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# ABSTRACTS

# SIXTEENTH JOINT LEPROSY RESEARCH CONFERENCE

## National Institutes of Health
## Bethesda, Maryland, U.S.A.
## 13–14 July 1981

## U.S.–Japan Cooperative Medical Science Program

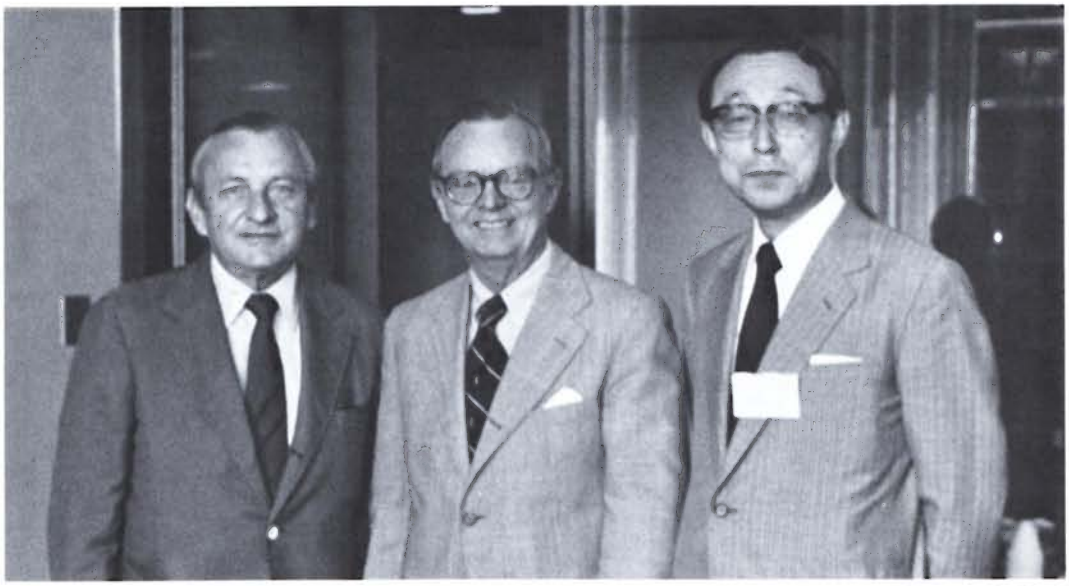
### OPENING REMARKS

On behalf of Dr. Gwinn, Dr. Beck, Dr. Jordan, and the National Institute of Allergy and Infectious Diseases, I would like to welcome you to the National Institutes of Health and to this, the 16th Joint Leprosy Research Conference of the U.S.–Japan Cooperative Medical Science Program. I would like to extend a special welcome to Dr. Suwa, Chairman of the Japanese delegation, and other members of the U.S. and Japanese delegation who are with us today.

Over the years we have seen many changes in these conferences. From beginnings in descriptive accounts of clinical and histopathologic observations and in relatively isolated experimental findings, we have seen systematic leprosy research progress rapidly. From humble beginnings as a poor cousin of tuberculosis, leprosy has now progressed to the frontiers of available general knowledge in such diverse areas as animal models, pharmacology, biochemistry, microbiology, and immunology. Increasingly, individual leprosy research efforts have become dependent on progress by others, both in areas specifically identified as leprosy research and in areas within general disciplines such as pharmacology, biochemistry, microbiology, and immunology. I know of no meeting which focuses the attention of the U.S. and Japanese scientists on current progress in leprosy research as much as these annual Leprosy Research Conferences. Through publication of our proceedings, the impact of these conferences is worldwide and difficult to overestimate.

As was so clearly stated by a former chairman of the U.S. Leprosy Panel, Dr. Shepard, in the 12th Joint Leprosy Research Conference in Boston in 1977, "Because leprosy is such a [widespread and] important medical and public health problem, the potential benefit from quality research is uncommonly large . . . . Leprosy is predominantly a disease of tropical countries. Leprosy research is predominantly an activity of developed countries. This geographic separation has, as one of its unfortunate consequences, a continuing pressure to reduce research on tropical diseases." At perhaps no time in recent history has the pressure to reduce research expenditures in tropical diseases been greater than it is in 1981. Hopefully, these annual conferences, of profound if not vital importance in the progress of leprosy research in our two countries, and, by extension, worldwide, will not fall victim to these pressures at this critical juncture when so much remains to be learned about this ancient disease.

—Robert C. Hastings, *Chairman*
*U.S. Leprosy Panel*



Left to right, Dr. Ivan Bennett, Chairman, U.S. Delegation; Dr. Richard Krause (center), Director, National Institutes of Allergy and Infectious Diseases, National Institutes of Health; and Dr. Norio Suwa, Chairman, Japanese Delegation.

Dr. Robert C. Hastings, Chairman, U.S. Leprosy Panel, and Dr. Masahide Abe, Chairman, Japanese Leprosy Panel.

Participants of the Sixteenth Joint Leprosy Research Conference

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# ABSTRACTS OF LEPROSY CONFERENCE

**Fukunishi, Y., Okada, S., Nishiura, M. and Kusaka, T.** Electron-microscopic and biochemical studies of the peribacillary substance of *M. leprae*.

When examined by the freeze-etching technique, spherical droplets always appeared specifically around *M. leprae* growing in lepra cells. For this reason it was thought that the material making up these spherical droplets might be closely related to the process of multiplication of *M. leprae* in the phagolysosomes of macrophages of host cells (human being, nude mouse, and armadillo). Judging from the electron-microscopic and cytochemical findings, it was thought that the spherical droplet was composed of a kind of glycolipid.

In order to get more detailed information, we analyzed the pattern of lipids contained in lepra cells of nude mice by means of gel-permeation columns (GPC) in high pressure liquid chromatography (HPLC). It is possible to presume the molecular weight of unknown lipids in GPC because the relation between the retention volume and the separation zone (peak) of each molecular weight is fixed in the system.

Judging from the results of these GPC in HPLC, substances with molecular weights of 1600, 1150, 500, and 300 were found specifically in the spherical droplets around *M. leprae* in host cells.

After purification of these unknown lipids with presumed molecular weights of 1600, 1150, 500 and 300, experiments will be done to study the biochemical components and constituent elements of the substances in these peaks using some additional detectors and mass-spectrometry.—[Leprosy Research Laboratory, Kyoto University School of Medicine, Kyoto, Japan; Department of Biochemistry, Kawasaki Medical School, Kawasaki, Japan]

**Fieldsteel, A. H. and Colston, M. J.** The intact mouse as a host for the detection of small numbers of viable *M. leprae* in the presence of large numbers of heat-killed *M. leprae*.

Not infrequently, when neonatally thymectomized Lewis rats (NTLR) are inoculated into the foot pads with large numbers of *M. leprae* ($>10^7$), fewer organisms are recovered when the animals are sacrificed a year or more later. This is due, in part, to the fact that a significant number of NTLR are not fully immunosuppressed, and that growth of the *M. leprae* to numbers as great as, or greater than, the original inoculum was prevented by this residual immunocompetence. However, it was still possible to prove that these inocula contained viable *M. leprae*, because subsequent subpassage of small numbers of organisms into intact mice gave unequivocal evidence of multiplication. These results suggested that it might be possible to use intact rodents for the detection of small numbers of viable *M. leprae* in the presence of large numbers of dead *M. leprae*.

Either $10^1$ or $10^2$ viable *M. leprae* were mixed with either $10^5$, $10^6$ or $10^7$ heat-killed *M. leprae*, followed by inoculation into both hind foot pads of normal BALB/c mice. Six months later half the mice in each group were killed, foot pads pooled, the *M. leprae* counted and subpassaged to another group of intact BALB/c mice. There was no evidence that replication had occurred in the original mice. Decreases in recovered organisms ranged from 5- to 418-fold. However, in each group, except those originally inoculated with killed *M. leprae* only, there were significant increases in numbers of *M. leprae* recovered from subpassage mice after six months.

In the same experiment, the remainder of the original mice were harvested one year after inoculation, and the organisms again subpassaged into normal BALB/c mice. Although there was still no evidence of direct growth there were significant increases in the counts between 6 and 12 months. Subpassage again resulted in unequivocal growth after 6 months.

Although the NTLR has proven to be a sensitive host for the detection of small numbers of *M. leprae* obtained from heavily infected NTLR undergoing chemotherapy, this sensitivity has appeared to be reduced when the source of *M. leprae* was human biopsy material rather than isogeneic tissue. Because the NTLR is not com-

pletely immunosuppressed, it seemed possible that the human skin triggered an immune response that nonspecifically inhibited the growth of small numbers of *M. leprae*. To test this hypothesis, we homogenized normal human facial skin, mixed it with heat-killed and viable *M. leprae* in the same proportion that they would be present in a biopsy specimen, and inoculated the mixture into the foot pads of NTLR. Unequivocal growth occurred from inocula of $10^1$ or $10^2$ viable *M. leprae* mixed with $5 \times 10^6$ heat-killed *M. leprae* and human skin. The same results were obtained when the inoculum contained skin and $10^1$ or $10^2$ viable *M. leprae*, and no heat-killed bacilli. However, no growth occurred in either group when the inoculum contained only $10^0$ viable *M. leprae* in addition to the skin and killed bacilli. On the other hand, inocula that contained no skin, but did contain $5 \times 10^6$ heat-killed *M. leprae* plus $10^0$ viable, or $10^0$ viable only, did replicate. These results indicate the possibility that replication of *M. leprae* might occur after the inoculation of a single organism, and that the human skin may have had a moderate effect on the number of *M. leprae* required to produce a foot pad infection. Both observations obviously need further confirmation.

The results reported here, although preliminary, are most encouraging. The remarkable ability of the intact mouse to discriminate between heat-killed and viable *M. leprae* means that unless it can be shown that drug-killed *M. leprae* behave in a fashion different from heat-killed organisms when inoculated into foot pads of intact mice, it should be possible to utilize the latter as substitutes for immunosuppressed rodents for the detection of small numbers of viable bacilli in patients undergoing chemotherapy.—[Life Sciences Division, SRI International, Menlo Park, California 94025, U.S.A.]

**Acknowledgment.** This work was supported in part by the U.S.–Japan Cooperative Medical Science Program, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland (Grant R22 AI-08417), and in part by a grant from the Chemotherapy of Leprosy (THELEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

**Meyers, W. M., Walsh, G. P., Brown, H. L., Binford, C. H., Gerone, P. J., Wolf, R. H., Gormus, B. J. and Martin, L.** Leprosy in the Mangabey monkey (*Cercocebus torquatus atys*, "Sooty Mangabey").

At the Fifteenth Joint Conference on Leprosy Research of the U.S.–Japan Cooperative Medical Science Program, we reported naturally-acquired leprosy in a mangabey monkey. This animal was imported from Central Africa and had never been experimentally inoculated with *Mycobacterium leprae*. The species of this animal has been established as *Cercocebus torquatus atys* and is commonly called the "sooty mangabey." The natural range of this species is from Nigeria to the Congo (Zaire) River. The initial clinical findings in January 1980 in this animal were as follows: extensive infiltrations of the skin of the face, nodular infiltrations of the ears and extensor surfaces of the forearms, no enlargement of peripheral nerves or paralytic deformities like those seen in subpolar-lepromatous to borderline-lepromatous leprosy ($LL_S$-BL) in man. Ultrastructural findings were consistent with lepromatous leprosy in man and with experimental leprosy in the nine-banded armadillo and nude mouse. Bacteriologic and immunologic studies failed to detect any differences between the acid-fast bacilli in tissues of this animal and known *Mycobacterium leprae*.

There was a gradual increase in the extent of the infiltrations of the skin noted on the initial examination. By February 1981 there were additional massive infiltrations on all extremities, especially the hands and feet, and the tail. The lesions on the face and ears were severely ulcerated. In December 1980 paralytic deformities were first noted and the monkey became increasingly lethargic and lost weight. There was paralysis of the intrinsic muscles of both feet and of the extensor digitorum and peroneal muscles. There was no apparent loss of function of the tibialis anterior muscle. Both feet were inverted and there were ad-

vanced flexion deformities of the toes. The monkey was thus forced to walk on the lateral aspects of the foot. This deformity, perhaps combined with loss of normal sensation and infiltration of the skin of that area provoked extensive ulceration of the foot. In the hands there was mild paralysis of the intrinsic muscles with early flexion deformities. Peripheral nerves of the extremities were palpable but were not believed to be enlarged.

On 12 February treatment of the infection in the index monkey was started with oral rifampin. The regimen was 10 mg/kg body weight daily for 28 days. Treatment was initiated because of the continued deterioration of the general health of the animal. At the end of 1 week of rifampin therapy the general health status was much improved. Much of this early improvement could be attributed to an effect on secondary bacterial infection. Biopsy specimens of infiltrated skin were obtained after 2 weeks of rifampin therapy. Histopathologic evaluation of these specimens revealed that all acid-fast bacilli (AFB) were nonuniformly stained or were granular. The cellular reaction at this early stage of chemotherapy did not show any significant differences from those observed in pretreatment specimens. The Morphologic Index (MI) of smears made from these specimens was reduced to near zero. Suspensions of organisms separated from post-treatment biopsy specimens have been inoculated into mouse foot pads to determine viability. Following treatment for 28 days with rifampin the monkey was started on DADDS therapy (20 mg every 77 days).

Two "sooty mangabey" monkeys were inoculated on 20 March 1980 with suspensions of organisms from the index monkey. Each monkey received $1.2 \times 10^9$ AFB intravenously and $3 \times 10^8$ AFB subcutaneously at each of five sites. On 29 July 1980 there were plaques measuring approximately 10 mm × 5 mm on each ear at inoculation sites. One of these was biopsied and showed histopathologic changes similar to those seen in early leprosy in the borderline-lepromatous area of the spectrum of the disease. These lesions continued to enlarge and biopsy specimens obtained on 12 February 1981 showed marked progression of the histopathologic changes. At this time, the changes were similar to those seen in specimens obtained from the index animal in January 1980. There were erythematous papules on the scrotum. Biopsy specimens revealed histopathologic changes typical of $LL_s$-BL leprosy.

The second "sooty mangabey" monkey developed lesions at the inoculation sites on the ears much more slowly. A biopsy specimen obtained on 12 February 1981 from one of the lesions showed histopathologic changes reminiscent of borderline leprosy in man.

Nine nine-banded armadillos were inoculated i.v. in January 1980 with $10^8$ AFB obtained from the index monkey. Four died (or were euthanized) with a disseminated disease that was clinically and histopathologically like that which follows inoculation with *M. leprae*; three are alive without lesions, and two animals died of unrelated causes.

On 10 December 1980 two "sooty mangabey" monkeys were inoculated intravenously and at multiple sites in the skin with first passage *M. leprae* (human origin) obtained from infected tissues of armadillos. By June 1981 there was early evidence of progressive lesions at inoculation sites.

Each of ten mice was inoculated in the foot pad with $5 \times 10^3$ AFB from the index monkey. After 8 months the mean foot pad count of these animals was $6.6 \times 10^6$ AFB.

Observations during the past year have indicated that the naturally-acquired leprosy in the "sooty mangabey" has been progressive. The cutaneous infiltrations became extensive and widely disseminated, most heavily on the nonhairy or lightly haired body surfaces. The deformities of the hands and feet was a striking development. There is early evidence that the disease may have been successfully transmitted to other "sooty mangabey" monkeys. Clinical and histopathologic observations on the two passage monkeys suggest that there may be a spectrum of forms of leprosy in the mangabey monkey.—[Armed Forces Institute of Pathology, Washington, D.C.; Delta Regional Primate Research Center, Covington, Louisiana 70433, U.S.A.]

**Acknowledgment.** These studies have

been supported in part by the Heiser Program for Research in Leprosy. The World Health Organization (IMMLEP), and the Damien-Dutton Society.

**Nakamura, M.** The effect of vitamins on serial cultivation of *M. lepraemurium* in axenic liquid medium and its implication in the culture of *M. leprae*.

The effect of vitamins on the growth of *M. lepraemurium* was reported at the U.S.–Japan meeting last year. Further studies have been carried out with the purpose of making the culture medium more sensitive and more potent. Since human leprosy bacilli certainly multiply in the mouse foot pad, particularly in nude mice, it is conceivable that *M. leprae* requires no compound which is specifically essential for its growth. If so, the way to attain cultivation of *M. leprae in vitro* might be to establish an extremely sensitive culture medium in which *M. lepraemurium* easily and abundantly propagates like *M. tuberculosis*.

Therefore, the effect of vitamins on the growth of *M. lepraemurium* was investigated. The results obtained indicate that significant growth stimulating effects were found with vitamins $B_6$, $B_{12}$, $K_3$, and PABA. A slight effect was found with biotin and vitamin $K_5$. No effects were noted with vitamin $B_1$, L, M, and P. These effects were determined on the basis of whether or not serial growth of *M. lepraemurium* could be maintained.

Vitamins having a significant effect on the serial growth of *M. lepraemurium* were selected and employed for a cultivation trial of *M. leprae*. Results are not as yet conclusive since the viability of the cultivated *M. leprae* has not been tested.—[Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan]

**Kvach, J. T. and Veras, J. R.** A fluorescent staining procedure for determining the viability of mycobacterial cells.

A fluorescent staining procedure has been developed which rapidly, accurately, and economically measures the viability of mycobacterial cells. *Mycobacterium smegmatis* and *M. phlei* have served as prototype organisms to establish conditions which insure optimal staining.

The staining method incorporates the use of the fatty acid ester fluorescein diacetate (FDA) and ethidium bromide (EB). Nonpolar, nonfluorescent FDA enters live cells where it is enzymatically hydrolyzed by acetylesterase to polar, fluorescent fluorescein which rapidly accumulates in the cytoplasm. These cells appear green when viewed under incident ultraviolet illumination. Ethidium bromide enters dead cells and intercalates between the bases of DNA molecules. These cells appear red-orange under UV illumination. Live cells are, therefore, identified on the basis of possessing acetylesterase and their ability to exclude EB whereas dead cells are identified on the basis of lacking acetylesterase and their inability to exclude EB. The feasibility of applying the staining procedure to *M. leprae* has been investigated and the results are encouraging. Our findings reveal that armadillo-derived *M. leprae* possess acetylesterase and therefore stain green. *M. leprae* cell suspensions exposed to adverse physico-chemical conditions give rise to high proportions of red-stained cells as would be expected if the cells were being killed. An alternative means of determining the viability of *M. leprae* appears to be feasible.—[Department of Pathobiology, Johns Hopkins University, Baltimore, Maryland 21205, U.S.A.]

**Dhople, A. M., Suchin, J. and Storrs, E. E.** The ATP content and other characteristics of *Mycobacterium leprae*.

This report presents for the first occasion data obtained from measuring the ATP content of *M. leprae* grown in the livers and spleens of nine-banded armadillos, in the foot pad of a nude mouse, and also from skin biopsy specimens of human leprosy patients. Significant differences were found to occur between the ATP content of *M. leprae* and *M. lepraemurium*.

*M. leprae* were harvested aseptically from spleens and livers of armadillos and from the foot pad of a nude mouse, and suspensions were prepared in phosphate buffer. Microscopic counts on these suspensions were $10^{10}$–$10^{11}$ AFB per gram of

tissue, with 35–50% of the organisms showing solid staining. The AFB in these suspensions gave growth curves that were identical to human *M. leprae*. The suspensions were then processed by existing methods to eliminate any host-derived ATP, and their intracellular ATP was determined using the firefly bioluminescent technique. The ATP content of *M. leprae* was 1.2–1.4 picograms per $10^6$ cells, which is about 50% of that found in *M. lepraemurium* cells isolated from infected mouse livers. Furthermore, these organisms, when diluted and washed by centrifugation at 4°C, did not lose any appreciable amount of ATP, suggesting strongly that *M. leprae* cells are different from those of *M. lepraemurium*.

When the contaminants were detected in these suspensions, they were treated with either 4% NaOH, 5% oxalic acid, or with saturated trisodium phosphate-zephiran. All three procedures yielded contaminant-free *M. leprae* suspensions. There was no loss in the ATP content of *M. leprae* after treatment with NaOH while the other two procedures resulted in about 10% loss of the original ATP.

When the AFB counts in suspensions from armadillo spleens and livers were low ($10^9$/gram and $10^8$/gram), the suspensions had to be treated first with trypsin and chymotrypsin to liberate all nonbacterial cells; these were then exposed to Triton X-100 to release all nonbacterial, host-originated ATP, which was hydrolyzed with ATPase before extracting bacterial ATP. This procedure resulted in only a 10% loss of the original ATP of *M. leprae*. However, when dealing with suspensions from human skin biopsy specimens ($\pm 1 \times 10^9$/gram), use of collagenase along with trypsin and chymotrypsin was essential. The ATP content of human-derived *M. leprae* (MI 10–12%) was 1.1–1.2 picogram per $10^6$ cells. The suspensions treated by different procedures described above, when inoculated into mouse foot pads, gave standard growth, indicating retention of viability of *M. leprae*.

The *M. leprae* suspensions prepared from spleens and livers of armadillos and purified by the above procedures were used to study their characteristics. Various biochemical tests performed indicated that *M. leprae* have a closer relationship with *M. vaccae* than any other mycobacteria. The endogenous dehydrogenase activity of whole cells of *M. leprae* was so strong that the reduction of triphenyl tetrazolium chloride in response to substrates was not observed with any of the TCA cycle intermediates. However, with the cell-free extracts, even though all the substrates accelerated formation of formazan, the response was maximum with citrate, malate, and pyruvate.

In other studies it was found that *M. leprae* possesses fumarate hydratase activity, and malate is produced from fumarate; it possesses succinic dehydrogenase activity, and fumarate is produced from succinate. There is also aconitate hydratase activity, and citrate is produced from cis-aconitate and isocitrate. Also, citrate synthase activity is present and citrate is produced from acetyl CoA and oxalacetate. Other enzymes in the TCA cycle enzyme system are currently under investigation. The preliminary studies clearly indicate that, in addition to cytochromes of the $a+a_3$ and b type, c-type cytochrome is also present in *M. leprae*.—[Medical Research Institute, Florida Institute of Technology, Melbourne, Florida 32901, U.S.A.]

**Abe, M., Ozawa, T., Minagawa, F. and Yoshina, Y.** Immuno-epidemiological studies on subclinical infection with *M. leprae*. II. FLA-ABS and lepromin tests in schoolchildren in the Miyako Islands.

For the purposes of detecting subclinical infection with *M. leprae* in individuals with low or no resistance against leprosy and of treating them before the occurrence of overt symptoms of leprosy, fluorescent leprosy antibody absorption (FLA-ABS) tests and lepromin tests using the Dharmendra antigen were conducted on the school children in three districts of the Miyako Islands in Okinawa.

Among 1379 school children examined in 1980, 158 (11.5%) had a subclinical neural sign such as the enlargement of a peripheral nerve without sensory loss; the percentage is higher in boys than in girls. The percentage of positive FLA-ABS tests was signif-

icantly higher in children with neural signs than in those without these signs, and this difference was more apparent in boys than in girls. Suspicious depigmentation of the skin was found in 39 cases, but it showed no correlation with the FLA-ABS test. The Fernandez reaction due to Dharmendra antigen showed a significant correlation with the tuberculin test in those school children without a history of BCG vaccination. This correlation was not significant in those school children vaccinated with BCG. Because of this cross reactivity, the Fernandez reactions did not correlate with the neural signs nor with the dermal signs. However, the usefulness of this reaction as an indicator of cell-mediated immunity was promoted by the specificity of FLA-ABS test.

Among 217 school children examined simultaneously with both tests, 58 showed positive reactions in both. Enlargement of peripheral nerves was found in 36 of these cases. The other 30 children showed a positive FLA-ABS test and a negative or doubtful Fernandez reaction. Neural signs were found in half of these cases. They must be carefully observed or should be preferably vaccinated because they have been infected with *M. leprae* without the acquisition of cell-mediated immunity to leprosy. The frequency of such cases in a certain population may be used as an index of susceptibility to leprosy, and this index will serve to reduce the scale of a vaccination trial.—[National Institute for Leprosy Research, Higashi-murayama-shi, Tokyo, Japan]

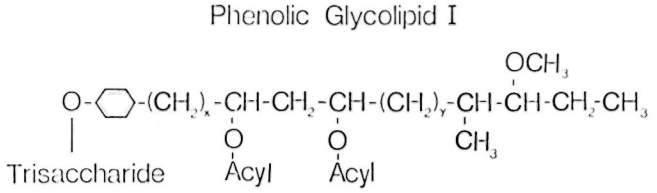
**Gillis, T. P. and Buchanan, T. M.** Antigenic relationships between *M. leprae* and other mycobacteria defined by absorbed leprosy sera and monoclonal antibodies.

We have reported previously that lithium acetate extracts of *M. leprae* may contain *M. leprae*-specific antigens. We have extended these studies to include 21 species of mycobacteria and have found that the immunoreactivity as defined by an absorbed reference leprosy sera (ARLS) is present in extracts from four nonpathogens, *M. gastri, M. nonchromogenicum, M. flavescens, M. gordonae,* as well as *M. lepraemurium* and *M. bovis* BCG. The other 15 species tested showed no immunoreactivity with ARLS and included five significant human pathogens (*M. tuberculosis, M. scrofulaceum, M. kansasii, M. marinum, M. intracellulare*). These results suggest that the extract from *M. leprae* contains antigen(s) that are shared in part with a limited number of species of mycobacteria and may contain antigenic determinants specific for *M. leprae*. In an attempt to delineate these antigenic relationships, we have prepared monoclonal antibodies to the lithium acetate extract of *M. leprae*. Spleen cells from immunized BALB/c mice were fused with mouse myeloma cells (NSI/1) to produce hybrid cells. Supernatant fluids from the resultant hybrids were screened for antibody by ELISA using three antigenic preparations, *M. leprae*, armadillo liver homogenate, and a carbohydrate-rich extract prepared from *M. smegmatis*. Initial screens showed that the majority of the hybrids were reactive with the *M. leprae* extract only; however, hybrids producing antibody which reacted with the differentiating antigens (i.e., armadillo antigen and *M. smegmatis* carbohydrate antigen) were detected.

Twelve separate clones were developed. Immunodiffusion precipitation and microzonal cellulose acetate electrophoresis analysis of ascites antibody were consistent with the production of monoclonal antibody of the IgG isotype by each clone. Further characterization of the immunoreactivity of each monoclonal antibody against 15 species of mycobacteria by ELISA showed that two of the twelve monoclonal antibodies reacted only with *M. leprae*. Two other clones reacted with all species tested and the remaining eight clones gave distinct reactive patterns, none of which could be considered specific to a given species.—[Immunology Research Laboratory, U.S. Public Health Service Hospital, Seattle, Washington 98114, U.S.A.]

**Hunter, S. W. and Brennan, P. J.** A novel glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity.

We are interested in the mechanism of pathogenesis of *Mycobacterium leprae* and in its antigenic composition. In particular,




THE FIGURE

we seek species-specific antigens which could be used for the development of diagnostic skin tests and serological tests and in exploring the disordered cellular immunity so characteristic of some forms of leprosy.

Leprosy is the most serious of a variety of diseases caused by nontuberculous (atypical) mycobacteria. Recently, we characterized the specific surface antigens of a host of atypical mycobacteria as "C-mycosidic" glycopeptidolipids, composed of an invariant monoglycosylpeptidolipid "core" and a variable species- or type-specific oligosaccharide (cf. J. Biol. Chem. **254** (1979) 4205; and Eur. J. Biochem. in press). Moreover, the C-mycosidic antigens comprise the bulk of the superficial cell wall sheath which surrounds certain atypical mycobacteria and which apparently shields them within phagolysosomes from lysosomal enzymes (cf. J. Bacteriol. **144** [1980] 814). This information prompted us to seek like substances and roles in *M. leprae*. Indeed we have reported that an impure apolar lipid fraction from *M. leprae* yielded distinct lines of precipitation with antisera from lepromatous patients and from infected armadillos, and did not react with antisera from patients with other mycobacterial infections (cf. Int. J. Lepr. **48** (1980) 382). Characteristics of the lipid fraction indicated that the active substance might be related to the aromatic glycolipids of mycobacteria, the so-called mycosides A, B, G.

A pure glycolipid has now been isolated from serologically active *M. leprae* lipid extracts. The infra-red spectrum of the lipid was characterized by the absorption bands of an aromatic nucleus (1510 and 1605 $cm^{-1}$). The ultra-violet absorption spectrum showed two very strong peaks at 338 nm and 274 nm (with a shoulder at 280 nm), again very indicative of the existence of a phenolic nucleus in the glycolipid from *M. leprae*.

The NMR spectra of the phenolic glycolipid showed a quadruplet at $\Delta$ 6.80–7.20 characteristic of a phenolic nucleus with its *para* hydroxyl linked to an aliphatic chain. A multiplet centered at $\Delta$ 4.84 may be attributed to protons in an ester configuration. Singlets and doublets in the range $\Delta$ 3.30–3.58 indicated a number of different methoxyl groups presumably these from sugars and the $\beta$-glycol substituent. Signals clearly attributable to anomeric protons were observed at $\Delta$ 4.25, 4.42, and 5.45. Clearly all of the evidence indicated that the lipid from *M. leprae* is a phenolic glycolipid closely related to the mycosides A, B, G from a variety of other mycobacterial species.

The key to the singularity of the glycolipid from *M. leprae* emerged when the sugar composition was examined. It contained three sugars, some of which had not previously been encountered in nature. They were identified as 2,3-di-*O*-methylrhamnose and 3,6-di-*O*-methylglucose. The complete structure of the glycolipid appears to be as shown in THE FIGURE. Thus, *M. leprae* contains a phenolic glycolipid the sugar composition of which is unique and is probably exclusive to *M. leprae*. Apparently the aglycone portion of the molecule is not unique to *M. leprae*; it is probably similar to that in *M. kansasii* and other mycobacteria.

However, the question of whether the type-specific phenolic glycolipid of *M. leprae* is the type-specific antigen has not yet

been clearly answered. Perhaps the most striking feature about this glycolipid is that it represents a surprisingly high 2% of the mass of the leprosy bacillus. Moreover, it is excreted into the infected liver in amounts (2.2 mg/g) which indicate to us that it may be responsible for the electron-transparent "foam" which surrounds the leprosy organism in infected tissue.—[Colorado State University, Fort Collins, Colorado, U.S.A.; National Jewish Hospital and Research Center, Denver, Colorado, U.S.A.]

**Quesada-Pascual, F., Gillis, T. P. and Buchanan, T. M.** Quantitation and characterization of armadillo proteins that contaminate *Mycobacterium leprae* during its purification.

Since *M. leprae* cannot be grown in culture media, quantity sufficient for immunochemical studies must be obtained from infected humans or animals. Currently the best source of *M. leprae* is from liver tissues of artifically infected nine-banded armadillos. Since liver components may contaminate *M. leprae* purified from these tissues, it would be useful to have assays to determine which antigens in the final *M. leprae* preparation are mycobacterial and which antigens are of armadillo origin. We have developed methods to quantitate armadillo liver protein contamination and to identify the specific armadillo liver proteins that remain as contaminates in each step of the Draper procedure to purify *M. leprae* from armadillo tissues.

Rabbit antiserum was prepared to a 30,000 × *g* supernatant of homogenized normal armadillo liver. This antiserum recognized more than 17 separate liver proteins, as demonstrated by the "Western Blot" method. In this technique normal liver homogenate was electrophoresed over an SDS-12.5% polyacrylamide gel, and a "blot" of this gel pattern was transferred by electrophoresis onto nitrocellulose paper. After washing the paper to remove SDS, the blot was reacted first with the rabbit antiserum, and then with $^{125}$I-labelled staphylococcal protein A to detect antigen-bound antibodies. Autoradiography of the treated blot indicated the subunit molecular weights of the specific armadillo liver proteins recognized by antibodies in the rabbit antiserum. In addition, liver homogenate was coated to polystyrene plates, and an ELISA immunoassay capable of detecting as little as 100 nanograms of contaminating liver antigen was developed using the same rabbit antiserum. Both the "Western Blot" and the ELISA methods were applied to each step of the Draper purification procedure.

The ELISA indicated more than 100 fold reduction in the amount of liver proteins that contaminate *M. leprae* from the initial to the final steps of the Draper purification procedure. The major steps to remove liver protein contaminants were the Percoll gradient centrifugation and the two-phase aqueous separation. The two-phase aqueous separation removed several low molecular weight contaminants. Only one protein, with a subunit molecular weight of approximately 70,000 remained as a contaminant in the final purified organisms. This was strongly recognized in "Western Blot" by the rabbit antiserum even though no particulate contamination was visible by light microscopy of aniline blue counterstained, acid-fast stained specimens. This protein may contaminate antigen extracts of *M. leprae* prepared from infected armadillo tissues or be present in these *M. leprae* preparations used for human injection. A more sensitive ELISA can now be developed directed specifically at this contaminant protein, and such an ELISA might be used routinely to quantitate the amount of armadillo liver protein contamination of different *M. leprae* organism preparations.—[Immunology Research Laboratory, U.S. Public Health Service Hospital, Seattle, Washington 98114, U.S.A.; Departments of Medicine and Pathobiology, University of Washington, Seattle, Washington 98195, U.S.A.]

**Miller, R. A. and Buchanan, T. M.** An enzyme-linked immunosorbent assay using arabinomannan from *M. smegmatis*: results in patients with tuberculosis and leprosy and in family contacts of leprosy patients.

A carbohydrate antigen composed predominantly of arabinomannan (AM) has been partially purified from *Mycobacterium smegmatis* by serial lipid extraction of

whole organisms (acetone, ether-ethanol, chloroform, chloroform-methanol) followed by denaturation of proteins in the residual material using 0.5 N sodium hydroxide at 70°C in a nitrogen atmosphere for five hr. After centrifugation and concentration, the supernatant underwent fractional ethanol precipitation with 30%, 60% and 80% ethanol (vol/vol). The crude precipitate from the 80% precipitation contained carbohydrate and protein in approximately a 1:3 ratio. Two sequential phenol-water separations yielded a product from the aqueous layer which was 95% carbohydrate by weight. SDS polyacrylamide gel electrophoresis followed by Coomassie blue and PAS staining and by "Western Blot" (this technique involves electrophoretic transfer of the gel pattern to nitrocellulose paper followed by serial reaction of the paper with pooled human leprosy sera and $^{125}$I-labelled staphylococcal protein A; autoradiography will then indicate bands corresponding to antigens recognized by specific antibodies in the sera) showed that the residual protein migrated as a diffuse band in the low molecular weight region of the gel and that no immunoreactive material was present on the "Blot" in a location corresponding to this band. Immunoreactivity on the "Western Blot" was concentrated in three distinct bands located in a region where only carbohydrates were present on staining. Thin layer chromatography of the hydrolysate of the polysaccharide revealed it was composed primarily of arabinose and mannose with trace amounts of glucose and galactose.

An enzyme-linked immunosorbent assay (ELISA) was developed using this AM antigen, and sera from normal controls (22), BCG vaccinated controls without evidence of active disease (16), patients with tuberculosis (25), patients with leprosy (89), and household contacts of leprosy patients (194) have been tested. Means and standard deviations of the $OD_{492}$ readings on 1:50 dilutions of sera tested using this ELISA were: normal human sera .157 ± .074, BCG vaccinated controls .261 ± .087, tuberculosis patients .491 ± .412, leprosy patients .584 ± .427, and household contacts of leprosy patients .168 ± .128. Both the tuberculosis group and the leprosy group were significantly different from the control population ($p < .01$). The BCG group and the leprosy group were also significantly different at the $p < .01$ level. Neither the BCG and control groups nor the tuberculosis and leprosy groups differed significantly. Using an upper limit of normal defined as the mean plus two standard deviations of the control group, 56% of the tuberculosis group, 67% of the leprosy group, and 7.2% of the household contacts had abnormally elevated titers. These 14 contacts with elevated antibody titers may be harboring previously undiagnosed mycobacterial infection and are the subject of ongoing investigation.—[Immunology Research Laboratory, U.S. Public Health Service Hospital, Seattle, Washington 98114, U.S.A.]

**Katoh, M. and Matsuo, Y.** Lysosomal enzyme activity in cultured macrophages infected with some species of mycobacteria.

Changes of lysosomal enzyme activity were assessed according to the method of Mead, *et al.*, using 4-methylumbelliferone-conjugated substrates in cultured mouse peritoneal macrophages infected with five species of mycobacteria. Infection with *Mycobacterium tuberculosis* raised acid phosphate activity in macrophages but not β-glucuronidase activity. The more heavily the cells were infected, the more highly the acid phosphatase activity increased. The effect of infections with *M. bovis* BCG and *M. microti* on the lysosomal enzyme activity in macrophages was similar to that with *M. tuberculosis*. The cells infected with *M. leprae* showed a significant decrease in the activity of both acid phosphatase and β-glucuronidase. No change was observed in acid phosphatase activity in the cells infected with *M. lepraemurium*. Infection with this microbe caused either no change or a decrease in the activity of both β-glucuronidase and N-Ac-β-glucosaminidase not only within the cells but also in the culture medium. The pattern of enzymatic response was of similar quality with both *in vivo* and *in vitro* grown *M. lepraemurium*.—[Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734, Japan]

**Kohsaka, K., Yoneda, K., Mori, T. and Ito, T.** Study of the chemotherapy of leprosy with nude mice. The effect of dapsone (DDS) on nude mice experimentally infected with *Mycobacterium leprae*.

Dapsone (DDS) has been widely used for the treatment of leprosy as a primary drug. It has been shown to have bacteriostatic or partially bactericidal effects on *M. leprae* in normal mice by several workers. At the Fifteenth Joint Conference on Leprosy Research, U.S.–Japan Cooperative Medical Science Program, we reported that the administration of DDS was not effective in suppressing the growth of *M. leprae* in nude mice in both preventive and therapeutic experiments. Several possibilities were pointed out for these results. The first question was whether or not the strains of *M. leprae* used in these experiments were DDS resistant. In this paper, the DDS sensitivity of these strains was determined in studies with normal mouse foot pad infections. DDS levels were determined in the serum and liver of nude mice fed the drug.

## MATERIALS AND METHODS

**DDS sensitivity test.** Two strains of *M. leprae* were tested. A bacillary suspension of *M. leprae* was prepared from nude mice previously infected with *M. leprae* derived from a previously untreated lepromatous leprosy patient. $5 \times 10^3$ bacilli were inoculated into the right hind foot pads of KK strain mice bred in our laboratory. The inoculated mice were divided into two groups; one group was treated with DDS (0.01% in the diet for 5–6 months) and the other was an untreated control group. The number of acid fast bacilli (AFB) in the infected foot pads was counted by Shepard's technique.

**DDS level in serum and liver.** Female BALB/c-nu/nu mice and female strain mice (both 4 months old) were fed with a diet containing 0.01% DDS. Blood was taken from 4 animals and pooled to separate serum. Livers of individual animals were removed immediately after the animal was sacrificed by drawing blood. DDS levels in 1 ml of pooled serum and 1 g of each liver were determined by the Schiff base formation method described by Levy and Higgins (Int. J. Lepr. **34** (1966) 411).

## RESULTS

Table 1 shows the results with Inoculum A. In the control group, $1.5 \times 10^6$ AFB were harvested at 6 months after inoculation, but in the DDS treatment group, only $6.4 \times 10^3$ AFB were recovered. Thus DDS significantly suppressed the growth of *M. leprae* in normal mice. This Inoculum A was the same strain of *M. leprae* used in the therapeutic experiment with nude mice reported at the last meeting (Table 2). As shown in Table 2, DDS was not effective in suppressing the growth of the same strain of *M. leprae* in nude mice.

Table 3 shows the results with Inoculum B. At 6 months after inoculation, $9.6 \times 10^5$ AFB were harvested from the control group and $4.6 \times 10^5$ AFB were recovered from the DDS treatment group. These results indicate that Inoculum B is a DDS resistant strain. This Inoculum B was the same strain of *M. leprae* used in the preventive experiment with nude mice reported previously (Table 4).

Table 5 shows the results of DDS determinations in the serum pools and livers. No significant differences were found in DDS levels between BALB/c-nu/nu mice and KK strain mice. These results indicate that the absorption and distribution of DDS is similar in nude mice and in normal mice.

TABLE 1. *Effect of DDS on* M. leprae *in normal KK strain mice.*

| Group | Inoculum | 6 months after inoculation[a] |
|---|---|---|
| Treated with DDS (0.01%)[b] | $5 \times 10^3$/0.05 ml | $6.4 \times 10^3$/foot pad |
| Untreated control | $5 \times 10^3$/0.05 ml | $1.5 \times 10^6$/foot pad |

[a] Harvest: average number of bacilli from 4 mice.
[b] Mice were given DDS for five months beginning three weeks after inoculation.

TABLE 2. *Therapeutic effect of DDS on experimental leprosy in nude mice.*

| Group | Inoculum | Start of treatment | Duration of treatment with DDS | |
|---|---|---|---|---|
| | | (7 mo. after inj.) | for 2 mo. (9 mo. after inj.) | for 10 mo. (17 mo. after inj.) |
| Treated with DDS (0.01%) (5 mice) | $1.7 \times 10^7$ (0.05 ml) | slight swelling of foot pads | — | $5.4 \times 10^8$/fp<br>$4.8 \times 10^8$/fp<br>$3.5 \times 10^8$/fp |
| Untreated control (7 mice) | $1.7 \times 10^7$ (0.05 ml) | $4.0 \times 10^7$/fp | $5.2 \times 10^8$/fp<br>$5.1 \times 10^8$/fp | — |

## DISCUSSION AND CONCLUSION

We have been attempting to study the chemotherapy of leprosy using experimental leprosy of nude mice. As we reported at the last meeting, we carried out two experiments: one was a preventive study and the other was a therapeutic study with DDS in nude mice with experimental leprosy. The results of both experiments indicated that treatment with 0.01% DDS in the diet for 10 months did not suppress the growth of *M. leprae* in nude mice. The two strains used in these two experiments were derived from previously untreated lepromatous patients, but there was a possibility that both of these strains might be DDS resistant. Therefore, we tested the sensitivity of these strains by normal mouse foot pad techniques. We have shown that the strain used in the therapeutic study is DDS sensitive, and the other strain, used in the preventive study, is DDS resistant. DDS levels in serum and in the livers of nude mice were not much different from the levels found in normal mice. Thus the absorption and distribution of DDS is similar in nude mice and in normal mice.

It is strongly suggested that the antileprosy effect of DDS may need the cooperation of T cell function. We would like to continue this study.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Suita City, Osaka, Japan]

TABLE 3. *Preventive effect of DDS on* M. leprae *Inoculum B in normal KK strain mice.*

| Group | Inoculum | Harvest after 6 months |
|---|---|---|
| Treated with DDS (0.01%) | $5 \times 10^3$/fp | $4.6 \times 10^5$/fp |
| Untreated control | | $9.6 \times 10^5$/fp |

TABLE 5. *DDS level in pooled serum and individual liver of nude mice and KK mice.*

| | BALB/c-nu/nu | KK strain |
|---|---|---|
| Serum | 1.6 μm/ml | 1.4 μm/ml |
| Liver | 3.3 μm/g | 2.8 μm/g |
| | 3.0 μm/g | 2.7 μm/g |
| | 2.8 μm/g | 2.7 μm/g |
| | 2.7 μm/g | 2.5 μm/g |

TABLE 4. *Preventive effect of DDS on* M. leprae *infection in nude mice.*

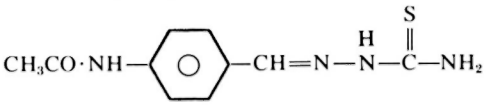
| Group | Inoculum | Duration of treatment with DDS (0.01% in the diet) | | |
|---|---|---|---|---|
| | | for 4 months 7 mo. after inj. | for 5 months 8 mo. after inj. | for 10 months 13 mo. after inj. |
| Treated with DDS (0.01%) (5 mice) | $2.7 \times 10^7$ (0.05 ml) | — | $5.2 \times 10^8$/fp | $1.0 \times 10^{10}$/fp<br>$2.1 \times 10^9$/fp<br>$1.3 \times 10^9$/fp |
| Untreated control (7 mice) | $2.7 \times 10^7$ (0.05 ml) | $8.5 \times 10^8$/fp | $3.3 \times 10^8$/fp | — |

**Jacobson, R. R.** Clofazimine after 16 years—the best antileprosy drug?

Since 1965 clofazimine (B663, Lamprene®) has been used both alone and in combination drug regimens to treat over 175 patients at Carville and supplied by us for use under our protocol to treat many others at various clinics throughout the United States. Clinical and bacteriological results have thus far been uniformly good. No strains of *Mycobacterium leprae* resistant to it have as yet been found and it appears to be at least as effective on a long-term basis as dapsone when used as monotherapy. The only serious toxicity problem to date has been in the small bowel and that is easily prevented if the use of prolonged very high doses is avoided. Even the pigmentation which is so distressing to many patients is not as long lasting as was once feared and the recent availability of a 50 mg capsule may make lower doses more convenient with improved compliance and less pigmenting effect. Clofazimine is clearly useful for control of erythema nodosum leprosum (ENL) and probably also for reversal reactions and these appear to be less common if clofazimine is used as initial therapy for typical leprosy cases. Though more costly than dapsone, its various advantages over that drug may make it more cost effective in the long run. Unfortunately to reduce cost and pigmentation still further and simplify its delivery under field conditions a trend toward the use of extremely low doses of the drug has begun to appear. Are our past mistakes with dapsone about to be repeated?—[National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

**Morrison, N. E.** Antileprosy activity of 2-acetylpyridine thiosemicarbazones.

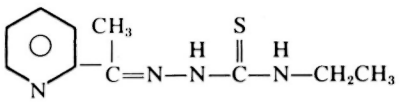
At the U.S.–Japan Cooperative Medical Science Program's 15th Joint Conference on Leprosy Research (1980) a new series of compounds, the 2-acetylpyridine thiosemicarbazones, were reported to inhibit the multiplication of *Mycobacterium leprae* in the mouse foot pad. Initially synthesized as antimalarials (cf. J. Med. Chem. **22** [1979] 865–883; 1367–1373), these thiosemicarbazones are different in structure to the series that gave rise to 4-acetamidobenzaldehyde thiosemicarbazone or thiacetazone (I) which has been used clinically in the treatment of leprosy as well as tuberculosis.



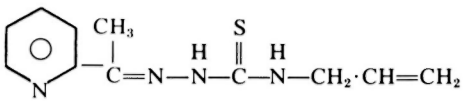
(I)

The present series relate to thiacetazone structurewise by inserting the 4-acetamido N into the phenyl ring to give a pyridine ring and translocating the thiosemicarbazone substituent from the 4- to the 2-position on the heterocyclic ring.

The mouse foot pad data have indicated that two of the newer thiosemicarbazones exert powerful antileprosy effects. The two most active 2-acetylpyridine thiosemicarbazones (structures II & III)



(II)



(III)

were substituted at the $N^4$ position with ethyl or allyl groups. The nature and size of the $N^4$ substituent were important determinants for optimal activity.

The initial findings for this new series ([3]) have now been extended in the following directions. a) A kinetic-type of Shepard experiment (cf. Int. J. Lepr. **35** [1967] 429–435) has been carried out in which the thiosemicarbazones were withdrawn from the mouse diet at day 180 and changes in the afb counts followed out to day 450. b) The extraction and measurement of residual thiosemicarbazone concentrations remaining in the mouse foot pad tissue at day 450 was carried out. c) Microscopic counting of the numbers of methylene blue positive rods that appeared to result from *M. leprae* exposed to thiosemicarbazone action during division inside the mouse foot pad. d) The structural recognition that increased substitution on the pyridine ring (Structures II & III) at the 6-position will further reduce mouse toxicity of this series of 2-acetylpyridine thiosemicarbazones.

It has been concluded from this data that

Structures II and III are more potent than thiacetazone in suppressing the multiplication of *M. leprae*. The prolonged suppression in the absence of the compound from the diet was not due to any significant concentration of the compounds remaining in the foot pad tissues. One effect of thiosemicarbazone action on *M. leprae* is to cause a loss of acid-fastness. A similar effect has been found *in vitro* when cells of *M. smegmatis* ATCC #607 were exposed to 2-acetylpyridine thiosemicarbazone growth inhibitory action. These data tend to suggest that one of the antibacterial effects of thiosemicarbazones is to interfere with the synthesis of wall-bound mycolic acids in *M. leprae* and other mycobacteria (cf. J. Gen. Microbiol. **121** [1980] 249–253; Trends in Biochem. Sci. **4** [1979] 280–282). It is not known whether this is a primary or secondary effect since it has been recently reported that a 2-acetylpyridine thiosemicarbazone type of structure will inhibit RNA synthesis in *E. coli* (American Soc. Microbiol. Abstracts, 81st Meeting [1981], 3).

Finally, drug design approaches are now being followed to optimize the position (2-, 3- or 4-) on the pyridine ring of the thiosemicarbazone substituent while retaining optimum diffusion parameters (log P) and reduced toxicity for mammalian tissues for this promising series of compounds (cf. Int. J. Lepr. **49** [1981] 180–186). [School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205, U.S.A.]

**Piper, L. M., Agrawal, K. C. and Hastings, R. C.** Anti-inflammatory immunosuppressive thalidomide analogs. Screening.

Thalidomide is remarkably effective in controlling erythema nodosum leprosum (ENL) reactions in lepromatous leprosy. Its mechanism of action in ENL has been extensively studied, and it appears to have two clinically relevant sites of action in ENL. One site involves inhibition of neutrophil chemotaxis in response to complement-derived chemotactic factors. This anti-inflammatory site of action is detectable in a whole animal model, the late (6 hr) carrageenan-induced rat paw edema. A second site of action of thalidomide in ENL appears to involve inhibition of IgM antibody synthesis. This immunosuppressive site of action is detectable in another whole animal model, splenic antibody or plaque forming cells (PFC) in mice four days after i.v. immunization with sheep erythrocytes.

Approximately 50 thalidomide analogs have been screened in these two assays for anti-inflammatory activity (carrageenan) and immunosuppressive activity (PFC) to date. Seven simple phthalimide derivatives have been screened. Both electron-donating (hydroxyl) and electron-withdrawing (nitro) substitutions near the imide bond system of phthalimide produce activity in PFC. None are active in carrageenan. Five thalidomide metabolites having intact phthalimide ring systems have been screened. Two, phthalyl-D,L-glutamic acid and D,L-N-$\alpha$-phthalylglutamine are active in PFC, but not in carrageenan. L-N-$\alpha$-Phthalylglutamine and D-N-$\alpha$-phthalylglutamine are not active in either assay, however.

Six other N-substituted phthalimides have been screened. N-Pthalimidophthalimide enhances PFC and is inactive in carrageenan. A pyridine N-oxide-substituted phthalimide, AH 3, has activity in both systems. A 2-amino-substituted pyridine derivative, AH 9, inhibits PFC but is not active in carrageenan.

Thirteen $\alpha$-substituted glutarimides with changes in the 6 membered phthalimide moiety have been studied. Six compounds involved predominantly changes in bulk of the 6 membered ring. To date, three of these, AH 12, taglutimide and AH 23, are active in PFC and one, AH 11, is active in carrageenan. Three compounds involved predominantly changes in polarity. None were active in carrageenan but E 35 (same polarity as thalidomide) and E 122 (more polar) are active in PFC while E 48 (less polar) is inactive. Four compounds involved electron donating substitutions (amino- or hydroxyl-) on the 6 membered phthalimide system. To date, three are active in carrageenan (4-hydroxythalidomide or AH 20, 3-hydroxythalidomide or AH 22, and 4-aminothalidomide or AH 13). One, AH 20, is active in PFC and one, 3-aminothalidomide or AH 14, enhances PFC.

Two compounds represent changes in the 5 membered ring system of the phthalimide moiety and two involve major alterations of the phthalimide system. Two of these, EM 12 and glutethimide, are active in carrageenan.

Two $\beta$ substituted glutarimides, E 350 and E 565 are active in PFC, but inactive in carrageenan. Three compounds with substitutions of the N-H in thalidomide with amino acids have been screened. One, the phenylalanine substituted analog, AH 4, is active in PFC but enhances carrageenan. Of two unrelated sedative-hypnotics, chlordiazepoxide is active in carrageenan but inactive in PFC while pentobarbital is inactive in carrageenan.

D,L-Thalidomide is active in both carrageenan and PFC. To date, L-thalidomide is active in PFC but inactive in carrageenan while D-thalidomide is active in both assays.—[Pharmacology Research Department, National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.; Department of Pharmacology, Tulane Medical Center, New Orleans, Louisiana 70112, U.S.A.]

**Agrawal, K. C., Hassan, K. M. and Hastings, R. C.** Structure activity relationship studies of thalidomide analogs as anti-inflammatory and immunosuppressive agents.

A systematic structure-activity relationship study has been initiated in an effort to develop an analog of thalidomide which may be effectively employed in the clinical management of erythema nodosum leprosum (ENL) occurring in lepromatous leprosy. This effort has been directed to design and synthesize analogs which may not possess the toxicity of thalidomide, manifested by teratogenicity and peripheral neuritis. The antiinflammatory and immunosuppressive activities of these agents have been determined by utilizing the carrageenan rat paw edema test and the Jerne plaque-forming cell (PFC) assay in mice, respectively, at various dose levels. The following observations were deduced:

a) Modifications made in the phthalimide portion of thalidomide by either removing or reducing the benzene ring, produced compounds which did not demonstrate any significant activity. However, insertion of an endoxy or an endomethylene bridge (taglutimide) in the hexahydrothalidomide molecule provided agents with significant activity in the PFC assay but with no activity in the carrageenan assay. In contrast, introduction of a single double bond in taglutimide restored the activity in the carrageenan assay. These results suggest the necessity of an unsaturated (—C=C—) linkage in the phthalimide portion for carrageenan activity.

b) The electronic effects were analyzed by synthesizing analogs containing $NO_2$, COOH, $NH_2$, or OH groups. The agents containing the electron donating groups such as 3 or 4-hydroxy and 4-aminothalidomide retained the anticarrageenan activity, whereas the analogs with electron withdrawing groups such as 4-nitro or 4-carboxy were not active in this assay system. However, the biological activity in the PFC assay did not correlate with electronic properties since not only the 4-nitro and 4-carboxy analogs but also the 4-hydroxythalidomide was active in this system.

c) N′-Substituted analogs of thalidomide synthesized by replacing the NH of the glutarimide portion with glycine, alanine, or phenylalanine were generally not active in either assay system except the phenylalanine analog which was active in PFC assay.

d) Modification of the glutarimide moiety by replacing it with succinimide, pyridine or with substituted pyridines produced agents with variable activity. The most active analog of this series was the compound containing the 1′-oxidopyridine moiety. This agent was active in both the carrageenan and PFC assay and appears to have potential for further testing.

The results suggest that modifications in the parent thalidomide molecule can be made which retain the antiinflammatory and immunosuppressive activity, but yet may be expected to alter the neurotoxic and teratogenic properties.—[Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112, U.S.A.; Pharmacology Research Department, National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

**Krahenbuhl, J. L. and Humphres, R. C.** Effects of foot pad infection with *Mycobacterium marinum* and *M. leprae* on natural killer cell activity as a manifestation of cell mediated immunity to local infection.

Natural killer (NK) cells appear to be a subpopulation of effector cells which, in

mice, are distributed in various lymphoid organs. The most apparent effector function of NK cells is a potent spontaneous cytolytic effect on a wide spectrum of neoplastic target cells with little or no cytolytic effect on normal target cells. NK cells are nonadherent, nonphagocytic, and lack complement receptors but possess low affinity IgG Fc receptors. They lack the surface immunoglobulins characteristic of B cells, possess a low density of Thy 1 antigen but can be found in nu/nu mice. Thus, the exact lineage of NK cells is unclear—it has been suggested that they could be pre-T cells or be promonocytes. Since NK cells appear to provide a low level of protection against certain tumors *in vivo*, it has been postulated that they play an important role in immunosurveillance against cancer. However, there is also evidence that suggests that NK cells play a homeostatic function in hemopoiesis or are important regulatory cells of the immune response.

In the present studies, measurement of NK cell activity was employed solely as a manifestation of a nonspecific host cell mediated immune (CMI) response to local (foot pad) infection with *M. marinum* or *M. leprae* or to foot pad vaccination with killed *M. leprae*.

To measure NK activity, a 4 hr cytolytic assay was performed in which release of $^{51}Cr$ from prelabeled YAC-1 target cells was quantitated as % release. Four different lymphoid cell populations were examined for NK cell activity: spleen, peritoneal cells (PC), draining popliteal lymph node (LN), and contralateral LN.

When the number of lymphoid cells obtained from a given anatomical compartment (spleen, PC, LN) were sufficient, the assay was performed separately with cells from individual mice. In the case of certain cell populations (PC, LN from normal mice, contralateral LN from infected mice) it was necessary to pool the cells from all the mice of each group. Regardless of whether the assay was performed on cells of a single mouse or a group, a range of effector to target (E:T) ratios (3:1, 6:1, 12:1, 25:1, 50:1, 100:1) was employed for each determination.

In the first series of experiments NK cell activity was examined at various times during the course of foot pad infection with *M. marinum* ($5 \times 10^3$ organisms in right hind foot pad [RHF]). Assays were performed at the peak of local *M. marinum* multiplication (day 10) and on day 17, when the number of viable *M. marinum* in the foot pad had markedly declined. In two separate experiments performed at 10 days after infection and in two separate experiments performed at 17 days, there was no difference in NK activity of splenic cells of infected compared with normal mice. In PC, NK activity was low in both groups but was slightly depressed ($p < .01$) in infected mice at 10 days and slightly elevated ($p < .05$) at day 17. Of the greatest interest were our findings when NK cell activity was measured in LN. In each of four separate experiments pooled popliteal LN cells from normal mice were shown to possess little or no NK cell activity—regardless of E:T ratio % release was between 0.5 and 2%. In each of 2 experiments performed 10 and 17 days after *M. marinum* infection respectively, LN cells from pools of the contralateral (LHF) popliteal LN also failed to show any NK cell activity (<1% lysis). Only in the cell population from the popliteal LN draining the *M. marinum* infected RHF was NK cell activity elevated. In four different experiments, carried out at 10 or 17 days after infection, NK activity in cells from the draining LN of 16 individual infected mice was markedly ($p < .001$) elevated (5 to 15% lysis at E:T of 25:1 or 50:1).

In related studies, experiments are being carried out with lymphoid cells of mice infected in the RHF with *M. leprae*. In neither of two experiments to date was there any indication that NK cell activity was elevated in spleen or LN at the point in the *M. leprae* growth curve when multiplication of the organism plateaued (day 136 and 138 respectively).

In a separate but related series of ongoing experiments, the effects of vaccination with killed *M. leprae* on various CMI responses are being determined. At 28 days after injection of $10^7$ heat killed armadillo-derived *M. leprae* into the RHF, there was slight enhancement of splenic NK cell activity in each of 5 individual vaccinated mice. Of greater importance however, appeared to be the local effects of vaccination of NK cell activity in the draining LN. NK cell activity in pooled LN cells from normal mice and pooled contralateral LN cells from vaccinated mice was <0.8% release

and <1.0% release respectively at an E:T of 50:1. In marked contrast LN from the RHF of vaccinated mice were enlarged and cells from 9 of 10 of these LN from individual mice possessed markedly enhanced NK cell activity ranging from 3 to 17% release.

These studies are still in their initial stages and more complete results will be available over the next few months. However, it appears at present that the analysis of NK cell activity in response to foot pad mycobacterial infection or inoculation with killed organisms may represent a sensitive method of determining host CMI response to local infection. The results obtained following injection of killed *M. leprae* are especially interesting since the assay for NK activity in spleen or LN cell population may correlate with the level of protection afforded by vaccination. Studies are currently underway to determine the level of protection induced by vaccination with varying doses of killed *M. leprae* administered alone or in combination with different immunopotentiating preparations (i.e., MDP and its analogs, *Corynebacterium parvum*). At various times after vaccination, the level of protection is being determined by challenge with viable *M. leprae* and by determination of NK cell activity in different lymphoid cell populations. The results of these studies will be presented.—[Leprosy Research Unit, United States Public Health Service Hospital, San Francisco, California 94118, U.S.A.]

**Humphres, R. C., Gelber, R. H. and Krahenbuhl, J. L.** Effector function of mononuclear cells from leprosy patients.

The effector function of mononuclear cells, monocytes, and monocyte-derived macrophages from leprosy patients is being evaluated by examining the following immune parameters: natural cellular cytotoxicity against tumor cells, mitogen induced lymphocyte blastogenesis, phagocytic and microbicidal activities, ability to produce lymphokines, and their responsiveness to lymphokine signals.

The putative impairment of the cell-mediated immune (CMI) response in leprosy patients has been suggested from observations of reduced reactivity to skin test antigens and depressed blastogenic responses in the presence of mitogens or antigens in these individuals. However, the basis for this impairment in leprosy patients has yet to be delineated with respect to the cell population(s) involved since an overall suppression of CMI is seldom observed. In this context the present study investigated another measure of human CMI, spontaneous cellular cytotoxicity effected by natural killer (NK) cells against the human myeloid cell line K-562, which has been shown to be highly sensitive to the lytic event attributable to NK cells. The NK cell activity in the mononuclear cells of 21 normal subjects, 18 lepromatous leprosy (LL) patients, 12 LL patients with erythema nodosum leprosum (ENL), and 8 borderline (BL) patients was examined. In parallel, the ability of these patients' mononuclear cells to undergo blastogenesis in the presence of the plant lectins, concanavalin A (Con A) and phytohemagglutinin (PHA), was also tested.

There was no significant difference in NK activity of LL patients and BL patients as compared to normal subjects at all effector : target (ET) ratios tested. Comparison of normal subjects and patients' mononuclear cells with respect to mitogen induced blastogenesis showed no significant difference. Of greater interest were the results obtained when NK cell activity was assessed in the 12 LL patients with ENL. In comparison to controls and patients with LL the NK cell activity in patients with ENL was significantly less. Although the treatment of choice for ENL consists of prednisone and/or thalidomide, evidence was found that the reduced NK cell activity in ENL patients was not due to the immunosuppressive effects of this therapy. Longitudinal studies of the NK cell response were carried out in one patient who was studied several weeks prior to the onset of ENL, at which time his NK cell activity was normal, and then at various intervals during the course of his ENL episode, when marked suppression of NK activity and subsequent recovery were observed. Remarkably high NK cell activity was observed in the cells of one individual with BL who was undergoing a severe reversal reaction and in a patient with LL complicated by Lucio's phenomenon.

Ongoing studies on the microbicidal capacity of monocytes and monocyte-derived macrophages of patients with LL will be presented as will be studies of lymphokine production and lymphokine response in mononuclear cells of patients with LL.—[Leprosy Research Unit, U.S. Public Health Service Hospital, San Francisco, California 94118, U.S.A.]

**Bullock, W. E., Watson, S. R., Makonkawkeyoon, S., Vithayasai, V., Jacobson, R., Schauf, V. and Nelson, K.** Impairment of immunoregulatory control over B lymphocyte function in lepromatous leprosy.

Lepromatous leprosy is characterized by polyclonal hypergammaglobulinemia and variable degrees of autoantibody formation. To explore these phenomena, peripheral blood leukocytes (PBL) from 10 lepromatous (L) donors and 19 healthy controls were tested for immunoglobulin production after pokeweed mitogen stimulation.

The response was measured by a reverse hemolytic plaque forming cell (PFC) assay using staphylococcal protein A conjugated to sheep erythrocyte targets and developing antiserum. The median PFC response of the L group was 23,820/$10^6$ cells (range 10,670–125,000) vs 6,990 PFC/$10^6$ cells (range 1540–15,380) for controls ($p < .01$). Calculation of the PFC/$10^6$ B lymphocytes also demonstrated a significant difference ($p < .01$). The PFC responses of four patients with BT or TT disease did not differ from the control group. The time course of the PFC response by PBL from normal controls and L patients was measured after 3, 5 and 7 days respectively in culture. Responses by PBL from L donors were substantially higher than those of normal cells at each time interval. Preliminary studies indicate that the spontaneous PFC responses of PBL from the patient and control groups do not differ significantly.

In three different cell mixing experiments, addition of increasing numbers of peripheral blood T lymphocytes ($10^3$ to $10^6$) from normal donors to $10^6$ PBL from L donors progressively reduced the very high PFC of the latter cells to normal levels. In the converse experiment, addition of either $10^3$ or $10^4$ blood T cells from L patients exerted little effect upon the PFC response by $10^6$ normal PBL. However, addition of $10^6$ lepromatous T cells greatly increased the responses by normal PBL.

These results demonstrate that there is B lymphocyte hyperactivity in patients with lepromatous leprosy; current data indicate that this may result from a deficiency of T suppressor cell regulation over helper T cell activity and/or B cell function.—[University of Cincinnati College of Medicine, Cincinnati, Ohio, U.S.A.; University of Chiang Mai, Chiang Mai, Thailand; National Hansen's Disease Center, Carville, Louisiana, U.S.A.; University of Illinois, Chicago, Illinois, U.S.A.]

**Mehra, V., Mason, L. and Bloom, B. R.** Evidence for *in situ* activation of suppressor T cells in lepromatous leprosy patients.

Although a number of mechanisms have been proposed to explain the failure of lepromatous leprosy patients to mount an appropriate cell-mediated immune response to the antigens of *M. leprae*, the precise mechanism underlying the immunological unresponsiveness is still unclear. One immunological mechanism that could explain the selective unresponsiveness of lepromatous leprosy patients to *M. leprae* antigens would be the presence of specific suppressor cells.

In an attempt to explore the possibility of existence of *M. leprae* induced suppressor cells in leprosy patients we developed an *in vitro* assay that measures the ability of Dharmendra lepromin to induce suppression of mitogenic response of lymphocytes to Con A. These studies indicate that lepromin induces the suppression of Con A response of mononuclear cells from lepromatous and borderline but not tuberculoid leprosy patients or normal donors. Two cell populations contributed to the lepromin induced suppressor activity, an adherent cell, possibly a monocyte, and a T cell.

In an attempt to characterize the suppressor T cells further, the T cells were separated into $TH_2+$ and $TH_2-$ subsets, identified by antiserum that selectively reacts with $TH_2+$ subset of human T cells, using

a fluorescence-activated cell sorter (FACS). When these $TH_2+$ and $TH_2-$ subsets were admixed with the mononuclear cells of normal individuals, lepromin induced the suppression of Con A response of normal mononuclear cells only in the presence of $TH_2+$ subset cells obtained from lepromatous and borderline leprosy patients, and not with $TH_2+$ subset cells of tuberculoid patients or normal donors. The $TH_2-$ subset, constituting approximately 70–80% of the T cells, failed to induce any suppression.

Since it is well known in mice and humans that mitogen and antigen activated T cells acquire both Fc receptors for IgG and Ia determinants, we have examined the $TH_2+$ and $TH_2-$ subsets for the presence of Ia-like determinants and Fc receptors for IgG, by using monoclonal anti-Ia and rosetting with IgG coated erythrocytes, respectively.

Lepromatous and borderline leprosy patients have elevated levels of $Ia^+$ T cells and most of these $Ia^+$ cells are found in the $TH_2+$ subset, whereas <5% of T cells from normal individuals exhibited Ia antigens, and they were distributed in both the $TH_2+$ and $TH_2-$ subsets. The T cells were also separated into $Ia^+$ and $Ia^-$ subsets, using FACS, and mixed with normal mononuclear cells to see if lepromin induced the suppression of mitogenic response of normal mononuclear cells to Con A in the presence of $Ia^+$ cells.

The results strongly suggest that the presence of Ia-like determinants on the $TH_2+$ subset may be an index of the degree of *in situ* activation of suppressor cells.—[Albert Einstein College of Medicine, Bronx, New York 10461, U.S.A.]

**Rea, T. H. and Yoshida, T.** Serum migration inhibitory activity in patients with leprosy.

Because leprosy and sarcoidosis have many similarities, and because the serum of patients with sarcoidosis has been found to have migration inhibition factor (MIF) activity, we sought evidence of migration inhibitory activity in the serum of patients with leprosy.

Inhibitory activity was assayed by comparing areas of migration of guinea pig peritoneal macrophages in media containing study or control serum. Results were computed as percent inhibition,

TABLE 6

| Group | Number | Inhibitory activity | | | | p-value compared with controls[a] |
|---|---|---|---|---|---|---|
| | | − | ± | + | ++ | |
| Controls | 25 | 21 | 2 | 1 | 1 | |
| All BT, BB, BL, LL | 54 | 13 | 14 | 14 | 13 | <.001 |
| BT, BB, BL, LL | | | | | | |
| No reaction | 41 | 13 | 13 | 8 | 7 | <.05 |
| In reaction | 13 | 0 | 1 | 6 | 6 | <.0001 |
| LL | | | | | | |
| No reaction | 12 | 3 | 5 | 2 | 2 | =.07 |
| In reaction | 9 | 0 | 1 | 4 | 4 | =.00002 |
| BT, BB, BL | | | | | | |
| No reaction | 29 | 10 | 8 | 6 | 5 | <.05 |
| In reaction | 4 | 0 | 0 | 2 | 2 | =.006 |
| PPD | | | | | | |
| 10 mm or more | 12 | 2 | 2 | 3 | 5 | <.001 |
| 4 mm or less | 11 | 2 | 3 | 3 | 3 | =.005 |
| DNCB | | | | | | |
| Positive | 12 | 3 | 0 | 3 | 6 | =.00008 |
| Negative | 18 | 3 | 7 | 3 | 5 | =.0092 |

[a] <, chi-square computed with Yates' correction; =, with Fisher's exact method.

100 − [(study area)/(control area) × 100], and are expressed as − 0–15%, ± 15–25%, + 25–35% and ++ 35–45%. For statistical analysis (chi-square) − and ± results were interpreted as negative and + and ++ as positive. Patients were classified according to Ridley's system as BT, BB, BL or LL. The reactional states encountered were Lucio's reaction and erythema nodosum leprosum (ENL) in LL and reversal reactions in BT, BB and BL patients.

The distribution of inhibitory activity among untreated patients and controls (dermatology clinic patients) is shown in Table 6.

Inhibitory activity was heat stable at 56°C, not dialyzable and upon Sephadex G-100 gel filtration the molecular weight of the active substance was between 10,000 and 30,000 Daltons, smaller than the active substance in sarcoidosis.

Inhibitory activity was significantly more common in the 54 untreated leprosy patients than in the 25 controls ($p < .001$). Among untreated patients positive (+ and ++) inhibitory activity was particularly common in those with reactional states, 12 of 13, in contrast to those without reactional states, 15 of 41, (p-values of $<.0001$ and $<.05$ respectively as compared with controls and a p-value of $<.001$ when comparing reactional with nonreactional patients). A firm association of positive inhibitory activity with reactional states was also present in treated patients, 6 of 7 with active reversal reactions ($p = .0002$) and 5 of 10 with active ENL ($p = .01$ compared with controls). In untreated patients the distribution of inhibitory activity was similar among tuberculin (PPD) reactors and nonreactors. Positive inhibitory activity was more common in dinitrochlorobenzene (DNCB) responders, 9 of 12, than in nonresponders, 8 of 18, a difference consistent with the previously reported high prevalence of DNCB responders among patients with reactional states.

In summary, migration inhibitory activity was found in the serum of patients with leprosy. The relationship, if any, of the substance producing this inhibitory activity to the lymphokine, MIF, is not known. The inhibitory activity in leprosy sera could not be correlated with either tuberculin or DNCB unresponsiveness. However, the inhibitory activity was strongly associated with reactional states of diverse types, but the nature of this association is not known.—[Section of Dermatology, LAC-USC Medical Center, 1200 N. State St., Los Angeles, California 90033, U.S.A.; Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032, U.S.A.]

## CLOSING REMARKS

Ladies and Gentlemen,

It is my great pleasure to give the closing remarks at the end of this meeting. On behalf of the Japanese participants, I extend our hearty thanks to Dr. Hastings, the Chairman of the U.S. Leprosy Panel, and to all the U.S. participants for organizing this meeting very well and for entertaining us very kindly. We were able to learn many new facts in every session of this conference and we were able to enjoy pleasant social evenings. We are very happy to meet again with Dr. Binford and Dr. Hanks, who have been leading and encouraging us since the first Joint Conference in Tokyo.

I would like to give my personal impressions of this Joint Conference. I was particularly interested and impressed by Drs. Gillis and Buchanan's presentation that they utilized *M. leprae* specific reference serum in my immunofluorescence test for their antigenic analyses by immunodiffusion and ELISA techniques, and that they have successfully demonstrated monoclonal antibodies to *M. leprae*. They have generously offered to supply this antibody to me so that I can utilize it as a new reference standard in the FLA-ABS test, as well as in another antibody assay under investigation. It is fresh in our memory that the successful transmission of *M. leprae* into armadillos and nude mice has brought about great advances toward the goals of our program, and it is still accelerating other studies on the prophylaxis and therapy of leprosy. These are only two examples of the numerous results obtained by the prompt interchange of scientific information made possible by these Joint Conferences over the last 16 years.

Someone may wonder if this period is too long for one project. But we know that leprosy is an ultra-chronic disease and that it takes a very long time to finish only one experiment. Leprosy research is often compared to a Marathon race. Each runner must keep his own pace so that he does not drop out of the race. I am still running at my pace, although sometimes it seems like jogging. Since our final goal is very far in the distance and very broad, we do not need to compete with each other on the same course; rather we must cooperate with each other, in order to reach the final goal as soon as possible, aiming at the eradication of leprosy from the world.

I must state another benefit of the program on this occasion. It is the visiting fellowships sponsored by the program, although they are limited to Japanese scientists. We were able to send many young scientists to institutions in the U.S.A. They were greatly encouraged by their visit, and they are now most active as the investigators of the Japanese Leprosy Panel. Visiting fellowships to Southeast Asian countries have been very appreciated by the doctors working at the National Leprosaria in understanding the present status of leprosy in these countries. Owing to these visiting fellowships, we have been able to promote a friendly understanding between our two nations.

Finally, I hope that our activities as stated above are justly evaluated by the committee and that further continuation of the Leprosy Panel will be approved after the evaluation. I wish to hold the 17th Joint Conference next year and to welcome you all in Japan. Thank you.

—Masahide Abe, *Chairman*
*Japanese Leprosy Panel*

# INTERNATIONAL LEPROSY ASSOCIATION

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