to which the macrophages adhered were removed, dried, fixed, and stained. Infected macrophages were observed microscopically. Liposomes were prepared by standard methods. Rifampin was soluble in chloroform. The killing effect of ofloxacin or chloramphenicol on *S. enteritidis* in macrophages was observed. Cultured macrophages were infected with *M. leprae* to a phagocytic index of 90. Three to five bacteria were phagocytized in a cell. The number of bacteria in phagocytic cells of the controls or of ofloxacin (40 μg/ml in medium) was increased 1 week after infection. On the other hand, the macrophages of mice treated with protamine or rifampin liposomes inhibited intracellular multiplication of *M. leprae*. *M. leprae* T-53 were grown about 3 weeks in human peripheral blood macrophages. This method can be applied for susceptibility testing of *M. leprae* to antimicrobial agents.—[Department of Microbiology, Kitasato University School of Medicine, Kitasato, Japan]

Kohsaka, K. and Ito, T. Effect of ofloxacin and minocycline on experimental leprosy.

Ofloxacin, a quinolone compound, is a synthetic antibiotic and has been used clinically, especially in the chemotherapy of infections of the urinary tract. The antimycobacterial effect of the drug *in vitro* and *in vivo* was also reported. We previously reported that ofloxacin is effective in suppressing the growth of *Mycobacterium leprae* in experimental leprosy with nude and normal mice. The efficacy, however, is variable and dependent on the manner of administration or doses of the drug, strain of nude mice, etc.

As mentioned above, it is confirmed that administration of a daily dose of 3 mg ofloxacin 6 times a week for 100 days markedly suppressed the growth of *M. leprae* in nude mice. Treatment with a 0.075% ofloxacin-containing diet was also as effective in suppressing the growth of the bacilli as in the same group dosed by the spoon method. Minocycline, 0.3 mg (equivalent to 75 mg/man) 5 times a week for 100 days, was slightly effective in suppressing the growth of *M. leprae* in the nude mouse. It seems that investigations of these drugs should be continued.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan]

Jacobs, W. R., Jr., Snapper, S. B., Lugosi, L. and Bloom, B. R. Development of genetic systems for the mycobacteria.

Requisite to a detailed understanding of the molecular basis of bacterial pathogenesis is a genetic system which allows for the transfer, mutation, and expression of specific genes. Genetic analysis of mycobacteria has been exceedingly difficult since the mycobacteria grow slowly and no natural efficient method of gene transfer within the mycobacterial species has thus far been found. Using a molecular genetic approach, we have developed both the vectors and the methodology for efficient gene transfer in the mycobacteria. Initially, a novel type of mycobacteriophage vector was developed, termed a shuttle phasmid. This hybrid shuttle vector replicates in *Escherichia coli* as a plasmid and in mycobacteria as a phage, capable of introducing foreign DNA into a wide variety of mycobacterial species. A set of shuttle phasmids, constructed from a temperate mycobacteriophage, retained their ability to lysogenize their mycobacterial hosts and could thus introduce foreign DNA stably into mycobacterial cells. An *E.*

coli gene conferring kanamycin resistance was cloned into these vectors and shown to express in the mycobacteria, thus providing the first selectable marker gene for subsequent genetic studies. Using kanamycin-resistance gene as a selection, the *M. fortuitum* plasmid pAL5000 replicon, and electroporation, a plasmid transformation system has been developed for both *M. smegmatis* and BCG. We now plan to use these phage and plasmid systems to analyze, genetically, the virulence attributes of the pathogenic mycobacteria. In addition, by introducing and expressing foreign antigens in BCG, we hope to develop a novel, recombinant multivaccine vehicle capable of conferring immunity to a variety of bacterial, viral, and parasitic pathogens.—[Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, U.S.A.]

Clark-Curtiss, J. E. A species-specific DNA probe as a candidate for diagnosis of leprosy.

Diagnosis of leprosy is made on the basis of the presence of characteristic lesions on the skin of susceptible individuals and, in cases of lepromatous leprosy, microscopic observation of acid-fast bacilli (AFB) that do not grow on conventional mycobacteriological media. However, since infection of individuals with *Mycobacterium leprae* may precede the development of symptoms by 3 to 10 years, it has long been desirable to develop a reagent that could be used to detect *M. leprae* infection and, thus, permit initiation of chemotherapy and/or immunotherapy at an earlier stage of the disease.

Among the recombinant molecules in the pYA626::*M. leprae* genomic library, a recombinant molecule (pYA1065), which hybridizes to 15 to 19 distinct bands of restriction endonuclease-digested *M. leprae* chromosomal DNA, has been identified. This recombinant molecule is specific for *M. leprae* DNA; no hybridization between the probe and chromosomal DNA from 15 different cultivable mycobacterial species has been detected, but the probe hybridized to the same restriction endonuclease-digested DNA fragments of four separate *M. leprae* isolates. The probe is sensitive to the point of detecting hybridization to an amount of DNA equivalent to that in approximately 4000 *M. leprae* cells. The probe can also hybridize to DNA from *M. leprae* cells in homogenized skin-biopsy material from human lepromatous leprosy patients. Thus, pYA1065 appears to be a good candidate for an effective diagnostic reagent.

The pYA1065 probe also has potential for use in monitoring the growth of *M. leprae* cells in mouse food pads, as an alternative to microscopic enumeration of AFB. In addition, hybridization between the pYA1065 probe and chromosomal DNA from putative *in vitro*-cultivated *M. leprae* cultures would be a useful test for confirmation of the identity between *in vitro*-grown cultures and *M. leprae* from human leprosy patients.—[Departments of Microbiology and Immunology and Biology, Washington University, St. Louis, Missouri, U.S.A.]

Acknowledgments. This work was supported by U.S. Public Health Service grant AI23470 from the National Institutes of Health.

Mohagheghpour, N., Munn, M. W., Gelber, R. H. and Engleman, E. G. Identification of a major immunostimulating protein from *M. leprae.*

The cell-mediated immune response to *Mycobacterium leprae* is believed to have a major influence on the clinical course of patients infected with this agent. Therefore, purified *M. leprae* proteins are essential probes for dissecting the T-cell immune responses to this bacterium. Use of murine monoclonal antibodies (MAB) and recombinant DNA expression libraries have led to the identification and initial characterization of a number of *M. leprae* proteins. However, the major immunogenic determinants of *M. leprae* remain obscure. With the goal of identifying such determinants, we initiated an effort to isolate native proteins from *M. leprae.*

Using a relatively gentle extraction procedure, we have isolated the native protein(s) from the pellet fraction of sonicated leprosy bacilli. This preparation, designated MLP, contains a major protein of molecular mass 35 kDa on the basis of SDS-PAGE analysis. SDS-PAGE of an identically treated sonicate of armadillo-liver cells (control-

protein) did not reveal a band in the region of 35 kDa. The dominant (35 kDa) protein in the MLP was recognized by a mouse MAB (ML03-A_1) which is known to react with a 35 kDa protein of *M. leprae*. In addition to the 35 kDa protein, this MAB reacted with a minor band of 70 kDa. The same 35 kDa and 70 kDa bands were precipitated by rabbit polyclonal antibodies generated against the MLP, and by 4 of 4 sera obtained from patients with lepromatous leprosy (LL). In contrast, neither rabbit anti-*M. bovis* sera nor sera from healthy controls recognized MLP, and only 1 of 4 sera from patients with borderline tuberculoid (BT) leprosy reacted with this protein.

MLP at concentrations of 0.5 μg/ml induced proliferation comparable to that elicited by 10 μg/ml whole *M. leprae* in PBL from BT patients, suggesting that MLP is a potent T-cell immunogen. Increasing the concentration of MLP to 10 μg/ml resulted in profound reduction of proliferation. Control-protein from armadillo-liver cells, however, did not produce significant stimulation (SI $\leq$ 2), indicating that the proliferative response to MLP is not due to contaminating xenogenic antigens. In further analysis of the immunostimulatory capacity of MLP, we observed that PBL from the *M. leprae*-nonresponder LL patients (SI $\leq$ 2), in contrast to the T cells from the responder lepromatous patients, failed to proliferate when stimulated with MLP. Thus, the profiles of the response of nonresponder LL patients to MLP and intact leprosy bacilli are identical. Apparently, the T-cell epitope associated with MLP is also expressed in BCG, since 6 of 8 BCG-vaccinated individuals demonstrated significant responses to MLP. In contrast, PBL from two healthy individuals who had not been previously exposed to *M. leprae* or BCG did not respond to MLP by proliferation (SI $\leq$ 2). These findings indicate that an epitope(s) associated with MLP is recognized by T cells of *M. leprae*-responder leprosy patients as well as healthy individuals vaccinated with BCG. Finally, in addition to stimulating T-cell responses from freshly isolated PBL, MLP also stimulated proliferative responses in *M. leprae*-reactive CD4+ T-cell clones. The possibility existed that components of the MLP preparation other than the major 35 kDa protein were responsible for the observed T-cell responses. To assess this possibility, we adopted the approach of Young and Lamb to isolate the separated protein bands from SDS-PAGE of *M. leprae*. While the overall responses of PBL to whole *M. leprae* antigens in cultures containing nitrocellulose membrane was reduced, the highest response (SI = 18.6), and the only response with SI > 2, was observed in the wells containing the 35 kDa protein.

Taken together, these findings suggest that the 35 kDa protein represents a major immunostimulatory component of *M. leprae*. The possibility exists that additional proteins present in the MLP preparation are capable of eliciting a T-cell response but failed to do so because they could not be recovered in sufficient quantity from SDS-PAGE. Even if such proteins are present in our preparation, the analysis of T-cell-stimulating activity recoverable from SDS-PAGE suggests that the 35 kDa protein is the major T-cell immunogen in MLP. The failure of T cells from the majority of LL patients to respond to whole *M. leprae* is thought to be a major contributing factor both to the massive accumulation of *M. leprae* organisms in the skin of LL patients and to the aggressive clinical course which characterizes these patients. Therefore, our finding that the same patients whose T cells failed to respond to whole *M. leprae* also failed to respond to MLP is of particular interest, since this suggests that MLP is not only a major immunostimulatory component of *M. leprae* but also that it is one of potential clinical relevance. In addition to serving as a probe in studies of the mechanism of *M. leprae*-specific T-cell anergy, the strong immunogenicity of MLP observed in a large panel of convalescent BT patients suggests that the 35 kDa protein may be a useful component in a vaccine designed to provide protection against infection with leprosy bacilli. The fact that this antigen is recognized by the T cells of BCG-vaccinated individuals, as well as leprosy patients, does not necessarily detract from its potential efficacy as a leprosy vaccine. Indeed, recent evidence suggests that the protective antigens of mycobacteria are those which are shared by other mycobacterial species, rather than species-specific

antigens. Studies have been initiated in a murine model of leprosy to test this possibility.—[Medical Research Institute of San Francisco, San Francisco, California, U.S.A.; Stanford University School of Medicine, Stanford, California, U.S.A.]

Acknowledgment. This work was supported by grant AI-22653 from the National Institutes of Health.

Hirata, T. and Chyugun, M. Cellular peripheral parts of the "so-called" leprosy bacilli in comparison with avian tuberculous mycobacterium and other microorganisms *in vitro* and/or *in vivo.*

The fine structure of the cellular peripheral parts of the leprosy bacilli is reported in comparison with other mycobacteria and other microorganisms *in vitro* and/or *in vivo.* It appeared that the cell wall of mycobacteria in lesions was thicker than *in vitro.* The cell wall and the cytoplasmic membrane in *Mycobacterium leprae* (in lepromatous leprosy patients) have been considered to be similar to those of other mycobacteria, but careful electron microscopic observations of *M. leprae* in human skin lepromas showed slightly different features when compared with the leprosy bacilli in nasal mucosal biopsies. The typical fine structures of the cell wall were observed in the bacillary cells of the mucosa, even though the ultrastructural features of the leprosy bacilli cell wall in the skin lepromas were much less clear.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Matsuo, E., Sasaki, N. and Skinsnes, O. K. *In vitro* binding of beta-glucuronidase to a mycobacterium HI-75.

Immunohistologic staining of leprous lesions demonstrated the immunoreactive sites for human beta glucuronidase (B-Gase) in the globi which led us to the revision of the origin of B-Gase which *Mycobacterium leprae* possess. The present study was conducted to investigate if B-Gase can combine with *M. leprae in vitro.* To perform the study we needed a certain amount of HI-75 as the alternate of *M. leprae* since it also seems to show nerve lesions in nude mice. We also studied the possibility of the enhanced growth of the bacilli by the split product by B-Gase.

HI-75 grown in Ogawa's egg medium were transferred to the modified Ogawa's medium containing both glucuronic acid (GA) and N-acetyl-glucosamine (NAG) (10 mg/ml) for quick growth and were compared with growth of the controls without these additives. The autoclaved bacilli were divided into groups: 1) untreated; 2) extracted with xylol; 3) treated with phosphate buffered saline (PBS), pH 7.2, containing 1 mM EDTA; 4) extracted with the mixture of chloroform, methanol, and water (CMW) at the ratio of 16:6:1 overnight; and 5) treated with EDTA-PBS, pH 7.2, containing trypsin before the suspension of 10% with the saline with 1 mM each of $CaCl_2$ and $MgCl_2$ (CM saline).

B-Gase purified from bovine liver, suspended with CM saline, and extracted crude from human kidney with EDTA-PBS were utilized. Each of the 0.25 ml suspensions of HI-75 and the enzyme solutions were placed in an incubator at 37°C for 1 hr. The suspension of washed bacilli was then mixed with the same volume of naphthol AS-B1 glucuronide solution, according to the method of Hayashi, *et al.* The precipitates turned red within 30 min at 37°C in those cases where B-Gase had already combined with HI-75.

The binding of B-Gase of bovine liver and HI-75 was observed in cases where HI-75 was treated with methods 1 and 4. HI-75 treated with method 3 showed weak colorization. When human kidney extract was utilized, the coloration was weak. In the cultivation, Ogawa's medium with GA and NAG showed much faster and better growth of the bacilli than the medium without these.

The *in vitro* binding seems to occur with the presence of $CaCl_2$ and $MgCl_2$, as shown by the first experiment. The reason for the influence of xylol is not clear. The incapability to destroy the substance responsible for the binding with the mixtures of CMW by the fourth experiment and the capability to do so with trypsin by the fifth experiment suggest that the substance responsible for the bindings might be protein rather than lipid. The weak binding of human B-Gase and HI-75 indicates the possible influence of EDTA. The requirement for B-Gase by

M. leprae in the host might be attributable to the requirement for glucuronic acid as shown by the culture study.—[Departments of Pathology, Kyorin University School of Medicine, Tokyo, Japan, and Sun Yat-Sen University of Medical Science, Guangzhou, China, and Tohoku Shinseien, Miyagi, Japan]

Quismorio, F. P., Jr., and Rea, T. H. Serum antiphospholipid antibodies in leprosy.

Antiphospholipid antibodies including anticardiolipin antibodies (ACA) and lupus anticoagulant (LA) are known to be associated with certain manifestations of systemic lupus erythematosus (SLE), such as recurrent thrombosis, thrombopenia, and fetal wastage. LA as well as antibodies to VDRL antigen have previously been reported in leprosy patients. We examined for the presence of ACA in leprosy patients to determine its frequency and clinical significance. We also compared the specificity and other characteristics of ACA in leprosy and that found in SLE.

IgM and IgG ACA were measured by an ELISA method and the results were expressed as phospholipid units (Ann. Rheum. Dis. 46:1, 1984). Serum titer was classified into negative, low, moderate, and high positive.

Grouping together negative and 1+ as "low level" responses and 2+ and 3+ as "high level" responses, 25 of 51 (49.6%) of lepromatous leprosy (LL) patients had high level IgM ACA. In contrast, only 3 of 23 (13.4%) of the borderline tuberculoid leprosy (BT) sera were so affected. "High level" responses of IgG ACA were less common in LL sera (13/51 or 25.4%) but were of similar prevalence in BT sera (3 of 23), when compared with the IgM ACA.

No association was noted between the presence or serum titer of IgM and IgG ACA with the occurrence of Lucio's phenomenon, erythema nodosum leprosum, with the duration of drug therapy, with a positive rapid plasma reagin test, or with positive antinuclear antibodies.

Absorption of leprosy as well as SLE sera with whole irradiated *Mycobacterium leprae* did not remove the IgM or IgG ACA, indicating that the antibody response is not to an antigen expressed on *M. leprae.* IgM and IgG antibodies to negatively charged phospholipids, phosphotidylserine and phosphotidylinositol but not to zwitterionic phospholipid, phosphotidylcholine were found in leprosy and SLE sera. The IgG ACA in leprosy belonged predominantly to the IgG3 subclass; in SLE the IgG subclass distribution was more polyclonal.

The biological significance of antiphospholipid antibodies in leprosy is not clear at this time. Because such antibodies are virtually universal in syphilis and not uncommon in infections including the acquired immunodeficiency syndrome, antiphospholipid antibodies may appear as part of the host response to the infection and tissue injury. On the other hand, the observed association between ACA and certain features of SLE suggests that the clinical significance of these antibodies in leprosy needs further investigation.—[University of Southern California School of Medicine, Los Angeles, California, U.S.A.]

Izumi, S., Fujiwara, T., Ikeda, M., Nishimura, Y. and Sugiyama, K. Clinical and seroepidemiological application of *M. leprae*-specific gelatin particle agglutination test (MLPA) for leprosy.

A novel gelatin particle agglutination technique (MLPA) was established and the potential fields of application of the test were investigated by using 1147 sera from Japan, 6 sera from Korea, and 163 sera from a leprosy-endemic area in Brazil. It was found that MLPA is useful for monitoring chemotherapy, predicting relapse, and detecting high-risk persons in household contacts.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan; Institute of Natural Science, Nara University, Nara, Japan; Fuji Rebio Inc., Hachioji, Japan; National Ohshima Seisho-en Hospital, Aji-cho, Japan]

Mehra, V., Bloom, B. R., Mandich, D., Hunter, S., Brennan, P. J., Torigian, V., Rea, T. H. and Modlin, R. L. Immunological significance of *Mycobacterium leprae* cell walls.

Development of a vaccine against leprosy hinges on the identification of antigens that stimulate protective cell-mediated immune

(CMI) responses. Cell walls from other mycobacteria have been shown to induce significant CMI responses. The present work explores the possibility that the cell wall of *Mycobacterium leprae* contains antigens important for CMI. *M. leprae* cell walls prepared by differential solvent extraction contained arabinogalactan, mycolates, peptidoglycan and, unexpectedly, large amounts of proteins. Purified cell walls stimulated proliferation of T cells from 25 tuberculoid but not 27 lepromatous leprosy patients, and elicited delayed-type hypersensitivity skin reactions in 40 guinea pigs and 5 patients sensitized to *M. leprae.* Analysis of the precursor frequency of antigen-reactive human peripheral T cells revealed that as many cells (~1/6000) proliferated to cell walls as to intact *M. leprae.* Sequential removal of mycolates and arabinogalactan resulted in a large peptidoglycan-protein complex which retained all of the immunological activity. This immunological reactivity was destroyed by proteolysis. T-helper clones (N > 100) derived from leprosy skin lesions proliferated in response to cell walls but not soluble or recombinant *M. leprae* antigens. Several of these T-cell clones recognized antigens of 16 kDa and 7 kDa contained on nitrocellulose transfers of *M. leprae* cell extract. These results suggest that a major effort is warranted to isolate and characterize the cell wall-associated protein antigens involved in CMI in leprosy.—[Section of Dermatology, University of Southern California School of Medicine, Los Angeles, California, U.S.A.; Department of Microbiology, Colorado State University, Fort Collins, Colorado, U.S.A.; Department of Microbiology, Albert Einstein College of Medicine, Bronx, New York, U.S.A.]

Hunter, S. W., McNeil, M., Stewart, G. and Brennan, P. J. Isolation and characterization of large-molecular-size and hydrophobic protein antigens from the cell walls of *Mycobacterium leprae.*

In a recent study with others (Melancon-Kaplan, *et al.,* Proc. Natl. Acad. Sci. U.S.A. 85:1917–1921, 1988), we demonstrated that certain reactivities crucial to the immune response in leprosy were associated with the cell wall "core" of *Mycobacterium leprae* and that the responsible component was protein in nature. We now describe the isolation by differential solvent extraction of a highly immunogenic, large-molecular-size, insoluble protein complex from *M. leprae,* freed of soluble proteins, mycolates, arabinogalactan, and appreciable peptidoglycan. The protein complex, called cell wall-protein (CW-P), is of relative molecular size 2×10^6–20×10^6 Da, is distinguished by a high content of Ala, Gly, Leu, Asx, Glx, and phosphorus, and represents over 7% of the bacterial mass. It is stable to a variety of dissociation and reductive processes. Monoclonal antibodies to CW-P also react with the known soluble 65 kDa protein of *M. leprae.* Conversely, antibodies that recognize internal epitopes within the 65 kDa polypeptide chain react with CW-P; however, antibodies that recognize the N and C termini of the 65 kDa molecule fail to react with CW-P. Thus, cell wall-protein prepared in this fashion is apparently composed, at least in part, of bound segments of 65 kDa. On the other hand, CW-P, when prepared from cell walls of *M. leprae* obtained by sucrose gradient density centrifugation, is virtually devoid of 65 kDa protein, but retains its potent immunoreactivity. R. L. Modlin and B. R. Bloom and colleagues have demonstrated that T-cell lines established to cell walls when tested for reactivity against sonicated *M. leprae* proteins separated by SDS-PAGE react preferentially to proteins of molecular weight 7 kDa, 16 kDa, and 28 kDa. Thus, the current image of CW-P is of a core of peptidoglycan to which are attached certain highly immunogenic polypeptides. Besides CW-P, cell walls of *M. leprae* contain a number of lipophilic proteins which maintain an avid association with the dominant cell envelope lipopolysaccharides, lipoarabinomannan and lipomannan.—[Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, U.S.A.]

Krahenbuhl, J. L., Sibley, L. D. and Chae, G. T. Induction of macrophage unresponsiveness to interferon-gamma by *Mycobacterium leprae* constituents.

A conspicuous feature of lepromatous leprosy (LL) in man is the abundance of macrophage-rich granulomas harboring enormous numbers of *Mycobacterium lep-*

rae. We have previously demonstrated that macrophages (MAC) isolated from the *M. leprae*-infected foot pads of athymic mice and heavily burdened with bacilli are defective in their ability to become activated (i.e., enhanced microbicidal capacity) in response to treatment with interferon-gamma (IFN-γ). This defective MAC response in experimental LL was localized to the MAC from the lepromatous lesion; MAC from another anatomical compartment (peritoneal cavity) from the same mice were fully responsive to IFN-γ. We have subsequently shown that a similar MAC defect can be induced in cultures of peritoneal MAC heavily infected *in vitro* with live, but not killed, *M. leprae*. Production of prostaglandin E2 (PGE2) by infected MAC appears to be an important, but not the only, mechanism of the refractory response to IFN-γ.

To further characterize defective MAC activation in LL, we have begun examining the effects of purified constituents of the cell walls of *M. leprae* and other mycobacteria on MAC effector function. Lipoarabinomannan (LAM), characterized recently by Brennan and his colleagues, is one major constituent of the cell wall that is widely distributed within the genus *Mycobacterium* and related genera and is the major carbohydrate containing immunogen recognized by sera from patients with tuberculosis and leprosy. The present study examines the capacity of LAM to inhibit IFN-γ-induced activation of the efferent functions of cultured mouse MAC and human monocyte-derived MAC (MdM).

Mouse MAC treated with LAM derived from *M. leprae* or *M. tuberculosis* failed to respond to IFN-γ with enhanced microbicidal capacity for *Toxoplasma gondii* or cytotoxic activity for tumor target cells. Likewise, treatment of MdM with LAM blocked the induction of enhanced microbicidal capacity by human IFN-γ. PGE2 production by LAM-treated MAC was not elevated. LAM treatments were not toxic to the MAC and did not affect their basal cell metabolism. Defective activation required pretreatment of MAC for 12–24 hr with the intact molecule and was not evident using deacylated LAM. Studies with ^{125}I-labeled IFN-γ showed that receptor binding and degradation of IFN-γ was normal in LAM-treated MAC despite their unresponsiveness to IFN-γ at doses 100× those which effectively activate normal MAC. These findings suggest that LAM, a major mycobacterial surface component, may contribute directly to the intracellular survival of *M. leprae* by inhibiting IFN-γ-mediated activation of the microbicidal capacity of macrophages.

Collectively, our studies of the functional capacity of mouse MAC in experimental LL indicate that defective MAC activation is a prominent feature of the highly localized conditions occurring within MAC-rich granulomas of the *M. leprae*-infected nu/nu foot pad or in MAC cultures that contain numerous live bacilli or the appropriate dose of LAM. Specific T-cell anergy in LL is probably the key defect in the pathogenesis of this disease, but it is possible that MAC defects may contribute as well since conditions that fulfill the essential features of localized defective MAC activation are clearly met in human LL. Although numerous reports substantiate that peripheral blood monocytes in LL have a normal microbicidal capacity and normal response to IFN-γ, our studies underscore the importance of local conditions on the functional capacity of tissue MAC in the lepromatous lesion itself. It is noteworthy that dissemination of opportunistic infection with *M. tuberculosis* and *M. avium-intracellulare* in the immunocompromised patient also leads to high tissue burdens of bacilli, a condition that may restrict local MAC function. Consequently, the capacity of purified LAM to inhibit MAC function may be a relevant component in the pathogenesis of such chronic mycobacterial infections in man.—[Immunology Research Department, GWL Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Fukutomi, Y., Inui, S. and Onozaki, K. Macrophage activation after phagocytosis of *M. lepraemurium* and *M. avium in vitro*.

It is well known that many monokines are produced by macrophages activated with various agents. In this experiment, production of one of the monokines—interleukin 1 (IL-1)—was observed after phagocytosis of heat-killed *Mycobacterium avium* and *M.*

lepraemurium. Resident peritoneal macrophages exhibited similar results. Enhanced IL-1 production occurred when an increased amount of the mycobacteria was added to the cultures. Maximum production of IL-1 was observed within 24 hr after phagocytosis, and the production was gradually lost with the lapse of time.

Ia antigen expression on macrophage cell surfaces was also examined. A significant reduction of Ia antigen was observed after phagocytosis, but the reduction was suppressed when indomethacin was present in the cultures. Therefore, the reduction of the antigen expression may have resulted from the production of prostaglandins by these cells. In contrast to the marked enhancement of Ia antigen expression in macrophages treated with lymphokines (LK), a lower induction occurred when the cells phagocytosed these mycobacteria before treatment with LK.

These results indicate that macrophages are able to activate and to secrete IL-1 following phagocytosis of these mycobacteria. However, the activation was a temporary event, and it seems likely that once macrophages phagocytosed these mycobacteria, the cells would no longer respond to LK.—[National Institute for Leprosy Research, Tokyo, Japan; Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan]

Kaplan, G. and Cohn, Z. A. Role of lymphokines in the regulation of cell-mediated immunity in leprosy.

The lack of accumulation of helper T cells in the lesions of lepromatous leprosy focused our attention on the ability of the dermal milieu of these patients to provide the necessary conditions for normal cellular immune responses. We wanted to establish whether CD4+ T cells were inhibited from emigrating into the site of *Mycobacterium leprae* infection and whether they could be retained and activate to release lymphokines in these lesions. For this purpose, we generated delayed-type hypersensitivity (DTH) reactions in the dermis of lepromatous patients by the use of a second party antigen, purified protein derivative of tuberculin (PPD). The nature and interaction of the cells accumulating in the DTH site were studied by immunocytochemistry at the light- and electron-microscopy levels.

Temporal longitudinal analysis of the DTH response to PPD in 150 lepromatous patients has shown that the majority of lepromatous patients normally respond to a second party antigen. During the first 18 hr after PPD administration, significant numbers of mononuclear leukocytes, many of them helper T cells, entered the lesions and their numbers increased, reaching a plateau at 96–120 hr. OKT8+ cells, initially predominant in the lesions, became a minor component of the lesions. Of particular interest was the appearance of potent accessory cells of the T6 phenotype in DTH reactions by 96 hr after antigen administration. These cells contained Birbeck granules, indicating that they were Langerhans' cells. As the lesions matured (40–60 hr), the overlying epidermal keratinocytes began to proliferate and by 67–72 hr the epidermis was thickened (two- to threefold). In addition, the phenotype of the keratinocytes overlying the delayed reaction was modified from Ia negative to Ia positive. This information, as well as the presence of an interferon-gamma (IFN-γ)-induced peptide (IP-10), suggested that IFN-γ was released locally at the site of DTH from less than 24 hr to more than 1 week after antigen administration. Many of the above-mentioned changes induced by PPD occur within the natural lesions of tuberculoid leprosy patients. The keratinocytes overlying the lesions are Ia positive, T6+ Langerhans' cells are prominent in tuberculoid granulomas, and the IFN-γ-induced peptide is expressed continually.

A reduction in the number of foam cells observed during the DTH to PPD in the lesions of lepromatous patients was of interest. Electron-microscopic studies revealed that at 72–96 hr after PPD administration, dead and damaged foam cells were prominent and cell organelles, *M. leprae* and its remnants were found in the extracellular space. In 80% of PPD-responsive patients a reduction in the numbers of acid-fast bacilli, ranging from 5000- to 10,000-fold, was observed locally at 21 days after PPD administration into a lepromatous lesion. The reduction in bacillary load was corre-

lated with an intense mononuclear cell infiltrate, the maintenance of a high CD4/CD8 T-cell ratio, the formation of granulomas, and the extensive destruction of previously parasitized macrophages. These results suggest that there may be a way to bypass nonresponsiveness to *M. leprae* with second party antigens.

In recent studies we have observed that it is possible to evoke a DTH-like response (mononuclear cell infiltrate) with recombinant IFN-γ and recombinant interleukin-2 (rIL-2). We have injected low doses of recombinant IFN-γ or IL-2 into the lesions of lepromatous leprosy patients and studied the reactions using immunocytochemistry and electron microscopy. The responses to IFN-γ and IL-2 resembled the local response to PPD. At 24 hr, the migration of large numbers of helper T cells and monocytes was already prominent and accompanied by induration. The majority of the lymphoid cells found in the lesions up to 21 days in response to IFN-γ injection were of the CD4 phenotype. However, in IL-2-injected sites the high ratio of CD4+/CD8+ cells did not persist beyond 1 week in most patients. The local administration of both recombinant lymphokines enhanced keratinocyte proliferation, Ia expression, and thickening of the epidermis. In contrast to the situation with DTH and IL-2 injection, no T6+ Langerhans' cells accumulated in the dermis in association with helper T cells in IFN-γ-injected sites.

A reduction in the number of foam cells and *M. leprae* was observed following IFN-γ and IL-2 administration. In INF-γ-injected macular lepromatous lesions, a reduction of 5000- to 10,000-fold in bacterial load was observed in 9/14 patients who received four to six 10 μg doses. A similar reduction in bacterial load was also observed in 12/14 patients injected with two to three 10 μg doses of IL-2.—[The Rockefeller University, New York, New York 10021, U.S.A.]

Nakamura, K. and Yogi, Y. Experimental inoculation with leprosy bacillus in various hybrid nude mice (continued): Results of CD-1 (ICR) hybrid nude mice.

We have successfully established an animal model for experimental lepromatous leprosy by using NFS/N nude mice originating from a Swiss colony and SHR nude rats originating from a Wistar colony. In addition, non-obese, diabetic (NOD) hybrid nude mice originating from Swiss-Webster mice (Jcl:ICR outbred strain) gave excellent results with the development of heavy lepromatoid lesions as compared with that of "resistant" Crj:CD-1 (ICR) or Jcl:ICR nude mice.

In this report, we compared the susceptibility of CD-1 (ICR) and Jcl:AF hybrid nude mice originating from the same Swiss-Webster (ICR outbred) mice with that of "resistant" CD-1 (ICR) nude mice.

We have established CD-1 (ICR) hybrid nude mice (M1) by mating the CD-1 female mice and NFS/N or N:NIH(s) male nude mice. Jcl:AF hybrid nude mice (IAI inbred female mice originating from Jcl:ICR outbred strain) were obtained from CLEA Company, Tokyo, Japan. CD-1 (ICR) hybrid nude mice (4–7 weeks old; 10 nude mice, respectively) and 10 CD-1 (ICR) nude mice 4–7 weeks old were used as control groups. The inoculum size was 5.2×10^5 bacilli/foot derived from hind foot passage of Crj:CD-1 (ICR) nude mice. The experiment with the Jcl:AF hybrid nude mice was performed separately. Ten Jcl:AF nude mice and CD-1 (ICR) nude mice (6 males, 7 females) 4–5 weeks old were used. The inoculum size was 7.8×10^5 bacilli/foot derived from passage of Jcl:ICR nude mice. The site of injection was the right hind foot pad. Mice were maintained in a vinyl isolator under specific pathogen-free (SPF) conditions.

At 434 days after injection, there were marked nodular lesions in the infected foot, developing to the lower leg and toes of CD-1 (ICR) × NFS/N and CD-1 (ICR) × N:NIH(s) hybrid mice (M1), indicating no significant differences against susceptibility to *Mycobacterium leprae* in either mouse group. In contrast, CD-1 (ICR) nude mice showed lesions with a slight swelling at the infected paws.

The swelling due to *M. leprae* growth in Jcl:AF hybrid nude mice gave excellent results as compared with CD-1 (ICR) nude mice at 300 days and 433 days after inoculation, as well as the CD-1 (ICR) hybrid nude mice mentioned above. Thus, CD-1

(ICR) hybrid nude mice and Jcl:AF hybrid nude mice were highly susceptible to *M. leprae* as compared with CD-1 (ICR) nude mice in spite of originating from the same Swiss-Webster mice (ICR outbred strain). The acid-fast bacilli (AFB) isolated from the swollen tissues were distinguished from other mycobacteria by the reinoculation test using Jcl:ICR normal mice which indicated persistent resistant infection only at the site of injection, loss of acid-fastness by pyridine extraction, and the cultivation on a modified Nemoto's egg yolk medium as well as on 1% Ogawa's medium at 33°C and 37°C for 3 months which showed no growth. In addition, sections of the inoculated feet of CD-1 (ICR) and Jcl:AF hybrid nude mice showed the presence of severe lepromatoid lesions, when stained with Fite-Faraco and hematoxylin-eosin, compared with the CD-1 (ICR) nude mice.

Therefore, we have established CD-1 (ICR) hybrid nude mice and Jcl:AF hybrid nude mice as a new model for experimental lepromatous leprosy, resembling the cases of ICR-Lasat mice and their athymic counterparts, and NOD hybrid nude mice as compared with that of "resistant" CD-1 (ICR) nude mice.

In spite of the fact that these mice originated from a Swiss-Webster colony, they gave excellent results with the development of marked lepromatoid lesions, resembling the NFS/N and N:NIH(s) nude mice originating from a Swiss strain. Thus, the formation of lepromatoid lesions in the Swiss and Swiss-Webster hybrid nude mice (M1) may be produced by the genetic background except for CD-1 (ICR) nude mice. The same may be true for SHR hybrid nude rats, originating from the Wistar colony, which differ from "resistant" WKY, WM, and LOU nude rats in experimental lepromatous leprosy.

In summary, we have established CD-1 (ICR) × NFS/N, CD-1 (ICR) × NIH(s) and IAIx NFS/N hybrid nude mice at the M1 circle (generation) as a new model for experimental lepromatous leprosy. The development of marked lepromatoid lesion formations in nude mice may be produced by the genetic background of the Swiss colony except for CD-1 (ICR) nude mice.—[National Institute for Leprosy Research, Tokyo, Japan]

Young, R. A. Mycobacterial genes and antigens.

A number of investigators have pooled their expertise in recombinant DNA expression technology and immunology to investigate the immune response to infection by *Mycobacterium leprae* and *M. tuberculosis.* Research that led to the isolation and identification of genes that encode major protein antigens of the etiologic agents of leprosy and tuberculosis is summarized. In addition, studies that have implicated some of these antigens in the cell-mediated immune response to infection are discussed.—[Whitehead Institute for Biomedical Research, Nine Cambridge Center, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.]

Nomaguchi, H., Matsuoka, M., Nakata, A. and Ito, T. Purification of 65-kDa protein of *Mycobacterium leprae* in *Escherichia coli.*

We recloned the gene for the 65-kDa protein of *Mycobacterium leprae* from the λgtll recombinant phase into a multicopy plasmid vector pUC8 for overexpression of the gene. The subclone that overproduces the 65-kDa protein was constructed as follows: A recombinant phage was isolated from lysogens of the λgtll phage clone Y4178, and the DNA was extracted by phenol. The DNA was digested with the restriction endonuclease *Eco*RI. Plasmid DNA of the vector pUC8 was purified from strain JM83 carrying pUC8. The DNA was treated with *Eco*RI and alkaline phosphatase. These DNA fragments from Y4178 and pUC8 were treated with the bacteriophage T4 DNA polymerase for ligation. After transformation into *Escherichia coli* JM83 and screening on plates containing ampicillin (200 μg/ml), X-gal (100 μg/ml), and IPTG (1–3 mM), nine white colonies carrying recombinant pUC8 DNA were isolated. Of the nine colonies, three (pUC8-N4, pUC-N5, pUC-N7) contained a 3.6-kb DNA for the entire 65-kDa protein gene. The orientation of the inserted DNA in pUC-N5 was the same as the promoter of *E. coli lac* gene on pUC8, and pUC-N4 and pUC-N7 were opposite.

The crude protein preparations from

JM83 cells carrying pUC-N5 and pUC-N7 were electrophoresed on SDS-12.5% PAGE, and stained with Coomassie blue. It has been demonstrated that the 65-kDa protein was overproduced in these cells under inducing and noninducing conditions with IPTG. The 65-kDa materials produced by the cells carrying the recloned plasmids were analyzed for their antigenicity in Western blot assay. The results with antibody 111E9 showed that there was a single strongly reactive species of about 65 kDa in the crude protein preparation (0.5 μl of protein preparation) with both orientations of the DNA fragment inserted in the vector under inducing and noninducing conditions. Smaller reacting species (<65 kDa) were also observed when large amounts of the extracts were used or when the protease inhibitor was omitted from the protein preparation. Thus, the 65-kDa protein gene can be over-expressed in *E. coli* regardless of its orientation with respect to promoters on the cloning vector.

A crude protein preparation from JM83 cells carrying pUC-N5 was affinity-purified with monoclonal antibody (MAB) 3A. The 65-kDa protein fractions by affinity column were single bands by direct staining of the gel with Coomassie brilliant blue. The affinity-purified 65-kDa protein fraction of *M. leprae* was recognized in Western blotting assays with MAB 111E9.

The affinity-purified protein was used with the skin test of response to *M. leprae* on mice. Weak reactions were observed not only at the skin-test sites but also at the site of inoculation 4 months earlier with the injection of 5 μg of the affinity-purified protein.—[Research Institute for Microbial Diseases, Osaka University, Osaka 565; National Institute for Leprosy Research, Higashimurayama-shi, Tokyo 189, Japan]

Barnes, P. F., Modlin, R. L. and Rea, T. H. A comparative study of the immunohistology of leprosy and tuberculosis.

The study of T lymphocytes from sites of disease activity in patients with tuberculosis and leprosy provides an opportunity to directly evaluate the human immune response to mycobacterial infection. Therefore, we investigated the phenotype and function of CD4+ pleural fluid cells from patients with tuberculous pleuritis. In addition, we compared the microanatomic distribution of CD4+ subpopulations in tissue granulomas from patients with leprosy and tuberculous pleuritis. In patients with tuberculous pleuritis, pleural fluid was selectively enriched with CD4+CDw29+ T lymphocytes, a subpopulation that includes "memory" T cells which have previously been exposed to antigen *in vivo*. In contrast, CD4+CD45R+ cells, thought to comprise "naive" T cells, were not concentrated in pleural fluid. Immunoperoxidase staining of pleural tissue confirmed the predominance of CD4+CDw29+ lymphocytes at the site of disease activity. Immunostaining of tissue from patients with leprosy revealed a predominance of CD4+CDw29+ cells in patients with tuberculoid, but not lepromatous leprosy. These immunohistologic findings suggest that the relatively effective cell-mediated immune response mounted by patients with tuberculous pleuritis and tuberculoid leprosy may be mediated through CD4+CDw29+ "memory" T cells. The immunologic unresponsiveness characteristic of lepromatous leprosy may reflect relative absence of this antigen-reactive subpopulation. To evaluate the functional role of CD4+CDw29+ cells, CD4+ subpopulations were evaluated for their ability to contribute to a cell-mediated immune response against *Mycobacterium tuberculosis* by assaying immune function *in vitro*. Pleural-fluid-derived CD4+CDw29+ cells, but not CD4+CDw29− lymphocytes, proliferated vigorously and produced high levels of interferon-gamma (IFN-γ) when stimulated with purified protein derivative (PPD). CD4+CDw29+ clones produced IFN-γ specifically in response to PPD but not to an irrelevant antigen, tetanus toxoid. IFN-γ levels, measured directly by radioimmunoassay, were markedly elevated in pleural fluid, compared to peripheral blood, suggesting production of this lymphokine *in vivo* at the site of tissue inflammation. The sum of these data indicate that, in tuberculous pleuritis, CD4+CDw29+ cells are selectively concentrated at the site of disease activity, produce IFN-γ, and are likely to play an important role in the local, human, cell-mediated immune response to *M. tuberculosis*. The immunohistologic similar-

ities between tuberculosis pleuritis and tuberculoid leprosy suggest that CD4+ CDw29+ cells may play a similar role in leprosy and, potentially, in other granulomatous diseases.—[University of Southern California-Los Angeles County Medical Center, Los Angeles, California, U.S.A.]

Izaki, S., Isozaki, Y., Tokairin, S., Segawa, I., Tanji, O., Hsu, P. S., Hibino, T. and Kon, S. Proteolytic and anti-proteolytic regulation in granulomatous tissue reaction.

We have previously demonstrated that proteolytic activities such as plasminogen activator and elastase are induced in the tissue extract of experimental granulomatous inflammation. The present paper demonstrates that anti-proteolytic reaction simultaneously takes place in the granulomas.

For the experimental granulomatous inflammation, a) 10^7 of *Mycobacterium lepraemurium* were subcutaneously inoculated into C57BL/6N, C57BL/6N(nu/+), C57BL/6N (nu/nu), and CBA/N mice to develop cutaneous granulomas of murine leprosy, and b) 50 cercariae of *Schistosoma mansoni* were intravenously injected into BALB/c, BALB/c (nu/+), and BALB/c (nu/nu) mice to develop a hepatic parasite egg granuloma of murine schistosomiasis. The granulomas were isolated and extracted with 0.05 M Tris-HCl + 0.1 M NaCl, pH 8.0, for the soluble fraction, and the precipitate further extracted with 2 M KSCN + 0.1% Triton X-100 for the bound fraction. Plasminogen activator and plasminogen activator inhibitor activity was assayed with ^{125}I-fibrin microplates, nonlabeled standard fibrin plates, and synthetic chromogenic peptide substrates. SDS-polyacrylamide slab get electrophoresis followed by enzymographic detection of plasminogen activator activity was utilized as well. Elastase and elastase inhibitor activity was assayed with ^{3}H-elastin and synthetic chromogenic peptide substrates.

In both murine leprosy and murine schistosomiasis, a modulation of the plasminogen activator activity was observed associated with the development of granulomatous inflammation. When plasminogen activator activity decreased, plasminogen activator inhibitor activity was, in turn, increased. However, in both animal models, immunosuppressive host animals did not show such proteolytic and anti-proteolytic modulation, but constantly exhibited plasminogen activator inhibitor activity. It is suggested that plasminogen activator associated with granulomatous inflammation is a urokinase-type plasminogen activator and is secreted from immunologically activated macrophages in the granulomatous lesions, and that plasminogen activator inhibitor is most likely the type 2 plasminogen activator inhibitor (PAI-2) of monocytes.

On the other hand, in both murine leprosy and murine schistosomiasis elastase activity found in the bound fraction of tissue extract did not show dynamic modulation during the development of granulomatous inflammation in either immunopositive or immunosuppressive host animals. Elastase inhibitor activity was localized in the soluble fraction and also did not show significant modulation. The biochemical characteristics of partially purified extracts suggest that the elastase detected in the bound fraction is a serine-proteinase-type elastase of the membrane fraction of monocytes, belonging neither to the pancreatic-type, neutrophil-type nor to the divalent cation-dependent macrophage-type elastase, and the elastase inhibitor found in the soluble fraction is an α1-proteinase inhibitor, possibly from blood serum and lymphocytes.

Different kinds of proteolytic and anti-proteolytic regulation systems were demonstrated commonly in murine leprosy and murine schistosomiasis. Since the plasminogen activator activates plasminogen to form plasmin, resulting in degradation of fibrin and other proteins, and elastase plays a role in the digestion of proteoglycan, fibrin, fibronectin, and collagen, as well as elastin, the overall proteolytic activity seems mainly toward fibrous matrix proteins in the inflammatory tissue. In fact, fibrin and elastin meshworks demonstrated by immunohistochemical and histochemical techniques were continuously remodeled during the development of granulomatous inflammation. However, for both plasminogen activator and elastase, we also found that specific regulatory inhibitors co-exist in the inflam-

matory tissue. Such proteinase inhibitors may have a role in the limitation of over-degradation of matrix proteins to preserve cell-matrix contact and to maintain organization of the granulomatous structure. In conclusion, two different kinds of proteolytic and anti-proteolytic systems to control the turnover of fibrous-matrix proteins were demonstrated during the development of granulomatous tissue remodelings.—[Department of Dermatology, Iwate Medical College, Iwate, Japan]

CLOSING REMARKS

Dr. Mori, Members of the Japanese Panel, Fellow Members of the United States Panel, Japanese Guests, American Guests, Guests from the People's Republic of China and from Thailand:

We are now closing the 23rd meeting of the leprosy component of the United States–Japan Cooperative Program in Medical Research. We are closing a meeting that has been a tremendous success; a success in all aspects, from the sylvan sedate surroundings of this beautiful new campus in the heart of the pride of Japan, old historical Nara. We thank Dr. Fujiwara; Mr. Suizu, President of Nara University; Mr. Oura, Chairman of the Board of Trustees of Nara University; and a host of support staff for the opportunity to meet here and for the delightful presentation of traditional Japanese dancing. We also thank Dr. Izumi for his support of Dr. Fujiwara's efforts. The meeting was also a success due to the care with which the two panels and Dr. Gwinn, in the case of the U.S. contingent, selected outstanding contributors with well-balanced presentations in relevant and topical areas.

We are particularly grateful to all of these speakers, especially those who traveled long distances at some personal inconvenience. And, last but not least, the meeting was a success due to each and every one of you, through your very presence and the excellent discussion and debate.

There is another more direct, more subtle reason for the success of this meeting, and it is due to the determined, impeccable leadership that Dr. Masahide Abe has given to leprosy research in Japan over many years. Dr. Abe retired from the chairmanship of the Japanese Leprosy Panel this year, but not from his involvement in leprosy work. As a mark of our esteem and appreciation of Dr. Abe, the U.S. Panel has decided to compile into a single volume, properly inscribed, the key publications generated by American participants in the U.S.–Japan Cooperative Leprosy Program during Dr. Abe's tenure as Chairman of the Japanese Panel. Ths volume is not ready at this time but will be presented to Dr. Abe at the International Leprosy Congress in The Hague this September.

Two years ago at our 21st meeting in Osaka, and last year at our 22nd Joint Conference in Bethesda, Maryland, there was a question mark, a note of trepidation over our proceedings. The searching eyes of Dr. Ivan Bennett, Dr. Shiro Someya, Dr. Tadao Shimao, and Dr. Edward Hook were on us as they reviewed past and projected activities of the U.S.–Japan Cooperative Leprosy Program as part of an overall review of the U.S.–Japan Cooperative Medical Science Program. These reviewers have now reported, and they are praiseworthy of past activities and, in addition, have provided us with excellent guidelines for our future endeavors. In the opinion of the reviewers, molecular biology, immunology, and the goal of an effective vaccine should be emphasized. However, significant criticism was identified by the reviewers which I should mention to you, and that was the virtual absence of close, intensive collaboration between Japanese and U.S. scientists in the area of leprosy research. This is a topic that each and every one of us should address as it relates to our own work. Both the Japanese and U.S. Panels will also address this matter, seeking help from Dr. Tohru Tokunaga in his capacity as Deputy Director-General of the National Institutes of Health and from Dr. Darrel Gwinn, Leprosy Program Officer at the NIH in Washington.

Thus, as we leave here, we can be satisfied with a job well done and with the challenge

of forging stronger ties and collaborations toward our common goal of the eradication of leprosy.

We now have the sea and sunshine of San Diego to look forward to. Accordingly: Rainen, San Diego (Next year, San Diego). Arigato. Sayonara.

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