ABSTRACTS

THIRTEENTH JOINT LEPROSY RESEARCH CONFERENCE

Osaka Riverside Hotel
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September 27–29, 1978

U.S.-Japan Cooperative Medical Science Program

FOREWORD

Ladies and Gentlemen, I welcome you, our colleagues from America, here in Osaka.

I wonder how many of you know that this town was once called "Naniwa" or "Namba." As I carry the latter as my family name, it seems to me quite proper that I should speak before anybody else and put aside a formal speech in order to play the part of a guide to this town.

This town, Osaka, happens to be the oldest town in Japan and was created by Emperor Nintoku in the early part of the 4th century, and remained for several centuries the center of the country.

Since ancient times, many immigrants and refugees continued to flow in from countries of the Asian continent by crossing the sea, and it was they who brought the Eurasian culture into this country, including Buddhism. Many valuable relics have been preserved up to the present and are contained in the famous "Shosoin" Temple in Nara, which is not far from here. Nara is another old town and worth visiting. Some of these relics were imported in this age and have been well conserved as national treasures. "Osaka" was "The New World" in those days.

The current Osaka is the most prosperous commercial center in our country, as well as the ancient cultural center. We have a saying that, "the people in Kyoto are extravagant in dress and that of Naniwa in food." I hope you will thoroughly explore this aspect.

If I carry on guiding too much, some of you might be tempted to stay here, which would be very awkward, so I had better return to the routine speech. There are severe difficulties in our way and there may be unforeseen obstacles. To overcome this, a propagation of new blood is required. It is our duty to carry out better research and offer better services, that is creation and practice of doctrine, placing focus on the developing countries. Only this will stimulate the entrance of new investigators and avail good successors. It seems meaningful that the present conference is convened here, this historical town of Osaka.

Now let us proceed to a fruitful discussion of the conference.

Thank you.

MASASHI NAMBA, *Chairman*
Japan Leprosy Panel

PROGRAM OF THE THIRTEENTH JOINT LEPROSY RESEARCH CONFERENCE

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ABSTRACTS OF LEPROSY CONFERENCE

Hirata, Tsunehiko. Electron microscopic observations of *Mycobacterium leprae* by means of serial ultrathin sectioning.

The purpose of the study was to describe the cytomorphologic aspects of cell division and the interrelationships of intracytoplasmic membrane systems of *Mycobacterium leprae* at the electron microscopic level by means of serial ultrathin sectioning.

When division was complete and an electron transparent zone separated the newly formed cells, the new electron-dense cell wall layer was found between the zone and the original cell wall. The mist-like layer was preserved in the final stage of septum formation, surrounding the whole leprosy bacillary cell. The cytoplasmic membrane against the cell wall of newly formed cells was smoother, continuously uniformly dense and well defined. Intracellular membranous organelles that conform to the definition of mesosomes in leprosy bacilli were seen as laminated structures and clusters of vesicles, which were connected to the cytoplasmic membrane adjacent to the cell wall. The diverse morphology of the membrane and its internal tubular components, as well as the very limited area of attachment with the cytoplasmic membrane, was clear in serial series of micrographs. Clumps of leprosy bacilli were confined to the cytoplasmic area of the host-cell, and the morphologic appearances of individual bacilli in the clump showed variety. Their forms were normal, rough, granular, fragmented, vacuolar, coccobacillary and so on.—[National Institute for Leprosy Research]

Okada, S., Okada, Y., Ogawa, T., Mori, T. and Nishiura, M. Electron microscopic study of colonies of *Mycobacterium lepraemurium.*

Colonies of subcultures of *Mycobacterium lepraemurium* grown on slants of Ogawa's egg yolk medium were observed with the scanning electron microscope. In rough type colonies, filaments, mainly 70-120 mμ in diameter, extended between bacilli; granules, mainly 100-200 mμ in diameter, were occasionally found on the surface of bacilli and filaments. Neither structure could be observed in smooth type colonies. In freeze-dried rough type colonies (which were therefore not exposed to organic solvent in the process of drying) the interbacillary filaments could also be observed. Moreover, filaments could be demonstrated in a rough type colony which was crushed gently, suspended in physiological saline solution, negatively stained with a solution of phosphotungustic acid, and observed with transmission electron microscopy.

When leprosy bacilli and murine leprosy bacilli that had been isolated from infected tissue were stained with ruthenium red dissolved in buffered fixatives, a coat of complex carbohydrate could be found at the circumference of the cell wall of ultrathin-sectioned bacilli with transmission electron microscopy, as S. Okada reported earlier. Much information suggests that ruthenium red has an affinity for complex carbohydrates, especially acid mucopolysaccharide. When colonies of murine leprosy bacilli were stained with ruthenium red, the bacilli showed the same coat as that of bacilli grown *in vivo*. The margin of the interbacillary filament was also stained with ruthenium red thus presenting a tubule-like appearance. From these facts it can be concluded that the interbacillary filament is not an artefact formed during the preparation of specimens for scanning electron microscopy.

The interbacillary filaments could be found in rough type colonies of murine leprosy bacilli that had been immersed in chloroform after dehydration with graded ethanol. Therefore, it can be said that the interbacillary filament in the colony of murine leprosy bacilli observed with the scanning electron microscope is different from the filamentous or tapelike substance found by Draper and Rees in murine leprosy bacilli from mouse tissues and regarded by them as mycoside C. Some strains with rough colonies on Ogawa's medium changed to the smooth type after repeated subculture. In such cases the interbacillary filament disappeared before the macroscopic change from rough to smooth type.

The coat visualized with ruthenium red stain could not be found in the shadow-cast

preparations of human or murine leprosy bacilli isolated from lesions. However, a coat with a jagged outer margin could be found on the outside of the cell wall in freeze-etched specimens. Therefore, it can be concluded that the coat has a concrete existence.

It was demonstrated that the homogeneous extracellular substance that fills up the space or valley between bacilli in colonies of murine leprosy bacilli is also a complex carbohydrate and connected with the coat of complex carbohydrate surrounding the cell wall of bacilli.—[Leprosy Research Laboratory, Kyoto University School of Medicine; Food Hygiene Center, Kitasato Institute, Tokyo; Department of Leprology, Research Institute for Microbial Diseases, Osaka University]

Harada, K., Gidoh, M. and Tsutsumi, S. Carbol fuchsin staining of mycobacteria in leprosy institutes of Southeastern Asia and Japan, especially the effects of "basic fuchsin" used in the carbol fuchsin formula.

We have examined the carbol fuchsin staining technics used in leprosy institutes in Southeast Asia and Japan, especially the staining effectiveness of the basic fuchsin used in the carbol fuchsin formula. Different staining procedures are used—hot or cold, with carbol fuchsin formulas in which the basic fuchsin absorption maxima vary from 546 to 556 mμ, different decolorizers, and different decolorizing times. With basic fuchsins that had a low absorption maximum, bacillary staining was always beaded, but with basic fuchsins that had a high absorption maximum staining was often solid. Therefore, it would be difficult to get consistent results in staining intensity and in the form of bacilli. Moreover the Bacterial and Morphologic Indices in different institutes could not be compared. Consequently, the carbol fuchsin stain as it is used is inconsistent and sometimes gives a false picture of the patient's true condition as judged by the Bacterial and Morphologic Indices. It may be that the periodic acid-carbol pararosanilin stain is the most adequate for demonstrating leprosy bacilli.—[National Sanatorium Tama Zensho-en, Tokyo; National Institute for Leprosy Research, Tokyo]

Nakayama, Tetsu and Endo, Hiroko. Acid-fast large bodies found in leprosy material.

During attempts to purify the *M. leprae* suspension obtained from infected armadillo liver, acid-fast large bodies (AFLB) were found in a fraction free of tissue components. An emulsified leproma was washed with distilled water and suspended in 0.5M solution of dibasic potassium phosphate and incubated at 27°C for one hour. By this procedure, nuclei of host cells were destroyed and most tissue components were dissolved. The suspension was then washed two or three times with water by centrifugation. The final pellet contained many AFLB with few bacilli.

AFLB were found in human lepromas, infected armadillo livers and infected mouse foot pad materials. In the case of murine leprosy, the same kinds of AFLB were found in the infected tissues (skin, liver, spleen, testicle, etc.). Moreover AFLB were observed in colonies of the cultivable variant of *M. lepraemurium* grown on Ogawa's egg yolk medium, even after many successive transfers. AFLB could be observed in leproma smears. AFLB was never found in the tissues of non-infected mice.

The natural shape of AFLB may be spherical, but when fixed to the slide it was round or elliptical. The average size was about 40 μm in diameter. There were large or small forms and irregular ones. Masses of AFLB were often observed; agglutination may have occurred during the centrifugation procedure. The surface material of the AFLB was thick, mucous, and strongly acid fast. This capsular substance was not soluble in alkali. The marginal part of the capsule was very often indistinct and weakly acid fast. The central part of mature AFLB was more thick and dense, and bounded by capsular substance with a thin membrane. Immature or incomplete AFLB lacked such a central part, and was thinly coated. There was no specific difference among AFLB of human, armadillo, and murine leprosy, but AFLB found in the infected mouse foot pad materials and in the colonies of the cultivable variant of *M. lepraemurium* on Ogawa's medium were mostly incomplete forms.

For characterization of the surface properties of AFLB, the method of counter-cur-

rent distribution in dextran and polyethylene glycol system was applied. AFLB distributed with the bacilli and were separated from the tissue components. This meant AFLB and the bacilli belonged to the same biological team.

A honeycomb matrix composed of hexagonal units was found as an interior structure of AFLB. The matrix substance was resistant to staining with carbol-fuchsin and showed double refraction. This property made it possible to confirm the localization of the matrix in AFLB with polarization microscopy.

Based upon these observations, it was concluded that the solid content in the unit of the matrix grew up as a bacillary form and came out of the unit. After releasing the bacilli, the matrix gradually dispersed.

Hyphae and macroconidia were occasionally found in human, armadillo or murine lepromas. Relating to those fungal cells, the established positions of AFLB and *M. leprae* might be clarified in the whole system of "Leprophyton" (Nakayama). At present the name "*Sporangium leprae*" (Nakayama) might be used, just as the technical term "*M. leprae*" is used.—[National Institute for Leprosy Research, Tokyo]

Mori, Tatsuo and Nyein, Mar-Mar. Studies on growth factors of *Mycobacterium lepraemurium.* II. Egg yolk protein fraction.

Since *Mycobacterium lepraemurium* grows on 1% Ogawa yolk medium, we have looked for a growth factor for *M. lepraemurium* in egg yolk. Minimal medium was prepared from two volumes of egg white and one volume of modified 1% Ogawa basal medium (1 gm KH_2PO_4, 1 gm sodium glumate, 8.5 gm glycerin and 1.6 mg hemin in 100 ml distilled water) adjusted to pH 6.1 with 1N HCl. Organic solvent-extractable substances of egg yolk, yeast extract, brain heart infusion broth, and water boiled extract of egg yolk were tested for growth factor using minimal medium, but no activity was seen with any of these substances. The organic solvent-extracted residue of egg yolk, however, showed a growth promoting activity, so we sought a growth factor in fractions of egg yolk protein. Egg yolk protein was hydrolyzed with 1 N NaOH, 1 N HCl, trypsin or pronase to remove colored substances and lipid; however, these treatments destroyed growth promoting activity. Eight ml of egg yolk mixed in 100 ml of egg white medium supported growth to a degree that was two-thirds of control 1% Ogawa yolk medium; even 1 ml of egg yolk gave twice the growth with minimal medium. Because sulfhydryl proteins of egg yolk might act by controlling the oxidation-reduction potential, parachloromercuric benzoate (PCMB), which is known to bind to SH groups, was tested; even 100 μg/ml of PCMB did not inhibit the growth of *M. lepraemurium,* however.

Egg yolk was separated into a water soluble and a water insoluble fraction. The water soluble fraction of egg yolk had growth promoting activity. The insoluble fraction supported growth in cooperation with a water-boiled extract of egg yolk. The water-boiled extract of egg yolk could substitute partially for yeast extract. We think that the water-insoluble fraction may contain a growth-supporting protein which controls the oxidation-reduction potential to that optimal for growth of *M. lepraemurium.* Dr. Koseki reported that *M. lepraemurium* can grow on Kirchner medium containing thioglycolate. As the cytochrome system of *M. lepraemurium* differs from that of the other mycobacteria, *M. lepraemurium* may require a specific oxidation-reduction potential in aerobiosis. Such a reducing egg yolk protein may be a growth factor for *M. lepraemurium.*—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Yamada-Kami, Suita City, Osaka; Bacteriology Research Division, Department of Medical Research, Ministry of Health, No. 5 Zafar Shah Road, Rangoon, Burma]

Dhople, Arvind M. and Hanks, John H. Continuous *in vitro* growth of *Mycobacterium lepraemurium.*

In 1972 Nakamura described a novel system in which *Mlm (Mycobacterium lepraemurium)* achieved one cycle of *in vitro* growth. This organism, our interim model for *M. leprae,* has been regarded as an obligate intracellular parasite for 69 years. The keys to the initiation of growth in the Nakamura system were: a) empirical adjustment of oxidation-reduction potential by means of restricted air volumes and the addition of sulf-

hydryl compounds, b) supplementing a synthetic base with seven compounds that compensate metabolic deficiencies, and c) incubation at 30°C. Growth at 30°C ceased after six weeks, serial transfers did not succeed and growth at 38°C could not be accomplished.

30°C. Optimization of the Nakamura system increased growth rates 3.3-fold, i.e., to eight times the rates in susceptible mice. The termination of growth and loss of growth potential after six weeks were due to instability of the supplements—six out of seven were chemically unstable. The most important ones have been successfully replaced by stable precursors or related compounds: cysteine by dithiothreitol, α-ketoglutaric acid by malic acid and hemin by delta-aminolevulinic acid. This permitted the growth of *Mlm* cells and retention of growth potential to eight weeks. Serial transfers each four weeks for 24 weeks (six growth cycles) expanded the original cell populations ten million times, but there was a steady decline in the growth potential (from 24x in first cycle to 9x during the sixth cycle).

38°C. Incubation of the foregoing system at 38°C increased elongation (unbalanced growth) of the cells and severely repressed successful cell divisions and growth rates. This temperature restriction revealed rate-limiting steps in metabolic and biosynthetic pathways. The decline in growth potential at 30°C indicated a requirement of unknown growth factors. These shortcomings were overcome by means of three elements: stable substrates, 20x the usual levels of magnesium and the addition of unknown compounds provided by yeast extract (0.7%) plus tryptic digests of casein (1.0%). Growth now occurs at ten times the rates in susceptible mice. During serial transfers of the cells to new medium each four weeks (total period 44 weeks, 11 growth cycles), cell populations expanded 4.6×10^{16}-fold without any loss of growth potential. Further there is no indication that *Mlm* can yield mutants that are even partially adapted to grow *in vitro*. This indicates that growth is now continuous at the body temperature of the natural hosts.—[This work has been supported in part by grants from NIAID, World Health Organization and The Damien-Dutton Society. Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, Maryland 21205]

Nakamura, Masahiro. Cultivation trials of *M. leprae* in cell-free liquid medium: enigmatic growth of *M. leprae* in ND-15 MT and ST medium.

No growth of *M. leprae* has been observed so far in the NC-5 system (Nakamura, M.. J. Gen. Microbiol. **82** [1974] 385-391) and the ND-5 system (Nakamura, M.. Proc. Jap. Acad. **51** [1975] 707-711) in which *M. lepraemurium* multiplies quantitatively.

Recently, however, enigmatic growth patterns of *M. leprae* were recognized in ND-15 (with oxaloacetate instead of d-ketoglutarate) containing malate or succinate and tyrosine according to preliminary microscopic observations. These culture media are referred to as ND-15 MT (malate, tyrosine) and ND-15 ST (succinate, tyrosine), respectively. The precise composition of the culture medium is as follows: 50 ml of ND-15 base containing 10 ml of calf serum, 6.25 mg adenosine, 1 ml of 0.125% NADH, 1 ml of 5% malate (or succinate), 1 ml of 5% tyrosine, 0.5 ml of 1.16% vitamin C. Four tenths ml of *M. leprae* suspension were added, and the inoculated medium was distributed in 7 ml amounts per tube and cultivated at 30°C. In order to evaluate the growth, culture tubes were centrifuged, and the sediments were resuspended in 0.1 ml formol-gelatine. Smears were made with a loop and stained with Ziehl-Neelsen and observed under a light microscope. The results obtained indicate that the number of clumps of *M. leprae* frequently increased in these media after three months or five months of cultivation. The possibility of contamination with cultivable mycobacteria could be eliminated by l-DOPA oxidase tests, tests of acid-fast extractability by pyridine, and by cultivation on routine culture media for mycobacteria.

Possible growth was frequently recognized when inocula of more than 10^7 *M. leprae* per ml were used. However, the results were not definite and were not reproducible. Therefore, in the future, it should be determined whether these findings represent the genuine growth of *M. leprae* or not by means of quantitative bacillary count, ATP ultrasensitive measurement, and other biochem-

ical analysis.—[Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan]

Nakamura, Kazunari and Yogi, Yasuko. The nude mouse as an experimental lepromatous leprosy model (continued): the enhancing effect of thymus cells in infected nude mice.

We had previously reported that lepromatous leprosy lesions were produced in all nude *(nu/nu)* mice inoculated with a number of *M. leprae* strains. The mice were reared in vinyl isolators under SPF conditions or in conventional animal rooms with air-conditioning or in rooms without air-conditioning. Autoclaved food pellets, drinking water and wood bedding were used in cages with isocaps. Serial passages in nude mice strains were successful. Inoculations were made in one or both hind feet or by various routes. Foot thicknesses were slightly less than approximately 2.0 mm, and systemic lepromatous leprosy lesions without ulceration were characterized bacteriologically and histopathologically.

In the present report, long-term observations were carried out to determine whether heavy lepromatous leprosy lesions developed in thymus-grafted nude mice as compared to that previously reported in CF#1-*nu/nu*. The 6 to 11 week old nude mice on a C57BL/6 background (F6) were maintained in vinyl isolators with SPF conditions. Thymus cells were obtained from heterozygous nude mice (*nu*/+) 7 to 14 days after birth, and 5.0×10^7 cells were inoculated intraperitoneally into infected nude mice five months after right hind foot pad inoculation with 5.0×10^6 *M. leprae* of nude-mouse passage strain K. Of 28 nude mice used there were 10 nontreated mice (4 male, 6 female), and 18 treated mice (10 male, 8 female). We compared the foot swelling of treated and nontreated nude mice and performed bacteriologic and histopathologic investigations and compared the findings with those reported in a similar experiment with CF#1-*nu/nu*. After receiving *M. leprae* by foot pad inoculation, foot swelling in the nontreated group was not observed six to seven months after inoculation. At seven to eight months a little foot swelling (thickness 0.3 mm-1.8 mm) was observed in this experiment.

In contrast the foot thickness of thymus-cell grafted nude mice was increased four weeks after thymus cell inoculation. In some males, thickness was increased more than in the females 12 weeks after thymus cell inoculation. Thus, the difference of sex was remarkable, as previously reported in CF#1-*nu/nu*. Some treated nude mice at 11 to 13 months after infection had gradual decreases in foot swelling, but other males and females had gradual increases in foot thickness to greater than 4 mm, as well as toe and joint swelling. The uninoculated left hind foot was swollen at that time (thickness: 0.3 mm). The bacillary counts in the inoculated foot were over 10^{12} bacilli, and over 10^9 bacilli per foot in the uninoculated foot. Histopathologic findings were similar to that reported previously for CF#1-*nu/nu*. The lesions developed in uninoculated preferred sites such as opposite foot, fore feet, tail, ear, eye, nose, cheek (mystacial site), lip, testis, epididymis, femoral, lumbar, and dorso-lumbar skin and muscle. Tremendous foot, toe and joint swelling were observed (foot thickness: 5-8 mm, weight: 0.5-0.7 gm) in some males and females. In addition, there was hypertrophy of epididymis and deferens and slight swelling of uninoculated fore feet and left hind foot. Thus the epididymis was preferred more than the testis after intravenous, intraperitoneal, and other routes of inoculation. No clinical ulceration due to *M. leprae* was observed at any time during these experiments. Furthermore, jumping behavior, priapism, and pigment loss were observed in some treated and nontreated mice.

These findings have led to the conclusion that a model of severe lepromatous leprosy (more severe than that reported previously in CF#1-*nu/nu* mice) was established in our new model in which thymus cells were grafted five to seven months after *M. leprae* infection in the foot pad.—[National Institute for Leprosy Research, Tokyo]

Sushida, Kiyo. Effects of ALS on the experimental transmission of leprosy bacilli in different mouse strains.

Studies of antilymphocyte serum (ALS) in Aj/e mice have been reported by Gray (1966). He showed that the injection of ALS

caused a profound fall in number of circulating lymphocytes. Similar results were shown in C57BL/6 and C3H/H3 strains of mice. It was suggested that these mouse strains treated with ALS might be used to establish leprosy infections in mice. Gaugas (1968) and Fieldsteel (1975) have already reported the experimental transmission of leprosy bacilli to mice treated with thymectomy and ALS.

The present study examined the possibility that antimouse (C3H/He) ALS be used for immunosuppressing four other strains of mice [C3H/H3, IH-F (ICR × C3H/He), IB-F (ICR × C57/BL/6), and dd] which would be inoculated into the testes with leprosy bacilli taken from lepromatous patients. When the strain was homologous with the ALS (C3H/H3), the mice lived until 5.5 months after inoculation of leprosy bacilli. Nevertheless, they lived for a long time after skin grafts (Chiba *et al,* 1971). That is, these ALS-treated mice might be more wasted after this bacterial infection than after the skin transplantation. The immunosuppressive effect of cross species ALS had already been shown with skin grafts (Winn, 1970).

The positive data in the present work consisted of abundant acid-fast bacilli found intracellularly as globi (+G). The effects of C3H/He ALS were different as far as strains were concerned: dd mice injected with ALS and infected with leprosy bacilli lived much longer than C3H/He mice. However, both C3H/He and dd mice failed to develop infection (+G) with leprosy bacilli. In the hybrid mice, IH-F mice reacted similarly to C3H/He as regards ALS and leprosy infections, and IB-F mice survived eight months longer than the C3H/He groups and were infected with leprosy bacilli (+G). It was very interesting that the positive data with C3H/He ALS were obtained only in heterologous mouse strains, but no definite conclusions can be formed at the present time. If the ALS were to be injected into the homologous mice strains, the dosage might need to be lower than the 0.1 ml used here. —[Department of Microbiology, Tokyo Women's Medical School, Tokyo]

Meyers, W. M., Walsh, G. P., Binford, C. H. and Brown, H. L. Leprosy-like disease (indigenous leprosy) in wild armadillos *(Dasypus novemcinctus).*

A naturally acquired leprosy-like disease, or indigenous leprosy, in seven nine-banded armadillos *(Dasypus novemcinctus)* in Louisiana was first reported by Walsh *et al* in 1975 (J. Reticuloendothel. Soc. **18:** 347-351). Sixty armadillos with indigenous leprosy have now been studied. The animals were captured by independent trappers and delivered to GSRI. Infected animals were from eleven sites in Louisiana and one site in Texas, 12 to 325 miles from GSRI. Smith (personal communication) and Kirchheimer (personal communication) have studied three similarly infected animals from Louisiana; and Fox (personal communication) and Anderson (personal communication) studied one armadillo each from Mississippi and Texas, respectively. Thus a total of 65 animals with indigenous leprosy are now known.

Diagnosis of indigenous leprosy was made by examining ear snips and smears and inguinal lymph nodes for acid-fast bacilli (AFB). Fifty-five of the 60 infected armadillos had AFB in the ears, and all of 48 animals so examined had AFB in inguinal lymph nodes. Histopathologic evaluations were made of tissues obtained at autopsy from 59 of the 60 armadillos. Our findings on 41 of these animals have been reported by Binford *et al* in 1977 (J. Reticuloendothel. Soc. **22:** 377-388), and the findings in the remaining 18 animals were similar. There were AFB in histiocytes, reticuloendothelial cells of liver and spleen, lining cells of pulmonary alveoli, vascular endothelial cells, and in nerves. Some macrophages were filled with AFB and there were globi. The tongue was regularly infected.

The acid-fastness of the AFB was extractable with pyridine and the organisms responsible for the infection were nonculturable. Suspensions of liver, spleen, lepromas and inguinal lymph nodes of 41 animals were inoculated onto mycobacteriologic media and incubated at 32° and 37° C. Small numbers of *M. avium-intracellulare* were cultivated from inguinal lymph nodes of 8 of the 41 animals, and the spleen of 1 of the 41 animals. Individual lepromins were prepared from lepromas or lymph nodes from six armadillos with indigenous leprosy. These lepromins were assayed in a total of 146 leprosy patients. Each of the specimens gave a pattern of Mitsuda reactions similar

to those provoked by lepromas of human origin: lepromatous patients gave negative or very weak reactions and tuberculoid patients gave strong reactions. Thirty-three purified suspensions of a total of 47 specimens from lepromas, lymph nodes, spleen and liver oxidized D-DOPA. We are not completely certain of the specificity of this oxidation reaction in the system we employed because some suspensions of normal armadillo tissue gave a positive reaction. This may have been an auto-oxidative process because boiled tissue was reactive. Although we believe it is possible, or even likely, that the bacillary suspensions oxidized D-DOPA, the possible participation of armadillo tissue components must be considered.

Three separate groups of eight, six and nine armadillos that had been screened for indigenous leprosy and found free of the disease were injected subcutaneously, respectively, with 4.0×10^8, 4.9×10^6, and 6.7×10^9 AFB from three naturally infected armadillos. Of these three groups of armadillos, respectively, four, three and five animals developed a widely disseminated mycobacteriosis that histopathologically and microbiologically was like that seen in naturally infected armadillos. Lepromin prepared from one animal gave typical Mitsuda reactions in leprosy patients. The first evidence of infection in the passage armadillos was a nodule at the sites of injection 9 to 14 months after injection. Widely disseminated disease developed 16 to 25 months after inoculation. Infection of the mammary gland was detected in one lactating armadillo with disseminated indigenous leprosy. This animal gave birth to a quadruplet set of infants four months after admission to the GSRI laboratory. All infants died within seven days and at autopsy there was no evidence of disease. The milk of the mother contained AFB. Histopathologic examination of a biopsy specimen of the nipple revealed numerous well-stained AFB, singly and in clusters in the walls and endothelial cells of blood vessels, and in the lining cells of the mammary ducts. This animal at autopsy had a far advanced, widely disseminated mycobacteriosis that was in all respects typical of indigenous leprosy.

The dimensions of the geographic area from which animals with the disease have been reported are approximately 300 to 400 miles (480×640 km). The size of the area argues strongly against a single-source origin of the infection. The elapsed time between the establishment of experimental leprosy in the armadillo and detection of the indigenous disease does not support the concept that there is an association between experimental leprosy and indigenous leprosy in the armadillo. Although direct evidence is lacking, we believe that the best explanation for the origin of the indigenous infection is that the armadillos became infected initially by contact with *M. leprae* of human origin and have propagated the infection by armadillo-to-armadillo passage. This could be by any of a number of routes: e.g., by the respiratory passages, by shedding of organisms from the tongue or open lesions, or in the milk from mother to young. Insects may play a role in transmission, either by biting or as part of the diet of the armadillo.

Studies on the epidemiology of indigenous leprosy are urgently needed. Information thus obtained may help establish modes of transmission of leprosy in man.—[Armed Forces Institute of Pathology, Washington, D.C. 20306; Gulf South Research Institute, New Iberia, Louisiana 70560]

Kohsaka, K., Yoneda, K., Mori, T. and Ito, T. The study of chemotherapy of leprosy with nude mice.

It was previously reported by the authors that lepromatoid lesions developed in nude mice inoculated with *Mycobacterium leprae;* acid-fast bacilli obtained from the lesion were identified as *M. leprae.* Furthermore, we reported that a new model of experimental leprosy in laboratory animals was established by the use of nude mice; successive transmission of *M. leprae* to other nude mice was confirmed by the experiment, and the reproducibility of transmission of *M. leprae* derived from several different patients to nude mice was also demonstrated. We therefore, attempted the application of the new model of animal transmission to the field of chemotherapeutic study of leprosy.

A relapsed LL patient was treated with rifampicin 450 mg daily for two months, and materials for inoculum were obtained by biopsy before treatment and after two

months of treatment. A BL patient was treated with 450 mg daily of rifampicin, and biopsy was done before treatment on day 4 with a two day intermission after two days of treatment, on day 9 with a two day intermission after one week of treatment, and after one month of treatment. In the experiment of chemoprophylaxis, the infected mice were given 0.5 mg (once), and 0.2 mg (6 days a week, for 2 weeks) of rifampicin orally; the doses of 0.5 mg and 0.2 mg in the mouse are equivalent to 1,500 mg and 600 mg in man. Clindamycin (0.2 mg) and DDS (0.03 mg) were also given to infected mice for one month, from day 20 after infection; the dose of 0.2 mg in the mouse is equivalent to 600 mg in man, and the dose of 0.03 mg is near to 100 mg in man. For examination, the number of bacilli in the foot pad at the site of inoculation was counted according to our modification of the method of Shepard, and the foot pads were examined histopathologically.

The results of the experiments indicated that rifampicin shows tremendous initial killing effect for *M. leprae;* the bacilli lost the infectivity for nude mice after only 2 days of administration with 450 mg of the drug to man. It was also suggested that single administration of 1,500 mg once and/or 600 mg daily for two weeks of rifampicin may be effective as chemoprophylaxis of leprosy. Chemoprophylactic administration with a daily dose of 0.03 mg of DDS and/or 0.2 mg of clindamycin for one month was not able to prevent the growth of *M. leprae* in nude mice.

It is hoped that the nude mouse technic will be adopted at many institutes, and the studies of chemotherapy and chemoprophylaxis of leprosy will be promoted rapidly by the use of nude mice.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka]

Fieldsteel, A. Howard and Levy, Louis. Combination dapsone-rifampin therapy in neonatally thymectomized Lewis rats (NTLR) chronically infected with *Mycobacterium leprae.*

In previously reported experiments, we had found that a single oral dose of 10 or 20 mg/kg of rifampin given to *M. leprae*-infected NTLR did not render *M. leprae* noninfectious for mice. It did result in an increase in the generation time of up to 30 days greater than that observed in controls, indicating that bacterial killing had occurred. However, a combination of the minimal effective dose (MED) of dapsone (DDS) given for 49 days in combination with a single dose of 10 mg/kg rifampin administered on the 35th day of dapsone therapy reduced the proportion of viable *M. leprae* by a maximum 1:100,000, indicating a remarkable synergistic effect.

Since one of our initial objectives was to produce a model of persisting *M. leprae,* we set up an experiment utilizing NTLR infected 13 months previously with 5×10^4 *M. leprae* in the foot pads. We gave 10 mg/kg rifampin with the brief course of the MED of dapsone with the expectation that this regimen would be nonsterilizing, but would appear to be fully effective when *M. leprae* from treated NTLR were inoculated into intact mice two weeks after cessation of treatment. Unfortunately, treatment with this regimen was not fully effective as assessed by mouse foot pad subinoculation. *M. leprae* harvested at the end of treatment multiplied in normal passage mice with the generation time (G) of 71.9 days. Mice inoculated with 5×10^3 *M. leprae* obtained from NTLR 78 days after rifampin treatment in the same regimen were negative for growth of *M. leprae* after one year, while another group of mice inoculated with *M. leprae* from the same NTLR gave a G of 49.6 days. *M. leprae* harvest from NTLR at 148, 175, 207, and 330 days were infective for intact mice, with Gs ranging from 24.9 to 32.5 days. Since Gs were equivalent to those obtained in mice inoculated from untreated NTLR, it could be assumed that there was complete regrowth from the initial killing of *M. leprae.* This experiment also included a group of NTLR that received 5×10^{-5} g% dapsone in the diet continuously until death, in addition to the 10 mg/kg rifampin treatment at 35 days. *M. leprae* from NTLR killed 37, 231, 246, and 330 days after rifampin produced no infection in intact mice. *M. leprae* from NTLR obtained 4 and 70 days after rifampin were infectious for mice.

In these experiments NTLR were simultaneously inoculated with between 5×10^5 and 1×10^7 *M. leprae* from the same animals

whose organisms were inoculated into intact mice. In all but one instance in which organisms failed to grow in mice, they were infectious for NTLR. In no instance did organisms fail to grow in NTLR when they were infectious for mice.

In a third experiment now under way the objective is to provide information on persistence of *M. leprae* that would be useful in the design of therapeutic regimens. In this experiment, the duration and frequency of rifampin were varied. The drug regimens and the results of passage to intact mice and NTLR are shown in the table below.

M. leprae from the group given one dose of 10 mg/kg rifampin on the background of the continuous MED of dapsone were infectious for mice 14, 32, and 123 days, but not 273, 284, and 303 days after rifampin treatment. In the group given two doses of rifampin on the background of the MED of dapsone, passages were positive in mice after 22 days (G = 93.8), but not after 149, 238, and 284 (6 month harvest only) days. Multiplication did occur in passage NTLR at 149 and 284 days. *M. leprae* from the group given 10 doses of RMP on the background of the continuous MED of DDS were not infectious for mice, but were infectious for NTLR at 8, 149, and 197 days.

Two NTLR from the group receiving 100 times the MED of DDS (5×10^{-3} g%) and one dose of 10 mg/kg of rifampin on day 35 were sacrificed 14 days after rifampin and on the 49th day of DDS treatment. The organisms were infectious for both mice and NTLR. *M. leprae* obtained 149 days and 168 days after rifampin treatment were not in-

TABLE 1. *Chemotherapy of* M. leprae *infection of neonatally thymectomized Lewis rats (NTLR).*

	No. of days[a]	Results of passage (generation time in days)[b]	
		Mice	NTLR
None	—	23.8	
5×10^{-5} g% DDS	14	34.1	294, 108, NM[c] (2)
continuously from day 1	32	67.6	NI[d]
+ 10 mg/kg RMP on 35	123	118	60.7 NM (2)
	273	>100[e]	P[f]
	284	> 100	P
	303	> 100	P
5×10^{-5} g% DDS	22	93.8	NI
continuously from day 1	149	NM	88.4, 154
+ 10 mg/kg RMP on	238	NM	NI
days 35 and 39	284	>100	134, NM
5×10^{-5} g% DDS	8	NM	94.1, 153, 184
continuously from day 1	72	NM	NM
+ 10 mg/kg RMP on days 35	149	NM	99, 104
to 39 and 42 to 46	197	NM	81.6, 73.4
5×10^{-3} g% DDS on days 1	14	35.3, 42.2	64.0, 88.6, 274
to 49	149	NM	47.6
+ 10 mg/kg RMP on day 35	168	NM	118
	281	>100	NI
	282	>100	P

[a] Time from first dose of rifampin (RMP) to harvest and passage.
[b] Mice inoculated with 5×10^3 AFB/foot pad; NTLR inoculated with between 10^5 and 10^7 AFB/foot pad.
[c] NM = failure of *M. leprae* to multiply on passage.
[d] No NTLR inoculated.
[e] >100 = no multiplication after six months; one year harvest pending.
[f] Harvest pending.

fectious for mice, but had a G of 47.6 days and 118 days, respectively, in passage NTLR.

Based on the preliminary results presented here, it appears that the best chance of obtaining a regimen that will produce persisting *M. leprae* is to give repeated doses of rifampin on the background of the MED of dapsone. In any event, it appears that the NTLR, because of its high degree of immunosuppression can be used to detect a small proportion of surviving *M. leprae* in inocula containing up to 5,000 times as many organisms as can be inoculated into intact mice. —[This work is supported by the U.S.-Japan Cooperative Medical Science Program, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education and Welfare (Grant R22 AI-08417, and by a grant from the World Health Organization). Life Sciences Division, SRI International, Menlo Park, California 94025; Hebrew University—Hadassah Medical School, Jerusalem, Israel]

Tsutsumi, Sadae and Morrison, Norman E. Studies on clofazimine mode of action.

Clofazimine has been found efficacious as an antileprosy drug, especially in the treatment of sulfone resistant leprosy. Clofazimine has the structure of a phenazine quinoneimine that readily undergoes reversible oxidation and reduction. During studies on cross resistance relationships using clofazimine-resistant mutants isolated in the laboratory, it became apparent that a one-way cross resistance was present between rifampin and clofazimine-resistant mutants. By contrast, rifampin-resistant mutants were fully sensitive to clofazimine. This latter observation, first seen in laboratory-derived mutants, has now been seen in rifampin-resistant *M. leprae* mutants isolated from clinically resistant patients and tested in the mouse foot pad at the USPHS Hospital, Carville, Louisiana.

Due to the fact that clofazimine will form complexes with DNA and that the degree of interaction is dependent upon the G + C content of the strand, the suggestion was put forth that clofazimine, or one of its metabolites, may interact with the high G + C mycobacterial strand to preferentially inhibit template function. Because of the one-way cross resistance relationship a possible likely result of strand binding was the inhibition of DNA-dependent RNA polymerase, the enzyme responsible for transcription.

The results of initial studies on clofazimine inhibition of the transcriptase enzyme will be reported. Single-stranded templates were used to measure transcription by the holoenzyme based on label incorporation from ^{14}C-ATP into the acid-insoluble RNA containing fraction. In comparing inhibitions produced by clofazimine versus rifampin, a known RNA polymerase inhibitor, it was evident that different mechanisms were in effect. For example, significant inhibition with clofazimine was found with templates containing a high G + C content. Transcription from such templates was inhibited some 50% of total count incorporated by clofazimine at 1.5×10^{-5}M by preincubation of the DNA template with the drug prior to the addition of holoenzyme. During the time course of the assay the clofazimine inhibition was not maintained, indicating that in the presence of holoenzyme a slow dissociation of the DNA-clofazimine complex occurred and transcriptase activity subsequently appeared. The reasons for this slow dissociation in relation to the mode of action of clofazimine will be discussed.—[National Institute of Leprosy Research, Tokyo; Department of Pathobiology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland]

Tsutsumi, S., Gidoh, M., Minagawa, F., Yoshino, Y., Narita, M., Kaniwa, S., Matsumura, J., Takitani, S. and Fukushi, K. Fundamental studies on immunopotentiative substances.

In order to find a promising method for clinical treatment of persistently positive cases (PP), immunopotentiation therapy by the polysaccharides (PSs) used in the cancer field has been examined. As a first step, the clinical treatment of a few PP patients by PSK was performed. PSK is PSs simply extracted from *Coriolus versicolor* which belongs to the basidiomycetes. It can be

orally administered. After finding a clinical process characterized by lowering in the occurrence of ENL and the temporary but marked increase in immunologic responsiveness, trials of other PSs, named OK-432 and TGDS (and intramuscularly administered) was performed. The results obtained were not hopeful, however. ATSO was purified from PSK by Terada *et al,* and found by Sato *et al* to have an effect superior to PSK on experimental cancer in rats. Chemical studies on acidic PSs of *Sasa senanensis* (PSs-SS) and the derivatization of BHP-activated dextran have been performed. The former had been studied for its anticancer effect. The latter was synthesized to find a promising substance of higher purity than plant source PSs; the mechanism of action is imagined to be nonspecific but to normalize the surface and then intracellular function of lymphocytes according to the general mechanism for the PSs series.

It was found that BHP-T (T: taurine as the ligand) and PSs-SS extracted by a Thin Channel under low temperature exhibited stronger immunopotentiation effects which were at the most comparable to ATSO when examined by an improved Cunningham method. The effect of BHP-T on guinea pigs was further examined by Yokomuro's method. Both of BHP-T and PSs-SS showed an antiexudative effect on carrageenin-induced acute edema in the rat; it was especially strong with the former, possibly as a result of the affinity to venular intima. The compounds showed no antifungal activities. The acute and subacute toxicities of BHP-T to mice were found to be trifling.

As a result of chemical studies on PSs-SS, sulfate possibly bound to glucose was presumed to be a component of PSs. Other components, arabinose, xylose, galactose and uronic acid, were measured gas-chromatographically. Free monosaccharides such as ribose and mannose could scarcely be detected in PSs. The molecular percent of amino acids of protein and the contents of alkalies in *Sasa senanesis* were also determined.—[National Institute for Leprosy Research, Tokyo; National Leprosarium, Tama Zensho-en, Tokyo; National Saitama Hospital; Bethlehem Hospital; Tokyo Science University; Nihon Medical College]

Okamura, Kazuko. Chemotherapy for leprosy and anterior uveitis.

The introduction of chemotherapy has had a profound effect on the treatment of leprosy lesions. Observed from the viewpoint of ophthalmology, small nodules of the lids, cornea, sclera and iris can be expected to regress slowly and ultimately almost to disappear with chemotherapy. However, chemotherapy has no effect on uveitis, and chemotherapy is contraindicated for exudative uveitis.

It is very important to consider the possible blindness of patients, because serious damage and reduced visual acuity follows chemotherapy. The clinical observations of anterior uveitis on 30 leprosy patients in the National Leprosarium of Tama-Zensho-en, Tokyo were made by means of the slit-lamp, and the clinical classification of leprosy anterior uveitis was attempted. Leprous uveitis in ophthalmological texts is equivalent to granulomatous. However, there is granulomatous and nongranulomatous uveitis as observed clinically. Therefore, leprous uveitis was classified in three types. The interrelations of the appearance of these kinds of uveitis and the skin conditions of patients were observed.—[National Leprosarium Tama-Zensho-en, Tokyo]

Mehra, Vijay and Bloom, Barry R. The induction of cell-mediated immunity to human *M. leprae* in the guinea pig.

Guinea pigs were immunized with intact or disrupted armadillo-grown human *M. leprae* administered in aqueous or oil vehicles. In addition, comparisons were made of route of administration and the effect of BCG and water-soluble adjuvants on the degree of sensitization. For assessing delayed-type hypersensitivity, guinea pigs were tested with various dilutions of *M. leprae* suspended in saline, water-soluble *M. leprae* extract, PPD and a water-soluble extract of normal armadillo tissue. The results demonstrated:

1. Under no conditions was any skin test reactivity found to normal armadillo tissue extract.

2. Significant degree of sensitization to both *M. leprae* and its water-soluble extract was achieved by sensitizing guinea pigs with

M. leprae suspended in Hanks solution or saline. All suspensions or emulsions were effective at sensitization, but appeared to be no better, and in general, slightly weaker, than simple inoculation in aqueous suspension. In the guinea pig, the intradermal route appeared to engender slightly better reactivity than did inoculation in the foot pad.

3. Autoclaved *M. leprae* in Hanks or saline inoculated intradermally appeared to be the most effective immunogen. This protocol does not require an oil vehicle, appears to be relatively safe, and should be considered for human sensitization studies.

4. Studies on sensitization of guinea pigs with a mixture of killed *M. leprae* and living BCG failed to demonstrate any significant adjuvant effect of BCG on sensitization to *M. leprae*. Indeed, there was some evidence of slightly decreased sensitization when *M. leprae* were given together with BCG. However, water-soluble cord factor appeared to potentiate significantly the sensitization to *M. leprae* in aqueous suspension.

5. The water-soluble *M. leprae* antigen extract appeared to be remarkably *M. leprae*-specific in that it gave significant reactions in guinea pigs sensitized to *M. leprae* and failed to show significant reactivity in guinea pigs immunized with BCG. This soluble type antigen offers the possibility of a useful *M. leprae*-specific skin test antigen preparation.

6. The minimum dose required for sensitization with *M. leprae* in aqueous suspension was 55 μg of purified bacilli.

7. Animals inoculated with *M. leprae* in saline or together with BCG showed positive skin test reactivity to the first skin test application made fully one year after initial sensitization.—[Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461]

Hastings, R. C., Shannon, E. J. and Job, C. K. Studies on the antigenic interrelationships of *M. leprae*.

Polar lepromatous leprosy (LL) is characterized by a permanent absence of detectable cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) to antigens of *M. leprae*. Although controversial, there is a school of thought which holds that this anergy on the part of LL patients is on a genetic basis, and that such individuals are, from birth, unable to respond with CMI to *M. leprae*. In order to be effective, a vaccine for leprosy must be capable of successfully inducing CMI to *M. leprae* in individuals who would ordinarily develop LL after infection with *M. leprae*. Only by doing so could the vaccine prevent LL and therefore eliminate the most bacilliferous cases that are the most likely to transmit the disease to others. We have reasoned that if the inability of LL patients to respond to *M. leprae* is on a genetic basis, then a vaccine based on *M. leprae* itself would likely be ineffective, and that the most promising candidate for a vaccine would be the mycobacterial species antigenically most closely related to *M. leprae* which could also immunize individuals genetically predisposed to developing LL. Inherent in this line of reasoning is that a species which is antigenically identical to *M. leprae,* or nearly so, may well be unable to immunize individuals genetically predisposed to developing LL. As the initial step in identifying a potential candidate for a vaccine, a ranking of mycobacterial species as to their antigenic relatedness to *M. leprae* as measured by CMI and DTH was attempted.

The model chosen for study consisted of Fort Detrick Duncan Hartley guinea pigs immunized with armadillo-derived *M. leprae*. The presence of CMI and DTH to integral *M. leprae* and to integral preparations of various cultivatible mycobacterial species were assessed with skin testing, macroscopically and histopathologically, and by lymphocyte blast transformation studies using lymph node cells challenged with integral antigens *in vitro*.

Doses of autoclaved, armadillo-derived *M. leprae* in Freund's incomplete adjuvant from 10^4 to 10^9 organisms per animal given in divided doses into the foot pads essentially did not result in immunization of these animals when they were tested four weeks later. Single doses of autoclaved *M. leprae* in Hank's balanced salt solution (HBSS) ranging from 10^4 to 10^9 organisms per animal did not result in reliable immunizations in our hands, except at relatively high doses. Autoclaved *M. leprae* given in doses of 10^4 to 10^9 organisms per animal in HBSS in two doses, separated by four weeks, and testing the animals four weeks after the second immunization, caused significant degrees of

CMI but was also associated with considerable humoral immunity as evidenced by neutrophilic, as well as round cell infiltrates of skin biopsy sites and Arthus-type reactivity at skin test sites four hours after challenge, as well as 24-hour reactivity. Immunization with viable armadillo-derived *M. leprae* in single doses of 10^4 to 10^8 organisms per animal in HBSS resulted in more satisfactory responses at higher doses with minimal evidence of humoral immunity. After priming the animals with viable (cryopreserved) *M. leprae* in the foot pads, boosting them four weeks later with viable (cryopreserved) *M. leprae* intradermally, and testing four to five weeks later, rankings of 25 species of mycobacteria were obtained relative to *M. leprae*. Rankings obtained by skin testing reflected Arthus type sensitization at both 4 and 24 hours after challenge and were not correlated with rankings obtained by lymphocyte blast transformation.—[Supported in part by grants from the World Health Organization (IMMLEP) and the Victor Heiser Award. Pharmacology Research Department, USPHS Hospital, Carville, Louisiana 70721. This investigation received support from the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Program for Research & Training in Trop. Dis., and the Victor Heiser Award]

Collins, F. M., Watson, S. B. and Morrison, N. E. Antigenic relationships between *M. vaccae, M. nonchromogenicum* and *M. leprae.*

The nature of the antigenic relationships which exist between *M. leprae, M. vaccae* and *M. nonchromogenicum* have been the subject of a number of investigations in recent years. *M. vaccae* is a rapid grower sometimes isolated from cattle whereas *M. nonchromogenicum* and its close relative, *M. terrae* are slow growing nonchromogens found only in soil and not apparently associated with human or animal diseases. Several isolates of *M. vaccae* have been obtained from cattle and opossums in New Zealand and these were compared with the TMC type strain by means of intravenous, intracutaneous (flank skin), subcutaneous (foot pad) or intramuscular inoculation into specific pathogen-free mice and strain 2 guinea pigs. The survival of the inoculum was determined by quantitative bacterial counts at weekly intervals up to two months. The behavior of the *M. vaccae* strains was compared with that of BCG Pasteur and BCG SM^R. The effect of increasing the number of viable *M. vaccae* introduced into C57Bl/6, DBA-2, A, C3H, Balb/c, CBA and outbred ICR mice was compared with that seen in athymic (nude) mice and their nu/+ littermates. Quantitative viable counts of the mycobacterial populations within the foot pads, popliteal lymph node, liver and spleen indicated that the *M. vaccae* strain dropped sharply in viability early in the infection in all of the mouse strains and in the guinea pigs. However, the liver and spleen populations in the intravenously challenged C57Bl/6J and Balb/c mice stabilized after seven days and persisted *in vivo* for several weeks. There was little observable difference between the growth curves for the *M. vaccae* strains introduced into the athymic and their nu/+ control littermates. The decline in viability by the type *M. vaccae* population in the mouse foot pad was quite rapid and was analogous to that observed for BCG SM^R. Somewhat surprisingly, rough translucent colony variants of *M. nonchromogenicum* and *M. terrae* survived better within the foot pads of the test mice than did *M. vaccae* although none of them caused significant systemic disease. None of the infected animals developed significant delayed hypersensitivity responses following the injection of 5 or 25 μg of the appropriate cytoplasmic antigen extracts.

In order to determine whether *M. vaccae* or *M. nonchromogenicum* possess sensitizing antigens, groups of B6D2 (C57Bl × DBA F_1 hybrid) mice and strain 2 guinea pigs were immunized with repeated intravenous doses of 10^8 live *M. vaccae* or *M. nonchromogenicum*. Other mice were injected subcutaneously with suspensions of 5×10^8 dead BCG SM^R, *M. vaccae, M. nonchromogenicum* or armadillo-derived *M. leprae,* suspended in Tween saline or in Freund's incomplete adjuvant. The killed vaccines were reinjected 14 days later. The animals were foot pad or skin tested weekly using graded doses of PPD or the corresponding cytoplasmic proteins derived from *M. vaccae, M. nonchromogenicum* or *M. leprae.* Quantitative measurements of the increased skin or foot pad thickness were made after 3, 6, 24, 48, or 72 hours and the nonspecific swelling seen in

age-matched unvaccinated controls was subtracted before the reactions were assessed for their specific and cross-reactive activity. Some of the mice were injected in the left hind foot pad with 5×10^8 dead intact *M. vaccae* or *M. leprae* cells (Mitsuda type antigens) and the swelling profiles were determined over the first 72 hours and then at weekly intervals for up to six weeks. The significance of these findings is discussed in relation to the existence of cross-reacting sensitins to *M. leprae* antigens in the various mycobacterial species tested.—[Trudeau Institute, Saranac Lake, New York; Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland]

Caldwell, H. D., Kirchheimer, W. F. and Buchanan, T. F. Identification of a *Mycobacterium leprae* specific protein antigen and its possible application for the serodiagnosis of leprosy.

Acetone-killed *M. leprae* separated from infected armadillo liver tissue without the use of proteases were treated with 0.2 M Lithium Acetate, 20mM EDTA, pH 8.8 solution and the concentrated antigen extract was analyzed by Ouchterlony immunodiffusion. The antigen extract gave a single immunoprecipitate when reacted with pooled lepromatous leprosy (LL) patients' sera made highly specific for *M. leprae* by adsorption. Apparently identical precipitates were produced by reacting the antigen extract with each of 15 treated LL patients, 5 of 7 patients with tuberculoid leprosy, and 3 of 4 *M. leprae* infected armadillos. Human control sera including those from 18 patients immunized with BCG, and 15 patients with brucellosis or nonspecific urethritis, and uninfected armadillo sera failed to react with the antigen. Identically prepared extracts of *M. smegmatis, M. phlei, M. vaccae, M. duvale,* and *M. diernhoferi* gave no immunoprecipitates with sera from LL patients or infected armadillos. Preliminary characterization indicates the antigen is protein since antigenicity was destroyed by pronase and/or heat treatment. The specificity of the protein antigen for *M. leprae,* and the presence of antibody to this antigen in patients with leprosy suggest a possible role for this antigen in the serodiagnosis of leprosy.—[Immunology Research Laboratory, USPHS Hospital, Seattle, Washington and Department of Medicine and Pathobiology, University of Washington, Seattle, Washington; Laboratory Research Branch, USPHS Hospital, Carville, Louisiana]

Abe, M., Yoshino, Y. and Saito, T. Immunologic and epidemiologic studies on subclinical infections with *M. leprae.*

The fluorescent leprosy antibody absorption (FLA-ABS) test was improved by absorbing the serum with the suspensions of BCG and *M. vaccae.* The test was positive in nearly 100% of the patients with bacillus-positive forms of leprosy, but negative in 18 patients with pulmonary tuberculosis in 50 healthy noncontacts and in 65 out of 66 patients in general hospitals. Therefore, the sensitivity and specificty of the FLA-ABS test was proved to be satisfactory for detecting sublinical infections with *M. leprae.*

The test was positive in 57 of 62 (91.9%) cases of household contacts. The mean antibody titer was higher in infants, in children and grandchildren of the patients, and in contacts of lepromatous patients than in comparison groups. Among 39 cases tested with lepromin, 7 cases showed doubtful Mitsuda reaction but were positive in the FLA-ABS test. Such cases should be carefully observed.

Among 15,000 schoolchildren in a leprosy endemic area, 173 children were tested with FLA-ABS because they had a palpable auricular nerve or suspicious skin eruption. A positive reaction was found in 63%. The percentage of positivity was slightly higher in the villages than in urban area, corresponding to the higher leprosy incidence rate in the former. The FLA-ABS test showed no correlation with the results of tuberculin test or a history of BCG vaccination in these children. Their sera were also examined by indirect immunofluorescence with the smear of BCG (BCG-FA test) and by the rubella virus hemagglutination inhibition test. However, these tests showed no correlation with the FLA-ABS test. Among 58 sera in which both FLA-ABS and BCG-FA tests were positive, 57 sera did not react with six strains of mycobacteria other than *M. leprae;* one case did and it was later found to be leprosy.

From these observations it is presumed that the rate of subclinical infection of *M. leprae* in the schoolchildren in this area is less than 1% and more than 200 times larger than

the leprosy incidence rate in this area.—[National Institute for Leprosy Research, Tokyo; National Leprosarium Okinawa Airaku-en]

Koseki, M., Sanada, K., Harada, K., Hazama, S. and Ozawa, T. Immunopathologic observations in long-term BI negative lepromatous leprosy patients.

The important problem for Japanese leprologists is how to prevent exacerbation in lepromatous leprosy patients who have been BI negative for a long time, more than ten years. If exacerbation will occur, the time of occurrence and method of treatment must be studied.

As the first step, immunologic and histopathologic examinations were performed. All cases were lepromatous leprosy patients who had been skin-smear negative for a long time (11 males, 12 females). The percentage of peripheral lymphocytes in the differential count of white blood cells was normal. The total serum protein and its different fractions in almost all cases were within normal limit. Alpha-1-globulin showed normal value in 12 cases and was decreased in others. Alpha-2-globulin was normal in 8 and increased in others. Beta-globulin was normal in 20 and increased in 3 cases. Gamma globulin was normal in 11 and increased in 12 cases. RA was positive in 3 cases and CRP in 8 cases. ASLO had normal values in all cases. Both antinuclear antibody and Coomb's tests were negative in all cases. Ig-G was normal in 18 cases, decreased in one, and increased in 4 cases. Ig-A was normal in 18 and increased in others. Ig-M was normal in 21 and increased in others. C3 was normal in 12 and increased in others. CH50 was normal in 12, decreased in 7, and increased in 4 cases. Twenty-two were positive in PPD skin tests, 12 with Dh-R, 16 with PHA, and 20 with DNCB.

Histopathologic findings on skin biopsy specimens from all patients were stained with H&E, Ziehl-Neelsen and the modified allochrome procedure. The epidermis showed thinning. The rete ridges were very short or absent. Liquefaction degeneration in the basal layer was observed in some. In the pars papilaris, perivascular and both intra- and subpapilary lymphocytic cellular infiltration were recognized. In the pars reticularis, cellular infiltration around vessels and appendages was also observed. Significant inflammatory or granulomatous cellular infiltration was not observed. The atrophy of skin appendages was remarkable.—[National Leprosarium Tama-Zensho-en, Tokyo; National Leprosarium Ohshima-Seishoen, Kagawa; National Institute for Leprosy Research, Tokyo]

Shepard, Charles C. and Minagawa, Fumishige. Further studies on the immunizations of mice with *M. leprae* antigen.

Our previous studies have shown that heat-killed *M. leprae* differ from other reported mycobacteria in being completely immunogenic when administered in aqueous media. Therefore, the preparation of *M. leprae* vaccines from armadillo-grown organisms appears technically feasible. The present studies were directed toward learning whether the methods in use for purification of *M. leprae* from armadillos were harmful to immunogenicity; only foot-pad-enlargement results are available at this time. Determination of the dose reponse curves showed that if the immunizing and eliciting dose exceeded 1×10^7 organisms the dose response was flat or inverse. Hence, the doses used for immunization were usually 1×10^7 and a smaller dose. The protease treatments used in purification (24 hours of pronase or trypsin and chymotrypsin) proved to be harmful to the immunizing or eliciting activity in most experiments. Presumably, the loss in activity is not so great as to be detectable in all experiments.

Five-minute treatment with trypsin, however, distinctly promoted immunogenicity, probably as a result of partial purification. The high degree of purification that is achievable with the two-phase polymer procedure (dextrose T500:polyethylene glycol) was not harmful either and sometimes increased immunogenicity. Gamma irradiation was not harmful at a dose of 0.25 megarads or, in a single experiment, in a dose of 2.5 megarads. The yields of bacilli by the two-phase polymer procedure before or after the five-minute trypsin treatment are low (about 20%) but complete after the 24-hour treatment with trypsin and chymotrypsin, so there is a question at the moment as to how to proceed with the development of a vaccine.—[Ack-

nowledgment. This work was partly supported a) by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases (NIAID) by means of an interagency agreement between the NIAID and the Center for Disease Control, and b) by the World Health Organization through its IMMLEP program. The infected armadillo livers were kindly supplied by Dr. W. F. Kirchheimer through contacts with NIAID and IMMLEP]

Patel, Parsottan J. and Lefford, M. J. Specific and nonspecific resistance in mice immunized with irradiated *Mycobacterium leprae.*

Following subcutaneous inoculation of irradiated *Mycobacterium leprae* (I-ML) into the hind foot pads of mice, there was inincreased resistance to *Listeria monocytogenes* at the immunization site, indicative of macrophage activation. In spite of the high level of localized macrophage activation, which was proportional to the immunizing dose of I-ML, no such activity could be demonstrated systemically in these mice, as evidenced by the absence of increased resistance to an intravenous challenge with *L. monocytogenes.* Under these conditions, I-ML immunized mice were nonetheless resistant to intravenous infection with either *M. tuberculosis* or *M. bovis,* BCG, and this immunity was transferred to normal recipients using spleen or lymph node cells. Neonatal thymectomy completely prevented the velopment of antimycobacterial immunity after immunization of mice with I-ML, but immunity was restored by an intraperitoneal infusion of syngeneic thymocytes. Systemic nonspecific resistance to a challenge with *L. monocytogenes* could be generated in I-ML-immunized mice, but not in normal controls, by an intravenous injection of either disrupted I-ML or PPD.

This study reveals that after subcutaneous immunization of mice with I-ML, there is local accumulation of activated macrophages at the inoculation site and a widespread distribution of lymphocytes which are sensitized to mycobacterial antigens. Nonspecific resistance is mediated by the former cells and specific antimycobacterial immunity by the latter.—[Trudeau Institute, Inc., Saranac Lake, New York 12983]