SECOND U.S. HANSEN'S DISEASE RESEARCH CONFERENCE

Baton Rouge, Louisiana 4–5 December 1986

PROGRAM 4 December 1986, Thursday

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ABSTRACTS SECOND U.S. HD RESEARCH CONFERENCE

Ell, S. R. Plague and leprosy in the Middle Ages: a paradoxical cross immunity?

In recent years, it has become well established that the diagnosis of leprosy in Europe became accurate by the 13th century, although only lepromatous leprosy was subject to the religious, legal, and social sanctions carried by that diagnosis. It is also clear that the incidence of leprosy began to decline in the 14th century. Since 1348 marked one of, if not the worst demographic disasters in European history, it has often been assumed that leprosy patients were killed off in such great numbers that leprosy began to decline as a result. This argument suggests persons in leprosaria as weak, malnourished, and ripe for any epidemic disease. A more systematic review of both medieval and modern (medical) evidence suggests that plague ought to have acted to increase the proportion of persons with leprosy and that leprosy's decline in Europe was well under way when the Black Death occurred

Anecdotal evidence from areas in which both plague and leprosy exist raises the possibility that leprosy confers at least partial immunity to plague. In Madagascar, for example, in this century a plague epidemic decimated the resident staff of a leprosarium without a casualty among the patients. Two more recent concepts help explain this. In the first place, many lepromatous leprosy patients mount exaggerated antibody responses to bacterial antigens. One organism for which this response has been well documented is Salmonella typhi, immunity to which confers immunity to plague. Secondly, plague victims are not typically malnourished or sickly. Yersinia pestis replicates very rapidly in humans, but is highly dependent on available host serum iron. For religious reasons, the diet of leprosy patients in the Middle Ages was one which would reliably lead to iron deficiency. Thus, there is a basis for the anecdotally observed immunity of some leprosy patients to plague.

It is also apparent that leprosy was already on the decline by 1300; that is a half century before plague struck Europe. The reasons for this decline are totally unknown. In 1300, half of the leprosaria beds in England were empty, having been full in 1200 when the population was smaller. In the midst of this decline, plague dropped population densities over 50% in many urban areas. To the extent that leprosy is a disease associated with overcrowding, plague backhandedly compromised the contagion of leprosy, while sparing persons suffering from the disease. At the same time, it is possible that healthy persons charged with provisioning leprosaria may have been killed off to a degree that many persons with leprosy died anyway.

This situation in which one disease confers immunity to a second, which nonetheless hastens the demise of the first, might plausibly be considered a paradoxical cross immunity.—[Department of Radiology, The University of Chicago Medical Center, Chicago, Illinois 60637]

Dhople, A. M. Thymidine uptake as an indicator of cell ability and *in vitro* growth of *M. leprae*.

The claim of the cultivation of any microorganism rests on two criteria: a) that there is definite unequivocal evidence of growth, and b) characterization of the cultivated organism identified as Mycobacterium leprae or undistinguishable from them. While there is no difficulty in establishing multiplication in fast-growing organisms, M. leprae presents problems not only because of its slow rate of multiplication but also because of its property of "clumping." In the absence of very evident growth, such as 100-fold multiplication, the criterion of assessing the multiplication of the organism by the existing methods as described by Hanks. Shepard and Rees is inadequate because it is extremely crude and does not take into consideration the "clumped" bacilli. Therefore, in addition to intracellular ATP, we decided to consider incorporation of labeled precursors of macromolecule as another sensitive criterion.

The significance of incorporation of ³H-thymidine by *M. leprae* cells has already been demonstrated by Khanolkar and also by Nath and co-workers. Since thymidine is a precursor of DNA but not RNA, its incorporation into bacterial DNA should represent a fundamental correlate (DNA synthesis) with viability of *M. leprae* and presumably provide an approach to evaluate at least the DNA-synthetic activity of this organism.

In order to use ³H-thymidine as an in-

dicator of bacterial viability and DNA synthesis, it is important to determine if the ³H-thymidine gets incorporated into bacterial DNA. For this purpose, three types of experiments were carried out: a) direct assay of 3H-thymidine uptake by DNA isolated from pre-labeled M. leprae, b) assay of thymidine kinase in M. leprae, and c) assay of thymidine phosphorylase in M. leprae. In our studies, we have shown that 17% of the total ³H-thymidine uptake by whole cells is attributed to the incorporation into bacterial DNA and only 1% into RNA. This finding was further substantiated by demonstrating the presence of thymidine kinase and the absence of thymidine phosphorylase in cell-free extracts of M. leprae. Similarly, in vitro-grown M. lepraemurium and M. lufu have the ability of 3 H-thymidine uptake, and possess thymidine kinase but not thymidine phosphorylase. On the other hand, uptake of ³H-thymidine by M. tuberculosis and M. avium was significantly lower, and both showed the presence of thymidine phosphorylase but not thymidine kinase. Finally, in a given culture medium for M. leprae, an excellent correlation has been demonstrated between intracellular ATP, bacterial DNA, ³H-thymidine uptake, and viability of M. leprae by the mouse foot pad technique.-[Medical Research Institute, Florida Institute of Technology, Melbourne, FL 32904]

Acknowledgment. This work was supported by the German Leprosy Relief Association, Würzburg, Federal Republic of Germany.

Franzblau, S. G. and Harris, E. B. Metabolic analyses of *M. leprae*: use in evaluation of *in vitro* culture media and drug activity.

The suitability of a number of (previously described) *in vitro* culture media for *Mycobacterium leprae* was determined by analysis of bacillary intracellular ATP. Values were determined at time intervals and compared to a Dubos-albumin control medium (DA). The rates of ATP decay in *M. leprae* incubated in Nakamura's NH-10 medium, Veeraghavan's V(L) medium, Dhople-Hanks medium, two "conditioned" media (derived from nerve cell supernatants) sim-

ilar to Mahadavan's, and three modified Sigma serum-free cell culture media were all more rapid than that obtained in DA medium. DA was thus employed as a basal medium in an in vitro system for screening for antileprosy drugs using ATP analysis. Three of four commonly used antileprosy drugs, as well as established antimicrobial agents acting on cell wall, nucleic acid and protein synthesis, and membrane function resulted in significantly accelerated decay of ATP compared to drug-free controls. A number of metabolic analyses are being employed in parallel to determine optimal cultural parameters for M. leprae: ATP content, uptake and incorporation of ¹⁴C-palmitate into phenolic glycolipid-I, uptake of ³H-adenosine and ³H-amino acids, uptake and oxidation of 14C glucose, and FDA/EB viability staining.-[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgment. This work was supported, in part, by NIH grant no. AI22492.

Harris, E. B., Franzblau, S. G. and Hastings, R. C. *In vitro* studies of phenolic glycolipid-I/¹⁴C-palmitate interaction and *Mycobacterium leprae* metabolism.

The metabolic activity of Mycobacterium leprae in an in vitro system was examined by utilizing the presence in M. leprae of a specific phenolic glycolipid (PGL-I). Bacteria derived from experimentally infected armadillo tissue and nude mouse foot pads were suspended in Dubos-albumin medium and incubated in the presence of ¹⁴C-palmitic acid. Using established procedures, total lipids were extracted from the reaction mixtures and PGL-I separated by column and thin-layer chromatography (TLC). The PGL-I fractions were recovered from the TLC plates and transferred to scintillation vials for radioactivity determination. The incorporation of 14C from palmitic acid into the PGL-I fraction increased linearly for 14 days, and was shown to be both pH and temperature sensitive. It was also observed that incubation under increased CO₂ tension markedly stimulated 14C incorporation into PGL-I. Various antileprosy agents were investigated for their effects on PGL-I synthesis using this system. The effectiveness of the drugs was reflected in the amount of ¹⁴C from palmitate incorporated into the PGL-I fraction. The data gathered from this study suggest a system for assessing the metabolic activity of *M. leprae* exposed to various experimental conditions.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgments. This work was supported, in part, by NIH grant no. AI22492 and NIAID Interagency Agreement no. Y01-AI-60001.

Foster, R., Sanchez, A., Lau, B. H. S., Small, C. S., Stuyvesant, W., Baldwin, B. and Foster, F. The effect of diet on the growth of *M. leprae* in mouse foot pads.

Diet was one of the earliest environmental factors suspected in the etiology of Hansen's disease. There has been recent interest in studying the possible association of diet with the disease. This study evaluates the possible role of diet affecting the growth of *Mycobacterium leprae* in the foot pads of mice on diets differing in fat and protein content.

Five semipurified diets contained 2% fiber, 1% vitamins, and 5% minerals. One diet contained dried pork (high fat); another dried, smooth-skinned, fresh-water barbel fish (low fat). These two diets were simulated in levels of fat and protein, using soy protein and soy oil (see table, soy "pork" and soy "fish"). One diet was formulated to contain low levels in both fat and protein content (lo-lo). One hundred ten BALB/c male, weanling mice were randomly divided into five groups of 21 mice each, and fed these different diets ad libitum. On day 10 of the diet, 1500 mouse-passaged M. leprae (obtained from Centers for Disease Control, Atlanta, Georgia) were inoculated into both hindfoot pads using a modified Shepard technique. The mice were individually identified and weighed periodically. Beginning at the third month, five mice/group were sacrificed monthly until the sixth month. Foot-pad harvests and smears were performed using Shepard's method on one hindfoot pad; the other foot pad was used for histology. Differences in the smear bacterial counts among diet groups increased

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with time. The six-month counts are shown below.

Diet	% Fat	% Protein	Mean (±5.0) AFB/ml
Pork	26.9	20.2	$8.49 (\pm 6.19) \times 10^{5}$
Soy "pork"	27.1	23.3	$6.41 (\pm 5.83) \times 10^{5}$
Barbel fish	5.6	31.0	$2.57(\pm 2.91) \times 10^{5}$
Soy "fish"	4.4	34.5	$2.54(\pm 1.78) \times 10^{5}$
Soy (lo-lo)	2.7	12.7	$7.13 (\pm 5.81) \times 10^{5}$

Microscopic study of the mouse foot pads failed to show any significant changes or acid-fast bacteria (AFB). No significant difference in AFB smear counts was obtained due to protein source (fish or pork) in this study. Therefore, diets with similar fat levels were combined. Significant differences (p =0.023) were observed between the high-fat (pork and soy "pork") and low-fat (fish and soy "fish") diet groups. The low-protein and low-fat diet (lo-lo) resulted in high bacterial counts, perhaps due to protein deficiency in the early stages of development of the mouse host.

These data suggest that high dietary fat or low dietary protein may influence the progression of Hansen's disease once it has been established in the host. More study is urgently needed to define the possible role of diet in Hansen's disease.—[Programs in International Health and Nutrition, School of Health, and Departments of Microbiology and Pathology, School of Medicine, Loma Linda University, Loma Linda, CA 92350]

Acknowledgments. This study was supported in part by the Leprosy Research Foundation and the Loma Linda Pathology Group, Inc.

Ohashi, D. K. The identification and characterization of acid-fast bacilli cultured on hyaluronic acid media and an evaluation of their etiologic role in leprosy.

Two strains of mycobacteria, Molokai-75 (MO-75) and Hawaii-75 (HI-75), were cultured from the tissues of Hansen's disease patients by Skinsnes who alleged them to be *Mycobacterium leprae*. These strains were examined and characterized by a variety of methods. They are acid-fast, pleomorphic bacilli which grow on Middlebrook and egg-based media. Both strains form typical my-

cobacterial microcolonies on Middlebrook medium. MO-75 develops mature colonies on Middlebrook 7H10 agar plates at 37°C at 5 weeks; HI-75 develops mature colonies at 3 to 4 weeks. MO-75 produces a lightvellow pigment and is a nonphotochromogenic strain; HI-75 develops a deep yellow pigment and is a scotochromogenic strain. In approximately 60 standardized determinative tests to evaluate phenotypic characteristics, MO-75 was found to be most similar to M. intracellulare, and HI-75 most similar to M. scrofulaceum. In Schaefer antisera, MO-75 agglutinates in type 7 sera; HI-75, in type 41 sera. Each strain differs in its susceptibility to different antibiotics. and neither is susceptible to low doses of dapsone or clofazamine. Examination of these strains by crossed immunoelectrophoresis (CIEP) using rabbit sera raised to killed whole-cell sonicates indicated that the strains did not share common antigens. Normal human sera and sera from lepromatous patients, incorporated in the intermediate gel of the CIEP systems, reacted with most antigens of both strains. CIEP analyses indicated MO-75 was more similar to an accepted strain of M. intracellulare than HI-75, and HI-75 was more similar to an accepted strain of M. scrofulaceum. In experiments to stimulate growth of these strains, MO-75 responded more to sulfhydryl-containing molecules contained in the media than did HI-75. Thin-layer chromatography of the type-specific C-mycosides demonstrated different mobilities for MO-75 and HI-75.

It was concluded that these strains are not identical to each other, as might be expected if they were the isolated form of M. leprae. As a result of this research, MO-75 and HI-75 are the best characterized of any mycobacteria isolated from lepromas. MO-75 and HI-75 resemble other descriptions of slowly growing scotochromogenic mycobacteria reported from lepromas based on rate of growth and pigmentation. The use of mycobacterial taxonomy to identify leprosyderived mycobacteria (LDS) is demonstrated. Characterization of LDS by methods such as these may eventually clarify the pathological significance of mycobacteria isolated from lepromas.-[Walter Reed Army Medical Center, Washington, D.C. 20307]

Khera, V. R. and Mahadevan, P. R. Studies of *in vitro*-cultivated isolates of *Mycobacterium leprae* from nodules of lepromatous leprosy patients.

Sixty-five isolates of Mycobacterium leprae were harvested from nodules of lepromatous leprosy patients. These isolates, cultivated in the conditioned medium obtained from mouse dorsal root ganglion cultures, were similar bacteriologically, biochemically, and immunologically during the first 3 to 4 transfers to the well-characterized strains of M. leprae. These in vitro-cultivated isolates, however, deviated from M. *leprae*-specific characteristics in their later transfers in the conditioned medium, but regained their M. leprae-specific characteristics after one in vivo cycle through the mouse foot pads (MFP). The regained characteristics were once again lost after 3 to 4 in vitro transfers. The acid-fast leprosy bacilli harvested from dapsone- or rifampinresistant patients also elicited growth in the conditioned medium containing the same dose(s) of dapsone (25 μ g/ml) or rifampin (4 μ g/ml). Likewise, these isolates demonstrated growth in MFP in drug-fed mice. All of the in vitro-cultivated isolates differed from atypical mycobacteria on the basis of mycolate profiles, protein patterns, growth and biochemical characteristics, and exhibited a Mitsuda response similar to M. leprae. Further genetic characterization of these isolates is in progress.-[Baylor College of Medicine, Houston, TX 77030; Foundation for Medical Research, Bombay, India]

Williams, D. L. and Gillis, T. P. Expression of recombinant 65 kD protein of *M. leprae* using the cloning vector pKK223-3.

Mycobacterium leprae protein antigens for the most part have remained either undefined, as to their immunogenic potential, or have been studied indirectly with immunologic reagents, such as monoclonal antibodies and T-cell clones. The production of *M. leprae* proteins as recombinant products has established a new source of protein antigens for study. Production of these recombinant proteins in a suitable expression system should provide sufficient quantities of native proteins and polypeptides for direct immunogenicity/antigenicity studies. Preliminary results of 65 kD protein production in Escherichia coli Y1089 recombinant lysogens (lysogenized with lambda gt11 clone Y3178 encoding 65 kD protein) indicated that the 65 kD protein was not produced as a fusion protein under the control of the lac Z promotor. Small but detectable amounts were observed by immunoblotting using monoclonal antibodies. In an attempt to increase the yield of 65 kD, we produced transformants in E. coli JM 105 using the recombinant plasmid pKK223-3 containing either a 3.6 kb EcoR1 restriction fragment from clone Y3178 (pKK223-3-3.6) or a 2.9 kb NruI restriction fragment (pKK223-3-2.9) derived from the purified 3.6 kb fragment. Transformants produced with the pKK223-3-3.6 plasmid showed increased 65 kD production as compared to recombinant lysogens. However, all transformants screened showed no enhancement of 65 kD production in the presence of IPTG, suggesting the absence of a fusion protein product. Further immunologic screening and DNA analysis of other transformants including pKK223-3-2.9 is currently in progress to test the feasibility of using "highlevel" expression vectors, such as pKK223-3, for enhanced production of 65 kD protein and other M. leprae proteins.-[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Clark-Curtiss, J. E. A molecular approach to epidemiological studies of leprosy.

The inability to cultivate Mycobacterium leprae by conventional laboratory techniques has made study of this organism's genetic and physiological capabilities extremely difficult. Moreover, there are presently no means to determine the extent of relatedness or divergence among M. leprae strains isolated from human patients in different parts of the world, nor is there much information regarding the relatedness of M. leprae to the armadillo-derived mycobacteria (ADM) described by Portaels, et al. (Ann. Inst. Pasteur 136A: 181) or to the M. *leprae*-like organism that causes leprosy in the mangabey monkey. Hybridization experiments between total chromosomal DNAs from human-derived M. leprae and M. leprae isolated from naturally infected armadillos have been reported to demonstrate essentially complete homology be-

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tween these organisms (Smith, *et al.*, Int. J. Lepr. **51**: 667).

Recombinant DNA technology has afforded a means to determine the functions of specific *M. leprae* genes by inserting fragments of the *M. leprae* chromosome into plasmid or bacteriophage lambda vectors that can then be introduced into *Escherichia coli*. Since *E. coli* is readily cultivable and genetically well characterized, the cloned *M. leprae* genes can be identified by their ability to complement mutations in the *E. coli* host strain or by immunological screening using monoclonal antibodies to specific *M. leprae* antigenic determinants or using polyclonal patients' sera.

In order to establish the extent of relatedness or divergence among M. leprae strains, I have chosen ten recombinant molecules from among the plasmid libraries prepared in my laboratory to use as probes in restriction fragment length polymorphism (RFLP) analyses. RFLP analysis is a technique by which small differences in the DNA sequences of two organisms can be determined by changes in sizes of DNA fragments generated by restriction endonuclease digestion. The ten recombinant molecules used in the studies were chosen at random and range in size from 1 to 3.3 kilobases. The M. leprae insert DNA from each of these molecules was separated from the vector, radiolabeled, and used as a probe in Southern hybridizations with chromosomal DNA from human M. leprae from two experimentally infected armadillos, M. vaccae, M. lufu, several ADM strains, and M. leprae purified from a naturally infected armadillo. The chromosomal DNAs were totally digested with the PstI restriction endonuclease (in separate reactions) prior to separation by agarose gel electrophoresis. The *M. leprae* insert that specifies citrate synthase (Jacobs, et al., PNAS 83: 1926) hybridized to single *PstI* fragments of all chromosomal DNAs tested, but only with DNA from the human M. leprae strains and the strain from the naturally infected armadillo did the probe hybridize to the same size fragment. Two other probes hybridized to fragments of M. vaccae, M. lufu, and M. *leprae* DNA and one probe hybridized to M. lufu and M. leprae DNA. In all cases, hybridization to the M. vaccae and M. lufu

DNAs was significantly less than to M. leprae DNA, and the homologous fragments were different in size from those of M. leprae, indicating polymorphism with respect to the PstI restriction sites. The six other probes hybridized only to DNA from human M. leprae and from the naturally infected armadillo M. leprae. In all hybridizations thus far, the probes hybridized to the same size fragment from human M. leprae strains and from the naturally infected armadillo M. leprae, confirming the identity of the two strains. Thus, RFLP analysis will be extremely useful in establishing molecular relationships among the genomes of M. leprae from different parts of the world.-[Departments of Microbiology and Immunology and Biology, Washington University, St. Louis, MO 63130]

Acknowledgments. This research was supported by the Chemotherapy of Leprosy (THELEP) and the Immunology of Leprosy (IMMLEP) components of the United Nations Development Program/World Bank/ World Health Organization Special Programme for Research and Training in Tropical Diseases.

Hunter, S. W., Chatterjee, D., Cho, S.-N., McNeil, M., Gaylord, H., Fujiwara, T. and Brennan, P. J. The cell envelope of *Mycobacterium leprae*.

See abstract in Int. J. Lepr. **54** (1986) 727–729.

Anderson, D. C., Young, R. A. and Buchanan, T. M. Synthesis of epitopes of monoclonal antibodies to the 65 kD protein of *Mycobacterium leprae*.

Using the predicted sequence of the 65 kD protein of *Mycobacterium leprae* (Mehra, *et al.*, PNAS **83** (1986) 7013–7017), peptides have been synthesized containing the epitopes for 9 of 14 different monoclonal antibodies which recognize this protein. The shortest peptide which tightly binds the IIIC8 monoclonal is DPTGGMGGMDF-amide, which half-inhibits binding in an ELISA at 30 ng peptide. Deletion of the N-terminal residue destroys potency, and deletion of the C-terminal phe increases the amount needed for half-inhibition to 700 ng. Oxidation of both methionines de-

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creases potency by over 100-fold, suggesting that these may be critical residues for binding to the antibody. The shortest peptide which tightly binds the IIC8 monoclonal is YEDLLKAGV-amide, which half-inhibits in an ELISA at 35 ng. Deletion of the N-terminal tyr decreases potency tenfold, and deletion of the next N-terminal glu drops binding another fivefold. These antibody epitope sequences represent refinements of the longer published epitope sequences of ASDPTGGMGGMDF and GEYEDLLK-AGVADP. Interestingly, the monoclonal F67-2, which inhibits interaction of IIC8 with the 65 kD protein in cross-competition studies (Buchanan, et al., unpublished data) appears to share a very similar epitope with IIC8 with the minimal sequence DLLKAGV. We have also approximately located the epitopes for six other monoclonals. The binding sites for these antibodies also appear to be sequential rather than assembled. Synthetic peptides and their variants may also prove useful in finding and studying critical sequences unique to M. leprae, as well as those which stimulate proliferation of helper/inducer T cells, which may be useful as skin test reagents or in vaccines.-[University of Washington, Seattle, WA 98144; Whitehead Institute for Biomedical Research, Cambridge, MA 02142; GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgments. This work was supported in part by the Immunology of Leprosy Component of the UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases, the Rockefeller Foundation Program for Research on Great Neglected Diseases, and by PHS Grant no. AI-23982 from NIAID.

Modlin, R. L., Bloom, B. R., Nelson, E. E., Shen, J.-Y., Gunter, J. R. and Rea, T. H. *In situ* and *in vitro* characterization of T-helper lymphocytes in leprosy.

See abstract in Int. J. Lepr. 54 (1986) 733.

Mshana, R. N. and Nilsen, R. Leprosy neuropathy: cells expressing MHC class II antigens in the lesion.

Without neurological complications, leprosy would mainly be a skin disease of only cosmetic importance. The pathogenesis of leprosy neuropathy is not fully understood but is generally thought to be the consequence of an immunopathological reaction. By using monoclonal antibodies to cell surface antigens, we have analyzed the local immune reaction in leprosy neuropathy lesions. It was found that BT nerve lesions differed from LL nerve lesions in having significantly larger numbers of Leu-3+ (helper/inducer) and Tac+ (IL-2 receptor) T cells, B cells (Leu-14), Leu-2a+ (suppressor/cytotoxic T cells) and cells expressing transferrin receptors were similar in both groups. HLA-DR and HLA-DQ antigens were found on Schwann cells, lymphocytes, macrophages and endothelial cells. Leu-6+ dendritic cells suggestive of Langerhans' cells were also seen in the lesions. Multibacillary nerve lesions from patients with paucibacillary cutaneous leprosy lesions had the same immunological reaction as LL nerve lesions. We believe this to be the first instance in which MHC class II antigens have been shown on Schwann cells. Furthermore, our demonstration of Langerhans' cells in intraneural leprosy lesions suggests that the pathogenesis of leprosy neuropathy is a more complicated immunopathological reaction than currently thought.-[GWL Hansen's Disease Center, Carville, LA 70721; Armauer Hansen Research Institute, Addis Ababa, Ethiopia]

Mshana, R. N., Hastings, R. C. and Krahenbuhl, J. L. Macrophages infected *in vitro* with live mycobacteria fail to express Ia antigen after stimulation with lymphokine.

In vitro cultured C3H/HeJ mouse peritoneal adherent cells were infected with live or heat-killed (HK) Mycobacterium kansasii, M. phlei, or M. scrofulaceum. Infected or noninfected cells were incubated with lymphokines generated by stimulating rat spleen cells with ConA. Ia-expressing cells were counted after staining with monoclonal antibody to Ia^k and developed with FITC or Texas Red conjugated goat anti-mouse IgG. Live but not HK bacilli were found to suppress lymphokine-induced Ia expression. The suppression was dose-dependent, and could not be overcome by prolonged

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incubation with supraoptimal doses of lymphokines. The antigen-presenting capacity of infected cells was assessed by the lymphocyte stimulation test. Adherent cells infected with live bacilli were found to induce a stronger response than cells infected with HK bacilli. The difference was particularly strong at low antigen doses. The implications of these findings on the pathogenesis of disseminated mycobacterial infections are discussed.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgment. The work was supported by NIH grant no. AI22442.

Levis, W. R., Meeker, H. C., Schuller-Levis, G., Gillis, T. P., Brennan, P. J. and Zabriskie, J. Monitoring of leprosy patients for antibodies to mycobacterial protein, glycolipid, and lipopolysaccharide antigens.

Detection of IgM antibodies to the *My*cobacterium leprae-specific phenolic glycolipid-I (PGL-I) has been a major advance in the serodiagnosis of leprosy, and may also be useful for monitoring disease activity. It is known that reactivity of PGL-I occurs in about 2%–5% of a healthy U.S. population and even higher in leprosy-endemic and hyperendemic areas. Comparable serological progress for other *M. leprae* antigens may, therefore, provide a useful adjunct to testing for antibodies to PGL-I.

In this study, we demonstrate by Western immunoblot and a competition antibody binding assay that armadillo-derived *M. leprae* protein antigen shows seroreactivity in patients with active multibacillary leprosy. In addition, it was seen that antibodies to *M. tuberculosis* lipoarabinomannan (t-LAM) may be useful in the detection and serologic monitoring of leprosy.

Methods

ELISA for antibodies to PGL-I and t-LAM. PGL-I was incorporated into liposomes with sphingomyelin, cholesterol, and dicetyl phosphate. Control liposomes were made without PGL-I. After coating ($2.5 \mu g/$ PGL-I/ml), plates were washed, then blocked with phosphate-buffered saline plus 3% bovine serum albumin (PBS-BSA). Sera, diluted 1:20 in PBS-BSA, were added in duplicate to wells containing PGL-I liposomes and control liposomes. Plates were then washed, and goat anti-human IgM (or IgG) peroxidase conjugate was added. Plates were again washed, and substrate solution was added [1.8 mM 2.2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) plus 0.1 mM H₂O₂ in phosphate buffer]. The reaction was stopped and read at 405 nm. Results were expressed as $\Delta E = E(PGL-I)$ liposome coat) – E(control liposome coat).

M. tuberculosis t-LAM was dissolved in carbonate-bicarbonate buffer (2 μ g/ml). Plates were coated; one half with the t-LAM solution and one half with coating buffer alone. After coating, plates were washed and blocked with PBS-BSA. Sera diluted 1:20, 1:100, and 1:200 (for detection of IgA, IgM, and IgG, respectively) were added in duplicate to t-LAM-coated and uncoated wells. Plates were washed, and goat anti-human IgG (or IgM or IgA) peroxidase conjugate was added. Plates were again washed, and the substrate solution (1.8 mM ABTS plus 0.1 mM H_2O_2 in citrate phosphate buffer) was added. Results were expressed as $\Delta E =$ E(t-LAM-coated wells) - E(uncoated wells).

Western immunoblot. Lyophilized M. leprae preparations were suspended in PBS at 2 mg/ml. From this, 0.6 mg was mixed in 300 μ l of 2× sample buffer and loaded onto a 12.5% gel. The material was electrophoresed for 3 hr at 46 mA, and then transferred across to nitrocellulose paper for 1 hr at 1 A. The nitrocellulose paper was cut into strips, and each strip was incubated with the appropriate human serum (1:100 dilution) for 2 hr at room temperature. Following appropriate washes, the strips were placed in the enzyme-conjugated anti-human IgG for 1 hr, followed by the appropriate substrate/dye mixture.

65 kD Competition antibody binding assay (CABA). The CABA was developed using the murine monoclonal antibody (MAB) IVD8 which reacts with an *M. leprae*-specific epitope found on a cell wall-associated protein of *M. leprae* ($M_r = 65,000$). The assay is designed to detect antibody in human serum which effectively competes for the binding of MAB IVD8 to the *M. leprae*specific epitope. *M. leprae* cell wall material was coated on microtiter plates at a protein concentration of 10 µg/ml. Plates were washed, then blocked with PBS-BSA. Na iodine-¹²⁵-labeled IVD8 and either patient's serum (1:20) or dilutent (PBS-1% BSA) were added to individual wells in quadruplicate. Plates were washed, and 10% sodium do-decylsulfate was added to each well. After 30 min, 100 μ l was transferred from each well to vials for scintillation counting.

Results

Western immunoblot detected primarily multibacillary leprosy. Fifteen of 29 LL and 7 of 7 BL patients tested exhibited at least one strong band. Only 2 of 11 BT and 0 of 8 controls showed any reactivity. The immunoblot banding pattern of leprosy patients, as measured by the number of bands, correlated directly with both the bacterial index (BI) and the level of anti-PGL-I IgM (r = 0.5318, p < 0.01 and r = 0.5560, p < 0.01, respectively).

The 65 kD CABA was found to detect active multibacillary leprosy as opposed to paucibacillary disease. Nineteen of 59 multibacillary patients were positive, while all 15 paucibacillary patients were negative, as were 17 contacts and controls. Patients positive for the 65 kD CABA had a significantly higher BI, anti-PGL-I IgM, and anti-PGL-I IgG than those who were negative. Among leprosy patients, significant positive correlations were found between the BI and 65 kD seroreactivity, and anti-PGL-I IgM and 65 kD seroreactivity (r = 0.3728, p < 0.05and r = 0.5354, p < 0.01, respectively, by Spearman rank order correlation).

IgM antibodies to t-LAM were seen primarily in multibacillary patients. IgG appeared highest in multibacillary patients, but was also seen in many paucibacillary patients. Serum IgA to t-LAM was seen less frequently.

Anti-t-LAM IgM was found to correlate with the BI (r = 0.4698, p < 0.001) more strongly than anti-t-LAM IgG (r = 0.2926, p < 0.05). One symptomatic contact of an LL patient found to be positive for anti-PGL-I IgM also had elevated levels of antit-LAM IgM and IgG ($\Delta E = 0.36$, 0.49, and 0.95, respectively, for anti-PGL-I IgM, antit-LAM IgM, anti-t-LAM IgG). A control serum donor found to be consistently positive for anti-PGL-I IgM was negative for antibodies to t-LAM.

Conclusions

Determination of antibody levels to M. leprae protein antigens and mycobacterial lipopolysaccharide antigens may prove useful as an adjunct to PGL-I in contact screening and in monitoring the disease activity in leprosy patients. The relative roles of glycolipid, protein, and polysaccharide antigens in the pathogenesis of leprosy need to be individually explored. Quantitative differences in exposure to these antigens in concert may be important in the type of subsequent immune responses.-[Institute for Basic Research in Developmental Disabilities, Staten Island, NY 10314; GWL Hansen's Disease Center, Carville, LA 70721; Colorado State University, Fort Collins, CO 80523; The Rockefeller University, New York, NY 10021]

Meeker, H. C., Schuller-Levis, G., Fusco, F., Giardina-Becket, M., Sersen, E., Brennan, P. J. and Levis, W. R. Monitoring of leprosy patients with antibodies to *Mycobacterium leprae* phenolic glycolipid-I and a synthetic glycoconjugate.

Studies to date indicate that antibodies to Mycobacterium leprae phenolic glycolipid-I (PGL-I) have considerable value in helping to monitor disease activity in leprosy patients and may be useful to detect new cases among contacts of patients. By determining levels of anti-PGL-I antibodies sequentially in leprosy patients' sera, information may be obtained on the course of the disease and treatment. In addition, sequential monitoring for antibodies to PGL-I may yield insight into the role of these antibodies in leprosy. To this end, we have performed sequential analysis on leprosy patients' sera for IgM and IgG antibodies to PGL-I by ELISA. Synthetic PGL-I glycoconjugates have been produced for the purpose of serodiagnosis of leprosy. Since supplies of armadillo-derived PGL-I are limited, PGL-I glycoconjugates may be important for largescale studies. To evaluate the usefulness of PGL-I glycoconjugates for patient monitoring, we have performed ELISAs with both a native PGL-I and a PGL-I glycoconjugate, ND-BSA, which contains the terminal disaccharide of PGL-I covalently linked to bovine serum albumin (BSA).

Methods

ELISA for antibodies to PGL-I using native glycolipid. PGL-I was incorporated into liposomes with sphingomyelin, cholesterol, and dicetyl phosphate. Control liposomes were made without PGL-I. After coating $(2.5 \,\mu g \, PGL-I/ml)$, plates were washed, then blocked with phosphate-buffered saline (PBS) plus 3% bovine serum albumin (PBS-BSA). Sera, diluted 1:20 in PBS-BSA, were added in duplicate to wells containing PGL-I liposomes and control liposomes. Plates were then washed, and goat anti-human IgM (or IgG) peroxidase conjugate was added. Plates were again washed, and substrate solution was added [1.8 mM 2.2'-azino-di(3ethylbenzthiazoline-6-sulfonic acid) (ABTS) plus 0.1 mM H₂O₂ in phosphate buffer]. The reaction was stopped and read at 405 nm. Results were expressed as $\Delta E = E(PGL-I)$ liposome coat) – E(control liposome coat). Sera with very high titers of anti-PGL-I IgM were assayed at higher dilutions, and ΔE readings were corrected to 1:20 by multiplying by the dilution factor.

ELISA for antibodies to PGL-I using synthetic glycoconjugate (ND-BSA). Microtiter plates were coated with ND-BSA in carbonate-bicarbonate buffer at a concentration of 0.5 µg/ml carbohydrate. Control wells were coated with a corresponding concentration of BSA. After coating, the plates were washed, then blocked with PBS plus 1% BSA plus 0.05% Tween 20. Sera, diluted as described earlier, were added in duplicate to ND-BSA and control wells, followed by washing, and the addition of goat anti-human IgM (or IgG) peroxidase conjugate. Plates were again washed, then the substrate solution was added, and ΔE values calculated as previously described.

Results

Using the ELISA with native PGL-I in liposomes, sequential analysis of serum anti-PGL-I IgM in leprosy patients who were at some point considered positive for anti-PGL-I ($\Delta E > 0.10$, dilution of 1:20) confirmed that most patients experienced a decrease of anti-PGL-I IgM with treatment. Thirty-five of 46 anti-PGL-I IgM-positive patients had a lower final ΔE than initial ΔE , while 11 of 46 increased in ΔE . Only one patient exhibited a strong increase in anti-PGL-I IgM ($\Delta E = 0.36$ to $\Delta E = 0.91$). This patient was taken off prednisone 2 months before an initial increase in anti-PGL-I IgM was seen. Rifampin was increased from 300 mg/day to 600/day 2 months before another increase was seen.

A symptomatic contact of an LL patient was found to have a positive anti-PGL-I IgM. This contact was then given dapsone 50 mg/day. A sequential serum sample taken 11 months later showed this contact had become negative for anti-PGL-I IgM (initial $\Delta E = 0.36$, final $\Delta E = 0.06$). Sequential monitoring of several patients who developed erythema nodosum leprosum (ENL) showed a drop in anti-PGL-I IgM level with the onset of ENL prior to starting thalidomide treatment.

In a group of 23 patients assayed by both native PGL-I ELISA and ND-BSA, a significant correlation was found between the rate of change of anti-PGL-I IgM and the bacterial index (BI) when assayed by the native PGL-I ELISA (r = 0.503, p = 0.013), but no significant correlation was found by the ND-BSA ELISA (r = 0.242, p > 0.05).

Conclusions

We conclude that sequential monitoring of PGL-I antibody levels may be valuable for following disease activity in leprosy patients. The finding that the decrease in anti-PGL-I IgM is greater in high BI patients when assayed by the native PGL-I ELISA may reflect clearance of large amounts of PGL-I from highly active patients during the course of treatment. Further study will be necessary to investigate the discrepancy between the native PGL-I ELISA and the ND-BSA ELISA.—[Institute for Basic Research in Developmental Disabilities, Staten Island, NY 10314; Colorado State University, Fort Collins, CO 80523]

Chehl, S., Gillis, T. P., Job, C. K. and Hastings, R. C. Attempts to produce erythema nodosum leprosum in *M. leprae*-infected nude nu/nu mice with monoclonal antibodies.

Erythema nodosum leprosum (ENL) is characterized histologically by swelling and edema of the endothelium with intense neutrophilic granulocyte infiltration of vessel walls. This histologic pattern strongly suggests that one component of the developing ENL lesion is an Arthus-like reaction. Immune complexes containing mycobacterial antigens have been detected in patients experiencing ENL reactions, and some investigators have detected deposition of immunoglobulins and complement components at the site of ENL lesions. Animal models currently used in leprosy research do not demonstrate an ENL-like reaction during infection and disease progression. The Mycobacterium leprae-infected nude mouse constitutes an animal model similar to multibacillary disease in humans, and is appropriate for some immunologic studies concerning aspects of multibacillary Hansen's disease, including ENL. Accordingly, we have attempted to induce ENL in M. leprae-infected nude mice using IgG and IgM monoclonal antibodies to either PGL-I or the 65 kD protein of M. leprae.

M. leprae-infected nude mice were injected either with ascites containing monoclonal antibody (intravenous) or with hybridoma cells (intraperitoneal) secreting monoclonal antibody in situ. Animals treated by intravenous injection were observed at 4, 6, and 24 hr after injection for detection of gross ENL signs (e.g., whole body erythema, inflammation/ulceration of infected foot, conjunctivitis). Animals were sacrificed 24 hr after injection of monoclonals, and the M. leprae-infected foot was examined histologically. Both gross and histologic observations were no different than untreated control mice or mice injected with a monoclonal antibody reactive with an unrelated antigen.

Nude mice injected with hybridoma cells producing monoclonal antibodies (anti-PGL-I, anti-65 kD, anti-Neisseria gonorrhea pili) were observed daily over a 4-week period for gross changes associated with ENL. Gross and histopathological observations were negative for ENL-like reactions, and no significant changes in bacterial load were observed. These results indicated that the presence of large amounts of complement-fixing antibody (anti-PGL-I or anti-65 kD protein) in the circulation was not able to induce an ENL-like reaction in M. leprae-infected nude mice. It is possible, however, that neither PGL-I nor the 65 kD protein is found in immune complexes associated with ENL. Alternatively, sequestration of antigen in phagocytic cells may block potential antigen-antibody reactions at sites important for initiation of ENL through deposition of immune complexes. Additionally, other immunologic mechanisms (e.g., antigen-reactive T cells) could provide the missing component which may act either prior to or in concert with immune complexes to produce ENL. Further analyses of possible mechanisms of ENL induction in the nude mouse model are currently under investigation.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgment. The work was supported by grant no. AI-22492 from the National Institutes of Health.

Sibley, L. D. and Krahenbuhl, J. L. Lymphokine response of lepromatous granuloma macrophages and macrophages infected *in vitro* with *Mycobacterium leprae*.

See abstract in Int. J. Lepr. **54** (1986) 725–726.

Sibley, L. D. and Krahenbuhl, J. L. Electron microscopic study of the intracellular fate of *M. leprae* in normal and activated macrophages.

The inability of the mononuclear phagocyte to cope with the leprosy bacillus is an obvious characteristic of lepromatous leprosy. Mycobacterium leprae replicates within macrophages, reaching enormous numbers in the lepromatous granuloma. In parallel with other studies in our laboratory that are focused on the microbicidal effects of activated macrophages the present work examines the capability of the macrophage to digest M. leprae. Studies were carried out with the electron microscope to determine the intracellular fate of the leprosy bacillus in normal and activated macrophages containing thorotrast as a secondary lysosome tracer.

Suspensions of *M. leprae* were obtained from the foot pads of infected nu/nu mice. These suspensions were enriched for metabolically active and presumably more viable organisms, as determined by quantitation of ATP activity, by passage through a Percoll gradient. Thin and thick sections (50–60 nm and 200–300 nm, respectively) were prepared from infected macrophage

monolayers and examined at various times after infection. Intracellular M. leprae surrounded by a characteristic electron transparent zone were contained within membrane-bound phagosomes of macrophages cultured in vitro for 1 to 6 days. In normal macrophages, a majority of phagosomes containing freshly isolated live M. leprae resisted fusion with thorotrast-labeled lysosomes. The extent of fusion was not significantly affected by pre-treatment of M. leprae with patient sera containing high titers of anti-M. leprae antibodies. In contrast, a majority of phagosomes containing gamma-irradiated M. leprae underwent lysosome fusion in normal macrophages. In addition, increased lysosome fusion was observed in phagosomes containing live M. leprae in macrophages activated by incubation with recombinant interferon-gamma. Increased fusion was associated with an increase in the number of fragmented and damaged bacilli, suggesting that increased digestion followed fusion of the lysosome with the phagosome. These studies suggest that activated but not normal macrophages may have an increased capacity for clearance of the leprosy bacillus.-[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgments. The work was supported by grants from the National Institute of Allergy and Infectious Diseases, AI-22442 and AI-22492, and the U.S.-Japan Cooperative Medical Sciences Program.

Ramasesh, N., Franzblau, S. G. and Krahenbuhl, J. L. The *in vitro* microbicidal effects of activated macrophages on *M. leprae.*

The inability to quantitate the viability of *Mycobacterium leprae* has vastly impeded progress in leprosy research. To monitor *M. leprae* viability, titrations which employ the Shepard foot pad technique are used. Clearly, there is a strong need to develop alternate, more rapid techniques to determine the viability of *M. leprae* in order to assess the efficacy of chemotherapeutic regimens or to explore the effector mechanisms of host resistance to leprosy. In the present study, several procedures have been employed in an attempt to explore the microbicidal effects of activated macrophages on the leprosy bacillus.

Suspensions of M. leprae were obtained from the foot pads of athymic nude mice infected 10 to 16 months previously. Following homogenization, suspensions of live M. leprae were quantitated and diluted in tissue culture medium RPMI-1640 supplemented with 20% fetal calf serum. Normal macrophages were obtained from the peritoneal cavities of unstimulated BALB/c mice. Activated macrophages, known to possess a potent microbicidal capacity against a broad spectrum of phylogenetically unrelated pathogenic microorganisms, were obtained from mice injected with killed Corvnebacterium parvum. Seven days after an intravenous or intraperitoneal C. parvum injection, peritoneal cells were harvested, washed, and allowed to phagocytize M. leprae in suspension for 1 hr at a ratio of 20 bacteria per macrophage. After 2 hr to allow macrophage attachment, nonadherent cells and extracellular bacteria were removed by washing, and the plates were reincubated.

Fluorescein diacetate: ethidium bromide (FDA:EB) viability stain. Briefly, this technique involves staining with the nonfluorescent fatty acid ester fluorescein diacetate in combination with ethidium bromide. Viable bacteria with an intact cell membrane convert FDA to fluorescein by means of a nonspecific esterase, and appear green when viewed with a fluorescent microscope. Since ethidium bromide enters only cells with damaged membranes and stains the nucleic acids, nonviable M. leprae stain orange under the fluorescent microscope. After an additional 24 or 120 hr, the infected cells were scraped off, lysed by sonication, and the bacteria washed and stained with FDA:EB. Smears of bacteria were prepared and examined by fluorescent microscopy. In a typical experiment 60% to 70% of the M. leprae stained as viable at time 0, and this proportion remained virtually unchanged during 5 or more days in culture in normal macrophages. In contrast, activated macrophages had a marked effect on the FDA:EB staining pattern of M. leprae. However, no effects were observed during the first 24 hr of association of M. leprae with activated macrophages. By day 5, however, over 60% of the organisms from activated macrophages could be classified as dead. Not shown in any of these data is the quality of green or orange staining of M. leprae. Whereas the majority of the greenstained M. leprae isolated from the mouse foot pads at 0 hr were stained solidly green and remained so when maintained in normal macrophages, virtually all of the organisms graded as green and thus viable in activated macrophages were actually stained a very beaded green or were dual-stained. It is likely that the beaded nature of the green fragmented forms correlates with their being damaged and, thus, less viable than their solid green counterparts. These data are consistent with the FDA:EB staining procedure being applicable to quantitation of activated macrophage microbicidal effects on the leprosy bacillus. These findings are currently being corroborated by two other approaches.

Quantitation of PGL-I synthesis by M. leprae in macrophage cultures. Within the host cell, M. leprae synthesizes large amounts of a characteristic phenolic glycolipid (PGL-I) shown to be biochemically unique and immunologically specific for M. leprae. The present study explores the biosynthesis of PGL-I by the leprosy bacillus in tissue cultures of mouse peritoneal macrophages. PGL-I synthesis was monitored in vitro by measuring the incorporation of radio-labeled palmitic acid (14-C PA) into the extractable PGL-I fraction of M. lepraeinfected macrophages. Following the lysis of normal or activated macrophages challenged with M. leprae, the bacilli were counted, their glycolipids extracted with chloroform and methanol, and separated by thin-layer chromatography. The amount of 14-C PA incorporated into PGL-I was quantitated by liquid scintillation counting. The identity of labeled PGL-I was confirmed by chromatography and immunostaining on polysulfone paper. Formalinkilled M. leprae failed to synthesize PGL-I, and rifampin treatment prevented PGL-I synthesis. In preliminary studies, activated macrophages also inhibited PGL-I synthesis. Additional studies are under way.

Quantitation of M. leprae ATP activity. In parallel with the above two approaches for measuring the effects of normal and acti-

vated macrophages on the membrane integrity and metabolic activity of the leprosy bacillus, studies are being carried out to explore the relationship between the ATP activity of *M. leprae* released from macrophages and their viability.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Acknowledgments. The work was supported by U.S. Public Health Service Grant AI-22442 from the National Institute of Allergy and Infectious Diseases and by the U.S.-Japan Cooperative Medical Science Program.

Collins, F. M., Stokes, R. W. and Takashima, T. Chronic *M. avium*-complex infections in athymic (nude) and T-cell depleted (Thxb) mice.

Mycobacterium avium, M. intracellulare, and M. scrofulaceum are opportunistic human pathogens which normally induce selflimiting infections in immunocompetent adults. Under certain circumstances, they can give rise to a progressive systemic disease involving the lungs, spleen, and gutassociated lymphoid tissues (GALT), usually after the normal T-cell defenses have been compromised in some way (Hodgkin's disease, silicosis, AIDS). The role of the T cells in limiting the systemic spread of M. avium-complex infections was examined in susceptible (BALB/c) and resistant (A/J) strains of mice infected aerogenically, intravenously, subcutaneously, or intragastrically. Virulent strains of M. avium and M. intracellulare multiplied more extensively in the lungs and spleens of the susceptible BALB/c mice, regardless of the route of challenge. However, avirulent M. intracellulare and M. scrofulaceum were eliminated from the tissues of both susceptible and resistant mice with no sign of systemic spread even in irradiated or cyclosporin-Atreated animals. Oral challenge of normal susceptible mice with virulent strains of M. avium and M. intracellulare resulted in the induction of progressive systemic disease within 30 days. However, the related avirulent serotypes were unable to establish a stable GALT infection even after multiple challenges in germ-free mice.

Challenge experiments were also carried

out in athymic (nude), thymectomized, irradiated, bone-marrow reconstituted (Thxb), and normal BALB/c mice. T-cell depletion markedly enhanced the severity of both M. avium 724 and M. intracellulare 673 challenges, compared to those seen in the immunocompetent controls. The lung count in aerogenically challenged nude mice was almost 2000 times that seen in the normal controls, and all of the athymic mice succumbed to the challenge infection within 90 days. The controls survived for at least 6 months. T-cell depletion did not enhance the growth or survival of the avirulent M. intracellulare 1406 or M. scrofulaceum 1306 in vivo.

Acquired resistance to an aerogenic M. avium challenge was restored to syngeneic Thxb recipients by the infusion of immune T cells harvested from BCG, H37Rv or M. avium-vaccinated donors. Peak production of immune T cells in the M. avium-infected donors occurred substantially later (4 to 6 weeks) than in mice receiving equivalent numbers of BCG or H37Rv (2 to 3 weeks). However, cross-protection could also be detected when memory T cells were harvested from isoniazid plus rifampin-treated donors. These memory T cells responded to crossreactive as well as specific sensitins, and the role of these antigens in triggering the protective T-cell response in appropriately immunized donors is discussed.-[Trudeau Institute, Inc., Saranac Lake, NY 12983]

Breger, D. Correlating Semmes-Weinstein monofilament mappings with sensory nerve conduction parameters in Hansen's disease patients.

The Semmes-Weinstein monofilaments are used for evaluating sensibility, offering a means to determine relative thresholds of touch-pressure sensation. They both evaluate the level of cutaneous sensation to touch-pressure, and provide a mapping of the sensory distribution of the hands and forearms. Comparison of sensibility mappings with nerve conduction measurements affords an even more complete picture of peripheral nerve problems than either test alone. Functional integrity of sensory neurons can be demonstrated by sensory conduction latencies and amplitudes of evoked responses during nerve conduction testing.

The data presented in this paper are drawn from measurements of two population samples of Hansen's disease patients. The first patient group is from the former USPHS Hospital in San Francisco. Included in this group studied were 14 patients with involvement of 77 individual peripheral nerves, including the ulnar, median and radial nerves. Subsequent data were taken from a sample of 14 cases from the patient population at Gillis W. Long Hansen's Disease Center, Carville. In the two groups, patients measured had both Semmes-Weinstein monofilament mappings and sensory nerve conduction tests as part of their routine evaluation.

The results and correlations between the monofilament mappings and sensory nerve conduction velocities are discussed. Although there are essential differences in these two tests, when used together they present a clearer picture of a patient's sensory status than either test used alone.—[Rehabilitation Research Department, GWL Hansen's Disease Center, Carville, LA 70721]

Bell-Krotoski, J. Monitoring of patient neural status during drug therapy-preliminary study.

This is the first of several reports on a pilot study to monitor patient neural status during various drug therapies. It is believed that careful monitoring of peripheral nerve changes during specific drug therapy can establish a relationship between a drug and its effect on neural status which will prove effective in improving patient treatment, and in preventing neural damage.

In this first report, data from 150 patients (300 extremities) is reviewed for a comparison of peripheral nerve involvement in the Hansen's disease population sampled. The sample was drawn from patients as listed alphabetically on the patient roster at the Gillis W. Long Hansen's Disease Center. For comparison purposes, the patients were broken down into groups according to their disease classification and specific pharmacological therapy. Each of the patients treated was given a sensory and motor evaluation. In addition, determinations were made as to deformity and peripheral nerve status

by a screening form developed for use in monitoring patient upper extremity neural status. Testing was staggered over a 1-year period concurrent with the patient's annual hand screen. Fifty of the patients were retested at the end of 1 year. Results from the first and second tests are compared and discussed, seeking early correlations of peripheral nerve changes with disease classifications and specific pharmaceutical agents. The sensory and motor exams in this report are of the abbreviated type. Detailed mapping of patient peripheral nerve status was also done on many patients. In a future report the abbreviated testing will be compared with detailed sensory testing and a determination made on whether the abbreviated exams can be used as good predictors of peripheral nerve changes.-[Rehabilitation Research Department, GWL Hansen's Disease Center, Carville, LA 70721]

Look, J. O. Ulcer border reshaping in the treatment of neuropathic plantar ulcers.

Look reports on a study done in the Congo during 1980–1984, involving not less than 125 neuropathic plantar ulcers on not less than 50 Hansen's disease patients. Most of these patients lived in isolated areas where hospitalization was not available, and were treated as ambulatory outpatients. Treatment by the usual methods of debridement and bandaging had produced virtually no healing of the ulcers which were referred to by the nationals as "unhealable ulcers." Look studied the treatment logic necessary for successful ulcer therapy in ambulatory patients. Trophic ulcers exist because of ischemic pressure which obstructs the inflow of arterial blood to the affected area. Therefore, any method which promotes healing must allow blood supply to the wound margin.

Three elements were determined to be necessary for successful treatment in its simplest form: a) The reshaping of ulcer margins to form a 45° angle from the level of healthy skin to the floor of the ulcer; b) The use of elastic (Ace) bandages to achieve a pumping action during ambulation which was believed to maintain a more oxygenated blood in the wound margins; c) The use of cast boots as a basic protective footwear prior to the availability of better footwear.

Look believes that the success achieved from reshaping ulcer margins at a 45° angle is based on physical principles which are discussed. Ischemia in the wound margin is caused by dynamic or static deformation and compression of this tissue.

Deformation of the wound margin occurs principally from shear forces which act on the wound margin along a line parallel to the direction of the force. By bringing the slant of the margin more parallel to the direction of the shear force, the negative effect of the shear force is attenuated.

Compression of the wound margin is inversely proportional to the resistance to compression provided at the cellular level. If the ulcer has a perpendicular border, the resistance to compression is approximately half that if the ulcer has a border slanted at 45°.

The improved healing obtained by the 45° angle wound margin is based on logical physical principles. The more resistance there is to deformation and compression, the less ischemia there will be at the wound margin. Virtually all plantar ulcers in ambulatory outpatients were healed. The exceptions were patients who severely abused the affected limbs. – [Hansen's Disease Consultant to the Ministry of Health, People's Republic of Congo, 1973–1977 and 1980–1984]

Nawoczenski, D., Birke, J. and Coleman, W. Effect of rocker sole design on plantar forefoot pressures.

The rocker sole design has been shown to be an important pedorthic procedure for the prevention and management of the neuropathic foot lesions commonly seen in Hansen's disease and diabetes mellitus. Rocker soles have been shown to reduce pressure and shear stress on the forefoot during walking. Several different rocker designs have been described in the literature. These designs have differed in the placement, radius of curvature, and angle of the rocker.

The purpose of this study was to compare the effectiveness of six rocker sole designs in reducing forefoot pressure. Twenty normal subjects walked in shoes modified with the following rocker designs: (R1) 0% radius, 30° angle and metatarsal head placement; (R2) 60% radius and 50% placement; (R3) 75% radius and 50% placement; (R4) 75% radius and 60% placement; (R5) 125% radius and 60% placement. Radius and placement were determined by a percentage of length of individual shoes to normalize for shoe size differences. Capacitive transducers were used to measure the pressure at four sites of the left foot. Mean relative pressure was reduced in the R1, R2, and R3 rockers as compared to the other designs.

This study defines rocker designs currently used in the management of neuropathic foot problems and shows their relative effectiveness in reducing pressure on the forefoot during walking.—[Rehabilitation Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Theriot, S., Birke, J., Lawrence, K. and Dressel, L. Reliability of the biothesiometer in measuring plantar vibratory thresholds.

Vibratory sensory testing is commonly used to monitor neural status in patients with peripheral neuropathy. The tuning fork method has many sources for error, and is not sensitive enough to distinguish between minimal changes in neural function. The biothesiometer was developed to give the clinician an instrument that offers quick and quantitative measurement of vibratory thresholds.

The purpose of this study is to determine the inter- and intra-tester reliability of this instrument in measuring the vibratory threshold on the plantar surface of the foot in normal subjects and Hansen's disease patients.

Twenty normal subjects and 35 medically stable Hansen's disease patients, ranging in age from the 20s to the 80s, were randomly selected to participate in the study. Two testers independently took three measurements of vibratory threshold at the pulp of the great toe, the first metatarsal head, the fifth metatarsal head, and the center of the calcaneus. An average for each area was determined. This procedure was then repeated on three consecutive days by the same two testers.

Data were analyzed using interclass correlation coefficients and 2-way repeated measures ANOVA's. Vibratory threshold means and standard deviations for each of the four sites were reported in both populations. The biothesiometer was found to be more reliable in normal subjects than in a patient population. Nevertheless, it should prove to be a helpful tool in assessing changes in neural status and identifying patients who are at risk of ulcerations on the plantar surface of the foot.—[Rehabilitation Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Mukherjee, A., Walsh, G. P., Gormus, B. J. and Meyers, W. M. Ultrastructural changes in the blood vessels in dermal lesions of leprosy.

Blood vessels play an important role in the dissemination of Mycobacterium leprae throughout the body and also in the development of granulomatous lesions at various sites. Our earlier studies on the mediumsize subcutaneous veins have shown consistent venous involvement with bacilli and granulomas in the vein wall. We have subsequently carried out a study of the dermal microvasculature in and around skin granulomas in leprosy patients, infected armadillos, nude mice, and the mangabey monkey. This comparative ultrastructural study of the microvasculature reveals more frequent endothelial cell bacillation in leprosy patients than in the experimental animals. The ultrastructural features in the small vessels near the granulomas in bacillated (LL and BL) cases also suggest a regular movement of bacilli from intravascular to extravascular compartments. This process probably occurs through a) migration of the bacilli-laden monocytes through the vessel wall or b) picking up of bacilli by endothelial cells and transfer to the subjacent pericytes. The endothelial cells do not seem to respond to the bacilli contained within them. Bacilli with well-developed electron-transparent zones are seen in the cell cytoplasm. No definite phagosomal membranes can be seen around the organisms, and no lysosomes are seen in the bacilli-laden endothelial cells. Nonlepromatous cases show an increase in the endothelial cell height which is mainly caused by an increase in the amount of intermediate filaments in the cytoplasm.

The results of this study indicate an active involvement of the microvasculature, and particularly the endothelial cells in bacillary movement and granuloma development in leprosy lesions.—[Division of Microbiology, Armed Forces Institute of Pathology, Washington, D.C. 20306-6000; Delta Regional Primate Research Center, Covington, LA 70433]

Acknowledgments. Nude mice tissues were contributed by Dr. Y. Fukunishi. The study was carried out during a National Institutes of Health Fogarty International Center Fellowship No. 1 F05 TW03595-01 B1-5.

Gormus, B. J., Wolf, R. H., Baskin, G. B., Greer, W. E., Walsh, G. P., Meyers, W. M. and Binford, C. H. Naturally acquired leprosy in sooty mangabey monkeys—a second case.

We have previously reported the existence of naturally acquired LL-type leprosy in a sooty mangabey monkey (*Cercocebus atys*). *Mycobacterium leprae* from that monkey (#A015) have been used repeatedly to experimentally transmit leprosy to other mangabeys and to rhesus (*Macaca mulatta*) and African green (*Cercopithecus aethiops*) monkeys.

We recently diagnosed subpolar LL-type leprosy in a second sooty mangabey (#G932) that had never been inoculated with *M. leprae* or with any infectious agent to our knowledge. This animal had been housed in direct contact with mangabey A015 at the Gulf South Research Institute in New Iberia, Louisiana. Mangabey A015 was subsequently transferred to the Delta Regional Primate Research Center in November 1979. Both A015 and G932 were imported to the U.S.A. from Nigeria, along with other sooty mangabeys, prior to 1977.

Mangabey A015 manifested LL leprosy in 1979, whereas G932 showed no clinical signs of leprosy until skin lesions appeared in 1986. It appears that G932 acquired leprosy by contact with A015; A015 may have contracted leprosy from contact with a human leprosy patient, another infected mangabey, or by other means. The history of the mangabeys prior to their shipment from Nigeria is unknown, however, precluding definite conclusions concerning the origins of their disease.

The existence of a second case of naturally acquired leprosy in a sooty mangabey offers strong support for zoonotic leprosy involving this primate species. Naturally acquired leprosy occurs in armadillos, and has been observed in a chimpanzee. The importance of zoonotic leprosy in the epidemiology of the disease is unknown, but evidence suggests that humans can acquire leprosy by contact with diseased armadillos. Because mangabeys are mild-mannered animals and are often kept as pets in leprosy-endemic areas of Africa, their susceptibility to leprosy could have serious implications for both humans and mangabeys.-[Delta Regional Primate Research Center-Tulane University, Covington, LA 70433; The University of Southwestern Louisiana-New Iberia Research Center, New Iberia, LA 70560; Armed Forces Institute of Pathology, Washington, D.C. 20306-6000]

Ohkawa, S., Martin, L. N. and Gormus, B. J. Mycobacterium leprae-induced lymphoproliferative response of experimental leprosy monkeys: regulatory role of monocyte and lymphocyte subsets.

We investigated the immunological status of seven normal control monkeys and 23 mangabey monkeys experimentally inoculated with mangabey-origin Mycobacterium leprae. Clinically, these monkeys were divided into three broad groups: a recently inoculated group, a resistant group, and a susceptible group. The resistant group included 11 monkeys, 7 of which have shown no clinical sign of disease to date and 4 of which had shown local disease that regressed spontaneously. The susceptible group included 8 monkeys, 5 of which have disseminated disease and 3 with local but stable disease. When peripheral blood mononuclear cells of these monkeys were cultured with Dharmendra-type human M. leprae, 1 of 7 normal monkeys, 4 of 4 of the recently inoculated group, 7 out of 10 resistant monkeys, and 3 of 8 susceptible monkeys showed significant responses.

In this experimental monkey model, we studied the regulatory role of lymphocyte and monocyte subsets *in vitro*. Lymphocytes were fractionated into OKT4-en-

riched cells and OKT8-enriched cells. Monocytes were fractionated into Fc receptor-positive (FcR+) cells and Fc receptornegative (FcR-) cells. Percentages of OKT8+ cells showed a good negative correlation with the lymphoproliferative responses to T-enriched cells supplemented with unfractionated monocytes. But the depletion of OKT8+ cells could not recover the response of nonresponding monkeys' lymphocytes. The resistant group and susceptible group did not differ in their percentages of OKT8+ cells. OKT8+ cells negatively regulate the response of lymphocytes, but the suppressor activity of OKT8+ cells is not the main cause of unresponsiveness in the susceptible group.

The FcR- monocyte population mainly includes antigen-presenting activity, but the percentage of FcR- monocytes showed a significant negative correlation with the lymphoproliferative responses in the resistant group. By depleting FcR+ monocytes from the culture, some susceptible monkeys showed a weak but significant response to M. leprae, while other susceptible monkeys remained unresponsive to M. leprae. Through this experimental system, we could find an immunological spectrum in experimental leprosy monkeys which is well known in human leprosy. The immunological defect of leprosy monkeys may exist in the OKT4+ lymphocytes or the FcRmonocytes.-[Delta Regional Primate Research Center-Tulane University, Covington, LA 70433]

Walsh, G. P., Meyers, W. M., Binford, C.
H., Baskin, G. B., Wolf, R. H., Gormus,
B. J. and Martin, L. N. Primary polyneuritic leprosy in nonhuman primates.

Primary neuritic leprosy is a form of the disease in which lesions are limited to nerves without obvious dermal lesions. It is a type of leprosy that is uncommon in humans.

We have previously reported the successful transmission of leprosy to African green monkeys (*Cercopithecus aethiops*), describing the early clinical manifestations of the disease. Recently, necropsy examination of these animals 5 years after inoculation with *Mycobacterium leprae* revealed a predominantly neuritic form of leprosy.

Three adult African green monkeys were

inoculated with *M. leprae* isolated from the sooty mangabey monkey with naturally acquired leprosy. The animals were inoculated intradermally at four different sites (eyebrow, upper lip, and the tips of each ear).

Twenty-four months after inoculation, all three animals had discrete nodules along the free margins of the ears. Histopathologic examination of biopsies of these lesions revealed changes consistent with those seen in human lepromatous leprosy. One of the animals eventually developed disseminated disease. The nodules on the ears of the other two animals regressed and were re-classified histopathologically as regressing borderline leprosy.

All three animals were necropsied 5 years after inoculation. The monkey with disseminated cutaneous lesions also had extensive involvement of major peripheral nerves and nasal mucosa. The two animals in which the ear lesions regressed had extensive lesions in all the major peripheral nerve trunks of the upper and lower extremities. Histopathologic evaluation of these nerves revealed severe infiltration with lymphocytes, histiocytes, occasional neutrophils, plasma cells, and rare giant cells. In some nerves there were patches of fibrosis. The acid-fast bacilli in these nerves were solidly stained, suggesting viability. There was no significant involvement of the liver, spleen, or other viscera. At necropsy, the infiltrate in all of these animals was interpreted histologically as having features of borderline leprosy.

These studies indicate that experimentally infected African green monkeys can develop a polyneuritic form of the disease, and this species may prove to be a model for the study of primary neuritic leprosy, a form of the disease rarely seen in humans.— [Armed Forces Institute of Pathology, Washington, D.C. 20306-6000; Delta Regional Primate Research Center-Tulane University, Covington, LA 70433]

Malaty, R., Beuerman, R. W., Rayfield, M. A. and Franklin, R. M. Infection of adult trigeminal neurons with *Mycobacterium leprae in vitro*—a model system.

Leprosy is the third leading cause of preventable blindness worldwide and a major cause of peripheral nerve damage. Infection of the trigeminal nerve, a purely sensory cranial nerve, is one of the pathways that leads to blindness in leprosy. The corneal nerves are affected in the early stages of the disease, resulting in corneal hypesthesia. The primary cause of visual impairment, however, is the chronic iritis of leprosy, which has been associated with a neuroparalytic process caused by early involvement of the iris nerves.

Study of the pathogenesis of ocular leprosy has been largely limited to eyes removed at autopsy or from patients with advanced disease, and the lack of a suitable model has markedly hindered progress in this area of research.

The purpose of our investigation is to develop an *in vitro* model system for studying the invasion and reaction of trigeminal neurons by the leprosy bacilli.

The trigeminal ganglia of adult rabbits were removed, enzyme dissociated, suspended in nutrient media, and dispersed on collagen-coated, six-well plates. Neuronal cell bodies attached to the surface after 3 to 4 days and elaborated neural processes, forming a dense neural feltwork. Fibroblasts also attached and replicated. After 7 days, the cell culture was inoculated with a suspension of Mycobacterium leprae prepared from foot pads of a M. leprae-infected athymic nude mouse. The suspension contained 6.3×10^8 acid-fast bacilli (AFB) per ml. Then 0.5 ml of 10⁴ and 10⁵ M. leprae per ml was added to each tissue culture well. Seventy-two hr later, the cell layer was fixed and stained with Ziehl-Neelsen. AFB were seen singly and in clumps within the neuronal cell bodies, fibroblasts, and between cells. Further studies with varying numbers of *M. leprae* and studies involving purified trigeminal neurons in culture are under way. Our results to date indicate that adult trigeminal neurons may phagocytize M. leprae in vitro and may be used as an in vitro model for further studies on host-parasite relationships in neural tissue and in the neuropathogenesis of leprosy.-[Louisiana State University Eye Center, New Orleans, LA 70112]

Vadiee, R., Shannon, E. J., Gillis, T. P., Harris, E. B., Mshana, R. N. and Hastings, R. C. The evolution of IgG and IgM antibodies to phenolic glycolipid-I in armadillos inoculated with *M. leprae*.

Plasma samples were collected from 30 armadillos (*Dasypus novemcinctus*) prior to intravenous inoculation with 6.1 \times 10⁸ \pm 2×10^8 (mean \pm S.D.) of *Mycobacterium* leprae. After inoculation, plasma samples were collected at 3-month intervals for a period of 1 to 2.5 years. IgG and IgM antibodies to phenolic glycolipid-I (PGL-I) were detectable after 200 days using an enzyme-linked immunosorbent assay. The absorbance values for IgG anti-PGL-I were significantly higher than IgM anti-PGL-I antibodies. To determine if there was a relationship between bacterial load and either IgG or IgM anti-PGL-I antibodies, the burden of M. leprae per g of liver tissue was quantitated at necropsy. The burden of M. leprae in live animals was arbitrarily assigned to be 1×10^8 per g of liver tissue. Using Spearman's rank order correlation coefficient, there was an inverse correlation (rho = 0.507, p < 0.01) with bacterial load and absorbance values for IgG anti-PGL-I antibodies. IgM anti-PGL-I absorbance values and bacterial load were less correlated (rho = 0.374, p < 0.05). It was concluded that the kinetics of IgG and IgM antibodies to PGL-I are useful in monitoring the progression of infection with M. leprae. In addition, low absorbance values for IgG anti-PGL-I antibodies are more predictive than IgM anti-PGL-I of high bacterial loads.-[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Truman, R. W., Hugh-Jones, M. E., Job, C. K. and Hastings, R. C. Seroepidemiology of leprosy in wild armadillos (*Dasypus novemcinctus*).

Other than man, the nine-banded armadillo (*Dasypus novemcinctus*) of the south central United States is the only highly endemic natural host of leprosy. The origin and risks of this infection are important basic scientific questions, and a better understanding of the reservoir of *Mycobacterium leprae* represented by armadillos is needed. Both histopathological examination of armadillo ear tissues and serologic screening by ELISA for IgM antibodies to the phe-

nolic-glycolipid-I (PGL-I) antigen of M. leprae appear to be good relative indices to estimate prevalence in armadillos. In a survey of 315 armadillos from Louisiana and Florida, enzootic disease was only detected among the Louisiana animals. Average antibody prevalence rates (11.1%) were five times higher than rates based on histopathological examination, indicating armadillos have a much greater experience with M. leprae than previously believed. Significant trends indicative of the origin of M. leprae in armadillos migrating from Texas could not be confirmed. Prevalence rates between groups of Louisiana armadillos were similar. Ratios of antibody and histologic prevalence may be affected by self-healing infections or population characteristics and environmental conditions influencing longevity of armadillos and disease transmission. Environmentally shed organisms may be involved in the transmission of leprosy in armadillos, and the PGL-I antigen is detectable in the environment of infected armadillos. Further population-based comparisons of enzootic prevalence over the range of armadillos will help to describe the origin and risks of this unique animal disease and may have significant impact on our understanding of leprosy in man.-[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721; Louisiana State University School of Veterinary Medicine, Department of Epidemiology and Community Health, Baton Rouge, LA 708031

Stallknecht, D. E., Truman, R. W., Hugh-Jones, M. E. and Job, C. K. Surveillance for *Mycobacterium leprae* infections in a community of nine-banded armadillos.

Over a 2-year period, population characteristics and leprosy prevalence were described in a community of armadillos occupying a 16.7 km portion of the Atchafalaya River levee in Pointe Coupee Parish, Louisiana. In the summer of 1984 and the spring of 1986, a total of 132 armadillos was sampled and released. Recapture analysis indicated a stable population averaging $261 \pm$ 62 armadillos in both years with a distribution of 1.6 animals/hectare using the levee habitat. Prevalence of IgM antibodies to the phenolic glycolipid-I (PGL-I) antigen of Mycobacterium leprae was not significantly different in the years studied (averaging 11.4%), but tended to be lower in the spring sampling (7.5%) than in the summer survey (16.3%). Histopathological examinations of ear biopsies indicated an average prevalence of 2.3%, and ratios of antibody prevalence to histologic prevalence remained similar. Leprosy-positive animals were distributed throughout the habitat, but there was increased disease detectability in areas where armadillos were more abundant, suggesting a tendency for density associations in the occurrence of armadillo leprosy or a source of sampling error for surveys employing nonsystematic methods.-[Louisiana State University School of Veterinary Medicine, Baton Rouge, LA 70803; Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]

Hastings, R. C., Sanchez, R. M. and Job, C. K. Congenital transmission of leprosy in armadillos.

In a recent publication, Brubaker, et al. reported 11 infants less than 1 year of age with leprosy diagnosed histopathologically. In five, the mothers had lepromatous disease. They emphasized the role of intrauterine exposure to *Mycobacterium leprae* or its antigens in the transmission of leprosy. Anti-*M. leprae* antibodies of IgM and IgA classes have been found in the cord serum of 30% to 50% of the babies born to mothers with lepromatous disease.

In this study, placentae and fetuses from three lepromatous armadillos were studied histopathologically. The uterine muscle, the endometrical tissue, and the placental villi showed infiltration by *M. leprae*. In 3 of the 12 fetuses examined, *M. leprae* were present inside the macrophages of the spleen.

These findings show that intrauterine infection of the armadillo fetus is clearly possible, and that congenital infection may also occur in humans.—[Laboratory Research Branch, GWL Hansen's Disease Center, Carville, LA 70721]