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U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

Program of Leprosy Symposium

FOREWORD

The Fifth Annual Leprosy Research Conference sponsored by the U.S.-Japan Cooperative Medical Science Program was held in Boston, Massachusetts, 24-26 April 1970. As previously, the program consisted essentially of proffered papers describing work completed during the previous year. The papers, and their abstracts published here, provide a measure of the status of leprosy research in the United States. Since 1965, leprosy research in this country has increased considerably, and a good part of the increase can be traced directly to the Program. There is now a widening interest in leprosy research, which fortunately has drawn new scientists into the field, and stimulated those already in the field to increase their efforts. These conferences have proven to be most valuable forums, providing, as they do, for the rapid exchange of new information and its discussion by a knowledgeable and interested group of colleagues.

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

Program of Leprosy Symposium

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Program of Leprosy Symposium

Fasal, P. The impact of recent leprosy research on patient management.

In January 1960, a Leprosy Clinic was inaugurated at the Public Health Service Hospital, in San Francisco. This clinic was elevated to a Leprosy Service in 1965.

A review of the experiences obtained with 288 patients having leprosy and seen between 1960-1970, demonstrated vividly the changes brought on by recent advances in research.

Individual patients as well as tables were used to illustrate changes in patient management.

Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. Properties of phenoloxidase in *Mycobacterium leprae.*

Our previous results demonstrated the presence of a characteristic phenoloxidase in *M. leprae.* Further studies were conducted to explore the nature of the enzyme in the leprosy organisms. In mushroom tyrosinase (with no monophenolase activity) and in melanoma extracts, the enzymes were inhibited with increasing substrate concentration. In *M. leprae,* no pronounced inhibitory effect was observed even at high substrate levels, indicating a greater affinity of the enzyme for the substrate.

The above observations could not be due to permeability barrier in the organisms, because the *M. leprae* preparations used had been disrupted by ultrasonic oscillation. We found ultrasonic oscillation to be a very effective procedure for rapidly breaking up the leprosy bacilli. To determine whether the phenoloxidase of *M. leprae* was soluble or particulate, the sonified suspension was subjected to ultracentifugation. Almost all of the activity was located in the particulate fraction.

The effect of cyanide on phenolase further demonstrated the higher affinity of the enzyme for the substrate in the case of *M. leprae.* Cyanide completely inhibited plant and mammalian tyrosinase; however, the leprosy organisms had to be preincubated with cyanide to produce even a partial effect. It has been reported that plant phenolase could oxidize NADH (DPNH) and NADPH (TPNH through the quinones formed in the reaction. Our results also show that a similar mechanism operates in *M. leprae,* indicating that substrates linked to NAD and NADP could be utilized by the organisms.

Several inhibitors of phenoloxidase tested prevented proliferation of *M. leprae* in the mouse foot pad. The effects of treatment with penicillamine and mimosine were particularly significant. These compounds produced total inhibition of plant and mammalian tyrosinase, but had no effect on *M. leprae;* they were oxidized by the organisms. Penicillamine and mimosine also did not arrest multiplication of the leprosy bacilli in the mouse foot pad. The results suggest that phenoloxidase probably has a role in the proliferation of *M. leprae.*

From the standpoint of chemotherapy, the most promising inhibitor of phenolase

was diethyldithiocarbamate. This compound inhibited the enzyme completely even in intact *M. leprae*. It not only prevented multiplication of the bacilli in the mouse foot pad, but was also effective in established infections in the mouse foot pad. These studies are still in progress.

Shepard, C. C., and McRae, D. H. A characteristic that varies between strains of *Mycobacterium leprae*.

On the basis of their behavior on passage in mouse foot pads, isolates of *M. leprae* may be arranged according to the average generation time (G) between inoculation and harvest (H) and the number of bacilli in the harvest, (Shepard, C.C. *et al.*, *J. Bact.* **89** (1965) 365-372). With different isolates, the median G varied from 18 to 42 days and the median H from $10^{5.6}$ to $10^{6.8}$, short generation times being associated with high harvests. "Fast" strains (short G and high H) usually did not occasion a cellular infiltrate (lymphocytes and macrophages) when their growth first reached detectable levels, whereas "slow" strains usually did. Bacillary harvests, routinely made in late log or early plateau phase, tended to have higher solid ratios in the case of "fast" strains.

The "fast" characteristic of a strain (a) did not change on continued mouse passage, (b) was usually the same in multiple isolates made from the same patient, and (c) was not associated with DDS-resistance or (d) with country of origin.

Since the properties of high harvest and minimal cellular reaction suggest lepromatous disease, and low harvest and lymphocytic infiltration suggest borderline disease, correlation with the type of patients' clinical disease is being looked for, although unsuccessfully so far. For example, the number of bacilli in the originating specimen (biopsy or nasal washing) does not correlate with the median H of the isolate.

McRae, D. H. and Shepard, C. C. Attempts to cultivate *Mycobacterium leprae* in tissue culture.

Tissue culture experiments were carried out in mouse macrophages by the technic of Chang (*Internat. J. Leprosy* **33** (1965) 586-588) and in the cells listed in Table 1 by the technic of Garbutt (*Internat. J. Leprosy* **33** (1965) 578-585). Inocula of *M. leprae* were highly purified suspensions (*J. Exper. Med.* **118** (1963) 195), from skin biopsies or mouse foot pads. Twenty-one mouse macrophage experiments were completed, but no evidence of bacillary increase was noted. The experiments by the Garbutt technic are summarized below:

This technic was usually successful in preventing large losses of bacilli, so that bacilli were usually detectable throughout the experiments. In some passages, bacillary increases were observed that were very probably technical artifacts since they were preceded or followed by passages with large decreases. In other instances, especially with 14pf cells, increases were observed during two or more consecutive

TABLE 1. *Tissue culture experiments with* M. leprae.

Cell	Species	Usual temp.	Total exper.	Exper. lasting > 100 days		
				No.	Duration	Passages
HEL	Human	33	7	5	249–315	6–8
14pf	Rat	33	7	5	218–332	11–16
FP	Mouse	30	8	5	167–280	3–10
IgH	Iguana	30	4	3	131–444	3–5
IgV	Iguana	30	13	11	118–463	2–8
P2H	Podocnemius	30	2	1	452	5
TKW	Terrapene	30	6	5	125–329	2–5

passages, sometimes even by both methods of measurement (bacillary counts and percentage of cells containing bacilli). However, doubt has now been thrown on even these results by mouse inoculation. In recent experiments involving FP and poikilothermic cells, mice have been regularly inoculated at each tissue culture passage. Bacillary viability was not demonstrable beyond 50 days, even in experiments in which later bacillary increases were observed in consecutive passages. Thus it would seem that in all *in vitro* cultivation experiments the bacillary viability should be monitored by mouse inoculations, since under many conditions viability does not seem to remain after two months.

Wiygul, W. C. and Rightsel, W. A. Studies on the phagocytosis of *Mycobacterium lepraemurium.*

Studies were conducted to compare phagocytosis of *M. lepraemurium* by mouse peritoneal macrophages, LM strain of mouse fibrocytes, and human embryonic skin cells. Cultures were prepared in Leighton tubes and incubated from 2 to 6 hours at 37°C. Following this period, the tubes were inoculated with *M. lepraemurium* at a multiplicity of 10 acid-fast bacilli (AFB) to one tissue cell and the tubes again incubated from 1 to 6 hours at 37°C. The cover glasses were removed, washed, fixed, stained, and intracellular bacilli enumerated.

Maximum phagocytosis was obtained when 4- to 6-hour cultures were inoculated with *M. lepraemurium,* and the infected cultures incubated an additional 4 to 6 hours. An examination of 500 cells on each of 5 cover glasses for a total of 2,500 cells yielded the following results: (1) 74.8% of the macrophages contained bacilli with an average of 5.68 AFB per cell; (2) 15.5% of the LM cells contained bacilli with an average of 1.70 AFB per cell; and (3) 20.6% of the human embryonic skin cells contained bacilli with an average of 1.81 AFB per cell.—[This investigation was supported by the United States-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 R22-A1-08051), Bethesda, Maryland 20014]

Rightsel, W. A. and Wiygul, W. C. Growth of *Mycobacterium lepraemurium* in millipore diffusion chambers.

Millipore diffusion chambers were used to study and compare the growth of *M. lepraemurium* in (a) the LM strain of mouse fibrocytes, (b) mouse peritoneal macrophages, and (c) cells derived from human embryonic skin (HES) when maintained in either mice, guinea-pigs, or in monolayers of the cell cultures in Petri plates. The experiments were terminated at selected time intervals, yields of acid-fast bacilli (AFB) determined, and generation times calculated. The average generation times were: (1) chambers with and without LM cells in mice, 9.9 and 11.0 days, respectively; (2) chambers with and without LM cells in guinea-pigs, 13.0 and 24.0 days, respectively; (3) chambers with LM cells in Petri plate cultures of LM cells 15.0 days; (4) chambers with and without macrophages in mice, 6.8 and 11.0 days, respectively; (5) chambers with and without macrophages in guinea-pigs, 21.0 and 24.0 days, respectively; (6) chambers with macrophages in Petri plate cultures of macrophages, 14.0 days; (7) chambers with and without HES cells in mice, 8.3 and 11.0 days, respectively; (8) chambers with and without HES cells in guinea-pigs, 9.6 and 14.4 days, respectively; and (9) chambers with HES cells in Petri plate cultures of HES cells, 12.9 days.

Experiments employing the diffusion chamber systems and maintained for 20, 30, 40 and 50 days yielded the following increase in numbers of AFB when chambers were inoculated with:

(1) macrophages and maintained in mice 7.6, 20.8, 27.2, and 30.7 fold increase.

(2) macrophages but maintained in guinea-pigs, 3.8, 5.4, 4.7, and 5.3 fold increase.

(3) macrophages and maintained in macrophage Petri plate cultures, 2.8, 5.4, 7.5, and 9.2 fold increases.
(4) LM cells and maintained in mice, 5.4, 8.3, 10.7, and 14.2 fold increase.
(5) LM cells and maintained in guinea-pigs, 3.8, 5.2, 7.1, and 9.8 fold increase.
(6) LM cells and maintained in LM cell Petri plate cultures, 2.3, 3.9, 6.0, and 8.4 fold increase.
(7) HES cells and maintained in mice, 5.1, 12.1, 22.2, and 28.4 fold increase.
(8) HES cells and maintained in guinea-pigs, 4.3, 7.8, 8.6, and 11.5 fold increase.
(9) HES cells and maintained in HES cell Petri plate cultures, 2.9, 4.6, 6.5, and 8.1 fold increase.
(10) no cells (cell-free) and maintained in mice, 3.3., 6.1, 10.1, and 12.5 fold increase.
(11) no cells (cell-free) and maintained in guinea-pigs, 2.7, 3.5, 4.8, and 5.8 fold increase.

Limited studies on the multiplication of *M. leprae* in diffusion chambers containing mouse peritoneal macrophages and maintained in mice for up to 77 days gave a 6.0 fold increase with an average generation time of 27.8 days.—[This investigation was supported by the United States-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 R22-AI-08051), Bethesda, Maryland 20014]

Andersen, R. N. and Chang, Y. T. Morphologic changes produced by various Ziehl-Neelsen staining technics on *Mycobacterium leprae* and *Mycobacterium lepraemurium*.

Nyka (*J. Bact.* 93 (1967) 1458) reported that the acid-fast appearance of *Mycobacterium tuberculosis* was enhanced if smears were treated with 10% periodic acid for 4 to 24 hours prior to Ziehl-Neelson (ZN) staining. In attempts to reduce the time required by this technic, the following modification was adopted in our laboratory. Smears, flooded with 2 ml. of 1% periodic acid, were heated gently until bubbles began to appear, allowed to stand for one minute without further heating, washed thoroughly and subjected to ZN staining. Morphologic comparisons were made of *M. leprae* and *M. lepraemurium* stained with the following technics: (1) cold ZN for at least 20 minutes at room temperature, (2) hot ZN staining by heating the stain (on smears) to boiling and allowing to stand for 5 minutes without further heating, (3) periodic acid followed by cold ZN staining, (4) periodic acid followed by hot ZN staining. All smears were destained with 1% HCL in 70% ethanol for a few seconds and counterstained with Loeffler's methylene blue. The percentage of solid bacilli was calculated from an average of two readings of 200 bacilli each. The solid category represents organisms of uniform shape which are stained homogeneously deep-red throughout the length of bacilli. The results are shown in the following table.

The highest percentage of solid bacilli was observed in the periodic acid-treated organisms of both *M. leprae* and *M. lepraemurium*. The organisms also appeared broader. After periodic acid, there was no marked difference in percentage of solid forms whether the bacilli were stained with the cold or hot ZN method. With ZN staining alone, heating appeared to affect the best staining of *M. lepraemurium*, while *M. leprae* were stained somewhat better with cold stain.

A large proportion of organisms, ranging from 8 to 85%, exhibited metachromatic granules in smears stained by the hot ZN method. These granules were observed occasionally with cold ZN staining, and were almost absent in periodic acid-treated organisms.

Apart from those bacilli categorized unequivocally as solid or nonsolid, a fairly large number of organisms were observed which were classified as nonsolid simply on the grounds that the stain was not taken up homogeneously; i.e., certain areas were

Organisms	Stored at −20° C, months	Solid bacilli, %				% metachromatic granules, hot ZN
		Cold ZN	Hot ZN	HIO_4 cold ZN	HIO_4 hot ZN	
M. leprae:						
TW 3	13	0.5	0	1.0	1.0	35.0
TW 4	12	2.0	0.5	11.3	8.3	28.5
TW 5	11	0.5	0.3	6.3	6.0	32.5
TW 6	10	1.0	1.5	6.0	6.0	73.0
TW 21	21 days	0	1.0	1.8	1.0	65.0
TW 20		0.8	0	16.5	21.0	76.0
TW 23		1.5	1.5	13.5	15.0	44.0
TW 24		1.0	1.0	6.0	7.0	34.0
Mouse foot pad, 8 months		7.0	3.0	48.0	50.0	8.0
M. lepraemurium (mouse, 135-day infection)		5.5	15.5	72.5	75.0	85.0

stained deeply while others were noticeably pale. They were observed in all stained smears, but were more numerous in those pretreated with periodic acid. The significance of these organisms as a potential source of bacillary growth should not be underestimated, since growth of the irregularly stained organisms have been observed with *M. lepraemurium* grown in macrophage cultures.—[Aided by Emmaus-Suisse grant through the World Health Organization]

Reich, C. V. The relationship between turbidity increases, microscopic counts, and viability in the genus *Mycobacterium.*

Bacteriologic reproduction during *in vitro* logarithmic growth is routinely accompanied by increases in three directly related but, not necessarily equivalent phenomena: microscopic counts of numbers of bacilli, increases in turbidity of the culture and, increases in the number of viable reproductive units. Since these are all measures of essentially the same biologic process, the validity of any procedure that is used for these determinations can be tested by a comparison of the growth curves produced by the three procedures with a single growing culture.

This effort was conducted using the potentially pathogenic mycobacterium "NQ" in order to take advantage of its apparent 100% acid-fastness in the logarithmic phase of growth, its smoothly dispersed growth and its reasonably short generation time (1.5 days). Microscopic counts, turbidities, and viabilities were taken with respect to duration of growth and these curves were compared.

The results indicated a marked disparity between microscopic counts of acid-fast stained aliquots and the number of viable reproductive units as determined by a tube dilution procedure (Poisson distribution). In the growth phase between mid-logarithmic growth and continuing past the stationary phase, the acid-fast counts represented 10% or less of the demonstrable viability. These differences were significant. The fact that the number of viable units was greater than the microscopic counts is in direct contradiction to the general findings with the microscopic count-viability relationship in data with Gram-staining organisms. This anomaly could be indicative that the acid-fast stain does not stain constantly representative portions of populations of mycobacteria, hence, it is possible that the acid-fast stain

may not be a valid means of making mycobacteria visible for quantitative microscopic evaluation.

Imaeda, T., Galindo, B., Kanetsuna, F. and Rieber, M. Biologic and ultrastructural characteristics of various mutants of mycobacteria.

The purpose of the present study is to isolate various types of mutants of standard strains of mycobacteria, and to examine their ultrastructural characteristics. The mycobacterial strains used were *M. tuberculosis* H37Ra, *M. smegmatis* ATCC 607, *M. smegmatis* 14468 and *M. phlei* ATCC 11758.

When *M. tuberculosis* and *M. smegmatis* 607 were seeded on brain-heart infusion or Penassay agar medium supplemented with glucose or glycerol, round yellowish-white colonies occur together with the rough surfaced, irregularly bordered colonies. These colony morphologies did not change during successive transfer on the same media. In contrast, the other two species, *M. smegmatis* 14468 and *M. phlei*, only form rough colonies under the same experimental conditions.

Ultrastructurally, the rough colony forming cells (R-clones) of all species show a three-layered cell wall. It consists of an outer nonosmiophilic fibrillar layer embedded in an amorphous substance, a middle osmiophilic saccule overlaid with a nonosmiophilic fibrillar network, and an inner layer of moderately osmiophilic matrix containing nonosmiophilic thick fibers. The cell wall of smooth colony forming cells (S-clones), which is much thicker than that of R-clone cells, is composed of two layers i.e., an outer nonosmiophilic fibrillar layer and an inner osmiophilic layer. Sometimes, flagella-like long tubular structures, which originate from the cytoplasmic membrane, protrude through the cell wall.

Of interest is the fact that S-clone cells contain small amounts of mycolic acid, which shows physical characteristics identical to that of R-clones of the same species. In addition, *in vitro* migration of alveolar macrophages obtained from rabbits sensitized with R-clone cells is inhibited by the S-clone cells of the same species. These data suggest that both S- and R-clones belong to the same species.

Since the S-clone cells are found to be insusceptible or resistant to some mycobacteriophages, to which the R-clones are susceptible, the S-clone cells are also selected by the mycobacteriophages after infecting the stock culture in which R- and S-clones may coexist.

Spontaneous mutation of the S-clone to large coccoids which are characterized by atypical thick cell walls with disordered septation occur at very low frequency. Treatment with large doses of ultra-violet light or with cycloserine is found to enhance this mutation. These coccoids are tentatively named "AWDS" mutants. The AWDS mutants liberate, spontaneously or following mitomycin C treatment, mycobacteriophages which infect certain species of R-clone cells of mycobacteria including their corresponding R-clones, but do not infect or lyse either S-clones or AWDS mutants. The cell walls of the AWDS mutants lack both fibrillar components and mycolic acids. It seems that the change of lysogenic state is related to the mutation of the S-clones (cryptic lysogeny) to the AWDS mutants (apparent lysogeny).

Although the S-clones have not been selected from the stock cultures of *M. smegmatis* 14468 and *M. phlei*, their AWDS mutants occasionally occur, spontaneously or upon UV irradiation, from their R-clones. The phages are inducible from the R-clones of these two species, while their AWDS mutants produce both infective and defective phages. Therefore, the function of defective lysogen(s) in the R-clones may be related to the alteration of the cell wall biosynthesis resulting in the formation of AWDS mutants.

The present results lead us to conclude that among mycobacterial populations there are at least three types of mutants or clones which are ultrastructurally different from each other. Their cell wall biosynthesis may be associated with the change of lysogenic state(s). It is also suggested that microorganisms ultrastructurally similar to S-clone cells or AWDS mutants, if they are obtained during the *in vitro* culture of human or murine leprosy bacilli should not

be discarded as contaminants, rather they should be carefully examined.—[This investigation was supported by the United States-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-07888), Bethesda, Maryland 20014]

Navalkar, G. and Warick, R. P. Studies on a temperature induced variant of *Mycobacterium balnei.*

Preliminary studies on *M. balnei* grown at 30°C and 37°C have shown that the enzyme activity of the strain grown at the higher temperature diminished significantly in regard to hydrolysis of various amides and in some instances the hydrolyzing capacity was absent. Also the 30°C cell extract had about 2.5 times more phosphoglucomutase and glucose-6-dehydrogenase activity than the 37°C cell extract. Protein estimates carried out on the concentrated culture filtrates and cell extracts showed that total soluble protein was about 50% higher in the 37°C culture than in identical preparations from the 30°C culture. Antigenic studies in progress at this time have also indicated differences between the parent strain and its temperature induced variant. The various changes already noticed in the mycobacterium when grown at the two different temperatures suggest the possibility of changes in its virulence and/or immunogenic faculties. It is visualized that these investigations will afford a better insight into the nature of infectious mycobacteria, in particular of organisms that have a predilection for low temperatures for both the *in vivo* and *in vitro* growths.—[This investigation was supported by the United States-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-08647), Bethesda, Maryland 20014]

Burchfield, H. P., Storrs, E. E. and Bhat, V. K. Analysis of DDS and MADDS in human plasma by gas chromatography.

Plasma (1 ml.) containing DDS and MADDS is extracted twice with ethyl acetate and the combined extract is washed successively with dilute sodium hydroxide and sulfuric acid solutions. The DDS and MADDS are then back extracted into 20% sulfuric acid. The solution is cooled, diazotized, and excess nitrous acid destroyed with sulfamic acid. KI is added to replace the diazonium groups with iodine atoms. DDS is converted to 4-iodophenyl sulfone and MADDS to 4-acetamido-4′-iodophenyl sulfone. These compounds are extracted into ethyl acetate and the solvent evaporated. The residue is hydrolyzed with sulfuric acid, then made basic and extracted into ethyl acetate. The extract is concentrated to a volume of 0.1 ml and a 5 μl. aliquot injected into a gas chromatograph equipped with an electron capture detector. Chromatography is carried out at 270°C on a two-foot column packed with 3% Versamid-900 on Gas-Chrom Q. Yields of 4-iodophenyl sulfone from DDS and 4-amino-4′-iodophenyl sulfone from MADDS are better than 80%. Sensitivity is of the order of 1-10 nanograms of each compound. No interfering peaks have been observed in any of the plasma samples examined thus far.—[This investigation was supported by the United States-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 (Contact PH-43-68-1272), Bethesda, Maryland 20014]

Harris, S. C. and Ribi, E. Separation of dapsone metabolites by centrifugal chromatography.

A rapid method for separation of 4,-4′-diaminodiphenyl sulfone (DDS), N-acetyl-DDS (MADDS), and N,N′-diacetyl-DDS (DADDS) has been developed. This technic eliminates the need for formation

of derivatives or for partition of the compounds between different solvents. Centrifugal force is used to accelerate migration of sample components through an ultra fine silica gel (particle width 150 Å). The apparatus used and details of the method have been previously described by Ribi *et al.* (*Ann. New York Acad. Sci.* **154** (1968) 41-57 and *J. Bact.* **102** (1970) 250-260.).

An artificial mixture of DDS, MADDS, and DADDS (individual components kindly supplied by Dr. A. J. Glazko, Parke, Davis & Co.) was dissolved in chloroform-methanol 65:25. Chloroform-methanol 97:3 was used for preparing the silica gel and for developing the chromatograms. The three components were separated into sharp bands after five minutes development at 2,500 x g. The bands, containing 10-20 γ of each component, were detected by either U.V.-illumination or the sulfuric acid-sodium dichromate charring method. By coupling elution of the bands with spectrophotofluorometry, quantitiative detection of nanogram quantities was possible.

Peters, J. H., Gordon, G. R., Ghoul, D. C., Levy, L. and Tolentino, J. G. Studies on the metabolism of dapsone in American and Philippine subjects.

Our earlier studies demonstrated that a direct parallel exists between capacities of normal subjects to acetylate isoniazid, sulfamethazine (SMZ) and dapsone (DDS) (Peters *et al. Fed. Proc.* **29** (1970) 803). Thus, the known genetic polymorphism for the acetylation of the first two drugs also applies to DDS. To examine the stability of the acetylation characteristic in normal subjects, we determined the plasma levels of DDS and monoacetyl DDS (MADDS) and the urinary excretion of DDS, MADDS, acid-labile DDS and MADDS, and acid-hydrolyzable conjugates of DDS in 6 subjects after four separate tests in each volunteer. Plasma and urine were obtained following oral administration of 100 mgm. DDS in the tests, which were separated by intervals of 2 to 4 weeks. The results clearly show that individuals' acetyation characteristics are stable and reproducible, Therefore, assignments of acetylator status from single tests are valid.

We have also examined the acetylation characteristics of an ethnically homogenous group of 25 subjects from Cebu, Philippines. The characteristics were determined from the urinary excretion of SMZ and acetyl SMZ following oral administration of SMZ (10 mgm./kgm.), and from the plasma levels of DDS and MADDS following oral DDS (50 mgm.) in seperate experiments. In these studies, also, a direct parallel between capacities to acetylate SMZ and DDS was found. Furthermore, levels of MADDS and ratios of MADDS:DDS were directly related, but no direct or inverse relationship could be discerned between levels of DDS and the ratios of MADDS:DDS. These results are similar to our earlier findings in a smaller group of American subjects.—[These investigations were 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 (Grants R22 AI-07801 and AI-08214, Order D-182741-O, and Contract NIH-69-2003), Bethesda, Maryland 20014]

Glazko, A. J., Dill, W. A. and Baukema, J. Some factors influencing absorption of parenteral sulfone drugs.

Following 300 mgm. I.M. doses of DADDS, the plasma DDS levels in man rose to a peak of 40-60 ng./ml. in 6 to 8 days, dropping to levels of 30-35 ng./ml. in the following week, where they remained for about 2 months (*American J. Trop. Med. & Hyg.* **17** (1968) 465). DADDS administered in 225 mgm. I.M. doses produced plasma levels of 20-25 ng./ml. in 1 week, and reached peak levels of about 30 ng./ml. in 4-6 weeks. In later time periods the levels were close to those observed with the higher dose. Since these products are mixtures of different crystal sizes, an attempt was made to distinguish between the effects of a "fine" particle size preparation *vs* a "medium" size preparation. Groups of 6 rhesus monkeys were given 50 mgm./kg. I.M. doses of these preparations. The initial plasma levels were about 4-fold greater with the "fine" preparation; these fell rap-

idly until the plasma levels were about the same for both preparations 6-8 weeks after dosing. Residual drug recovered from the injection sites 14 weeks after dosing represented 5-10% of the "fine" DADDS, and 18-39% of the "medium."

Using a repository sulfone derivative with somewhat greater water solubility than DADDS, Cl-608 [4'-4''-(p-phenylene-bismethylidyneimino - p-phenylenesulfonyl) -bis-acetenilide] in 225 mgm. I.M. doses produced higher plasma levels in man over the first 6-week period than equivalent doses of DADDS. The levels were about the same for the two preparations in later time periods. Residual drug at the site of injection in rat muscle was less than 1% of the dose in 8 weeks. About 50% of I.M. DADDS was recovered from rat muscle in 10 weeks, representing a slower rate of removal from the injection site.

Promin (glucosulfone sodium), a highly water-soluble sulfone derivative was administered I.M. to rats in doses of 50 mgm./kgm. It disappeared rapidly from the injection site with an estimated half-life of 15 minutes. This was accompanied by a sharp rise in the plasma and tissue levels of DDS and MADDS. Equivalent I.M. or per-oral doses of DDS produced similar levels. The half-life was about 10 hours in both experiments. The long half-life thus appears to be characteristic of DDS.—[This investigation was supported by the United States-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 (Contract PH-43-68-979), Bethesda, Maryland 20014]

Biggs, J. T. and Levy, L. Plasma protein binding of dapsone (DDS) and monoacetyl dapsone (MADDS) in rapid and slow acetylation phenotypes.

Plasma protein binding of DDS and MADDS has been investigated using an ultra-filtration technic. The degree of binding of DDS and its chief metabolite is of interest since there are several ways in which protein binding may affect DDS metabolism. Protein binding may play a role in the long half life of DDS and the virtual absence of MADDS in the urine. Although Gelber *et al.* (in press) have shown that after ingestion of either DDS or MADDS, constant and reproducible MADDS/DDS ratios are established within 4 to 6 hours which correspond with results obtained after acetylation phenotyping with sulfamethazine and isoniazid. Protein binding could be responsible for the individual variation in the MADDS/DDS ratio.

When binding of either compound is studied by the addition of different amounts of drug to plasma samples *in vitro*, binding of both DDS and MADDS appears to be constant over a wide range of concentrations. Preliminary work indicates that MADDS, the more tightly bound of the two compounds, does not significantly displace DDS previously bound to plasma proteins.

Volunteer subjects and leprosy patients previously phenotyped as rapid or slow acetylators ingested 50 or 100 mgm. of DDS. Blood samples were drawn and the MADDS/DDS ratio and per cent binding of each of the compounds was determined. MADDS appears to be 99 to 100% bound in all individuals studied regardless of acetylation phenotype, which may account for its virtual absence from the urine. DDS binding appears to be less complete and ranges from 70 to 80%. No relationship between the per cent DDS bound and the MADDS/DDS ratio appears to exist. Thus, individual variation in protein binding does not account for the differing MADDS/DDS ratios observed.—[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, Welfare (Grant R22-A1-07801), Bethesda, Maryland 20014]

Levy, L. Death of *Mycobacterium leprae* in mice, and the additional effect of dapsone administration.

The rate of killing of *M. leprae* by untreated immunologically normal mice has been measured by means of mouse passage

of organisms obtained from mouse foot pad harvests performed at weekly intervals after the plateau phase of bacterial multiplication had been attained. Death of *M. leprae* has been shown to occur by exponential decay, with a half-disappearance time of viable *M. leprae* of about 14 days.

The effect of dapsone, added to the mouse diet in a concentration of 0.1% just before maximal multiplication had occurred appears to be one of prolongation of the lag phase of multiplication of *M. leprae*.

These results were compared with the effect of dapsone administered by the kinetic method of Shepard (*Internat. J. Leprosy* **35** (1967) 429-435). The measurement of the half-disappearance time of dapsone in the male BALB/c mouse, in collaboration with Dr. John H. Peters, Stanford Research Institute, Menlo Park, California, permits a more precise interpretation of the results of the kinetic experiments. In these latter experiments, evidence of persisting bacteriostasis (analogous to lengthening of the lag phase) may be inferred.—[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 R22-A1-07801), Bethesda, Maryland 20014]

Morrison, N. E. and Dewbrey, E. E. Sulfone resistance in *Mycobacterium sp.* 607.

A series of 4:4′-diaminodiphenyl sulfone (DDS) resistant mutants have been isolated from *Mycobacterium sp.* 607. The mutants were isolated by step-wise selection in increasing concentrations of DDS in liquid medium. Studies have shown that significant cross-resistance occurs to all of the sulfonamides tested. No significant cross-resistance was found to antimycobacterial drugs such as isoniazid, streptomycin, viomycin, kanamycin, thiacetazone, D-ethambutol, rifampicin and the riminophenazine, B.663. A noteworthy feature of the sulfonamide cross-resistance was marked potentiation in the minimal inhibitory concentration (MIC) of the sulfonamide as compared to the sulfone MIC to block growth of the resistant organisms. The relationships between sulfonamide MIC's dissociation constants and liposolubilities for the DDS sensitive and resistant mutants have indicated fundamental changes associated with membrane penetration and/or active site binding has occurred with the emergence of DDS resistance. Bacteriologic data have demonstrated that p-aminobenzoic acid (PABA) reverses the growth inhibition by DDS. The reversal was competitive. The emergence of sulfonamide cross-resistance and the PABA competitive reversal of DDS action supports the hypothesis that DDS inhibits mycobacterial growth through antifolate activity.—[This investigation was supported in part 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-08416), Bethesda, Maryland, 20014]

Trautman, J. R. and Jacobson, R. R. Present status of the original group of twenty-two patients who received sulfone therapy for leprosy.

The treatment of leprosy was revolutionized by the introduction of sulfone therapy by Faget in 1941. The purpose of this paper is to describe the present status of the 22 patients with lepromatous leprosy who were included in the initial investigation of the effects of promin in leprosy. (Faget, G.H., Pogge, R.C., Johansen, F.A., Dinan, J.F., Prejean, B.M. and Eccles, C.G. The promin treatment of leprosy. A progress report. Publ. Hlth. Rept. **58** (1943) 1729-1741. Reprinted in: *Internat. J. Leprosy* **34** (1966) 298-310).

The current study encompasses a review of the clinical and laboratory data, with special emphasis on treatment received, adequacy of treatment, and, if inadequate, the reasons therefor.

Of the original 22 patients, 13 are living. Ten have active leprosy, and all of these either have sulfone resistant disease or are suspected of having it. Initially, response to promin therapy was generally dramatic, but the overall course of the majority of

those patients now living has been unsatisfactory.

An analysis of the information available strongly suggests that only those patients with lepromatous leprosy who received continuous sulfone therapy in dosages at least equivalent to the maximal dosages of DDS in use today achieved a state wherein their disease was inactivated and remained so. Irregular therapy, or therapy with inadequate dosages of sulfones, probably was responsible for the emergence of sulfone resistant leprosy.

Chang, Y. T. Effect of B.1912, a new riminophenazine derivative in murine leprosy.

The effect of a new riminophenazine derivative, B.1912 (2-anilino- 3-cyclohexylimino- 5-phenyl-8-chloro-3, 5-dihydrophenazine), was compared with B.663 in murine leprosy in mice. Four experiments were performed. In the first 2 experiments animals were treated on the day of infection and continued for 3 weeks in one experiment and 3 months in the other. The third experiment involved delayed treatment in which drugs were given 2 months after infection and continued for 3 months thereafter. In experiment 4, animals were treated for the first 5 months then observed for another 5 months without treatment to see if there was any bactericidal activity. Two different doses of each drug were used.

When drugs were administered from the beginning of the infection, both B.663 and B.1912 showed marked suppressive activity. All drugs revealed an equal activity except the smaller dose of B.1912, which was slightly less effective. In the third experiment, leprous growth was held in check by the larger dose of the drugs, while the smaller dose showed only a slight suppressive activity. In the fourth experiment, increase in both leprosy indices and bacillary counts was observed in animals treated with the smaller dose of the drugs. With the larger dose, proliferation of bacilli was indicated in the bacillary counts, but not in leprosy indices. It appeared that neither drug exhibited bactericidal activity. Both drugs were well tolerated in the long-term experiments.

Both drugs produced marked coloration. Difference in the degree of coloration between the two phenazines was not observed until the end of the 3-month experiment. At this time, coloration of animals receiving the larger dose of B.1912 was more marked than those receiving the same dose of B.663. Since coloration depends on drug accumulation, the extent of coloration should bear a direct relationship with the drug level in tissues. However, this does not seem to apply to phenazine compounds. The color of B.663 is medium orange in comparison to the orange-red B.1912. An equal coloration in the animals should indicate that the tissue concentration of the lighter dye is higher than the deeper one. A deeper coloration in the orange-red dye-treated animals might represent a tissue level less than or approaching that in the lighter dye-treated animals. Since tissue coloration at the end of three weeks was similar with both drugs, it would indicate that the rate of accumulation of B.1912, the deeper dye, was less than B.663, the lighter one. In other words, it appears that B.1912 actually exhibited a lower tissue concentration than B.663.

Difference in coloration of animals was also observed 5 months after discontinuation of the drugs in the last experiment. The drug coloration was almost completely eliminated in animals receiving B.1912, while definite coloration was still observed in those receiving B.663. This indicated that the rate of elimination of B.1912 was faster than B.663. Perhaps the cause of less accumulation of B.1912 was chiefly due to faster elimination.

These results suggest that B.1912 showed a rate of drug accumulation less than, and a rate of drug elimination faster than B.663 in mice. When the full dose of drugs was used, B.1912 was as effective as B.663 in the treatment of murine leprosy.

Skinsnes, O. K. and Yamashiro, K. M. Morphology and pathogenesis of peripheral nerve involvement in leprosy.

Twenty-five sciatic and 24 ulnar nerves derived from 14 autopsies on Chinese subjects were studied by sequential sectioning involving a total of 1,778 tissue blocks. Of

these 6 ulnar nerves included total lengths from brachial plexus to the wrist and 5 sciatic nerves were complete from the sciatic plexus through the posterior tibial nerve to the ankle level. The remainder of the specimens consisted of varying lengths of the same nerves. Twelve of the autopsies were on lepromatous patients. Additionally there was 1 intermediate and 1 tuberculoid case. Limited material from the latter two instances precluded definitive conclusions. In the lepromatous material, varying degrees of leprous lesions, as determined by foam cell infiltration and confirming acid-fast bacilli demonstrations, were found throughout the lengths of these nerves. These included significant lesions in those areas where the nerves lie deep to covering muscles.

The morphologic pattern of lesion distribution was compared with, and found similar to, the pattern of distribution of a series of lymphomas and carcinomas permeating abdominal nerves and peripheral nerve trunk of the extremities. This comparison suggested, that in addition to probable involvement of peripheral nerves in leprosy by blood born dissemination, direct extension of infection to the nerves may occur by lymphatic dissemination from skin lesions via lymphatic channels coursing with cutaneous nerve connections to the neural lymphatic plexus accompanying the peripheral nerve trunk. Demonstrations of acid-fast bacilli in perineural and intraneural lymphatics of cutaneous nerves in lepromatous leprosy were presented in support of the concept.

It was recalled that in first infection type tuberculosis there is lymphatic dissemination of bacilli to regional lymph nodes and that such dissemination is not generally seen in second infection type tuberculosis when some degree of immunity and hypersensitivity has been established. It was suggested that, immunologically, lepromatous leprosy is analogous to an ongoing first infection type in tuberculosis whereas tuberculoid leprosy is analogous to second infection type of tuberculosis. In the light of this analogy, it is possible that in lepromatous leprosy continuous lymphatic dissemination of infection to peripheral nerves may take place while such dissemination in tuberculoid leprosy might be expected to occur only in the first weeks of infection before the establishment of enhanced cellular immunity and the development of specific hypersensitivity to *M. leprae*.

There is a further possibility that lipid breakdown products of leprosy bacilli may promote lymphatic drainage in a fashion analogous to that of the intestinal lacteals.—[This investigation was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education, and Welfare (Grant AI-04353), Bethesda, Maryland 20014]

Drutz, D. J. and Cline, M. J. Polymorphonuclear leukocyte and macrophage function in leprosy.

Lepromatous leprosy is characterized by a generalized impairment of lymphocyte-mediated immune phenomena similar to that described in sarcoidosis and Hodgkin's disease. While there is no clear evidence that lepromatous leprosy patients share the increased susceptibility to infection common to the latter two diseases, there is some suggestion in the literature that both tuberculosis and disseminated vaccinia may occur more commonly in lepromatous than tuberculoid leprosy patients.

In order to assess the cellular antimicrobial mechanisms in leprosy patients, three sets of studies were carried out. Polymorphonuclear leukocyte (PMN) function was evaluated by the ability of these cells to kill yeast-phase *Candida albicans* using the technic of Lehrer (*J. Bact.* **98** (1969) 996). In these studies, PMN's from most patients with tuberculoid, borderline, and lepromatous leprosy phagocytized and killed the test organism as efficiently as normal PMN's when studies were carried out in pooled homologous serum from normal donors. Three patients (2 lepromatous; 1 borderline), 2 of whom were receiving steroids for reactive states, had abnormally low PMN candidacidal activity. This phenomenon is under further investigation.

In the second set of studies, the ability of human macrophages derived from peripheral blood monocytes to kill the fac-

ultative intracellular bacterium *Listeria monocytogenes* was evaluated. Monocytes were isolated from human blood by the method of Cline (*J. Exper. Med.* **128** (1968) 1309), which takes advantage of their selective buoyancy in albumin gradients. Macrophages were derived from these cells in tissue culture, the medium consisting of McCoy's medium, 30% pooled serum from normal AB blood donors, and penicillin and streptomycin or penicillin alone. Macrophages from tuberculoid, borderline and lepromatous patients were equally capable of killing *Listeria* and were superior in nearly all cases to macrophages derived from normal donors whether cells had been grown initially in penicillin-streptomycin or penicillin-containing medium alone. Listericidal activity was unrelated to type or duration of chemotherapy or to presence of the various reactive states.

In the third set of studies, the relative ability of macrophages derived from tuberculoid and lepromatous patients to digest washed, heat-killed *M. leprae* isolated from infected mouse foot pads was evaluated. Beiguelman, as well as Barbieri and Correa (*Internat. J. Leprosy* **35** (1967) 377-381) have claimed that macrophages derived from lepromatous leprosy patients are less capable than tuberculoid macrophages of digesting dead leprosy bacilli. In our studies, leprosy bacilli underwent equivalent fragmentation in tuberculoid and lepromatous macrophages over a period of 3 weeks of intracellular residence. In no case did bacilli disappear from cells. Impressive giant cell formation occurred in cultures derived from both tuberculoid and lepromatous macrophages when leprosy bacilli were present, but not in control cultures devoid of the bacteria.

Thus, in studies to date, we can find no evidence for *in vitro* differences between tuberculoid and lepromatous macrophages despite impressive evidence for differences in lymphocytes between these disease forms. Further studies of the role of lymphocyte-macrophage interaction in host defense against intracellular microorganisms such as *M. leprae* are in progress.

Han, S. H., Weiser, R. S. and Kau, S. T. Prolonged survival of skin allografts in leprosy patients.

It is generally recognized that immunologic abnormalities involving delayed hypersensitivity occur in leprosy patients, especially those with lepromatous leprosy. Since acquired allograft immunity is largely of a cellular nature and since acquired antitissue cellular immunity and delayed hypersensitivity appear to be obligatorily interdependent, it is logical to expect that allograft immunity would be impaired in leprosy.

Thirty well-nourished young, adult male volunteers were employed; 10 had lepromatous leprosy, 10 had tuberculoid leprosy, and the remaining 10 were normal, healthy subjects. All leprosy patients were under DDS treatment. The subjects were divided into experimental groups of 6. Grafting within each group involved reciprocal skin grafts transferred between 2 normal subjects and 2 skin grafts transferred from each of the 2 normal subjects to patients, one graft to a lepromatous patient and the other to a tuberculoid patient.

Donors were carefully screened, and no donor whose SGOT titer exceeded 40 units was employed. Histocompatibility typing was not done. Grafting was conducted by the method of Converse and Rappaport. The rejection interval was defined as the shortest interval when $\frac{2}{3}$ or more of the graft showed evidence of circulatory stasis. A few biopsied specimens taken early in graft rejection showed a histologic picture which was similar in all groups. The mean survival times of grafts were: normal subjects, 11.22 days; lepromatous patients, 15.2 days and tuberculoid patients, 13.44 days. Analysis by "student t test" showed that the average differences between all groups were statistically significant. The results showed clearly that allograft immunity is impaired significantly in the two polar forms of leprosy, impairment being greatest in lepromatous leprosy. The results are compatible with previous observations that other expressions of nonspecific immunologic impairment related to delayed hyper

sensitivity are greater in lepromatous than in tuberculoid leprosy.—[This investigation was supported by the United States-Japan Cooperative Medical Sciences Program administered by the National Institute of Allergy and Infectious Disease of the National Institutes of Health, Department of Health, Education, and Welfare (Grant AI-08211), Bethesda, Maryland 20014; and a grant from the National Science Council, Taipei, Taiwan, Republic of China]

Rees, R. J. W. Review of some recent studies on leprosy, 1969-1970.

Dapsone resistant studies. Since 1964, on the basis of clinical and mouse foot pad infections, 22 patients with dapsone resistance have been detected at our Leprosy Research Unit in Malaysia. Bacilli from 20/22 patients were resistant to dapsone levels in the mouse (0.01% in the diet; 1.5 μg./ml. in the serum) equivalent to patients receiving 100 mgm. dapsone/day. Three out of nine strains tested multiplied in mice fed 10 times greater levels of dapsone. Analysis of 21 of these patients show a history of prior sulfone treatment of 5-16 years (mean 11). Nine of the 21 (43%) received solapsone as well as dapsone for a part of this period, the remainder received dapsone throughout. In the period covered only 10% of all patients in Malaysia received solapsone. Currently the distribution of slow-fast acetylation phenotypes to isoniazid are being studied in 16 of these patients in collaboration with Dr. G. A. Ellard (Rees, Pearson and Waters).

Determination of the MIC of sulfadimethoxine and sulformethoxine against *M. leprae* in the mouse. The minimal inhibitory concentration (MIC) for sulfadimethoxine was 0.01% in the diet giving a plasma concentration of 37 μg./ml. and for sulformethoxine 0.04% and 62 μg./ml. respectively. Therefore these two long-acting sulfonamides are far less active against *M. leprae* than dapsone with the equivalent figures of 0.0001% and 0.018 μg/ml., respectively. We have already shown cross resistance between dapsone-resistant strains of *M. leprae* and these two long-acting sulfonamides. While our results confirm the antileprosy activity of these two sulfonamides their MICs are close to the concentrations of the drugs obtained in man on standard doses compared with dapsone levels in the serum 100-fold higher than the MIC obtained in patients on standard dapsone therapy. For these reasons we conclude that treatment with these long-acting sulfonamides may much more readily induce sulfone resistant strains of *M. leprae* than with dapsone treatment. (Ellard and Rees).

Ability of peripheral monocytes from leprosy patients to "digest" *M. leprae in vitro*. We have failed to confirm the claim of Barbieri, Correa and Beiguelman that blood monocytes from tuberculoid but not lepromatous, patients are capable of digesting heat-killed *M. leprae in vitro* based on changes in the numbers and morphology of acid-fast bacilli within monocytes from seven fully validated pairs of tuberculoid and lepromatous patients (Godal and Rees).

The role of lymphokines and lymphocytes in mycobacterial infections. Antigen stimulated lymphocytes have been shown to release soluble substances which activate macrophages leading to their proliferation or inhibition of their migration. It has been hypothesized that lymphokines play a role in cell-mediated immunity, although they have not hitherto been shown to inhibit growth of bacteria within macrophages. Our present studies have shown that when lymphokines are added to cultures of peripheral blood-derived macrophages infected with vole bacilli or *M. lepraemurium*, the macrophages are not only activated but are then capable of completely inhibiting multiplication of their intracellular load of mycobacteria.

Similarly macrophage activation was obtained in cultures of blood-derived macrophages exposed to *M. leprae in vitro* from patients with tuberculoid leprosy (high resistant form) in the presence of lymphocytes. No such activation was obtained in the absence of lymphocytes. Under similar conditions no activation was observed in cultures of macrophages from patients with lepromatous leprosy (low resistant form of the disease). These results underline the presence of a lymphocyte-macrophage deficiency in patients with lepromatous leprosy. (Godal and Rees).

Recent studies on *M. leprae* in thymectomized-irradiated mice. It has now been established that the intraperitoneal inoculation of *M. leprae* in thymectomized-irradiated mice results, by 15 months or later, in a progressive infection confined to the same specific sites, i.e., skin of nose, ears and foot pads and peripheral nerves, as obtained in similar mice inoculated intravenously. Thus the intraperitoneal route provides a much more practicable site of inoculation for obtaining heavily and systemically infected mice than the intravenous route, since the latter requires much greater purification of bacilli to avoid immediate toxic manifestations from the homogenates. It has also been shown that the intratesticular inoculation of *M. leprae* in thymectomized-irradiated mice not only leads to high yields of bacilli locally but eventually to spread of infection to all sites of predilection. Mice inoculated intravenously or intraperitoneally also, by 15 months and later, give high yields of bacilli in the testes, comparable to those obtained in now well established sites of predilection (Rees).

Peripheral nerve damage in normal and thymectomized-irradiated mice inoculated with *M. leprae*. The peripheral nerves of all mice whether normal or immunologically suppressed and by whatever route inoculated eventually (12 months onwards) become infected with *M. leprae*. Bacilli are always found within Schwann or perineural cells. These bacilli do not necessarily lead to degeneration of nerve fibers. However, with passage of time there is in all these mice histologic evidence of increasing nerve degeneration. Eventually (20 months onwards) nerve damage can be sufficiently advanced to manifest as deformities of the hind-legs, which we have designated "foot-drop." This is best manifest by holding the mouse vertically at the nape of the neck and root of the tail. In this position a mouse with severe sciatic nerve damage is unable to retain the hind leg in flexion with the toes spread. As the mouse tires in this position these manifestations become exaggerated often with brief periods of ankle clonus. Histologically such severe nerve damage is associated with gross loss of nerve fibers within the endoneurium, collagen replacement and destruction of the perineurial sheath by fibrous tissue. All these manifestations replicate completely the severe nerve damage seen in patients with leprosy. Systematic studies at the level of the light and electron microscope at different stages are indicating that the primary lesion is of the perineurium. Once the perineurium is damaged nerve fibers within the endoneurium undergo degeneration. These manifestations resemble most closely the changes seen in congenital hypertrophic peripheral neuropathy. Thus we believe that the mouse is again providing important new information on the pathogenesis of neural damage in leprosy (Weddell and Rees).

Dill, W. A., Chucot, L. and Glazko, A. J. A new analytical procedure for B.663 (clofazimine).

B.663 is a riminophenazine derivative [2-(*p*-chloroanilino)-5-(p - chlorophenyl-3, 5-dihydro-3 - (isopropylimino) - phenazine] which is effective in the treatment of lepromatous leprosy (Brown and Hogerzeil, *Leprosy Rev.* 33 (1962) 6). Unchanged drug appears to accumulate in tissues, where it may be present partly as a colorless reduced form (Barry *et al., Nature* 179 (1957) 1013). Plasma levels have not been measured in the absence of a satisfactory analytic procedure. The present report outlines a highly sensitive analytic procedure which yields good recovery of B.663 from plasma and tissue homogenate:

(a) Take 3 ml. plasma + 3 ml. of 1% "Maxatase" (Pfizer) in 2% borax; or 1 ml. 1:10 tissue homogenate + 1 ml. enzyme-borax mixture. Incubate at 25°C for 30 minutes.

(b) Add 12.5 ml. n-heptane. Extract by shaking for 10 min. in a glass-stoppered tube.

(c)Transfer 10 ml. of the heptane layer to a fresh tube, add 3 ml. 1M citric acid and extract drug into the aqueous layer by shaking.

(d) Transfer 2 ml. of the citric acid layer to a fresh tube, add 2 drops of 3% titanous chloride + 1 drop 6N sulfuric acid.

(e) Heat mixture on a steam bath at 100°C for 10 min. Cool.

(f) Add 1.5 ml. 2-ethylhexanol and extract by shaking.

(g) Separate the 2-ethylhexanol layer; measure fluorescence at 366 mμ emission (Aminco-Bowman).

Normal plasma specimens containing 0.1-1.0 μgm. of B.663 per ml. are used as standards. The fluorescence is directly related to drug concentration over a range of 0-3.0 μgm./ml. Additional work will be needed to see if the leuco form of the drug is extracted quantitatively under the same conditions as the colored form, and to determine whether any metabolites of B.663 interfere with this analytic procedure.

Rat experiments with 50 mgm./kgm. peroral doses of B.663 produced peak plasma levels of 0.41-0.44 μgm./ml. in 8-24 hours; drug levels in the liver were about 23 μgm./gm. in 4 hours, falling to 6 μgm./gm. in 24 hours and 4 μgm./gm. in 72 hours. Four peroral doses of 25 mgm./kgm. each, administered perorally to rats over a 3-day period, produced highest levels 4 hours post-dose in body fat (170-220 μgm./gm.), with progressively lower levels in the liver (90 μgm./gm.), lung (30-50 μgm./gm.), kidney (30-40 μgm./gm.), spleen (12-13 μgm./gm.), heart (6-26 μgm./gm.), and skeletal muscle (4 μgm./gm.). The pattern of metabolic disposition suggested by this study is one of drug accumulation in body fat, followed by redistribution into other tissues and slow elimination.—[This investigation was supported by the United States-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 (Contract PH-43-68-979), Bethesda, Maryland 20014]

Han, S. H., Weiser, R. S. and Ling, P. P. Inhibition of macrophage migration by lymphocytes from leprosy patients in the presence of PPD and extracts of *Mycobacterium leprae*.

The observation that in lepromatous leprosy delayed sensitivity to lepromin is essentially absent and that delayed sensitivity responses to other allergens is severely depressed prompted us to investigate the inhibition-of-macrophages-migration responses in leprosy patients. Since it is difficult to obtain cell mixtures from man containing sufficient numbers of macrophages, the tests were conducted with human peripheral blood lymphocytes mixed with guinea-pig macrophages. The standard inbition-of-macrophages-migration test was conducted using PPD and an extract of sonicated *M. leprae* prepared by the method of Olmos Castro and Arcuri "leprolin."

The results showed that inhibition of migration of macrophages in the cell mixture containing lepromatous lymphocytes was depressed to a slight degree by the addition of leprolin. The depression of macrophage migration by leprolin was marked in the cell mixtures containing tuberculoid lymphocytes. The depression of migration effects obtained using PPD were similar to those obtained with leprolin except that they were slightly greater.—[This investigation was supported by the United States-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-08211), Bethesda, Maryland 20014]

Han, S. H., Weiser, R. S. and Tseng, J. J. Lymphotoxin production in leprosy patients.

Granger and coinvestigators have shown that when normal lymphocytes are stimulated with PHA or immune lymphocytes are stimulated with specific antigen they synthesize and liberate "lymphotoxin," which in high concentrations produces nonspecific killing of various cultured cells. Lymphotoxin production requires protein synthesis but not DNA synthesis. The capacity to produce lymphotoxin is completely lacking in the case of thymocytes and appears to be greatly reduced in the case of lymphocytes from Hodgkin's disease patients and patients with lymphatic leukemia. Since lymphocyte blastogenesis by PHA is reduced in leprosy, it is logical to expect that lymphotoxin production by lymphocytes would be impaired as well.

Lymphocyte suspensions were prepared

from heparinized venous blood from leprosy patients and normal subjects by the use of PVP and osmotic shock to eliminate red cells. The lymphocytes were suspended in Medium 199 containing 30% human AB serum. Various concentrations of PHA-M were added and the cells were incubated at 37°C for five days in roller cultures. Supernatants were assayed for lymphotoxin on Hela cells using vital stains for estimating cell killing. Lymphotoxin production by PHA-stimulating lymphocytes was severely depressed in the case of lepromatous leprosy and moderately depressed in the case of tuberculoid leprosy.

The depressed activities of lymphocytes from leprosy patients with respect to transformation and lymphotoxin production in the presence of PHA or specific antigen is probably related to the depressed capacity of leprosy patients to acquire and express delayed hypersensitivity.—[This investigation was supported by the United States-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-08211), Bethesda, Maryland 20014. We thank Dr. T. S. Yu, Director of Taiwan Provincial Lo Sheng Leprosarium and his associates for their kind cooperation and generous assistance]