

## ABSTRACTS

### TWELFTH JOINT LEPROSY RESEARCH CONFERENCE

Park Plaza Hotel  
Boston, Massachusetts  
September 27-29, 1977

U. S.- Japan Cooperative Medical Science Program

#### FOREWORD

The Twelfth Joint U.S.-Japan Leprosy Research Conference was held in Boston, Massachusetts on September 27-29, 1977. The joint tuberculosis conference was held simultaneously in an adjoining room, and on the last day the two groups met together to hear a symposium on mechanisms of immunological tolerance. Only the abstracts from the leprosy research conference can be included here.

There are some helpful parallels between leprosy and tuberculosis, so it is useful from time to time to facilitate exchange of information between the two fields. Leprosy research for the last two decades has shown, however, that the similarities between the two diseases are limited and unpredictable. In fact, the two etiologic agents seem to share only those properties that are common to all mycobacterial species. Two decades ago, one frequently heard that application of the ideas of tuberculosis to leprosy would yield results of great benefit, but in general this has not been true, and the major advances in leprosy research in recent years have, in fact, depended upon the acquisition of specific knowledge about *M. leprae*. As a result one now comes to look upon attempts to combine leprosy and tuberculosis research as attempts to save money by reducing research in the two fields. Leprosy is predominantly a disease of tropical countries, and leprosy research has been predominantly an activity of developed countries. This geographical separation has as one of its unfortunate consequences a continuing pressure to reduce research on tropical diseases. That the pressure can be countered successfully is evidenced by the fact that leprosy research has survived, and sometimes even flourished. Because leprosy is such an important medical and public health problem, the potential benefit from quality research is uncommonly large.

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

## ABSTRACTS OF LEPROSY CONFERENCE

**Nakamura, Masahiro.** Enriched growth of *M. lepraemurium* in flask cultures.

It has been reported that the NC-5 medium permits the *in vitro* growth of *M. lepraemurium* (*Mlm*). When a bacterial suspension of *Mlm* is inoculated into this medium, the cells quantitatively multiply for up to six weeks when incubated at 30°C. Thereafter degenerative morphologic changes in the cells take place and gradually the cells lyse. ND-5 medium was developed in order to eliminate these unfavorable findings. For it, Dubos medium was used as the base instead of Kirchner medium.

This paper is to present (i) physiologic characteristics of *Mlm* in addition to those previously reported, and (ii) a method to obtain large numbers of bacilli that can be used for biochemical studies and as inoculum for subcultivation trials. The two basal media used were Kirchner in the NC system and Dubos in the ND systems;  $\alpha$ -ketoglutarate was incorporated as an additive in subsystem 5 and oxaloacetate in subsystem 15. The results obtained indicated that *Mlm* multiplied more abundantly when the cells were grown in a 100 ml flask than in a 50 ml flask; both sizes of flasks contained 42 ml of culture medium. Furthermore, the bacilli grown in the ND-15 medium in 100 ml flasks multiplied well and maintained their physiologic characteristics.—[Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan]

**Dhople, Arvind M. and Hanks, John H.** The problem of obtaining macroscopic growth of *Mycobacterium lepraemurium* *in vitro*.

In 1972 Nakamura reported that *Mlm* (*Mycobacterium lepraemurium*) initiates extracellular growth in the NC-5 medium. Growth was limited and serial subculture could not be accomplished. Since confirming these observations we have optimized factors that influence the growth of *Mlm* in the Nakamura system at 30°, succeeded partially in serial transfers of the cultures and sought to explain the termination of growth after six weeks, i.e., when cell populations have expanded approximately 24-fold.

Exhaustion of the medium and restricted

oxygen supply and the accumulation of inhibitory compounds which arise during the deterioration of the medium did not explain the termination of growth, since 100 times the routine inoculum expanded functional biomass to the usual limits and during the usual periods of time.

A foremost limitation is the lability of the supplements required by the *Mlm*. For example, uninoculated medium lost its growth promoting quality after incubation for three weeks at 30°. Addition of fresh supplements to the pre-incubated medium reestablished the standard growth rates. Renewals of medium each two weeks maintained maximum growth rates for six weeks, though it did not sustain the earlier growth rates. Pyruvate and the sulfhydryl compounds (cysteine) must be freshly dissolved and filtered for each experiment. When incubated individually for ten days the percent of original activity retained by other compounds was:  $\alpha$ -ketoglutarate 17%, hemin 48%, cytochrome C 66% and fetal calf serum 87%. In the inoculated medium the bacterial cells apparently conserve the original compounds (or intermediates) and grow at full rates for four weeks. The most labile and important compound,  $\alpha$ -ketoglutarate, has been replaced with malate, with a 20% gain in cell yields. Experiments in progress include the possibility of replacing pyruvate with acetate and hemin and/or cytochrome C with  $\alpha$ -amino levulinic acid, a precursor of heme compounds. Nakamura's mineral-deficient base is being replaced with one that provides a complete battery of minerals, citrate as chelator, ammonium ions to facilitate transamination and both acetate and malate as stable supplements.

Other investigations have uncovered two principles that facilitate the foregoing inquiries.

1. "Vitreous Mice." After substituting 0.7 mM DTT (Dithiothreitol) for the labile cysteine and thioglycolate used hitherto, cells are maintained with an essentially constant ATP and growth potential during an interval of six weeks after active growth has ceased. These stable stocks of depleted cells provide inoculums that shorten current experiments by eliminating the early periods of growth.

**2. Incubation at 38°.** Incubation at this temperature severely handicaps cells in the original NC-5 medium. The usefulness of additional compounds or complexes (e.g., 0.5% yeast extract and 2% trypticase) can be quantitated within two to four weeks at 38° more decisively than at 30° for eight weeks.—[Supported by the U.S.-Japan Cooperative Medical Science Program, Grant No. 8866-07. JHU-LWM Leprosy Research Laboratory, School of Hygiene and Public Health, Baltimore, Maryland 21205]

**Kirchheimer, W. F., Sanchez, R. M., Pasqua, J. P. and Walsh, T.** Quantitative aspects of experimental infection of armadillos with *Mycobacterium leprae*.

One objective requiring knowledge of quantitative aspects of experimental leprosy in armadillos is an integral part of our attempts to study fundamental aspects of leprosy such as validity of the genetic hypothesis and the mechanism of resistance (susceptibility). We give arbitrarily "resistance" a quantitative connotation hoping that at a dose of *Mycobacterium leprae* infectious for only 10% armadillos the assumed cell differences in susceptibles and resistants become measurable. We have determined that the desired bacterial dose falls below  $10^5$  intracutaneously inoculated *M. leprae* A.

Another quantitative aspect concerns production of large numbers of high quality leprosy bacilli in as short a time as possible to supply WHO and others with Lepromin A and infected tissues to IMM LEP and NIAID. In our experience the most suitable way to accomplish the desired objective is by intravenous inoculation of several hundred million *M. leprae* A (*M. leprae* H?). The rate of infection (disseminated leprosy) approaches under these conditions 100% in less

than two years. Approximately 50% armadillos carry billion amounts of *M. leprae* per gram of liver, spleen, lymph nodes and subcutaneous lepromas in around 12 months. As one example times of harvests and amounts of *M. leprae* harvested are shown in Table 1 (below). In this experiment eight armadillos were challenged. Only one showed no signs of disseminated infection after 612 days (infection rate 88%).—[USPHS Hospital, Carville, Louisiana 70721]

**Morrison, Norman E. and Marley, G. M.** Studies with the clofazimine analog, B1912.

B1912 has been suggested as a candidate drug for testing in human leprosy. In the absence of pre-clinical data an investigation of the antimetabolic effects of the compound was undertaken.

Cell monolayers of a human epithelial cell line were prepared on cover slips in Leighton tubes. The cells were maintained in Eagle's minimal essential medium supplemented with amino acids and 5% fetal calf serum. Cell monolayers were exposed to varying concentrations of B1912 (1-10  $\mu$ g/ml), added in DMSO, and the mitotic index of the adhering cells was quantitated after fixation using the aceto-orcein staining procedure. It was found that metaphase and anaphase were severely depressed with increasing concentrations of B1912. The antimetabolic effect was expressed between prophase and metaphase in the cell cycle. This was consistent with earlier data which indicated that B1912 formed stable complexes with human DNA. It is suggested that B1912 acts as a metaphase inhibitor producing lethal effects in rapidly dividing cells. The antimetabolic effect was seen with concentrations of B1912

TABLE 1. Number of leprosy bacilli per gram of tissue of armadillos infected intravenously with  $2.0 \times 10^8$  *M. leprae*.

Days after infection	Liver	Spleen	Lymph node	Subcutaneous leproma
308	$1.5 \times 10^{10}$	$3.7 \times 10^{10}$	$4.4 \times 10^{10}$	
363	$2.5 \times 10^9$	$7.2 \times 10^9$	$2.4 \times 10^8$	$3.5 \times 10^{10}$
369	$6.1 \times 10^{10}$	$5.5 \times 10^9$	$4.0 \times 10^8$	$9.1 \times 10^8$
383	$6.3 \times 10^9$	$4.8 \times 10^{10}$	$4.0 \times 10^{10}$	$6.1 \times 10^{10}$
384	$4.1 \times 10^{10}$	$7.3 \times 10^{10}$	$8.7 \times 10^9$	
390	$9.0 \times 10^9$	$1.8 \times 10^{10}$	$4.7 \times 10^{10}$	

that could be attained in human serum.

Further confirmation of the nucleotoxicity of B1912 was found in feeding experiments with pregnant mice. When mice were fed (0.05% diet) at 50 mg/kg/day for five days during midpregnancy a complete interruption of pregnancy occurred resulting in fetal death and resorption. No maternal deaths occurred. It was evident that placental transfer of B1912 had taken place in the aborted fetus. This anti-embryogenesis effect was consistent with the antimetabolic effect found in tissue culture. It is evident that a considerable amount of pre-clinical testing will be required before B1912 can be safely tested in human volunteers. The compound appears to have greater toxicity than that found for clofazimine.—[This investigation was supported by Biomedical Research Support, Grant FR-05445 from the Biomedical Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health, Department of Pathobiology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland]

**Fieldsteel, A. H., Tse, P., Cheng, M. and Levy, L.** Criteria for multiplication of *Mycobacterium leprae* in neonatally thymectomized Lewis rats.

With the more common use of immunosuppressed animals in leprosy research, it is becoming increasingly necessary to establish criteria for multiplication in these animals. These criteria may not necessarily be the same as for intact animals. Presumably, all of the animals in current use (thymectomized-irradiated mice, neonatally thymectomized rats, athymic nude mice) maintain some degree of immune competence. Therefore, there may be a limit to the number of *M. leprae* that can be inoculated—either because they will elicit some sort of immune response or because they will not multiply due to the fact that the number of organisms in the inoculum is so large that multiplication could not be detected.

We have had considerable experience with neonatally thymectomized Lewis rats (NTLR) infected with large numbers of *M. leprae*. From these animals we have passaged between  $1 \times 10^7$  and  $5 \times 10^7$  *M. leprae* into the foot pads of NTLR. Mostly, these large numbers of organisms were obtained from NTLR that had been treated with ri-

fampin or dapsone or combinations of the two drugs. Therefore the proportion of viable *M. leprae* might be expected to drop to an undetectable level if relatively few organisms were inoculated. We did not know if large inocula would evoke an immune response, but we did know that dead *M. leprae* were cleared out of the tissues relatively rapidly. We therefore attempted to determine if we could demonstrate either unequivocal growth of the large inocula or the presence of viable *M. leprae*, detectable by further passage of the harvested organisms into intact mice. In a number of instances we made multiple passages of a given inoculum in several concentrations ranging from  $5 \times 10^5$  to  $5 \times 10^7$  per foot pad. The purpose was to determine if we could get direct evidence from the smaller inocula in case we failed to do so with  $10^7$  or greater organisms. The animals were killed between 6 and 26 months after inoculation.

The results could be divided into three categories. The first group consisted of 11 NTLR in which there was at least a 10-fold increase in the inoculum, thus satisfying the criterion of absolute increase in numbers.

The second group consisted of 33 NTLR in which there was less than a 10-fold increase over the inoculum, but no animal yielded less than  $10^7$  *M. leprae* per foot pad or fewer than were inoculated. The average increase was 3.9-fold, with a range of 1.2 to 9.1. Passages from nine of these harvests were made in intact mice and all of the inocula multiplied. However, there were five additional rats in Group 2 in which mouse passage was not made because smaller inocula from the same animals increased at least 10-fold. Also, in Group 2 there were five NTLR in which there was a 5.85- to 9.08-fold increase in numbers. These harvests were not tested for viability because the increases occurred relatively early after inoculation and because of the large number of organisms harvested. It seemed almost certain that these inocula were also viable, especially since they contained many solid staining bacilli. Thus we felt, with a reasonable degree of confidence, that when  $10^7$  or more *M. leprae* were inoculated into the foot pads of NTLR and at least that number could be recovered six or more months after inoculation, the inoculum probably contained viable organisms. If it can be demonstrated consis-



tently that failure of such large inocula to increase does not mean lack of viability, it would mean that the number of *M. leprae* that could be inoculated into the foot pads of NTLR to detect a very small proportion of viable organisms could be increased many times over the number currently used.

The third group is perhaps of greatest interest. It consisted of 31 NTLR that were inoculated in the foot pads with 1 to  $5 \times 10^7$  *M. leprae* with recovery of fewer organisms than the number inoculated. At least 26 of the 31 rats could be shown—by either direct or indirect methods—to yield viable AFB (acid-fast bacilli) regardless of the numbers harvested. That included the two harvests that yielded the fewest AFB ( $1.84 \times 10^5$  and  $2.05 \times 10^5$ ).

As previously noted, our practice was to make multiple passages into NTLR of at least two concentrations of *M. leprae* derived from drug-treated NTLR. Although the results presented here are retrospective in the sense that had we set out to determine the ideal or maximum inoculum of *M. leprae* for NTLR, we would not have included animals that had received chemotherapy. Therefore, we can draw few (if any) conclusions from the failure of various inocula to replicate. However, several positive conclusions can be drawn despite the obvious drawbacks. Regardless of the number of *M. leprae* inoculated into the foot pads of the NTLR (i.e., up to a known maximum of  $5 \times 10^7$ ), if at least  $10^7$  organisms are recovered six months or longer after inoculation, it is probable that multiplication has occurred. Further, the results also suggest that when  $10^7$  or more *M. leprae* are inoculated into the foot pads of NTLR and there is a decline in numbers to less than  $10^7$ , further passage into intact mice would be worthwhile before deciding that the original inoculum was not viable. In our experiments, 13 of 14 such inocula proved infectious for mice. Lastly, because NTLR probably are not completely immunosuppressed, inocula of  $10^7$  or greater may evoke enough of an immune response to account for the results described here. Therefore, an inoculum of  $5 \times 10^5$  carried out simultaneously with a larger inoculum may prove to be directly infectious without the necessity of making subpassages to intact mice.—[This work was supported by the U.S.-Japan Cooperative Medical Sci-

ence Program, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education and Welfare (Grant R22 AI-08417). Life Sciences Division, SRI International, Menlo Park, California and Hebrew University-Hadassah Medical School, Jerusalem, Israel]

**Gelber, Robert and Levy, L.** Further studies of dihydrofolate reductase inhibitor activity on the multiplication of *M. leprae*.

New bactericidal agents are required to confront the dual problems of bacterial resistance and persistence in lepromatous leprosy. Previously we reported activity of a series of dihydrofolate reductase inhibitors (2, 4-diamino-5, 6-substituted quinazolines) alone and in combination with dapsone against *M. leprae* infection of the mouse foot pad (IJL 44:124). The quinazolines were screened by the kinetic technic of Shepard (IJL 35:429), i.e., mice were fed drug-containing diets from day 60 to day 150 following foot pad infection. During early drug administration some of these agents prevented multiplication of *M. leprae*, but later, while the mice were still receiving the drug, multiplication of *M. leprae* occurred at the normal rate.

Studies of various dietary concentrations of two of these agents (5,6-dimethyl-2, 4-diaminoquinazoline (SRI 68) and 5-methyl-6-*n*-propyl-2, 4-diaminoquinazoline (SRI 105) with a minimally effective dietary concentration of DDS (0.0001%), using the kinetic technic, have been completed. During drug administration both quinazolines, with DDS, acted synergistically; the number of *M. leprae* decreased, suggesting bactericidal activity. In general, lower concentrations of quinazoline were more active than higher concentrations. Following discontinuation of SRI 105 plus DDS, delay in resumption of multiplication did not occur. However, there was no multiplication of *M. leprae* for at least 45 days following the discontinuation of one concentration of SRI 68 plus DDS. These results suggest that SRI 105, together with DDS, is bacteriostatic, while SRI 68 combined with DDS has bactericidal potential. To quantitate the actual lethal potential of SRI 68 and SRI 105 (0.001% and 0.003%) alone and in combination with DDS (0.0001%), the proportional

bactericide technic (I.R.C.S. Med. Sci. 2: 1037) was employed. Using this method mice were inoculated in the foot pads with  $10^4$ ,  $10^3$ ,  $10^2$ , and  $10^1$  *M. leprae* and fed a drug-containing diet for the next 60 days; *M. leprae* were counted one year after the completion of therapy and the percentage of organisms killed was calculated by a most probable number calculation. SRI 68 and SRI 105 together with dapsone were 89% to 96% bactericidal, while the quinazoline alone was not bactericidal at either concentration. Unexpectedly, the very low dietary concentration of dapsone was similarly bactericidal to the combined regimen, and hence synergism could not be demonstrated.

Two other quinazolines were studied alone at dietary concentrations of 0.003% and in combination with 0.0001% DDS in the mouse foot pad by the previously described kinetic technic of Shepard. One of these was provided by Rozman, 2,4-diamino-6-(2-naphthylsulfonyl)quinazoline, and is in phase 2 IND studies (personal communication, 1975). The other is one of a series synthesized by Hynes, 2,4-diamino-6[2-(3,4-dichlorophenyl)acetamido]quinazoline ( $H_1$ ). This compound was previously found active *in vitro* against *Cryptococcus neoformans* (Proc. Soc. Exp. Biol. Med. 151:173) and *Candida albicans* (Proc. Soc. Exp. Biol. Med. 153:230) (acute i.p. toxicity in mice > 500 mg/kg (Hynes, personal communication, 1977). Both appeared synergistic with dapsone;  $H_1$  (in combination with dapsone), the only one of the two where there were enough animals remaining to quantitate the growth delay following cessation of therapy, caused a growth delay of 100 days. Such growth delay suggests that  $H_1$  together with dapsone is even more bactericidal than the previously studied quinazolines.—[Leprosy Research Unit, PHS Hospital, San Francisco, California 94118]

**Colston, M. J., Ellard, G. A., Pattyn, S. R. and Hilson, G. R. F.** The activity of ethionamide and prothionamide in the chemotherapy of experimental leprosy.

Several recent reports have emphasized the growing problem of dapsone resistance in leprosy and the need to use combinations of two or more drugs in order to decrease the rate of relapse due to drug-resistant *M. leprae*. For the treatment of previously un-

treated patients, one of the drugs given must be dapsone, while the most widely recommended companion drugs are rifampicin and clofazimine. The use of rifampicin, however, is severely limited by its high cost, while many patients find clofazimine unacceptable because of skin discoloration. Thus there is a requirement for other effective antileprosy agents to be used in combination with dapsone in the initial treatment of lepromatous patients, and to be used in combination with other drugs in the treatment of dapsone-resistant patients.

Although ethionamide ([ $\alpha$ -ethyl] thisonicotinic acid) has been shown to be active against *M. leprae* in the mouse foot pad, its MIC has not been determined. This paper describes the determination of the minimum inhibitory and minimum bactericidal concentrations of ethionamide and of its propyl analogue, prothionamide, against *M. leprae* using the mouse foot pad technic. The minimum effective dose (MED) of ethionamide against three strains of *M. leprae* was determined as 0.01%. The MED of prothionamide against two strains of *M. leprae* was determined as 0.01%, and against a third strain 0.03%. The bactericidal activity of both compounds against *M. leprae* was assessed. Using the "kinetic" technic, the compounds were found to be purely bacteriostatic at 0.03%, but at 0.1% a significant degree of bactericidal activity was observed. Using the "proportional bactericidal test," both compounds were found to exert an identical bactericidal effect with 45 days administration of 0.1% producing a 98.6% killing effect. The serum levels in mice of ethionamide and prothionamide were determined using a radioactive assay procedure. There was no significant difference between serum concentrations of ethionamide and those of prothionamide. Mouse serum levels were proportional to dietary concentrations, with mice fed on 0.2% drug having a drug serum concentration of approximately 1.0  $\mu$ g/ml. This gives a value for the MIC of ethionamide and prothionamide of approximately 0.1  $\mu$ g/ml, with both compounds showing significant bactericidal activity at serum concentration of approximately 0.5  $\mu$ g/ml. Peak serum levels in man produced by well tolerated doses of ethionamide and prothionamide (500-750 mg per day) are reported as 3-5  $\mu$ g/ml, suggesting that peak serum levels exceed the MIC against *M. lep-*

rae by a factor of 30, and that bactericidal serum levels are maintained for at least part of the 24 hour cycle. These values were compared with the values obtained for other antileprosy agents.

Finally, because of several reports of cross-resistance of *M. tuberculosis* to ethionamide and thiacetazone, two strains of *M. leprae* obtained from patients who had relapsed during treatment with ethionamide were screened for sensitivity to ethionamide, thiacetazone and thiambutosine. Both strains were found to grow in the presence of all three drugs, suggesting that ethionamide-resistant strains of *M. leprae* are also resistant to thiambutosine and thiacetazone.—[St. George's Hospital Medical School, London SW17 0QT; Royal Postgraduate Medical School, London W12 0HS; Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium]

**Goucher, C. R., Peters, J. H., Gordon, G. R., Murray, J. F., Jr., Ichikawa, W., Welch, T. M. and Gelber, R. H.** Chemical and bacteriological assays of rifampin, rifampin-quinone, and desacetyl-rifampin.

Rifampin (rifampicin, RFM) is a semisynthetic antibacterial agent that is active against a wide variety of microorganisms including *Mycobacterium tuberculosis* and *M. leprae*. RFM exerts its biologic activity by inhibiting the DNA-dependent RNA polymerases of bacteria. Mammalian RNA polymerases are not affected by this drug. The potent inhibitory action of RFM against the growth of gram-positive bacteria can be used as a basis for bioassay techniques (Arzneim.-Forsch. **12a**, p 1907, 1971; J. Clin. Pathol. **27**, p 457, 1974). This bioassay, however, when applied to biological samples that have not previously undergone chemical separation, is not capable of distinguishing RFM from its active metabolites. The biologic activity of RFM metabolites has not been carefully quantitated, although RFM-quinone and desacetyl RFM (DARFM) both prevent bacterial growth. The analysis of RFM and DARFM by spectrophotometric methods is limited by the close similarity of their absorption spectra, as well as their modest molar absorptivities (Chest. **61**, p 526, 1972).

Murray *et al* (The Pharmacologist **17**, p 266, 1975), using high pressure liquid chrom-

atography (HPLC), successfully separated RFM and DARFM. In this chemical analysis, samples are treated with ascorbic acid to reduce RFM-quinone or DARFM-quinone, if present, to the corresponding hydroquinones prior to analysis. This chemical analysis proved both rapid and specific. We set up and tested the plate-diffusion bioassay of Dickinson *et al* (J. Clin. Pathol. **27**, p 457, 1974) for RFM. Regression analysis of the relationship between  $\log_{10}$  levels of RFM, RFM-quinone, and DARFM and the diameters of growth inhibition zones of *Staphylococcus aureus* yielded linearity from 0.03 to 2.0  $\mu\text{g}$  per ml. In concentrations exceeding 2  $\mu\text{g}$  per ml, zone diameters deviated from linearity. In this bioassay, RFM-quinone was equal in inhibitory activity to RFM, and the DARFM exhibited an activity one-fourth that of RFM and RFM-quinone.

To compare the methods, blind analyses were performed at the PHS Hospital (bioassay) and at SRI (chemical assay) of RFM samples (in 5% human albumin) prepared independently at the two institutions. Recoveries by both procedures on the groups of six samples ranging in concentration from 0 to 10  $\mu\text{g}$  RFM/ml were quantitative with the HPLC method, yielding a slightly higher mean (99.6% vs. 97.8%) and a narrower range (92 to 107% vs. 78 to 118%) than the bioassay. Results of applying both procedures to the assay of RFM levels in plasma of leprosy patients receiving RFM yielded a linear correlation ( $r=0.994$ ), clearly confirming the results obtained in prepared samples. Also, concurrent analyses of 11 plasma samples from rats receiving RFM yielded a linear correlation ( $r=0.918$ ) between the two methods of assay.—[This work was supported by NIH Grants AI 07801 and AI 08214. Leprosy Research Unit, PHS Hospital, San Francisco, California 94118, and Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025]

**Tsutsumi, S., Gidoh, S., Narita, M., Koide, S. and Funazu, T.** The characteristic anti-inflammatory effects of several antileprosy drugs.

The efficacy of DDS in various exudative but noninfectious diseases cannot be explained merely by the inhibition of folate synthesis. Recently, Katabira *et al* of the

Japanese dermatological field found a clue to the answer to this question in a marked inhibitory effect by DDS on carrageenin-induced acute rat edema (CARE). We have examined in more detail the anti-inflammatory effects of several representative anti-leprosy drugs by authentic pharmacodynamic test methods.

1. **CARE.** The inhibitory effect by DDS was clearly seen when the dose of DDS was greater than 4 mg/kg. In agreement with the report by Katabira *et al*, the effect of 10 mg DDS/kg was so marked that it was almost comparable to that seen with the same dosage of Indomethacin (IDM). However, when observations were continued for 24 hours, it was found that the inhibition of DDS rapidly decreased after 8 hours, in contrast to the inhibition of IDM. Katabira *et al* reported that after a single dose of 150 mg of DDS to humans the nitroblue tetrazolium test (NBT) was inhibited, but after a continuous seven days of administration of the same dosage it was enhanced. This relationship between the two experimental results suggested to us that in the earlier stage there was a stabilizing effect on macrophage (MØ) lysosomal membrane or an anti-exudative effect on venular intima, and in the later stage there was an accelerative effect on the NAD-NADH system inside the MØ. We named this double effect of DDS a tidal wave action.

Among other tested compounds, sulfadimethoxine and thalidomide exhibited a clearly detectable inhibition. However, when comparing these two, the inhibition by the former showed a peak at the bradykinin phase, in contrast with that by the latter which continued up to 24 hours. In the case of sulfamethoxy-pyridazine and promizole the inhibition was similar to that by sulfadimethoxine but lower. The effect of DDS in dosages of 10, 30, and 100 mg/kg was higher than that of the others at 100 mg/kg, except for IDM.

2. **Permeability skin test with the use of Pontamine Sky Blue 6B.** The venular permeability of pigmented guinea pigs or rabbits was tested by intracutaneous injection of several chemical mediators separately. The drugs tested were given by the oral route, but when histamine or serotonin was used as the chemical mediator, they were mixed together even in intracutaneous injections but in low concentrations. As a

result the following tendency was noticed.

a. The approximate order of the sensitivity of a chemical mediator to the inhibition by a drug was: bradykinin  $\gg$  serotonin  $>$  histamine.

b. The approximate order of drug inhibition on the permeability increase by a chemical mediator was: DDS  $>$  thalidomide, TV 1322  $>$  B663. In the case of TV 1322 the dose was 3 mg/kg of IDM-glutarate. The dosage of the other drugs was 100 mg/kg.

c. RFP enhanced permeability by the histamine test. Result (a) was coincident with both facts, i.e., the inhibition peak in the case of sulfonamide-type drugs in CARE was found at the bradykinin phase, and the chemical mediator playing the main role in CARE is bradykinin.

3. **Stabilization of rat erythrocyte membranes.** The fresh rat erythrocyte was used as a suitable and favorable model of lysosomal membrane. A definite heat and hypotonic shock was given to the erythrocyte. The hemolysis which occurred was markedly inhibited by TV 1322 in various concentrations. Thalidomide showed inhibition and minimum inhibition at 1/4000 M. B663 in high concentration weakly inhibited the hemolysis. DDS showed no inhibition.

4. **Whittle test.** The inhibitory effect of drugs on pigmental exudation into mouse peritoneal cavity was examined. In this test also, a marked effect by TV 1322 and a weak one by B663 were observed. Clindamycin enhanced the hemolysis. Thalidomide and DDS showed no inhibition.

5. **Mizushima's protein denaturation test.** Except for IDM, none of the tested drugs showed inhibition.

6. **CMC pouch method.** After four repeated experiments, it was concluded that DDS weakly inhibits the emigration of leukocytes into CMC pouch for four hours after the dose, a time when the DDS per 1 mg of protein in the exudate of the CMC pouch becomes maximum. Thalidomide inhibited the leukocyte emigration and TV 1322 did so more markedly.

7. **Adjuvant-induced arthritis (AIA).** More than ten rats (Sprague-Dawley, male, 150 gm or greater) per group were used. The results were as follows.

a. DDS markedly exacerbated AIA during the last week.

b. B663 markedly inhibited AIA, but it



was somewhat inferior to TV 1322 of the lower dosage.

c. Clotrimazole also showed a marked inhibition. These results suggested the following mechanism.

DDS: in view of characteristic inhibition of CARE by tissue accumulation after the repeated dosage, the tidal wave action of DDS may bring about some immunologic disorders especially of suppressor T cells. In the case of leprosy there may be a further increase of the tidal wave action by acceleration of MØ function through phagocytosis and pinocytosis of leprosy bacilli. Thus, immunologic disorders may be increased even though the drug has antibacterial activity.

B663: this drug may normalize the immunologic disorders caused by MØ function accelerated by leprosy bacilli, or by leprosy bacilli + DDS by decreasing the RES function.

Clotrimazole: the effect on AIA is a complicated problem. However, an effect on an early hemolysis stage, such as the myeloblast, must be considered.

**8. Neither DDS nor B663 showed an influence on the serum concentration of cyclic AMP.** In any event the pharmacodynamic activity of antileprosy drugs is only partial when compared to those of the usual steroidal or nonsteroidal anti-inflammatory drugs. The present findings indicate that in the future the pharmacodynamic activity of antileprosy drugs should be considered.—[National Institute for Leprosy Research, Tokyo, and National Leprosarium, Tama Zensho-en, Japan]

**Peters, J. H., Murray, J. F., Jr., Gordon, G. R. and Jacobson, R. R.** Metabolic-bacteriologic relationships in the chemotherapy of lepromatous patients with dapsone or dapsone-rifampin.

Combination chemotherapy has become increasingly important for the treatment of lepromatous leprosy because of the two problems of bacterial resistance and bacterial persistence during monotherapy. Dapsone (DDS) resistance has been reported in as many as 13% of lepromatous patients; persistence of *M. leprae* susceptible to DDS or rifampin (RFM) has been demonstrated in tissues of lepromatous patients receiving optimal monotherapy for many years (Gelber, *Int. J. Lepr.* **44** [1976] 369).

Previously, we reported that lepromatous patients receiving a combined DDS-RFM regimen exhibited marked and consistent decreases in levels of DDS and monoacetyl DDS (MADDS) in plasma, skin, and nerve compared with the levels found in these tissues in patients receiving DDS alone (*Fed. Proc.* **36** [1977] 996). Thus, we confirmed and extended the observations of Gelber and Rees (*Am. J. Trop. Med. Hyg.* **24** [1975] 963) that RFM increased the clearance of DDS from plasma. In other work, we found that RFM did not influence the binding of DDS and MADDS by plasma proteins.

In the current studies, we measured levels of DDS and MADDS in plasma and in two skin sites (exhibiting high and low degrees of bacterial infiltration) from 13 patients receiving 50 or 100 mg DDS daily. Four of those receiving 50 mg DDS alone were also studied following administration of 50 mg DDS with 600 mg RFM. Samples were collected four hours after administration. Extent of bacterial infiltration was measured and expressed as the Bacteriologic Index (BI). Levels of DDS and MADDS in the plasma and autoclaved skin specimens were determined by our published procedure (*J. Chromatog.* **107** [1975] 67). To eliminate possible differences due to varying water content of tissues, different doses of DDS, and different body weights of the patients, we measured protein levels of all samples and expressed the drug levels in units of ng of DDS or MADDS per mg of protein per mg of DDS administered per kg of body weight or ng/mg protein/dose.

Plasma levels of DDS in all patients ranged from 3.1 to 29.4 ng/mg of protein/dose. Levels of DDS in skin were comparable, ranging from 2.1 to 23.8 ng/mg of protein/dose. These values showed that DDS readily penetrated into skin sites of both high and low bacterial infiltration. MADDS levels in skin (0.2 to 8.7) were lower than those found in plasma (0.9 to 43.4).

Examination of the percentage change in BI, DDS, MADDS, and acetylation ( $\text{MADDS} \times 100 / \text{MADDS} + \text{DDS}$ ) values in skin sites of high and low bacterial infiltration in each patient indicated a direct relationship between the change in BI and changes in MADDS levels ( $r = 0.561$ ;  $p < 0.02$ ) and acetylation ( $r = 0.667$ ;  $p < 0.005$ ). Thus, a lower BI in a skin site was accompanied by a lower MADDS level and

lower acetylation. Alternatively, lower acetylation of DDS may also be interpreted as increased deacetylation of MADDs to DDS. No relationship between change in BI and DDS level was apparent ( $r = -0.245$ ; NS).

In those four patients, studied both on DDS alone and on DDS plus RFM, we found that RFM caused consistent decreases of DDS and MADDs in both plasma and skin. Also, RFM decreased the acetylation of DDS in all plasma samples and in seven of eight skin specimens. Separate evaluations of this group receiving both DDS and RFM showed that RFM did not alter the relationships, in skin, between change in the BI and changes in MADDs levels and acetylation noted for all patients. Measurement of total sulfones by hydrolysis in plasma of these four patients receiving DDS showed that DDS and MADDs were the only sulfones present, confirming earlier conclusions by us and others. However, hydrolyzed samples of plasma from these patients receiving DDS and RFM yielded increases in total sulfones ranging from 37% to 55% (mean, 53%) higher than the sum of DDS and MADDs measured before hydrolysis. The identity of the increased acid-hydrolyzable DDS in these patients has not yet been established.—[This work was supported in part by NIH Grant AI-08214, Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025; and Medical Department, PHS Hospital, Carville, Louisiana 70721]

**Jacobson, Robert R. and Hastings, Robert C.** Primary sulfone resistant leprosy.

Since 1972, routine mouse foot pad drug sensitivity testing has been performed on most new cases with borderline and lepromatous leprosy referred to the U.S. PHS Hospital in Carville, Louisiana. In the past all isolates of *Mycobacterium leprae* from new cases were fully sensitive to 0.0001% dapsones in the diet of the mouse; that is, no growth occurred. In the last five years however, bacilli from nine new cases have shown growth at this or higher levels of dapsones in the diet. We believe that this probably represents the first evidence of primary sulfone resistance. It is likely that this is a result of these patients having contracted their disease from a long-standing active case infected with sulfone resistant bacilli, although this has not been conclusively proven in any instance since their contacts are usually un-

known. Two of the cases also demonstrated some clinical evidence of sulfone resistance as did another case on whom mouse foot pad studies were not done prior to the start of treatment. Primary sulfone resistance represents a potentially serious problem of major proportion and the treatment of these cases poses a dilemma for both the physician and the patient.—[USPHS Hospital, Carville, Louisiana 70721]

**Collins, Frank M. and Morrison, Norman E.**

A mouse infection model of lepromatous leprosy.

*M. marinum* is an alternative model for studying the immune response to leprosy. Its low temperature optimum results in the selective infection of the foot pad even in intravenously infected mice. Unlike the systemic disease, the foot pad infection persisted for many months, eventually stabilizing at about  $10^3$  bacilli per pad. A  $10^8$  *M. marinum* inoculum was reduced by 99% in the liver and spleen within 30 days but viable bacilli persist in the foot pads and tail skin of the mice for up to 18 months. The infected mice did not develop DTH to PPD-S, PPD-Y or PPD-M, despite evidence of an early CMI response on the part of the host defenses.

Mice infected with  $10^5$ - $10^6$  viable *M. simiae* by the i.v., subcutaneous and aerogenic routes showed long persisting populations in the liver, spleen and lung for up to 18 months (the time limit of the experiment). No substantial change in viability up or down occurred when the i.v. route was used. There was no specific DTH at any time during the study. When  $10^6$  organisms were injected into one hind foot pad, there was an initial period of inactivation of the local inoculum, but eventually the foot pad and popliteal node counts stabilized and persisted at a low level for many months. Several thousand viable organisms were recovered from the foot pad 18 months later suggesting that if a local CMI response was responsible for the early decline in viability (beginning about day 30 and complete by day 50-60) this cellular response was unable to eliminate an established infection. Histologic examination of the foot pads and spleen showed minimal signs of inflammation and granuloma formation. The mice were unresponsive to PPD-Simiae and to PPD-S. Depending upon the inoculum size and the route of inoculation, the host response was more or less sup-

pressed. The larger inocula behaved as though they were nonimmunogenic for the mouse. The persisting *M. simiae* infection bears similarities to that of human lepromatous leprosy. Attempts to induce a persisting infection with *M. vaccae* and *M. nonchromogenicum* either by intravenous or subcutaneous inoculation were unsuccessful making it less likely that these organisms will induce an effective cross protection against *M. leprae*. Analogies between the *M. simiae* infection in mice and the corresponding *M. leprae* infections in man will be discussed.—[Trudeau Institute Inc., Saranac Lake, New York, and Johns Hopkins University, Baltimore, Maryland]

**Poulter, L. W. and Lefford, M. J. L.** The generation of cell-mediated immunity to subcutaneous *Mycobacterium lepraemurium* infection and its subversion by the systemic spread of infection.

Using a sonicated preparation of *M. lepraemurium* (*Mlm*) a significant increase in foot pad swelling was elicited in mice infected with *Mlm* five weeks previously. This foot pad reaction exhibited all the characteristics of a DTH reaction. As primary *Mlm* infection progresses, however, DTH reactivity is lost. It has been found that there is a temporal relationship between this loss of both DTH and resistance and the multiplication of the organism at the original subcutaneous site of inoculation. Further experiments showed that the development of DTH reactivity could be totally suppressed by deliberately infecting animals intravenously. This suppression was found to be dependent on systemic administration of *Mlm* rather than the overall host burden of mycobacteria. Splenectomy failed to reverse this effect and no humoral suppressive factors were detectable in the serum of these animals.

Comparisons were drawn between the deliberate suppression of DTH by intravenous inoculation and the natural suppression of DTH that occurs in the progressive stages of the disease. It emerged that the level of systemic antigen (rather than initial *Mlm*) may well be responsible for the subversion of DTH reactivity.—[Trudeau Institute, P.O. Box 59, Saranac Lake, New York 12983]

**Akiyama, Takehisa and Esashika, Ikuko.** Experimental murine leprosy: induction of

depressed cell-mediated immunity in ICR mice infected with large doses of murine leprosy bacilli.

Using the macrophage migration inhibition test, induction of a depressed state of cell-mediated immunity against not only infection-associated antigens but also an unrelated protein antigen was observed in mice of the rather resistant C57BL/6 and ICR strains, after inoculation with 10 to 100 times the conventional dose of murine leprosy bacilli. More interestingly, however, specific antibody formation in the ICR mice immunosuppressed with large doses of the bacilli seemed to be enhanced. Such animals possessing generalized depression of cell-mediated immunity and enhancement of specific antibody formation simultaneously appear to be invaluable as models of human lepromatous leprosy.

Furthermore it was shown that a marked restoration of cell-mediated immunoresponsiveness was brought about in the majority of ICR mice inoculated first with large doses of the bacilli, but followed in three to seven days with five successive daily injections of 0.5 KE OK-432, an inactivated and lyophilized preparation of a low virulence strain of *Streptococcus pyogenes*. These findings led us to consider a possible use of immunopotentiators such as OK-432 in adjunct immunotherapy of human leprosy. For details see: Akiyama, T. and Esashika, I. Experimental murine leprosy: induction of depressed cell-mediated immunity in ICR mice infected with large doses of murine leprosy bacilli and immunopotentiating action of OK-432. *Kitasato Med. J.* 7 (1977) (in press). —[Department of Microbiology, Kitasato University, School of Medicine, Sagami-hara-shi, Kanagawa-ken, Japan]

**Makino, M., Mori, T., Nyein, M.-M. and Ito, T.** Antigenic substances of *Mycobacterium lepraemurium*. 1. Isolation and purification of  $\alpha$ -antigen from *Mycobacterium lepraemurium*.

Ogawa *et al* succeeded in culturing *M. lepraemurium* on 1% egg yolk medium; Mori *et al* have confirmed the finding. It is possible now to obtain a large amount of *M. lepraemurium* without contamination with tissue components. We cultured a large amount of *M. lepraemurium* Hawaiian strain by this

method and obtained a cell extract from the bacilli by grinding in a mortar.

In this report we present evidence that from the cell extract of *M. lepraemurium* we could obtain antigenic substances and a material with  $\alpha$ -antigenicity to the rabbit. We named this antigenic substance as  $\alpha$  (Im). It migrated in SDS polyacrylamide electrophoresis as a clear single band. The molecular weight of  $\alpha$  (Im) was estimated to be between 25,000 to 35,000 by comigration with purified diphtheria toxin. The continuous absorption spectrum of  $\alpha$  (Im) in the ultraviolet region showed a maximal absorption at 275 nm. The evidence strongly suggested that  $\alpha$  (Im) is a highly purified single protein. Some characterization of  $\alpha$  (Im) has been accomplished.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka, Japan]

**Abe, Masahide and Yoshino, Yuji.** Antigenic specificity of *M. leprae* by indirect immunofluorescence.

The fluorescent leprosy antibody absorption (FLA-ABS) test has been developed as a new, simple and sensitive serological test for detecting anti-*M. leprae* antibodies in leprosy sera. In order to increase the specificity of this test two strains of mycobacteria, BCG and *M. vaccae* which gave the strongest cross-reaction with pooled serum of lepromatous patients, were used for the absorption of serum. Sonicated suspensions of these bacilli were added to the serum and the supernatant after centrifugation was diluted with phosphate-buffered saline containing cardiolipin and lecithin. The antibody titer to *M. leprae* did not drop significantly after this absorption, but no reaction with absorbed serum at a dilution of 1:40 or higher was observed with any other mycobacteria examined so far. Therefore, the serological specificity of *M. leprae* was also demonstrated by indirect immunofluorescence. This technic was applied to identify acid-fast bacilli grown in infected armadillos and it gave the same result as direct staining with anti-NE fluorescent antibody on most of the specimens sent from other laboratories.—[This study was supported by U.S.-Japan Cooperative Medical Science Program and by WHO IMMLEP Project. National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan]

**Shepard, Charles C.** The immunogenic competence of killed *M. leprae*.

At the Tokyo conference last year, we reported that the immunogenicity of *M. leprae* was not impaired when the organisms were killed by heat or phenol. In most experiments the immunogenicity was moderately, but significantly, increased when the immunizing suspension was heated to 100°C or 121°C. Most of the results reported then had to be with vaccination against infectious challenge with *M. leprae*, but the degree of protection was found to parallel the degree of enlargement in lymph nodes that drained the intradermal vaccine site. We also reported preliminary observations on the foot pad enlargement that followed the injection of  $1 \times 10^7$  *M. leprae* into the foot pads of intradermally immunized mice. The readings at 48 or 72 hours have been used to compare the potency of antigens.

In further experiments with foot pad enlargement we have observed that heat, up to 121°C, increased the potency of *M. leprae* somewhat, either as immunizing antigen or as eliciting antigen. When *M. leprae* was broken up (in the Mickle tissue disintegrator) the immunizing activity was lost, but the eliciting activity was not. Neither the cell wall fraction nor protoplasmic fraction was immunogenic. Both were active as eliciting antigen, but the cell wall fraction was more active. The effects of the diluent and the purification procedures have been studied in several experiments. Usually Hanks balanced salt solution has been a better diluent than phosphate buffered saline. The purification procedure in standard use in our laboratory involves a five minute treatment with trypsin (short trypsin procedure). A procedure in use in much of IMMLEP's work involves a 24-hour treatment with trypsin and yields a pure product. As judged with an eliciting antigen consisting of intact *M. leprae* purified by the short trypsin procedure, the *M. leprae* purified by the longer enzymatic treatment did not lose immunogenicity. The longer treatment appeared however, to decrease the activity of *M. leprae* somewhat as eliciting antigen.

A confounding factor is the inhibitory effect of doses larger than  $1 \times 10^7$  *M. leprae* as immunizing and eliciting antigen. Thus with  $1 \times 10^7$  *M. leprae* as immunizing antigen, the response to  $1 \times 10^7$ ,  $2 \times 10^7$ , and



$4 \times 10^7$  *M. leprae* as eliciting antigen increased with dosage. With  $4 \times 10^7$  *M. leprae* as immunizing antigen, however, the response to these amounts of eliciting antigen decreased with dosage. With  $2 \times 10^7$  *M. leprae*, the response was mixed. An analogous relationship held for varying amounts of immunizing antigen. Thus for more accurate work, it would be necessary to count the bacilli accurately and to employ at least two doses of antigen to determine the slope of the dose-response curve.—[Center for Disease Control, Atlanta, Georgia]

**Patel, P. J., Lefford, M. J. and Mackaness, G. B.** Induction of cell-mediated immunity to *Mycobacterium leprae*.

The immune response of mice to armadillo-derived, irradiation-killed *Mycobacterium leprae* (I-ML) was investigated. Following injection of 100  $\mu$ g I-ML into the left hind foot pad (LHFP) of mice, a state of cell-mediated immunity (CMI) was engendered to antigens of *M. leprae*. The evidence for CMI was as follows: a) development of delayed-type hypersensitivity (DTH) to both human tuberculin PPD and soluble *M. leprae* antigens, b) T-lymphocyte-dependent macrophage activation at the inoculation site, c) specific systemic resistance to the cross-reactive species *M. tuberculosis*, and d) immunopotentiality of the DTH response to an unrelated antigen. The CMI induced by I-ML in aqueous suspension was greater than that obtained with the same antigen in water-in-oil emulsion, even though the latter generated a more severe reaction at the site of immunization. I-ML also induced a stronger CMI response than the corresponding dose of heat-killed BCG.—[Trudeau Institute, Saranac Lake, New York 12983]

**Buchanan, Thomas M.** Immunochemical and structural integrity of protein antigens of mycobacteria during separation from armadillo liver antigens.

Accepted procedures for isolation of *Mycobacterium leprae* from armadillo liver tissue involve pronase in addition to collagenase, triton X-100 and EDTA washes. We utilized surface radiolabeled ( $^{125}$ I) and unlabeled *Mycobacterium smegmatis* mixed 5% by weight (lg) with radiolabeled ( $^{131}$ I) and unlabeled normal armadillo liver tissue (19g) to assess the effect of purification proce-

dures on mycobacterial antigens. The purification procedure of Draper produced significant separation of armadillo liver tissue from *M. smegmatis*. However, approximately 20% of the surface proteins of *M. smegmatis* were lost following triton X-100 EDTA washes, and about 17% of the radioactively tagged *M. smegmatis* proteins were lost to lower phase after collagenase treatment. These losses occurred with minimal effect on the antigenicity of residual surface proteins of *M. smegmatis*. Most significantly, the pronase treatment caused substantial loss in antigenicity and loss of nearly half of the radio-labeled proteins to the lower aqueous layer. Furthermore, sodium dodecyl sulfate polyacrylamide gels demonstrated almost complete destruction of labeled surface proteins. The normal pattern of three major proteins with approximately 60 to 120,000 subunit molecular weights was altered to only small molecular weight fragments with subunit sizes of approximately 6,000 to 14,000 daltons following pronase. For immunochemical analysis of potentially significant surface protein antigens of *M. leprae*, methods of purification of the leprosy bacillus from armadillo liver must be developed. Results from our studies of alternative purification procedures will be presented.—[University of Washington, Seattle, Washington]

**Bloom, Barry R. and Mehra, Vijay.** The induction of delayed-type hypersensitivity to human *M. leprae* in guinea pigs.

Guinea pigs were immunized with intact or disrupted armadillo-grown human *M. leprae* administered in aqueous or oil vehicles. In addition, comparisons were made of route of administration and the effect of BCG and water-soluble adjuvants on the degree of sensitization. For assessing delayed-type hypersensitivity, guinea pigs were tested with various dilutions of *M. leprae* suspended in saline, water-soluble *M. leprae* extract, PPD and a water-soluble extract of normal armadillo tissue. The results demonstrated:

1) Under no conditions was any skin test reactivity found to normal armadillo tissue extract.

2) Significant degree of sensitization to both *M. leprae* and its water-soluble extract was achieved by sensitizing guinea pigs with *M. leprae* suspended in Hanks solution or sa-

line. All suspension or emulsions were effective at sensitization, but appeared to be no better, and in general, slightly weaker than simple inoculation in aqueous suspension. In the guinea pig, the intradermal route appeared to engender slightly better reactivity than did inoculation in the foot pad.

3) Autoclaved *M. leprae* in Hanks or saline inoculated intradermally appeared to be the most effective immunogen. This protocol does not require an oil vehicle, appears to be relatively safe and should be considered for human sensitization studies.

4) Studies on sensitization of guinea pigs with a mixture of killed *M. leprae* and living BCG failed to demonstrate any significant adjuvant effect of BCG on sensitization to *M. leprae*. Indeed, there was some evidence of slightly decreased sensitization when *M. leprae* were given together with BCG. However, water-soluble cord factor appeared to potentiate significantly the sensitization to *M. leprae* in aqueous suspension.—[Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461]

**Hastings, Robert C. and Morales, Melvyn J.**

The effect of thalidomide on neutrophil function.

Earlier studies with thalidomide have indicated that the drug has, as one of its sites of action in *erythema nodosum leprosum* (ENL), a purely anti-inflammatory action. In these experiments with the carrageenan rat paw edema model, thalidomide inhibited edema formation in doses equivalent to those used clinically in patients with ENL. This anti-inflammatory activity was abolished in animals depleted of neutrophils by methotrexate pretreatment. Thus, thalidomide's activity in this model depended on neutrophils. We have now examined in more detail the action of thalidomide on neutrophils.

Thalidomide does not induce overt neutropenia clinically, therefore its action on neutrophils presumably involves functional changes. We have not been able to show any effect of thalidomide on either the classical or the alternate pathway activity of the complement system, therefore presumably the generation of chemotactic factors from complement proceeds normally in the presence of thalidomide. Thalidomide inhibits chemo-

taxis of human neutrophils *in vitro* in response to complement derived chemotactic factors from zymosan activated autologous serum. The drug does not have demonstrable activity in: a) an *in vitro* system of phagocytosis of opsonized zymosan particles, b) the release of lysosomal enzymes *in vitro* from intact human neutrophils during phagocytosis of zymosan, c) the release of lysosomal enzymes from isolated guinea pig peritoneal exudate neutrophilic granules *in vitro*, or d) the osmotic lysis of erythrocytes *in vitro* as a measure of nonspecific membrane effects. On the basis of data available to date, one of the relevant sites of action of thalidomide in ENL is in purely inflammatory events and involves the neutrophil. The drug appears to act at this site by inhibition of neutrophil chemotaxis.—[Pharmacology Research Department, USPHS Hospital, Carville, Louisiana]

**Namba, Masashi and Kobayashi, Shigenobu.** Bulbar palsy syndrome of leprosy due to cranial nerve involvement and its suppression.

Complications of the cranial nerves of leprosy are rarely observed in lepromatous and borderline cases. Such symptoms are liable to emerge and are severe in borderline cases in the subsided quiescent stage. Six cases have been reported. The typical syndrome is marked bulbar palsy together with other findings suggestive of VII, IX, X, XI cranial nerve involvement. Two cases were fatal despite all available treatment and were autopsied; the findings were reported in the past. The other four cases had good improvement. Though the reaction was usually well suppressed by dexamethasone and the symptoms improved moderately, some sensory and motor disturbance was irreversible. Irregular treatment or prompt increase of the drug dosage is undesirable, for it may give rise to a potentiation of the reaction in borderline cases. Other drugs such as salvarsan, iodine, chemotherapeutics and antibiotics have at times had the same effect. In all cases several prodromal signs were observed; among them neuralgia in cranial nerve areas was the most common.

General symptoms of borderline reaction should be looked for, such as reactivation of eruptions and enlargement of peripheral cranial and spinal nerves. The eruptions are

macular or serpiginous with red hue. The only way to overcome this medical emergency is by the withdrawal of all current therapeutics and suppression by corticosteroids and sophisticated supportive care. Though the emergence of this syndrome may be rare, it is sometimes fatal. We must study this severe host parasite interaction more, especially its treatment and prevention.—[National Institute for Leprosy Research, Tokyo, and National Sanatorium, Kryu Rakusen-en, Japan]

**Shannon, E. J., Miranda, R. O. and Hastings, R. C.** Inhibition of *de novo* antibody synthesis by thalidomide.

Thalidomide is highly effective clinically as an anti-inflammatory agent in a complication of leprosy called *erythema nodosum leprosum* (ENL) which is an Arthus type hypersensitivity reaction. As part of a study on the mechanism of action of thalidomide in ENL, the effect of the drug on *de novo* antibody (Ab) synthesis has been determined in locally-bred Swiss-Webster mice by the Jerne plaque technic and serological assays.

The primary antibody response as measured by splenic antibody forming cells, and hemagglutinin and hemolysin titers four days after i.v. injection of an ED50 dose of sheep erythrocytes (E) is almost exclusively IgM. Thalidomide significantly inhibited IgM antibody formation to this thymic dependent antigen in concentrations of 0.003%, 0.01%, and 0.03% w/w in powdered diet if fed for 5 or 7 days prior to immunization with E. As measured by <sup>14</sup>C thalidomide blood concentrations, these doses of thalidomide in mice are equivalent to therapeutic doses in humans. In leprosy patients being treated with thalidomide for ENL, there is a selective decrease in serum IgM concentrations by radial immunodiffusion compared to leprosy patients not receiving thalidomide. Since a depression in IgM synthesis was demonstrated we also attempted to elucidate the immunocompetent target cell(s) effected by this drug. Thalidomide in concentrations of 0.03%, 0.01% and 0.003% w/w in a seven day diet did not affect reticuloendothelial system clearance of colloidal carbon, in fact at the highest concentration (0.03%) there was enhanced clearance. Thus, thalidomide did not exert an effect on antigen clearance by macrophages.

Thalidomide did not influence the serological IgM response of BALB/c mice to pneumococcal type III polysaccharide—a thymic independent antigen. Thus, the target cell does not seem to be a B cell nor does thalidomide seem to nonspecifically inhibit synthesis of  $\mu$  chain immunoglobulins. One of the clinically relevant sites of action of thalidomide in ENL is to inhibit IgM type antibody synthesis. The drug's target site among the macrophage and antibody forming, helper, and suppressor immunocompetent cells remains to be elucidated.—[Pharmacology Research Department, USPHS Hospital, Carville, Louisiana]

**Kohsaka, K., Mori, T. and Ito, T.** Nude mice for research in leprosy.

At the last meeting in Tokyo, we reported (Kohsaka *et al.* Lepromatoid lesion developed in nude mice inoculated with *M. leprae*. Manuscripts of Joint Conference on Leprosy, U.S.-Japan Cooperative Medical Science Program, Tokyo, 1976; and La Lepro 45 [1976] 177-187) that lepromatoid lesions developed in nude mice inoculated with *M. leprae*, and the acid-fast bacilli obtained from the lesions were identified as *M. leprae*. The successful passage of *M. leprae* into the other nude mice was demonstrated by the experiment, and the reproducibility of transmission to nude mice was also shown.

Successive transmission of *M. leprae* was carried out three times by the foot pad technic with the organisms that proliferated in nude mice. *M. leprae* derived from five lepromatous patients were also inoculated into foot pads of the mice. Infected animals were maintained in vinyl (plastic) isolators under SPF conditions.

Macroscopic swelling of the infected foot pads of all animals was found at the tenth month after infection, and a lepromatoid lesion was seen at the site of the inoculation. At the same time the bacterial harvest was  $3.6 \times 10^8$  from a foot pad. The body temperatures of nude mice (BALB)/c-nu/nu and normal litter mates (BALB)/c-nu/+ were examined with an electronic thermometer, and no significant difference was found between the two. At the tenth month after inoculation, *M. leprae* were detected in the skin of low temperature parts of the body, but none were detected in the skin of higher temperature parts. *M. leprae* were seen in

the lung, liver and spleen but not in the kidney. *M. leprae* from all five different patients were successfully transmitted into foot pads of the nude mice. The maximum yield of *M. leprae* was  $1.1 \times 10^{10}$  from a foot pad at the eighth month after infection.—[Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan]

**Bullock, Ward E.** Evolution of suppressor cell populations in experimental mycobacterial infection.

Immunosuppressor activity of considerable potency and complexity is generated during the course of chronic, progressive infection of C3H/Anf mice by *M. lepraemurium*. From the 4th through 10th week, spleen cells from infected mice moderately suppress the direct plaque forming cell (PFC) response of normal spleen cell cultures to sheep red blood cells (SRBC). Suppression at this stage of infection is mediated by cells with the properties of macrophages. A marked increase in splenic suppressor activity at 10–11 weeks is associated with the appearance of a second suppressor cell sub-

population composed of T lymphocytes. Suppressor cells are not present in peripheral lymph nodes until terminal infection at 22–25 weeks.

Whole spleen cells from 16-week infected mice suppress both inductive and proliferative phases of the immune response to SRBC *in vitro* whereas a purified T-cell subpopulation suppresses only the T-cell dependent, inductive phase. Suppressor spleen cells depress the T-independent antibody response most severely, but there is also a direct effect upon B cells as shown by moderate suppression of responses to TNP-LPS and DNP-Ficoll. Cell-cell contact is required for maximal immunosuppression, however, suppressor spleen cells appear to generate a soluble suppressor factor(s) that induces depression of moderate severity. Of potential importance is the temporal association between the appearance of suppressor T cells in the spleens of infected mice at 10–11 weeks and an acceleration of clinical deterioration.—[Division of Infectious Diseases, Department of Medicine, University of Kentucky, College of Medicine, Lexington, Kentucky]