### ABSTRACTS

# NINTH ANNUAL LEPROSY RESEARCH CONFERENCE

Stanford Research Institute Menlo Park, California May 15–16, 1974

#### FOREWORD

We have had annual leprosy research conferences now since 1965. The conferences have proven to be most useful, providing as they do: review of research progress, an informed discussion of results, and an opportunity to plan collaborative work for the future. This year because of restrictions on budget and travel, we were not able to have the usual financial support, but many of us felt that we could not afford to miss a meeting. Many were not able to attend, but some sent an abstract to provide a note on their progress. We also relaxed the rule on the number of presentations by one person when only one of the co-authors could be present. The meeting was held in the San Francisco Bay area because there are more people engaged in leprosy research on the southern peninsula than in any other area of the United States. We are indebted to the Stanford Research Institute for their hospitality and the use of the conference facilities and to the Center for Disease Control for reproduction of the book of abstracts for the meeting.

> CHARLES C. SHEPARD Conference Chairman

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## Program of the Ninth Annual Leprosy Research Conference

#### McCormick, Joseph B. and Feldman, Roger A. Leprosy in the United States, 1971-1972.

There were 268 cases of leprosy reported in the United States and Puerto Rico in 1971 and 1972. Two hundred twenty-three cases (83% of the total) were reported from three states: California (92), Texas (55), and Hawaii (76). Sixty-nine percent of the cases were born outside the United States. A significant increase occurred in the number of patients born in the Philippines and American Samoa, from a total of 36 cases in 1969 and 1970 to 90 cases in 1971 and 1972. Fiftyfive of 77 (70%) cases born in the United States never lived outside the United States boundaries. Of the 268 cases, lepromatous leprosy occurred in 51%, tuberculoid in 26%. dimorphous in 16%, and indeterminate in 7%. Thirty-seven of the 55 cases born in the United States never lived outside of the United States, and had no history of leprosy in another member of their family. Twentysix of these 37 cases (70%) had lepromatous or dimorphous leprosy, and 11 of the 37 patients (30%) had indeterminate or tuberculoid leprosy. The mean age at diagnosis of the 37 patients with no known contact to leprosy was 45.8 years, compared to a mean age at diagnosis of 33.8 years in those cases born in the United States but who had either lived in endemic areas outside the United States or who had a history of another family member with leprosy. Two hundred twenty-three of the total 268 cases (83%) were confirmed by biopsy. Six new cases of leprosy were diagnosed as a direct result of routine examination by a state health official of family contacts of known cases of leprosy.-[Center for Disease Control, Atlanta, Ga.]

#### Harris, E. B. and Prabhakaran, K. Uptake of radioactive DOPA by *Mycobacterium leprae in vitro*.

We reported previously the oxidation of tritium-labeled DOPA by *Mycobacterium leprae*, separated from lepromatous human tissues as well as from organs of experimentally infected armadillos. The present study deals with the uptake and binding of DOPA

by the leprosy bacilli. The organisms were obtained from lepromatous human spleen removed at autopsy. The *M. leprae* preparations were tested for their ability to oxidize D-DOPA and tritiated DL-DOPA. For comparison, cultured melanoma cells were employed. The melanocytes oxidized L-DOPA and labeled DL-DOPA; however these cells show very little activity towards D-DOPA.

The organisms were incubated with tritiated (1.0 µCi/2 ml) DL-DOPA (pH 6.8) for 120 minutes at 37°C. After incubation, the reaction mixtures were centrifuged, the supernatant decanted and the sediment was washed four times with deionized distilled water. This procedure removes the free DOPA and also the tritiated water formed in the oxidation of the substrate. Radioactivity in the first supernatant as well as the subsequent washings was assayed; the final supernatant showed very little activity above the background counts. The washed sediment was suspended in deionized distilled water, and the suspension was lyophilized. The lyophilized material was resuspended in water and the radioactivity present was assayed in a Beckman Model LS-250 Liquid Scintillation Counter.

With the melanocytes, similar procedures were followed, except that incubation with the tritiated (10.0  $\mu$ Ci/10 ml) substrate was carried out in the culture flasks with the cells adhering to the growth surfaces. The cells were scraped off and all the washings were done using the culture medium for suspending the cells. The final sediment was suspended in water, for lyophilization and liquid scintillation counting.

The results showed that *M. leprae* as well as melanocytes take up radioactive DOPA. Heating the cell cultures and the bacilli produced considerable impairment of their ability to bind the substrate.

*M. leprae* and melanoma cells possess *o*-diphenoloxidase. A cell line of fibroblasts developed from armadillo skin did not oxidize DOPA and also showed no uptake of the radioactive substrate. Myocardial cells are known to contain catecholamines; cultured turtle-heart cells were found to bind tritiated DL-DOPA.—[USPHS Hospital, Carville, La.]

Fieldsteel, A. Howard. The neonatally thymectomized rat: a highly sensitive host for the detection of small numbers of viable *M. leprae* in the presence of large numbers of dead *M. leprae*.

The intact mouse is highly susceptible to foot pad infection with M. leprae, the minimum infectious dose being 3 to 30 organisms; however, the biggest drawback to the use of intact mice for the study of M. leprae infection is in the evaluation of the results of chemotherapy. The efficacy of various drug regimens is generally demonstrated in terms of initial killing of M. leprae as evaluated in the mouse foot pad. M. leprae recovered from biopsy specimens taken periodically during treatment is inoculated into mice. Primarily because a small inoculum of M. leprae must be used  $(5 \times 10^3)$  due to the "ceiling effect" seen in intact mice, the sensitivity of the intact mouse is severely limited even though decrease in viable M. leprae of up to 99.9% can be detected. Since a patient with lepromatous leprosy may enter treatment with 1011 to 1012 viable M. leprae, the infection is by no means eradicated when viable organisms are no longer detectable by the mouse foot pad technic.

We have shown that cooler sites on the body of the neonatally thymectomized Lewis rat are highly susceptible to M. leprae infection after either local or intravenous injection; however, it was not known if small numbers of viable M. leprae could be detected in the presence of large numbers of dead organisms in a situation similar to that which might exist during or after drug therapy. Accordingly, groups of neonatally thymectomized Lewis rats were inoculated with a mixture of 107 heat-killed M. leprae and varying numbers of viable M. leprae ranging from 25 to 1,250 per site. Each rat was inoculated in four sites, males in hind foot pads and testes, females in hind foot pads and ears. Control groups consisted of thymectomized rats inoculated with killed inoculum only and thymectomized rats inoculated with corresponding concentrations of viable M. leprae only. When it was necessary to check viability of harvests, passage was made to foot pads of intact BALB/c mice.

No viable *M. leprae* were recovered from the killed inoculum itself either after inoculation into thymectomized rats or on passage to mouse foot pads. On the other hand, *M.*  *leprae* was able to multiply in all of the other groups both in the presence and absence of the killed organisms. These results will be discussed in detail, but from the data it seems likely that the neonatally thymectomized rat should also prove to be a highly sensitive host for the detection of small numbers of *M. leprae* in the presence of large numbers of drug-killed *M. leprae.*— [This work is supported by the U.S.-Japan Cooperative Medical Program, Grant AI-08417. Life Sciences Division, Stanford Research Institute, Menlo Park, Ca. 94025]

Evans, Michael J. Mononuclear cell turnover in mouse foot pads infected with Mycobacterium leprae.

This study was designed to establish the technics necessary for the evaluation of mononuclear (MN) cell turnover in the mouse foot pad infected with *M. leprae*, and to provide insight into the turnover kinetics.

BALB/c mice infected five months earlier in both hind foot pads with  $5 \times 10^3$  M. leprae were injected intraperitoneally with 100 µCi 3H-TdR, and pairs of mice were sacrificed at intervals of one hour and 1, 7, 11, and 14 days after injection. Both hind foot pads were removed, fixed, embedded, and prepared for light and electron microscopic autoradiography. Initially, foot pad sections were analyzed to determine the distribution and the types of labeled cells. Low-power light micrographs were taken of the areas to be studied. These areas were then scanned with a magnification of 1,000X, and the position of each labeled cell in the cellular infiltrate was noted and marked on the corresponding low-power micrograph. To study the kinetics of MN cell turnover in the cellular infiltrate, the number of labeled cells present at each time interval was estimated by counting labeled and unlabeled MN cells at a magnification of 1,000X. All MN cells in a field were counted and scored; the slide was then moved until a new field was in view, and the counting process was then repeated. At least 500 cells were scored in each section, requiring the examination of 15 to 20 fields. The results from the study of both foot pads were combined, and the number of labeled cells per 1,000 MN cells was determined for each animal.

At the time the animals were sacrificed, the infection was late in its plateau phase, and most of the cells containing organisms appeared activated, by morphological criteria. Labeled cells were found distributed throughout the infiltrate; certain areas appeared to contain more labeled cells than others, suggesting that these cells were not randomly distributed. The population of labeled cells was composed of lymphocytes, monocytes, and macrophages. None of the labeled cells contained organisms at any of the times studied. At one hour after injection of the 3H-TdR, an average of 16.7 MN cells per 1,000 cells was labeled. At one day, 29.4 labeled cells were scored per 1,000 cells; this value decreased to 15.9 at 11 days and 17.0 at 14 days. Not only did the proportion of labeled MN cells appear to decrease, but the number of grains per labeled cell appeared also to decrease.

These preliminary results suggest that: 1) the MN cells of the infected mouse foot pad divide; 2) they can increase in number by proliferation of the existing MN cells, and perhaps also by migration of new cells to the site; 3) cells containing *M. leprae* do not divide; and 4) new cells do not appear to be phagocytizing *M. leprae.*—[This work is supported by the U.S.-Japan Cooperative Medical Program, Grant AI-10110. Life Sciences Division, Stanford Research Institute, Menlo Park, Ca. 94025]

#### Navalkar, R. G., Levy, L., Patel, P. J., Dalvi, R. R. and Dhople, A. M. Immune response to slow growing mycobacteria.

Plaque-forming cells (PFC) in the spleen of mice infected with slow growing mycobacteria such as M. leprae, M. lepraemurium and M. ulcerans were enumerated by localized hemolysis in agar gel, using sheep red blood cells (SRBC) coated with homologous and heterologous antigens. In the primary response, maximal levels of antibody forming cells varied with each species in respect of time of appearance and the secondary response appeared to be higher in magnitude than the primary one. Hemagglutinin determinations using 2-mercapto-ethanol treated sera indicated the absence of 7S antibodies, thus suggesting the secondary response to be an enhanced IgM type rather than a true IgG reaction.-[This investigation was supported by the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program administered by Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases (Grants R22 A1-08647 and R22 A1-07801), Bethesda, Md. Meharry Medical College, Nashville, Tenn.; Leprosy Research Unit, USPHS Hospital, San Francisco, Ca.; JHU-LWM Leprosy Research Laboratory, Baltimore, Md.]

#### Morrison, N. E., Congdon, C. C., and Collins, F. M. Effect of thymosin on BCG infection in the T-lymphocyte depleted mouse.

It has previously been reported that BCG produces a fatal mycobacteriosis in mice depleted of T cells by adolescent thymectomy, irradiation and bone marrow reconstitution. When compared to normal controls an increased number of BCG organisms was found in the lungs, bone marrow, lymph nodes and circulation of the T cell deficient animal. In contrast to the normals such animals did not develop peripheral tuberculin hypersensitivity when measured in the foot pad.

A thymosin fraction was prepared from calf thymus, the active principle of which is known to be an acidic polypeptide with a molecular weight of 12,600. Since it has been reported by Bach *et al* that *in vitro* incubation of mouse bone marrow cells with thymosin will result in the appearance of a subpopulation of spontaneous rosette-forming cells containing T cell markers, such as sensitivity to anti-theta serum and azathioprine, it was of interest to see if thymosin would influence T cell dependent immune responses against BCG infections in the T cell depleted mouse.

Thymosin was injected into normal and T cell depleted animals for an eight day period prior to intravenous challenge followed by an eight day period post-challenge. The multiplication of BCG in the lungs, spleen and liver was followed over a 90 day period.

Thymosin treatment of the T cell depleted animal prevented the occurrence of a fatal BCG mycobacteriosis in that no deaths occurred during the experimental period. Changes in the viable counts of BCG in the lungs, spleen and liver were consistent with the eventual emergence of an immune response that controlled the infection. These results are consistent with the claim that a thymosin-sensitive subpopulation of T cell precursors can be matured into a functional state in the absence of the thymus to express some aspects of cell-mediated immunity.— [JHU-LWM Research Laboratory, Baltimore, Md.; Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn, and Trudeau Institute, Saranac Lake, N.Y.]

Shepard, Charles C. Reinfection and vaccine versus *M. leprae* infections in mice.

In *M. leprae* infections in mice we have very consistently found protection after vaccination with living BCG, even in doses as low as  $10^{6.0}$  bacilli. In contrast in an early experiment we found no evidence of protection following an *M. leprae* infection of the opposite rear foot pad; the first infection contained an average of about  $10^{6.0}$  bacilli. In recent years, however, several other laboratories have reported definite reinfection immunity.

In a new experiment we were careful to time the appearance of the plateau phases accurately, and in some groups we counted bacilli in individual mice. Some mice received injections of *M. leprae* or BCG at the time the plateau was attained in the first hind foot pad. The challenge to all groups was given one month after the appearance of the first plateau. Immunity was judged by harvests at the time of appearance of the second plateau and again three months later. In the reinfected group, harvests of individual mice showed that nearly every reinfected mouse had some degree of immunity. The immunity was nearly complete if the bacilli in the first foot pad numbered more than 10<sup>5.8</sup>, but it was often partial when they numbered less than 105.8. A level of immunity equal to that observed in the reinfected mice was seen in a group of previously uninfected mice that received M. leprae that had been harvested from the first infection, purified, and concentrated so that the number of M. leprae injected into the foot pad (1063) was equal to that in the first injection. A group receiving 107.0 BCG only in the foot pad also had approximately the same immunity, but a group receiving a mixture of 10<sup>6.3</sup> *M. leprae* and 10<sup>7.0</sup> BCG in the foot pad had less protection. BCG given intradermally was apparently as protective as the same number given into the foot pad, but the early and late harvests gave different results. The best immunity was seen in reinforced mice that had also

received BCG given intradermally or into the previously infected foot pad.

Although BCG and M. leprae are related antigenically, the BCG could theoretically have had its protective effect without BCGsensitized T lymphocytes actually reacting with M. leprae antigens, since BCG-vaccinated mice have been shown to have activated macrophages generally, not just in local areas. Theoretically death of M. leprae occurs in activated macrophages, but the activation might occur elsewhere with subsequent migration into the infected area, or it might occur locally as a result of contact of sensitized T lymphocytes with M. leprae antigens .- [This work was partially supported by an interagency agreement between the National Institute of Allergy and Infectious Diseases (National Institutes of Health) and the Center for Disease Control. Atlanta, Ga.]

Ng, Herman and Levy, Louis. Further studies of the protection conferred by prior mycobacterial infection of mice and the failure of adoptive transfer.

Earlier studies demonstrated that mice previously infected in one foot pad with Mycobacterium marinum resist challenge in the opposite foot pad with M. marinum or M. leprae. This finding, together with Fenner's demonstration that naive mice were not protected against M. marinum challenge by the administration of serum from previously infected mice suggested that the protection against both homologous and heterologous challenge resulted from a cellmediated immune response. We have therefore attempted to protect naive mice against mycobacterial challenge by the transfer of splenic lymphocytes from previously infected mice.

In a series of experiments, splenic lymphocytes obtained from mice previously infected with *M. marinum* or *M. leprae* were administered to naive mice. Lymphocytes were harvested at intervals after primary infection; 10<sup>6</sup> to 10<sup>8</sup> cells were administered intravenously (iv) or intraperitoneally (ip), and  $10^7$  lymphocytes were injected into the foot pad from 90 days before to 7 days after challenge with *M. marinum*. In one experiment, the foot pads of naive mice that had received 10<sup>7</sup> or 10<sup>8</sup> lymphocytes iv or ip from donor mice infected with *M. marinum* seven months earlier were significantly less swollen than those of mice receiving only the challenge inoculum and those of mice challenged after the transfer of cells from previously uninfected donors. Other attempts to provide protection against challenge either with *M. marinum* or *M. leprae* by the transfer of splenic lymphocytes from mice previously infected with *M. marinum* or *M. leprae* have failed.

Although the mean of the measurements of foot pad thickness was sometimes smaller in mice receiving lymphocytes from previously infected donors than in control animals, the great variation in foot pad thickness among mice of the same environmental group made for wide confidence limits around the mean values, rendering apparent differences not significant. In an effort to develop a more discriminating model, we studied the mortality of mice after iv challenge with M. marinum. The mortality was found to vary with the size of the challenge dose, and mice previously infected with M. marinum but not those previously infected with M. leprae were found to resist the iv challenge with M. marinum. However, the ip transfer of 108 splenic lymphocytes from mice previously infected with M. marinum or M. leprae failed to protect naive mice against the iv M. marinum challenge.

There appear to be at least two explanations for the failure of adoptive transfer of protection against mycobacterial challenge. The transferred cells may have included too small a proportion of specifically sensitized lymphocytes, either because of the timing of lymphocyte harvest relative to primary infection, or because the spleen is not an optimal source of sensitized cells following local infection in the foot pad. Alternatively, it may be that protection against mycobacterial challenge requires the presence of antibody in addition to sensitized lymphocytes. Further studies are in progress.-[This work was supported in part by the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program, administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases (Grant R22 AI-7801). Leprosy Research Unit, Public Health Service Hospital, San Francisco, Ca. 94118]

Welch, T., Levy, L., Ng, H. and Spitler, L. Preliminary studies in vitro of cell-mediated immunity in *M. leprae-* and *M. mari*num-infected mice.

Because splenic lymphocytes from mice previously infected with Mycobacterium marinum or M. leprae failed to protect naive mice from mycobacterial challenge, we began studies to assess the ability of splenic lymphocytes to respond to mycobacterial antigens in vitro. At various times after infection with either M. marinum or M. leprae, mice were killed by cervical dislocation, and the spleens aseptically removed. Splenic lymphocytes were combed into RPMI 1640 tissue culture medium supplemented with 2% penicillin-streptomycin solution, 1% 1-glutamine and 5% fetal calf serum v/v. The lymphocytes were pooled from three to four mice and filtered through a nylon wool column to remove cellular debris, effete cells, macrophages and polymorphonuclear leukocytes. Cell suspensions were allowed to remain on the column for 20 minutes and then washed through with 50 ml of medium. The purified lymphocytes were washed once with medium and cell counts made. At this time, at least 95% of the cells were lymphocytes and more than 99% remained viable. Cell suspensions were diluted to 2-3 × 106/ml and dispensed in 1 ml lots into sterile test tubes. Three tubes of cells were left without antigen as base line control cultures, and triplicate tubes of cells were stimulated with some or all of the following antigens: M. marinum, M. leprae, PPD. Triplicate tubes were also stimulated with PHA. Cells were incubated at 37° in a 5% CO<sub>2</sub> - 95% air mixture for 48 hours, 0.1 microcurie of 14C thymidine was added to each tube, and the cultures reincubated for 16 hours. At this time the cells were harvested and the amount of incorporated radioactive label assessed by liquid scintillation.

Lymphocytes from M. marinum infected animals could be shown to respond at significant levels to M. marinum antigen as soon as two weeks after infection, with stimulation ratios remaining high as long as six months post-infection. However, lymphocytes from M. leprae infected mice did not show significant stimulation until four months after infection. Stimulation ratios remained elevated for at least two months in these mice, the period during which the M. leprae are being killed.

Indirect macrophage migration inhibition tests were done on the supernatants from splenic lymphocytes cultured at 5-10 × 106 lymphocytes/ml, using normal guinea pig macrophages as indicator cells. The same medium as that used for stimulation was used, except that the fetal calf serum contained in the medium had been previously shown to support the migration of guinea pig lymphocytes. Migration inhibitory factor (MIF) was produced as early as two weeks after infection with M. marinum, Supernatants from M. leprae infected mice have not been found to contain MIF. However, at this time all supernatants have not been tested.

Antigens prepared from armadillo M. leprae seem to be less effective in stimulating lymphocytes or in producing MIF. This may be due to toxicity inherent in armadillo v material since supernatants from these preparations (without bacilli) appear to decrease thymidine incorporation of lymphocytes. [This work was supported in part by the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program, administered by the Geographic Medicine Branch. National Institute of Allergy and Infectious Diseases (Grant R22 AI-07801), and by NIH Career Development Award AI-43012 to Dr. Spitler. Division of Immunology, Department of Medicine, University of California, San Francisco; Leprosy Research Unit, Public Health Service Hospital, San Francisco, Ca.]

Bullock, W. E., Evans, P. D. and Wyatt, C. R. Depletion of lymphocyte subpopulations in the spleen by murine leprosy.

Murine leprosy is a chronic granulomatous infection of lymphoid tissues and involves periarteriolar lymphocyte sheaths of the spleen. C3H mice were inoculated iv with 1 × 108 Mycobacterium lepraemurium. At 8, 16 and 23 weeks post-inoculation, infected and age-matched control mice were sacrificed. Whole spleens were prepared for total nucleated and differential cell counts. Lymphocytes bearing the  $\theta$ -isoantigen were quantitated by a 51Cr release cytotoxicity assay and the response of spleen cells to phytohemagglutinin P (PHA) was measured. Mean numbers of nucleated cells increased to a maximum of 2.8 × 108 at 16 weeks in infected spleens but had declined

by 23 weeks (2.1 × 108 cells). The mean cell count in control spleens at 23 weeks was 1.4 × 10<sup>8</sup> cells. The proportion of lymphocytes by differential count in infected spleens at 23 weeks was 15-24% vs 58-70% in normal spleens. Frequencies of  $\theta$ -positive cells (% lysis) declined after the eighth week of infection to a mean of  $5.5 \pm 1.4\%$  vs  $22.9 \pm 4.1\%$ in control spleens at 23 weeks. Responses to PHA by cells from infected spleens were also markedly decreased at 23 weeks. The results indicate that murine leprosy causes progressive loss of lymphocytes including T cell subpopulations from the spleen.-[Research supported by NIH Grant AI-10094. University of Kentucky College of Medicine, Lexington, Kentucky]

Coleman, M. B., Morales, M. J., Botero, G. I. and Hastings, R. C. The effect of thalidomide on a quantitative precipitin reaction *in vitro*.

Thalidomide is effective in suppressing the clinical manifestations of *erythema nodosum leprosum* (ENL). ENL is considered by many to be a manifestation of an Arthus or immune complex phenomenon. One of the crucial steps in the sequence of events occurring in an Arthus or immune, complex phenomenon is the combination of an antigen with its antibody and the formation of a precipitate. Thalidomide is known to chemically interact with various amines and proteins. The present experiments test the hypothesis that thalidomide inhibits ENL by preventing the precipitation of antigenantibody complexes.

Rabbit antibody to bovine serum albumin (BSA) was purified from commercial antiserum by affinity chromatography and labeled with <sup>125</sup>L BSA was labeled with <sup>131</sup>L Quantitative precipitin curves were established and zones of approximately 50% antigen excess, optimum proportions, and approximately 50% antibody excess determined by liquid scintillation spectroscopy. The effects of thalidomide (10 4 to 10 9M) on the quantity of antigen and antibody in the supernatants and precipitates in each zone were determined.

Thalidomide has no effect on the quantity of BSA or antibody to BSA precipitated in any of the three zones of the quantitative precipitin reaction. These data provide no support for the hypothesis that thalidomide affects the precipitation of an antigen and its antibody in ENL.—[Supported by the U.S.-Japan Cooperative Medical Science Program, Grant AI-10103. USPHS Hospital, Carville, La.]

Drutz, David J. and Bodel, Phyllis. Mechanisms of endogenous pyrogen production in patients with leprosy: Why are patients with uncomplicated lepromatous leprosy afebrile?

The primary mechanism for fever production in infectious diseases involves the elaboration of endogenous pyrogen (EP) by phagocytic leukocytes with subsequent stimulation of temperature-regulating centers in the hypothalamus. The most effective stimulus to the production of EP *in vitro* is phagocytosis; the monocyte (MN) is five to ten times as effective as the polymorphonuclear leukocyte (PMN) in this regard.

In patients with uncomplicated lepromatous leprosy (LL), both PMN's and MN's, as well as fixed reticuloendothelial phagocytes contained large numbers of leprosy bacilli. Nevertheless, in the absence of "reactions," patients with LL are not febrile. Studies were therefore carried out in order to determine whether EP production is defective in the leukocytes of patients with LL, and whether *M. leprae* is an apt stimulus for EP production.

Buffy coat leukocytes from two patients with uncomplicated LL who were bacteremic were incubated overnight in the presence or absence of heat-killed S. aureus strain 502A (10-20 cocci/leukocyte). LL leukocytes produced as much pyrogen as similar numbers of leukocytes from normal blood donors in the presence of staphylococci. Neither LL nor normal leukocytes produced EP in the absence of S. aureus. Conversely, MN's obtained from three normal donors and incubated overnight with heat-killed M. leprae (3 bacilli/leukocyte) produced large amounts of pyrogen. Supernates from the washed bacilli did not cause release of pyrogen when incubated with leukocytes.

These studies indicate that LL leukocytes are fully capable of pyrogen production, and that *M. leprae* is an effective stimulus for EP production *in vitro*. It is possible that absence of fever in patients with LL may relate to a selective defect of T lymphocytes. In vitro studies designed to investigate this possibility are in progress.—[University of California School of Medicine, San Francisco, Ca.; Yale University School of Medicine, New Haven, Conn.]

Rea, T. H., Quismorio, F., Nies, K., Harding, B., Disaia, P., Levan, N. and Friou, G. Intradermal antigens, epicutaneous haptenes, T cell counts, B cell counts, lymphocyte transformation and autoantibodies in one group of patients with lepromatous leprosy.

Our initial observations in lepromatous subjects of normal responsiveness to intradermal antigens and of normal sensitization to an epicutaneous haptene (Rea et al, Clin. Res. 20 [1972] 214; Nalick et al, Am. J. Obstet. Gynecol. 118 [1974] 393) was contrary to the many reports of a nonspecific impairment of cell-mediated immunity (CMI) in patients with lepromatous leprosy. Such contrary findings prompted further study to learn if any nonspecific deficiency of CMI could be demonstrated in our group of patients. Furthermore, because these patients appeared to be early in the course of their illness, i.e., ambulatory outpatients, median age of 31, little neurologic deficit, scant or absent nodular lesions, and the common presentation with erythema nodosum leprosum with few or no other signs of lepromatous leprosy, we reasoned that study of this group would help determine if the reported nonspecific deficiency of CMI was a predisposing cause for lepromatous leprosy or a result of the disease.

Forty-two patients were studied: 22 men and 20 women. Forty-one were born or raised in Mexico. Forty-one were classified as LL by the clinical criteria of Ridley and Jopling; one was classified as LI. It was not possible to do all tests on all subjects.

Intradermal antigens. Forty-two lepromatous patients received intradermal antigens. Control patients, 85 for tuberculin and 35 for the rest, were from the dermatology clinic and all were born in Mexico. At both the 5 mm and 10 mm induration level of responsiveness, there was no significant difference between the two groups for streptokinase/ streptodornase, mumps antigen, trichophyton, candida antigen or histoplasmin. Lepromatous patients showed less responsiveness to tuberculin 5 tu PPD-pill, p value was less than 0.05, suggesting perhaps a specific deficiency of CMI but no nonspecific deficiency.

Epicutaneous sensitization. Sensitization to 2,4-dinitro-1-fluorobenzene (DNFB) was attempted in 22 lepromatous subjects and was successful in 19 for a sensitization rate of 86%; the "normal" control sensitization rate was 100% (15 patients with cervical dysplasia); the "abnormal" control sensitization rate was 38% (74 patients with invasive cervical carcinoma). Because DNFB might be too potent an immunogen/antigen, 2,4dinitro-1-chlorobenzene was used to attempt to demonstrate diminished responsiveness. Using a sensitizing dose of 2,000 mg and challenging at 21 days with 0.1, 0.05, and 0.025 mg DCNB, 9 of 12 lepromatous subjects have been sensitized and 13 of 15 controls (dermatology clinic patients). There is no "significant difference" between the two groups as the p value is greater than 0.1 with any of the three challenge doses used, thus failing to demonstrate diminished CMI.

T cell and B cell counts. Circulating thymus-dependent lymphocytes, T cells, were counted by the method of sheep red blood cell (SRBC) rosette formation. Ficoll-Hypaque gradient separated lymphocytes, 0.4 ml of  $5 \times 10^6$ /ml, were incubated with 0.4 ml of 0.5% SRBC suspension, at 4°C for 20 hours, then smeared, stained and counted; a SRBC-rosette being a lymphocyte with three or more attached SRBC. Controls were from normal individuals counted on the same day as lepromatous subjects. Fifteen lepromatous subjects gave a mean T cell count of 70.1%, ± 6.1; 18 control subjects gave a mean of 68.8% ± 7.7.

Circulating bursa-equivalent lymphocytes, B cells, were measured by incubation Ficoll-Hypaque gradient separated lymphocytes with fluorescein-isothiocyanate conjugated sheep anti-whole human immunoglobulin at  $4^{\circ}$ C for 20 minutes; then viewing under ultraviolet light, counting the cells with fluorescent spots on their surfaces. Controls were run on the same day as the patients. Fifteen lepromatous subjects gave a mean B cell count of  $21\% \pm 7.6$ ; 13 control subjects gave a mean of  $24\% \pm 5.2$ .

Total lymphocytes, estimated by a routine WBC and differential done at the time of the T and B cell study, were within the range of normal.

No nonspecific immunologic abnormality

is found on the basis of either T cell or B cell counts.

Lymphocyte transformation. Lymphocyte transformation was studied using phytohemagglutinin (PHA), Difco, Bacto-phytohemagglutinin-M lot #589700, at concentrations of 1/10, 1/100 and 1/1000 in both autologous serum and fetal bovine serum (FBS). All specimens were run in triplicate, and controls (Mexican-born hospital patients) were run on the same days as leprosy patients. The uptake of tritiated thymidine after seven days' incubation at 37°C was the measure of transformation.

Using FBS the means and standard deviations of the logarithms of the counts per minute were: at PHA 1/10, leprosy  $3.93 \pm$ 0.28 and controls  $3.71 \pm 0.30$ ; at PHA 1/100, leprosy  $3.30 \pm 0.46$  and controls  $3.19 \pm 0.33$ ; at PHA 1/1000, leprosy  $2.76 \pm 0.41$  and controls  $2.97 \pm 0.27$ ; p values greater than 0.05, 0.5 and 0.2, respectively.

The differences in counts obtained using FBS and autologous serum in both lepromatous subjects and controls appeared to be random.

To the extent that PHA responsiveness is a measure of CMI, no evidence for impaired CMI in lepromatous leprosy is found.

Autoantibodies. We have included a search for autoantibodies in this present battery of studies because we were curious to learn if this group of patients with no demonstrable nonspecific deficiency of CMI would show the high incidence of autoantibodies reported by others in patients with lepromatous disease.

Eleven autoantibodies were sought in 32 patients using a latex fixation test for rheumatoid factor, an indirect immunofluorescent spot test for antinucleoprotein and anti-DNA (DNP/DNA) and a composite tissue slide of rat stomach, diaphragm, kidney and liver for autoantibodies directed against nuclei, mitochondria, cytoplasm, glomerular basement membrane, parietal cells, smooth or skeletal muscle and reticulin. Twenty dermatology clinic patients matched for age, sex and birth in Mexico were one control, and thirty dermatology clinic patients born in Mexico and without any suspicion of an immunologic illness were the other control. No differences with a p value of 0.1 or less were found between the lepromatous patients and the controls. Thus no abnormality of autoantibody production is found.

No correlations, segregation or differences could be found on the basis of therapy, presence or absence of ENL, DNCB/DNFB unresponsiveness, weak responses to intradermal antigens, etc. Thus there appeared to be no "immuno-deficient" subgroup.

We have been unable to demonstrate any nonspecific deficiency of CMI or abnormality of autoantibody production. Our findings are similar to those of Convit et al (Int. J. Lepr. 39 [1971] 556; 40 [1972] 4) and Salazar-Mallen et al (Alergia 18 [1971] 185) and give rise to a thesis: a nonspecific deficiency of CMI (or excessive autoantibodies) is neither prerequisite to nor a necessary consequence of lepromatous leprosy. The many reports of others gives rise to an antithesis: a nonspecific deficiency of CMI (or excessive autoantibodies) is a common finding in lepromatous leprosy. Consideration of differences between our patients and those of others gives rise to a synthesis(s): synthesis A; since our patients and those of Convit are evidently early in the clinical course of their illness, the changes noted by others may be secondary to longstanding disease. Synthesis B; since our patients and those of Salazar-Mallen et al are ethnically similar to one another but different from those of most other reports, differences in immunologic phenomena may be secondary and represent the influence of ethnic factors upon disease expression. Synthesis C; reasoning by analogy with the hypothesis of Lawrence and Zweiman (Trans. Assoc. Am. Physicians 81 [1968] 240) who argue that the nonspecific loss of CMI in sarcoidosis is due to preoccupation of the CMI apparatus with Kveim antigen, we envision our patients to have polar lepromatous leprosy with a CMI apparatus which completely ignores antigens of M. leprae (either tolerance or absence of "natural factor") and is therefore free to respond normally to other antigens; we envision the patients of others to have less than polar lepromatous leprosy with a CMI apparatus which can recognize, however feebly and ineffectively, the antigens of M. leprae as foreign, leading to preoccupation with such antigens and eventually preempting other CMI responses.-[Section of Dermatology, Section of Clinical Immunology and Rheumatic Diseases and Department of Obstetrics and Gynecology, University of

Southern California/Los Angeles County Medical Center, Los Angeles, Ca.]

Quismorio, F., Rea, T., Levan, N. and Friou, G. Immunoglobulin deposits in clinically normal skin of lepromatous leprosy patients.

Immunoglobulin deposits have been described in the dermal-epidermal junction of clinically involved skin in lepromatous leprosy (Bullock et al, Am. J. Trop. Med. Hyg. 23 [1974] 23). These deposits appear as characteristic "banding" by direct immunofluorescence with monospecific anti-IgM antiserum labeled with fluorescein. Similar immunoglobulin deposits have been reported in systemic lupus erythematosus (SLE) and in bullous pemphigus, although IgG and C3 have also been detected in the deposits in these latter two conditions. The immunoglobulin deposits in SLE have been shown to contain both antinuclear and antibasement membrane antibody activities. It is conceivable that the deposits in leprosy are autoantibodies directed against cutaneous antigens. To elucidate the nature of these deposits, we have studied biopsies of clinically normal skin in lepromatous leprosy and tested for circulating antiepithelial antibodies in the sera of these patients.

Punch biopsies were obtained from clinically normal skin of 13 patients with lepromatous leprosy, 10 of whom had erythema nodosum leprosum. Although clinically normal, sections of the skin specimens showed granulomas [lepromas]1 with foamy histiocytes and acid-fast bacilli on conventional histopathology. Cryostat sections were studied by direct immunofluorescence using fluorescein labeled monospecific antiserum to human IgG, IgM, IgA, IgD, IgE, C3 and Clq. Serum antibodies to basement membrane and to intercellular substance were tested by indirect immunofluorescence using rabbit or guinea pig esophagus as substrate. Sera from 32 lepromatous leprosy patients (which includes the 13 with skin biopsies) were examined for these antibodies.

Immunoglobulin deposits were detected in 10 of the 13 skin biopsies. In five, the deposits consisted of a finely granular "band" of IgM similar to that previously described by Bullock and co-workers. IgG was also seen in the dermal-epidermal deposit in one

<sup>1</sup> Editor inserted. IJL 38 (1970) 203-206.

patient. In five patients, IgM deposits were found along dermal collagen fibers. Small discrete aggregates of IgA were present in the dermis in two patients. Tissue bound IgD, IgE, C3 and C1q were not found. The specificity of the fluorescent staining was confirmed by conventional blocking experiments using unlabeled specific antiserum.

Antibodies reactive with intercellular substance were present in 9 of 32 lepromatous leprosy sera. These antibodies belonged to the IgG class and appeared similar to that seen in pemphigus vulgaris. The antibody was not inhibited by the addition of lepromin, tuberculin or trichophyton. In three of the nine patients, IgG antibody reacting with the basement membrane of the esophagus was also seen.

Our studies indicate that tissue bound immunoglobulins are present not only in the involved skin but also in clinically normal skin in lepromatous leprosy. The antibody activity of these deposits is not known. It is possible that these represent in vivo bound antibodies to skin antigens. Although we found serum antibodies reactive with epithelial antigens, these were not identical to those present in the skin. While the localization of skin deposits suggests that these were IgM antibody to basement membrane determinants, the circulating antibody was an IgG reacting with intercellular substance. However, it is also possible that these deposits are high affinity low titered antibodies which are cleared rapidly from the circulation by binding to the basement membrane. Elution studies similar to those performed in SLE skin are necessary to further elucidate the biological significance of these deposits.-[Section of Clinical Immunology and Rheumatic Disease and Section of Dermatology, Department of Medicine, University of Southern California School of Medicine, Los Angeles, Ca.]

Hastings, R. C., Morales, M. J., Slack, C. E. and Jacobson, R. R. Dialyzable transfer factor in lepromatous leprosy toxicity studies.

Considerable interest has been generated by reports of several investigators who have administered immunotherapy to patients with leprosy (Paradisi *et al*, N. Engl. J. Med. **280** [1969] 859; Bullock *et al*, N. Engl. J. Med. **287** [1972] 1053; Lim *et al*, Clin. Im-

mun. Immunopathol. 1 [1972] 122). Concern has been justifiably expressed by some workers as to the potential hazards of this new approach to therapy (Godal et al, N. Engl. J. Med. 288 [1973] 741; Crawford, C. L., N. Engl. J. Med. 288 [1973] 741). The purpose of the present report is to present preliminary toxicity data gathered during the course of treating three polar lepromatous leprosy patients with dialyzable transfer factor (Lawrence and Valentine, Am. J. Pathol. 60 [1970] 437). The material was prepared from healthy lepromin (Fernandez as well as Mitsuda) skin test positive hospital personnel by modifications of the method of Schulkind et al (Cell. Immunol. 3 [1972] 606). The donors were screened for the presence of hepatitis-associated antigen by counterelectrophoresis (Hyland Laboratories). Thyoglycollate cultures were made at five steps in the procedure and sterility in preparation was ensured prior to administration of the material. Each injection was Millipore filtered (0.45µ pore size) prior to lyophilization, a two step (48 hours followed by 24 hours) dialysis was employed; and a final Millipore filtration (0.2µ pore size) was performed immediately after reconstitution with 3.0 ml sterile, nonpyrogenic water and before subcutaneous injection into the subjects. Each patient received transfer factor (TF) from approximately 7 × 109 lymphocytes given in 36 divided doses over a 12 week period. All three patients improved clinically and bacteriologically. All three patients experienced histologically documented reversal reactions with onset at 19, 23 and 35 days after TF injections were begun. In each case the reversal reaction continued for the remainder of the trial (and beyond).

Daily clinical examinations did not reveal any lasting adverse reactions to the TF injection. There were absolutely no local reactions to the material (a total of 108 doses injected) other than those which could be elicited by injecting the same volumes of sterile water. Daily testing of hand and foot strength revealed a transient, very mild, right ulnar nerve paresis in one patient which cleared spontaneously in three days and was associated with the peak intensity of his reversal reaction. From days 38-42 of the trial this patient had low-grade fever (maximum 100.6°F orally) associated with his reversal reaction. Otherwise there were no clinically detectable changes in motor strength or sensory examinations, and no constitutional signs. Detailed eye examinations were kindly made by Dr. M. Brand at intervals throughout the study and no changes were noted. Similarly electrocardiograms and chest x-rays remained normal.

Laboratory data obtained at two week intervals revealed no clinically significant changes in hemoglobin, hematocrit, white blood cell counts, or differential counts, blood urea nitrogen, serum creatinine, alkaline phosphatase, SGOT, total and direct bilirubins, total serum protein and albumin, and creatinine clearance. Abnormal values which remained stable included low hemoglobins, a tendency to a left shift in the white blood cell differential counts, low serum albumins with corresponding elevations in serum globulins, and a tendency for lowered creatinine clearances.

Motor nerve conduction velocities of the ulnar (3 segments), median (2 segments) and common peroneal nerves obtained at intervals during the study revealed no significant changes. Abnormal values were confirmed to measurements in the left ulnar nerve of one patient who had a stable complete left ulnar nerve paralysis clinically before TF was begun.

In three polar lepromatous leprosy patients treated with substantial doses of TF. we have found no evidence of inherent toxicity in the material either locally or systemically. The potentially hazardous reversal reactions which occurred in all three patients were mild, have required no specific therapy, and have resulted in no lasting adverse effects. Given the experience that corticosteroids are effective in suppressing spontaneous or chemotherapy-associated reversal reactions, and in reversing damage incurred by these reactions when given promptly and in adequate dosages, we feel that dialyzable TF can be administered with reasonable safety to lepromatous leprosy patients under intensive medical observation. In our opinion the recognized hazards of TF and reversal reactions do not appear to be excessive under these conditions in view of the potential benefits to the patientsubjects involved .- [USPHS Hospital, Carville, La.]

Jacobson, Robert R. Treatment of leproma-

#### tous leprosy with rifampin.

Since 1971, 25 patients with lepromatous leprosy have been treated with either 300 mg or 600 mg of rifampin alone or in combination with other drugs. Skin lesions appear to clear equally well with any of the rifampin regimens. Data on the fall in the Bacteriologic and Morphologic Indices and the length of time elapsed until no growth is obtained in the mouse foot pad system, however, suggests but does not yet prove that the 600 mg dose of rifampin may be superior to the 300 mg dose.

The resolution of skin lesions appears to be faster with rifampin than with most other forms of therapy. The rate of clearance of bacilli from the skin may also be somewhat more rapid, but follow-up will have to be continued for at least several more years to determine whether this is indeed the case. *Erythema nodosum leprosum* has occurred in just over half of our cases and has generally been managed satisfactorily with thalidomide. Side effects from rifampin have not as yet been a problem. It has thus far proven to be an excellent antileprosy drug, but its high cost may limit its application. —[USPHS Hospital, Carville, La.]

#### Gordon, G. R., Ghoul, D. C., Murray, J. F., Jr., Peters, J. H. and Levy, L. Tissue levels of dapsone and monoacetyldapsone in rats and mice receiving dietary dapsone.

The use of the mouse as an experimental animal for pharmacologic studies of antileprosy drugs as well as for the detection of dapsone-resistant M. leprae is well established. It also has been demonstrated that the Lewis rat can serve as a host for the multiplication of M. leprae. To determine the relationship between plasma and tissue levels of dapsone (DDS) and monoacetyl DDS (MADDS), we measured the concentration of these sulfones in plasma and tissues of male Lewis rats and male BALB/c mice receiving DDS in the diet. All measurements were performed using our chromatographic-fluorometric method, which is specific for DDS and MADDS at nanogram levels.

When rats were fed a diet containing 0.005 gm% DDS, mean plasma levels at seven days were 157 ng/ml and 44 ng/ml for DDS and MADDS, respectively. Nearly identical levels of both compounds were found at 21 days. Tissue-to-plasma ratios of DDS at both time

periods were 0.7 for muscle and 1.0 for testes. Ratios of 1.2 to 1.8 were found for ear, nose, and body and scrotal skin. The highest ratios found were 2.2 for foot pads and tail skin. Tissue-to-plasma ratios for MADDS were similar to those found for DDS, and the extent of acetylation of DDS to MADDS in tissues was similar to that found in plasma.

Mice fed a diet containing 0.0025 gm% DDS exhibited plasma levels at 7, 14, and 21 days averaging 180 ng/ml and 9 ng/ml of DDS and MADDS, respectively, with minimal differences among the various times. Tissue-to-plasma ratios of DDS at all time periods ranged from 0.6 to 0.9 for brain, muscle, and testes and from 1.3 to 1.6 for ear, foot pads, skin, and fat. The highest ratio found was 3.5 for liver. Tissues exhibited low levels of MADDS, as did plasma.

Both species exhibited the same levels of DDS and MADDS in plasma and tissues at seven days as those found at later times, indicating rapid equilibration and no accumulation of the sulfones. The plasma levels found on these diets were consistent with our earlier observations. Because we previously demonstrated that diet and plasma levels of DDS are directly related, we would expect tissue levels to show the same direct relationship. Previous estimations of the minimal inhibitory concentration (MIC) of DDS for M. leprae in tissues of rats and mice were based on the assumption that tissue-toplasma ratios are one. Our findings that most tissue-to-plasma ratios are >1 suggest that the MICs of DDS for M. leprae extrapolated from plasma data are too low by a factor no greater than two.-[This research was supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by NIAID (Grant AI-08214). Stanford Research Institute, Menlo Park, Ca.; Public Health Service Hospital, San Francisco, Ca.]

Levy, Louis. Dapsone susceptibility of Mycobacterium leprae as a determinant of patient response to acedapsone.

In the course of an earlier study (Shepard et al, Am. J. Trop. Med. Hyg. 21 [1972] 440) of previously untreated patients with multibacillary leprosy, the rate of response to therapy with acedapsone (4,4'-diacetamidodiphenylsulfone, DADDS) 225 mg administered intramuscularly every 77 days was

measured by inoculation of mice with Mycobacterium leprae recovered from skin biopsy specimens obtained at intervals during treatment. It was demonstrated that M. leprae from three patients lost their infectivity for mice as rapidly as did those from control patients treated with dapsone in full dosage, whereas the rate of killing of M. leprae was much slower in the remaining seven patients. The staging of a trial of acedapsone therapy of multibacillary leprosy in Cebu, the Philippines, as part of the collaborative effort there of the U.S. Leprosy Panel and the Leonard Wood Memorial, provided the opportunity for a prospective study of the importance of several patient and organism variables.

Previously untreated patients with multibacillary leprosy who volunteered to participate in the current drug trial in Cebu were randomly assigned to one of three regimens, one of which consisted of acedapsone 225 mg every 11 weeks. Skin biopsy specimens were obtained for mouse inoculation before treatment, and at intervals of one, three and six months after beginning treatment. Isolates of M. leprae in mice were passaged each to a group of 60 mice; passage mice were administered drug-free diet or meal containing 0.00001%, 0.00003%, or 0.0001% dapsone for about 150 days beginning with the day of inoculation; all mice were fed drug-free diet after dapsone administration was stopped. Harvests were performed at intervals from all groups of mice, permitting the detection of multiplication of M. leprae during dapsone administration as well as the construction of growth curves. Twenty-five patients are enrolled in this study; partial or complete results, now available for 14 patients, are summarized in the accompanying table.

The results suggest that the patient strains vary considerably in susceptibility to dapsone. Five of 14 strains were completely inhibited by the lowest concentration of the drug, and eight were not inhibited at all. All were fully susceptible to 0.0001% dapsone, 11 of 14 showing bactericidal-type activity at this concentration. The rate at which a previously untreated patient's *M. leprae* are killed during antimicrobial therapy with acedapsone appears independent of the degree of susceptibility of the organisms to dapsone.—[This work was supported in part by the U.S. Leprosy Panel of the U.S.-Japan

Patient strain	Isolatea	Inhibition by DDS				
		0.00001%	0.00003%	0.0001%		
Most	rapid responders (neg.	for mouse after o	ne month DADDS	5)		
Elle	#1	0 <sup>b</sup>	++	+++		
Magl	#1	0	±	++		
Pena	#1	0	±	++		
Rapid respond	ers (pos. for mouse afte	er one month, neg.	after three months	s DADDS)		
Diwa	#1	+ •	++	++		
	#2	+	++	++		
Rica	#1	. ++	+++	+++		
	#2	+	+++	+++		
Sala	#1	++	+++	+++		
	#2	+	+++	+++		
Suar	#1	0	+++	+++		
	#2	±	++	+++		
Slow resp	onders (pos. for mouse	after three month	s, neg. after six mo	onths)		
Alin	#1	++	+++	+++		
	#2	++	+++	+++		
Cond	#1	0	++	+++		
	#2	±	- ++	+++		
Illu	#1	++	. +++	+++		
	#2	+	+++	+++		
	#3	0	+++	+++		
Mahu	#1	0	++	+++		
	#3	±	++	+++		
Ordo	#1	0	++	+++		
0100	#2	õ	++	+++		
	Poor responders (po	s. for mouse after	six months)			
Garn	#1	+	+++	+++		
21414.42	#2	+	++	- +++		
	#3	0	++	++		
	#4	0	++¢	++*		
Merc	#1	++	+++	+++		
	#2	+	+++	+++		
	#3	++¢	++ °	++*		

Response	10	DA	DDS	VS	suscepti	bility	to	DDS.
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"Isolate #1 was obtained from the pretreatment specimen, #2 from that after one month of treatment, and #3 from that after three months of treatment.

<sup>h</sup>Inhibition is scored as follows, primarily according to the results of the harvest performed just before stopping dapsone administration:

 $\begin{array}{l} 0 \geq 5 * 10^5 \ M. \ leprae \ per \ foot \ pad \\ \pm < 5 * 10^5 \ but \geq 10^5 \ M. \ leprae \ per \ foot \ pad \\ + < 10^5 \ but \geq 5 * 10^4 \ M. \ leprae \ per \ foot \ pad \\ + < 5 * 10^4 \ M. \ leprae \ per \ foot \ pad \end{array}$ 

bactericidal-type activity-that is, a growth delay after stopping drug treatment longer than can +++ be attributed to the presence of the drug.

<sup>c</sup>Because the study has not been completed, bactericidal-type activity (+++) cannot be excluded.

Cooperative Medical Science Program, administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases (Grant R22 AI-07801 and IAA -AI -73-9910). Public Health Service Hospital, San Francisco, Ca. 94118]

Peters, J. H., Gordon, G. R., Murray, J. F., Jr. and Levy, L. Metabolic disposition versus therapeutic response to acedapsone.

Twenty-two previously untreated multibacillary leprosy patients who volunteered

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to participate in a drug trial at Leonard Wood Memorial in Cebu City received 225 mg acedapsone (DADDS) intramuscularly every 11 weeks. After the first dose of DADDS, plasma samples were taken at 1, 3, 5, 7, 9, and 11 weeks for determination of levels of DADDS, monoacetyldapsone (MADDS), and dapsone (DDS). Skin biopsy specimens were obtained for mouse inoculation before treatment and after one, three, and six months of treatment. At the end of the six month treatment period, all patients were tested for acetylator phenotype using an oral dose of 10 mg sulfamethazine (SMZ) per kg. In addition, their characteristics for the metabolism of DDS were determined following an oral dose of 50 mg DDS.

Analyses for DADDS, MADDS, and DDS after DADDS treatment were performed by our modified chromatographic-fluorometric procedure, now sensitive to 0.1 ng/ml. Other assays were performed by published procedures.

Mean plasma levels of DADDS, MADDS, and DDS at one week were 7, 26, and 33 ng/ml, respectively; peak levels of 10, 37, and 45 ng/ml were recorded at three to five weeks; and thereafter the mean levels declined slowly to 5, 22, and 25 ng/ml, respectively, at eleven weeks. From the logarithmic decline during 7 to 11 weeks, we calculated half-times of disappearance (T1/2) of 47, 42, and 42 days for DADDS, MADDS, and DDS, respectively. At all time periods, the extent of hydrolysis of DADDS to MADDS and DDS was approximately 90%. The extent of acetylation of DDS to MADDS averaged 42% (range, 26% to 56%) in the 22 patients.

Tests for acetylator phenotype with SMZ in 21 of the patients showed that 16 (76%) were rapid acetylators and 5 (24%) were slow acetylators. The 16 patients of the rapid phenotype acetylated 25% to 48% of the test dose of DDS, and the 5 slow acetylators, 17% to 26%. The average acetylation of DDS by the total group was 33%-substantially below the average acetylation of DDS by the same group when DADDS was administered. Percentages of acetylation of SMZ, of DDS after DDS, and of DDS after DADDS all were significantly correlated. T1/2 values for DDS and MADDS after DDS were not correlated with acetylation capacities. The mean T1/2 of DDS was 31 hours (range, 21 to 46 hours), and the mean  $T_{2}^{1/2}$  of MADDS was 35 hours (range 26 to 60 hours) in the 21 patients. Individual  $T_{2}^{1/2}$  values of DDS after DDS, and of DDS after DADDS were not correlated.

Response to DADDS was estimated from results of the tests of multiplication of the patients' M. leprae in the mouse foot pad. Grades I, II, and III of response were defined as no multiplication after one, three, and six months of treatment, respectively. Slowest response-i.e., multiplication after six months of treatment-was designated Grade IV. Of 15 patients for whom tests of response are completed, the following indicates type of response, number of patients, acetylator phenotype, and range of T1/2 values of DDS after DDS: Grade I-3, rapid, 25 to 29 hours; Grade II-4, 3 rapid and 1 slow, 21 to 30 hr; Grade III-6, 5 rapid and 1 slow, 26 to 34 hours; and Grade IV-2, rapid, 44 to 46 hours. Thus far, no consistent relationships between therapeutic response and metabolic characteristics are apparent. -[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by NIAID, NIH (Grant AI-08214 and Contract NIH 69-2203). Stanford Research Institute, Menlo Park, Ca.; Public Health Service Hospital, San Francisco, Ca.]

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

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

We can now report the complete results of these analyses in 447 patients who began DADDS treatment in 1967. These results have been divided into the following groups: a) all patients; b) patients classified by disease type; c) paucibacillary (I, BT, TT, BT/TT) patients with subdivisions by year of healing; and d) multibacillary patients with subdivisions by type of response, i.e., good or poor response with or without infiltration. The group, taken in its entirety, showed mean levels of DADDS, MADDS, DDS, and total sulfones of 17.5, 27.2, 31.3, and 67.6 ng/ml, respectively, in plasma samples taken 75 days after DADDS injection. These levels, which can be considered the minimal levels, are substantially above the established minimum inhibitory concentration of 1 to 10 ng/ml for DDS against M. leprae in the mouse and rat foot pad test system.

We compared the mean values of the entire New Guinea group with sulfone levels found in plasma taken 77 days after DADDS injection in a group of 20 Filipino patients who had received only one DADDS treatment. All the sulfone levels, with the exception of MADDS, were significantly, but not substantially, higher in the patients from New Guinea. The MADDS level was the same for both groups. Because the sulfone levels were essentially the same in both the Filipino and New Guinea groups, and because the latter group received a substantially larger number of injections, we have concluded that repeated doses of DADDS every 75 to 77 days do not cause an accumulation of sulfones in plasma.

The results in the New Guinea group showed that an inverse relationship exists between plasma sulfone levels (except DADDS) and the body weights of the patients. The lack of correlation between DADDS and body weight suggests that DADDS plasma levels are not subject to the normal weight-related metabolic influences, but depend only on physical factors such as solubility in fluids bathing the injection site. Comparison of the sulfone levels within the paucibacillary group showed that no relationship existed between the year in which the patient healed and the sulfone levels. Nor was any relationship found between sulfone levels and type of disease in paucibacillary patients. The sulfone levels in healed paucibacillary patients were also compared with the corresponding levels in patients ex-

hibiting multibacillary disease, but no differences were evident. The sulfone levels in poor responders of the multibacillary group were compared with those in good responders of the same type, but no correlation could be ascertained.—[This work was supported in part by the U.S.-Japan Cooperative Medical Science Program, administered by NIAID (Contract NIH 70-2283) Stanford Research Institute, Menlo Park, Ca.; Department of. Public Health, Territory of Papua New Guinea; Center for Disease Control, Atlanta, Ga.]

#### Russell, D. A., Shepard, C. C., McRae, D. H. and Vincin, D. R. Present status in the acedapsone therapeutic trial in the Karimui, and its relationship to other trials.

The latest assessment in the Karimui trial was made in November 1973. A total of 337 patients have been treated since the start of the trial in 1967. Of these, 23 were classified as burnt-out TT or polyneuritic patients at the outset, and most of them continue to have deformities or patches of altered skin pigmentation, as might be expected, and are classified clinically as stationary. Excluding these, 281 had paucibacillary leprosy (1, TT, TT/BT, and BT), and 273 are now considered healed clinically; the remaining eight are classified as stationary or healing and improving. In short, the clinical response in paucibacillary leprosy has been satisfactory.

The multibacillary patients were followed with skin smears. There were 28 previously untreated patients who had high enough BI's to allow determination of solid ratios. Initially all 28 responded to treatment with early disappearance of solid bacilli and the usual gradual decrease in BI. By 1972, however, solid bacilli had reappeared in the smears of five patients. This occurred after their BI's had decreased 1.83-3.50 units; their BI's then ceased to decrease. Skin biopsy specimens were obtained for mouse inoculations. Two did not contain detectable numbers of bacilli, and mouse inoculations were negative. Three contained detectable bacilli; mouse inoculations proved positive and the bacilli were provisionally sensitive to 0.0001% DDS in the diet. There has been time for passage of one strain, and it has been confirmed to be sensitive to DDS. Plasma sulfone determinations by Peters and

colleagues showed normal levels in these five patients; thus they apparently have always had drug concentrations in their tissues several times the MIC demonstrated for their strains.

The BI's of the other 23 of the 28 patients responded satisfactorily, and their BI's were zero or approaching zero in 1972. Analysis of the results in these patients showed that the rate of fall in BI was a function of the Ridley-Jopling classification, of the height of the initial BI, and of the duration of treatment. The curve of decrease was S-shaped in many patients, and, in patients with more lepromatous diagnoses and higher initial BI's, the fall in BI was somewhat slower and the period of most rapid fall occurred later. Maximal rates of decrease for individual patients were nearly all between 1.5 and 3.0 BI units per year. To have stated the decrease in BI as an average from the beginning of treatment would have obscured the rapidity of decrease during particular intervals.

The finding of viable but apparently DDSsensitive bacilli in 5 of 28 patients seems to be interpretable only as long-term survival (without multiplication) of a small fraction of DDS-sensitive organisms, which become demonstrable only when a sufficient number of nonsolid bacilli have disappeared. It seems possible that bacterial killing would have commenced again with continuance of the same therapy. Nevertheless from these results we could conclude that for multibacillary leprosy acedapsone therapy was not optimal by itself. Consequently we have added a 90-day course of rifampin (600 mg daily) for each multibacillary patient, without interruption of acedapsone injections. The early response since rifampin has been favorable.-[Center for Disease Control, Atlanta, Ga.; Department of Public Health, Port Moresby, Papua New Guinea]

Russell, D. A., Worth, R. M., Jano, B., Fasal, P. and Shepard, C. C. Acedapsone in the preventive treatment of leprosy: three years after the last shot.

The design, execution and two year posttreatment incidence of leprosy in this field trial of DADDS in Micronesia has been reported elsewhere (1.2). Mass treatment with DADDS was offered to about 1,500 people in three villages with a high prevalence of leprosy in the Ponape District of Micronesia during 1967–1970. Annual reexamination of this entire population for leprosy since 1967 has revealed the following incidence (Fig. 1).



FIG. 1. Leprosy incidence among Pingelapese people (1968-1973) by histological type.

Of the nine new cases appearing since the end of mass DADDS, only one (and he with a proven sulfone-resistant strain of M. leprae indicated by an asterisk on the figure) is among the 915 people who received nearly all 15 injections of DADDS in 1967-1970. The other eight cases (all with a history of eight or fewer injections) were among the approximately 630 people who received 12 or fewer injections. There is now a significantly lower incidence (p .01) of leprosy among those who received a full three year course of DADDS. No cases have yet appeared among those 300 or more children born since 1968. Annual surveillance of this population will continue.-[This work was done under Contract NIH-NIAID-72-2075 (HEW)]

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- Dhople, A. M. and Hanks, J. H. Energetics of *Mlm (Mycobacterium lepraemurium)* during the transition from *in vivo* to extracellular growth.

LSM (Memo L-531, February, 1974) reported our confirmation that the Nakamura system permits the *in vitro* growth of *Mlm*. Several Japanese colleagues already had reached this conclusion.

In order to facilitate such investigations we outline three propositions. 1. Metabolic or biochemical indicators of physiologic / states are more significant than microscopic or plate counts: a) because the essentials can be learned without waiting for growth to occur, and b) because decreases in competence can be quantitated as readily as increases. 2. In the absence of high plating efficiency, growth potentials (ATP per viable cell) cannot be determined. 3. Given sources of both nitrogen and carbon and relatively constant conditions, the ATP per culture, or aliquot, measures the FBm (functional biomass), the parameter that should correlate with plate and microscopic counts (see LSM Memo L-538, February, 1974).

In earlier communications it was shown that the FBm of Mlm in Oiwa's medium deteriorated in the same fashion as the respiration and HTC documented by Gray and Hanks. In the recent ATP experiments the three-day data demonstrated that the FBm in Nakamura's semisynthetic base (E-K) fell to 50% of the original. The addition of the supplements (complete NC-5) reduced the physiologic "sag" by 50%. Within ten days the FBm in NC-5 had returned to 100% of the energy levels possessed by Mlm during growth in CFW mice, i.e., the cells had successfully reconstructed the leaky "in vivo" type membranes into those which are competent for extracellular growth. Within 20 days the rate of expanding FBm had improved slightly. Fetal calf serum was significantly superior to goat serum. Thus, the goals of such experiments had been accomplished in less than three weeks.

In further investigations recalculation of correction and conversion factors demonstrated that FBm and the microscopically measured total biomass increase at parallel rates. The conditions and components in NC-5 medium are being classified into three categories: inhibitory, beneficial and essential. The effects of CO2 and temperature and more precise quantitations of the effects of components in the NC-5 medium will be available at the time of the U.S.-Japan meeting. A table or figure will be submitted as an addendum at that time.-[JHU-LWM Leprosy Research Laboratory, School of Hygiene and Public Health, Baltimore, Md. 21205]

Rightsel, W. A. and Sawyers, M. F. The synthetic capabilities of mouse macrophages following *in vitro* infection with *Mycobacterium leprae* and *Mycobacterium lepraemurium*.

The natural host for both M. leprae and M. lepraemurium is the monocyte, and there is little doubt that M. leprae thrives inside human macrophages in vivo. Several reports have demonstrated the cultivation of M. lepraemurium in long-term tissue cultures of mouse peritoneal macrophages; more recently, we achieved a 32-fold increase with this organism using a specialized diffusion chamber technic in which M. lepraemurium was inoculated into the chambers with macrophages and then maintained for 50 days in the mouse. Since M. leprae has never been successfully cultivated in vitro, the macrophage has been used to study the host-parasite relationship in comparison with that of murine leprosy.

It is our general hypothesis that cellular growth is controlled primarily by macromolecular synthesis and, hence, studies using the macrophage may provide direct information relative to the physiological blocks of these *in vivo*-grown host-dependent microbes. Therefore, the synthetic capabilities of mouse peritoneal macrophages inoculated with either *M. leprae* or *M. lepraemurium* were studied by following the incorporation after pulse labeling of <sup>3</sup>H-leucine into protein and <sup>3</sup>H-uridine into ribonucleic acid (RNA).

After infection with M. leprae, the macrophages showed an immediate inhibition of protein synthesis that progressively decreased as indicated by the uptake of labeled leucine. On the other hand, macrophage cultures inoculated with M. lepraemurium showed an immediate uptake of the precursor that continued for four days followed by a gradual and continuous decrease on incorporation of the labeled compound. Hence, these two organisms showed different effects on the protein synthetic capabilities of infected macrophages. Neither of these results corresponded to that of macrophages inoculated with inert latex particles or uninoculated controls, both of which exhibited similar recurrent kinetic patterns. In contrast, no difference was observed in the RNA synthesis of macrophages inoculated with either M. leprae, M. lepraemurium, or

inert latex particles as indicated by kinetic patterns similar to uninoculated controls during the uptake of labeled uridine over a 12-day period. These methods using radioactive precursors have provided an in vitro tissue culture system to study and compare the effect on the macromolecular synthesis of macrophages infected with these two fastidious acid-fast mycobacteria.- [This investigation was supported by the U.S.-Japan Cooperative Medical Science Program administered by NIAID (Grant R22-AI-08051), NIH. Department of Pathology, Baptist Memorial Hospital and Department of Microbiology, University of Tennessee Medical Units, Memphis, Tenn. 38146]

#### Skinsnes, Olaf K. and Matsuo, Eiichi. Contrasting morphologies of reactional states in leprosy.

In 1964 the hypothesis, now quite generally accepted, was advanced that tuberculoid reactions are primarily expressions of delayed-type hypersensitivity, that *erythema nodosum leprosum* is essentially the manifestation of antigen-antibody reactions or immune complex disease and that reactions in dimorphous leprosy contain elements of both types of phenomena.

Necropsy studies of two young Chinese men, aged 16 and 24 years, one having tuberculoid reaction and the other acute *erythema nodosum leprosum* are presented to demonstrate the contrasting morphologic manifestations in these two conditions. Since the two patients each committed suicide during acute reactional episodes and were young persons, a unique opportunity is presented to obtain some concept of the systemic extent of such reactions.

The necropsy findings of the tuberculoid cases, as related to leprosy findings, showed: 1) severe granulomatous neuritis of sciatic, popliteal and ulnar nerves as well as of cutaneous nerves; 2) focal interstitial granulomatous myocarditis, hepatitis, orchitis and nephritis. All these latter lesions were minimal, scattered and focal.

The clinical lepromatous patient's skin lesions histopathologically were BL rather than classical LL. The reactional manifestations, however, were histopathologically characteristic of the responses seen in immediate type hypersensitivity. There was: 1) reactional inflammation, pronounced, with arteriolitis in sciatic and ulnar, as well as cutaneous nerves; 2) fibrinoid arteriolitis in pulmonary septa, heart, liver, spleen, pancreas and kidneys. The fibrinoid material was PAS positive and compatible with immune complex deposition.

The renal lesions in the tuberculoid case were scattered and minimal and could be expected to heal without scarring. The vascular lesions in the BL cases were pronounced and involved primarily the afferent glomerular and interlobular arterioles. There was slight deposition of PAS positive material in glomerular capillaries and Bowman's capsule.

The findings in this single case suggest that the major immune complex reaction in ENL-type response occurs in preglomerular arterioles and not in glomeruli. If this be generally true then the general lack of necropsy findings of classical glomerulonephritis morphology in lepromatous leprosy, except occasionally, may be explained. The vascular lesions described could well lead to the vascular thickening and obliteration as well as glomerular scarring that has more generally been described in lepromatous leprosy necropsy series and characteristic of "end stage kidneys."-[ALM Leprosy Atelier, Department of Pathology, University of Hawaii School of Medicine, Honolulu, Hawaii. NIH Grant AI-10034]

#### Walsh, G. P., Storrs, E. E., Burchfield, H. P. and Vidrine, M. F. New statistics on the incidence of leprosy in the armadillo.

It was recently reported that 40% of a group of 20 armadillos inoculated in 1970 and 1971 had developed leprosy over a period of 3 to 3.5 years (Science 183 [1974] 851-852). This group was comprised of random-bred animals that had been captured as adults in the wild.

In a more recent study, a group of 24 animals was inoculated intradermally with M. *leprae* obtained from an infected armadillo. This group was composed of animals that although not bred in captivity, were raised in captivity and their selection for the study was based on the requirement that they not exceed 1.5 years of age. It was felt that young animals should be used since in all likelihood, they would be immunologically less mature than full grown adults and perhaps more susceptible to infection. In addition, it was felt that the use of an armadillopassed strain might also reduce the time required for the development of the disease.

Biopsy specimens taken six months after inoculation were positive for AFB in 4 of the 24 animals while our latest results show that 15 of the 24 animals (62%) are positive for AFB at 14 months post-inoculation. It should be emphasized that this statistic is not final and it would not be unreasonable to assume that one or more of the nine animals that are negative at this time will become positive in future months.

It should also be mentioned here that in a related experiment, all three animals that were inoculated intravenously developed a more severe and disseminated infection than intradermally-inoculated animals. Thus the route of inoculation should also be considered in determining susceptibility.

These results reinforce the use of the armadillo as a model for the study of leprosy. By using younger animals that are probably less immunologically capable than adults, the susceptibility of this species is significantly greater as manifested by a shorter incubation time and a greater percentage of animals developing infection.

Therefore, while successful breeding in captivity is still very desirable and should be vigorously pursued, the high degree of susceptibility among young random-bred animals will allow extensive use of this animal species as an experimental model in the study of leprosy.—[Gulf South Research Institute, New Iberia, La.]