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ABSTRACTS

SEVENTEENTH JOINT LEPROSY RESEARCH CONFERENCE

Saito Hohon Kaikan
Honcho 2-Chome, Sendai-shi, Japan
25–27 July 1982

U.S.–Japan Cooperative Medical Science Program

OPENING REMARKS

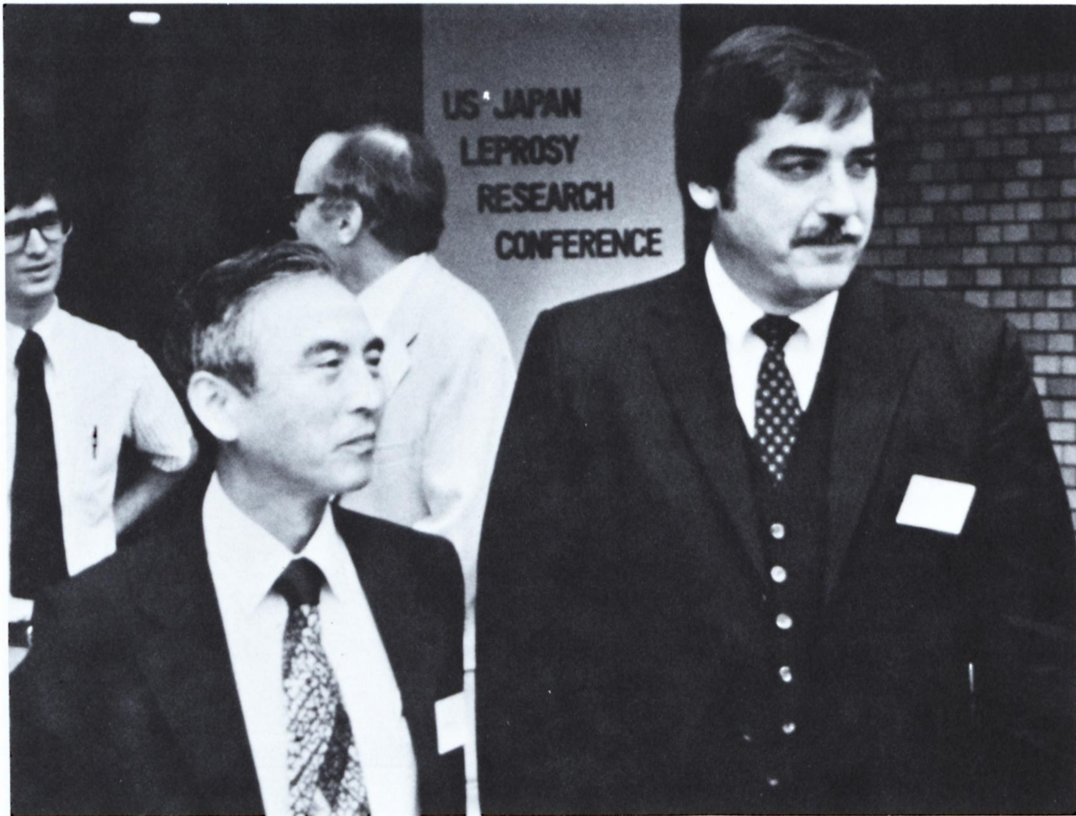
On behalf of the Japanese Leprosy Panel, U.S.–Japan Cooperative Medical Science Program, I have the honor and the pleasure of giving the opening address at this, the Seventeenth Joint Conference on Leprosy Research.

First of all, I am happy to bring you good news. Last year, this panel was reviewed by the Joint Committee reviewers, and they recommended further continuation of the leprosy panel. This recommendation was accepted at the Eighteenth Joint Committee meeting held in Tokyo last month. The tuberculosis panel was also reviewed at the same time. According to the report of this meeting, the committee recommended that the leprosy panel and the tuberculosis panel have a joint meeting of at least one day to discuss a common research problem, for example, the immunology of mycobacterioses, which is very actively being investigated by the two panels. We have experienced such joint meetings several times in the past, and it is certainly worthy of consideration in the next year. I have been told personally by members of the committee that such a joint meeting does not mean a fusion of the two panels in the near future.

May I express my hearty gratitude to all the participants. Thirty beautiful papers were submitted for this conference, and delivered to you at registration. I also thank all the guests who have accepted our invitation in spite of their busy schedules and a trip of long distance. You are proof that leprosy is still a disease attracting many investigators. In addition, I hope this conference will be successful, encouraging more scientists to join our panel for the study of leprosy. This is one of the important reasons why the joint conference is held in different places each year.

I must mention why I chose Sendai for our meeting place this year. Sendai is the biggest city in Tohoku, in the northeastern part of the Japanese mainland, with a population of nearly 642,000. Since 1957, Sendai has had a "sister city" relationship with Riverside, California. Although new leprosy cases are scarcely found now in this area of Japan, a national leprosarium, Tohoku Shinsei-en, is located only 60 km from here. The director of this leprosarium is Dr. Tokuzo Yokota—a son of Dr. Kensuke Mitsuda, a famous pioneer leprologist. There was also a leprosy research laboratory at Tohoku University, but this laboratory has been changed to the study of other fields of medicine.

In ancient times, this area was a native, undeveloped land very far from the capital. However, many visitors and workers came from the West and had a good friendship with the local people, as written in an old poem in the Mannyoshu. We can also think of the past through many poems written by the travellers. One of them was Basho Matsuo who



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

Boat sight-seeing tour to Matsushima.

Participants of the Seventeenth Joint Leprosy Research Conference.

wrote a book entitled "The Narrow Road to a Far Province." We can find traces of him at several places in this area. The ancient narrow road has now been changed to a broad road, as represented by the opening of Tohoku Shinkansen. All of these things may attract us to this beautiful city, Sendai.

Accordingly, I hope you will be greatly encouraged, not only by this conference, but also by every experience during your stay in Sendai.

Thank you.

—Masahida Abe, *Chairman*
Japanese Leprosy Panel

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ABSTRACTS OF LEPROSY CONFERENCE

Hirata, Tsunehiko. Cytology of leprosy bacilli.

The purpose of these studies is to describe the fine structure of the micro-cytological association of nuclear apparatus and membranous structure and the intracytoplasmic inclusions of leprosy bacilli.

Morphological studies of leprosy bacilli undertaken with the electron microscope make it clear that the nuclear apparatus appears in ultrathin sections as an area of variable shape and size, filled with delicate fibers not delimited from the cytoplasm by a membrane. The apparatus is often in contact with an intracytoplasmic membranous structure, a so-called mesosome.

The intracytoplasmic inclusions of leprosy bacilli are mostly homogeneous and spherical, and they do not uniformly exist in the bacillary cells. They do not appear to be delimited by a membranous structure and apparently have no internal structure.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Koseki, Y., Watanuki, M., Yamazaki, T., Sanada, K. and Ogawa, T. The egg-yolk reaction produced by human and murine leprosy lesions and its cultural significance on primary isolation.

Fresh human and murine leprosy lesions inoculated onto Ogawa egg-yolk (EY) medium without decontamination treatment produced various reactions, especially when the pH of the medium was adjusted to below 6.0. The EY reaction was studied using a newly devised EY agar slant. The EY reaction was composed of three stages: first, formation and diffusion of a zone of grayish-white precipitate around the inoculum; second, formation of a sharply demarcated translucent zone; and finally, various transient phenomena within the second zone and close to the inoculum. The reaction took place most efficiently at pH 5.5 or less with both human and murine lepromas. In the pH zone of 5.6 to 6.3 the rate and intensity of the reaction varied inversely with the pH of the medium, with no reaction at pH 6.4 or greater. The EY reaction was also given by a cell-free extract of human leproma and even by normal tissues (liver and spleen) of mice, although the reaction produced by the latter was much slower and weaker than that obtained by murine leprosy lesions.

Thin layer chromatography of lipids extracted from the reaction-producing and control media showed that at least three enzymatic activities were involved in the reaction: 1) phospholipase A, 2) lipase, and 3) acyl transferase. Dapsone (DDS) and rifampin had little effect on the reaction produced by human leproma. Among seven protease inhibitors tested, only pepstatin had a marked inhibitory effect on the reaction produced by murine leprosy lesions. It inhibited not only the first EY reaction, but also completely inhibited the second reaction. A very limited reaction resembling the third one was produced. Extracts of the liver from *Mycobacterium lepraemurium* (*Mlm*)-infected mice invariably showed higher activities of five acid hydrolases, acid phosphatase, arylsulphatase B, β-glucuronidase, acid lipase, and cathepsin D, than did liver extracts from control animals. Although treatment of murine leprosy lesions with NaOH at final concentrations of 0.5% (w/v) or less had little effect on either the reaction or the subsequent growth of the bacilli, treatment with final concentrations of 1% or 2% completely inhibited the EY reaction and had a remarkably beneficial effect on the growth of *Mlm*.

The conclusions are as follows: the EY reactions produced by human and murine leprosy lesions are due mainly to elevated activities of lysosomal enzymes. EY reactions are detrimental to the growth of *Mlm* on primary isolation. It is suggested, therefore, that procedures for the pretreatment of human lepromas be aimed at avoiding damage from lysosomal enzyme activities insofar as possible.

Regarding the inhibition of the EY reaction by pepstatin, it should be noted that there are similarities between the chemical structures of pepstatin and mycoside C_2. Mycosides of the C_2 type are present in the capsule of *Mlm in vivo*, and possibly could

act as inhibitors of cathepsin D.—[National Institute of Health, Tokyo 141, Japan; National Leprosarium Tama-Zensho-en, Tokyo 189, Japan; The Kitasato Institute, Tokyo 108, Japan]

Nakamura, Mashiro. Stimulation of the growth of *Mycobacterium lepraemurium* in cell-free liquid medium by aspartic acid, sucrose, liposome, thymidine, and deoxyuridine (NDLASU medium).

ND-5 medium definitely permits the primary growth of *Mycobacterium lepraemurium* (*Mlm*) from *in vivo* sources, but it is unsatisfactory for prolonged growth. This is indicated by the difficulty of subcultivation of *Mlm* in this medium. Hanks, Dhople and Funk achieved continuous growth of *Mlm* by substitution of stable ingredients for unstable ones contained in the original Nakamura system. In addition, they added lecithin, cholesterol, and nucleotides to the medium, thus improving the medium to make it more satisfactory for *in vitro* cultivation.

The present data indicate that a remarkable growth stimulation of *Mlm* was observed by simply adding DL-aspartic acid to the medium. Hence, it was necessary to modify the original ND-5 medium. Along the course of these experiments, the culture medium has been improved by additions of aspartic acid, sucrose, liposomes, thymidine, and deoxyuridine, respectively. Finally, a cell-free liquid culture medium having the following composition was established and referred to as NDLASU medium. When a suspension of *Mlm* containing approximately 10^6 organisms is inoculated into NDLASU medium and cultivated at 30°C, a quantitative multiplication of *Mlm* takes place for up to four weeks of incubation. Later clusters of *Mlm* form. This is a typical growth pattern of mycobacteria and is similar to those seen with *M. tuberculosis* and other cultivable mycobacteria.

NDLASU

Base: Dubos powder 0.5 g, adenosine 4.0 mg, thymidine 8.0 mg, thioglycollate 1.0 mg, succinamide 42.0 mg, dextran 82.0 mg, DL-aspartic acid 10.0 mg, sucrose 430.0 mg, liposome-4 (egg lecithin 80 mg + lecithin 40 mg in 2 ml $CHCl_3$, evaporated, resuspended in 10 ml water with ultrasonication) 0.6 ml, water 38.0 ml, pH 6.2. The final solution is autoclaved.

Supplements: Deoxyuridine (5 mg/ml water) 1.0 ml, vitamin K_3 (1 μg/ml) 0.5 ml, PABA (1 mg/ml) 0.5 ml, calf serum 5.0 ml. All of these are aseptically added.—[Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan]

Matsuo, Y. Use of cycloheximide for cultivating *Mycobacterium leprae* and *Mycobacterium lepraemurium* in cell culture.

In order to maintain the cells infected with *Mycobacterium leprae* and *M. lepraemurium* in good condition as long as possible, cycloheximide, a specifically suppressive antibiotic for the protein biosynthesis of eukaryotic cells, was incorporated into the culture medium. The antibiotic at a concentration of 0.1 μg per ml in the medium delayed the division of A31 cells significantly, but did not kill them. The infected cells seldom floated off the substratum, and could be held for ten weeks or more without changing the medium frequently. The results obtained with *M. lepraemurium* are satisfactory for the present. Successful subcultures of this microbe have been continued so far up to the tertiary culture, and the fourth one is under way. An overall generation time of the bacilli was estimated at 22.1 days for a period covering 35 weeks. The intracellular bacilli often appeared in bundles arranged very close to each other. The advantages of the cycloheximide treatment are that the technique is very simple, frequent changes of the medium are not needed, and the results obtained are highly reproducible. However, the growth of *M. leprae* has not yet been demonstrated in this cell culture system.—[Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734, Japan]

Nomaguchi, H., Kohsaka, K. and Mori, T. The relationship between pathogenicity in mice and the ability of *M. lepraemurium* to grow in tissue culture cells.

Murine leprosy bacilli of the Hawaii strain (*Mycobacterium lepraemurium-in vivo*) and

cultured murine leprosy bacilli from rough colonies of the Hawaii strain grown on Ogawa egg yolk medium (*M. lepraemurium*-Ogawa) both produced lepromas in CBA mice (pathogenic bacilli). However, the bacilli from smooth colonies on Ogawa egg yolk medium (*M. lepraemurium*-Ogawa) did not produce lepromas in CBA mice (nonpathogenic bacilli). The adherence of these bacilli to the host cells and their ability to grow in tissue culture cells were examined.

M. lepraemurium-in vivo and *M. lepraemurium*-Ogawa were inoculated into A31 monolayer cells. At intervals, the infected cells were washed with PBS, fixed and stained, and the number of bacilli that adhered to the cells was scored. The adherent ratio of *M. lepraemurium-in vivo* was higher than *M. lepraemurium*-Ogawa. The rate of adherence of *M. lepraemurium*-Ogawa 5th on Ogawa egg yolk medium (5th cultural passage of Hawaii strain on Ogawa egg yolk medium) was higher than *M. lepraemurium*-Ogawa 10Y (more than ten-year-old cultural passage of Hawaii strain on Ogawa egg yolk medium). Thus, the adherence rate of *M. lepraemurium* to A31 cells decreased with time in culture on Ogawa egg yolk medium.

The relative growth ability of *M. lepraemurium-in vivo* and *M. lepraemurium*-Ogawa in tissue culture cells was examined. *M. lepraemurium-in vivo*, *M. lepraemurium*-Ogawa 2nd (from rough colonies in the 2nd cultural passage on Ogawa medium), and *M. lepraemurium*-Ogawa 5th (from rough colonies in the 5th cultural passage on Ogawa medium) multiplied well in tissue culture cells, but *M. lepraemurium*-Ogawa 10Y did not grow well in tissue culture cells.

On the other hand, after prolonged incubation in tissue culture cells, even *M. lepraemurium*-Ogawa 10Y became adapted to the culture cells. These adapted bacilli proliferated well in culture cells (*M. lepraemurium*-Ogawa 10Y-A31).

M. lepraemurium-Ogawa 10Y were inoculated into nude mice. Five months later, the bacilli were transferred onto Ogawa egg yolk medium although no increase in the number of bacilli had been observed in the nude mice. Three months later, rough and smooth colonies were isolated on Ogawa egg yolk medium.

M. lepraemurium-in vivo and *M. lepraemurium*-Ogawa 5th produced lepromas in CBA mice, but *M. lepraemurium*-Ogawa 10Y did not. In nude mice, *M. lepraemurium*-Ogawa 10Y did not produce lepromas at seven months after inoculation. *M. lepraemurium*-Ogawa 10Y-A31 were inoculated into nude mice and CBA mice. After 51 days, the bacilli did not increase in number in these mice. Bacterial elongation was observed in the nude mice, but not in the CBA mice.

The ability of *M. lepraemurium*-Ogawa or *M. leprae* to interfere with the adherence rate and the growth ability of *M. lepraemurium-in vivo* was examined by co-cultivation methods. No interference was observed. The problem of restoring the pathogenicity of these attenuated bacilli is discussed.—[Research Institute for Microbial Diseases, Osaka University, Yamadakami, Suita, Osaka, Japan]

Hastings, R. C. and Morales, M. J. Observations, calculations, and speculations on the growth and death of *M. leprae in vivo*.

Recently we have followed, in some detail, the pattern of growth of *Mycobacterium leprae* in athymic, nude mice. In inspecting the data and calculating conventional generation times for *M. leprae*, an inconsistency became apparent as follows:

In our hands, the apparent generation time for *M. leprae* in nude mice at the interval between day 90 and day 180 after inoculation is approximately 12 days. If at a given time in the logarithmic phase of growth there were ten *M. leprae* and one stained solidly [Morphologic Index (MI) = 10%], then, after a doubling time or generation time for the total number of acid-fast bacilli, there would be a total of 20 *M. leprae*. If the MI remained constant at 10%, two of these 20 bacilli would stain solidly.

Conventionally, only the solidly staining acid-fast bacilli are capable of multiplying. If a doubling of the total number of acid-fast bacilli occurs and the MI remains at 10%, then the two solidly staining bacilli at the end of the doubling time came from the one solid bacillus originally present. Conventionally, the non-solidly staining bacilli

do not multiply, but are slowly cleared from the body. Therefore at the end of the doubling time, the nine non-solidly staining acid-fast bacilli present originally could have remained and could therefore still be present. At the end of one doubling time for the total number of acid-fast bacilli (12 days), therefore, one can account for 2 solidly staining bacilli and 9 non-solidly staining bacilli, for a total of 11 acid-fast bacilli. There are, by definition, a doubling of the original ten total acid-fast bacilli, and therefore a total of 20 total bacilli are present at the end of the doubling time. The question then arises as to where do the extra nine non-solid bacilli come from. There would appear to be at least three possibilities: a) Non-solid bacilli can replicate. b) Non-detectable precursors can develop into non-solidly staining acid-fast bacilli. c) The generation time of solidly staining acid-fast bacilli, considered alone, is much more rapid than the overall estimate based on the doubling of the total number of acid-fast bacilli. A corollary of the third possibility is that the spontaneous conversion of solid to non-solid (presumed viable to presumed non-viable) bacilli is relatively rapid.

Intuitively, it seems logical that in this example, in order for 1 viable acid-fast bacillus to generate 2 viable acid-fast bacilli plus 9 non-viable acid-fast bacilli at the end of one generation time of approximately 12 days (a net increase of 10 acid-fast bacilli), it would have to undergo division 10 times, and 9 of the daughter cells would have to die. Thus intuitively it would seem that viable *M. leprae* may be able to divide at a rate of about once a day, and that the average viable *M. leprae* may live for only a little longer than a day.

We then derived more precise estimations, in brief, as follows:

The total number of acid-fast bacilli for each nude mouse at various time intervals after inoculation with *M. leprae* was experimentally measured. The proportion of solidly staining acid-fast bacilli in each of the animals was similarly experimentally measured. The product of the two gave the total number of solidly staining acid-fast bacilli per animal at each time interval.

Assuming a single compartment model, and ignoring the rate of elimination of non-viable bacilli, the overall growth of *M. leprae* in nude mice could be expressed as a simple first order generation of bacilli based on the total number of acid-fast bacilli:

$$\frac{dA}{dt} = k_a * A_t \quad \text{or} \quad A_t = A_0 * e^{k_a * t}$$

where the rate of change of the total number of bacilli, dA/dt, is a function of the apparent generation constant (k_a) and the total number of bacilli present at any given time (A_t). A_0 refers to the original number of bacilli present and e is the base of the system of natural logarithms. The above relationships can be simplified to:

$$k_a = \frac{\ln(A_t/A_0)}{t}.$$

Knowing A_t, A_0, and t, various k_a values can be calculated for each of the time intervals for each of the groups of nude mice which had received different inocula of *M. leprae*. The steepest part of the growth curve was between 90 days and 180 days after inoculation, and this was taken to be the logarithmic phase of growth of the bacilli. The mean k_a for the total number of bacilli in the interval between day 90 and day 180 after inoculation with *M. leprae* in these animals was 0.0567 day^{-1}. This yielded a mean doubling time of 12.5 days for the total number of acid-fast bacilli in these nude animals at this time interval.

We then postulated a two compartment model for the growth of *M. leprae* in nude mice. We assumed that only solidly staining bacilli (L_t) were viable and proliferating. We assumed that these viable bacilli were being generated with a generation constant (k_{in}), were being killed or spontaneously dying with a killing constant (k_k), and were transformed into non-solidly staining bacilli (D_t) which were presumed incapable of replication and which were eliminated from the body or at least from detectability with an elimination constant (k_e). We assumed that all three processes (generation, killing, and elimination) followed first order kinetics. Based on the above assumptions, the total number of acid-fast bacilli (A_t) is the sum of those which are alive or stain solidly (L_t) and those which are dead or stain non-solidly (D_t) or:

$$A_t = L_t + D_t$$

If m is taken to be that propor-

tion of the bacilli which stain solidly, $\frac{\text{(Morphologic Index)}}{100}$, then:

$$L_t = A_t * m$$

We assumed that the actual rate of change in the total number of acid-fast bacilli could be described by the algebraic sum of the rates of change in the numbers of live bacilli (L_t) and the rates of change in the numbers of dead bacilli (D_t). The rates of change of live bacilli (dL/dt) can be described by the difference between their generation ($k_{in} * L_t$) and their killing ($k_k * L_t$). The rate of change of dead bacilli (dD/dt) can be described as the difference between the production of dead bacilli from live bacilli ($k_k * L_t$) and the rate of elimination of dead bacilli ($k_e * D_t$). Thus:

$$\frac{dA}{dt} = k_a * A_t \quad \text{and}$$

$$\frac{dA}{dt} = (k_{in} * L_t) - (k_k * L_t) + (k_k * L_t) - (k_e * D_t)$$

Equating these two expressions for dA/dt and cancelling:

$$k_a * A_t = (k_{in} * L_t) - (k_e * D_t)$$

Substituting for L_t and D_t in terms of A_t, cancelling, and rearranging, the following relationship can be shown:

$$k_{in} = \frac{k_a + k_e - (k_e * m)}{m}$$

Thus the generation constants for viable bacilli (k_{in}) can be determined from the calculated apparent generation constants for the total number of bacilli (k_a), the proportion of solidly staining acid-fast bacilli which were observed (m), and the elimination constant for non-viable bacilli (k_e). It was therefore necessary to estimate k_e. No data are available from nude mice for this purpose. To arrive at an estimate of k_e, we made three assumptions. Firstly, we made the assumption that the rate of elimination of dead bacilli from nude mice should be approximately equivalent to the rate of elimination of dead bacilli from humans lacking cell-mediated immunity to *M. leprae*. Secondly, we assumed that effective chemotherapy essentially converts all *M. leprae* into dead bacilli. Finally, we assumed that the rate of fall in the Bacteriologic Index (BI) as measured by skin scrapings for acid-fast bacilli in humans was related to the rate of fall in the total body load of *M. leprae*. We therefore analyzed the rate of change of the BI in two groups of polar lepromatous leprosy patients receiving effective chemotherapy with dapsone (DDS) and clofazimine, respectively. These calculations yielded a mean k_e value of 0.00278 day^{-1}. Using this value, we then calculated generation constants (k_{in}) based only on solidly staining bacilli and the assumptions and equations mentioned above. The mean k_{in} for solidly staining bacilli in nude mice in the interval from day 90 to day 180 after inoculation is 0.6874 day^{-1}, yielding a mean doubling time of about 1.00 day (1.097 days).

We next needed to estimate k_k to completely describe the two compartment model. This was done as follows:

By the same reasoning as that used in the single compartment model of the total number of bacilli, we calculated an apparent generation constant (k_b) for viable bacilli based on the numbers of solidly staining bacilli (L_t) calculated for each animal at each time interval. This single compartment model ignores the rate of killing of live bacilli.

$$\frac{dL}{dt} = k_b * L_t \quad \text{or} \quad L_t = L_0 * e^{k_b * t}$$

This apparent generation constant (k_b) for solidly staining bacilli is 0.0548 day^{-1}, yielding an apparent mean doubling time of 12.9 days for live *M. leprae* for the interval between day 90 and day 180 after inoculation.

The rate of change in the number of live acid-fast bacilli using a two compartment model can be expressed as follows:

$$\frac{dL}{dt} = (k_{in} * L_t) - (k_k * L_t)$$

Equating this expression with that based on the apparent generation constant (k_b):

$$\frac{dL}{dt} = k_b * L_t = (k_{in} * L_t) - (k_k * L_t)$$

This can be rearranged and simplified to solve for k_k:

$$k_k = k_{in} - k_b$$

Using the above equation, values for the killing constants for solidly staining bacilli (k_k) were calculated. In the interval between day 90 and day 180 after inoculation the mean k_k value is 0.6326 day^{-1}, yielding a mean half-life of 1.20 days for a viable *M. leprae* organism.

In summary, based on the above assumptions and calculations, and assuming the logarithmic phase of growth of *M. leprae* in the nude mouse model is from day 90 to day 180, the generation constant for viable *M. leprae* is approximately 0.6874 day^{-1} which yields a generation time for viable *M. leprae* of 1.097 days or 26.3 hours. This is in keeping with slow growing mycobacteria in general, the generation time of *M. tuberculosis* being approximately 18 hours. The killing constant for *M. leprae* is 0.6326 day^{-1}, yielding a half-life of solidly staining or viable bacilli of 1.20 days or 28.9 hours. The elimination constant for dead *M. leprae* is estimated to be 0.00278 day^{-1}, yielding a half-life of dead organisms being cleared from the body (or from detectability) of 249 days.

Based on these relationships one can calculate a MI which represents an equilibrium insofar as the total body load of *M. leprae* is concerned. This value is 0.403%.

Incubation times for primary polar lepromatous leprosy can be calculated based on the mean BI and the mean MI of newly admitted polar lepromatous patients at Carville. These values are in reasonable agreement with the conventional incubation times for clinical leprosy of 3–5 years.

Estimations can be made of the probable size of the initial effective inocula of *M. leprae* by setting time to be the conventional incubation periods of 3–5 years. The initial effective total inocula are on the order of 10^4–10^5 total bacilli.

Calculations can be made as to the probable number of drug resistant mutants of *M. leprae* originally present in a polar lepromatous patient who relapses on monotherapy with various single drugs. These are on the order of 1 in 10^5 organisms which are initially resistant to streptomycin, 1 in 10^7 initially resistant to rifampin, and 1 in 10^9 initially resistant to ethionamide. The calculations suggest that secondary sulfone resistance usually develops during the course of chemotherapy and is not the result of selection of a pre-existing resistant mutant.

These calculations can also be used to predict the effects of different MIs on the BI over time in a polar lepromatous leprosy patient.—[National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

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Yoneda, K., Kohsaka, K., Miyata, Y., Mori, T. and Ito, T. The study of leprosy chemotherapy in nude mice. Effect of dapsone (DDS) on nude mice experimentally infected with *Mycobacterium leprae* (3).

We have studied the effect of dapsone (DDS) on *Mycobacterium leprae* in nude and normal mice. Normal mice were inoculated with a small number of bacilli (5.0×10^3) per foot pad and nude mice were infected with both a small number (5.0×10^3) and a large number (8.0×10^6) of *M. leprae*. Two strains of *M. leprae* were used (Izumi and Naha-10.30) both of which were originally derived from untreated leprosy patients and subsequently maintained by passage in nude mice in our laboratory.

M. leprae (5.0×10^3) were inoculated into the right hind foot pads of nude mice, and the mice were divided into two groups. One group was the untreated control and the other group was given 0.01% (w/w) DDS in the diet from the day following the inoculation until the termination of the experiment.

When nude mice were inoculated with 5.0×10^3 *M. leprae* harvested from the Izumi strain in second passage, six-month harvests in the untreated controls yielded 1.6×10^6 bacilli per foot pad and 6.0×10^5 bacilli could be detected in nude mice that had been treated with 0.01% DDS. When nude mice were inoculated with 5.0×10^3 *M. leprae* from the Izumi strain in third passage, nine-month harvests in the untreated controls yielded 2.1×10^6 bacilli per foot pad. At the nine-month harvest, 8.8×10^4 bacilli could be detected in the group

treated with 0.01% DDS. When mice were inoculated with 5.0 × 10^3 *M. leprae* from the Naha-10.30 strain in sixth passage, nine-month harvests in the untreated controls yielded 1.3 × 10^5 bacilli per foot pad and at nine months 1.4 × 10^5 bacilli could be detected in the group treated with 0.01% DDS.

M. leprae were inoculated into the foot pads of normal mice using inocula of 5.0 × 10^3 from the Izumi and the Naha-10.30 strains, respectively. For each strain the mice were divided into two groups. One group was the untreated control and the other was given 0.01% DDS in the diet beginning the day following the inoculation. In both the control groups, the bacillary populations reached above 10^6, but in the groups treated with 0.01% DDS no AFB were found during the counting procedure at the six-month harvests.

When nude mice were inoculated with 8.0 × 10^6 *M. leprae* from the Izumi strain maintained in third mouse passage, nine-month harvests in the untreated control group yielded 7.2 × 10^8 bacilli per foot pad and 4.9 × 10^6 bacilli could be detected in the nude mice that had been treated with 0.01% DDS. When nude mice were inoculated with 8.0 × 10^6 *M. leprae* from the Naha-10.30 strain maintained in sixth mouse passage, nine-month harvests in the untreated control group yielded 2.4 × 10^9 bacilli per foot pad. At nine months, 4.0 × 10^6 bacilli could be detected in the nude mice that had been treated with 0.01% DDS. Considering the size of the inoculum per foot pad and the proportion of the inoculum retained at the local site ("fix ratio"), the number of bacilli did not change in these nude mice during the administration of 0.01% DDS. Further studies are necessary to completely account for these phenomena.—[National Leprosarium Ohshima Seisho-en, Ajicho, Kida-gun, Kagawa, Japan; Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan]

Kohsaka, K., Yoneda, K., Ito, T. and Tanabe, S. Inoculation of *Mycobacterium leprae* into the "rhino mouse."

In 1940, Howard reported a mouse mutant, "rhino" (hr^{rh}), characterized by a marked wrinkling and thickening of the skin without hair. Rhino mice have a number of abnormal characteristics. The skin abnormalities appear with aging. Changes in the thymus, lymph nodes, and spleen occur with the skin abnormality. The thymus degenerates at an early stage and later disappears. At the same time, enlargement of lymph nodes and splenomegaly occur with depletion of lymphocytes. In the other organs, collagen disease-like, morphological changes with functional abnormalities of antibody formation have been shown. The life space of the rhino mouse is reportedly about 500 days in specific pathogen-free (SPF) and 200 days in conventional environments, but the rhino mice used for our experiments could survive for 13 to 15 months under conventional conditions.

In 1976, the successful transmission of *Mycobacterium leprae* to nude mice was reported by us and by Colston and Hilson. We are attempting to find another model of experimental lepromatous leprosy with a laboratory animal other than the nude mouse. Preliminary results with rhino mice inoculated with *M. leprae* are presented.

Five 8-month-old rhino mice (hr^{rh}) of genetic background A/H, bred in our laboratory by Tanabe, were used. They were housed under conventional conditions. A suspension of *M. leprae* was prepared from a nude mouse foot pad infected with *M. leprae*, and 2.0 × 10^7 bacilli in volumes of 0.05 ml were inoculated into each of the right hind foot pads of the mice.

All of the five infected rhino mice were dead or were killed from 130 to 235 days after inoculation. The mice were examined bacteriologically and histopathologically; 3.4 × 10^7, 6.8 × 10^7, 6.9 × 10^7, 8.6 × 10^7, and 9.5 × 10^7 *M. leprae* were recovered from the infected foot pads of the five mice, respectively, and numerous acid-fast bacilli were seen in the histopathological specimens. The numbers of bacilli harvested were only slightly higher than the inoculum and were much lower than the 10^9 bacilli which would have been expected in nude mice. Nevertheless, the results suggest that *M. leprae* proliferated in the foot pads of these rhino mice because the "fix ratio" of *M. leprae* at the site of inoculation is approximately 30% of the inoculum.

It seems that the rhino mouse, as well as the congenitally athymic nude mouse, will be useful for leprosy research. If the rhino mouse develops lepromatous lesions, it will be an interesting animal for the transmission of *M. leprae* because it has a thymus in its earlier stages of life.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; National Leprosarium Ohshima Seisho-en, Ajicho, Kida-gun, Kagawa, Japan; Department of Bacteriology, Osaka University Medical School, Osaka, Japan]

Meyers, W. M., Walsh, G. P., Binford, C. H., Brown, H. L., Wolf, R. H., Gormus, B. J., Martin, L. N. and Gerone, P. J. Multibacillary leprosy in unaltered hosts, with emphasis on armadillos and monkeys.

Despite major advances in recent years, there remains an urgent need for an ideal experimental animal model for leprosy. The ideal model would be an immunologically unaltered animal that manifests the entire spectrum of the disease, reactional episodes, and peripheral neuritis with deformity. Most studies on the chemotherapy, pathogenesis, and epidemiology of leprosy have focused on patients with multibacillary leprosy. Multibacillary leprosy has now been observed in the following animals in which the immune system has not been artificially altered: armadillos, nude mice and rats, chimpanzee, and monkeys.

In the nine-banded armadillo (*Dasypus novemcinctus*), lepromatous leprosy develops in up to approximately 90% of animals inoculated intravenously with 10^8 leprosy bacilli. In the clinical and histopathologic evaluations of approximately 500 infected nine-banded armadillos, we have observed only lepromatous leprosy without spontaneous regression of disease. Intravenously inoculated animals develop the disease earlier, and the disease is more rapidly fatal than in armadillos inoculated intracutaneously; they demonstrate heavy infections of the liver, spleen, and lymph nodes. Apart from low body temperature, susceptibility factors have not been identified. Chemotherapy, immunotherapy, and vaccination studies on leprosy using this model are urgently needed.

Seven-banded armadillos (*D. hybridus*) and eight-banded armadillos (*D. sabanicola*) are susceptible to lepromatous leprosy but have been studied only in a preliminary manner. The eight-banded armadillo may also manifest hyperergic forms of leprosy.

Disseminated leprosy has been established in the nude mouse in several laboratories, and there are preliminary studies suggesting that similar but less pronounced infections may develop in nude rats. Although artificially unaltered, these congenitally athymic animals have an established immunologic defect, limiting their applicability to leprosy in humans.

Disseminated leprosy has been seen in a single chimpanzee and thus far attempts to transmit leprosy to other chimpanzees has not been successful. Although further studies are of interest in this species, there is no evidence that this animal is highly susceptible.

We have now observed disseminated leprosy in two species of monkey—the sooty mangabey (*Cercocebus atys*) and rhesus (*Macaca mulata*). The original female mangabey monkey was infected naturally. Histopathologically, the disease was near to the lepromatous form, and responded initially to rifampin and diacetyl diaminodiphenyl sulfone (DADDS) therapy; however, viable organisms have recently been recovered from nasal secretions of this animal. The etiologic agent cannot be differentiated from the leprosy bacillus by standard criteria. The organism is sensitive in the mouse foot pad at the 0.01% dapsone (DDS) dietary level but not at the 0.001% and 0.0001% level. Paralytic deformities of the hands and feet developed approximately 13 months after diagnosis. Two male animals of the same species inoculated with the etiologic agent from the index animal, and two other males of the same species inoculated with known human leprosy bacilli, have developed borderline-lepromatous to lepromatous leprosy. All animals were inoculated with 10^9 organisms intravenously and 10^7 organisms at five intracutaneous sites. Initial clinical lesions appeared approximately six months after inoculation, and the disease has progressed slowly over the ensuing 16 to 24 months.

Two 8-month-old rhesus monkeys, one male and one female, were inoculated with

organisms from the original naturally infected mangabey monkey. At 15 months post-inoculation, the male had infiltrated lesions of the skin of the ears and face at inoculated sites, and of the skin of the scrotum, arms, legs and tail at uninoculated sites. Histopathologically, the lesions resembled leprosy in the lepromatous area with invasion of cutaneous nerves. Suspensions of acid-fast bacilli separated from tissue oxodized DOPA and their acid-fastness was extractable with pyridine. The organisms were noncultivable on routine mycobacteriologic media. Additional studies on the identification of the organisms are in progress. At 20 months post-inoculation, there is marked progression of disease. The female animal does not demonstrate clinical lesions at 20 months post-inoculation.

A variety of models are now available for experimentation on multibacillary leprosy. The armadillo should be more fully utilized, particularly in chemotherapy and immunotherapy studies. We speculate that the mangabey and rhesus monkey will answer the long-sought need for a primate model and, when fully developed, should provide highly meaningful information on all aspects of leprosy in humans.—[Armed Forces Institute of Pathology, Washington, D.C. 20306, U.S.A.; Delta Regional Primate Research Center, Covington, Louisiana 70433 U.S.A.]

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Nakamura, K. and Yogi, Y. The nude mouse as an experimental lepromatous model (continued): The NFS/N nude mouse as a new model using the intra-upper lip inoculation method.

We have successfully established the BALB/c-nude mouse as an animal model for experimental lepromatous leprosy using the right upper lip (mystacial vibrissae) as the inoculation site. In this report, we compared the susceptibilities of various strains of nude mice by using NSF/N-nu, C57BL/6-nu, CBA/N-nu, C3H/HeN+MTV (having murine tumor virus)-nu, and BALB/c-nu strains. We compared sites of inoculation, namely of the upper lip site, with a technique of inoculating two sites on the hind foot, respectively.

The *Mycobacterium leprae* strain used was a "virulent" strain with which, in some BALB/c-nu mice, a slightly swollen lepromatoid infected hind paw develops at 6–7 months after the foot is injected with 10^5–10^6 bacilli. Four 10-week-old nude mice on various backgrounds (NFS/N, CBA/N, C57BL/6, C3H/HeN+MTV, and BALB/cA) were used. The injected site was the right upper lip (mystacial vibrissae located part); the control groups were injected in "two parts of the hind foot." In the latter, inoculations were made both at a dorsal site through the upper joint of the right hind foot, and at a plantar (foot pad) site. After receiving *M. leprae* in the right upper lip (inoculum size 1.8×10^6 bacilli), lip swelling of NFS/N-nu mice occurred with excellent results, that is to say, the lips of all tested mice were swollen. In contrast, in BALB/cA and C57BL/6 nude mice, only a slight swelling appeared, as previously reported. Lip swelling of C3H/HeN nude mice occurred sooner than that of CBA/N nude mice. In NFS/N nude mouse experiments, lepromata gradually developed and spread to the neighboring sites, such as the opposite lip, the lower lip, and the nose. After 20 months of infection, a tremendous development of lepromatoid lesions appeared on the infected upper lip and the neighboring sites, such as the opposite lip, lower lip, nose, eye, head, root of the ear, and neck in two out of four surviving NFS/N nude mice. In contrast, in the CBA/N nude mouse and the C3H/HeN nude mouse lepromata did not develop. Excellent foot swelling occurred in NFS/N-nu and C3H/HeN-nu mice after inoculation of 1.0×10^6 *M. leprae* into the foot using the two-site technique outlined above. With BALB/cA-nu, C57BL/6-nu, and CBA/N-nu mice, only slight swelling of the infected foot was observed in some of the tested mice. Later in the infec-

tion, CBA/N nude mice were obtained with excellent swelling. However, in C57BL/6 nude mice and BALB/cA nude mice there was poor swelling throughout, just as was the case with these strains after upper lip inoculation. In some NFS/N nude mice, a marked swelling of the popliteal lymph nodes as well as infected foot occurred, with large numbers of acid-fast bacilli having invaded the popliteal lymph nodes.

In some of the C3H/HeN-nu and CBA/N-nu mice, after 18 months of infection, paw ulceration occurred. In some NFS/N-nu mice, after 20 months of infection, a tremendous development of severe lepromatoid lesions was noted. These lesions appeared on the infected paw and at lumbar and abdominal sites with ulceration. The ulcers had more tendency to heal in the NFS/N-nu mice than in the C3H/HeN-nu and CBA/N-nu animals. At 20 months, the swollen foot of the NFS/N-nu mice weighed 3.1 g and contained 5.3×10^{11} bacilli per g.

In comparing BALB/cA-nu, C57BL/6-nu, C3H/HeN-nu, CBA/N-nu, and NFS/N-nu mice for susceptibility to the growth of *M. leprae*, the NFS/N-nu mouse was confirmed as a powerful tool as an experimental leprosy model using the upper lip inoculation method. With hind foot infections, C3H/HeN-nu, CBA/N-nu, and NFS/N-nu mice developed heavier infections than C57BL/6-nu and BALB/cA-nu mice. Thus, the development of a heavy lepromatoid formation in nude mice was influenced by the genetic background of the animals.

In summary, we have established the NFS/N-nu mouse as a new model using an intra-upper lip inoculation method. We have compared NFS/N nude mice with BALB/cA-nu, C57BL/6-nu, CBA/N-nu, C3H/HeN+MTV-nu mice by both upper lip and foot inoculations. It is quite possible that these nude mice may be a source of *M. leprae* for vaccine studies and for the preparation of lepromin (lepromin-M) and leprosin (leprosin-M). This source would have an advantage over *M. leprae* from armadillos because of the lack of problems which could arise due to naturally occurring leprosy-like diseases in armadillos.—[National Institute for Leprosy Research, Tokyo, Japan]

Nakamura, K. and Yogi, Y. The hereditarily athymic asplenic (LASAT) mouse as an experimental lepromatous leprosy model (continued): Role of the spleen in the formation of the lepromatoid lesions.

In 1980, we reported preliminary studies of *Mycobacterium leprae* infections in LASAT mice at the First Congress of Hansenology of the Endemic Countries, Rio de Janeiro, Brazil. Afterwards, we reported preliminary studies on the second passaged infection in LASAT mice compared with BALB/c nude mice. In this report, we wished to determine the role of the spleen in the formation of the lepromatoid lesions. We used LASAT mice and compared them with splenectomized nude mice of the strains BALB/cA-nu, C3H/HeN-nu, NFS/N-nu, C57BL/6-nu, and CBA/N-nu.

The strain of *M. leprae* used was "virulent" strain obtained from BALB/c nude mouse passage. Four 10-week-old nude mice on the various backgrounds, i.e., BALB/cA, C57BL/6, NFS/N, C3H/HeN+MTV, and CBA/N, and four 11-week-old LASAT mice on a BALB/c (N4) background were used. Splenectomy was done about four weeks after birth. The mice are maintained in vinyl isolators under specific pathogen-free (SPF) conditions.

After receiving 1.8×10^{6} *M. leprae* in the right upper lip, lip swelling developed in the LASAT mice and in the BALB/cA and C57BL/6 nude mice at 248 days and 353 days after infection. In contrast to NFS/N-nu mice, later gross lesions did not develop in LASAT mice or in C3H/HeN-nu, and CBA/N-nu mice. At that time (353 days after infection), bacillary counts were over 10^{11} bacilli/g in the LASAT mice. In some LASAT mice, a slight but not progressive ulcer developed. There was marked individual variation among LASAT mice as to the development of lip swelling in comparison to NFS/N nude mice. Splenectomy of nude mice enhanced the bacillary counts and lip swelling, particularly in CBA/N-nu and C3H/HeN-nu animals. Lepromatoid swelling in some LASAT mice was faster than that of BALB/cA-nu mice. The "normal" hind feet of LASAT mice supported bacillary growth better than "abnormal" hind feet of these animals.

After right hind foot pad inoculation (two parts of a hind foot method), inoculum size: 1.6×10^6 bacilli, foot swelling in some LASAT mice appeared with excellent results compared with BALB/cA and C57-BL/6 nude mice. In some LASAT mice slight ulcer formation occurred on the infected foot. These ulcers did not develop as severely as those of C3H/HeN, NFS/N, and CBA/N nude mice, however. At this time, the weight of the leproma was 0.78 g and the bacillary counts were over 10^{11} per g. Thus the LASAT mice had marked lepromatoid lesions which were never seen in BALB/cA and C57BL/6 nude mice with long-term observation. In addition, a marked individuality occurred among LASAT mice as to foot swelling in comparison with NSF/N nude mice. Furthermore in some LASAT mice having "abnormal" hind feet, we observed swelling of the infected foot and toe 320 days after both hind feet were inoculated.

In splenectomized nude mice, bacillary counts of the infected foot were enhanced; furthermore, foot swelling was faster than that in non-splenectomized nude mice. Swelling in splenectomized CBA/N-nu mice was particularly stronger than that of control CBA/N-nu mice. Thus, the lack of spleen may be allowing enhanced growth of *M. leprae*. It was previously demonstrated that the spleen may be important in regulating the development of lepromatoid lesions at an early stage of the infection. Unfortunately, with long-term observation, a high incidence of tumor occurred in these mice with marked enlargement of the lymph nodes, such as the mesenteric nodes. Thus, the secondary complication or age-dependent immunobiology may influence resistance to the development of lepromata at late stages of the infection.

In summary, the lack of a spleen may be important in regulating the development of lepromatoid lesions in early stages after infection. Additionally, the development of lepromatoid lesions may be influenced by the genetic backgrounds of the LASAT mice.—[National Institute for Leprosy Research, Tokyo, Japan]

Nakamura, K. and Yogi, Y. The nude rat as an experimental lepromatous leprosy model (continued): The effects of carrageenans, splenectomy, and treatment with thymus cells.

We have studied the effects of carrageenans and the effect of treatment with thymus cells on nude rats infected with *Mycobacterium leprae*. Furthermore, the effect of splenectomy of the nude rat was studied.

We confirmed that there are "virulent" and "low-virulent" strains of *M. leprae* in nude rats. The strain used was a "virulent" strain obtained from Rowett nude rat passage (second passage strain). Four 6-week-old Rowett nude rats were used in all experiments. Splenectomy was done at four months and five months after right hind foot infection. Thymus cells were obtained from heterozygous rats (nu/+) at 7–14 days after birth; 5.0×10^7 cells were intraperitoneally inoculated in the infected nude rats at 5 months, 6 months, and 10 months after infection. Carrag(h)eenan (type II, believed to be essentially iota-carrageenan, Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was administered both interperitoneally (i.p.) and in the right hind foot two days before infection with *M. leprae* and compared with a group only treated i.p. *M. leprae* were inoculated by the "two parts of the hind foot" method, in which the inoculum was administered both at a dorsal site through the upper joint of the right hind foot and at a plantar site.

At 187 days after infection subcutaneously in the right hind foot with 1.8×10^6 *M. leprae* the nude rats treated with carrageenan (i.p. and hind foot) developed lepromatoid swelling of the infected foot. In the carrageenan-treated nude rats, 1.9×10^9 bacilli were found 187 days after the inoculation, while bacillary counts in control nude rats were 6.0×10^7 bacilli. Later, at 363 days after the inoculation, marked, thick nodules appeared on the infected foot and toe. At that time, the weight of the swollen foot was 5.8 g and the number of AFB was approximately 10^{11} bacilli per g. There was invasion of the perineurium of nerves in the infected foot by AFB. In some carrageenan-treated nude rats, multiple nodular swellings occurred on the hind foot 388 days after infection. By 17 months after inoculation, a marked infection had appeared on

the mouth, face, ears, at lumbar sites, and the tail, with marked multiple nodular infiltrations occurring on the skin of the lumbar area, the infected paw (including femur) and the root of the tail.

Also in carrageenan (i.p. only) treated nude rats with right hind foot pad inoculation, systemic infection occurred with marked nodular swelling of the infected foot and small nodules on the opposite (uninfected) hind foot. Later, in some of the animals, marked multiple nodules developed at uninfected sites such as the fore feet, the mouth, face, ears, at lumbar sites, and such. At 236 days after carrageenan (i.p. only) treated nude rats received 1.8×10^6 *M. leprae* in the right fore foot, marked swelling of the infected fore foot occurred. After carrageenan (i.p. only) treated nude rats received 2.0×10^6 *M. leprae* in the testis, a number of acid-fast bacilli were obtained in testis tissue. Fifteen months after carrageenan (i.p. only) treated nude rats received 1.0×10^6 *M. leprae* at the upper lip inoculation site, a marked nodular swelling with ulceration appeared. Splenectomy enhanced *M. leprae* growth in nude rats.

Carrageenan-treated Rowett nude rats and splenectomized nude rats may be useful models of lepromatous leprosy.

Nude rats were inoculated in the right hind foot with 8.5×10^5 *M. leprae*. Heterozygous thymus cells were given and swelling in the infected right hind foot developed four weeks later. Compared to controls, bacillary counts were reduced 10- to 100-fold in animals receiving thymus cells. These results are in contrast to our earlier findings of enhanced growth of *M. leprae* following this treatment in nude mice.

The Rowett nude rat may be a suitable model for immunotherapeutic as well as chemotherapeutic studies. *M. leprae* carrageenan-treated nude rats may be a possible source of lepromin-R and leprosin-R.—[National Institute for Leprosy Research, Tokyo, Japan]

Kashiwabara, Y. and Nakagawa, H. Phospholipid deacylating activities in murine leprosy bacilli.

Enzyme activities which hydrolyzed acyl groups of phospholipids in murine leprosy bacilli grown in host tissues were examined using various [^{14}C]-labeled phospholipids as substrates.

The particulate fraction of murine leprosy bacilli grown in host tissues contained phospholipid deacylating activities with acidic pH optima. It hydrolyzed a homologous series of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine at similar rates. It also hydrolyzed 1-acyl and 2-acyl GPCs (sn-glycerol 3-phosphocholine) more rapidly than phosphatidylcholine.

Ca^{++} did not stimulate either diacyl- or monoacyl-hydrolase activity. Fe^{+++} inhibited the hydrolysis of phosphatidylcholine, but caused little or no inhibition of the deacylation of 1-acyl- and 2-acyl-GPCs. Triton X-100 caused a little stimulation of the deacylation of 1-palmitoyl-phosphatidylcholine but no stimulation of those of 2-oleoyl- and 1-stearoyl phosphatidylcholine in the concentration range of 0.1% to 0.5%, suggesting that the effect of this detergent was affected by the fatty acyl moieties esterified to phospholipids.

The particulate fraction produced fatty acid and lysophospholipid when incubated with diacylphospholipids, suggesting the presence of both phospholipase A_1 and A_2 activities in the membrane fraction of murine leprosy bacilli grown in host tissues.

Some properties of the phospholipid deacylating activities were found to be different from those reported in mammalian cells as well as those in some bacteria.

Phospholipid deacylating activities with similar acidic pH optima were also detected in the particulate fraction of cultivated murine leprosy bacilli (*M. lepraemurium* Hawaiian-Ogawa). Some properties of the phospholipid deacylating activities of the cultivated murine leprosy bacilli were compared with those of host-grown *M. lepraemurium*.

Fe^{+++} inhibited the deacylation of diacylphospholipids by the cultivated murine leprosy bacilli more strongly than that by the host-grown bacilli.

Triton X-100 stimulated deacylation of three kinds of phosphatidylcholines by the particulate fraction of the cultivated murine leprosy bacilli and, again, the effect of this detergent was affected by fatty acyl moieties esterified to phosphatidylcholine.

The particulate fraction of the cultivated

murine leprosy bacilli also produced fatty acid and lysophospholipid when incubated with diacylphospholipids, suggesting the presence of phospholipase A_1 and A_2 activities.

The particulate fraction of host-grown murine leprosy bacilli rapidly hydrolyzed various phosphatidylcholines at similar rates, while that from the cultivated bacilli showed substrate specificity toward the fatty acyl moiety of the phosphatidylcholines, suggesting that the substrate specificities of the enzyme activity in the particulate fraction of host-grown and the cultivated bacilli were different.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Fukunishi, Y., Okada, S. and Nishiura, M. Biochemical study of the peribacillary substance of *M. leprae*.

Gel-permeating high performance liquid chromatography (HPLC) of acetone-soluble lipids of experimental armadillo lesions caused by *M. leprae* of various different origins (naturally acquired leprosy-like disease of monkey, human leproma, and naturally acquired leprosy-like disease of armadillo) was performed.

Characteristic patterns of peaks and retention volumes were obtained with all the varieties of armadillo lesions mentioned above. Peaks at retention volumes common to all varieties of armadillo lesions are 2000 (fraction 1), 1100 (fraction 2), 900 (fraction 3), 700 (fraction 4), and 310 (fraction 5).

Fraction 3, fraction 4, and fraction 5 were also found in murine lepromas and normal mammalian tissues, but fraction 1 and fraction 2 were not found in these tissues.

Mass spectrometry was performed on all fractions obtained by HPLC of all three varieties of armadillo lepromas.

Characteristic mass spectra of fraction 3, fraction 4, and fraction 5 were obtained in all three varieties of armadillo lepromas. However, mass spectrometry of fraction 1 and that of fraction 2 were not successful due to the difficulty of ionizing these fractions.—[Seisho-en National Leprosarium, Kagawa Pref., Japan; Leprosy Research Laboratory, Kyoto University, Kyoto, Japan]

Nishiura, M. and Fukunishi, Y. Comparative study of the nerve lesions of experimental animals inoculated with *M. leprae*.

Peripheral nerve lesions of nude mice inoculated with *M. leprae* were examined 1 year to 1 year and 8 months after the inoculation.

Leprosy bacilli were found in perineurial cells, endothelial cells, Schwann cells, and axons of the myelinated nerve fibers inside the leproma of the foot pads. Almost all bacilli in these host cells were solid.

In the proximal portion of the sciatic nerve, bacilli were found chiefly in the macrophages of the endoneurial space. Bacilli were not found in the perineurial cells of the sciatic nerves.—[Leprosy Research Laboratory, Kyoto University, Kyoto, Japan; Seisho-en National Leprosarium, Kagawa Prefecture, Japan]

Tsutsumi, S. and Gidoh, M. Fundamental studies on the immunomodulative control of leprosy by pharmacotherapeutic means.

Immunomodulation may have important values for the treatment of leprosy: a) control or even the prevention of erythema nodosum leprosum (ENL), b) preservation of host-mediated drug efficacy even under the progress of immunological disorders by leprosy, and c) lessening the depressive effects of drugs on cell-mediated immunity (CMI).

The endeavor has been continuing by a method of rosette formation between rabbit red blood cells (RRBC) and guinea pig lymphoid cells (GPL), the latter collected from immunodepressed (from one to three weeks after thymectomy) or from recovered (two months or more after thymectomy) animals. The former animals were appropriate for determining an immunopotentiative effect and the latter for an immunodepressive effect.

Immunopotentiative effect. The effect was detected as a stimulative effect on the recovery of depressed CMI.

1) An immunomodulative drug CCA significantly increased rosette formation at a dosage of 60 mg/kg. But, it was not significant at 30 mg/kg; whereas clofazimine (B663) increased rosette formation even at

this dosage. Nevertheless, the possibility of CCA in combination with chemotherapy was proposed, due to its actions resembling those of B663 but with no pigmentation.

2) Two newly synthesized amphoteric dextrans (ADx) named Compd. V and VI with molecular weights of the dextran (Dx) parts of 7.68×10^4 and 2.5×10^5, respectively, were examined. The latter significantly increased rosette formation after the intramuscular injection of 15 or 30 mg/kg, once every other day for 13 days (a total of six times). It was comparable to the effect of ATSO, a 1-3 β-glucan in an oral dosage of 30 mg/kg once every day for the same period of time.

3) Three intramuscular doses of 15 mg/kg of Compd. VI or 0.1 mg/kg of B512F given in the axillae close to the axillary lymph nodes as a solid in oil type (S/O) emulsion over a 13-day period did not increase the rosette formation.

4) ADx inhibited carrageenan-induced rat acute edema (CRAE) in contrast to the stimulation by Dx. In spite of the lowering in the amphotericity of Compd. VI (due to the decrease in the introduction of both acidic and basic radicals into Dx) compared with those of the previously synthesized four ADx, Compd. VI still showed, though weakly, the inhibitory effect on CRAE.

5) Paralleling the lowered amphotericity, the toxicity of Compd. II to mice was lower than the toxicities of the previous four ADx.

6) Relating to 4), the analgesic action of dapsone (DDS) was examined at 50 mg/kg. DDS showed a weak peripheral analgesic action.

7) The thymosine fraction extracted from calf or guinea pig thymus glands was injected subcutaneously in the axillae close to the axillary lymph nodes of immunodepressed animals in the daily dosage of 5 mg per animal covering 13 days. A significant potentiation was found only in the calf thymosine group.

Immunodepressive effect. This effect was determined in recovered animals.

1) The order of the depressive effect was: (mg/kg, dose route, dose frequency/13 days, MTX (0.4, oral, 13), Imuran [5, oral, 4 (per 9 days)] > DDS (20, oral, 13), Ifosfamide (15, oral, 13) > rifampin (30, oral, 13). Sulfadimethoxine (100, oral, 13) was inactive.

2) Cyclophosphamide (CP) in the dosage of 200 mg/kg once intraperitoneally at two days prior to thymectomy merely depressed CMI after the thymectomy. The depression was found to be continued even at 27 days after the thymectomy.

3) The intraperitoneal injection of antithymocyte globulin purified from rabbit serum (which had a total *in vitro* cytotoxicity equivalent to the killing of 1.56×10^9 guinea pig thymocytes) completely abolished rosette formation in recovered animals. This effect was only partial when it was given to smaller intact guinea pigs.

GPL collected by Ficoll Paque were further fractionated on a Percoll gradient. The highest rosette percent was found in the T cell layer.

Discussions were presented regarding a) the usage of this rosette formation method not only in the leprosy field but also in wider biomedical fields where the screening of immunosuppressors is needed and b) the change in the concept of antileprosy chemotherapy, which seems to be too partial to bacteriological views.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Tsutsumi, S. and Gidoh, M. Fundamental studies on the possibility of perineurotropic derivatives of acidic anti-inflammatory drugs for treatment of leprous neuritis.

Although Minauchi reported the usage of Carbamazepine for the control of leprous neuritis, an optimum regimen for the treatment of peripheral neuritis has not been established. For this reason, derivatizations of the COOH moiety in acidic anti-inflammatory drugs to $COOR_1$ (ester-type) and COR_2 (amide-type) were performed. The synthesized derivatives were: Brufen (BF): $R_1 = CH_2CH_2NEt_2 \cdot HCl$ (BFDEAE); $R_2 =$ PABA ethyl ester (PABAEE) amide (I), anthranilic acid ethyl ester (AAEE) amide (II), Flurbiprofen (FBF): $R_2 =$ PABAEE amide (III), AAEE amide (IV), mefenamic acid (MA): $R_1 = CH_2CH_2NEt_2 \cdot HCl$ (MADEAE), Carprofen: $R_2 = NHCH_2CH_2NEt_2$ (V), Ketoprofen: $R_2 =$ PABAEE amide (VI), and AAEE amide (VII). The bio-

availabilities of these compounds were compared with their starting acidic anti-inflammatory drugs regarding anti-inflammatory and local anesthetic effects. The results were:

1) BFDEAE exhibited not only an anti-inflammatory effect comparable to BF but also a strong local anesthetic effect which was found to be 20-fold stronger than that of procaine·HCl. BFDEAE also showed a weak antihistaminic action.

2) BFDEAE was rapidly hydrolyzed to BF in rat plasma. But, the acute toxicity after intraveneous injection was higher with BFDEAE than with BF, and was comparable to that of procaine·HCl.

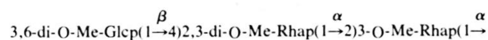
3) The derivative of Carprofen (V) also exhibited a strong anesthetic effect. The infiltration local anesthetic effect was found to be twofold stronger than that of Xylocaine. But, perhaps due to its strong surfactant activity, the dropping of V in high concentrations onto the cornea of guinea pigs caused apparent corneal muddiness. Neither antiphlogistic nor analgesic action was found in V; whereas a compound named BFDEAEAM which was derived from BF in a fashion similar to the derivatization of V from Carprofen exhibited both actions comparable to BFDEAE. Thus, it was found that these actions of procaine- or procainamide-type derivatives were dependent upon the starting anti-inflammatory drug. For example, both the antiphlogistic and local anesthetic effects of MADEAE were weaker than those of MA and Xylocaine, respectively.

4) Among I–IV, VI, and VII, both the inhibitory actions on carrageenan-induced rat acute edema and the analgesic actions examined by the Randall-Selitto method were found only in the PABAEE amides (I, III, and VI); while those activities of PABAEE itself were negligible. But, the effect of I, III, and VI were somewhat weaker than those of BF, FBF, and Ketoprofen, respectively, and none of them exhibited local anesthetic effect.

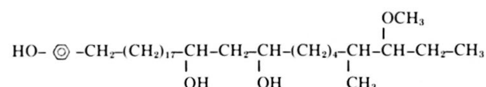
5) Discussions were presented about the possibility of using these types of derivatives in the treatment of peripheral neuritis.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Hunter, S. W., Fujiwara, T. and Brennan, P. J. Structure and antigenicity of the specific phenolic glycolipid antigens and a related diacyl phthiocerol in secretions from *Mycobacterium leprae.*

In a recent publication, we reported on the presence of a specific phenolic glycolipid (Phenolic Glycolipid-I) in *Mycobacterium leprae* and in infected armadillo tissues [cf. Hunter, S. W. and Brennan, P. J. J. Bacteriol. **147**(1981)728–735]. It had an inherent oligosaccharide, composed of 3-O-Me-rhamnose, 2,3-di-O-Me-rhamnose, and 3,6-di-O-Me-glucose, glycosidically linked to the phenol substituent. The structure of the oligosaccharide has now been determined, by partial acid hydrolysis, permethylation, ^{1}H- and ^{13}C-NMR as:

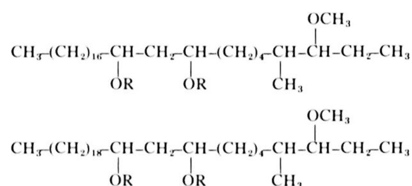


(assuming that the glucose substituent is in the D-enantiomeric configuration, and the two methylated rhamnoses are L). Acid hydrolysis of deacylated Phenolic Glycolipid-I yielded a phenolic phthiocerol "core," and mass spectrometry and proton NMR of the permethylated "core" proved the following structure:



Combined GLC-mass spectrometry showed three tetra-methyl branched "mycocerosic" acids attached to the hydroxyl groups of the phthiocerol substituent with MW (as methyl esters) of 466, 494 and 522. These have been identified as 2,4,6,8-tetramethyl hexacosanoate, 2,4,6,8-tetramethyl octacosanoate, and 2,4,6,8-tetramethyl triacontanoate.

A second phenolic glycolipid (Phenolic Glycolipid II) was also recognized among materials secreted by *M. leprae* into armadillo tissues. It was found to be identical to Phenolic Glycolipid I in all respects except that the terminal sugar is 6-O-Me-glucopyranose. Finally, quite large quantities of two diacyl phthiocerols were found in these secretions, the full structural details of which have been elucidated:



in which R, the acyl substituents, are the same three found in the phenolic glycolipids (see above).

Phenolic Glycolipids I and II are immunologically active, reacting with rabbit antiserum to *M. leprae* and with sera from lepromatous leprosy patients in ELISA systems and by gel diffusion of liposomes containing the glycolipids.

There is a strong circumstantial case indicating that the phenolic glycolipids in combination with the diacyl phthiocerols are the "peribacillary substance," "small spherical droplets," "foamy structures," "capsular material," "electron transparent zone," long described by electron microscopists. The function of these substances is presumably to provide passive protection to the resident *M. leprae* within the habitually inimical milieu of the phagolysosome.—[Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, U.S.A.]

Umland, E. T., Brennan, P. J., Tung, K. S. K. and Teuscher, C. Antibody responses of leprosy patients to *Mycobacterium leprae* glycolipid-1 antigen.

Many investigators have detected antibodies to many antigens of *Mycobacterium leprae* and other mycobacteria in sera from leprosy patients. We have studied the antibody response to a purified, structurally characterized glycolipid surface antigen unique to *M. leprae*. *M. leprae* glycolipid-1 has been isolated and characterized by Hunter and Brennan (cf. J. Bacteriol. **147** (1981) 728–735). In a solid-phase radioimmunoassay (RIA), IgG class antibodies have been detected by ^{125}I labeled *Staphylococcus aureus* Protein A. Since leprosy patients have antibodies that react with *M. kansasii* mycoside-A as well as *M. leprae* glycolipid-1, a serum is classified as positive only if it reacts more strongly with the *M. leprae* antigen. Antibody levels are expressed as the ratio of uptake of label by test serum to the mean uptake by normal sera.

In sera from untreated Thai leprosy patients, the following correlation between clinical and histopathologic classification and presence of IgG antibody specific to *M. leprae* glycolipid-1 was found:

	Patients positive/ Patients tested
TT (polar tuberculoid)	7/8
BT (borderline tuberculoid)	10/15
BB (borderline)	0/1
BL (borderline lepromatous)	2/2
LL (polar lepromatous)	9/9

The overall positive rate was 80%. At least 4 of the 7 negative results are considered to be false negatives: the TT patient was later sero-positive; 2 BT patients and the BB patient had acid-fast bacilli in histopathologically compatible skin biopsy specimens. The clinicopathologic status of the remaining three BT patients is inconclusive. As has been described in previous studies, tuberculoid patients tend to have less serum antibody than lepromatous patients. The mean (± standard deviation) antibody level of BL and LL patients was 10.8 ± 4.6; while that of BT and TT patients was 2.2 ± 1.9. In addition, immune responses to *M. leprae* glycolipid-1 appear to play some role in the evolution of erythema nodosum leprosum (ENL) reactions in leprosy patients. The antibody level of six ENL patients was 3.7 ± 2.0. In one patient who developed an ENL reaction, his antibody level dropped sharply from 17.0 in a specimen collected before treatment to 2.8 at the onset of his ENL reaction.

Healthy persons closely exposed to leprosy patients over an extended period of time are known to develop immune responses to leprosy bacilli, suggesting that they may have had a subclinical infection. Six of 12 (50%) family contacts of leprosy patients and 2 of 21 (10%) hospital personnel in a leprosy hospital in Thailand are positive. There is no apparent relationship between the type of leprosy of the patient and the incidence of sero-positivity in this small group of family contacts.

In summary, a RIA for antibody to a

unique and probably pathogenetically important surface component of *M. leprae* is under development. The sensitivity of the RIA for antibody of IgG class is 80% in single serum samples from untreated patients with clinical disease and is 74% in untreated patients with tuberculoid leprosy. The antibody level in LL and BL patients is significantly greater than that of TT and BT patients. A proportion of healthy contacts (10% to 50%) also have detectable antibodies. Of 55 contacts and non-contacts without clinical disease, eight are sero-positive, giving a specificity of 85%. Patients with ENL reactions appear to have significant changes in antibody level prior to the onset of the reaction.—[Department of Pathology, University of New Mexico School of Medicine, Albuquerque, New Mexico, U.S.A.; Department of Microbiology, Colorado State University, Fort Collins, Colorado, U.S.A.]

Miller, R. A., Gillis, T. P. and Buchanan, T. M. Immunochemical characterization of antigens of the leprosy bacillus, including production and initial characterization of monoclonal antibodies to *Mycobacterium leprae*.

The leprosy bacillus contains numerous protein, polysaccharide, glycolipid and lipid antigens. Relatively little is known about the pathogenic role of these antigens or how they interact with the human immune system. There exists chemical evidence for a specific trisaccharide found on the phenolic glycolipids I and II of *Mycobacterium leprae*, but little other information is available to indicate which antigens are unique to *M. leprae*, and which are shared with other microorganisms. Our laboratory is utilizing immunochemical techniques and hybridoma monoclonal antibody technology to better define these antigens.

The leprosy bacillus was isolated from a total of 1.5 kg of non-irradiated infected armadillo tissues, using the Draper 1979 purification procedure on 60 separate occasions. Analysis of the initial homogenization and subsequent purification steps by SDS-PAGE indicated that more than 90% of surface-exposed proteins of *M. leprae* were degraded during the purification procedure. Of several protease inhibitors tested, 0.1 M benzamidine most effectively blocked the formation of split products, resulting in substantial retention of the surface proteins on the purified organisms. These surface-exposed proteins had subunit molecular weights of approximately 36,000, 44,000, 53,000, 64,000, and >90,000. These proteins were analyzed by the Western Blotting Methodology, with a potent antiserum to normal armadillo liver antigens. Most of the approximately five major surface proteins in the purified organisms were of *M. leprae* origin, and a single protein of approximately 75,000 daltons subunit molecular mass appeared to be of armadillo liver origin, and remained bound to the leprosy bacilli during purification.

The arabinomannan polysaccharide of mycobacteria was purified from *M. smegmatis* by delipidation of whole organisms, alkaline extraction of the polysaccharide, and removal of the contaminating protein by precipitation with phenol. The purified product contained <3% protein by weight, which existed in a low molecular weight degraded form, and the remaining material was a polysaccharide of two to three sizes, and consisting almost exclusively of mannose and arabinose in a 1:1 ratio, as analyzed by thin-layer chromatography. This antigen was adapted to a quantitative ELISA assay performed on microtiter plates. Analysis of *M. leprae* in infected tissue suspensions and in purified organisms indicated that the arabinomannan antigen was exposed on the surface of purified whole bacilli. Also, more than 90% of this antigen remained in fractions that did not contain bacilli, following the Draper 1979 purification procedure. New purification procedures are needed to allow isolation of this and other *M. leprae* antigens that have been released from degraded bacilli into host tissues. Treatment of the purified arabinomannan by protease, DNAse, mannosidase, or periodate, with subsequent evaluation of the antigens' integrity by Western Blotting, using pooled LL sera, indicated that only periodate destroyed the antigen. This supported the carbohydrate nature of the antigen and suggests that arabinose is the immunodominant determinant.

Monoclonal antibodies have been prepared to *M. leprae* since they allow considerable immunochemical characterization,

even without extensive purification of these antigens. This is an advantage especially in leprosy research where many *M. leprae* antigens are available only in small quantities. Approximately 30 monoclonal antibodies have been prepared which react with *M. leprae*. Donor spleens for the hybridoma fusions were obtained from BALB/c mice immunized with crude cellular extracts of *M. leprae*, with whole *M. leprae* purified by the Draper procedure including 0.1 M benzamidine to minimize denaturation of antigenic surface proteins, or with arabinomannan purified from *M. smegmatis*, or from NHI or BALB/c mice infected with *M. lepraemurium*. NS-1 myeloma cells were used in all fusions. Antibody specificity of clones derived from these fusions was tested by enzyme-linked immunosorbent assay (ELISA) against sonicated extracts of *M. leprae* and other mycobacteria, arabinomannan from *M. smegmatis*, and normal armadillo liver tissue, and by both ELISA and indirect immunofluorescence against glutaraldehyde-fixed whole *M. leprae* and *M. lepraemurium*. Analysis of the results from the different assays indicates that 11 are directed at intracellular antigens while several of the remainder appear to recognize surface components. Two antibodies directed at cell-wall-associated proteins recognized *M. leprae*, but not 18 other species of mycobacteria, including most mycobacterial pathogens. Two other antibodies were directed at the arabinomannan antigen and recognized most mycobacteria. One antibody was directed at a 68,000 dalton protein common to *M. leprae*, *M. flavescens*, *M. gastri*, and *M. gordonae* when analyzed by Western Blotting. A second monoclonal antibody directed at this same protein apparently recognizes a different epitope since it recognizes only two of these species in addition to *M. leprae*. Other antibodies react with both normal armadillo liver tissue and with *M. leprae* purified from armadillo liver by the Draper procedure. These antibodies may recognize the principal liver contaminant(s) present on purified *M. leprae*. Of the 11 antibodies directed at intracellular or non-exposed cell-wall antigens, 8 were IgG and 3 were IgM. In contrast, of the 19 antibodies that react in an ELISA test using whole organisms, and presumably recognize surface antigens, all were IgM.—[Immunology Research Laboratory, Seattle Public Health Hospital, University of Washington, Seattle, Washington, U.S.A.; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Sengupta, U., Sinha, S., Ramu, G. and Desikan, K. V. Antigenic analysis of *M. leprae* in lepromins with reference to the delayed type of hypersensitivity.

Electrophoresis of leprosin-A and the sonicate of Dharmendra antigen (DA) revealed the presence of a common protein band extending as a continuous streak from the antigen well towards the anode. This band was also noted in the DA supernatant.

Immunoelectrophoresis of Dharmendra antigen and Mitsuda lepromin against sera of lepromatous leprosy (LL) patients showed the presence of an antigen near the well. However, although this antigen gave a precipitin reaction, it was not identifiable by any staining methods available for protein, lipid, and carbohydrate.

Using pooled LL sera as antibody in two-dimensional immunoelectrophoresis, DA sonicate revealed a thick precipitin line near the origin well. Besides this, we have also observed a faint precipitin line in the anodal region. Leprosin-A, on the other hand, gave six precipitin lines. However, the autoclaved leprosin-A gave only one precipitin line near the well.

The anionic components and cationic components were eluted from the gel after electrophoresis, concentrated, and further used for skin delayed type hypersensitivity (DTH) reactions in leprosy patients. All the 24 tuberculoid leprosy patients showed a positive DTH response with the anionic component at 48 hr. With leprosin-A and DA, four and one patients showed no response, respectively.

The cationic component of DA evoked a small reaction, erythema without induration, in 14 out of 24 tuberculoid leprosy patients. The peak skin reaction was noted at 10–15 hr.

The control elute did not induce any skin reaction.

Histological analysis of the biopsies of skin tests performed with the anionic component showed a DTH type of response with principally lymphocytic and mononuclear

cell infiltrations. However, the cationic component evoked a reaction which was infiltrated mainly by polymorphonuclear cells. Besides these polymorphonuclear cells, a few lymphocytes and mononuclear cells were also noted.

Analysis of the anionic and cationic components of Dharmendra antigen revealed that the skin DTH inducing antigen(s) resides/reside in the anionic region. When the anionic component of the gel section was further divided into equal halves and their antigen elutes were used for skin testing, it was noted that both the fractions were equally potent in evoking the skin DTH response. The cationic component, on the other hand, did not appear to take part in the DTH reaction. The small reaction evoked by this component attracted neutrophils at the site of reaction.—[Central JALMA Institute for Leprosy, Agra-282001, India]

Akiyama, T., Esashika, I. and Yamaura, N. Suppressor cells in experimental murine leprosy.

In previous studies, we noted that whole spleen cells from several inbred strains of mice infected subcutaneously with a low dose (5.8×10^5) of *Mycobacterium lepraemurium* (*Mlm*) exhibited consistently a development of cell-mediated immunity as measured by MIF-induced inhibition of macrophage migration. In contrast, intravenous infection with the same amount of *Mlm* into these animals led to markedly diminished MIF-activity, suggesting a development of suppressor cells in their spleens. In the present studies, we sought for a systematic appraisal of the effect of varying amounts of *Mlm* on the immunoregulatory system in three strains of mice with distinct susceptibility to the infection. CBA/J, C3H/HeN, and C57BL/6N mice, which are highly susceptible, moderately susceptible, and relatively resistant, respectively, were used with the following results:

1) Whole spleen cells were studied from CBA/J mice infected i.v. with either low or high doses (2.9×10^7) of *Mlm*, and C3H/HeN mice infected through the same route with a low dose of *Mlm*. MIF-activity was restored by pretreatment of these cells with anti-Lyt-2.1 serum plus complement, but not by pretreatment with carrageenan. This suggests that a subpopulation of T cells was responsible for the suppression observed.

2) A marked splenomegaly in parallel with unresponsiveness was noted in C3H/HeN mice infected i.v. with a high dose of *Mlm* and in C57BL/6N mice sacrificed after i.v. injection with a low dose of viable *Mlm*. Pretreatment of their spleen cells with either an antiserum specific for suppressor T cells in the presence of complement or carrageenan improved their response to some extent. Neither treatment completely abrogated the suppressive activity, however, indicating the presence of two distinct suppressor systems operating in their spleens.

3) Splenomegaly also occurred in C57BL/6N mice sacrificed after i.v. infection with a high dose of *Mlm*. The suppressor activity of unfractionated *Mlm*-spleen cells was retained after treatment with anti-Lyt-2.2 serum plus complement, but was almost completely removed after depletion of macrophages, suggesting that the unresponsiveness observed was mediated exclusively by suppressor macrophages.

4) The target activity of suppressor macrophages seemed to be nonspecific, because they were capable of inhibiting the MIF-activity of syngeneic responding cells against an infection-associated antigen as well as a nonassociated antigen. On the other hand, the target activity of suppressor T cells was specific to an antigen used for the sensitization of the donor animals.

5) Indomethacin added to the culture medium (0.5–1.0 μg/ml) led to a marked restoration of the responsiveness of the spleen cells from C57BL/6N mice infected with a high dose of *Mlm*, but failed to completely abrogate the suppressive activity of the macrophages. This suggests the presence of indomethacin-sensitive as well as indomethacin-resistant suppressor macrophage subpopulations within the spleen.—[Department of Microbiology, Kitasato University School of Medicine, Sagamihara, Kanagawa-ken 228, Japan]

Izumi, S., Sugiyama, K., Ohkawa, S. and Matsumoto, Y. *In vitro* enhancement of lymphoproliferative response to *M. leprae* by the inhibition of biosynthesis of prostaglandins.

The regulatory function of prostaglandins (PG) on the lymphoproliferative response to *Mycobacterium leprae* was investigated in five healthy contacts and 13 leprosy patients by the inhibition of biosynthesis of PG. The mononuclear cells from all 5 healthy contacts and 12 out of 13 leprosy patients showed significant increases in proliferation following the addition of two widely used inhibitors, indomethacin and aspirin. This enhancing effect of indomethacin was abolished when macrophages were depleted from the mononuclear cells. The enhancement recovered when the T cells were reconstituted with 10% or more macrophages. These results indicate that macrophages play an important role in prostaglandin-mediated regulation of cell-mediated immune responses to *M. leprae* both in healthy and in leprosy individuals.—[Leprosy Research Laboratory, Kyoto University School of Medicine, Sakyo-ku, Kyoto, Japan; National Ohsima Seisho-en Leprosy Hospital, Ajicho, Kida-gun, Kagawa, Japan]

Scollard, D. M. and Gardner, I. D. Effect of *M. leprae* on phagosome-lysosome fusion in human macrophages.

The interaction between *Mycobacterium leprae* and macrophages is of great interest because although macrophages are successfully parasitized by *M. leprae* in some patients, these cells are also generally regarded as the effector cells that eliminate *M. leprae* and as important regulators of subsequent immunologic interactions. Studies of other facultative and obligate intracellular parasites have demonstrated that in some cases they are able to delay or prevent fusion of the phagosome with lysosomes, providing one possible explanation of their ability to survive and multiply within host macrophages.

We have examined the interaction between *M. leprae* and normal human macrophages *in vitro*. *M. leprae* were obtained directly from skin biopsies of active lepromatous patients. After determining that no bacterial contaminants were present, the mycobacteria were counted and their integrity determined according to the Morphologic Index (MI) routinely used in the leprosy clinic. Peripheral blood monocytes from healthy volunteers were obtained after centrifugation on Ficoll Hypaque, and cultured on carbon-coated cover slips in medium containing autologous plasma. After two days, the adherent cells were labelled with ferritin and on the third day, they were exposed to *M. leprae* or control organisms for three hours.

The cultures were examined at various intervals to determine the phagocytic activity of the cells. Five-day cultures were embedded in Spurr medium and thin sections were studied to ascertain the presence or absence of ferritin in the phagosomes containing *M. leprae*.

Ferritin was found in 156 of 172 such phagosomes, indicating phagosome-lysosome fusion in 91% of instances following phagocytosis of *M. leprae*. The bacilli observed on these phagolysosomes usually showed evidence of damage, possibly as a result of lysosomal enzyme activity. The implications of these observations on phagosome-lysosome fusion were discussed.—[Department of Pathology, Hong Kong University, Hong Kong]

Modlin, R. L., Hofman, F. M., Bakke, A. C., Horwitz, D. A., Taylor, C. R. and Rea, T. H. T cell subsets in the peripheral blood and tissues of patients with leprosy.

Immunologic mechanisms are probably important in the pathogenesis of many aspects of leprosy. Recent studies have emphasized that suppressor cells may be critical in modulating some host responses. Monoclonal antibodies directed against T cell subsets provide a new tool for the identification of helper/inducer and suppressor/cytotoxic cells in the blood and tissue of man. Using monoclonal antibodies we have studied T cell subsets in the peripheral blood and tissues of patients with leprosy.

MATERIALS AND METHODS

Patients were classified by Ridley's system. The 14 tissues studied were: 4 tuberculoid (TT/BT), 2 reversal reaction (1 BT, 1 BB, both upgrading), 1 borderline with lepromatous features (BL), and 7 lepromatous (LL), 5 with and 2 without erythema nodosum leprosum (ENL). Six pa-

THE TABLE. *Summary of peripheral blood T cell subsets.*

Patient group	Median therapy in years	Mean lymphs per mm³ ± S.D.	Mean Pan T per mm³ ± S.D. (%)	Mean T helper per mm³ ± S.D. (%)	Mean T suppressor per mm³ ± S.D. (%)	Mean helper/ suppressor ratio (± S.D.)
Normal control N = 20		2300 ± 550	1513 ± 350 (75)	965 ± 218 (48)	347 ± 100 (22)	2.1 ± 0.65
All active leprosy patients N = 27	1	1934 ± 901	1196[a] ± 612 (59)	665[b] ± 407 (34)	504 ± 282 (26)	1.43[b] ± 0.69
Active LL without ENL N = 9	1.7	1543[b] ± 697	903[b] ± 527 (58)	516[c] ± 318 (32)	387 ± 240 (24)	1.36[b] ± 0.44
Active LL with ENL N = 12	2	2238 ± 991	1350 ± 660 (57)	841 ± 511 (35)	575 ± 296 (26)	1.48[a] ± 0.75
Active tuberculoid (TT/BT) N = 4	0	1656[a] ± 441	1159[a] ± 276 (70)	562[c] ± 173 (35)	501 ± 140 (31)	1.2[b] ± 0.53
Active reversal reaction (1 BB, 1 BB/BT) N = 2	0	2413 ± 1636	1542 ± 1151 (63)	650[a] ± 190 (32)	599 ± 616 (21)	2.0 ± 1.7
Inactive LL N = 7	8	2035 ± 717	1276 ± 639 (58)	827 ± 462 (42)	439 ± 271 (21)	2.0 ± 0.94
Systemic lupus erythematosus N = 27		1475[b] ± 790	1040[c] ± 545 (72)	470[c] ± 295 (33)	510 ± 365 (34)	1.2[c] ± 0.5

[a] $p < 0.05$ as compared with controls.
[b] $p < 0.01$ as compared with controls.
[c] $p < 0.001$ as compared with controls.

tients were treated, but the results in these cases did not differ from the eight untreated individuals.

The following specificities were sought in both blood and tissues: Pan T cell (Leu 1, OKT3), T helper/inducer (Leu 3, OKT4, and OKT3), T suppressor/cytotoxic (Leu 2, OKT8). The following specificities were also sought in tissues: HLA-DR (Ia), thymocyte (OKT6), natural killer (HNK1) and macrophage (OKM1).

Peripheral blood T cell subsets were measured by flow cytometry using an Ortho Spectrum III cytofluorograph, the primary antibody being conjugated with fluoroscein isothiocyanate. Frozen sections were stained using a modified immunoperoxidase technique, the biotin-avidin complex, with diaminobenzidine as the chromogenic substrate.

RESULTS

Peripheral blood studies. As shown in The Table, patients with leprosy, as compared to normal controls, have a deficiency in the T helper/inducer subset with or without an associated lymphopenia and Pan T cytopenia. This helper/inducer deficiency was present when all 27 active patients were taken together, but was particularly well developed in the tuberculoid and lepromatous without ENL groupings. The helper/inducer deficiency was not present in active lepromatous patients with ENL, nor in inactive patients with prolonged dapsone (DDS) therapy. The low helper-to-suppressor cell ratio found in all the active leprosy groupings was attributable to the low number of helper cells, except in the active lepromatous with ENL group, where an increase in the suppressor cells was obviously

important. Patients with systemic lupus erythematosus (SLE) also showed a helper/inducer deficiency in association with a lymphopenia, Pan T cytopenia and a low helper : suppressor ratio.

Tissue studies. The tuberculoid tissues showed the anticipated organization of epithelioid cell aggregates surrounded by a mantle of lymphocytes. Lymphocytes bearing the Pan T antigen were present in the mantle and also present, less densely but regularly, within the epithelioid cell aggregates. Lymphocytes staining for the T helper/inducer antigen were found within the epithelioid cell aggregates, but showed no predilection for the mantle. In contrast, lymphocytes having the T suppressor/cytotoxic antigen were predominantly in the mantle. There were twice the number of helper as compared with suppressor cells. HNK1-positive cells were relatively few in number, were present in small aggregates, and were confined to the mantle. Approximately half of the epithelioid cells stained with the OKM1 antibody, and these were arranged in clusters. The scattered lymphocytes present within the epidermis stained with Leu 1, Leu 2 (or OKT8), or Leu 3 in approximately equal numbers.

In the seven lepromatous specimens the Pan T, helper, and suppressor phenotypes were diffusely present within the histiocytic granulomas, without any mantle or epidermal staining. The five ENL lesions showed a 2:1 predominance of helper cells; whereas that of the two without ENL was 1:1.

In the one BL tissue were an appreciable number of HNK1 positive cells, approximately 10% of lymphocytes, and more T cells than in lepromatous tissues. However, all the subsets were similarly admixed with the mononuclear phagocytes.

In reversal reaction the Leu 1-, Leu 2- or Leu 3-staining cells were diffusely distributed in the granulomas of both specimens. No mantle was present, but lymphocytes did stain in the epidermis.

Virtually every cell in all the granulomas bore the Ia antigen, as did fibroblasts, particularly in lepromatous tissues.

In the epidermis of tuberculoid specimens was heavy staining for Ia and OKT6 antigens, previously shown to be borne by the Langerhans' cells. Also, compared with normal and lepromatous tissues, the numbers of OKT6-positive cell bodies and associated dendrites were increased in tuberculoid lesions.

There was no apparent relationship between the ratio of helper and suppressor phenotypes in the blood and their comparative numbers in tissue specimens.

DISCUSSION

Peripheral blood. The selective deficiency of T helper/inducer cells in patients with leprosy observed here may be of importance in understanding some of the immunological phenomena in leprosy. The absence of the helper/inducer deficiency in patients with ENL suggests that helper/inducer cells may have some role in the pathogenesis of this reactional state. The presence of a similar deficiency of helper/inducer cells in patients with SLE suggests that the immunological abnormalities shared by these two illnesses, such as autoantibody formation, hyperimmunoglobulinemia, circulating immune complexes, and impaired cell-mediated responsiveness, may have a common cellular basis.

Tissue studies. Two immunohistological patterns emerged from this study. In tuberculoid leprosy the helper/inducer cells were among the aggregated epithelioid cells, but the suppressor/cytotoxic cells were predominantly in the mantle. Our results show that the helper/inducer cells are in intimate contact with epithelioid cells, perhaps presenting antigen and stimulating epithelioid maturation. The suppressor/cytotoxic cells are at the periphery of the granuloma, perhaps limiting its extent. The lepromatous and reversal reaction tissues did not show this architectural separation of T lymphocyte subsets. Instead helper/inducer and suppressor/cytotoxic cells were admixed with the mononuclear phagocytes. In lepromatous tissues, a consequence of close proximity of suppressor and helper cells might be an inhibition of mononuclear phagocyte maturation to bacteriolytic epithelioid cells, permitting the florid bacillary proliferation. In reversal reactions, a consequence of the lack of a mantle of suppressor cells might be the associated tissue destruction. Whatever the functional consequences of these differing immunohisto-

logical patterns will prove to be, it is of considerable interest that two patterns have been identified, and more may be recognized, as new antigens are recognized and as other segments of leprosy's granulomatous spectrum become available for study, as is inferred from the relatively large numbers of HNK1-positive cells in the one BL specimen. Ridley has shown that early tuberculoid lesions may be intra-epidermal. The increased number of Langerhans' cells and the presence of lymphocytes in the epidermis of tuberculoid lesions may reflect the host response to intra-epidermal bacilli. Consistant with this is the widely recognized ability of Langerhans' cells to function as macrophages.—[Departments of Dermatology and Pathology, Los Angeles County/University of Southern California Medical Center, Los Angeles, California 90033, U.S.A.]

Matsuo, E., Yamada, K., Sasaki, N. and Skinsnes, O. K. Immunohistopathologic study of β-glucuronidase and lysozyme in human leprosy skin lesions.

Unlike other infectious diseases but similar to some thesaurimoses (storage diseases), the deposition of lipid-acid mucopolysaccharide (AMPS) complex has been observed in lepromatous and not in tuberculoid types of leprosy. Since the pathogeneses of thesaurimoses are often the genetic absences of enzymes capable of degrading the deposited substances in the cells, a similar absence of enzymes might be present in the lepra cells. In this study, we observed the presence of β-glucuronidase (B-Gase) and lysozyme in leprous skin lesions including tuberculoid, borderline, and lepromatous types, since B-Gase participated in the degradation of AMPS, and lysozyme is known to damage bacterial cell walls.

The materials consisted of formalin fixed and paraffin embedded tissues from 13 cases of tuberculoid, borderline, and treated lepromatous leprosy skin biopsies and nine cases of tuberculosis as controls. The sectioned and rehydrated tissues were stained for B-Gase and lysozyme by means of immunoperoxidase methods, with or without trypsin treatment of the tissue sections.

Heavy accumulation of B-Gase was noted only in the spindle or oval cells, which could be young epithelioid cells, in the granulomata of tuberculoid and borderline but not in lepra cells of lepromatous and borderline leprosy. In granulomata of tuberculosis, no distinct accumulation of B-Gase positive cells similar to that of tuberculoid leprosy was identified.

Heavy accumulation of lysozyme was noted in epithelioid and Langhans' giant cells of the granulomata of tuberculoid and borderline leprosy and of tuberculosis and not in lepra cells of lepromatous and borderline leprosy.

The above results clearly indicate that in the granulomata of tuberculoid leprosy the epithelioid cells and Langhans' giant cells have a great deal of lysozyme which is able to destroy bacterial cell walls. These findings might explain the restriction of the growth of *Mycobacterium leprae* in cases of tuberculoid leprosy. However, the same types of cells in the granulomata of tuberculosis also contain significant amounts of this enzyme. Therefore, this finding is not peculiar to leprosy. Nevertheless, it seems to be very important that the lepra cells do not contain this enzyme since the absence of this cell wall digesting enzyme might enable *M. leprae* to grow in lepra cells.

The presence of spindle or oval cells which could be young epithelioid cells showing B-Gase concentration seems to be peculiar to tuberculoid leprosy since these cells were not observed in either lepromatous leprosy or the granulomata of tuberculosis. This enzyme might participate in the mechanisms to eliminate AMPS in tuberculoid leprosy, and lower concentration or absence of it might enable the lepra cells to hold the saccharide, thus permitting the growth of *M. leprae* without digestion. These findings indicate the need for analysis of more cases by the same type of method in order to achieve a better understanding of the pathogenesis of leprosy.—[Department of Pathology, Kyorin University School of Medicine, Tokyo, Japan; Department of Pathology, National Institute for Leprosy Research, Tokyo, Japan; Department of Pathology, University of Hawaii School of Medicine, Honolulu, Hawaii, U.S.A.]

Naka, S. O. and Douglas, J. T. Leprosy ELISA antigen coating buffers and antigens: Comparison of *M. leprae* whole organisms with other mycobacteria.

Using a peroxidase conjugated ELISA, *Mycobacterium leprae* and several other mycobacteria were compared as antigens for the detection of antibody in leprosy patients. In addition, three methods were compared for antigen coating efficiency to microtiter plates.

Whole cells of *M. smegmatis*, *M. triviale*, *M. vaccae*, and *M. scrofulaceum* were suspended in coating buffers at a concentration of 0.15 OD units at 420 nm. The pH 8.2 buffers were 0.15 M Na borate, and 0.01 M ammonium acetate/carbonate. Coating was accomplished by incubating at 37°C for 3 hr or drying at 37°C for 24 hr. It was found that the best coating occurred with the volatile ammonium acetate/carbonate, resulting in $\geqslant$1.5-fold increase in reactivity. With pooled and individual patient sera diluted 1:200, of the non-*M. leprae* mycobacteria *M. smegmatis* was the most reactive, with ELISA OD 492 values being 1.5 to 5 times higher than the other antigens. It was found that autoclaving *M. smegmatis* increased reactivity an additional $\geqslant$1.4-fold, allowing detection of antibody titers of $\geqslant$1:12,000.

M. leprae isolated from armadillo tissue was similarly coated in the volatile buffer at a concentration of 0.07 OD units at 420 nm. Compared with autoclaved *M. smegmatis* at an equal concentration as measured by OD 420, *M. leprae* was found to be twice as reactive. Autoclaving *M. leprae* increased its reactivity. Both *M. leprae* and autoclaved *M. smegmatis* preferentially detected elevated antibody levels in multibacillary leprosy cases. Paucibacillary cases usually had low levels of antibody as detected by these antigens. The levels of antibody in most of the paucibacillary leprosy cases were similar to that of healthy controls. There were no apparent differences in the ability of *M. leprae* or autoclaved *M. smegmatis* to make distinctions between paucibacillary and multibacillary leprosy cases.—[Department of Microbiology, University of Hawaii, Honolulu, Hawaii, U.S.A.]

Abe, M., Ozawa, T., Minagawa, F. and Yoshino, Y. Immunoepidemiological studies on subclinical infection with *M. leprae*. III. Geographic distribution of FLA-ABS test positive responders with special reference to their possible source of infection.

The percentage of positive fluorescent leprosy antibody absorption (FLA-ABS) tests showed significant differences among the inhabitants of different districts in Okinawa. Provided that this percentage indicates the frequency of subclinical leprosy infections, the ratios of the prevalence of subclinical infection per new case with leprosy in the same district ranged from 836 to 2329. The differences in these ratios may be partially due to differences in the age and sex of the individuals examined, because the percentage of positive FLA-ABS responders was significantly higher in schoolchildren and in males than in adults and in females. Adults in rural districts showed higher positive percentages than those of urban adults. The distribution of positive and negative responders in two hamlets where the incidence of leprosy was relatively high suggested the localization of positive responders surrounding houses in which a leprosy case had recently been found and also the distribution of positive responders in the remote houses. A similar percentage of positive responders was also found in the other hamlets where no case of leprosy was reported recently. Therefore, a possible source of infection to these positive responders might be from the environment rather than from direct contact with leprosy patients.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

Bharadwaj, V. P., Ramu, G. and Desikan, K. V. Immunoepidemiological studies on subclinical infection in leprosy in household contacts in India—a preliminary report.

The results of simultaneous testing with FLA-ABS and lepromin in the contacts of multi- and paucibacillary forms of leprosy were as follows. Among 139 contacts of multibacillary forms of leprosy, both tests

were positive in 52 contacts and both were negative in 5. Sixty-four contacts were FLA-ABS positive and lepromin negative. In 18 contacts, the FLA-ABS was negative and the lepromin positive. On the other hand, among 34 contacts of paucibacillary forms of leprosy, the numbers of contacts showing the same results as above were 7, 11, 8, and 8, respectively. Therefore, the FLA-ABS test has been found to detect a greater percentage of subclinical infection among contacts of multibacillary cases who have a greater risk of getting infected than the contacts of paucibacillary or nonlepromatous cases. Those cases who are positive in FLA-ABS and have a negative lepromin response appear to be at higher risk of developing serious forms of the disease. Follow up of the contacts in our study has revealed that many of the cases from the group showing FLA-ABS positivity and lepromin negativity have already progressed to clinical disease in comparison to the other groups.

Another interesting finding is that FLA-ABS positivity appears much earlier in young contacts than the lepromin skin test response. This observation establishes the importance and significance of the FLA-ABS test in the diagnosis of subclinical infection and more so in young contacts. The temporal relationship of humoral and cellular response in contacts merits a special study by serial testing both FLA-ABS and lepromin tests among contacts of the 0 to 5-year-old group.—[Central JALMA Institute for Leprosy, Agra-282001, India]

Vithayasai, V., Nelson, K., Schauf, V., Bullock, W. E., Smith, T., Keuchler, C., Zankel, S. and Scollard, D. The clinical and epidemiological features of leprosy in two resettlement villages.

The care of leprosy patients in isolated locations has been a common practice worldwide throughout history from the Roman era and medieval times to the present. Although an isolated village may provide a leprosy patient with a sheltered supportive environment, where leprosy and any resultant deformities are more accepted by fellow villagers, such isolation can carry the risk of poorer treatment compliance and separation from regular medical and public health care and supervision. If in a leprosy resettlement village drug compliance is uncertain, leprosy prevalence high, and drug resistance present, there may be an increased risk to healthy individuals. There is particular reason to be concerned about exposure of healthy children, since they may have very close contact with infectious individuals. If children develop leprosy, they may have lifelong disease.

The resettlement villages in our study were established about 30 years prior to our survey. Both leprosy patients and healthy persons, including family members of patients, live in these villages. Medical care for the village residents is obtained when patients travel to the nearest large city, located about 70–150 km distant, or when health care workers visit their villages.

In order to determine the clinical and epidemiological features of leprosy in these populations, we evaluated 1210 persons (98% of the residents) living in two northern Thai resettlement villages. Of these 1210 villagers, 326 persons (26.9%) had either active leprosy or a history of the disease and 884 (73.1%) had neither. After a village census each resident had a medical history and physical examination. Additionally, patients and residents with findings consistent with leprosy had slit skin smears.

Children under 16 represent a large proportion (over one third) of the population residing in these two villages. The median age was about 25 years; 14.2% of the residents were 50 years of age or older. Many households were not simple nuclear families. Over half of the residents (57.8%) had at least one blood relative with leprosy.

Many of these villagers with leprosy had not received regular chemotherapy in recent years. Although 287 (88%) of the leprosy patients had had symptoms for 15 or more years, only 74 (22.7%) had taken dapsone (DDS) regularly for at least five years. DDS therapy had been started prior to 1969 in 243 persons, 81.5% of those with a history of leprosy prior to our survey.

We detected 20 new cases of leprosy in these villages; all were of tuberculoid type (BT or TT). Of the previously known cases,

188 (57.7%) were lepromatous (LL or BL), and 138 (42.3%) were tuberculoid (BT, TT) or indeterminant. The Bacteriologic Index (BI) was positive (≥1.0) for 58 and the Morphologic Index (MI) was positive (≥1%) for 12 of these previously diagnosed individuals. The high prevalence of BI and MI positive skin smears among patients several years after their original diagnosis and the high prevalence of new, previously undiagnosed cases indicate that chemotherapy was not entirely effective in this population.

The children in these villages had a high risk for developing leprosy. There were six male and one female children aged 9 to 16 years with new findings consistent with tuberculoid leprosy. The absence of cases in children under nine years of age is consistent with the reported mean incubation period of seven years for children exposed in infancy [cf. Int. J. Lepr. **39** (1971) 745–749]. These children had resided in the resettlement villages for 9–15 years. Four of these children were born in resettlement villages. All but one of the seven children had a blood relative with leprosy or household contact with leprosy patients. Households with at least one patient account for 65% of all households in these two villages.

There were 335 children at risk between the ages of 5 and 16, and the attack rate in this group is 7/335 or 21/1000. Between ages 5 and 16, tuberculoid leprosy was present in 37.2/1000 boys (6 of 161) and in 5.7/1000 girls (1 of 174). The rate of leprosy in this cohort could increase with additional incubation and exposure unless all affected villagers receive appropriate chemotherapy.

The children living in these leprosy resettlement villages are at high risk for tuberculoid leprosy. The prevalence in children aged 5 through 14 years living in a leprosy endemic area of rural northeast Thailand in 1972 was 3.2/1000, which was considerably less than the leprosy prevalence of 21/1000 we have observed [cf. Int. J. Lepr. **34** (1966) 223–243]. The fact that all of the children in the study with leprosy were of tuberculoid type is consistent with other studies finding that most children developing leprosy have the milder forms of the disease [cf. Carec Surveillance Report **6** (1980) 1–3 or PAHO Bull. **14** (1980) 301–303].

The increased risk to the children in leprosy resettlement villages could result from contact within the household or in the village with incompletely treated patients. The risk of transmission of leprosy to healthy people may substantially increase in leprosy resettlement villages in which there is not meticulous attention to appropriate chemotherapy. Treatment compliance and chemotherapeutic efficacy require careful periodic supervision and evaluation. Surveillance for new cases is required. In addition, such villages where treatment compliance has been poor may serve as "natural laboratories" in which to observe, quantitatively, the genetic and environmental risk factors in leprosy transmission and disease in man.—[Chiang Mai University and Chiang Mai/Illinois Leprosy Research Project, Chiang Mai, Thailand]

CLOSING REMARKS

Ladies and Gentlemen:

It has been a great privilege to once again participate in an annual U.S.–Japan Leprosy Research Conference. Once more, we have had the opportunity to meet together, to bring each other up to date on our research findings, and to renew warm, personal relationships. At this Seventeenth Joint Conference, we have been privileged to again hear an excellent group of papers containing a wealth of new information.

As already mentioned by Dr. Abe in his opening remarks, the U.S.–Japan Leprosy programs were reviewed last year. It is gratifying that the progress being made has been recognized by the U.S.–Japan Delegation, and that we can look forward to a continuation of this excellent program. We look forward to welcoming you to the Eighteenth Joint Conference in the United States next year, and we look forward to meeting in conjunction with our colleagues in tuberculosis.

On this occasion I would like to pay tribute to a member of the U.S. Leprosy Panel who was scheduled to be with us here but who died earlier this year, Dr. Howard Fieldsteel. Howard courageously and silently fought cancer for many years without complaint. It is difficult to accept that illness finally bested Howard Fieldsteel. Certainly none of his colleagues who competed with him scientifically, intellectually, or in the appreciations of the joys of life, were able to best this remarkable man. He is, and will be, missed.

Finally, on behalf of the U.S. participants, I would like to express my appreciation for the hospitality of our Japanese hosts in their language.

(The following words poorly pronounced in phonetic Japanese.):

How do you do, ladies and gentlemen. It was a great opportunity and honor to meet with all of you on this occasion. With your cooperation, I am sure we can be successful in expanding the knowledge of Hansen's Disease.

Thank you very much for your warm hospitality.

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