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Program of Leprosy Conference

Tepper, B. S. and Varma, K. G. Metabolic activity of *Mycobacterium lepraemurium.*

Metabolic studies with purified suspensions of mouse-grown *Mycobacterium lepraemurium* have continued to provide information on the biochemical capabilities and the physiological status of the organism.

The radioactive tracer technics used previously to demonstrate metabolic activity in *M. lepraemurium* have been improved. By measuring $^{14}$CO$_2$ resulting from the oxidation of $^{14}$C-labeled substrates, *M. lepraemurium* has been found capable of oxidizing acetate, fumarate, glutamate, glycerol, α-keto glutarate, and succinate. Similar studies have shown that glucose and asparagine are not oxidized by *M. lepraemurium.*

In repeating some of these experiments, markedly different degrees of metabolic activity were observed. Review of the procedures indicated that low activities were associated with *M. lepraemurium* derived from pelvic fatty pads lesions, while high activity occurred on occasions when organisms from infected livers were included. When separate suspensions of bacilli were prepared from the fatty pads and livers of the same group of mice the liver-grown bacilli were found consistently to oxidize acetate and glycerol at higher rates than fatty pad bacilli. These results may afford new insights into the physiological states of the bacilli in vivo and may suggest optimal sources of bacilli for experimental purposes.

Drutz, D. J. and Cline, M. J. Tritiated thymidine labeling of *M. leprae* in the human lepromatous macrophage.

Untreated lepromatous leprosy (LL) patients have $10^3$ to $10^6$ or more acid-fast bacilli (AFB) per ml of blood, largely within monocytes; such blood is infective for the mouse foot pad.

Utilizing technics previously described from this laboratory, monocytes were separated from the blood of bacteremic LL patients and inoculated into Leighton tubes containing 30% normal human AB serum in McCoy's medium. Cells were cultured for one to three weeks at 31° or 37° C. Large numbers of monocytes and their resultant macrophages harbored AFB. Cultures were exposed to methyl-tritiated thymidine (20 Ci/mM) at varying intervals; coverslips and supernatants were separately harvested after further intervals of incubation and radioautographs were prepared.

Nuclear labeling of glass-associated macrophages was negligible and general background was low. As nearly as 6 days after initiation of tissue culture, grain accumulations were apparent over AFB in globi. Singly occurring bacilli were seldom convincingly tagged and the relationship of bacterial morphology to thymidine incorporation could not be clearly ascertained.

Neither the temperature of macrophage incubation nor the presence of antibiotics (penicillin, nystatin) had any consistent effect on bacterial labeling.

Appropriate cultures of all tubes and reagents were sterile, although cultivable mycobacteria were specifically sought. Cultures of normal donor macrophages failed to reveal AFB when processed in the same manner as the LL cells.

It thus appears that *M. leprae* are capable of incorporating tritiated thymidine, presumably during DNA synthesis, under conditions obtaining in their host LL macrophages, and that globi represent colonies of viable microorganisms. These findings support our previous observations that viable AFB are present in the bloodstream of untreated LL patients.

Application of these technics to studies of foot pad-derived AFB in human macrophage cultures appears promising. Thus, tritiated thymidine labeling may provide a useful technic for evaluating the viability, or at least the DNA synthetic ability, of the leprosy bacillus (in vitro).*

Nishihara, M., Uehira, K., Hasegawa, T. and Takeuchi, M. The morphological differences between human and murine leprosy bacilli as revealed by the freeze-etching technic.
Freeze etching is a technique suitable for the study of the surface structure of bacterial cells. We have developed a new model of freeze-etch microtome, and with this apparatus we have examined the morphological difference between human and murine leprosy bacilli.

This freeze-etch microtome is composed of two triangular brass blocks. The lower triangular block is the specimen stage and the upper block has a cutting blade. After a small chip of human or murine leproma, which has been kept previously in 20% glycerol, is set in the specimen stage of the microtome, the whole metal block microtome is cooled to -196 °C in liquid nitrogen. Later, the cooled microtome is placed in the bell jar of the vacuum evaporator. When a vacuum of 10⁻⁵ Torr has been attained, the specimen stage is slowly heated until the temperature of the specimen is raised to -95 °C. At this moment, the specimen is cut with the blade of the upper block, and later, the exposed surface of the specimen is etched for 1-2 minutes in the vacuum. The replication of the exposed tissue surface and the removal of the tissue are carried out as in the usual freeze-etching technique.

A striking difference was found by this method in the so-called "electron-transparent-zones" (E.T.Z.) of human and murine leprosy bacilli. The E.T.Z. of human leprosy bacilli is composed of spherical droplets. These spherical droplets accumulate around human leprosy bacilli and form the intra-cytoplasmic foamy structures. Outside the cell wall of human leprosy bacilli, there are no membranous structures that surround the bacillary body directly. A membranous structure can be seen only around each foamy structure, and the substructure of this membrane is composed of irregularly arranged granules.

On the other hand, the E.T.Z. of murine leprosy bacilli is actually composed of lamellar band- or membrane-shaped crystals. Each crystal has straight parallel striations and has a tendency to split along these striations. Similar crystalline structure was never encountered in human leprosy bacilli.

**Evans, M. J. and Levy, L. L.** Ultrastructural changes in the cells of the mouse foot pad infected with *Mycobacterium leprae*.

* The purpose of these experiments was to describe ultrastructural changes in cells infected with *M. leprae* in the mouse foot pad during the log phase of multiplication and the death phase. To accomplish this, we inoculated a group of BALB/C mice in the right hind foot pad with 5 x 10⁶ organisms. The mice were sacrificed in pairs at 86, 90, 106, 125, 142, 150, 165, 180, 196, 210, 225, 242, 250, and 268 days after inoculation and prepared for electron microscopy by standard techniques. This sampling schedule allowed us to study the tissue during the log phase of multiplication (120 to 150 days) and the death phase (168 to 196 days) (Levy, L., Proc. Soc. Exp. Biol. Med., 135, 745, 1970).

During the early growth phase of *M. leprae* in the mouse foot pad, few organisms can be detected. These present are found in macrophages of the loose connective tissue. There are usually a few organisms in each infected macrophage. The organisms are within the cytoplasm and bound by a single membrane. The cytoplasm of the macrophage is less dense around the organism. There are few lysosomes and the bacteria do not appear to be degenerating.

At the peak of the growth phase, many macrophages containing *M. leprae* are found in the loose connective tissue. The organisms within a macrophage are bound by either a single or double membrane. There is an increased number of vacuoles which are also bound by a double membrane and lysosomes. Many of these organisms appear to be degenerating. The vacuoles usually contain a dense granular material similar to the degenerating organisms. The macrophage may also contain a large cluster of organisms. These are not bound by a double membrane.
in the cytoplasm which are bound by a single membrane. These do not appear to be degenerating. Occasionally, organisms are encountered in the sarcoplasm of striated muscle. They are usually bound by a single membrane and do not appear to be degenerating.

These studies show that during the death phase of *M. leprae* in the mouse foot pad there are ultrastructural changes in macrophages associated with degeneration of organisms and formation of lysosomes.

Oiwa, K., Yamada, H. and Ozato, K. Behavior of mitochondria and lysosomes of mouse macrophages infected with *M. lepraemurium*.

Peritoneal macrophages obtained from the H strain of mice one and ten weeks after intraperitoneal inoculation with Hawaii strain of rat leprosy bacilli were observed with the electron microscope. Peritoneal macrophages of normal mice and those obtained three days after casein injection (ip) were also examined as controls. The macrophages were fixed with OsO₄, embedded in methacrylate, and thin sections cut of randomly selected cells, and electron-microphotographed. The area of cytoplasm of the cells was measured with a planimeter, and the bacteria, mitochondria, and lysosomes were counted.

It was found that with the increase of the number of bacilli contained per 100 μ² of cytoplasm, the number of mitochondria contained in the same area decreased. Lysosomes did not increase in number in infected cells, and were rarely found in cells ten weeks after infection. Other morphologically interesting findings were also recognized and will be reported.

Kawaguchi, Y. Superinfection with leprosy bacilli in mice.

The multiplication of leprosy bacilli in the mouse foot pad, with yields not exceeding 10⁶ bacilli, was first reported by Shep- ard and has been confirmed by Rees and many others. It is assumed that this limitation on multiplication results from immunity that develops in the hosts. Therefore, superinfection with leprosy bacilli was investigated in mice, with reference to the development of immunity in the hosts, by means of the mouse foot pad system.

Before the experiments of superinfection were conducted, the susceptibility of various inbred mouse strains to foot pad infection with leprosy bacilli was examined. Significant but slight differences were revealed among these strains. On the basis of these findings, out of 12 tested strains three characteristic ones, DDD, C57BL/6 and KK, were used for the superinfection experiments. In the present report, the results with DDD and C57BL/6 strains are reported.

Female mice of the DDD strain were previously inoculated with doses of 2.9 × 10⁶ bacilli into right hind foot pad. 30 weeks later, were superinfected with doses of 2.7 × 10⁴ bacilli into left hind foot pad. From 10 to 40 weeks after superinfection, there was no significant multiplication of the bacilli in the left hind foot pads of superinfected or control mice. It is obvious from these findings that definite suppression of multiplication of the superinfecting bacilli was not found in this experiment. However, it is considered that these findings are due to slower multiplication of the bacilli in DDD mice, and are not concerned with the development of immunity in the hosts.

Female mice of the C57BL/6 strain were inoculated with doses of 5.0 × 10³ bacilli into right hind foot pad and were superin­fected with doses of 2.2 × 10⁴ bacilli into left hind foot pad 25 weeks after primary infection. Significant multiplication of the bacilli was observed in the left hind foot pads of control mice. In contrast, bacillary multiplication was found in only one mouse with superinfection. It is apparent from these observations that, in the case of the C57BL/6 strain, an established infection with the bacilli in one foot pad suppressed remarkably the multiplication of the bacilli inoculated later into the other foot pad.

On the other hand in the case of superinfection made five weeks after primary infection, no significant differences were found in multiplication of the bacilli in the left hind foot pads between the superinfected and control mice. These findings led us to a view that when the primary infection is not established, it cannot significantly suppress
the multiplication of superinfecting bacilli even in C57BL/6 mice. Ito, T., Kohsaka, K. and Kishi, Y. Effect of BCG vaccination on the cell culture of M. lepraemurium. The effect of BCG vaccination on the multiplication of M. lepraemurium in cell culture was examined. Mice were immunized with 0.2 mg of lyophilized BCG vaccine by subcutaneous inoculation. Peritoneal macrophages were taken from immunized and non-immunized mice after seven weeks of vaccination. The peritoneal macrophages from immunized and non-immunized group were mixed with M. lepraemurium, Hawaii strain. The mixture was introduced in diffusion chambers made of a plexiglass ring and mulipore filter Type GS. The chambers were maintained in test tubes with tissue culture media at 37°C, and/or implanted in peritoneal cavities of BCG immunized or nonimmunized mice. Bacillary count of the contents of chambers was examined. Direct challenge with small number of M. lepraemurium to immunized and non-immunized mice was also carried out. Limited suppression of the development of murine leproma in the immunized group was observed by direct challenge experiment. No difference, however, was noted between chambers with macrophages from immunized mice and those with normal macrophages as regards multiplication of M. lepraemurium in either the in vitro or the in vivo experiment. One shot of BCG vaccination was not found to give sufficient cellular immunity against M. lepraemurium to mouse. Rees, R. J. W., Beddingius, J. and Weddell, A. G. M. Studies to determine the pathogenesis of neural damage in mice infected with M. leprae. The predilection of M. leprae for peripheral nerves results eventually in the development of neuropathy in all forms of untreated leprosy in man and once established is responsible for the irreversible deformities associated with the disease. Studies on the pathogenesis of leprosy neur-
severe leprosy neuropathy, and both markers were found by light and electron microscopy within capillary endothelial lining cells and within the endoneurium. The significance of these findings will be discussed.

Speers, W. C. and Morrison, N. E. Conformation of the active site of mycobacterial dihydrofolate reductase.

Dihydrofolate reductase has been purified from DDS-sensitive, DDS-resistant and rifampin-resistant Mycobacterium sp. 607. The enzyme represents the second site of sequential blockade of the de novo mycobacterial folate pathway.

Aspects concerning the conformation of the active site binding region have been studied by the inhibitor profile method whereby structure-activity profiles for inhibition of the purified enzyme are compared with inhibition of whole cell multiplication.

It is apparent that active site binding represents the major determinant of efficient antifolate activity and that the conformation of the hydrophobic region adjacent to the active site plays a major role in the binding of the antifolate to the mycobacterial enzyme.*

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Examination of the capacities of 19 normal subjects to acetylate dapsone (4,4'-diaminodiphenyl sulfone, DDS), sulfamethazine (SMZ), and isoniazid (INH) in man yielding a differentiation of subjects into rapid or slow acetylator phenotypes also applies to DDS. (Golber et al., Clin. Pharmacol. Therap. 12, 225, 1971.) Further tests of the parallel between SMZ and DDS acetylation in 50 Philippine subjects yielded the same results. Plasma levels of monooacetyldapsone (MADDS) were found to be directly related to percentage acetylation in these 50 subjects; plasma levels of DDS were unrelated to the capacities of these individuals to acetylate.

Further refinements of our analytic procedures provided the ability to measure DDS and MADDS at nanogram levels in plasma and the repository form of DDS—i.e., 4,4'-diacetamidodiphenyl sulfone (DADDS)—at the same sensitivity (Gordon et al. 6th Annual Leprosy Research Conference, Atlanta, Georgia, March 10-12, 1971). Applying these techniques to plasma from subjects receiving DDS or DADDS showed that DADDS was not a metabolite of DDS but was present in plasma following intracellular administration of DADDS. Levels of DDS and MADDS found after DADDS treatment classified these patients as the same acetylator phenotype as when they received DDS, SMZ, or INH. The average amount of DADDS in the percentage of total DDS, MADDS, and DADDS in seven plasma samples was 13% indicating that 87% of the total DADDS was hydrolyzed to DDS or MADDS.

We have attempted to evaluate the therapeutic implications of differing acetylation capacities for DDS by comparing acetylator phenotype with response to therapy with DADDS and with the emergence during sulfone therapy of DDS-resistant Mycobacterium leprae. No relationship between therapeutic response to DADDS therapy and acetylator phenotype was found. Thus, of seven rapid acetylators studied, five responded poorly to therapy; of three slow acetylators, two responded poorly.

Furthermore, of 14 patients harboring M. leprae that were proved resistant to DDS, we found eight to be rapid and six to be slow acetylators. These preliminary results suggest that acetylator phenotype exerts no effect on response to sulfone therapy.*

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DADDS (4,4'-diacetamidodiphenylsulphone) is an insoluble derivative of dapsone which provides a continuous low blood dapsone concentration following intramuscular administration. A clinical trial of the drug in a dosage of 225 mg every 77 days, carried out in Cebu, demonstrated an effect in lepromatous leprosy equal to that of dapsone in an oral dose of 100 mg six days per week, as measured by the criteria of disappearance of acid-fast bacilli (AFB) from the nasal washings and of a fall of the solid ratio of Mycobacterium leprae recovered by skin scraping. Because of the interest in this drug as a means of treating hard-to-reach populations, a trial of DADDS treatment has been carried out in San Francisco, employing the more sensitive criterion of infectivity of the M. leprae for the mouse foot pad.

Fourteen patients with previously untreated lepromatous leprosy were assigned randomly to DADDS or to dapsone 50 mg daily by mouth, in a ratio of three DADDS to each dapsone subject. Skin biopsy specimens were obtained at intervals and sent by air on wet ice to Atlanta, where mouse inoculation was carried out. The response of the bacilli to treatment was measured by two criteria: (1) "incubation period," taken as the time from inoculation to the first appearance of AFB in histologic sections of the mouse foot pad; and (2) the generation time (G), the number of days from inoculation to mouse harvest divided by the number of doublings of M. leprae. An incubation period longer than 12 months and a G > 100 days indicate that the M. leprae in the inoculum were not infective for the mouse (and were, therefore, presumed dead).

The results are summarized in the accompanying table. The response to treatment of the three patients on dapsone was not different from that of 12 previous DDS patients treated and studied similarly. For all 15 dapsone patients, the mean incubation period became longer than 12 months in about three months of treatment, and the mean G reached 100 days slightly earlier. Three of these 10 DADDS patients responded as rapidly as any of the dapsone patients, and 7 responded more slowly.

The rate of killing of M. leprae during DADDS treatment is thus slower on the average than that during dapsone treatment. A few patients, however, responded as rapidly to DADDS as if they had been treated with dapsone. The slower response to DADDS may well result from the lower blood (and tissue) dapsone levels provided; those following DADDS are about 1/20th those following 50 mg dapsone daily.

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<th>First negative specimen by histol.</th>
<th>Last positive specimen by harvest</th>
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</table>

Mean duration of treatment (in days) at time of

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of patients in group</th>
<th>Mean duration of treatment (in days)</th>
<th>at time of</th>
<th>Last positive specimen by histol.</th>
<th>First negative specimen by histol.</th>
<th>Last positive specimen by harvest</th>
<th>First negative specimen by harvest</th>
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<tr>
<td>Dapsone</td>
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<td>100</td>
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<td>105</td>
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<tr>
<td>Dapsone</td>
<td>10</td>
<td>140</td>
<td>&gt;179†</td>
<td>105†</td>
<td>&gt;141†</td>
<td>&gt;179†</td>
<td></td>
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<tr>
<td>DADDS</td>
<td>10</td>
<td>140</td>
<td>&gt;179†</td>
<td>105†</td>
<td>&gt;141†</td>
<td>&gt;179†</td>
<td></td>
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<tr>
<td>Rapid responders</td>
<td>7</td>
<td>191</td>
<td>&gt;224‡</td>
<td>138</td>
<td>&gt;224‡</td>
<td>&gt;224‡</td>
<td></td>
</tr>
</tbody>
</table>

* Incubation period < 12 months.
* Generation time < 100 days.
* Controls in this DADDS trial.
* Present controls plus all previous patients treated and studied the same way.
* Because the last specimens obtained from one or more of the patients in this group were still "positive," "conversion" to "negativity" was not demonstrated (although in each case the infectivity had markedly decreased). The number shown is the mean for the patients who did convert; and the actual mean is expressed as being greater than this value.
sone per day. We are seeking an explanation of the individual differences in response to DADDS.

In summary, therapeutically important killing of M. leprae occurs during the initial stages of DADDS therapy; the killing appears, however, to be slower than that in the initial stages of dapsone therapy in conventional dosage.

Stors, Eleanor E. The armadillo as an animal model in leprosy studies.

Dr. Kirchheimer and I are going to give a brief report on our findings in a program studying the research potential of the armadillo in leprosy studies.

The nine-banded armadillo, Dasypus novemcinctus, is a mammal of the order Edentata, related to sloths and anteaters. Armadillos are found exclusively on the American Continents, and the nine-banded armadillo is the only species found naturally in the United States. This animal has several characteristics making it unique for use in various types of research. Among these features are (1) regular production of monzygous quadruplet young; (2) a low body temperature (around 32-34°C); (3) a long life span of 10-15 years; and (4) buildup of an oxygen debt. We at Gulf South Research Institute now have a research program with the Animal Resources Branch of the Division of Research Resources of NIH to develop the armadillo as a laboratory animal [Grant No. RR00455].

We also have a grant program in cooperation with Dr. Kirchheimer of the USPHS Hospital in Carville to study the armadillo as an animal model in leprosy transmission studies, supported by the Center for Disease Control in Atlanta [Grant No. CO0476]. We are now completing our second year of this program and have inoculated a total of 76 armadillos with Mycobacterium leprae. Inoculum has been obtained from untreated patients supplied by Dr. Binford and Dr. Kirchheimer, from a patient with apparent DSS resistant bacilli, from mouse foot pad inoculum supplied by Dr. Louis Levy from the USPHS Hospital in San Francisco, and from armadillo passage inoculum.

Most armadillos were inoculated intracutaneously on the abdomen, ears, or between the bands. Several armadillos were inoculated on the foot pad and several received intravenous injections of inoculum.

At the end of six-month and twelve-month periods, biopsy sections have been taken at the site of inoculation on many of the inoculated animals. Several of these biopsies have shown acid-fast bacteria.

On February 10, 1970, four animals were inoculated intradermally from biopsy material supplied by Dr. Binford. This biopsy material (S45) was sent from Surinam. The inoculum, worked up at Carville, had a count of 8.9 ± 0.4 × 10^8 B/ml with an MI of 3%. Each armadillo was inoculated intradermally in the right and left abdomen and in both ears with 0.1 ml of inoculum at each site. In May of 1971 armadillo Number 8 showed large palpable granulomas at inoculation sites on the abdomen and the ears. Biopsies taken on May 24, and on July 1, showed large number of acid-fast bacteria. The armadillo died on July 16, 1971. Slides showing armadillo Number 8 during the last stages of the disease were presented. Dr. Kirchheimer will now present data which he has gathered on this armadillo.

Kirchheimer, W. F. and Stors, E. Attempts to establish the armadillo (Dasypus novemcinctus Linn.) as a model for the study of leprosy. Report of lepromatoid leprosy in an experimentally infected armadillo.

An armadillo (Dasypus novemcinctus) developed lepromatoid infection with M. leprae approximately 14 months after inoculation of leprosy bacilli, from an untreated case of lepromatous leprosy, into the skin of its abdomen and earlobes. The diagnosis of lepromatous leprosy is supported bacteriologically by over 1,000-fold increase in the inoculation sites of acid-fast bacteria, which do not grow on mycobacterial culture media and which oxidize D-dopa. In addition, these acid-fast bacteria have been found in great numbers at a skin site remote from the inoculated area. The remote skin site was of normal appearance. The inoculated skin sites were converted into massive nodular lesions. The acid-fast bacteria were intracellular, and typical lepra...
cells made up much of the lepromas. Bacilli were also seen in cutaneous nerves. It is too early yet to evaluate the results of the mouse foot pad inoculations of the bacilli. 


Dapsone (DDS) has been a useful drug in leprosy treatment and has also been shown to be partially effective as a chemoprophylactic drug in two controlled trials in India. DDS has the disadvantage, however, of requiring frequent dosage, which gives rise to problems of patient cooperation. Elslager and his co-workers have developed a repository sulphone, Acedapsone (DADDS), by adding an acetyl group to each end of the DDS molecule. This results in a very slow, steady release of DDS over a period of 77 days, with DDS blood levels averaging about 50 mg/ml during that time. Shepard has shown that DADDS used in this way is effective against Mycobacterium leprae in mice and in a small field trial in man in the Philippines. Russell and his co-workers have recently confirmed this in a larger field trial in New Guinea.

We have been giving DADDS at the usual dose of 225 mg in adults or 150 mg in young children every 75 days for three years to a population of 1500 highly in-bred people in three small villages in Micronesia. This population has had a high prevalence of leprosy for at least ten years, with an annual incidence of about 11 new cases per year during the period 1963-1967. DADDS injections were started in the autumn of 1967, and the entire population was reexamined by the same leprologist in the autumn of 1969, 1969, and 1970. Six new cases appeared during 1969, none in 1969, and none in 1970.

The 68 active cases of leprosy in this population were also started on DADDS at the same time, and all have shown improvement except for:

1. One lepromatous patient who already had been on oral DDS for five years without improvement, but who is now improving on B663;
2. One indeterminate patient without previous treatment whose two small macules have not clinically changed during the three years of DADDS, but her biopsies have shown a histologic shift from an indeterminate to a tuberculoid pattern.
3. All cases continue on DADDS (except for the one on B663). All other DADDS injections in the population were stopped in the autumn of 1970. The population will be reexamined periodically for ten years to see if new cases resume. We have had no problem with ENL reactions, toxicity, fetal deformity, stillbirth, or lack of cooperation.


The repository sulphone DADDS (acedapsone) is receiving therapeutic trial in the Karimui in New Guinea. Because of the remoteness of the area, no other anti-leprosy drug is suitable. All of the 429 cases of diagnosed leprosy are being treated with DADDS. An injection of 225 mg of DADDS is given every 75 days, and, at appropriate intervals, clinical examinations are made and skin smears (6 sites) are made from smear-positive patients. The treatment has been well-received, and the injections have been given regularly. The latest examination for which clinical data have been analyzed was at 750 days after the start of therapy, and the clinical response and incidence of ENL were what would be expected in patients being treated with 50 to 100 mg of daily dapsone. Bacterial examinations are available from skin smears made at 1100 days (3 years). There were 28 previously untreated patients, who had sufficient bacilli in their skin smears to allow solid ratios (MI's) to be assessed at the beginning and at least one later date. In these patients the average BI's (Bacterial Indices by the Ridley scale) decreased from 3.61 to 0.78, a decrease of 2.83 units which corresponds to a loss of 650-fold. All patients have had distinct decreases in BI, and none have had
significant increases at any interval. Before treatment not all 58 patients had significantly elevated solid ratios, but in all those that did the solid ratios decreased satisfactorily, and 150 days after start of treatment the solid ratios of all patients were near base-line levels. A few sites had ratios of 1-2% at 375 and 600 days, but by 750 days no solid bacilli were observed at any site. At 1100 days the smears of one patient had a low elevation of the solid ratio, and steps are being taken to establish whether or not her bacilli are resistant to dapsone. #

Shepard, C. C. The action of rifampin on M. lepraes.

Rifampin is a semi-synthetic antibiotic derived from rifamycin which is produced by Streptomyces mediterranei. It has a broad antibacterial spectrum that extends to many mycobacteria, and it is now being used extensively in tuberculosis. Its toxicity is low on daily administration and after intake of about 50 mg/kg its half-life in the blood of mice and of man is about three hours. In our laboratory the minimal effective oral dosage against M. lepraes in three separate experiments has been 0.01% of drug in the diet, a dosage that produces blood levels of several micrograms per milliliter. Other laboratories have found lower figures; the reason for the discrepancy is not known. The most interesting feature of the action of the drug against M. lepraes is the rapidity of its bactericidal action. In several experiments the drug was started on about the 70th day after infection. When 0.01% in the diet was then given for 72 days, no subsequent growth of M. lepraes occurred. When 0.03% was given for 21 days, no subsequent growth was seen in one experiment, and in another the growth was delayed 294 days. When 0.03% was given for two days, growth was delayed for 156 days; in comparison dapsone must be continued for two to three months to cause the same degree of growth delay. In another experiment in progress rifampin was administered by gavage on the 70th day; a single dose of 10 mg/kg had some antibacterial effect and increasing doses up to 67.3 mg/kg had increasing effect. Combinations of rifampin and dapsone are being tested in mice; results show that the rapid killing effect of rifampin is not antagonized by dapsone. In human leprosy, combinations of rifampin with dapsone, administered as dapsone or as DADDS, might provide the advantages of both drugs, e.g., the very low frequency of drug-resistant mutants to dapsone and the rapid killing of rifampin. The theoretical benefits of several regimens will be discussed. #

Yokota, T. Leprosy cases resistant to chem/other.

* Among patients with lepromatous leprosy in our leprosarium, there were, as of January 1968, a total of 185 patients, 138 males and 47 females, who had relapses during treatment. Most of these patients, who had been treated with DDS-derivatives, mainly in the form of intravenous injection of Promin, were transferred to other drugs. By this means, most of them improved, even though gradually, both clinically and bacteriologically.

A part of them, however, presented new nodules and infiltrations repeatedly over more than five years and showed a Bacteriological Index above three at each examination. Such difficult cases amount to a total of 66, 54 males and 12 females, a figure that corresponds to 6.4% of all the lepromatous patients within our leprosarium. Obviously resistant cases are more frequent among the males than the females.

Details about these 66 cases are as follows: The number of years that elapsed from the beginning of the treatment to the relapse is from six to ten in 33.3% of the cases and from 11 to 15 years in 56.1%. If we classify these cases according to the drugs that were administered up to the relapse, 52 cases were on Promin, 11 on Promizole, cne on Prothyl and two cases had stopped receiving any treatment for more than four years. If we assume that a patient should receive 100 injections of Promin per year on the average as standard dosage, 32.7% of the cases satisfy this standard whereas 67.3% do not. Thus, we cannot deny that many cases of relapse are due to unsatisfactory treatment. The above data also show that even regular treatment *
could not prevent relapses in some of the cases.

As to the patients' attitudes after relapse, they may be divided as follows: 56.1% continue receiving regular treatment; 36.4% are obliged to have irregular treatment because of ENL, neuritis or disturbances of the digestive tract, and 7.5% lost interest in receiving treatment. Those who are serious about their treatment are given various drugs such as Ciba 1906, streptomycin, Ranamycin and DDS. But even then, there are cases that show no improvement. This suggests that drug resistance has occurred.

During the course of this present investigation, our attention was drawn to the fact that there were found among the 66 difficult cases, four sets of siblings, nine patients in all, who are either brothers or brother and sister. Accordingly we cannot but assume that there are constitutional factors determining the aggravation or the chronicity of symptoms.


The first cases of possibly sulphone resistant leprosy appeared in Sungai Buloh in 1961, and by 1964 there was evidence from both clinical study and sensitivity tests using the mouse foot pad that some, at least, of these patients were highly resistant to DDS.

Since then new cases have continued to appear, and the number has greatly increased in the past two or three years. It is now clear that sulphone resistant leprosy is likely to become a major problem in the treatment of lepromatous leprosy.

The clinical features of such patients on first presentation, together with the common findings during the course of a period of trial treatment with DDS in full dosage, will be described, drawing on our experience with the first 60 patients referred to us with prima facie evidence of sulphone resistance.


When lymphocytes from an animal sensitized to have delayed-type allergy (and thus cellular immunity in a broader sense) are placed in contact with the sensitizing antigen, a substance is said to be released that will inhibit the migration of macrophages of the same animal, or those of normal homologous or heterologous animals. The migration inhibition test utilizes this phenomenon in vitro for immunological studies. In the present study, an investigation was made to ascertain the applicability of the migration inhibition test for various studies of lepromin reaction.

Lymphocytes obtained from the peripheral blood of leprosy patients were mixed with the macrophages derived from the ascites of normal guinea pigs at a ratio of 1:4 to 1:8. The mixture was put into capillaries and centrifuged. Those parts of the capillaries containing cells were removed by cutting, placed in small Petri dishes containing culture medium containing either one of Dh antigen or OT, and incubated at 37°C for 48 hours. Areas of cellular migration were measured and compared with those of controls, and the inhibition of migration was expressed as the ratio.

Inhibition of migration was almost absent when the lymphocytes of T-type patients negative in both lepromin and tuberculin reactions were used in culture medium containing either Dh antigen or OT. In contrast, when the lymphocytes of lepromin positive and tuberculin positive T-type patients were used, a marked inhibition of migration was observed in the culture medium containing either Dh antigen or OT. The results were the same with lymphocytes of normal individuals positive in both lepromin and tuberculin reactions.

Fieldsteel, A. H. and McIntosh, A. H. Enhanced multiplication of Mycobacterium lepra in immunologically suppressed rats.

The susceptibility of four strains of rats to foot pad and testis infection with M. lepra has been investigated. In immunologically intact animals infection by either route, in all of the strains (Sprague-Dawley, Fischer, Buffalo, and Lewis), was erratic and
unpredictable. Irrespective of the quantity of the initial inoculum the level of multiplication of *M. leprae* rarely reached or exceeded 10^6.

Thymectomy at less than 24 hours after birth had no effect on the course of infection in Sprague-Dawley rats. Neonatal thymectomy in Fischer and Buffalo rats had a marked effect on foot pad infection, but not on testis infection. Yields of *M. leprae* from the foot pads of these animals reached 10^6 or greater after inoculation of 5 × 10^5 to 2.5 × 10^6 organisms. Antithymocytic serum (ATS) in addition to neonatal thymectomy did not significantly enhance *M. leprae* infection in Buffalo rats.

Lewis rats responded somewhat differently to infection with *M. leprae*. After foot pad inoculation the maximum number of organisms was 2.5 × 10^6. 60 weeks after inoculation. The peak in the thymectomized group was 2.5 × 10^6 organisms 69 weeks after inoculation. Acid-fast bacilli in the foot pads from rats which were thymectomized and given ATS reached a peak of 9.8 × 10^6 by the 69th week after inoculation.

Infection in the testes of Lewis rats followed a pattern similar to that in the foot pads. Maximum numbers were found at 60 weeks in the thymectomized-ATS treated group when the average number of acid-fast bacilli per testis was 2.9 × 10^6.

[Supported by the U.S.-Japan Cooperative Medical Science Program, Grant AI-08417]


In carrying out experiments in the transmission of *Mycobacterium leprae* to inbred CBA/J mice, using the thymectomy—900r method (Rees et al., Nature 215 (1967) 599), comparison was made between the restoration of hematogenesis by transfusing syngeneic marrow and shielding a portion of the animal's bone marrow.

In the three experiments to be reported, inbred CBA/J mice, at approximately six weeks of age, were thymectomized by a suction technic, and two or three weeks later irradiated (900r). During irradiation, in approximately one half of the thymectomized mice one femur was shielded by a band of lead 1.6 mm thick. The non-irradiated animals were given syngeneic bone marrow transfusions (marrow from the femurs of one done to three recipients).

In each experiment normal CBA/J mice were similarly inoculated in order to provide a base line for the growth of *M. leprae* in non-immunosuppressed animals.

Within a few days after irradiation, the mice were inoculated with *M. leprae* obtained from biopsies of lepromatous patients. In approximately one half of the animals, the inoculations were in foot pads, and in the other animals, carried out intravenously. In two groups, ears were inoculated in addition to foot pads. Because the evaluation of the results was to be done entirely by histopathology and not by counting, 65,000 or more bacilli were inoculated into foot pads.

Results. At 7-months post-inoculation, in the group of animals protected by shielding, 60-90% survived, while in the animals receiving bone marrow transfusions no survival in one experiment was 20-40%, and in the other two experiments, the rate was 0-5%.

In the majority of the animals that survived seven months, the enhancement of the growth of *M. leprae* was very impressive in each group.

In the normal animals at 7-10 months, post-inoculation, examination of the Fite-Faraco stained sections with a 10x objective and 10x oculars, growth was recorded when a few clusters of cells containing acid-fast bacilli were observed. According to our scale, in normal mice the growth when observed was 0.5-1+.

An example of the results is shown below in a group of 17 immunosuppressed mice protected by shielding a femur and inoculated in the hind feet. This group survived 7-13 months post-inoculation.

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<td>17</td>
<td>1</td>
<td>1</td>
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</table>
In the above table, a grade of 3+ was applied to histologic sections of the feet, which under the 10% objective showed extensive involvement by cells loaded with acid-fast bacilli. In the sections graded 3+, usually, there was much leprous involvement of metatarsal bone marrow, striated muscle, and nerves. In the sections graded 4+, there was almost total replacement of all tissues by cells containing acid-fast bacilli. The involvement was so great that the acid-fast section appeared red when observed without magnification.

In these experiments, the poor survival rate of the transfused animals may have been related to our experience with bone marrow transfusion. However, the excellent survival and enhancement of the growth of M. leprae in the shielded animals gives hope that this easily applied method of lead shielding will provide a useful method for experimental leprosy. With the shield procedures, successful immunosuppression procedures should be accomplished in random bred mice. The method should also be applicable to immunosuppression methods in animals, such as the hamster, in which intravenous administration of bone marrow is very difficult.

Immune status of thymectomized radiation chimeras was measured at approximately one month intervals for six months after thymectomy. The purpose was to show cellular and humoral immune responses and anatomical condition of lymphatic tissue following the period when M. leprae would be given to BC3F1 mice. Thymectomy preceded lethal total-body X-radiation by approximately ten days. After 950R exposure five million syngeneic bone marrow cells were administered intravenously. Allogenic tail skin grafts were used to measure cellular immunity. Rat erythrocyte antigens measured humoral immunity. Gross and cellular pathology examinations of lymphatic tissue revealed the anatomic conditions of lymphatic tissue. Normal mice were used as controls.

In the above table, a grade of 3+ was applied to histologic sections of the feet, which under the 10% objective showed extensive involvement by cells loaded with acid-fast bacilli. In the sections graded 3+, usually, there was much leprous involvement of metatarsal bone marrow, striated muscle, and nerves. In the sections graded 4+, there was almost total replacement of all tissues by cells containing acid-fast bacilli. The involvement was so great that the acid-fast section appeared red when observed without magnification.
strongly histoincompatible skin grafts for an extended time.

Various lymphocyte and thymocyte cell doses (1, 10, 25, 50, and 100 × 10^6/injection) were injected into rabbits according to the adjuvant method ([1960] 217) for the production of primary and secondary serum pools. Of each serum 0.5 ml was injected I.P. into A/He mice (10-15/group) on days −1 and +2 relative to C3H/He skin grafting on day 0. Primary low dose (10-50 × 10^6 cells/injection) antilymphocyte sera (ALS) were more immunosuppressive than all other ALS pools. Primary low dose ALS was also the least toxic of all ALS sera.

Comparable low dose antithymocyte (ATS) sera (10-50 × 10^6 cells/injection) were not very immunosuppressive. The most potent and least toxic of all the sera (ATS and ALS) was produced with 100 × 10^6 thymocytes/injection. The 100 × 10^6 ATS had a median survival time (M.S.T.) of 46.7 days. The longest low dose ALS M.S.T. was 38.5 days. Normal M.S.T. 10.3 days. The most immunosuppressive sera had the lowest leukoagglutination, hemagglutination and cytotoxicity titers. These sera had the least amount of antibody to mouse serum protein.

An indirect leukoagglutination test was developed which showed an excellent correlation between in vitro agglutination and in vivo immunosuppressive ability. Lymphocytes and thymocytes coated with various dilutions (1:2,000-1:30,000) of ALS or ATS were reacted with a 1:100 dilution of a goat anti-rabbit IgG serum (Miles Lab.) and then read for agglutination. By means of indirect leukoagglutination lymphocyte-specific immunosuppressive IgG molecules can be detected.

The most immunosuppressive serum was administered to thymectomized C3H recipients of A/He skin grafts. Doses of 0.25 and 0.50 ml of serum were injected into thymectomized C3H recipients (15-20/group) on days −1 and +2 relative to skin grafting on day 0 and pulsed weekly thereafter. Fifty percent (50%) of the 0.5 ml pulsed animals and 20% of the 0.25 ml pulsed animals had intact grafts on day 100. Thus, weekly serum pulser pulsed thymectomized recipients can overcome strong histocompatibility differences and achieve a prolonged immunosuppressive state.

Abe, M., Minagawa, F., Yoshino, Y., Ozawa, T., Saito, N., Ohsawa, Y., Nakamura, K., Hsiu, S., Yogi, T., Sasaki, N. and Kawazu, K. Immunochemical reactions of mice infected with M. leprae and the effect of immunosuppressive treatments on the host-parasite relationships. Immune response of mice against M. leprae infection and the effect of immunosuppressive treatments of these animals were examined, as a preliminary study for experimental lepromatous mice infected with M. leprae. Mice of dd-strains were divided into three groups: animals in group 1 were thymectomized at four weeks age after birth, then on and after four weeks injected intraperitoneally with rabbit IgG globulin against mouse thymocytes (ATG) at certain intervals; mice in group 2 were injected with cortisone (25 mg/Kg) and Imuran (30 mg/Kg); and mice in group 3 as a control received intraperitoneal injection of physiological saline. About half the number of mice in each group was challenged with M. lepraemurium (5.8 × 10^6 per site) into the right foot pad and peritoneal cavity, respectively, at one week after initial injection of the immunosuppressive agents. At 0 time, 3, 4 and 6 months after the challenge, three animals of each group were sacrificed to examine humoral antibody titers, skin hypersensitivity known as cellular immune response, and bacterial count of infected foot pad. Histopathologic observations were also performed on the foot of challenged mice, lymph nodes and viscer al organs.

The number of lymphocytes in peripheral blood of animals in group 1 and group 2 diminished soon after the initial injection of immunosuppressive agents, but recovered gradually after one month. Rejection of skin homografts occurred within 15 days in the animals of group 2 and group 3, whereas in group 1 was delayed so extraordinarily that survival longer than one hundred days could be observed on two of three animals. Production of hemolysis by spleen cells and serum hemolysin titer against sheep erythrocytes were sup-
pressed extremely in the animals of group 1. Anti-murine leprosy bacilli antibody was detected by indirect fluorescent antibody technique in sera of mice in group 2 and group 3 after the lapse of one month from the day of challenge. However, in the serum of animals in group 1 this antibody could not be found even up to the end of six months. Cutaneous reactions to the heated suspension of *M. lepraemurium* as well as contact skin hypersensitivity with chlorodinitrobenzene (CDNB) were inhibited only in the animals of group 1. Hemolysin production and contact-allergy with CDNB in the control group showed no significant difference between the animals whether they were infected or not.

Macrosopic changes in the regions infected with *M. lepraemurium* were barely recognized up to the end of six months after the inoculation. However, bacterial count in the foot pad of mice in group 1 tended to increase from three to six months after inoculation and reached to the average value of $1.2 \times 10^9$ at the end of six months. This value in group 2 varied with the month, but finally rose to the level of $1.2 \times 10^8$. In group 3 wide variation in the count was noted at the end of six months, the count being from less than $10^9$ to more than $10^9$. In mice of this group dissemination of bacilli to the bone marrow was localized mainly in the foot. In contrast with this, typical murallepromas were found in the iliac and femoral marrows of the animals in group 1 and group 2. Metastasis of bacilli to lymph nodes in remote regions as well as to visceral organs such as spleen, liver and lungs was most striking in mice of group 1.

* From these observations it was found that the immunosuppressive effect was more remarkably displayed by the combination of thymectomy and ATG than by cortisone and Imuran. Humoral and cellular immune responses were equally suppressed by the former treatment whether they were related or not related to the infection by *M. lepraemurium*. Observations on the control group seemed to be not consistent with the findings by others that cellular immune responses in murine leprosy are suppressed as a result of disease.


Transfer factor (TF) or whole leukocytes from donors with delayed hypersensitivity to antigens of *Mycobacterium leprae* were employed in an effort to reconstitute delayed allergy in nine patients with lepromatous leprosy who were anergic to these antigens. A mean of $4.1 \times 10^8$ lymphocytes was given to five patients and four received TF from equivalent cell numbers. A 0.1 ml "local" injection was given intradermally on the forearm and the remaining volume (1.4-5.3 ml) delivered subcutaneously to deltoid sites. One to six days post-transfer, six of nine patients experienced erythematous and indurative changes within lepromatous skin infiltrates. By day 12, these exacerbated lesions regressed. Simultaneously, *erythema nodosum* was precipitated in four patients, fever and arthralgias in three. Within seven days post-transfer, five patients converted from anergy to weak allergic reactivity as measured by 48 hour skin test reactions to *M. leprae* antigens. Test reactions at "local" transfer sites and at "remote" sites were of equal magnitude. A sixth patient became skin test positive at the "local" site only, reverting to negative seven days later. Biopsies of skin tests performed pre- and post-transfer in three patients showed increased perivascular lymphocytic infiltration in two post-transfer specimens. Repeated lymphocyte cultures from the nine patients after transfer demonstrated no increase of "H-thymidine uptake when incubated with antigens of *M. leprae*. All patients have been observed 1 to 25 years after transfer experiments. As yet, neither dramatic deterioration nor improvement has been noted.

The results indicate that TF may stimulate transient immunologic reactivity of low magnitude in lepromatous leprosy. The therapeutic promise of TF in single dosage appears limited. [Research supported by NIH grant AI-07964]

Ridley, D. S. Review of the five-group system for the classification of leprosy according to immunity. (Table)
### Five or Seven Group Classification of Leprosy

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<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td><strong>Bacilli in nose</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Bacilli in granuloma</strong></td>
<td>0</td>
<td>0-1</td>
<td>1-3</td>
<td>3-4</td>
<td>4-5</td>
<td>5-6</td>
</tr>
<tr>
<td><strong>% fall biopsy index in 6/12</strong></td>
<td>100</td>
<td>80</td>
<td>45</td>
<td>35</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td><strong>Epithelioid cells</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Langhans giant cells</strong></td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Eosinophil cells</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Foam cells</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+/±</td>
<td>±</td>
</tr>
<tr>
<td><strong>Erosion of epidermis</strong></td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Infiltration sub-epid. zone</strong></td>
<td>+</td>
<td>+</td>
<td>+/±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Nerve destruction (skin)</strong></td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

This table does not include histologic features which occur during reactions.