U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

Seventh Annual Leprosy Research Conference

Stanford Research Institute Menlo Park, California

February 29 - March 1, 1972

FOREWORD

The Seventh Annual Leprosy Research Conference was held at the Stanford Research Institute, Menlo Park, California. This one, and the previous U.S. annual conferences, were sponsored by the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program, which is administered by the Geographic Medicine Branch of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. As usual, the program consisted essentially of reports of leprosy research completed during the previous year and it thus provided a review of progress in leprosy research by U.S. leprosy scientists and their collaborators overseas. As before, only one presentation was allowed for each participant. The Japanese also have a national meeting on leprosy held in the spring each year. In addition to the national meetings, a joint Japan-U.S. leprosy research conference has been held each year; attendance by members of the visiting country is somewhat restricted because of the expense of foreign travel. These leprosy research conferences have afforded a focus of interest and information that has been essential to the growth of leprosy research. By the publication of the abstracts we hope to make the information available to our colleagues in other countries.

CHARLES C. SHEPARD, Chairman U.S. Leprosy Panel

Program of the Seventh Annual Leprosy Research Conference

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Program of Leprosy Conference

Feldman, R. A. Leprosy in the United States, 1969-1970.

There were 233 cases of leprosy reported in the United States, including Puerto Rico, in the years 1969-1970; 87% of the cases (202) were reported from seven areas: California, Florida, Hawaii, Louisiana, New York City, Texas and Puerto Rico. Of the 233 cases reported in the two year period, 69 (29.6%) were born in the United States, including Puerto Rico and 121 (52%) were born in the four countries of Mexico, Philippines, Cuba and Samoa. Lepromatous and dimorphous leprosy were diagnosed in 155 (68%) of the 227 patients whose clinical type was reported. In 198 the diagnosis instances, was biopsy confirmed, and only 16 patients had no biopsy performed or no biopsy information was recorded. Ninety-six of the 233 patients were hospitalized at one of three Public Health Service hospitals, while 138 either were not hospitalized or were seen in medical schools or general hospitals.

Prabhakaran, K., Harris, E. B., and Kirchheimer, W. F. The nature of the phenolase enzyme in *Mycobacterium leprae*.

Besides *o*-diphenoloxidase (tyrosinase, phenoloxidase), several other enzymes and copper proteins can oxidize phenolic substrates to pigmented products. It is known that diphenols like 3,-4-dihydroxyphenylal-anine (DOPA) are oxidized by peroxidase. There are reports in the literature that, along with tyrosinase, peroxidase is involved in the conversion of DOPA to melanin pigment in the human skin. It was of interest to study whether the dopa oxidase of *M. leprae* is a peroxidase or a true diphenol oxidase.

Commercially available horseradish peroxidase oxidized DOPA even in the absence of peroxide, due to contamination of the preparations with tyrosinase. Heating the enzyme $(70^{\circ}C)$ inactivated the tyrosinase, but retained the peroxidase activity. Both catalase (from beef liver) and peroxidase could oxidize DOPA in the presence of hydrogen peroxide. Without peroxide, these enzymes showed no oxidation of DOPA. Peroxide was not required for the activity of *o*-diphenoloxidase. *M. leprae* oxidized DOPA without added hydrogen peroxide, and also when catalase was added to destroy any endogenous peroxide. Earlier results had shown that *M. leprae* possesses both catalase and peroxidase. A few nonpathogenic cultivable mycobacteria tested had high catalase activity but no peroxidase.

Because o-diphenoloxidase is a copper protein, two copper proteins, ceruloplasmin and hemocyanin, were tested to see wheth. er they would oxidize DOPA. At low concentrations at which tyrosinase was active, neither ceruloplasmin nor hemocyanin showed any activity. Both the copper proteins at high concentrations stimulated oxidation of DOPA. However, this was found to be nonenzymatic, because they showed the same effect even after heating at 100°C. Exposure to high temperatures inactivated o-diphenoloxidase.

Laccase (*P*-diphenoloxidase) is another enzyme which oxidizes phenolic substrates. Hydroquinone is oxidized by laccase but not by tyrosinase. It was found that the phenolase of **M. leprae** does not oxidize hydroquinone.

Diethyldithiocarbamate (DDC) was the most potent inhibitor of the phenoloxidase enzyme in the leprosy bacilli. Three groups of mice were treated with DDC, diasone or rifampin directly in the foot pads, six months after inoculation of the foot pads with M. leprae. After six months of treatment, no significant reduction in the number of organisms was found in the mouse foot pads. The bacilli recovered from the control and the treated mice were reinoculated into new groups of mice. Six months later, the organisms from untreated mice had multiplied normally. Only a slight increase in number was noted in the case of organisms obtained from mice treated with diasone. On the other hand, bacilli from animals which received DDC or rifampin showed no multiplication. DDC is a copper

chelator. Another copper chelator, pencillamine, which did not inhibit phenolase of *M. leprae*, had no effect on multiplication of the organisms in mouse foot pads.—[This study was supported by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-07890)]

Dhople, A. M. and Hanks, J. H. Fate of Mycobacterium lepraemurium in diffusion chambers incubated in vitro and in vivo.

ATP is the source of biologic energy and assayable in picogram amounts. This criterion of growth potential has now been refined to a degree which is adequate to evaluate claims of limited microscopic growth of Mlm (Mycobacterium lepraemurium) in vitro and in vivo. Mlm suspensions were sealed in diffusion chambers and were incubated (a) in vitro, as per Oiwa's method, in test tubes containing Oiwa's medium renewed three times a week, and (b) in vivo, in the mouse peritoneal cavity by Rightsel. Microscopic counts and ATP determinations were conducted to learn whether either of the reports on extracellular growth of Mlm could be substantiated.

The in vitro chambers in 15 imes 100 mm test tubes at 37° were supplied routinely with 2 ml of Oiwa medium (base plus supplements, plus 10% bovine serum, plus 10% filtered mouse brain extract). Controls included: (a) the bacteria centrifuged against the bottom of 10 mm test tubes, with media renewed by methods that did not disturb the bacteria; (b) the basal medium only; (c) mouse brain extracts not filtered and mouse brain extracts pasteurized; (d) the foregoing four media not renewed; and (e) heat-killed bacteria. Results were assessed by pooling the contents of three chambers or tubes at each sampling period.

Since the results in the different experimental conditions and media agree closely, averages of all variables were combined. After 17 days the bacterial counts were 72% of the original, while the ATP per cell had fallen to 10% of the original. Equivalence was observed between diffusion chambers and test tubes and between the basal medium and supplementation with the various mouse brain extracts. Renewals of media three times a week did not sustain the energetics of the bacilli.

The *in vivo* chambers supplied by Dr. Rightsel had been maintained in the mouse peritoneal cavity for 50 days. The bacterial counts increased 3.4-fold and the ATP levels 2.5-fold. After correcting for the decreased length of the incubated bacilli, the actual increase in biomass was 2.7-fold. This, insofar as it is known, represents the first validation of the extracellular growth of an "intracellular" parasite.—[This work was supported by the U.S.-Japan Cooperative Medical Science Program administered by National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-08866)]

Imaeda, T. and Hawley, R. The possible / role of prophage(s) in mycobacterial mutation.

In our previous reports, the coccoid mutation of mycobacteria was explained in terms of the presence of prophage(s) in the wild type. The present study gives additional information which supports our interpretation on the relationship between prophage(s) and mycobacterial mutation.

The presence of prophage(s) in the wild type of Mycobacterium smegmatis ATCC 607 was suggested by the following experimental data: (1) although the wild type does not produce any infective phage particles with several inducing agents, bacitracin induces phage-like particles; (2) isoniazid treatment results in the formation of the coccoid mutant which liberates the infective mycobacteriophage MC-4, spontaneously or upon induction; (3) although the parental wild type is susceptible to MC-4 at 37°C, the immunity to this phage is exhibited at 43°C; and (4) restriction and exclusion systems do not seem to function in the parental wild type at the higher temperature.

The coccoid mutant is characterized by disordered septation, absence of both mycolic acid and mannose in the cell wall, rapid growth (four times faster than the wild type), and release of infective mycobacteriophages.

On the other hand, we obtained MC-4 lysogenized strains which confer immunity to MC-4 at 37°C. Interestingly enough, no infective phage particle can be induced from these strains, nor do they show any of the metabolic changes mentioned in the case of the coccoid mutant.

Based on these results, we assume that metabolic changes in the coccoid mutant may be related to the change of prophage function, with special reference to the replication gene function.

Reich, C. V., Abalos, R. and Madarang, M. A quantitative comparison of standard Ziehl-Neelsen vs. Nyka (periodate treated) stained smears from leprosy patients.

The Ziehl-Neelsen stain is routinely applied for differentiation of mycobacteria from other microorganisms and for visualization and microscopic quantitation of mycobacteria for clinical and research purposes. Recently there has been some evidence (Nyka. J. Bact. 93 [1967] 1458; Reich, Internat. J. Leprosy 39 [1971] 25) to indicate that some members of a given mycobacterial population might not be stained by this procedure. Nyka termed these cells "chromophobic" and demonstrated that they could be made visible by pretreatment with periodic acid.

This study was undertaken to determine whether the chromophobic characteristic was evident in leprosy specimens. Duplicate standardized skin scrape smears were made from lesions of 40 patients. On 37 other patients, selected lesions were biopsied. The biopsies were processed by preparing suspensions in buffered saline using Ten Broeck grinders. Duplicate smears of ten lambda aliquots of the suspensions were prepared. One smear was stained by the procedure of Shepard and McRae [Internat. J. Leprosy **36** (1968) 78] and the duplicate smear was stained in the same manner following overnight treatment with periodic acid. These smears were coded and given blind to two technicians for counting. After the data was collected the standard (untreated) smear was treated overnight in periodic acid and again stained. This slide was recoded and recounted.

One . hundred forty-seven periodate treated smears were compared with seventy-five routine Ziehl-Neelsen stains. The data showed eleven periodate treated smears with acid-fast cell counts below an accepted variation of \pm 30% of the standard Ziehl-Neelsen stain. Sixty-eight within accepted variation and sixty-eight periodate treated smears had over 30% more acid-fast cells than the standard. Of the eleven smears showing a reduction in acidfast bacilli, ten were slides that were restained after routine processing. These results would lend support to a conclusion that some leprosy bacilli are chromophobic to the routine Ziehl-Neelsen stain.-[Supported in part by the World Health Organization]

Chang, Y. T. and Andersen, R. N. Motion picture studies on macrophages in mouse bone marrow cultures.

Since *M. leprae* is an obligate intracellular parasite of macrophages, a thorough understanding of the function of the host cells is of paramount importance. One disadvantage in the study of macrophages is the lack of a long-term culture model for continued observation of the activity of macrophages. Such a model is now available in the cultivation of mouse bone marrow cells and a simple technic for longterm cinemicrography which has recently been developed in this laboratory.

This presentation is a 15 minute movie, showing various functions of macrophages, including cell division, cell death, phagocytosis, garbage removal, zeiotic bleb formation, and an intimate relationship with colonies of granulocytes. An interesting phenomenon observed in certain large, round cells was the formation of numerous tiny vacuoles which fused into very large vacuoles which subsequently collapsed. This cycle of formation and collapse of the vacuoles repeated for many hours, even for days, and seemed to be a normal function of these cells. Although the function of this phenomenon is not clear, it might be of importance in furnishing some clue in studies of lepra cell formation.—[Aided in part by Emmaus-Suisse through the World Health Organization]

Skinsnes, O. K. and Sakurai, I. Leprosy, lipids and lipophages.

Relatively few analyses of lipids in leprosy lesions are available despite the ever obvious lipid storage aspect of the lepromatous deficiency. Such studies as have been published have been based on histochemical technics and quantization is not recorded.

Histochemical and thin-layer chromatographic lipid analyses, with quantization, will be briefly presented and compared. These indicate that the major lipid content differences lie in a markedly high content of glyco- and phospholipids in lepromatous tissues as compared to tuberculoid lesions and normal skin. It is noted, however, that these methods of analysis probably do not measure or attack the true lipid storage problem. Both histochemical and chromatographic technics measure lipid fractions after variously separating them from the lipid complexes of their origin. This is suggested by some variable findings between the histochemical and chromatographic methods. It is probable that the lipids measured are components of unidentified waxes and it is possible that their measurement is a false indication of the true status of lipids in lepromatous tissues. It is likely that the detection technics accomplish a chemical breakdown of waxy substances which the lepromatous macrophages are incapable of, or very slow in accomplishing. The actual in vivo lepromatous tissue content of phospholipid may thus consist of wax-bound phospholipid and not free phospholipid. This may explain the absence of epithelioid cell formation and true granuloma structure, despite high phospholipid content in lepromatous lesions, if the earlier observations of Refvem are correct. He concluded, after an extensive review (Acta Med. Scand. Supp., 1954, pgs. 9-146), that epithelioid cells and granuloma formation occur in tissues where phospholipids are deposited as a result of any of several possible mechanisms.—[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grants AI-08644 and AI-10034)]

Kirchheimer, W. F. and Storrs, E. E. Leprosy in experimentally infected armadillos.

Histopathologic and bacteriologic postmortem findings on armadillo GSRI 8 are presented. This particular animal had been inoculated 520 days before its spontaneous death with 8.9 \times 10⁶ leprosy bacilli into each of two cutaneous sites at the abdomen and with an equal-number of bacteria into both earlobes. Materials and methods and findings during the life of this animal were described by W. F. Kirchheimer and E. E. Storrs (Internat. J. Leprosy 39 [1971] 692-701). This report also deals with development of a spreading form of leprosy in an additional armadillo, GSRI 5. The final part of this report gives some survival data of leprosy bacilli at the inoculation sites of 17 armadillos.

Histopathologic and bacteriologic postmortem findings for armadillo GSRI 8. Intracutaneous inoculation of viable (mouse foot pad multiplication) leprosy bacilli resulted in systemic leprosy. Bacteria were found in the blood, bone marrow, lymph lungs (macrophagic nodes, meninges, pneumonitis), spleen, liver, uninoculated skin, a nodular skin lesion in an uninoculated site, and hind foot pads (Table 1). In the gastrointestinal tract, in the kidneys, pancreas, testicle, adrenals, diaphragm and heart muscle, leprosy bacilli were present in relatively small numbers. There was no sign of central nervous system involvement except in a small part of the cauda equina of the spinal cord. Nerve tissue per se of the peripheral nerves was not involved, but

perineurium and loose connective tissues were heavily invaded. The uveal tract of the eye showed lepromatous involvement. Enumeration of the bacteria in various tissues showed heavy bacterial loads and for skin where comparable data are available was much higher than in human lepromas $(2.0 \times 10^{10}/\text{gm vs. } 10^7 \text{ to } 10^8 \text{gm})$ (Table 1). Viable bacteria (mouse foot pad multiplication) were found in liver, lymph node and skin. Reasons why bacteria from spleen and lung failed to proliferate are speculative (Table 2). Slides were shown to illustrate the histopathologic and bacteriologic findings. The occurrence of a leprous pneumonitis is the most unique finding. Remarkable are the number of bacteria. Worthy of discussion is their apparent failure to multiply apart from macrophages.

Leprosy in armadillo GSRI 5. This armadillo had been inoculated with the same bacteria, the same dose at the same time and at the same sites as armadillo GSRI 8. Numerical increases of the bacteria at the inoculation sites, their presence in the blood stream and in uninoculated skin signalize spreading disease. The bacterial load in the skin of this armadillo is only a fraction of that found in armadillo GSRI 8. Histopathologic evaluation of biopsy material shows strong lymphocytic response in some places with lepra cells not present universally. Bacteriologically and histologically this animal shows a response of partial resistance. Slides were shown to illustrate the bacteriologic and histologic findings.

Viability of leprosy bacilli at the inoculation sites of 17 armadilos. Except in armadillos GSRI 1-4, all leprosy bacilli used for infecting the armadillos were viable (mouse foot pad multiplication). Bacterial survival from 7 to 18 months seems to have occurred in the inoculated skin in 6 out of 17 armadillos. Slides were shown to illustrate these findings.-[The work was supported by Research Grant CC-00476 from the Center for Disease Control, Atlanta, Georgia and by the Japan-U.S. Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-03636)]

TABLE 1. Number of M. leprae per gm of autopsy material.

Right earlobe	$2.0 imes 10^{10}(7)$
Lymph node (groin)	$8.9 imes 10^{9}(15)$
Spleen	$6.6 imes 10^9(21)$
Brain	$6.2 imes 10^5(38)$
Uninoculated skin	$1.1 imes 10^9(12)$
Lung	$3.9 \times 10^8(12)$
Liver	$2.2 imes 10^8(33)$
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Numbers in parentheses are Morphologic Indices.

TABLE	2.	Mul	tip	ication	of	M	۱.	leprae	e from
autopsy	ti	ssue	of	armadi	illo	8	in	the	mouse
				foot pa	d.				

Tissue origin of bacteria	Number of bacteria inoculated per foot pad	Number of bacilli har- vested per foot pad after six months
Inoculated skin (right abdomen)	$1.2 imes10^4$	$9.8 imes10^5$
Uninoculated normal appearing skin	$1.0 imes10^4$	$1.4 imes10^6$
Liver	$1.6 imes10^4$	$9.1 imes10^5$
Spleen	$1.0 imes10^4$	$5.8 imes10^3$
Lung	$1.2 imes10^4$	$5.6 imes10^3$
Brain	$1.0 imes10^4$	0
Inguinal lymph node	$1.5 imes10^4$	$1.1 imes10^6$

Evans, M. J. and Levy, L. Fate of *M. leprae* inoculated in the mouse foot pad.

The purpose of these experiments was to determine what happened to *M. leprae* inoculated in the mouse foot pad. To accomplish this a series of experiments were performed. In the first group of experiments, mice were inoculated in both foot pads with 1.40 to 2.64×10^6 organisms. The animals were sacrificed at intervals from 2 hours to 27 days after inoculation. Foot pads from some mice were prepared for microscopy and the others used to determine how much of the original inoculum could be recovered.

The microscopic results of these first experiments, which utilized normal BALB/c and thymectomized-irradiated B6C3F1 mice, showed that the tissue of normal BALB/c mice responded first with an influx of polymorphonuclear cells and later lymphocytes and monocytes. The latter formed a diffuse infiltrate in the tissues. The organisms were found within these phagocytic cells and in the interstitial space. No organisms were observed in striated muscle fibers of tissues studied. In thymectomizedirradiated animals the initial reaction was the same, however the mononuclear response did not persist after seven days.

Studies to show the number of organisms which could be recovered from the foot pad after inoculation revealed there was a large loss of the original inoculum from the foot pad. At two hours after inoculation about 30% of the organisms could be recovered from the foot pad. About the same number or less were recovered at later times. The mean percentage of organisms recovered was 19.7 with a range of 4.8% to 39.1%.

In a second group of experiments, normal BALB/c mice and thymectomizedirradiated B6C3F₁ mice were inoculated in the foot pad with 5×10^3 *M. leprae.* No recovery of organisms was attempted. Microscopy of the foot pad tissue revealed changes similar to those seen in previous groups. First there was an influx of polymorphonuclear cells followed by lymphocytes and monocytes. The response was very slight when compared with the groups inoculated with 10^6 organisms, and after the seventh day no inflammatory cells were found in the tissues.

In order to estimate the loss of organisms from a small inoculum $(5 \times 10^3 \text{ cells})$, an inoculum using radioactive particles in place of organisms was prepared. Normal BALB/c mice were inoculated in the foot pad with Tc^{99m}-sulfur colloid which had been filtered so as to provide particles ranging from 0.2 to 0.8 μ in diameter. The colloidal suspension was diluted so as to provide inocula of about 5×10^3 or 10^6 particles per foot pad. Foot pad tissue and inguinal nodes were obtained one and two hours after inoculation and prepared for scintillation counting. Recovery of inoculated particles was about 30% for the small inoculum and 20% for the large; no concentration of the radioactive material was found in the regional lymph nodes.

These studies have shown that; (1) the tissue of the mouse foot pad responds to inoculation of M. leprae with an influx of polymorphonuclear cells followed by mononuclear cells, (2) in immunologically depressed animals and those given a small inoculum the mononuclear response does not persist, (3) the organisms present are contained within phagocytic cells, and (4) the organisms in the foot pad at this time represent only about 30% of the original inoculum .- [This work was supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grants AI-10110 and AI-07801)]

Yang, H. Y., Su, W. P. and Skinsnes, O. K. Effects of neonatal thymectomy on experimental infections of *M. lepraemurium* and *M. leprae*.

Newborn mice were thymectomized and infected with M. lepraemurium or M. leprae by the intraperitoneal route within 24 hours after birth. Control animals were sham-thymectomized and similarly infected. Mice infected with M. lepraemurium were used primarily to study peritoneal macrophage response. Thymectomized mice showed impairment of macrophage response in the peritoneal cavity and an early dissemination of infection to viscera (spleen and liver). At 10 weeks of age the bacillary count of tissue (spleen and liver) homogenate revealed 10 times the number of bacilli in the thymectomized group as in the controls.

Mice infected with M. leprae (27% solid form) were sacrificed at 1, 2, 4, 6, 8 and 10 months of age and bacillary counts of tissue homogenate (spleen, liver, lung and kidney) were compared in the thymectomized, sham-thymectomized and nonthymectomized groups. In thymectomized animals, the total number of bacilli in vis-

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cera increased slowly and reached a peak at six months of age. Thereafter, the number of bacilli began to decrease in coincidence with the recovery from the reduced macrophage response in the peritoneal cavity. In sham-thymectomized and nonthymectomized animals, however, visceral bacillary count showed a steady decrease of both the solid form and the non-solid form bacilli. At six months of age the difference in bacillary count between the thymectomized and sham-thymectomized animals was approximately 100-fold.

The results suggest that neonatal thymectomy reduces host resistance to obligate intracellular mycobacterial infection and reduces host macrophage response to the infection. These effects lasted for about six months in mice during which time there was an increase of *M. leprae* bacilli in the visceral organs.—[This investigation was supported in part by a grant from the University of Hawaii and in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-10034)]

Fieldsteel, A. H. Mycobacterium leprae infection in neonatally thymectomized rats. Effect of radiation and antithymocytic serum.

Neonatally thymectomized Lewis rats treated with antithymocytic serum (ATS) have been shown to be highly susceptible to foot pad and testis infection with M. *leprae* (Fieldsteel and McIntosh, Proc. Soc. Exp. Biol. Med. **138** [1971] 408.

In a more recent experiment with Lewis rats we attempted to find the best method of immunosuppression for promoting maximum growth of *M. leprae*. Twelve months after inoculation with 6.5×10^4 *M. leprae* the only definite conclusion that could be drawn was that neonatal thymectomy alone was highly effective. Maximum numbers of *M. leprae* in the hind foot pads of the untreated rats was 1.38×10^6 . In contrast, there was a maximum of 1.04×10^9 acid-fast bacilli in the hind foot pads and testes

of a rat which received no treatment beyond thymectomy. This was a larger number of organisms than was found in rats which additionally received either ATS, radiation, or both. It also reflected the fact that the testes contained 6.84×10^8 organisms which was almost twice that found in any other group. The largest number of M. *leprae* found in the foot pads was 6.06×10^8 in a thymectomized rat given five daily doses of 2.4 ml of ATS starting on the day of inoculation of M. *leprae*. To date, spread of M. *leprae* to uninoculated sites has been observed only in thymectomized-ATStreated, or thymectomized-irradiated rats.

It also appears that *M. leprae* infection may be enhanced in neonatally infected rats exposed to as little as 450 r given four months after infection. Ten months after inoculation with 2×10^4 *M. leprae* the testes of one rat yielded 8.82×10^8 acidfast bacilli. By comparison the testes of thymectomized-irradiated (950 r) BC3F₁ male mice have shown only a tenfold increase over the inoculum. This cannot be ascribed to effects of heavy radiation on the viability of the testes since testes of thymectomized-irradiated (925 r) Lewis rats have yielded as many as 4.60×10^8 *M. leprae.*

It is still too early to know which is the best method of immunosuppression, or if a fulminating lepromatous type of disease will result in these rats.—[Supported by the U.S.-Japan Cooperative Medical Program administered by the National Institute of Allergy and Infectious Diseases, National Institute of Health (Grant AI-08417)]

Congdon, C. C., Payne, H., Gengozian. N. and Urso, P. Immune status at twelve months in thymectomized radiation chimeras.

The immune status of $BC3F_1$ mice at twelve months after thymectomy, irradiation and syngeneic bone marrow transplantation was measured using anatomic, humoral and cellular measures of immunity. Body weight at the twelve month interval averaged 19 grams compared to 39 grams in normal control mice. An extreme wasting

syndrome in certain mice contributed to low average weight in the experimental groups. Spleen and peripheral lymph node weights were likewise lower in the experimental mice compared with control animals. Autopsy and cellular pathology study failed to explain the extreme wasting in certain experimental mice. Thymus dependent areas of lymphatic tissues were depleted of lymphocytes in the experimental group but the number of germinal centers was equal to control values. Tail skin allografts at the twelve month interval showed an average 20 day mean rejection time in thymectomized chimeras. Control mice rejected at 10 days. Both primary and secondary humoral antibody production were deficient in the experimental group for rat red blood cell antigens. In control mice the mean primary and secondary log₂ titers were 8 and 14 compared to 1 and 1 at the third weekly bleeding after antigen in the thymectomy group.

One other piece of data about the thymectomized radiation chimera is the primary *in vitro* response of dispersed spleen cells to sheep red blood cell antigens at two to three months after thymectomy. Hemolytic plaque formation was about one half control values in the short-term culture situation.—[The Oak Ridge National Laboratory is operated by Union Carbide Corporation under contract with the U.S. Atomic Energy Commission]

Bullock, W. E. Perturbation of lymphocyte recirculation in leprous rodents.

The capacity of thoracic duct lymphocytes (TDL) from normal Lewis rats to recirculate through lymphoid tissues of rats infected with *Mycobacterium lepraemurium* was measured. A mean of 1.3×10^6 ³H-uridine labeled TDL per gm body wt was given i.v. to four matched pairs of infected and normal rats. Lymphocytes were then collected from thoracic duct fistulas (TDF) every eight hours for three days. The mean peak (16-24 hr) lymphocyte output (LO) of normals was 22.0 \times 10⁶/hr and 17.7 \times 10⁶/hr in infected rats; their outputs were also below normals at all

other time intervals. By radioautography, the ratio of labeled lymphocyte output (LLO) from infected vs. normal rats at 24 hours was 0.56. Four pairs of rats were depleted of lymphocytes by TDF three days prior to i.v. injection of labeled TDL. In lymph collected every four hours thereafter for 48 hours, the mean LO of normals was 5.4 imes 10⁶/hr at 0 hours with a peak of 17.4×10^6 /hr at 16 hours. LO of infected rats at 0 hours was 4.4 imes 10⁶/hr and increased only to 7.1 \times 106/hr at 16 hours. The ratio of LLO from infected vs. normal rats at 16 hours was 0.41. Two pairs of lymphocyte-depleted rats were sacrificed 24 hours after injection of 51chromiumlabeled TDL. Mean radioactivity per 100 mg of spleen and lymph nodes from infected rats was 38.5% and 35.0%, respectively, of that in normals. Results show impaired TDL recirculation in rats with murine leprosy, possibly due to entry-block at lymph nodes and splenic white pulp.-[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-10094)]

Krahenbuhl, J. L., Remington, J. S., and Levy, L. Protection of mice against multiplication of *Mycobacterium leprae* by chronic infection with *Toxoplasma* gondii.

Previous studies from this laboratory have shown that mice chronically infected with the protozoan parasite Toxoplasma gondii are nonspecifically resistant to a variety of phylogenetically unrelated intracellular pathogens such as Listeria monocytogenes, Salmonella typhimurium, Brucella melitensis, Cryptococcus neoformans and Mengo virus. The present study was performed to determine if this nonspecific resistance extends as well against Mycobacterium leprae. BALB/c mice infected i.p. with the relatively avirulent C56 strain of Toxoplasma were challenged 40 days later in both hind foot pads with 5 \times 10³ M. leprae. Harvests of M. leprae 17 to 30 weeks after challenge revealed marked resistance to M. leprae growth in Toxoplasma infected mice. M. leprae multiplied in these mice, but at a rate much slower than in controls. The doubling time during logarithmic multiplication of M. leprae was approximately 37 days in Toxoplasma infected mice, compared with 13 days in control animals.

Additional experiments were carried out to test the local effects of a booster injection of Toxoplasma antigen administered into the M. leprae infected foot pad. Toxoplasma infected mice which had harbored M. leprae infections in both foot pads for 197 days received Hanks' balanced salt solution (BSS) in both infected foot pads. Thirteen days later M. leprae was harvested from foot pads, counted, diluted to provide a standard inoculum, and injected into passage mice. Compared to the BSS-injected controls, injection of Toxoplasma antigen produced a marked loss of viable M. leprae. Following passage, the time required to reach the plateau value of 10⁶ bacilli per foot pad was determined for harvests from the antigen and BSS-injected mice. Recipients of organisms harvested from antigenboosted foot pads required 166 days to reach this value, while those receiving M. *leprae* from the opposite foot pad required 131 days. These values are markedly different from the control value of 113±8 days obtained in recipients of M. leprae from BSS-injected animals, and demonstrate a profound killing effect by the booster injection.

To determine whether *M. leprae* infection induces nonspecific resistance, experimental and control mice were challenged with *Listeria monocytogenes* 15, 30, 90, 110, 140 and 190 days after infection with *M. leprae.* In addition to i.p. challenge from ILD¹⁰⁰ at each time period, some of the mice were injected i.v. with Listeria and their spleens cultured for viable organisms. Peritoneal macrophages were also cultured *in vitro* and challenged with Listeria. Based on these three parameters, an increased resistance to Listeria was not observed in *M. leprae* infected mice.

The enhanced microbicidal properties of the "activated" macrophage appears to be the effector of nonspecific resistance to unrelated intracellular organisms. Maintenance of a population of activated macrophages depends upon the presence of specifically sensitized lymphocytes and the persistence of the immunizing antigen. It appears then that macrophages activated by a chronic Toxoplasma infection may be capable of infiltrating the foot pad tissues infected with M. leprae. These cells either kill the. M. leprae or are inhospitable for M. leprae multiplication. The inability of M. *leprae* infected mice to show resistance to an unrelated intracellular bacterium (Listeria) could be due to the localized nature of the infection. Although the antigen persists for months, sufficient numbers of lymphocytes may not be stimulated, or subsequent circulation of either sensitized lymphocytes or activated macrophages in sufficient numbers to produce the desired effect may be prevented.-[Supported by The John A. Hartford Foundation, Inc. and grants from the National Institute of Health (No. 04717) and U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-07801)]

Drutz, D. J. and Gutman, R. A. The kidney in leprosy: an immunologic target organ.

The kidneys of leprosy patients are considered to function normally unless amyloidosis supervenes. However, recent studies indicate that both glomerular and distal renal tubular function may be impaired, presumably on an immunologic basis.

Eleven of 636 leprosarium patients had urine findings suggestive of active glomerulonephritis; urine abnormalities correlated strongly with a history of *erythema nodosum leprosum* (ENL). Of eight outpatients with active ENL, seven had hematuria, proteinuria, and red cell or hemoglobin casts. Lowered serum complement (C3) levels were recorded in one of two patients with biopsy-proven proliferative glomerulonephritis. Urine abnormalities were rapidly reversed with steroid therapy. Thus, glomerulonephritis may be a frequent unrecognized complication of ENL. Its relationship to circulating immune complexes remains to be demonstrated.

Ten leprosy patients were unable to lower their urine pH below 5.5 after oral NH4C1 (means pH 5.75 \pm 0.21). In contrast, 37 other patients and 30 normal control subjects achieved mean minimal pH's of 5.23 \pm 0.21 and 5.11 \pm 0.19, respectively. The acidification defect was unrelated to the type of leprosy [5 lepromatous (LL), 4 borderline tuberculoid (BT), 1 tuberculoid (TT)], type or duration of chemotherapy or the serum gamma globulin, immunoglobulin, cryoglobulin, antinuclear antibody, rheumatoid factor, AS-O or C-reactive protein levels. Pitressin-stimulated urinary concentrating capacity was abnormal (below 700 milliosmols/kg of water) in seven patients, two of whom had impaired urinary acidification. Five of the seven had LL; four had ENL. Percutaneous renal biopsy was conducted in fifteen patients, two of whom had abnormal acidification. In none were peritubular lymphocyte infiltrates or parenchymal invasion by M. leprae seen.

Renal tubular acidosis has been reported in a variety of diseases sharing only hypergammaglobulinemia in common. Under some circumstances, lymphocytic infiltration of peritubular tissues has been described. Although some patients with leprosy cannot acidify or concentrate their urine normally, the explanation appears unrelated to serum globulin levels or anatomic changes in the kidney. Although these defects of distal tubular function are felt to have an immunologic basis, their precise nature is not clear at this time.-[Supported in part by funds provided by the Bureau of Medicine and Surgery, Navy Department, for Work Units MR 005.09-0055 and T 022]

Gelber, R. H., Epstein, W. V., Fasal, P. and Drutz, D. J. Detection of circulating immune complex-like activity in the sera of patients with *erythema nodosum leprosum*.

Erythema nodosum leprosum (ENL) is characterized by clinical, histologic, and

immunologic features highly suggestive of antigen-antibody complex deposition in tissues throughout the body. Although cryoglobulins, antinuclear factors, rheumatoid factors, and thyroglobulin antibodies occur in lepromatous sera, their presence has no clear relationship to the occurrence of ENL. Serum complement (CH50 and C3) has been reported as both high and low during ENL reactions.

The gel precipitin reaction, utilizing the Clq component of complement (the site through which the first component of complement combines with gamma globulin or specific antibody), has proved useful in demonstrating the presence of circulating immune complexes in systemic lupus erythematosus (SLE). This precipitin reaction is positive in the majority of sera from hypocomplementemic patients with active SLE, but negative with sera from inactive normocomplementemic SLE patients and the vast majority of patients hospitalized with other disorders.

Studies were performed seeking circulating immune complexes in the sera of lepromatous leprosy patients with ENL, Eleven of 37 serum specimens in 6 of 12 ENL patients were positive for Clq precipitins. In contrast, only one of 39 sera from 12 patients with uncomplicated lepromatous leprosy produced a precipitin line with Clq. Of 7 sera obtained from 3 borderline lepromatous patients, one was positive for Clq precipitins; this patient was experiencing a severe downgrading reaction. None of 15 sera obtained from 8 patients with borderline or borderline tuberculoid leprosy produced precipitin lines with Clq. When serial sera were examined before, during, and after ENL, Clq precipitins were temporarily associated with the ENL reaction.

These results indicate that circulating material exhibiting behavior consistent with immune complexes is present in the sera of patients with lepromatous or nearlepromatous leprosy. Clq precipitin activity in the serum appears to bear a particular relationship to ENL and may be predictive for the ENL reaction.—[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-10288) and in part by the National Institute of Arthitis and Metabolic Diseases (Grant 1229)]

Chiller, J. Cellular basis of immunological unresponsiveness.

In mice, lymphocyte cooperation appears to be required for the formation of humoral antibodies to a variety of different antigens and appears to be the cellular basis for the carrier specificity required for the secondary response to a hapten. This collaboration involves cells derived from differing primary lymphoid tissues, namely from the thymus (T cells) and from the bone marrow (B cells). The exact manner by which cooperation occurs is not yet defined but it is finalized by the specific formation of immunoglobulin by B cells. In order to study the specificity of each lymphocyte population, the effect of immunologic unresponsiveness on B and T cells can be assessed in a system of absolute tolerance such as in the induction of unresponsiveness to heterologous serum proteins.

In the present experiments, a monomeric form of human gamma globulin (HGG) was used to confer in adult mice a complete, specific and long lasting unresponsive state. Using a system of cellular reconstitution of lethally irradiated mice, the effect of unresponsiveness to HGG was found to be specifically directed at both cellular levels (B and T). However, each cell population displayed a distinct kinetic pattern of unresponsiveness as well as a marked difference in the dose range of antigen necessary to induce tolerance. Unresponsiveness in T cells occurred very rapidly (24 hrs) and was maintained for a long period of time (> 100 days) before finally waning. In contrast, B cells required a longer induction period (8 days) and lost unresponsiveness at a much earlier time (50 days) than did B cells. The whole animal displayed a kinetic pattern of unresponsiveness superimposable on that observed for T cells, suggesting that a state of tolerance may exist in which only one cell

population (T cells) maintains the state. Conditions in which the dose of antigen used to induce tolerance was limiting also appeared to result in "split" cellular unresponsiveness that is only at the level of T cells. This phenomenon provides an insight in the cellular dynamics which occur in immunological conditions which terminate the state of unresponsiveness such as autoimmunity.

Morrison, N. E. Antimycobacterial activity of phenazine compounds.

The synthetic phenazine derivative, B.663, has become accepted as a first line antileprosy drug of proven use in DDSresistant cases. More recently a newer synthetic derivative, B.1912, has been suggested for clinical trials.

Investigation has been carried out to establish cross resistance relationships using drug resistant mutants isolated from Mycobacterium sp. 607. It has been found that significant cross resistance occurs between B.663 and B.1912. Furthermore, a unidirectional cross resistance occurs between B.663 and the rifamycins including the semisynthetic derivative, rifampicin. This fact thus suggests that B.663 inhibits the same enzyme site, DNA-dependent RNA polymerase, as rifampicin although possibly through a different mechanism. Evidence will be presented showing the spectrophotometric formation of DNA-B.663 complexes as a possible basis for antipolymerase activity.

A structure-activity series for various synthetic phenazine dyes has established a N-10-phenyl substituent on the phenazine ring as a requirement for enhanced antimycobacterial activity. Although phenazine antibiotics such as pyocyanine, chlororaphin, iodinin, myxin, griseolutein, tubermycin and lomofungin are microbially produced, none of these compounds contain a N-10-phenyl substituent. These data thus indicate new structural and mechanistic aspects to the design of synthetic phenazine antileprosy drugs.-[Supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-08416)]

DeGraw, J. I., Brown, V. H., Colwell, W. T. and Morrison, N. E. Development of drugs for selective inhibition of the mycobacterial folate pathway.

The *de novo* folic acid pathway in mycobacteria is known to consist of two key enzymatic steps. These are the condensation of *p*-aminobenzoic acid with hydroxymethylpterin pyrophosphate to form dihydrofolic acid and the subsequent reduction to tetrahydrofolate, the active "one-carbon" transfer agent necessary for biosyntheses. Ideally one would like to achieve a sequential blockage of both steps with attendant synergistic effect on bacterial growth inhibition and decreased probability of emergence of drug resistance.

Candidate drugs of the 2,4-diamino bicyclic pyrimidine class, designed to inhibit the above steps, have been synthesized and tested in the Mycobacterium sp. 607. Data are presented for whole cell growth inhibition in DDS sensitive and resistant strains. In addition data have been obtained for activity against the folic reductase enzyme isolated from this organism. The results obtained in this model system are being used as a guide to further drug development and the selection of candidates for more critical evaluation against M. leprae in the mouse foot pad.-[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Contract NIH 70-2286 and Grant AI-08214)]

Jacobsen, P. L. and Levy, L. The antimycobacterial activity of hydnocarpic acid.

Previous work in this laboratory showed that the crude salts of chaulmoogra oil, injected in a dose of 0.9 gm/kg three times weekly, inhibited the growth of *Mycobac*- *terium leprae* in the mouse foot pad. This finding along with an extensive review of the literature on chaulmoogra oil from 1904 to 1955 convinced us that a study of the antimicrobial spectrum and mechanism of antimicrobial action of this oil was warranted.

The early work, though chemically very thorough, is difficult to interpret because of the limited bacteriological and biochemical knowledge of the genus of mycobateria available at that time. Some of the studies do not identify the bacteria used, except as to genus, and others identified cultivable bacteria as *Mycobacterium leprae*.

Fifty-five strains of mycobacteria were screened using 3 and 30 μ g/ml of hydnocarpic acid (HYD), one of the two active components of chaulmoogra oil. Threequarters of the species studied were inhibited completely or partially (growth rate $\leq 50\%$ of control rate), at the 30 μ g/ml drug concentration.

Biotin has been shown to antagonize completely the inhibition of multiplication caused by HYD. Succinate and several fatty acids, products of carboxylation reactions requiring biotin as a coenzyme, and uracil, guanine, and adenine, metabolic intermediates found to be deficient during biotin deprivation of rats, were tested as possible antagonists of HYD. Adenine and guanine at concentrations of 20 μ g/ml partially reversed the HYD inhibition. The other compounds tested had no effect.

Mycobacterium intracellulare, which is sensitive to HYD, has been used to establish an experimental infection in mice. A study measuring bacterial counts from the spleens of both drug treated and untreated infected mice is now in progress.— [Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-07801)]

Gordon, G. R., Murray, J. F. Jr., Ghoul, D. C., Peters, J. H., Fieldsteel, A. H. and Levy, L. Rats in leprosy research: Metabolic disposition of dapsone in normal and infected animals.

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The susceptibility of the rat to infection with M. leprae has been reported (Fieldsteel and McIntosh, Proc. Soc. Exp. Biol. Med. 138 (1971). Since the rat may offer certain advantages over the mouse as an animal model for leprosy research, we have determined the metabolic disposition of dapsone (DDS) and monoacetyl DDS (MADDS) in Buffalo and Lewis rats. By a chromatographic-fluorometric method (Murray et al., J. Lab. Clin. Med. 78 [1971] 464) levels of DDS and MADDS were determined in plasma obtained 2, 4, 6, and 8 hours after intraperitoneal injection of DDS (1.0 mg/kg) or MADDS (1.2 mg/kg).

After DDS, female rats of both strains exhibited plasma levels of DDS that were higher at all times than those found in male rats. In Buffalo rats, the difference averaged 2.4-fold; in Lewis rats, 4.6-fold. Female rats also acetylated DDS more extensively than did males at all times. Average percentages of acetylation were: 9 Buffalo, 53; & Buffalo, 15; & Lewis, 40; and & Lewis, 23. After MADDS, female rats again exhibited higher levels of the administered drug than did males. However, percentage deacetylation of MADDS was consistently lower in the females. These data show that rats concurrently acetylate DDS and deacetylate MADDS. They are, therefore, qualitatively similar to man.

Half-times of disappearance of DDS and MADDS, respectively, found in these studies were: \circ Lewis, 6.8 and 3.4 hours; \circ Lewis 5.0 and 1.9 hours; \circ Buffalo, 6.7 and 5.5 hours; and \circ Buffalo, 5.1 hours (MADDS not studied). In thymectomized \circ Buffalo rats, the half-life of DDS was 6.4 hours, which did not differ from that found in intact \circ rats.

Based on the above metabolic parameters, we formulated diets for feeding to Lewis rats that would be expected to provide plasma levels of DDS above and below the known minimum inhibitory concentration of DDS for *M. leprae* (≤ 10 ng/ml) in mice. Plasma levels of DDS were determined at weekly intervals in normal male and female Lewis rats maintained four weeks on diets containing five levels of DDS ranging from 5×10^{-6} to 5×10 gm⁻⁴%. Levels of DDS in plasma were constant during the times studied and ranged from 1 to 12 ng/ml in males and 2 to 50 ng/ml in females. The highest plasma levels in both sexes were obtained in rats receiving 5×10^{-4} gm% DDS; the lowest measurable plasma levels were obtained in males receiving $5 imes 10^{-5}$ gm% DDS and in females receiving 1.6×10^{-5} gm% DDS. In both sexes, plasma levels of DDS were directly related to the dietary level of DDS. Studies are in progress in male Lewis rats that have been inoculated with M. leprae and that are being maintained on diets containing four levels of DDS ranging from 1.5×10^{-5} to 5×10^{-4} gm%. At the 63rd and 93rd day of the experiment, plasma levels of DDS were found to be nearly identical, on all diets, to those found in uninoculated rats.-[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grants AI-07801, AI-08214, and AI-08417)]

Peters, J. H., Gordon, G. R., Karat, A. B. A., and Meyers, W. M. Metabolic disposition of dapsone in Indian and African subjects.

Earlier, we showed that the wellestablished genetic polymorphism for the acetylation of drugs such as isoniazid and sulfamethazine (SMZ) also applies to dapsone (DDS) in American (Gelber *et al*, Clin. Pharm. Therap. **12** [1971] 225) and Philippine subjects (Peters *et al*, Internat J. Leprosy **38** [1970] 348).

Studies are now in progress in subjects of other countries, such as India and Africa, where leprosy is prevalent. Twenty-nine Indian subjects have been tested for acetylation capacities using both SMZ and DDS. For the former drug, tests on both plasma and urine were performed after an oral dose of 10 mg SMZ/kg. After 50 mg DDS orally, plasma samples were obtained after 4, 6, 8, 24, and 48 hours. In both cases, parent and acetylated drugs were measured in

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all samples obtained. Results from analyses of plasma after SMZ showed that 14 (48%) were slow, 3 (10%) were intermediate, and 12 (41%) were rapid acetylators. Data from the analysis of urine after SMZ or of plasma after DDS clearly differentiated slow from rapid acetylators but did not separate slow from intermediate acetylators. A reassessment of 10 slow acetylators of the group of 19 American subjects studied earlier showed that 2 were intermediate acetylators. No intermediate subjects could be identified in the group of 32 Philippine subjects studied previously. In the Indian subjects, no relationship between acetylation capacity and level of DDS or half-times of disappearance of DDS and MADDS was found. However, acetylation capacity and levels of MADDS were directly related. The results of these evaluations are essentially the same as we had observed in American and Philippine subjects earlier.

The initial study in 20 African subjects resulted from the combined needs to get plasma samples from African patients analyzed for DDS and to study populations in areas highly endemic for leprosy. From 57 plasma samples collected 8 to 96 hours after doses of 25 mg DDS or 50 mg DDS, we could tentatively identify 13 slow and 7 rapid acetylators. Subsequently, 21 patients were tested after an oral dose of 10 mg SMZ/kg. Results of analyses of both plasma and urine classified this group into 12 slow and 9 rapid acetylators. Five of the original group of 20 patients were members of the second group of 21 patients. As in all populations studied previously, a direct parallel existed between acetylation of SMZ and DDS in these five subjects-two were slow and three were rapid acetylators.

Broadening of our earlier evaluations of the relationship between acetylator status and the emergence of DDS-resistant M. *leprae* in patients (Peters *et al*, 6th Joint Leprosy Res. Conf., Bethesda, Md., July 26-28, 1971) showed that, of 21 patients, only 6 (29%) were slow acetylators. The high prevalence of the rapid acetylator phenotype (71%) in this group suggests a direct relationship between the rapid phenotype and the emergence of DDS- resistant *M. leprae.*—[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Contract NIH 70-2283)]

McRae, D. H., Russell, D. A., Scott, G. C., Vincin, D. R., and Shepard, C. C. Four year results in the therapeutic trial of acedapsone (DADDS) in the Karimui, New Guinea.

All patients with leprosy in the Karimui are being treated with acedapsone. More than 430 completed their fourth year of treatment in November 1971, and their clinical responses, with one exception, were deemed satisfactory. There were 28 previously untreated patients who had enough bacilli in their skin smears to allow monitoring of their solid ratios, and the results with these patients are being covered in this report. The response in BI at four years, with two exceptions, continues to be satisfactory, and eight BI's were negative (100 fields examined in each of six smears for each patient). Solid ratios (MI) which as previously reported dropped satisfactorily in all patients in the early part of the trial, can now be determined in only a few patients. In the MI's done at four years, the only solid bacilli seen were in the two patients whose BI responses were also unsatisfactory. One of the patients is a thirty year old female who responded satisfactorily at first, but was observed to have some solid bacilli at three years and four years. At three years her BI was the highest of the group and her clinical condition was thought to have progressed. A serum sample, taken 75 days after the previous DADDS injection, was found by J. H. Peters to have 22 ng DDS/ml, a level indicating satisfactory absorption and deacetylation. Bacilli from skin biopsy specimens, taken in September 1971, have been inoculated into mice for tests of DDS-resistance, but results are not yet available. Another patient was found to have a few solid bacilli at four years; his clinical condition was apparently satisfactory at that time.

Thus, overall, the patients have responded well to acedapsone. One, or possibly two cases with DDS-resistance may have appeared during the four years of therapy; satisfactory, but more expensive and inconvenient nonsulfone therapy can be provided for them. The convenience and certainty of administration of acedapsone have made therapeutic coverage possible for a population who could not otherwise have been reached.

Shepard, C. C., Levy, L. and Fasal, P. Rapid bactericidial effect of rifampin on *M*. *leprae*.

Leprosy responds very slowly to current therapy, so there is a special need for more rapidly effective drugs. Of the drugs tested to date, rifampin has been found the most rapidly bactericidal. In mice it exerted a bactericidal-type effect with single administration by gavage on the 70th day after infection; the effect increased in the range 10 to 40 mg/kg. A very good result was seen when 25 mg/kg was given on the 70th and 140th day after infection. In man the drug was also rapidly effective. Results are completed on seven patients with lepromatous disease treated with 600 mg rifampin daily; the viability of the bacilli in the skin lesions was tested by inoculation of mice. Infectivity for mice had disappeared in the first specimen collected after the start of therapy-at seven days in six patients and fourteen days in one. In four control patients treated with 50 mg dapsone daily, infectivity for mice was lost much more slowly, and in one was still present, though decreased, 112 days after the start of treatment. The rate of loss of infectivity with dapsone was in accord with our previous experience in 14 other patients treated the same way and followed by the same methods. The present results and those obtained previously illustrate the efficiency of mouse inoculations for monitoring therapeutic regimens in lepromatous patients. Dapsone, clofazimine, acedapsone, and rifampin have given results that are characteristic for each drug.-[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant AI-07801), and an interagency agreement between NIAID and the Center for Disease Control]

A collaborative effort of the U.S. Leprosy Panel and the Leonard Wood Memorial (presented by L. Levy). Chemotherapy trials in leprosy. I. Five clofazimine regimens in lepromatous leprosy.

Several years ago, the U.S. Leprosy Panel and the Leonard Wood Memorial began a series of chemotherapy trials in leprosy, which were carried out in Cebu, Philippines. The first trial, the results of which are nearly complete, involved the study of five clofazimine (B663) regimens in 48 patients with previously untreated lepromatous leprosy. The five regimens were: (1) 200 mg six days per week; (2) 100 mg three days per week; (3) 300 mg once weekly; (4) 600 mg once every two weeks; and (5) 600 mg on each of two consecutive days every four weeks. Regimen 1 is a standard regimen. Regimen 2 was chosen as one which would be effective at Sungei Buloh, as measured by the decrease of the MI, but which might be expected to be less effective than the standard regimen. The intermittent regimens were designed to deliver the same average dosage as Regimen 2.

The trial of intermittent clofazimine regimens was undertaken because of our desire to develop treatment programs which were both effective and convenient. We thought to take advantage of the accumulation of this drug in the body. An earlier trial had suggested a delay in the onset of killing of Mycobacterium leprae after clofazimine administration to patients with lepromatous leprosy was started; the delay appeared consistent with the need to saturate body stores with the drug. Because large intermittent dosage offered a means of hastening the saturation process, we thought that such treatment might prove not only convenient but even more effective than standard treatment.

Suitable patients were admitted to the Eversley Childs Sanitarium and assigned randomly to one of the five regimens. The response to treatment was measured primarily by means of mouse foot pad inoculation of the *M. leprae* recovered from skin biopsy specimens obtained before treatment and after two, four and six months of treatment. Mice were inoculated in Cebu, and duplicate inoculations were performed in San Francisco for 90% of the specimens. Measurement of infectivity is interpreted as a decrease in the proportion of viable *M. leprae* in a standard inoculum.

Mice were inoculated with the organisms recovered from 192 skin biopsy specimens. The results of the study of 80% of these specimens are now complete. Regimens 1 and 2 appear about equally effective; in both cases, loss of mouse infectivity had occurred after about two months of treatment on the average. Regimens 4 and 5 appear less effective; longer than three months of treatment were required on the average for loss of infectivity of the recovered *M. leprae* for the mouse. The efficacy of Regimen 3 appears to be intermediate.

The results are not consistent with the notion that body stores of the drug are readily available for antimicrobial activity.-[The trial is a collaborative project of the U.S. Leprosy Panel, U.S.-Japan Cooperative Medical Science Program, Geographic Medicine Branch, National Institute for Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, and the Leonard Wood Memorial (LWM), Washington, D.C. The trial protocol was developed by a Chemotherapy Committee, which also supervised the conduct of the trial. Members of the committee are C. H. Binford, R. S. Guinto, L. Levy, C. C. Shepard, and J. G. Tolentino. Other participants in this investigation are R. Abalos, E. de la Cruz, T. T. Fajardo, Jr., J. N. Rodriguez, and G. P. Walsh. The trial was carried out at the Eversley Childs Sanitarium, Cebu, with the assistance of Dr. A. P. Perez, Director of the Sanitarium, and the Philippine Government. This project is supported by Contract NIH 69-2003 between the NIAID and the LWM]

Monaco, A. P., Gozzo. J. J. and Wood, M. L. In vitro titration of antilymphocyte serum bound to lymphoid cell membranes as an indicator of *in vivo* immunosuppressive ability.

A major problem in the use of heterologous antilymphocyte serum (ALS) for production of immune suppression in mice and other species has been variability of *in vivo* immunosuppressive potency of various sera. Experiments aimed at the *in vivo* growth of the leprosy bacillus which utilize ALS for prolonged periods to induce longterm immune suppression may suffer from variability of potency in batches of ALS used over long periods of time. An *in vitro* assay which correlated directly with *in vivo* potency would aid in standardization and maintenance of a given level of immunosuppression over long periods.

In this study 20 rabbit anti-mouse lymphocyte serum (RAMLS) pools were produced and tested for their ability to prolong skin grafts. A/jax mice received 0.5 ml RAMLS on days -1 and +2 relative to grafts with C3H/He skin on day 0. The normal median survival time (MST) in this H-2 combination was 10.2 \pm 0.3 days. A wide range of immunosuppressive potencies was achieved with the various sera studied. Their in vivo effectiveness was correlated with a simple and reproducible in vitro assay which measures the ability of RAMLS to bind to lymphocytes. In this assay various dilutions of RAMLS (1/1000 to 1/512,000) were incubated with 7×10^{6} lymphoid cells. Washed RAMLS-treated lymph node cells were then incubated with a specific goat anti-rabbit IgG serum (GARIgG). Washed RAMLSand GARIgG-treated cells were then reacted with tannic-acid treated sheep erythrocytes coated with normal rabbit serum. A mixed agglutination reaction was thus achieved. Serum end-point titers were then read visually after cells were allowed to settle as in a passive hemagglutination assay. RAMLS titers greater than 1/128,000 showed MST values greater than 40 days. Sera with titers of 20-50,000 showed MST values from 25 to 35 days. Sera with titers less than 1/1,000 were not immunosuppressive at all. A perfect and direct correlation of *in vitro* activity with this assay with *in vivo* potency was obtained.

The use of this assay for titration of RAMLS to be used to enhance the growth of leprosy bacilli *in vivo* in ALS-treated mice may lead to consistent and more highly reproducible experimental results— [Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases (Contract NIH 70-2285)]

Glasgow, L. A. and Bullock, W. E. Impairment of interferon production in mice infected with *Mycobacterium lepraemurium*.

Since mononuclear cells within the reticuloendothelial system are a major source of interferon production, studies were performed to determine if parasitization of these cells with *M. lepraemurium* is associated with deficiencies of interferon production in addition to the abnormalities of cellular immunity previously demonstrated. Groups of control and infected CF_1 mice were inoculated with polyionosinic:polycytidylic acid (poly I:C), endotoxin or chikungunya virus (CV). Sera were assaved by the 50% plaque inhibition technic with vesicular stomatitis virus as challenge virus in L-929 cells. Interferon response to poly I:C in leprous mice was unaffected; it was two to fourfold higher than normal in response to endotoxin. After i.p. inoculation with 4×10^6 PFU of CV, the mean serum interferon level in control mice at peak response (6-7 hours) was 3,000 units/ml. The mean serum level in infected mice was 445 units/ml. Peritoneal exudate cells harvested from infected and control animals were assaved for interferon production after exposure to CV in vitro. In two experiments, interferon production by cells from infected mice was 15% and 30% respectively of that by normal cells. The results indicate that interferon production in response to challenge by CV is suppressed in leprous mice. Thus, cellular defenses may be inhibited at multiple levels as a consequence of intracellular infection by M. lepraemurium.