## ABSTRACTS

# EIGHTH JOINT LEPROSY RESEARCH CONFERENCE

TraveLodge at the Wharf San Francisco, California July 30–August 1, 1973

U.S.-Japan Cooperative Medical Science Program Geographic Medicine Branch National Institute of Allergy and Infectious Diseases National Institutes of Health

#### FOREWORD

An important element of research progress as we know it today is the research conference, especially one that is not too big or too formal. There is rapid communication and informed discussion, both formal and informal. Moreover, in these days of increased joint authorship, much collaborative work is planned.

The past joint U.S.-Japan leprosy research conferences have been very useful in these ways, and this year's conference was no exception. There was important progress reported in the three principal areas of leprosy research—cultivation and transmission, chemotherapy and pharmacology, and immunology. We were especially pleased with the increased participation by immunologists, and we hope to see this participation grow in the future.

The costs of travel restrict international participation, but we hope by publishing these abstracts to make the essential information more widely available.

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

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## Program of Leprosy Conference

#### Feldman, Roger A. Leprosy in Louisiana, 1855-1970. Epidemiologic study of longterm trends.

A well-known focus of leprosy in Louisiana was studied from 1855-1970. A decrease in incidence was observed which began before the use of chemotherapy. Many of the cases had onset in a limited area of Louisiana. French Louisiana, and in this area there was a high incidence of multiple family cases. Overall attack rates were found to be decreasing for each cohort of birth, and within cohorts after 1920, the highest attack rates were in the age group 10-19. A high attack rate was found for cases diagnosed in the years 1900-1929, probably a result of better reporting. Although persistent for a long period, the focus remained limited in geographic extent, and seems to be disappearing.

Hanks, J.H. and Dhople, A.M. Energetics of host grown microbes. Physiologic investigations with *M. lepraemurium* as an interim model for *M. leprae*.

ATP provides a biologically significant tool for quantitating either functional biomass or growth potential. Ultrasensitive detection of ATP plus a novel extraction of microbial ATP have decreased the hitherto required number of bacterial cells 60-fold. A nonenzymatic, 400 time safety factor eliminates host ATP. Thus, it is now possible to investigate the energetics of host-dependent microbes. Specifications of the ATP system, and the sensitivity required for work with *M. leprae* will be given.

The validity of ATP data from noncultivated mycobacteria was confirmed by means of microscopically counted *M. lepraemurium* cells and plate counted *M. phlei* cells under identical conditions, which included cells stressed and conserved as will be outlined in the physiologic studies below. One study alone demonstrated some 100 close relationships between ATP per aliquot and the number of viable cells (functional biomass) and consistent yields of ATP per cell (index of growth potential).

*M. lepraemurium*, the least penetrable of the pathogenic mycobacteria, was used as a

model while investigating two of the primary questions pertaining to host grown microbes, namely the following:

1. Whether all intracellular bacteria are leaky. The leaching rates of "in vivo-type" and "in vitro-type" cell membranes was compared, using *M. lepraemurium* and *M. phlei* cells de-energized at  $0.5^{\circ}$  during the dilution steps in a procedure that purifies *M. lepraemurium* cells. The *M. lepraemurium* membranes leached ATP ten times more rapidly that those of *M. phlei* and retained only 66% of the initial ATP. This denotes a disastrous loss of smaller cofactors and metabolites.

2. Whether the leakiness of intracellular bacteria can be compensated by providing external supplies of microbial cofactors and metabolites to equilibrate their metabolic pools. Refrigeration of *M. lepraemurium* for eight months in a mixture of membrane stabilizers and microbial cofactors conserved 93% of the original ATP per cell. Cells frozen at  $-76^{\circ}$  retained 37%. Projected decay rates suggested that the equilibration of metabolic pools may be superior to freezing during the first five years of storage.

ATP data have substantiated reports by Rightsel and Wiygul and Ito and Kishi that M. lepraemurium is capable of slow cellular growth when encased in diffusion chambers incubated in the peritoneal cavities of mice. This indicates that in vitro cultivation of M. lepraemurium should be feasible as an interim step in defining the physico-chemical parameters to be maintained during similar investigations with M. leprae. The use of ATP data to define the functional biomass and growth potential of M. leprae during the progression, regression and therapy of leprosy will be discussed. - [This investigation was supported by the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare, Grant AI-08866]

# Broman, R. L., Keele, B. B., Jr. and Tepper, B. S. Enzymatic evidence for an aerobic metabolism in *M. lepraemurium*.

A major problem in the cultivation of *M.* lepraemurium and *M.* leprae has been to de-

termine whether these organisms are aerobic or anaerobic. The probability of an aerobic metabolism for M. lepraemurium was supported by the reports of Mori et al [Internat. J. Leprosy 39 (1971) 796] that cellfree extracts contain b1 and a2-type cytochromes. Mori et al [Internat. J. Leprosy 39 (1971) 813] detected many of the enzymes associated with the tricarboxylic acid cycle in extracts of M. lepraemurium, however, these investigators, and later Donowa et al (Leprosy Scientific Memoranda, Sept. 1972), could not detect pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase activity, both enzymes essential for an operative tricarboxylic acid cycle. In our studies of M. lepraemurium extracts we have found both pyruvate dehydrogenase and a-ketoglutarate dehydrogenase activity. These results, together with our radioisotope tracer studies [Tepper and Varma, J. Gen. Microbiol, 73 (1972) 143] provide evidence of carbon flow from glycolytic pathways through acetyl-CoA into a complete tricarboxylic acid cycle.

Further evidence of an aerobic metabolism in M. lepraemurium is provided by our demonstration of superoxide dismutase activity. This enzyme appears to be vital to microorganisms that metabolize oxygen and, with catalase, has been found in all organisms that contain cytochrome systems. The primary physiological function of superoxide dismutase has been postulated to be the protection of oxygen metabolizing organisms against the detrimental effects of the biologically produced superoxide free radical [Mc-Cord, Keele and Fridovich. Proc. Nat. Acad. Sci. 68 (1971) 1024]. Superoxide dismutase activity in M. lepraemurium is at least four times greater than the activities of the aerobic organisms that have been tested and twelve times the activity found in M. phlei.

#### Prabhakaran, K., Harris, E.B. and Kirchheimer, W.F. Radioisotope assay of odiphenoloxidase in M. leprae.

Mycobacterium leprae separated from infected human skin, spleen and testes was shown to possess a characteristic enzyme which converted several phenolic substrates to quinones in vitro. No other mycobacteria tested so far showed o-diphenoloxidase activity. The phenoloxidase in M. leprae was demonstrated by the Warburg manometric method (where oxygen-uptake of the bacilli in the presence of substrate was measured), or by the spectrophotometric procedure (where absorbance maxima of the quinones formed were determined). Since these methods require large amounts of organisms which are not readily available, more sensitive technics had to be developed.

In the present study, M. leprae was separated from leprous human spleen (removed at autopsy), and from tissues of armadillos (which developed the spreading form of the infection, subsequent to inoculation with the leprosy bacilli). The organisms were disrupted by ultrasonic oscillation and the particulate fraction was collected by ultracentrifugation. The phenoloxidase activity was firmly bound to particulate elements in the bacterial cell. In some experiments, the enzyme was released into the supernatant fraction by treating the particles with the anionic detergent, sodium dodecyl sulfate. Tritiated DL-DOPA was used as substrate for assay of o-diphenoloxidase of M. leprae and also of mammalian and plant tyrosinases. In this procedure, the enzyme was incubated with the 3H-labeled substrate. Oxidation of the tritiated diphenol results in the formation of radioactive water and quinone. Radioactivity of the water was determined in a Beckman LS-250 liquid scintillation counter. The results showed that the leprosy bacilli readily oxidized tritium-labeled DOPA. The radioisotope tracer technic being highly sensitive, metabolic activities of M. leprae can be studied, using small amounts of material.

The organisms obtained from leprous human tissues, armadillo tissues and from mouse foot pads oxidized D-DOPA, indicating that the enzyme is distinct from tyrosinase present in mammalian melanocytes, which is relatively specific for L-DOPA.

Mori, Tatsuo. Cultivation of *M. lepraemu*rium on Ogawa's yolk medium and properties of the cultivated *M. lepraemurium*.

1. Primary isolation of *M. lepraemurium* was highly successful on Ogawa's medium under condition of low temperature and a long incubation.

2. Five bacilli of cultivated *M. lepraemu*rium gave rise to a leproma in the subcutaneous tissue of the mouse.

3. Results of amidase test of cultivated *M. lepraemurium* were as follows: urease (-), nicotinamidase (+), pyradinamidase (+).

4.  $QO_2$  of cultivated *M. lepraemurium* was  $1.7 \mu$ l/mg hour.

5. Glycerin and sodium glutamate were not oxidized with cultivated *M. lepraemurium* by Warburg manometric method.

Dr. Ogawa reported the cultivation of M. lepraemurium on Ogawa's yolk medium, however, the isolation coefficient is very low. As the growth of *M. lepraemurium* does not change at 37° C or 33° C and the culture medium will be more stable at 33° C than 37° C, we tried a primary isolation of M. lepraemurium at 33° C. Maintenance in low temperature may be adequate to prevent a degradation of culture medium from long-time incubation; fairly good results were obtained. Primary isolation of M. lepraemurium was positive in materials from nine of ten mice bearing subcutaneous murine lepromas. The negative mouse appears positive now but I have not yet identified it.

When cultivated M. lepraemurium, passaged 11 generations, was inoculated into the subcutis of mice, more than 104 bacilli were recovered from a subcutaneous leproma. When the same bacilli were implanted in the mouse peritoneal cavity in a millipore filter chamber together with mouse peritoneal cells, a growth of M. lepraemurium was found in the diffusion chamber. These M. lepraemurium grown in cell culture induced a subcutaneous leproma with the inoculation of only five bacilli. Since the minimal infective dose of animal passaged M. lepraemurium was five bacilli, it is concluded that the passaged acid-fast bacillus may be M. lepraemurium.

The results of amidase test of the cultivated *M. lepraemurium* described in the summary was similar to that of *M. avium*. Cultivated *M. lepraemurium* did not oxidize glycerin and sodium glutamate by the Warburg manometric method; the result was the same in the case of *in vivo* grown *M. lepraemurium*. The QO<sub>2</sub> of  $1.7\mu$ l/mg hr of cultivated *M. lepraemurium* was comparable to the  $4\mu$ l/mg hr of *in vivo* grown *M. lepraemurium*.

Nakamura, Masahiro. Factors affecting the growth of *M. lepraemurium* in the NC-5 medium and a subculture trial.

It is obvious from the papers already reported that *M. lepraemurium* is able to quantitatively multiply, without cells, in the NC-5 medium which is composed of enriched Kirchner medium containing goat serum and  $\alpha$ -ketoglutaric acid, cytochrome C, hemin, as well as 1-cysteine HCl. In addition, it was noted that the propagated bacilli maintained their pathogenicity to mice characteristic of murine leprosy. No growth and no pathogenicity of bacilli cultivated in the Kirchner medium under the same condition as the NC-5 medium were demonstrated.

In this paper, some factors are reported, especially the pH of the culture medium and the inoculum size, affecting the growth of bacilli in this cell-free medium.

Bacilli used were the Hawaiian strain of M. *lepraemurium* experimentally developed in subcutaneous tissues of  $C_3H$  mice. The growth of bacilli was observed by the silicon-coated slide culture method and estimated by the bacillary counting method previously described.

1. Optimal pH of the culture medium. The pH of basal media which are enriched Kirchner medium containing 0.005% calcium pantothenate was adjusted to 6.0, 6.3, 7.0, 7.3, and 7.6. After sterilization by autoclaving, additives such as goat serum,  $\alpha$ -ketoglutaric acid, cytochrome C, hemin, and 1-cysteine HCl were aseptically added. The best result was obtained when the bacilli, which were smeared on a silicon-coated slide, were incubated in the NC-5 medium whose basal medium was adjusted to pH 7.3.

2. Relationship between the inoculum size and the growth rate of M. lepraemurium. It was found that the increase of the number of bacilli cultivated in the NC-5 medium depended significantly upon the inoculum size of the bacilli; when the number of bacilli at "0" time was  $9.8 \times 10^6$  per ml of culture medium, the increase rate was remarkably low and indefinite. On the other hand, a smooth and definite growth curve could be obtained when the starting number of bacilli was  $9.8 \times 10^4$ , 100 times less than before, per ml of the medium.

3. A subculture trial. The silicon-coated slide with smear, cultivated in the NC-5 medium, was transferred to newly prepared NC-5 medium every one or two months. No transfer group was used as control. After a total of six months' cultivation, the growth pattern of each experimental group was compared. The result obtained apparently indicated that abundant multiplication of the bacilli was observed when the slide was transferred to a fresh medium every month, whereas no multiplication took place in the case of transfer through the enriched Kirchner medium which was used as control.

Hirata, Tsunehiko and Nakayama, Tetsu. Origin of capsular structure around leprosy bacilli in the host cell. Electron microscopical observation.

The purpose of this summary is to describe ultrastructural changes of *M. leprae* and *M. lepraemurium* during growth in their host cells.

It was assumed that the process of cell division, in the case of both human and murine bacilli, generally took place as follows:

At first, the division site on the surface of the cell wall became weak, and then the cytoplasmic membrane adjacent to this wall site distorted towards the center of the cytoplasm. Thus, bridge formation occurred and some of mesosomes were arranged parallel to the edge of bridge. They seemed to play a role in the organization of a cross wall.

After the construction of a cross wall (septa), the old wall around the two new halves of the daughter cell separated and were released from the new cell wall.

The structure of these disrupted old walls disappeared gradually. Meanwhile, they remained only as a thin layer, called "inner boundary layer," and finally became amorphous substances.

On this basis, it can be postulated that the capsular structure around leprosy bacilli in lepromas mostly originates from the bacterial side. —[This work is partially supported by a grant from U.S.-Japan Cooperative Medical Science Program and by useful suggestions from Dr. J. Hanks]

Nishiura, Mitsugu. Complementary double replica method for the study of human and murine leprosy bacilli.

The complementary double replica method is a technic in which both complementary freeze fracture surfaces of the specimen are replicated. After preparing replicas of both fracture surfaces, convex and concave images of the same bacillus must be collected on the fluorescent screen of the electron microscope. Application of this technic to the study of human and murine leprosy bacilli revealed interesting findings.

In the freeze fracture of human leprosy bacilli, the cleavage takes place at the outer surface of the cell wall. On the convex image of the bacillus, band structures are most often found on the surface of the cell wall. The fibrous texture of the cell wall is also clearly visible, but each fiber constituting the cell wall is about 70 Å thick. On the concave cast of the bacillus, the band structure and fibrous structure are shown as shallow grooves in freeze fracture replicas. These fine structures on the concave cast of the bacillus disappear after freeze etching, and the surface becomes smooth. On the contrary, convex bacillary cell walls do not change their cell wall texture after freeze etching. This proves that the concave cast of human leprosy bacilli is made up of the ice of glycerol-saline impregnating the tissue, and there is no cell wall material remaining in the concave complementary cast.

In freeze fracture of murine leprosy bacilli, the cleavage takes place between multilayered crystalline membranous structures around the cell wall of the bacillus. Also the cell wall surface is often exposed where part of the membranous structures has been removed. The concave cast also shows membranous structures which do not disappear after freeze etching. Most of the band structures of murine leprosy bacilli are usually not located on the outermost layer of the cell wall, but are shallowly embedded in the cell wall. In such cases, a thin layer of fibrous structure of the cell wall covers the band structures. Thicker fibrous structure (180 Å) is often observed on the surface of murine leprosy bacilli, and in such cases band structures seem to be composed of the same fibers.

#### Chang, Y.T. and Andersen, R.N. Animalto-animal variations in the growth of *M*. *lepraemurium* and *M. leprae*.

1. The animal-to-animal variation in the growth of *M. lepraemurium* was studied in a total of 14 strains of inbred mice. The intraperitoneal inoculum consisted of  $10^9$  organisms, and the growth was evaluated by estimation of the gross lesions of various sites and organs three months later. Only the CBA/J strain of mice revealed a uni-

form, highly reproducible growth among animals.

2. More studies were made with the CBA/J mice, using the survival time of animals as the criterion for growth evaluation. A total of 100 mice was used. The average survival time was decreased from two years to  $127.8 \pm 9.6$  days.

3. The effect of various doses of DDS was tested in CBA/J mice, using the survival time as the criterion for growth evaluation. The survival time was significantly increased by the lowest dose (0.001%) in the diet (P<.052).

4. The animal-to-animal variation in the growth of M. lepraemurium in foot pads was studied in a total of 16 inbred strains of mice. An inoculum of 5 × 103 organisms was injected and the bacterial counts were made five months later. Marked growth variations were observed, not only among various strains of animals, but also among the individual animals within the strain. The average bacterial count per foot pad varied from 3 × 106 to 50 × 106 organisms among various strains of animals. The difference between the lowest and the highest bacterial counts in the animals within each strain ranged from 4-fold to 266-fold. The standard deviations of bacterial counts of all 16 strains were very large, the lowest being 38% of the count. In eight strains the standard deviations were larger than the corresponding average bacterial counts.

5. The effect of various doses of DDS was studied in the foot pad-*M. lepraemurium* model in two strains of mice. Only the largest dose used, 0.1% in the diet, showed an activity of statistical significance (P < .01). The foot pad model appears to be 100 times less sensitive than the intraperitoneal-survival time model described in 3.

6. The variations on the growth of M. leprae in foot pads of CBA/J mice were studied in one experiment. Organisms,  $5 \times 10^3$  (biopsy material from Surinam), were injected into the foot pads of both left and right hind legs. Bacterial counts made at intervals of one to two months revealed an average count of  $1.8 \times 10^6$  organisms per foot pad at the end of nine months. The effect of DDS, B663 and B1912 (0.01% in the diet each) was studied. The bacterial counts varied markedly among the four animals of each group, and the standard deviation of each group was larger than the corresponding bacterial count. Although the average counts of all three treated groups were definitely smaller than the control animals, the differences were not statistically significant (P > .10).

7. Two weeks after the above experiment, i.e., at the end of 9.5 months, another 20 control animals were sacrificed and the bacterial counts were made on both the left and right foot pads. Marked variations of the counts were observed not only from animal to animal (maximal difference of 143-fold), but also from left to right foot pads (maximal difference of 25-fold). The average bacterial count of the whole group (40 foot pads) was  $24.4 \times 10^4$  organisms. The standard deviation was  $29.4 \times 10^4$ , which was larger than the average bacterial count.

It was concluded that the growth of *M.* lepraemurium and *M.* leprae in mouse foot pads did not follow the normal (Gaussian) frequency distribution. Because of this finding, interpretation of the results by statistical analysis observed from foot pad experiments is doubtful. —[Aided partly by Emmaüs-Suisse through the World Health Organization]

#### **Fieldsteel, A. Howard and Gartner, Susan.** Murine models for the study of *M. leprae* infection.

In our search for a suitable murine model for the study of long-term chronic M. leprae infection, we have investigated the use of immunosuppressed mice and rats. BALB/c mice thymectomized at five to seven days of age were slightly more susceptible to foot pad infection with M. leprae than intact BALB/c mice. In the latter, after inoculation with  $7.6 \times 10^3$  M. leprae, the infection peaked at 1.6 × 106 organisms per foot pad four months after inoculation, whereas in the thymectomized mice, the peak of 1 × 107 per foot pad was reached 11 months after inoculation. Thymectomized mice were also given various regimens of antilymphocytic serum (ALS) for up to six months in weekly doses of 0.5 ml. Fifteen months after inoculation, the number of organisms in these thymectomized groups was still slowly increasing, with a high of 2.9 × 107 per foot pad. At that time, the number of organisms in the mice receiving no treatment beyond thymectomy had fallen to  $2.2 \times 10^6$ , and the organisms did not appear viable.

Growth of *M. leprae* in the testes of BALB/c mice was erratic. No growth occurred in the testes of intact mice, and growth in testes of thymectomized mice was detected only 11 months after inoculation  $(1.7 \times 10^7 \text{ per testis})$ . In the thymectomized ALS-treated mice, the maximum number of *M. leprae* per testis was found between 11 and 15 months after inoculation, and averaged between  $5.7 \times 10^6$  and  $1.9 \times 10^7$  per testis.

The most promising results to date are those using neonatally thymectomized rats inoculated intravenously with M. leprae. Groups of these rats were further treated with either antithymocytic serum for six months or given two doses of 350 r X-irradiation four and eight months after inoculation. Neither of these additional treatments apparently had any further enhancing effect on the infection by the 14th month after inoculation with  $1.65 \times 10^7$  M. leprae. At that time, the average number of M. *leprae* in the tissues of the thymectomized rats was as follows: foot pads, 2.94 × 108; ears, 1.75 × 107; nose, 1.83 × 107; tail, 9.8 \* 108; scrotum, 1.33 × 106; testes, 1.15 × 106; body skin, 0. No growth of M. leprae could be detected in any of the same tissues of intact rats.

It appears that the Lewis rat, with only a minimal amount of immunosuppression namely, neonatal thymectomy—can be made highly susceptible to infection with *M. lep-rae*, and thus may become an experimental model for the study of human leprosy. —[This work was supported by the U.S.-Japan Cooperative Medical Program. Grant AI-08417]

Storrs, E.E., Burchfield, H.P. and Walsh, G.P. Report on the first four groups of armadillos to be inoculated with *M. leprae.* 

During December of 1969 through June of 1970, four groups of armadillos totaling 24 animals were inoculated with leprosy bacilli obtained from various sources. The inoculum used for the first group did not multiply in the mouse foot pad controls nor did it produce leprosy in any of four animals injected with it. Therefore, these animals will not be considered further except to illustrate the longevity of the armadillo in captivity.

The remaining three groups were inoculated with *M. leprae* from two human sources and one mouse foot pad source. At least one animal in each group developed leprosy. Of the 20 animals inoculated with viable bacilli, seven (35%) developed lepromatous leprosy as evidenced by the presence of enormous numbers of acid-fast bacilli, development of open lesions, and the formation of subcutaneous lepromas. In six of these seven cases diagnoses of lepromatous leprosy were substantiated by detailed histopathologic examination following autopsy. The seventh animal is still alive 40 months postinoculation. Two of the animals (10%) harbor acid-fast bacilli but have not as vet developed gross evidence of disseminated leprosy. However, one of these was not positive for AFB until three years after inoculation, indicating that the disease develops very slowly in some cases.

Six of the seven animals which developed lepromatous leprosy have died from the disease or complications resulting from it and have been autopsied. The average time from inoculation to death was 28 months and the median time 31 months. Five of these animals which died 26 to 34 months following inoculation yielded 854 g of lepromas containing an estimated 17 g of *M. leprae*. An animal severely infected with leprosy which died 15 months postinoculation had not at that time developed extensive lepromas.

Holding a total of 24 armadillos for an average of 3.2 years would correspond to 922 armadillo months. The six animals which died from causes related to leprosy reduced this figure to a net of 825 armadillo months. Six animals died from other causes during the experiment, reducing the total by 55 months, or 7% of the net armadillo months available. The average postinoculation life span of the six animals which died from other causes was 28 months.

In addition to the first 20 animals which were inoculated with viable *M. leprae*, other inoculated armadillos have developed leprosy. However, only these first three groups have been held long enough to estimate the severity and incidence of disease in adult wild-caught animals inoculated with bacilli unadapted to this species.

Even after 3 to  $3\frac{1}{2}$  years an endpoint has not been reached as is evidenced by the fact that a lepromatous animal is still alive 40 months after inoculation. In addition, the two animals harboring AFB could still develop lepromatous leprosy given a longer incubation period.

#### Binford, C.H., Issar, S.L., Storrs, E.E. and Walsh, G.P. Leprosy in the nine-banded armadillo (*Dasypus novemcinctus*). Summary of post-morten observations made on seven animals.

The histopathologic findings in the first armadillo that was infected with *Mycobacterium leprae* have been published [Kirchheimer, W. F., Storrs, E. E. and Binford, C. H. Internat. J. Leprosy **40** (1972) 229-242]. We have now had the opportunity to study seven more armadillos that on autopsy showed severe, widely disseminated infection with *M. leprae*.

Histopathologically the disease in these armadillos closely duplicated lepromatous leprosy in man. Of special significance was the similarity of the involvement of dermal and large nerves to lepromatous neuritis in man. The leprotic involvement of skin, lymph nodes, larynx, nose and eyes resembled human leprosy.

The lesions of liver and spleen while histopathologically similar to lepromatous leprosy in man, were in the majority of the armadillos more severe than usually observed in man.

Presumably because of the relatively low body temperature in the armadillo there was, in several animals, leprotic involvement of lungs, meninges and in one animal extension of the infection into the submeningeal cerebral cortex.

In some of the animals many lepromatous nodules containing large numbers of bacilli were found in the loose axillary and inguinal tissues, and over the entire back underneath the carapace. No borderline or tuberculoid features were observed in any of these animals. —[This work was supported in part by Research Grant CC-00476, Center for Disease Control, Atlanta, Georgia and by the U.S.-Japan Cooperative Medical Science Program administered by NIAIA (grants AI—7266 and 11,620)]

#### Sasaki, Norisuke and Namba, Masashi. Histopathological studies on leprotic bulbar palsy.

We have investigated the pathogenesis of this disease and previously reported on the clinical course and the pathologic studies of two cases of leprotic bulbar palsy. The most severe focus was found in the nucleus ambiguus showing localized minimal epithelioid cell granulomas and lymphocytic or monocytic infiltration in the areas of the granulomas. With regard to its pathogenesis we concluded that it is a histological manifestation caused by an immunological reaction between release of an antigenic substance originated from leprosy bacilli and proliferation of antibody producing cells. Therefore, when the immunity of the host is temporally accelerated, as in borderline cases, it is dangerous if an antibiotic or treatment for acceleration of immunity is used carelessly.

Results of further investigations will be reported as follows:

- 1. More definite histopathologic pattern of the focus.
- 2. A few granulated bacilli and some acidfast granular substance in the foci.
- Secondary demyelination and axonal changes.
- 4. No remarkable changes in the nucleus facialis.
- 5. Correlation between bulbar changes and peripheral nerves.
- Rightsel, W.A., Sawyers, M.F. and Peters, J.H. Chemotherapeutic activity of sulfones and rifampin on growth of *M. lepraemurium* in macrophage diffusion chamber cultures.

A cell-impermeable diffusion chamber technic has been developed that lends itself for growth studies of *M. lepraemurium*. This system now has been applied to chemotherapeutic studies with this organism since we previously demonstrated a seven- to ninefold increase of acid-fast bacilli (AFB) when diffusion chamber cultures of macrophages were maintained on monolayer Petri plate cultures of the tissue cells. Hence, the technic provides a method for an *in vitro* evaluation of antileprosy drugs when not under the influence or metabolism of an animal host.

The sulfones, especially 4,4'-diaminodiphenyl sulfone (DDS), have been widely used in the treatment of leprosy, and more recently rifampin (RMP) has been shown to be an effective antileprosy drug. Also, there have been numerous reports dealing with evidence of the *in vivo* formation of 4-amino-4'-acetamidodiphenyl sulfone (MADDS) from DDS as well as deacetylation of MADDS by the animal host indicating a balance between the process of acety-

lation and deacetylation. As a result, it has not been established whether MADDS possesses inherent antileprotic activity, or whether it must be hydrolyzed to DDS in order to express its activity. In fact, it has been demonstrated that MADDS is deacetylated completely to DDS in the mouse [Levy et al. Proc. Soc. Exp. Biol. Med. 140 (1972) 937] and, hence, there is no method to determine if this sulfone derivative itself is active against either M. leprae or M. lepraemurium. Therefore, these studies have utilized the diffusion chamber technic with M. lepraemurium in Petri plate tissue cultures to evaluate the chemotherapeutic activity of MADDS when outside the influence of the animal host. In addition, the activity of RMP has been compared with the sulfones in this system.

Results of dose response studies indicate that 0.12  $\mu$ g/ml DDS partially inhibits growth of M. lepraemurium, whereas MADDS at this same concentration has little or no effect. Also, RMP at 0.012  $\mu g/ml$ partially inhibits growth of the organism using the in vitro diffusion chamber system. When equivalent molecular weights of DDS and MADDS were compared for activity, the DDS suppressed growth of the AFB, whereas MADDS was inactive and permitted vields similar to the untreated control chambers. The tissue culture fluids were analyzed for DDS and MADDS at each media change for six weeks. These analyses showed that no MADDS was formed in the samples containing DDS and that essentially no DDS was formed in the samples containing MADDS.

These results indicate that the diffusion chamber technic can be adapted to evaluate the chemotherapeutic activity of antileprosy drugs against *M. lepraemurium* and provide for the first time a method to compare these drugs outside the metabolic influence of the animal host. —[This investigation was supported by the U.S.-Japan Cooperative Medical Science Program administered by NIAID (grants R22-AI-08051, -08214, NIH)]

Murray, J.F., Jr., Gordon, G.R., Peters, J.H., Levy, L. and Prochazka, G.J. Metabolism and clearance of dapsone, monoacetyldapsone and acedapsone in Filipino leprosy patients receiving acedapsone.

To define the role of metabolism of the

repository drug acedapsone (DADDS, 4,4'diacetamidodiphenyl sulfone) in leprosy chemotherapy, we studied 20 patients receiving 225 mg of DADDS, intramuscularly. Plasma levels of DADDS, monoacetyldapsone (MADDS), and dapsone (DDS, 4,4'diaminodiphenyl sulfone) were determined by modifications of our earlier chromatographic-fluorometric procedure [Murray et al. J. Lab. Clin. Med. 78 (1971) 464] now sensitive to 0.1 ng/ml. Plasma levels of DADDS, MADDS and DDS were measured biweekly beginning one week after initial DADDS treatment. Mean plasma levels of DADDS, MADDS and DDS were 6, 23, and 29 ng/ml, respectively, at one week. Mean peak levels of 10, 36, and 45 ng/ml at three weeks declined slowly to means of 5, 23, and 25 ng/ml at 11 weeks. The DDS level was never less than 14 ng/ml in any patient. During the study period, DADDS was hydrolyzed to MADDS and DDS extensively (≥90%). The mean acetylation of DDS to MADDS for all patients following DADDS treatment was 42 ± 1% (S.E.) with a range of 32-56%. The mean half-times of disappearance (T<sup>1</sup>/<sub>2</sub>) for DADDS, MADDS, and DDS were 44, 54 and 50 days, respectively.

Previously, we showed that the genetic polymorphism for the acetylation of sulfamethazine (SMZ) also applies to DDS in Filipino subjects [Peters *et al.* Am. J. Trop. Med. Hyg. **21** (1972) 450]. To extend these observations to DADDS treatment, we determined the acetylator phenotype of each patient to the current study after an oral dose of SMZ (10 mg/kg). Determinations of the extent of SMZ acetylation in plasma showed that of the 20 subjects, 16 (80%) were rapid and 4 (20%) were slow acetylators. Acetylation of SMZ was also measured in urine, and these values were found to be directly related to those found in plasma.

Subsequently, DDS acetylation was determined in plasma obtained 8, 24, 32, and 48 hours after an oral dose of 50 mg DDS. These determinations also provided sufficient data points for calculations of the T<sup>1</sup>/<sub>2</sub> values of DDS and MADDS in these patients. The mean acetylation of DDS to MADDS was  $33 \pm 2\%$  (S.E.) and ranged from 24-48%, significantly lower than that found after DADDS treatment in the same patients.

Acetylation capacities for SMZ and for DDS after either DDS or DADDS treatment

were all positively correlated, showing that the patients' different acetylation capabilities were also exhibited after DADDS administration. No relationship was found between acetylation capacity and the  $T\frac{1}{2}$  of DDS in these patients, which ranged from 21 to 46 hours and averaged 31 hours.

The bacteriologic response of the patients' *M. leprae* to the DADDS treatment was measured in the mouse foot pad test system. No relationship between response and any measured metabolic parameter was found. —[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program, NIAID Grant AI-08214]

Gordon, G.R., Murray, J.F., Jr., Peters, J.H., Russell, D.A. and Vincin, D. R. Plasma levels of sulfones in patients of Papua, New Guinea, receiving acedapsone. Correlation with clinical assessment.

Since November 1967, the leprosy patients in the Karimui of Papua, New Guinea have been treated with 225 mg acedapsone (DADDS) every 75 to 77 days. In July 1972, nearly all the current 460 patients were bled immediately before the next regular injection of DADDS, and some again about two weeks later. Heparinized plasma was immediately prepared in the field and shipped frozen to SRI's Menlo Park laboratories where the concentrations of DADDS, monoacetyldapsone (MADDS), and dapsone (DDS) were determined by modifications of our chromatographic-fluorometric procedure [Murray et al. J. Lab. Clin. Med. 78 (1971) 464] now sensitive to 0.1 ng/ml.

We can now report results of these analyses in 286 patients who began DADDS treatment in November 1967. Of these, clinical assessment in December 1972 showed that 259 patients exhibited paucibacillary, and 27, bacilliferous disease. In the latter group, residual infiltration was noted in nine patients. No significant differences were noted in the mean levels of DDS, MADDS, or DADDS in plasma obtained at 75 to 77 days from the different groups of patients. Levels of DADDS, MADDS, and DDS in all 286 patients at this time averaged 17.3, 26.0, and 30.5 ng/ml, respectively. The lowest level of DDS was found in 8.9 ng/ml, substantially above the minimal inhibitory concentration (MIC) of DDS for M. leprae, which is about 4 ng DDS/ml [Peters et al,

Seventh U.S.-Japan Cooperative Conference on Leprosy, Tokyo, 8-10 November 1972]. In plasma obtained from 85 patients (77 paucibacillary and 8 bacilliferous) two weeks after DADDS administration, again no diseaserelated differences were noted in the sulfone levels. Mean levels of DADDS, MADDS, and DDS for all 85 patients were 19.2, 41.1, and 47.4 ng/ml, respectively. These results from both plasma samplings indicate that DDS levels do not fall below the MIC of DDS for *M. leprae* at any time during regular treatment with DADDS.

Comparisons of these sulfone levels in patients receiving DADDS for approximately five years with the levels found in Filipino patients after initial treatment with DADDS (Murray *et al*, this conference) suggest that long-term DADDS treatment yields 2- to 3fold higher plasma levels of DADDS, but only moderately higher levels of DDS and MADDS. —[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by NIAID (Contract NIH 70-2283)]

#### Russell, D. S., Worth, R. M., Jano, B., Fasal, P. and Shepard, C.C. Acedapsone in the preventive treatment of leprosy.

Trials of preventive treatment using oral DDS in children with household exposure to lepromatous leprosy have shown only partial success in reducing the subsequent attack rate among these children, perhaps due to the difficulty of maintaining consistent dosage over a long period of time. During three years (autumn 1967-autumn 1970) the entire population of about 1,500 Pingelapese people (Ponape, Micronesia) with a high prevalence of leprosy (about 7%) was offered acedapsone injections every 21/2 months (total of 15 injections in three years). Annual leprosy examinations of the entire population have been carefully done since 1967. The incidence of new cases per sixmonth period has been:

Number of new cases per six-month period



Of the five new cases developing in 1971-72, four are among 648 people who had taken less than the full course of acedapsone during the period of mass treatment and only one is among the 923 people who completed all 15 injections. The number of new cases is still too small to test for significance of difference of risk between these two groups.

Shepard, Charles C. Effects of the various combinations of clofazimine, dapsone, ethionamide and rifampin on *M. leprae.* 

Several drugs appear now to be effective against M. leprae in that they are bactericidal for M. leprae as judged in kinetic experiments in mice and in short-term trials in man. Combinations of them may be more effective in man than any of them used singly, so combinations were tested in mice in kinetic experiments to look for possible antagonistic or additive effects. The dosages used were: clofazimine (B663), 0.004%; dapsone (DDS), 0.01%; ethionamide (Eth), 0.1%; rifampin (RMP), 20 mg/kg. The first three were given as additions to the diet from the 70th-91st day after infection, and the last by gavage on the 90th and 91st day. No strong interactions were seen. Among the combinations involving RMP, some increased growth delay was observed with six of the seven possible combinations; among those not involving RMP, increased growth delay was seen in one of the four possible combinations. In an earlier experiment involving these four combinations [Internat. J. Leprosy 40 (1972) 33] additive effects were more common.

There are objectionable features to B663 and Eth so combinations of RMP and DDS are more likely. Since the rate of bactericide is much more rapid with RMP, the effects of timing were studied with RMP being given 0, 38, and 77 days after the start of DDS in a low dosage that caused bacteriostasis but not much bactericide. RMP appeared to be fully active on the *M. leprae* in DDS-induced bacteriostasis.

The advantages of combinations of antibacterial drugs involve the following theoretical mechanism: a) sequential action such as is seen with the synergism between the 2,4-diaminopyrimidines and sulfonamides or sulfones; b) overcoming the effects of heterogeneity in the microenvironment of the organism, that is, one drug may act in a location where another is not effective; c)

overcoming the effects of heterogeneity in the physiologic state of the organism, that is, a fraction of the bacteria escape one drug by virtue of a latent state in which, however, they may still be susceptible to another drug; and d) overcoming the effects of true drug resistance of genetic origin. Probably the experimental design tested only mechanism a) very effectively. The other mechanisms probably were not explored because, with these drugs they involve only very small fractions of the bacterial population. -[This work was supported in part by an interagency agreement with the National Institute of Allergy and Infectious Diseases. National Institutes of Health, under the U.S.-Japan Cooperative Medical Science Program]

Ozaki, Motoaki. Therapeutic trial of rifampicin (RFP) combined with DDS therapy in lepromatous leprosy. Observation of twelve months.

This trial has been carried out by 13 national leprosaria and the National Institute for Leprosy Research since October 1971. Observations have been made clinically, bacteriologically, histopathologically and serologically at regular intervals through the trial. The present paper, however, is a report of our clinical observation.

Pure lepromatous cases were selected and divided into following five groups, i.e., Group A, B, C and D. Group A consisted of untreated new cases and Group B of relapsed cases. Group C consisted of so-called resistant cases; they were divided furthermore into C-1 and C-2 according to the duration of their past treatment. Group C-1 consisted of cases with poor improvement in BI though treated regularly for more than five years. Group C-2 showed worse clinical features after regular treatment of more than one year. Group D were cases who were confronted with blindness.

The number of cases in this trial was 76. Numbers of cases in each group were as follows: Group A, 4; Group B, 9; Group C-1, 55; Group C-2, 5; and Group D, 3.

We administered RFP 450 mg orally before breakfast in a single dose. Two different methods of administration were adopted and compared; twice a week and six times a week. We made a rule to use DDS concurrently with RFP except in Group D. The dose of DDS was gradually increased and reached the maximum dose of 75 or 100 mg daily at the ninth week.

Among the 76 cases, 40 cases finished the scheduled administration of RFP for 12 months. Seventeen cases dropped from the trial for various reasons. In the remaining 19 cases the treatment has not reached 12 months yet. Clinical results of the 40 cases were as follows:

sixth months after beginning administration. It is noticeable that these cases were found only in the two day group. This symptom was suspected to be an allergic reaction to RFP, but in some cases it was difficult to distinguish clinically allergic response to the drug from ENL.

Gastric disturbances were the most frequent side effect during the treatment, but they were not so severe. Three cases com-

Two day method.						
Group	Effective	Excellent	No change	Reaction	Worse	Total
A	1					1
В	6	(4)				6
C-1	18	(7)		(1)		18
C-2	1	24110		20.00		1
D						0
Total	26	(11)		(1)		26

Figures in parentheses are doubly listed.

Six day method.

Group	Effective	Excellent	No change	Reaction	Worse	Total
A						0
В						0
C-1	13	(3)		(2)		13
C-2	1					1
D						0
Total	14	(3)		(2)		14

Figures in parentheses are doubly listed.

As shown in the above tables, all of the 40 cases who were given RFP for 12 months showed effective results. In Group C-1 with poor response to sulfone treatment in the past, RFP was very effective. As for Group A, B, C-2 and D, even though their numbers were too small to provide a clear conclusion, favorable results were also obtained. We could not find statistically significant differences between the two day method and the six day method as to clinical effect, fall of BI and MI and frequency of ENL.

The reasons why administration of RFP was stopped in 17 cases were as follows: ENL or eye complications in eight cases, borderline reaction in two cases, gastric disturbances in two cases, extra-medical reason in one case and high fever shortly after the intake of RFP in four cases. This symptom of high fever shortly after the intake of RFP was observed mainly in the fifth and plained of itching or paresthesia after intake of RFP. Transient increase of S-GOT and GPT values was observed in two cases. One case showed thrombocytopenia, which recovered after the administration of RFP stopped.

The fall of MI during the treatment was satisfactory in almost all cases. Decrease of BI in 12 months was slight but statistically significant. These data in detail and some clinical features of the patients in the course of treatment were shown at the conference.

#### Hastings, Robert C. and Morales, Melvyn, J. Studies on thalidomide's site of action in erythema nodosum leprosum.

From the clinical observations that thalidomide benefits *erythema nodosum leprosum* (ENL) patients within 24-48 hours [Sheskin, J. and Convit, J. Hautarzt 17

(1966) 548-549; Hastings et al. Clin. Pharmacol. Ther. 11 (1970) 481-487], it may be assumed that the "rate-limiting" step(s) inhibited by thalidomide most likely lie in the efferent limb of the humoral immune system or in the subsequent immunologically induced inflammation. Thalidomide inhibits various phases of carrageenan-induced rat paw edema [Winter, C. A., Risley, E. A. and Nuss, G. W. Proc. Soc. Exp. Biol. Med. 111 (1962) 544-547], a nonimmunologically mediated inflammation. Carrageenan rat paw edema has at least three phases [DiRosa, M., Papadmitrion, J. M. and Willoughby, D. A. J. Pathol. 105 (1971) 239-256]. The edema developing one hour after carrageenan injection is largely mediated by mast cell contents, principally 5-hydroxytryptamine (5HT) and to some extent, histamine, and is inhibited by thalidomide. Thalidomide inhibits both 5-HT and histamine in isolated perfused smooth muscle preparations, however, the potent anti-5HT agent, methysergide, is not active clinically in ENL patients. Antihistamines are known to have little if any anti-ENL effect clinically. Thus, an antihistamine or anti-5HT action does not explain thalidomide's anti-ENL activity.

The edema developing three hours after carrageenan is mediated primarily by bradykinin [DiRosa et al. Op. cit.] and perhaps complement [Willoughby, D.A., Coote, E. and Turk, J. L. J. Pathol. 97 (1969) 295-305], and is inhibited by thalidomide. Thalidomide does not significantly inhibit bradykinin in isolated perfused smooth muscle preparations and has no demonstrable effect on complement activity in vitro or to decrease in vivo blood levels in rats, or in humans [Sagher, F., Sheskin, J., Zlotnick, A. and Turk, J. L. Internat. J. Leprosy 39 (1971) 541-553]. Thus, there is no evidence that an antibradykinin or anticomplement action accounts for thalidomide's clinical effectiveness in ENL.

The edema developing six hours after carrageenan is mediated by prostaglandins and polymorphonuclear leukocytes (PMN's) [DiRosa *et al. Op. cit.*] and is also inhibited by thalidomide. Thalidomide significantly inhibits prostaglandin E<sub>2</sub> and prostaglandin  $F_{2\alpha}$  in isolated perfused smooth muscle preparations. Indomethacin and aspirin are potent inhibitors of prostaglandin synthetase [Vane, J. R. Nature (New Biol.) **273** (1965) 1084-1090 and 1143-1149], however, they are of limited efficacy in ENL clinically. In animals depleted of PMN's by pretreatment with methotrexate, indomethacin inhibits paw edema developing six hours after carrageenan while thalidomide is not active. Thalidomide's clinical action is thus probably not due to inhibition of prostaglandins.

The mechanism of PMN-mediated tissue destruction is ultimately lysosomal. Corticosteroids presumably are effective in ENL as well as in other inflammatory conditions by virtue of stabilizing lysosomal membranes [Weissman, G. New Engl. J. Med. 273 (1965) 1084-1090 and 1143-1149]. Thalidomide stabilizes light mitochondrial fraction rat liver lysosomes in vitro in concentrations attainable in vivo clinically. It has greater efficacy in stabilizing human liver lysosomes in vitro in similar concentrations. On the basis of experimental evidence to date, we believe the most likely site of thalidomide's action in ENL is on lysosomal membranes. By stabilizing the M. leprae-containing phagolysosomal membranes of lepromatous macrophages, thalidomide would reduce the release of the antigen presumably responsible for initiating ENL. By stabilizing the lysosomal membranes of PMN's, thalidomide would inhibit the release of extracellular enzymes responsible for the ultimate tissue destruction of ENL. -[Supported by the U.S.-Japan Cooperative Medical Science Program, Grant AI 10103]

#### Levy, Louis and Ng, Herman. Homologous and heterologous immunity in mouse foot pad infections with *Mycobacterium marinum* and *Mycobacterium leprae*.

Leprosy workers have studied the mechanism by which multiplication of *Mycobacterium leprae* in the mouse foot. pad is limited for several reasons. A more detailed knowledge of this mechanism in the mouse may lead to a better understanding of the failure of this mechanism to operate in patients with lepromatous leprosy, and might permit the testing of procedures designed to increase the immune responsiveness of leprosy patients. Such studies might also lead to a method of immunosuppression that would permit the development of better animal models of human leprosy.

Because of some of the practical difficulties encountered in studying M. leprae infection of mice, a model of this infection would be useful. The self-limited character of the disease process that follows inoculation of mouse foot pads with Mycobacteriummarinum, and the demonstration by Fenner [Am. Rev. Tuberc. **76** (1957) 76] of homologous and heterologous immunity in this process suggests that studies of M. marinum infection of the mouse foot pad might produce information useful in understanding M. *leprae* infection. That M. marinum infection of the mouse foot pad is more easily studied than M. *leprae* infection recommends it as a model for immunological studies.

Preliminary experiments with *M. marinum* were carried out to examine the correspondence between measurements of foot pad thickness and those of the numbers of acid-fast bacilli (AFB) and of colony-forming units (CFU). Although the measurement of foot pad thickness lacked great precision, it proved to be a good index of the numbers of AFB and CFU early in the course of the infection. No influence on the process by mouse age or sex could be demonstrated. As was shown earlier by Fenner [Am. Rev. Tuberc. 73 (1956) 650] inoculum size influenced the rapidity with which the process developed, but not its severity.

Studies of homologous and heterologous immunity were then performed. As Fenner had already demonstrated [Am. Rev. Tuberc. 76 (1957) 76], prior infection of one hind foot pad with M. marinum protected mice against challenge with M. marinum in the other hind foot pad. In addition, prior infection of one hind foot with M. marinum protected mice against challenge with M. leprae in the other hind foot pad. Similar experiments in which mice were first infected with M. leprae produced interesting results. Prior infection of one hind foot pad with M. leprae protected mice against challenge with M. leprae in the other hind foot pad; the protection was evident as early as 90 days after the first infection. Prior infection of one hind foot pad with M. leprae protected mice against challenge with M. marinum in the same foot pad, but not in the other foot pad.

Studies of protection by prior infection with *Besnoitia jellisoni* against challenge with *M. marinum* and *M. leprae*, and studies of protection by prior infection with *M. marinum* against challenge with *Listeria monocytogenes* have also been carried out. These will be reported separately [Krahenbuhl, J. L., Remington, J. S. and Levy, L. Paper presented at 8th Joint Conf. on Leprosy, San Francisco, July 1973]. —[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md., Grant R22-A107801]

#### Evans, Michael J. and Levy, Louis. Cellular response of the mouse foot pad to M. marinum.

The purpose of this study was to determine the cellular response of the mouse foot pad to an inoculum of Mycobacterium marinum. Several workers have shown that M. marinum multiply quickly in the mouse foot pad, reach a plateau, and then decline in numbers as killing of organisms begins. The killing of organisms is thought to have an immunological basis. In the present study,  $5 \times 10^3$  organisms were inoculated in the foot pads of locally bred BALB/c mice. At daily intervals of 2 through 8 days and at 11, 16, 18 and 22 days the animals were sacrificed. Some foot pads were prepared for electron microscopy, and the others for determining growth curves. The results of studies concerning growth curves are presented in another paper. The present study describes changes in the foot pad at the light and electron microscope level and compares them to previous results obtained from an M. leprae infection in the mouse foot pad.

At two and three days after inoculation, there was only a slight tissue response observed. This consisted of a few small cellular infiltrates composed of two cell populations, mononuclear macrophages and granular leucocytes. Only an occasional organism was observed. At four through eight days, there was a massive increase in the size of the cellular infiltrate and the number of organisms. This infiltrate was composed of mononuclear macrophages and granular leucocytes and persisted throughout the 22 days studied.

At the ultrastructural level, the organisms in mononuclear macrophages and granular leucocytes appeared solid. They were found contained in phagosomes and also free in the cytoplasm at three through eight days. During this time the macrophages did not appear to be activated. By 11 days there was evidence of activated macrophages in the infiltrate. This was illustrated by an increase in lysosomes and fusion of the lysosomes with organisms. This condition increased in intensity and by 18 days there were numerous examples of degenerating organisms in the cellular infiltrate. Many of the cells were also degenerating. Ultrastructural changes such as these have been associated with the onset of cellular immunity.

Compared to the *M. leprae* infection, several differences were noted: 1) The persistence of granular leucocytes in the *M. marinum* infection, 2) the presence of organisms free in the cytoplasm and also within phagosomes during the multiplication phase, 3) the occurrence of unusual lysosomes at 11 through 22 days, and 4) the lack of foamy vacuoles in macrophages during the later phase of the infection.

Krahenbuhl, J.L., Remington, J.S. and Levy, L. Studies of nonspecific resistance to *Mycobacterium leprae* and *Mycobacterium marinum* associated with chronic infection with *Besnoitia jellisoni*.

In a previous presentation before this group, we reported that mice infected with the protozoan Toxoplasma gondii were markedly resistant to foot pad infection with Mycobacterium leprae. Moreover, a booster injection of toxoplasma antigen administered into the infected foot pad further enhanced resistance to M. leprae. In the present report, similar studies were carried out to test the effects of chronic infection with Besnoitia jellisoni, on M. leprae infection in mice. BALB/C mice were challenged with  $5 \times 10^3$ M. lepare in both hind foot pads 40 days after infection with Besnoitia. Harvests of M. leprae 15 to 30 weeks after challenge revealed marked resistance to M. leprae growth in Besnoitia-infected mice. M. leprae multiplied in these mice, but at a rate much slower than in control mice. Whereas in control mice the number of M. leprae per foot pad 15 and 19 weeks postchallenge was 4.3 × 10<sup>5</sup> and 1.8 × 10<sup>6</sup> respectively, the number of organisms in the foot pads of Besnoitia-infected mice 15 and 22 weeks postchallenge was 2.1 × 104 and 1.5 × 105 respectively. When similar experiments were performed to test for nonspecific resistance to foot pad challenge with Mycobacterium marinum in Besnoitia-infected mice, there appeared to be no effect.

In related studies, foot pad infection with *M. marinum* did induce significant resistance to systemic challenge with *Listeria monocytogenes*, however. This resistance was transient, being lost by the 29th day after *M. marinum* infection. Whereas in our previous report we observed that *M. leprae* infection did not induce nonspecific resistance to systemic challenge with *L. monocytogenes*, in the present study such resistance was demonstrated in *M. leprae*-infected mice when the mice were boosted up with *M. marinum* antigen five days prior to challenge with Listeria.

Navalkar, R. G., Levy, L., Patel, P.J. and Dalvi, R.R. Immunologic studies on leprosy—plaque-forming cells in mice immunized with *M. leprae*.

Plaque-forming cells (PFC) in the spleen of mice immunized with M. leprae cell extract and also infected with M. leprae by the foot pad technic of Shepard were enumerated by localized hemolysis in gel, using sheep red blood cells (SRBC) coated with M. leprae cell extracts derived from infected human and armadillo tissues and the various antigen positive fractions obtained by column chromatography. In the primary response, the maximal level of PFC production was reached at ten days in both groups. Higher peaks of PFC levels were seen at five days in the spleen in the secondary response. The results in both groups appeared to parallel each other despite one being a nonliving challenge and the other a multiplying infection. The number of PFCs seen with the armadillo-derived cell extract coated SRBC were not significantly different from those observed with the SRBC coated with cell extract derived from human leproma, although such differences were noted with SRBC coated with other mycobacterial antigens.

Yang, Hong-Yi and Skinsnes, Olaf K. Light and electron microscopic study of dermal hypersensitivity and leprous reaction in leprosy.

Skin hypersensitivity tests of 42 leprosy patients (16 lepromatous and 26 tuberculoid) were studied with both light and electron microscopy. Four different antigens were used and a total of 63 skin biopsies (Table) were examined.

Dharmendra lepromin	20	6
Mitsuda lepromin	8	1
Tuberculin (PPD)	7	8
BCG	6	7
Total	41	22

Biopsies were taken at intervals from 24 hours to four weeks after the challenge. Results revealed the following changes:

- A. Lepromin tests—(Dharmendra and Mitsuda)
  - Early reactions to lepromin in both tuberculoid and lepromatous patients were perivascular fibrin deposit; lysis of basement membrane of venules and capillaries; hypertrophy of endothelial cells; infiltration by polymorphonuclear leukocytes; and increase of interstitial mast cells and degranulating mast cells.
  - Moderate and prolonged vascular damage occurred only in tuberculoid patients.
  - 3) Late reaction (after two weeks) was seen only in tuberculoid patients. It consisted primarily of a dense infiltration of lymphocytes and an increase in mast cell degranulation and disruption of collagen tissue. Macrophages were infrequently seen but those present were characterized by enlargement of the Golgi apparatus and hyperplasia of endoplasmic reticulum.
  - 4) Cellular infiltrates in the lepromin reaction regressed rapidly in lepromatous patients leaving occasionally discernible small clusters of vacuolated macrophages and swollen fibroblasts at perivascular areas.
- B. PPD tests and BCG inoculation
  - Skin reactions to PPD and BCG were not consistent in relation to the lepromin tests in either the lepromatous or the tuberculoid patients. Reactive cases in both lepromatous and tuberculoid patients showed a predominant lymphocytic response and tissue mast cell response to PPD tests and a predominant epithelioid cell response to BCG inoculations. Two lepromatous patients reacted with an unusual nodular accumulation of vacuolated and foamy reticuloendothelial cells at second and third weeks after BCG inoculation. These vacuolated cells con-

tained intracytoplasmic lipid droplets, myelin-figures and degenerated bacillary debris.

A comparison was made between these induced dermal allergic responses and leprous reactions of five tuberculoid and eight lepromatous patients. There were remarkable morphologic similarities. The immunopathologic relationship of these results will be discussed.

#### Smith, G. S., Walford, R. L., Prochazka, G. J. and Shepard, C. C. Histocompatibility antigens in leprosy.

An association between HL-A antigens and the occurrence of a variety of diseases has been demonstrated. In particular some of the diseases have a clearly demonstrated hereditary component, such as certain examples of psoriasis (HL-A17) while some have an origin or component of disturbed immune function, such as lupus erythematous (HL-A8, W15), and Hodgkin's disease (HL-A5, W18).

The documented disturbances of immune function in leprosy, and the differences of immune disturbance between lepromatous and tuberculoid leprosy warranted a search for possible associations with HL-A antigens.

Materials and methods. Blood specimens of 50 normal Filipinos and 82 patients of the Cebu region were sent to Los Angeles where lymphocytes were isolated and stored in liquid nitrogen until tested. Microlymphocytotoxic testing was used to identify 26 main HL-A specificities and some additional subdivisions using sera of the Vth International Workshop. Leprosy patients were identified and classified as to type by the Cebu leprosarium staff.

*Results.* The HL-A antigen frequencies of the controls, total patients and the lepromatous and tuberculoid subtypes are compared in Table 1. No statistically significant differences in antigen frequencies are seen between the controls and total patients or those with the tuberculoid form of the disease. The patients with lepromatous disease differ from the controls in that HL-A10 [and subgroup HL-A10.2 (W26)] is increased, p < .05 uncorrected. A similar increase of HL-A4, p < .05 uncorrected, is noted in the patients with lepromatous disease when compared to the normal Filipinos. HL-A5

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	Cabu	Patients			
HL-A antigen	, controls N = 50 percent	Total N = 82 percent	Lepromatous N = 38 percent	Tuberculoid N = 44 percent	
HL-AI	0	1.2	2.6	0	
HL-A2	16	22.0	15.8	27.3	
HL-A3	2	1.2	0	2.3	
HL-A9	62	69.5	76.3	63.6	
HL-A10 W25 W26	16 0 14	30.5 2.4 29.3	36.8 0 34.2	25.0 4.6 25.0	
HL-A11 W19	30 22	47.6 20.7	44.7 15.8	50.0 25.0	
HL-A4(4a)	50	67.1	73.7	61.4	
HL-A6(4b)	72	68.3	63.2	72.7	

 

 TABLE 1. Frequencies of HL-A antigens of the first locus among normal Filipinos and patients with Hansen's disease.

Italicized comparisons, p < 0.05 uncorrected.

 TABLE 2. Frequencies of HL-A antigens of the second locus among normal

 Filipinos and patients with Hansen's disease.

	Cebu		Patients			
HL-A antigen	controls N = 50 percent	Total N = 82 percent	Lepromatous N = 38 percent	Tuberculoid N = 44 percent		
HL-A5	4	8.5	0	15.9		
W5	0	1.2	0	2.3		
W18	2	3.7	0	6.8		
HL-A7	2	7.3	5.3	9.1		
HL-A8	0	0	0	0		
HL-A12	2	2.4	2.6	2.3		
HL-A13	4	0	0	0		
HL-A14	0	0	0	0		
W15 (LND)	26	34.2	36.8	31.8		
HL-A17	8	8.5	10.5	6.8		
HL-A27	8	1.2	0	2.3		

Italicized comparisons, p<0.05 uncorrected.

is not significantly different between the controls and total patients, but there is a clear difference in HL-A5 frequency between the two types of leprosy, p < .05 uncorrected, Table 2. When the p value of all the above findings are corrected for the number of antigens examined the differences are not statistically significant.

Discussion. The enigmas of leprosy are numerous. Skin reactions to lepromin and serum antimicrobial antibody titers among normal individuals in endemic areas have shown some predictive value in determining the degree of individual susceptibility and the type of leprosy contracted. Evidence exists of an hereditary or familial pattern in these immune reactions. Furthermore, individuals with lepromatous leprosy have circulating antimicrobial antibodies and immune complexes, and partial loss of cellular immune function which are not found in persons with tuberculoid leprosy. Changes in individual cases from the tuberculoid to the lepromatous forms of the disease, are accompanied by the above immune function alterations. Partial reversal of these changes has been observed in some cases of lepromatous leprosy brought under adequate control by drug therapy. On the basis of these observations it was felt that examination of the HL-A antigens among patients with Hansen's disease would be informative.

Comparisons of the Cebu control population to the total patients and each form of the disease revealed no statistically significant differences in HL-A antigen frequencies when corrected for the number of antigens tested. Uncorrected values do show an increased frequency of HL-A10 and subtype HL-A10.2 (W26) in the total patients and particularly in those with the lepromatous form of the disease.

HL-A5 is the only antigen in this study with possible importance in distinguishing between the lepromatous and tuberculoid forms of leprosy. While the corrected p value is not significant, additional cases and controls might confirm this suggestion. Consideration of the 4c cross-reacting group of HL-A5, W5 and possibly W18 (Table 2) shows an even more pronounced difference between the patients with the two polar forms of the disease.

In retrospective studies of this type the choice of an appropriate control population presents special problems of selection. Hence, one can expect to find only gross differences between patients and normal individuals of similar genetic origin. This problem is especially pronounced when dealing with a highly polymorphous antigen system such as HL-A and the relatively limited number of patients available.

Similar studies of additional populations of different HL-A genetic composition are required to determine if HL-A plays any role in susceptibility to Hansen's disease, or if it influences the propensity for the disease to be manifest in the tuberculoid or lepromatous form.

#### Morrison, N. E., Congdon, C. C. and Collins, F. M. Specific recall immunity in the T-cell deficient mouse.

Previous work had indicated that T-cell deficient mice produced by thymectomy and irradiation plus bone marrow reconstitution were unable to survive intravenous challenge with vaccinating doses of BCG. The mortal BCG infections were consistent with an absence of antimycobacterial immunity to attenuated organisms due to lack of adequate numbers of specifically committed small lymphocytes necessary for the mediation of an activated macrophage population.

This paper focuses on the question of whether T-cell deficient animals were able to develop enhanced immunity recall or immunologic "memory" to mycobacterial reinfection. For this purpose normal and T-cell deficient animals were BCG challenged by the aerogenic route and at the appropriate time rechallenged with the virulent Erdman strain of M. tuberculosis. Differential plate counts of viable organisms and measurements of delayed-type hypersensitivity (DTH) indicated very clearly that the T-cell deficient animal was unable to develop any significant recall immunity or peripheral DTH on subsequent rechallenge. These data thus are consistent with a T-cell role in the development of antimycobacterial recall immunity.

#### Congdon, C. C., Payne, H. S., Gengoziare, N. and Urso, P. Further studies on the thymectomized radiation chimera.

Under an Interagency Agreement between the USAEC and NIH, thymectomized radiation chimeras and various control mice produced in Oak Ridge were sent to investigators working on leprosy or closely related diseases. Additional experimental and control BC3F1 mice were kept in Oak Ridge for immune status determination. During the three year period of the agreement 3947 experimental and 3532 control mice were shipped to specially designated leprosy workers. Immune status of mice kept in Oak Ridge involved study of mortality, autopsy, skin graft rejection and serum antibody response. Cumulative mortality in 124 experimental mice showed~25% mortality at 360 days after thymectomy and 50% mortality at 415 days. Eighty-five percent of experimental mice were dead at 545 days. During this same period only 5% of 146 control mice died. Experimental animals examined at 18 months after thymectomy and irradiation still showed the effect of thymectomy on lymphatic tissues at autopsy. At the same time interval there was delay in skin allograft rejection and serum antibody production was still not equal to the control animal

level. Extreme wasting in experimental mice was occasionally observed. Preliminary evidence indicated that the thymectomized radiation chimera is especially susceptible to mouse hepatitis virus: A histologic study of white pulp blurring and germinal center production in experimental animals showed these cellular immune parameters to be markedly reduced when a planned antigenic challenge was presented. When the thymectomized radiation chimera was produced using allogeneic marrow instead of the usual syngeneic material an unusually low serum antibody production to red blood cells was obtained. Antibody response in vitro in thymectomized radiation chimeras made with allogeneic marrow was less than those made with syngeneic marrow. - [Research jointly sponsored by USAEC and NIH under contract to Union Carbide Corporation]

Abe, M., Minagawa, F. and Yoshino, Y. Studies on fluorescent leprosy antibody absorption (FLA-ABS) test. The change of antibody titer in relation to clinical pictures.

The indirect fluorescent antibody technic was applied to the detection of anti-*M. leprae* antibodies which remain in leprosy serum after absorption of cross-reactive antibodies to the other mycobacteria. Since this test, named FLA-ABS test, was found to be specific to leprosy, further investigation has been initiated for the purpose of establishing a new serodiagnostic test and of clarifying the immunopathologic significance of this antibody.

The average antibody titer was slightly higher in untreated new lepromatous cases than in relapsed or unimproved lepromatous cases under treatment and much lower in inactive lepromatous cases in retrogressive or quiescent stages. In borderline leprosy, the titer was variable with respective cases, the average being nearly the same as that of inactive lepromatous cases but still higher than that of tuberculoid.

Most of active lepromatous cases were treated with the combined use of DDS and rifampicin under the team on "a therapeutic trial of leprosy with rifampicin" in national leprosaria and the antibody titers were examined at three month intervals for a period of one year. The antibody titers tend to drop clearly up to the sixth or ninth month with the improvement of symptoms, as reported separately. However, the titer showed some increase at the twelfth month. A similar concave curve of antibody titer was frequently observed in the patients who got *erythema nodosum leprosum* (ENL) during the treatment.

The antibody titers of some patients with ENL or borderline reaction were examined by means of fluorescent antibodies monospecific to IgG, IgM and IgA, respectively. IgG antibody was found in six of eight patients with ENL, while the ratio was 10/21 in lepromatous patients without ENL. However, the ratio of antibody titers, IgM/IgG, was not significantly different between the two groups. IgA antibody was found in a patient who experienced borderline reaction during the treatment with DDS and rifampicin.

Park, B., Good, R. A. and Lim, S. D. Studies with the NBT dye reduction test in leprosy.

The nitroblue tetrazolium dye test has proved to be an extremely useful clinical test for distinguishing bacterial from nonbacterial infections and bacterial infections from a variety of mesenchymal and malignant diseases. It thus seemed of interest to study the nitroblue tetrazolium dye test in patients with various forms of leprosy. We had hoped that the NBT test might prove useful in evaluating therapy and the extent of systemic involvement in this disease. To our astonishment, we found that the NBT test was negative in all forms of leprosy including the lepromatous form. In tuberculosis the NBT dye test is positive in active and progressive disease and particularly in the miliary form. Thus, in leprosy where extensive phagocytosis is present, we encounter a bacterial disease, involving extensive phagocytosis in which the NBT dye test is not a reliable indicator of the presence of the bacterial infection. Nor is the phagocytic process associated with this form of bacterial disease revealed as in the phagocytic process with other bacterial disease.

Although further studies are needed to assess the basis of this observation, it seems likely that lack of involvement of the polymorphonuclear leukocytes in the phagocytic process in leprosy is revealed by these findings. This finding may have importance in

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the therapy of leprosy since the polymorphonuclear leukocytes exclude all antibiotics, including rifampicin and thus protect intracellular organisms which they have ingested, from killing by antibiotics. But the lack of involvement of the polymorphonuclear leukocytes in the phagocytic process in this disease is not due to depression of cells themselves since they respond vigorously after stimulation with endotoxin.

#### Han, S.H., Weiser, R.S., Wang, J.J., Tsai, L.C. and Lin, P.P. The behavior of leprous lymphocytes and macrophages in the macrophage migration-inhibition test.

The male volunteers studied included 16 tuberculoid and 22 lepromatous patients and 18 healthy subjects ranging from 30 to 50 years of age. All patients were or had been under treatment with DDS or B663; their disease status was judged by skin biopsy, physical findings and the lepromin test.

Collection of human lymphocytes and macrophages and guinea pig macrophages was accomplished by procedures in common use. The test medium was medium 199 supplemented with 20% heat-inactivated normal human AB serum. The leprolin was prepared by sonification of bacillary concentrates from biopsy specimens (not treated to kill the organisms) followed by millipore filtration  $(0.45 \,\mu)$ . The migration-inhibition tests were conducted by the standard procedures of David, Thor, and Dray.

Migration indices of normal guinea pig macrophages in the presence of leprous lymphocytes and leprolin (100 µg/ml) showed that whereas migration of macrophages was depressed 20% in the presence of tuberculoid lymphocytes, no significant depression occurred in the mixtures containing lepromatous lymphocytes or the control normal lymphocytes. This result indicated that only tuberculoid lymphocytes were sensitive to leprolin. Migration indices of leprous monocytes in the presence of tuberculoid lymphocytes and leprolin showed that a uniform depression of migration of 20% occurred in all three mixtures containing lepromatous, tuberculoid and normal macrophages respectively. This result indicated that macrophages of all three kinds (tuberculoid, lepromatous and normal) were uniformly responsive to MIF.

Migration indices of leprous and normal

monocytes in the presence of normal lymphocytes and leprolin were unaffected and indicated that normal lymphocytes are not sensitive to leprolin, that leprolin is not toxic to monocytes and that in the absence of MIF all three kinds of macrophages migrate uniformly and normally.

Results similar to those above were obtained using leprous monocytes in the presence of lepromatous lymphocytes and leprolin except that for inapparent reasons the migration of tuberculoid monocytes was slightly but significantly depressed.

The results indicate that, whereas tuberculoid lymphocytes are specifically sensitive to leprolin and can produce MIF in its presence, lepromatous lymphocytes are not sensitive to leprolin and fail to produce MIF in its presence. The results also indicate that lepromatous and tuberculoid macrophages are not lacking in their capacity to respond to MIF. --[This investigation was supported by the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases (Grant No. AI-08211) of the National Institute of Health, Department of Health, Education, and Welfare, USA, and a grant from the National Science Council, Taipei, Taiwan, Republic of China]

#### Rocklin, Ross E. and Brouillard, Robert. V Lymphocyte and monocyte function in patients with Hodgkin's disease.

It has long been appreciated that patients with Hodgkin's disease develop a defect in cellular immunity. Although the exact nature of this defect has not been well-defined some investigators have suggested that thymusderived lymphocyte function is depressed. However, it was recently demonstrated (Churchill et al, Internat. Symposium on Hodgkin's Disease, 1972) that in vitro lymphocyte function (macrophage MIF production and lymphocyte transformation) may be normal in some Hodgkin's patients with cutaneous anergy. These results suggested that perhaps a defect in mediator production other than MIF, an abnormality in macrophage function, or in the inflammatory response per se might account for their cutaneous anergy. In the present study the production of lymphocyte mediators (MIF and chemotactic factor for macrophages), activation of blood monocytes by lymphocyte mediators, and the inflammatory response following application of croton oil was evaluated in 15 untreated patients with Hodgkin's disease.

Five of the patients were found to be responsive to one or more skin test antigens (PPD and SK-SD) and ten were found to be unresponsive to these antigens. Lymphocytes from skin test positive patients produced MIF and transformed in vitro in response to PPD and SK-SD to the same extent as skin test positive normal subjects. Lymphocytes from nine anergic patients also produced MIF and transformed in response to SK-SD and/or PPD to the same extent as skin test positive patients and normal subjects. Furthermore, lymphocytes from these nine anergic patients produced comparable amounts of a chemotactic factor for macrophages (assayed by Dr. Peter Ward) as did lymphocytes from normal subjects. Eight patients (five skin test positive, three anergic) developed erythema and vesicle formation following the cutaneous application of croton oil to the same extent as did eight normal subjects. Using methods recently described [Rocklin et al, Fed. Proc. (Abst) 32 (1973) 988], monolayers of blood monocytes were prepared from 15 Hodgkin's patients and 12 normal subjects and incubated in vitro for two to three days in the presence of control and MIF-rich whole supernatants or Sephadex G-100 fractions. The extent of monocyte activation following incubation with lymphocyte mediators was evaluated by measuring cell adherence (ug cell protein or DNA content/dish) and glucose oxidation (glucose-1-C14 to C14O2). The results demonstrated that monocytes from Hodgkin's patients were activated to the same degree by lymphocyte mediators as monocytes from normal subjects (see table below).

Of particular interest was the finding that autologous MIF preparations from two anergic patients with normal lymphocyte function were effective in activating their monocytes. In addition, autologous MIF from another anergic Hodgkin's patient with depressed lymphocyte function did not activate his monocytes but homologous MIF did promote activation of the patient's monocytes. These findings indicate that the presence or absence of lymphocyte induced MIF correlates with monocyte activation in the same patient. In conclusion, lymphocyte and monocyte function as well as the inflammatory response appears to be normal in some anergic patients with Hodgkin's disease. Therefore, the cellular-immune defect in these patients is subtle and perhaps not detectable by the assays presently available for measuring cellular immunity. Alternately, substances may be present in vivo which inhibit the function of mediators such as MIF and chemotactic factor but which do not interfere with the in vitro detection of these mediators.

#### Good, R. A., Gajl-Peczalska, K., Kiszkiss, D., Choi, Y.S. and Lim, S.D. Perturbations of lymphocyte populations in leprosy.

Studies of the circulating lymphocyte populations have been carried out in 36 patients with various forms of leprosy. T-cell numbers have been evaluated by two separate technics. These included spontaneous nonimmune rosette formation with sheep red blood cells, and the cytotoxic effect of a specific anti-T-cell antiserum. Further T-cell functional responses were evaluated by quantifying the response of the lymphocytes to phytohemagglutinin in whole blood. Bcell numbers were evaluated by enumerating

Unfractionated	Cell Adherence	Glucose-I-C <sup>14</sup>
supernatants	ug protein or	oxidation nmoles/
(MIF/control)	DNA/dish	mg protein/min
Hodgkin's patients	35% increase	98% increase
Normal subjects	42% increase	110% increase
Sephadex G-100 fractions		
Hodgkin's patients	51% increase	116% increase
Normal subjects	58% increase	120% increase

the percentages and absolute numbers of lymphocytes that possess surface immunoglobulins of the three major classes, IgM, IgG and IgA.

Patients with active lepromatous leprosy showed lower than normal numbers of Tcells and markedly increased numbers of Bcells. T-cell responses to PHA were also much lower than normal. Effective treatment of lepromatous leprosy by injections of lymphocytes from a series of donors each of which was mismatched with the recipient and with each other at major histocompatibility determinants corrected this immunological imbalance.

Bullock, W., Dwyer, J. and Fields, J. Disturbance of the ratio between blood thymus-dependent (T) and bone marrow derived (B) lymphocytes in leprosy.

The abnormalities of cell mediated immunity (CMI) associated with lepromatous leprosy suggest disturbed T-cell function. T:B lymphocyte ratios were determined in peripheral blood of 13 normal and 17 lepromatous subjects by marking B cell immunoglobulin receptor sites with 125I-labeled rabbit antihuman IgM serum. Simultaneously, lymphocytes from these subjects were stimulated with phytohemagglutinin M (PHA) and pokeweed mitogen (PWM). In vitro studies were then correlated with clinical and histologic criteria of disease severity. Results indicate that B cells constituted a significantly higher percentage of lymphocytes in lepromatous than in normal blood (p < 0.01). In 8 of 17 cases, the percentage of B cells ranged from 40% to 80% whereas the normal range was 20-33% of B cells. Conversely, the percentage of lymphocytes forming spontaneous rosettes with sheep red blood cells [thymus derived (T) lymphocytes] was significantly reduced (p < .01) in peripheral blood of seven lepromatous patients (range 10-55%) compared with ten normal controls (range 53-75%). PHA-induced DNA synthesis by lymphocytes from patients was significantly reduced (p < .01) at three days of culture but not at seven days. However, four of the six patients with more severe lepromatous leprosy had abnormal PHA responses at seven days and higher percentages of B cells. Responses to PWM by lymphocytes from lepromatous patients were generally higher than normal but the

difference was not significant. In general, the *in vitro* abnormalities of lymphocytes from these cases were concordant with the presence of lepromatous infection in its most severe clinical form. This association was not invariable as T:B cell ratios and responses to mitogens were normal in two patients with severe leprosy. It is concluded that the nonspecific disturbance of CMI in some patients with lepromatous leprosy may result from a reduction of circulating T-cells by destruction or impedance to recirculation rather than from immunosuppression of intrinsically normal T-cells. —[Research supported by N1H, Grant AI-10094]

Rea, Thomas H. and Levan, Norman E. The concordance of tuberculin responses with the presence or absence of skin ulcerations in lepromatous leprosy.

Cell-mediated immunity (CMI) was measured in 34 patients with lepromatous leprosy using a battery of seven soluble, microbiological antigens and epicutaneous, allergic sensitization to di-nitro-fluoro-benzene. No significant differences were found between the total population of LL patients and the controls. LL patients without necrotizing skin lesions had a significantly diminished reactivity to tuberculin when compared to controls and when compared to LL patients with necrotizing skin lesions.

#### Tuberculin reactivity 10 mm induration or greater

#### Positive Negative

Control	29	39
Total LL	8	26
LL without necrosis	3	24
LL with necrosis	5	2

No differences were found on the basis of gender, sulfone therapy, thalidomide therapy or the presence or absence of *erythema nodosum leprosum*. Because of the numerous statistical comparisons made in this study the argument for "significance" cannot be made on a mathematical basis alone. We regard it to be peculiarly fortuitous that significant differences were found only with a mycobacterial antigen; furthermore our results are similar to those of Waters and Ridley [ Internat. J. Leprosy **31** (1963) 418-436] who report five of six patients with nec-

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rotizing skin reactions to be tuberculin positive. Finally our results parallel those of Salazar-Mallen et al [Alergia 18 (1971) 185-191] who report normal reactivity to histoplasmin and coccidioidin but diminished reactivity to tuberculin. The failure to demonstrate nonspecifically diminished CMI is similar to that of Convit et al [Internat. J. Leprosy 39 (1971) 556-564]; this failure might be attributable to ethnic factors, i.e., Mexican-born patients have not been extensively immunologically studied, or might be attributable to age, i.e., the median age of the present group, 31, is appreciably lower than that reported in other studies suggesting that duration of disease might have a role in explaining these differences. It is concluded that LL patients are a composite of two populations: the absence of necrotizing skin lesions defines a population that has a specifically diminished tuberculin reactivity and the presence of necrotizing skin lesions defines a population that has normal tuberculin reactivity.

#### Spitler, Lynn E. Transfer factor therapy.

Transfer factor is a dialyzable extract of sensitized leukocytes, which transfers reactivity from skin test positive donors to skin test negative recipients. Transfer factor supplied by our laboratory has been used therapeutically to induce cellular immunity in 78 patients around the world. Many patients received multiple doses of transfer factor ranging from one unit given every six months for three years to one unit every week for six months to as much as eight units per week for a brief period. A total of 299 units of transfer factor have been given.

Diseases in which transfer factor has been effective in prophylaxis against infections or in therapy include the Wiskott-Aldrich syndrome, severe combined immunodeficiency disease, mucocutaneous candidiasis, chronic active hepatitis, coccidioidomycosis, dysgammaglobulinemia, Behcet's disease, aphthous stomatitis, linear morphea, familial keratoacanthoma and malignancy.

Not all patients in each disease category respond to the administration of transfer factor, and this has led to the recognition of different forms of the various diseases and that these diseases may have different pathogenetic mechanisms although they may clinically appear similar.

#### Jacobson, R. R., Slack, C. E., Morales, M. J., Kirchheimer, W.F. and Hastings, R.C. Transfer factor in polar lepromatous leprosy. A preliminary report.

Several groups have administered either intact leukocytes or dialyzable transfer factor [Lawrence, H.S. and Valentine, F.T. Am. J. Pathol. 60 (1970) 437-450] to patients with leprosy in an effort to stimulate cellmediated immunity toward M. leprae [Bullock, W.E. et al. New Engl. J. Med. 287 (1972) 1053-1059; Lim, S. D. et al. Clin. Immunol. Immunopathol. 1 (1972) 122-139; Paradisi, E.R. et al. New Engl. J. Med. 280 (1969) 859-861]. In the present study, dialyzable transfer factor has been given to a 67 year old Latin American male patient as the only therapy for his sulfone-resistant polar lepromatous leprosy. In a period of 12 weeks the patient received transfer factor from a total of 7.44 × 109 lymphocytes in 37 divided doses from 14 healthy hospital personnel donors. The donors had a mean of 10.9 years of continuous exposure to active leprosy patients; had a mean Fernandez skin test reactivity of 9.36 mm induration and a mean Mitsuda reactivity of 7.57 mm induration to dilute integral lepromin (20 × 106 acid-fast bacilli/ml). After 15 injections (35 days) of transfer factor, the patient developed a reversal reaction of the type described clinically by Bullock et al [Op. cit.]. This reaction subsided in its original intensity but continued throughout the remainder of the trial. The patient showed clinical improvement with flattening of his nodular lepromatous skin lesions. Skin scrapings showed a fall in the Morphologic Index from a mean of 1.17% to 0 by day 61. The Bacteriologic Index fell from an average of 4.17+ to a low of 3.33+ by day 61 and then appeared to plateau and rose to 3.83+ by the last week of the trial. Skin biopsies revealed lymphocytic infiltration of the dermis in association with the clinical reversal reaction and a reduction in numbers of acid-fast bacilli in the areas of lymphocytic infiltrate. Base line skin biopsies revealed 59.9% of the dermis to be involved in the lepromatous infiltrate, the Bacteriologic Index was 6+ and the Morphologic Index was 5% with a classification of lepromatous disease. After transfer factor 20.8% of the dermis was involved in the lepromatous infiltrate; the Bacteriologic Index was 6+; and the Morphologic

Index was 4% and the classification remained lepromatous leprosy. After transfer factor, the lepromin skin test remained negative both at 48 hours and at three weeks. Multiple studies were negative for ocular, peripheral nerve, cardiovascular, renal, or hepatic toxicity to transfer factor. In this patient, under these conditions of administration, transfer factor appears to have modest antibacterial effectiveness as the sole treatment of sulfone-resistant, polar lepromatous leprosy. This effect is mediated by or is associated with an influx of lymphocytes into the lepromatous lesions. The material had no detectable local or systemic toxicity in this individual.

# Lim, S.D. and Good, R.A. Treatment of leprosy by cellular engineering.

After analyzing extensively the immune status of patients with lepromatous leprosy, we decided to determine whether immunotherapy could be applied to achieve bacteriological, cellular, histological and clinical improvement in patients with leprosy. Thus far, we have treated 13 patients, 11 with lepromatous leprosy, one tuberculoid, and one a mixed form of the disease. Treatment included weekly or biweekly injections with leukocytes from individual donors each of which was mismatched with one another and with the recipient patients by major histocompatibility factors. The treatment periods lasted two to four months. Patients in the first six instances could not be treated effectively with current chemotherapeutic regimens because they had developed lepra reactions which required large doses of adrenal steroids for control. Treatment produced prompt clinical histologic and bacteriologic improvement. Of the astonishing results obtained in these patients, five cases were treated without chemotherapeutic agents. Two patients were then treated under rigid supervision in the highly controlled environment of a clinical research center at the University of Minnesota. Informed consent for the therapeutic trial was obtained in each instance. One of the latter two patients had borderline leprosy, and the other lepromatous leprosy. All cases showed improvement of the state of the disease, histological improvement in lymph nodes and skin, and dramatic improvement in bacterial and biopsy indices.

These observations which now need controlled clinical trial suggest that lepromatous, tuberculoid and mixed forms of leprosy can receive significant benefits from immunotherapy. Since the donors of the leukocytes in the cases treated in Minnesota were both tuberculin and lepromin skin test negative, the beneficial influence of immunotherapy cannot be attributed to specific transfer of delayed allergy as our results seem in concert with those of Mudd and Yeatts, who have observed that nonspecific immunotherapy may offer much in the treatment of leprosy. Among the most dramatic influences of this form of immunotherapy in leprosy was the correction toward normal of a disbalance of the lymphoid system revealed by the high numbers and percentages of Blymphocytes and the low numbers and percentages of T-lymphocytes.

# Dwyer, John and Kantor, Fred S. Production of nonspecific anergy in guinea pigs.

Attempts to develop an animal model of the more specific anergy of the cellular immune system seen in chronic granulomatous diseases of man have been most successful in guinea pigs. Injection of 100 mg to 5 mg of BGG and HSA into guinea pigs immunized with these antigens and egg albumin (OA) in Freund's complete adjuvant eight days earlier resulted in a marked reduction of the ability of these animals to develop a delayed hypersensitivity (DH) skin response to any of these immunogens. The reduction of DH to PPD and OA represents nonspecific anergy which will last three to four days following a desensitizing injection. Daily desensitizing doses of HSA and BGG will consistently extend the period of generalized anergy to ten days at which time responsiveness to OA and PPD but not HSA and BGG returns. Immunocompetent cells transferred from an immunized guinea pig into a desensitized animal lose their ability to passively transfer DH. Antibody studies show that the loss of nonspecific desensitization after ten days results from the development of tolerance to the desensitizing antigen. Sequential immunization and desensitization with a number of antigens can perpetuate nonspecific anergy indefinitely. This suppression of cellular immunity seems to be an active phenomenon perhaps mediated through suppressor T-cells. The better immunized an animal, the more easily is anergy induced. Serum factors controlling lymphocyte proliferation and lymphokine production similar to those described in leprosy, sarcoidosis and syphilis are being sought in this model.

Rightsel, W.A., Sawyers, M.F. and Yang, G.C.H. Macromolecular syntheses of mouse macrophages infected with *Myco*bacterium leprae and *Mycobacterium lep*raemurium. (Read by title)

Since M. leprae has never been successfully cultivated in vitro, the host-parasite relationship has been studied in comparison with that of murine leprosy or M. lepraemurium. In addition, it is recognized that the natural host cell for each of these acid-fast organisms is the macrophage and, hence, should support the metabolic activity of the organisms. Furthermore, the ribonucleic acid (RNA) synthesis seems to be inhibited in mouse macrophages infected with M. lepraemurium [Ozata, K. and Oiaw, K. Infect. Immun. 5 (1972) 255]. Also, we previously demonstrated a seven- to nine-fold increase of M. lepraemurium when diffusion chamber cultures of macrophages were maintained on monolayer Petri plate cultures of the tissue cells. Therefore, it seemed logical to study the synthetic capabilities of these tissue culture preparations following in vitro infection with either M. leprae or M. lepraemurium. In each instance, the M. leprae organisms were freshly harvested bacilli that had been extracted from human biopsies. The M. lepraemurium organisms were freshly harvested bacilli that had been extracted from a three to four month infected fatty pad. In order to study the biochemical changes that occurred in tissue cultures of mouse macrophages after inoculation with acid-fast organisms, the incorporation by pulse labeling of <sup>3</sup>H-leucine and <sup>3</sup>H-uridine into trichloracetic acid precipitable fractions was used to measure the total protein and RNA syntheses respectively.

Studies on the synthetic capabilities of tissue cultures of mouse peritoneal macrophages following inoculation with *M. leprae* suggest that protein synthesis is immediately inhibited and progressively diminishes over a period of 12 days. On the other hand, macrophage cultures inoculated with *M. lepraemurium* show a continuous increase in protein synthesis abilities of engulfed macrophages. Neither result is like that of the uninoculated controls or macrophages inoculated with inert latex particles which show similar recurrent kinetic patterns.

No difference was observed in the RNA synthesis of uninoculated macrophages or macrophages inoculated with M. leprae or inert latex particles as indicated by their similar kinetic pattern over a 12-day period. In contrast, macrophages infected with M. lepraemurium show inhibition of RNA synthesis after four days' incubation. These methods provide an in vitro tissue culture system to study the effect of the obligate intracellular parasites on the macromolecular synthesis of infected macrophages. - [This investigation was supported by the U.S.-Japan Cooperative Medical Science Program administered by NIAID, Grant R22-AI-08051, NIH]