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ABSTRACTS

EIGHTEENTH JOINT LEPROSY RESEARCH CONFERENCE

National Institutes of Health
Bethesda, Maryland, U.S.A.
3–4 August 1983

U.S.–Japan Cooperative Medical Science Program

OPENING REMARKS

Good morning, Ladies and Gentlemen:

It is a great honor to again have the privilege of welcoming you to a U.S.–Japan Leprosy Research Conference. As always, it is a great pleasure to see our Japanese colleagues. We eagerly look forward to learning of their findings since our last conference in Sendai, and to renewing our friendships.

This, the Eighteenth U.S.–Japan Leprosy Research Conference, is again being held at the National Institutes of Health and, again, this location has allowed a larger number of participants to attend than might have otherwise been possible.

This conference is unusual in several respects. Firstly, as occurred in 1977 in Boston, we are privileged to again be meeting in conjunction with the U.S.–Japan Tuberculosis Research Conference. This provides us with a great opportunity to meet with our colleagues in tuberculosis, exchange information and ideas, share common problems, and hopefully hasten the day when both of these dreaded mycobacterial diseases are controlled in the world.

Secondly, this conference is unusual in that we are fortunate to have the participation of a distinguished group of leprologists from India, and other guests from abroad. We extend a warm welcome and look forward to your participation in the conference.

Finally, I would like to express our deep appreciation to Dr. Bill Jordan, Dr. Darrel Gwinn, and the staff of the U.S.–Japan Cooperative Medical Science Program for again superbly organizing a U.S.–Japan Leprosy Research Conference. From the abstracts, exciting advances are occurring on both sides of the Pacific. I look forward to hearing this excellent group of papers.

Thank you very much.

—Robert C. Hastings, *Chairman*
U.S. Leprosy Panel

Participants of the Eighteenth Joint Leprosy Research Conference.

PROGRAM OF THE EIGHTEENTH JOINT LEPROSY RESEARCH CONFERENCE

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ABSTRACTS OF LEPROSY CONFERENCE

Stoner, G. L. Prospects for a *Mycobacterium leprae* vaccine against leprosy after Madras.

In the wake of the failure of BCG to prevent tuberculosis in the WHO-sponsored Madras trial, there is widespread skepticism about the efficacy of any other mycobacterial vaccine, especially a leprosy vaccine prepared from killed *Mycobacterium leprae*. This skepticism is understandable because *M. tuberculosis* and *M. leprae* are similar organisms structurally and are closely related antigenically. Furthermore, the fact that tuberculosis can be described as having an immunopathological spectrum analogous to the well-established leprosy spectrum is widely accepted as evidence that the two diseases are comparable immunologically. Other similarities include the chronic nature of both diseases, the fact that some of the same drugs are effective against both organisms, and the fact that the same vaccine (BCG) has been used against both diseases.

These similarities have tended to obscure the fact that these two pathogens must differ in subtle, but significant, ways because they actually produce two quite different diseases. In addition to the obvious difference of the tissue predilection of the two organisms, they differ with respect to their cultivability, their generation time, and their growth in animal hosts. But most importantly, the two diseases differ fundamentally with respect to their immunology. First, tuberculosis appears to be an opportunistic infection. In contrast, a successful *M. leprae* infection may not depend on a generalized debilitation of the host. Rather, an *M. leprae* infection probably establishes itself by inducing an antigen-specific unresponsiveness in individuals who are otherwise quite healthy. Secondly, although both diseases show an immunopathological spectrum, there is a crucial difference between them with respect to the source of infection within that spectrum. Leprosy infection is transmitted largely from lepromin-negative, multibacillary lepromatous patients. In contrast, the totally anergic disseminated tuberculosis infection, i.e., acute miliary tuberculosis, is not an important source of infection within the community. The major source of infection is the intermediate-type cases, many of whom are Mantoux-positive.

What are the implications of these differences for the prospects of a leprosy vaccine? Both of these differences suggest that *M. leprae* may be a better vaccine against leprosy than BCG is against tuberculosis. If a leprosy vaccine can induce specific cell-mediated immunity in susceptible individuals prior to natural infection, it should be effectively expressed in healthy individuals who might otherwise contract leprosy. Moreover, in some of those individuals vaccinated too late to prevent disease, a vaccine might precipitate a non-infectious borderline disease which could otherwise have developed into a communicable lepromatous infection at a much later time. This would effectively interrupt the transmission of the infection.

Clearly, whatever the implications of the Madras trial for the use of BCG as a tuberculosis vaccine, the Madras results have no direct bearing on the prospects for the control of leprosy with an *M. leprae* vaccine. Tuberculosis and leprosy are so different immunologically that a leprosy vaccine should be tested in a controlled field, and then judged on its own merits.—[Laboratory of Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205, U.S.A.]

Ridel, P.-R., Johl, J. S. and Krahenbuhl, J. L. Effects of vaccination with *Mycobacterium leprae* on local cell-mediated immunity.

In contrast to the marked effects of disseminated infection with certain intracellular pathogens (Toxoplasma, Listeria, BCG), on natural killer (NK) cell activity in various anatomical compartments (spleen, blood, peritoneal cavity), local infection of the mouse foot pad with *Mycobacterium marinum* or local treatment with suspensions of killed *M. leprae* enhanced only the

NK cell activity of the mononuclear cells from the draining lymph node. This effect was best demonstrated with mononuclear cells from popliteal lymph nodes and clearly was a localized effect. In BALB/C or B6C3F1 mice treated in the foot pad with killed *M. leprae* no significant increase in NK activity was observed in cells from either the spleen or the peritoneal cavity. Enhanced NK activity was observed only in the ipsolateral but not the contralateral popliteal lymph node.

The present report is concerned with additional studies which have been performed to further evaluate the effects of local administration of *M. leprae* on local host cellular response. Experiments were carried out with BALB/C and C3H/HeN mice, low and high NK responders, respectively. Because the local cell-mediated immune response (CMI) in leprosy is largely characterized by the close interaction between the leprosy bacillus and host macrophages, and macrophages are known to play important afferent and efferent roles in CMI, our continued attempt to modulate local CMI included treatment with suspensions of normal syngenic thioglycolate-induced peritoneal macrophages harboring intracellular *M. leprae*. In addition to NK response, a second manifestation of non-specific CMI was studied, the lymphoblastic transformation (LBT) of T cells cultured with the mitogen conconavalin A.

At 14 and 28 days after local treatment, NK activity was significantly elevated in ipsolateral lymph nodes from BALB/C or C3H/HeN mice treated with *M. leprae* alone. However, in both strains, treatment with *M. leprae*-burdened macrophages markedly enhanced local NK response above that seen after treatment with *M. leprae* alone. This enhancement was noted to persist for at least seven weeks after treatment. In the case of C3H/HeN (but not BALB/C) mice, significant enhancement of NK activity was observed in cells from the spleen as well as the draining popliteal lymph node.

In the case of the mitogen-induced LBT by local lymph node T cells, a suboptimal dose of ConA was employed, resulting in little or no response by normal lymph node cells or cells from popliteal nodes draining foot pads treated with macrophages. In contrast to our findings with NK activity, only treatment with macrophages harboring *M. leprae* produced a striking enhancement of the LBT response to ConA. Local injection of *M. leprae* alone had no effect.

To further evaluate enhancement of the local T cell response, an *M. leprae* antigen-specific LBT was performed with lymphocytes from the ipsolateral lymph nodes and spleens of treated C3H/HeN mice. The local specific LBT was significantly elevated in mice treated with *M. leprae* alone or with macrophages harboring *M. leprae*, but significant LBT to *M. leprae* antigen by lymphocytes from the spleen was observed only with mice treated with macrophages harboring *M. leprae*.

Additional studies on the incidence of the antigen-specific cell activation are under investigation to determine if there is a modification of the course of the infection with *M. leprae*. These above studies clearly indicate the important role of the macrophage in presentation of *M. leprae* antigen for optimum enhancement of CMI locally and in the induction of a systemic memory cell population specific to *M. leprae* antigen. Further investigation is necessary to understand the mechanism(s) underlying these phenomena.

This work was supported by Grant AI 19069 from the National Institute of Allergy and Infectious Diseases.—[National Hansen's Disease Center, Carville, Louisiana 70721; SRI International, Menlo Park, California 94025, U.S.A.]

Shepard, C. C. Comparative immunogenicity of BCG and *Mycobacterium leprae* in normal and in *M. leprae*-tolerant mice.

The BCG strain of *Mycobacterium bovis* is the only cultivable mycobacterium we have found that in mice provides solid protection against *M. leprae* infection or sensitization to *M. leprae*. The vaccination of mice is carried out with an optimal dose; whereas vaccination of humans will have to be carried out with a dose that produces an acceptable level of side effects (presumably local reactions). Consequently we compared the immunizing potency of heat-killed *M. leprae*, live BCG, and a mixture of the two intradermally (id) in a titration ranging from $10^{7.6}$–$10^{5.2}$ acid-fast bacteria (AFB). At 28

days, some groups were challenged with 10^7 heat-killed *M. leprae* for measurement of foot pad enlargement (FPE) and others were challenged with 5000 *M. leprae* in mouse passage for measurement of protection against infection. Local reactions to vaccination were also observed. The overall result was that the three vaccines gave similar results when given at the same dose. At the lowest dose, however, the mixture gave superior protection. In the lower dose range the *M. leprae* vaccine produced less local reaction, and consequently, with vaccines giving intermediate reactions (averaging 0.5–1.5 mm) the *M. leprae* and the *M. leprae* + BCG vaccine gave somewhat more protection than the BCG-only vaccine.

In a second experiment we compared the three vaccines in normal mice and in mice rendered tolerant by intravenous injection of *M. leprae*. The tolerant mice are analogous to Mitsuda-negative humans in the sense that they, in contrast to normal mice, fail to develop local 28-day reactions to id *M. leprae*. Of course, one hopes that a leprosy vaccine will prevent the appearance of clinical leprosy in Mitsuda-negative, clinically normal persons. FPE measurements showed that the normal mice were well sensitized by all three vaccines and that the intravenous injection induced good *M. leprae* tolerance (rendered the mice unresponsive to id vaccination with *M. leprae*). The tolerant mice that received BCG or BCG + *M. leprae* id, however, were moderately sensitized to the *M. leprae* challenge; the FPE in these groups averaged about 0.5 mm compared to about 1.0 mm in normal mice receiving the same vaccines. Protection against infection was different. The tolerant mice responded poorly to all the vaccines, although the normal mice were well protected. Thus, the results indicate a dissociation between immunity to infection and Mitsuda-type skin reactivity in tolerized mice.—[Leprosy Section, Respiratory and Special Pathogens Laboratory Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333, U.S.A.]

Rea, T. H., Modlin, R. L., Bakke, A. C., Horwitz, D. A. and Taylor, C. R. Tissue and blood T lymphocyte subsets in erythema nodosum leprosum and reversal reactions.

Because the reactional states of leprosy are important causes of tissue injury, and because they are probably immunologically mediated, we have sought data concerning the distribution of T cell subsets in the blood and tissues of patients with erythema nodosum leprosum (ENL) and reversal reactions, hoping to clarify their underlying mechanisms.

Patients were classified according to the criteria of Ridley. Tissue T cell subsets were studied in frozen sections by the sequential application of primary mouse monoclonal antibody, peroxidase conjugated goat anti-mouse and a chromogenic agent, aminoethyl carbazol. Quantitation was done by estimating the labelled cells as a percentage of all cells of the infiltrate. Blood T cell subsets were determined by flow cytometry. The T cell specificities sought were a pan T phenotype (Leu 1, Leu 4 or OKT 3), a helper/inducer phenotype (Leu 3 or OKT 4) and a suppressor/cytotoxic phenotype (Leu 2 or OKT 8) in tissues or blood, respectively. When both were determined, blood and tissue specimens were obtained within 1 hr of each other.

The peripheral blood studies are summarized in Table 1. Lepromatous patients without reaction were significantly and proportionately lymphopenic, pan T cytopenic, helper/inducer cytopenic and suppressor/cytotoxic cytopenic, but without abnormality if expressed as a percentage. ENL patients did not differ significantly from the controls.

The 8 patients with reversal reactions (2 BT, 2 BB, 4 BL), 7 untreated and 1 relapsing, had a significantly decreased mean helper/inducer value, both in absolute number and percent, without other abnormalities.

Morphologically the distribution of T cell subsets in tissues was similar in lepromatous patients with or without ENL, i.e., both the helper/inducer and the suppressor/cytotoxic phenotypes were admixed with the foamy histiocytes. In reversal reaction tissues there was always some expression of segregation of T cell subsets similar to that seen in nonreactional tuberculoid (TT/BT, BT) disease, i.e., the suppressor/cytotoxic

TABLE 1. *Mean (±S.D.) values of blood lymphocytes, T cell subsets and helper : suppressor ratios.*

	Lymphs	Pan T	Helper/ inducer	Suppressor/ cytotoxic	H:S ratio
Controls (22)	2582 ± 941	1671 ± 643	1027 ± 403	683 ± 333	1.7 ± 0.5
LL no ENL (18)	1611 ± 764[b]	1034 ± 531[a]	595 ± 364[b]	422 ± 238[a]	1.5 ± 0.6
LL with ENL (18)	2032 ± 840	1317 ± 557	845 ± 409	519 ± 266	1.7 ± 0.8
Reversal (8)	2249 ± 805	1324 ± 670	618 ± 162[b]	620 ± 343	1.3 ± 1.0
BT (18)	2269 ± 769	1534 ± 544	896 ± 414	592 ± 225	1.5 ± 0.8

[a] $p < .01$ as compared with controls.
[b] $p < .001$ as compared with controls.

phenotype being restricted to the lymphocytic mantle at the periphery of the granuloma, but the helper/inducer cells being admixed with aggregated macrophages. Each of the four BL patients with reversal reaction showed some segregation; whereas none of the four nonreactional BL tissues showed any segregation. Overall the segregation was not as well developed in reversal reactions as in nonreaction TT/BT or BT.

Table 2 summarizes the values for T cell subsets in tissues. ENL tissues compared with nonreactional lepromatous tissues showed a smaller percentage of the suppressor/cytotoxic subset and a larger percentage of the helper/inducer subset. Also, the tissue helper : suppressor ratio was significantly larger in ENL tissues. These relationships indicate an important role for cell-mediated immunity in the pathogenesis of ENL. Blood differences are consistent with this view.

In plotting the tissue helper : suppressor ratio against the blood helper : suppressor ratio, it was evident that there was no relationship between the two in any category studied. For example, the four ENL patients with extreme blood helper : suppressor ratios, 0.6, 0.6, 3.3 and 3.5, had tissue helper : suppressor ratios that were close to each other and to the mean, 2.0, 2.5, 1.7 and 2.3, respectively. This is consistent with a lymphocyte traffic pattern in tissues of selectivity of entry, retention, or exit, or some combination thereof.

Because tissue morphology shows that reversal reactions and BT leprosy share in common the segregation of T cell subsets, and because both reversal reactions and BT leprosy are associated with resistance, the segregation pattern may be the microanatomical inscription of effective resistance. Because there is strong evidence that reversal reactions are cell-mediated immune responses, the isolated helper/inducer cytopenia in reversal reactions is most easily understood as relfecting a high turnover of this phenotype, occurring as part of intensive stimulation of macrophages. The low helper : suppressor ratio in the tissues of reversal reactions can be interpreted similarly.—[Departments of Dermatology and Pathology, Los Angeles County/University of Southern California Medical Center, Los Angeles, California 90033, U.S.A.

TABLE 2. *Mean (±S.D.) values (percent) of T cell subsets in tissue infiltrates, and means of helper : suppressor ratios in tissue and blood.*

	Pan T	Helper/ inducer	Suppressor/ cytotoxic	Tissue H:S	Blood H:S
Reversal (7)	46 ± 162	29 ± 15	21 ± 9	1.6 ± 0.6[a]	1.5 ± 0.9
BT (15)	50 ± 18	43 ± 17	20 ± 8	2.2 ± 0.6	1.2 ± 0.5
BL (4)	46 ± 23	33 ± 17	24 ± 6	1.7 ± 1.4	1.4 ± 0.9
LL no ENL (10)	39 ± 11	19 ± 9	34 ± 13	0.6 ± 0.4	1.7 ± 0.6
ENL (12)	37 ± 17	32 ± 9[b]	15 ± 3[b]	2.1 ± 0.4[b]	1.7 ± 1.0

[a] $p < .02$ as compared with BT.
[b] $p < .001$ as compared with LL—no ENL.

Chehl, S. K., Shannon, E. J., Job, C. K. and Hastings, R. C. Reversal reactions in *Mycobacterium leprae*-infected nude mice.

Reversal reactions are manifestations of delayed hypersensitivity to *Mycobacterium leprae* and are thought to be accompanied by manifestations of effective cell-mediated immunity (CMI) as measured by bacterial clearing. Reversal reactions have been produced in thymectomized irradiated mice harboring large numbers of *M. leprae* by syngeneic leukocyte infusions (Rees and Weddel, Ann. N.Y. Acad. Sci. **154**: 214, 1968). Congenitally athymic nude (nu/nu) mice have been shown to be susceptible to *M. leprae* infections [Kohsaka, *et al.*, Lepro **45**:177, 1976; Colston and Hilson, Nature **262**:736, 1976; Nakamura and Yogi (personal communication, LSM, May 1976)] and can harbor large numbers of bacilli. These multibacillary nude mice have been used in adoptive transfer experiments with leukocytes from immunocompetent, heterozygous (nu/+) mice (Nakamura and Yogi, Int. J. Lepr. **47**:105, 1979). We have studied the induction of reversal reactions in a quantitative fashion in *M. leprae*-infected nude mice by infusing allogeneic splenic leukocytes from naive and *M. leprae*-immunized heterozygote donors.

Dose responsive inductions of reversal reactions, apparent by foot pad inflammation and swelling, decreased Morphological Indexes of the bacteria and mononuclear cell infiltrations histopathologically, were observed in both naive and immunized cell transfer experiments. The effective dose 50% (ED_{50}) in response to immunized cell infusions was considerably lower than that observed for unimmunized cells. The model would appear to be useful in evaluating potential antileprosy vaccines.—[Laboratory Research Branch, National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Watson, S. R., Bullock, W. E., Nelson, K. E., Schauf, V., Gelber, R. and Jacobson, R. R. Studies on Interleukin 1 production by peripheral blood mononuclear cells from leprosy patients.

The complex interactions of the immune response are initiated and controlled, in part, by soluble mediators produced by cells of the immune system. Interleukin 1 (IL-1) is such a factor that is produced by macrophages and has a variety of immunoregulatory effects. IL-1 is released from either antigen- or mitogen-stimulated macrophages and acts upon T cells that are in the early G_1 stage of the cell cycle, preparing them to respond to antigens or secondary mediator signals. One of the major activities of IL-1 is to induce the synthesis and secretion of Interleukin-2 (IL-2). IL-2 is essential for the continued proliferation of T cells. Thus, there is a link between IL-1 and IL-2 that is essential for the amplification of immune responses.

We have investigated the production of IL-1 by peripheral blood mononuclear (PBM) cells from leprosy patients in order to explore the hypothesis that the deficient cell-mediated immune (CMI) responsiveness of patients with lepromatous disease may result, in part, from impairment of IL-1 production.

IL-1 production by PBM cells from 17 patients with leprosy was studied. This group included 5 TT/BT, 2 BB and 10 BL/LL patients, ranging in age from 19–69 years. All patients were either untreated or had received anti-mycobacterial therapy for less than one week at the time of assay. Normal controls were age- and sex-matched.

PBM cells were separated from heparinized blood on Ficoll-Hypaque, washed and resuspended in Mishell-Dutton medium using RPMI 1640 as a base. Cells were added to 35 mm tissue culture plates at a concentration of 2×10^6 cells/ml and a total volume of 2 ml. After 4 hr incubation at 37°C the nonadherent cells were removed by vigorous washing. Fresh medium was added to the adherent cell population and a variety of inducing agents added to the culture—LPS 20 μg/ml or ConA 2 μg/ml or phorbol myristate acetate (PMA) 100 ng/ml. Twenty-four hours later, the supernatants were removed and dialysed extensively against phosphate buffered saline (PBS). IL-1 was assayed in a co-mitogenic assay system utilizing C3H/HeJ thymocytes cultured in the presence of a suboptimal concentration of PHA. The amount of IL-1 in the supernatant was quantitated by comparison with a standard preparation.

The PBM cells from all seven patients in the TT/BT and BB groups produced IL-1

when stimulated. However, we observed that 3 out of 5 patients in the TT/BT group and 1 of 2 BB patients produced IL-1 spontaneously. Three of the ten BL/LL group did not produce detectable IL-1 in response to IL-1-inducing agents and none of the cells from these patients spontaneously secreted IL-1. Cells from all 17 normal blood donors secreted IL-1, including those controls that had been shipped via air freight along with cells from patients. Normal cells did not release IL-1 spontaneously.

Although the patient population studied was small, the following tentative conclusions can be drawn:

a) The production of IL-1 by stimulated PBM cells from patients with tuberculoid and BB leprosy does not differ from that of normal controls. Moreover, spontaneous release of IL-1 by PBM cells *in vitro* from three of seven patients in this group suggests possible pre-activation *in vivo* of PBM cells from these patients.

b) PBM cells from seven of ten patients with BL/LL leprosy produced IL-1 in amounts that fell within the normal range. In these patients, therefore, defective amplification of the T cell mediated immune response as a direct consequence of impaired IL-1 production by PBM cells appears to be unlikely. On the other hand, three of ten such patients failed absolutely to produce IL-1 in response to a variety of stimuli. Additional studies will be required to confirm these findings and to evaluate the immunobiological significance of failure to produce IL-1 among patients with lepromatous leprosy.

This work was supported by NIAID Grant AI 16308.—[University of Cincinnati College of Medicine, Cincinnati, Ohio; University of Illinois Medical School, Chicago, Illinois; Mary's Help Hospital, Daly City, California; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Kaplan, G., Nogueira, N. and Cohn, Z. A. Studies on leprosy: A. Structural: The nature of infiltrating cells in the cutaneous lesions of leprosy. B. Immunological: Gamma-IFN production in lepromatous and tuberculoid leprosy; restoration of production in lepromatous patients with IL-2.

A. The dermal lesions of 27 patients with leprosy have been examined by immunofluorescence and transmission electron microscopy. The patients exhibited a spectrum of disease from polar lepromatous to polar tuberculoid with intermediate stages in various states of therapy and relapse. The nature and quantities of inflammatory cells and bacteria have been determined ([1,2]).

Lepromatous leprosy is characterized by cutaneous infiltrates containing predominantly parasitized foam cells with large, multibacillary vacuoles. *Mycobacterium leprae* were found intracellular within membrane-bound vacuoles. The osmiophilic bacteria had free ribosomes and membrane-like structures in their cytoplasm, and an outer double membrane. The bacteria were surrounded by an electronlucent halo which could be produced by the extraction of material of a lipid nature during the processing of the tissue for electron microscopy. Evidence for phagosome-lysosome fusion was obtained by cytochemical staining of the lysosomal enzyme acid phosphatase. The intact and partially degraded *M. leprae* were embedded in an amorphus matrix which could be of bacterial and/or host origin. Additional membranes were observed within the vacuoles, similar to those found in autophagic vacuoles. No extracellular bacteria were evident. Only small numbers of scattered lymphocytes were found, mostly of the Leu 2a/OKT 8 T cell subset. As one approached the borderline state, smaller numbers of bacilli were present as singlets and doublets in small vacuoles of macrophages. The more reactive forms showed increasing bacillary fragmentation, an occasional epithelioid cell and an increase in the numbers of lymphoid cells specifically of the Leu 3a/OKT 4 T cell subset. At the tuberculoid end of the spectrum, clear evidence of an exuberant lymphocyte response was evident. Large numbers of T cells with extremely long and complex filipodia were closely associated with epithelioid and multi-nucleated giant cells. Many of the mononuclear phagocytes appeared nonviable and areas of necrosis were evident. Bacillary remnants were scarce and the cytoplasms of the epithelioid cells contained many stacks of endoplasmic reticulum and mitochondria.

These observations are consistent with the

idea that both macrophages and T cells become activated as one approaches the tuberculoid pole of leprosy. We suggest that Leu 3a/OKT 4 helper cells may be capable of driving the effector function of mononuclear phagocytes. This would lead to a significant microbicidal effect on *M. leprae*, perhaps through the production of toxic oxygen intermediates.

B. Antigen and mitogen-induced gamma-interferon (γ-IFN) release was studied in peripheral blood mononuclear cells from 15 leprosy patients. Nine out of nine lepromatous patients (LL and BL) failed to release γ-IFN in response to stimulation by either antigen (*M. leprae*) or mitogen (ConA) (mean titer: 2.6 U/ml, range 0–16 U/ml). In contrast, cells from tuberculoid patients (TT and BT) displayed considerable levels of γ-IFN release under the same experimental conditions (mean titer: 200 U/ml, range 128–512 U/ml). Normal controls failed to respond to *M. leprae* (0–16 U/ml) and displayed good responses to ConA (128–256 U/ml). One patient without a histological diagnosis and one BB patient displayed intermediate levels of γ-IFN release (32 U/ml and 64 U/ml, respectively).

The responsiveness of the lepromatous patients for γ-IFN release could be restored by the addition of a partially purified IL-2 preparation and *M. leprae* antigen, but not by IL-2 alone.

The fact that T cells of patients with lepromatous disease fail to produce the macrophage-activating factor, γ-IFN, may explain the heavy bacillary load found in these patients. Therapeutic restoration of γ-IFN, IL-2, or both may be possible in the future.—[Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021, U.S.A.]

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Lefford, M. J., Skoff, R. P., Skoff, A. M. and Liu, K. M. Peripheral neuropathy in mice infected with *Mycobacterium lepraemurium*.

During the past several years many strains of mice have been examined with respect to their susceptibility to *M. lepraemurium* (Mlm). In the course of a recent study, it was noted that one mouse strain developed weakness and then paralysis of the hind quarters. This syndrome occurred in CBA/J mice and has never been observed in many other strains including C57BL/6, BALB/c, DBA/2, C3H/HeJ, SJL, and F_1 hybrids derived therefrom.

The genetic control of this syndrome is extremely fine, since other strains of the CBA background (CBA/N, CBA/CaJ and CBA/H-T6J) are either unaffected or much less severely affected. By contrast, at least 75% of intravenously infected CBA/J mice exhibit paralysis during the 2–3 weeks before they die. Paralysis occurs regardless of the infecting dose of the Mlm, in the range of 10^6 to 10^9 bacilli intravenously, and is also seen after foot pad infection, but less consistently. Paralysis occurs in adult-thymectomized/lethally irradiated/bone marrow reconstituted mice, but at a lower frequency, suggesting that T lymphocytes may facilitate paralysis but are not absolutely necessary. Experiments to confer paralysis by transfer of lymphoid cells are still in progress.

Macroscopic examination of the central and peripheral nervous systems of affected mice revealed no abnormalities. Similarly, no pathology was evident from examination of 5 μ thick paraffin wax sections, stained with hematoxylin and eosin. Plastic-embedded sections were then examined by light and electron microscopy. These revealed patchy, irregular loss of myelin in nerves arising from the lumbo-sacral plexus. The spinal cord itself was not involved nor were nerves of the brachial plexus. Tuberculoid granulomas have not been observed; neither are Mlm bacilli visible in the affected parts of the nerves, including the Schwann cells.

Five possible causes of the paralysis have been considered:

a) direct infiltration of the nervous system

with Mlm or Mlm-induced granulomatous lesions;

b) a cell-mediated immunity-hypersensitivity phenomenon analogous to acute experimental allergic neuritis;

c) a humoral antibody-mediated neuropathy;

d) a toxic neuropathy induced by some secreted product of the Mlm bacilli, and

e) the activation by Mlm infection of some underlying latent disease, e.g., a virus infection.

The present evidence does not support hypotheses a and b, but the remaining possibilities are being investigated.

The interest of these observations lies in the fact that, regardless of the underlying mechanism, there exists in experimental animals a neurological syndrome caused or precipitated by mycobacterial infection.—[Department of Immunology and Microbiology; Department of Anatomy, Wayne State University School of Medicine, Detroit, Michigan 48201, U.S.A.]

Sankaran, K., Hoffeld, J. T., Chaparas, S. D. and Oppenheim, J. J. Increased H_2O_2 production by splenic murine macrophages associated with susceptibility to *Mycobacterium lepraemurium* infection.

C3H/HeJ and C57BL/6J strains of mice are, respectively, susceptible (lepromatous) and resistant (tuberculoid) to *Mycobacterium lepraemurium* and have served as models of these forms of leprosy infection. Injection of the C3H/HeJ mouse with *M. lepraemurium* results in a disease analogous to human lepromatous leprosy. In both cases, macrophages are capable of ingesting these bacilli but are not effective in killing them. In both cases, macrophage lysosomal enyzme activities and the capacity to generate oxygen radicals have been reported by others. This study was undertaken to determine whether quantitative differences in capacity to generate hydrogen peroxide might account for the susceptibility of C3H/HeJ and the resistance of C57BL/6J to *M. lepraemurium* infection.

The *in vitro* release of hydrogen peroxide from peritoneal or splenic macrophages from these strains was tested after *in vivo* intraperitoneal (i.p.) administration of thioglycollate, *Propionibacterium acnes* (*C. parvum*), *M. lepraemurium*, or *M. bovis* (BCG). Four days before harvesting *M. lepraemurium* or BCG sensitized cells the mice were given thioglycollate i.p. The *in vitro* effect of the addition of *M. lepraemurium* to cultures of such macrophages was also studied. Hydrogen peroxide assays were performed after triggering with phorbol myristate acetate (PMA) either immediately after harvesting the macrophages from the animals or after 24 or 72 hr of incubation.

Splenic macrophages from C3H/HeJ mice previously injected i.p. with *C. parvum*, *M. lepraemurium* or BCG produced nearly two times more hydrogen peroxide than splenic cells from C57BL/6J mice at the time of harvesting. The amount of hydrogen peroxide production by splenic macrophages from C3H/HeJ mice at 24 hr and at 72 hr remained consistently higher than that from C57BL/6J mice.

The effect of *in vitro* stimulation of macrophages by *M. lepraemurium* was then examined. *M. lepraemurium* inhibited the release of hydrogen peroxide by splenic macrophages from C57BL/6J to a greater degree than that from C3H/HeJ, both after 24 and 72 hr of incubation.

The splenic macrophages from C3H/HeJ mice appear to have an increased capacity to produce hydrogen peroxide. This may be due to their reduced bactericidal capacity and consequent failure to eliminate the *M. lepraemurium*, which also would result in more prolonged exposure and consequent activation of their macrophages. Alternatively, it has been suggested that the high susceptibility to *M. lepraemurium* infection in C3H/HeJ mice may be due to excessive superoxide dismutase (SOD) activity of their splenic macrophages. High levels of SOD leads to the conversion of superoxide to hydrogen peroxide. The hydrogen peroxide may have toxic consequences and can interfere with the host defense to *M. lepraemurium*. Finally, the high concentration of catalase present in *M. lepraemurium* may permit the bacilli to resist bactericidal consequences of hydrogen peroxide. These possibilities are currently under study.—[Laboratory of Microbiology and Immunology, Clinical Investigations and Patient Care Branch, National Institute of Dental Re-

search, National Institutes of Health; National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland; Laboratory of Molecular Immunoregulation, BRMP, FCRF, National Cancer Institute, Frederick, Maryland, U.S.A.]

Dhople, A. M. Application of ATP assays to patient care in leprosy.

The inability to cultivate *Mycobacterium leprae* has been a major bottleneck in leprosy research, especially in detecting dapsone-resistant cases without using the mouse foot pad technique. Because of the ubiquitous distribution and metabolic importance of adenosine triphosphate (ATP), it is considered as an indicator of viability of *M. leprae,* and this is used to obtain fast and reliable information on the status of *M. leprae* obtained from biopsy specimens from leprosy patients under chemotherapy and to identify dapsone-resistant cases at very early stages.

Biopsy specimens from 14 randomly selected lepromatous leprosy patients, both untreated and treated with dapsone, were obtained. The ATP content of 1 million *M. leprae* from untreated cases ranged from 1.09 to 1.38 picograms with no correlation with the Morphological Index (MI); furthermore, these *M. leprae* gave standard growth curves in the foot pads of mice. Three of these patients were followed again 4–7 months after initiating dapsone treatment. *M. leprae* from two patients did not show any ATP and also failed to multiply in mouse foot pads, while those from a third patient showed the same levels of ATP and were found to be "intermediate resistant" to dapsone. On switching this patient to rifampin for three months, his *M. leprae* did not show any ATP and also failed to multiply in the mouse foot pads.

Of the 5 patients who have been receiving dapsone for 2–3 years, *M. leprae* from 3 did not contain ATP and failed to multiply in the mouse foot pads, while those from the other 2 contained normal levels of ATP and gave standard growth curves in foot pads. *M. leprae* from these two patients also were found to be "indetermediate resistant" to dapsone. When given rifampin for three months, *M. leprae* from these two patients failed to demonstrate any viability as determined by the ATP assay technique and confirmed further by mouse foot pad inoculations.

Studies have been initiated on lepromatous leprosy patients wherein a biopsy specimen is taken before treatment and at four- to six-week intervals for up to 4–6 months after initiating dapsone treatment. *M. leprae* from these biopsy specimens are then used for ATP as well as mouse foot pad assays. Thus far we have completed ATP studies on ten patients from Argentina, Brazil, India, and Surinam. Of the ten patients, the ATP content of *M. leprae* in eight patients has declined progressively to 4%–15% of the original levels, suggesting loss of viability. In the remaining two patients, *M. leprae* still contained approximately 75% of the original levels (before treatment) of ATP, suggesting resistance to dapsone.

These findings suggest that the ATP assay technique may be a useful tool for determining responses of *M. leprae* to a given antileprosy treatment instantaneously at an early stage of therapy.

This work has been supported by the National Institute of Allergy and Infectious Diseases and the THELEP component of the World Health Organization Special Programme for Research and Training in Tropical Diseases.—[Medical Research Institute, Florida Institute of Technology, Melbourne, Florida 32901, U.S.A.]

Nakagawa, H. and Kashiwabara, Y. Metabolism of glycerolipids in *Mycobacterium lepraemurium*; biosynthesis of phospholipids.

Mycobacteria contain large amounts of lipids, including both phospholipids and triglycerides, and so they are considered to have a complex lipid metabolism. However, little knowledge has been obtained about the biosynthesis of glycerolipids in mycobacteria.

We found four phospholipid biosynthetic enzymes in a cell-free extract of host-grown *Mycobacterium lepraemurium.* CDP-diglyceride synthetase, phosphatidylserine synthetase, phosphatidylserine decarboxylase, and phosphatidylglycerophosphate synthetase catalyzing the synthetic reactions of CDP-diglyceride, phosphatidylserine, phosphatidylethanolamine, and phospha-

tidylglycerophosphate, respectively, were identified in a cell-free extract of *M. lepraemurium*. CDP-diglyceride is known as a key intermediate in bacterial phospholipid biosynthesis. Phosphatidylethanolamine is one of the major phospholipids of *M. lepraemurium*, and phosphatidylserine is a precursor substrate of phosphatidylethanolamine in *M. lepraemurium*.

The biosynthetic pathways for phospholipids found in *M. lepraemurium* were similar to those of *Escherichia coli* and other bacteria, but were different from those of higher organisms.

The pH optimum for CDP-diglyceride synthetase was 6.5–7.5. Mg^{++} stimulated the activity. The Km values for CTP and phosphatidic acid were 1 mM and 2 mM, respectively. The pH optimum for phosphatidylserine synthetase was 8–9.5. The enzyme activity strongly required Mn^{++} (Co^{++}). The Km values for L-serine and CDP-diglyceride were 0.05 mM and 0.3 mM, respectively. The pH optimum for phosphatidylserine decarboxylase was 7.5–8. The Km for phosphatidylserine was 0.2 mM. The pH optimum for phosphatidylglycerophosphate synthetase was 8.5. The enzyme activity strongly required Mn^{++} or Zn^{++} (Mg^{++}) and Triton X-100, respectively. The Km values for sn-glycerol-3-phosphate and CDP-diglyceride were 0.09 mM and 0.03 mM, respectively. Experiments to show subcellular distributions demonstrated that these enzymes were located in the membrane.

The enzyme properties of both phosphatidylserine synthetase and phosphatidylglycerophosphate synthetase using a common substrate, CDP-diglyceride, were compared with each other to elucidate the regulation of the two enzymes for phospholipid synthesis. The effects of metal ions, especially the concentration of Mn^{++} and the presence(s) of materials like Triton X-100, in addition to the fatty acyl groups of CDP-diglyceride, seemed to be involved in the regulating mechanisms of both enzymes in *M. lepraemurium*.

Comparison of the properties of these enzymes of *M. lepraemurium* with those of other bacteria showed that there were both similarities and differences. In phosphatidylserine synthetase, two different types of properties are seen in Gram-negative and Gram-positive bacteria. This enzyme of *M. lepraemurium* was similar to that of the Gram-positive one.

Since phospholipids are membraneous components and have an influence on the nature of membranes, the elucidation of the phospholipid metabolizing enzymes of *M. lepraemurium* was considered to be important in order to understand the nature of cell membranes characteristic for parasitic, pathogenic mycobacteria. (Abbreviations: CDP = cytidine 5′-diphosphate; CTP = cytodine 5′-triphosphate; sn = stereospecific number)—[National Institute for Leprosy Research, Higashimurayami-shi, Tokyo, Japan]

Nakamura, M. Biochemical properties of *Mycobacterium lepraemurium* cultivated in cell-free liquid medium.

It was previously demonstrated that liposome, aspartic acid, and sucrose stimulated the growth of *Mycobacterium lepraemurium* (Mlm) in cell-free liquid medium. ND-5 medium was modified by these ingredients and NDLAS medium was established. After this presentation, it was found that the growth of Mlm was more stimulated by 10% egg yolk than by liposome. The present paper is to report morphological features and biochemical features, such as a cord factor, composition of protein, and plasmids in Mlm grown in the medium containing egg yolk instead of liposome.

A cord factor could be extracted from *in vitro*-grown Mlm and was determined to be identical to that from *in vivo* bacilli reported by Goren, *et al.*

There was a difference in the number of polypeptides observed by SDS polyacrylamide gel electrophoresis between *in vitro*- and *in vivo*-grown Mlm, namely the number of polypeptides in *in vivo*-grown Mlm was less. On the other hand, there was not much difference between *in vivo*- and *in vitro*-grown Mlm in the amino acid composition of whole cells. These findings possibly suggest that *in vivo*-grown Mlm may be more rigid than *in vitro*-grown Mlm. There was a lot of similarity of polypeptide bands between *in vitro*-grown Mlm and *M. avium*. The results obtained from the experiment on plasmid extraction suggested the presence of plasmids in Mlm grown *in vitro* un-

der a certain condition.—[Department of Microbiology, Kurume University School of Medicine, Kurume 830, Japan]

Ozawa, T., Kikuchi, I., Sasazuki, T., Sanada, K. and Koseki, M. Immunogenetical studies of leprosy in Japan.

Genetic control of the clinical course of leprosy was investigated using 43 unrelated patients with leprosy and 54 members of multiple-case families. HLA-DR2 and MT-1 were significantly increased in 23 unrelated patients with tuberculoid leprosy compared to normal controls (relative risk = 3.08, $\chi^2 = 5.09$; relative risk = 9.30, $\chi^2 = 6.18$, respectively). Seven out of 13 affected sib-pairs with lepromatous leprosy shared two HLA haplotypes; six shared one HLA haplotype. This haplotype distribution significantly differed from the random distribution ($\chi^2 = 7.60$, D.F. = 2, $p < 0.025$).

Tuberculoid patients showed higher immune responses to *Mycobacterium leprae* antigen *in vitro*; whereas lepromatous patients did not show any response to this antigen. The nonresponsiveness of lepromatous patients was antigen specific. The antigen-specific nonresponsiveness to *M. leprae* was controlled by T lymphocytes and not by macrophages. T lymphocytes from tuberculoid patients showed strong responses to *M. leprae* antigen in the presence of HLA-DR identical or haploidentical lepromatous patients' macrophages. On the other hand, T lymphocytes from lepromatous patients failed to respond to this antigen, even in the presence of HLA-DR identical or haploidentical tuberculoid patients' macrophages. These observations suggested that an HLA-linked, disease susceptibility gene controlled the clinical course of leprosy through immune regulation by T lymphocytes.—[National Institute for Leprosy Research; Department of Genetics, Medical Research Institute, Tokyo Medical and Dental University; National Leprosarium Tama-Zenshoen, Tokyo, Japan]

Miller, R. A., Gillis, T. P., Khanolkar, S. R., Young, D. B. and Buchanan, T. M. Further studies of monoclonal antibodies directed against defined mycobacterial antigens present in *Mycobacterium leprae* and other mycobacteria.

A collection of murine monoclonal antibodies have been produced that are directed against protein and polysaccharide antigens of *Mycobacterium leprae*. Many of the monoclonals recognize antigen epitopes which appear to be widely distributed within the mycobacterial genus; others have a more restricted distribution, and two are specific for *M. leprae*. The majority of those whose specificity has been identified bind to epitopes present on a 68,000 dalton protein, with several of the same monoclonal antibodies also binding to a protein moiety migrating at approximately 59,000 daltons. Another monoclonal antibody was specific for arabinomannan, a carbohydrate antigen found throughout the mycobacterial genus, and another appeared to be specific for a low molecular weight protein of approximately 14,000 daltons.

The eight monoclonal antibodies to the 68,000 dalton protein were produced from two separate hybridoma fusions. In one, the mice were immunized with partially purified intact *M. leprae* and in the other, a lithium acetate extract of *M. leprae* plus the cell wall pellet served as the immunogen. Identification of antigen specificity was performed by Western Blot and GIRA methodologies. Three of the antibodies were IgM. These three antibodies recognized both the 68,000 and 59,000 dalton proteins. Using a supernatant from sonicated mycobacteria as antigen in an ELISA test, two of these antibodies strongly recognized all 18 species of mycobacteria tested, including *M. leprae*. The third IgM antibody bound very poorly to the *M. leprae* sonicate but recognized with strong and nearly equal affinity the remaining 17 species. The five remaining monoclonal antibodies to the 68,000 dalton protein were all IgG, and varied in their degree of specificity for *M. leprae*. Three reacted with between three and six other species of mycobacteria, and two out of these three bound to both the 59,000 dalton and 68,000 dalton proteins. The last two were highly specific for *M. leprae* and bound to only the 68,000 dalton protein. None of the IgG monoclonals were widely crossreactive, in contrast to the IgM monoclonals. The IgG monoclonals, even when present in great excess, did not interfere with the binding of the IgM monoclonal antibodies. Treatment of the antigen preparation with proteinase

K abolished binding by all the monoclonals, but oxidation with sodium metaperiodate had no effect. Surface immunofluorescence could not be demonstrated for any of these monoclonal antibodies when tested with intact bacilli. The IgG monoclonal antibodies to the 68,000 dalton protein appear promising tools for the purification of this protein through techniques of specific immunoadsorption.

The monoclonal antibody directed against arabinomannan was obtained from a hybridoma fusion using spleen cells from a mouse immunized with partially purified arabinomannan from *M. smegmatis* suspended in incomplete Freund's adjuvant. It was an IgM monoclonal and reacted in ELISA with soluble antigen fractions from all 18 species of mycobacteria tested. The specificity of the monoclonal antibody was demonstrated by both Western Blot and GIRA analysis.

Two discrete, but somewhat diffuse bands in the 60–85,000 daltons region of the gel were recognized by both techniques. These bands were analogous to those seen when immune human sera was reacted with purified arabinomannan in a Western Blot. Binding of the monoclonal to microtiter plates coated with purified arabinomannan was inhibited by pre-incubation with mycobacterial arabinomannan, but not by D-arabinose, D-mannose, yeast mannan, or plant arabinogalactan. Using ascites diluted 1:2500 in an ELISA assay with plates coated with purified arabinomannan, 50% inhibition was produced by pre-incubation with 275 ng of purified arabinomannan, and 20% inhibition resulted from 30 ng. Surface immunofluorescence could not be demonstrated with *M. leprae* organisms purified by the Draper 1979 procedure.

Finally, a single monoclonal antibody was identified on Western Blot as specific for a low molecular weight antigen of approximately 14,000 daltons.

Research into the roles played by the specific components of *M. leprae* in the overall pathogenesis of infection (e.g., attachment, intracellular survival, virulence, etc.) have been hampered by the lack of monovalent antisera or purified antigens. Monoclonal antibodies such as those described above may prove useful in future studies by allowing blockage of specific antigenic sites on the bacilli, and by facilitating purification of important antigenic proteins.—[Immunology Research Laboratory, Seattle Public Health Hospital, University of Washington, Seattle, Washington; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Acknowledgments. This research was supported by the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, by the Rockefeller Foundation Program for Research on Great Neglected Diseases, and by the Heiser Foundation Fellowship Program in Leprosy.

Atlaw, T. and Roder, J. C. The production of human monoclonal antibodies against *Mycobacterium leprae.*

The hybridoma technique has provided monoclonal antibodies for the diagnosis and potential therapy of many infectious diseases and as molecular probes for elucidating the biological mechanisms of infection. Leprosy is a very poorly understood disease that poses severe health problems, mainly due to the lack of reliable and sensitive diagnostic methods for early detection. We report here a system which has the potential for generating human monoclonal antibodies against species-specific antigens of *Mycobacterium leprae* for potential use in 1) the early diagnosis of leprosy, 2) isolation of antigen(s) for vaccines, and 3) studies of idiotype regulation.

B cell lines from lepromatous leprosy (LL) patients were established by Epstein-Barr virus (EBV) transformation of peripheral blood lymphocytes. These LL-lines were fused with the GM-1500-derived, B-lymphoblastoid cell line, KR-4, which is ouabain resistant and HAT sensitive [D. Kozbor, A.E. Lagarde, J.C. Roder (1982) Proc. Natl. Acad. Sci. U.S.A. **79**:6651–6655]; 10^7 KR-4 cells were fused with 10^7 LL cells in the presence of 45% polyethylene glycol (w/v), and the hybrid cells were selected in hypoxanthine/aminopterin/thymidine(HAT) medium containing 10 μm ouabain. Supernatants from surviving hybrids were tested for anti-*M. leprae* antibody production using the enzyme linked immunosorbent assay (ELISA). Positive hybrids were sub-

cloned and tested for anti-*M. leprae* antibody production. In the ELISA screening tests, we used three different *M. leprae* antigen preparations, namely *M. leprae* soluble sonicates (MLS), *M. leprae* SDS extracts of the pellets obtained from centrifugation of sonicates (MSE), and a purified phenolic glycolipid preparation from *M. leprae* (M-GLIP), kindly provided by Dr. Patrick Brennan. Out of 1760 hybrid supernatants tested against MLS and MSE, 131 wells were positive for anti-MLS antibody production and 240 were positive for anti-MSE antibody production. Upon partial cloning and testing of the positive hybrids, we found that out of 3049 partial clones, 217 were positive for MLS and 340 for MSE antigens. One hundred seventy-six hybrids were screened for anti-M-GLIP antibody production and nine were found to be positive. Twenty-three out of 264 partial clones were positive for anti-M-GLIP positivity. Following final cloning, we found 148 (out of 2200) MLS positive, 211 (out of 2200) MSE positive, and 21 (out of 168) M-GLIP-positive clones.

In preliminary experiments to test the degree of crossreactivity of our *M. leprae*-reactive clones, we screened supernatants from 122 clones against the MLS and MSE antigen preparations and 48 clones against the M-GLIP antigen from four other mycobacteria (MTB, M.BCG, *M. smegmatis* and *M. phlei*). Six were specific for *M. leprae* protein antigens. From 48 clones screened for M-GLIP, none showed specificity for *M. leprae*. The major isotype of immunoglobulin produced by the *M. leprae*-reactive clones was found to be IgM and up to 10 μg/ml of this immunoglobulin was produced by these clones.

Human monoclonal antibodies specific to *M. leprae* will have a diagnostic potential in clinical and subclinical leprosy infections. Furthermore, the use of human monoclonal anti-idiotypic antibodies as therapeutic agents in certain clinical cases is a matter for consideration.

This work was supported by the World Health Organization (WHO).—[Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada]

Abe, M., Yoshino, Y., Minagawa, F., Miyaji, I., Sampoonachot, P., Ozawa, T., Sakamoto, Y. and Saito, T. Salivary immunoglobulins and antibody activities in leprosy.

The technics of immunodiffusion and the fluorescent leprosy antibody absorption (FLA-ABS) test were used for determining the levels of IgG, IgM, and IgA and their antibody activities against *Mycobacterium leprae* in the serum and the saliva from a total of 110 patients with leprosy of which 50 were lepromatous, 24 borderline, and 36 tuberculoid. The serum IgG, IgM, and IgA levels were not significantly different among these patients. In saliva, IgM was detected in only two cases with lepromatous leprosy and in three cases with tuberculoid disease. The concentrations of IgG and IgA in saliva and their ratios to those in serum were also not significantly different according to the classification of leprosy.

The percentages of positive FLA-ABS tests in the serum and saliva were compared by using fluorescent antibodies monospecific to IgG, IgM, and IgA, respectively. The results indicated that *M. leprae*-specific antibodies in the serum were mainly found in the IgG and IgM classes and less frequently in IgA. The IgG antibodies were found more frequently in lepromatous and borderline patients than in tuberculoid patients. Salivary IgA antibodies against *M. leprae* were found in a significant number of the specimens; whereas IgG and IgM antibodies were scarcely found. The percentage of positive reactions caused by salivary IgA antibodies was higher in tuberculoid and borderline leprosy cases than in lepromatous patients. A significant number of patients with tuberculoid and borderline leprosy secreted *M. leprae*-specific IgA antibodies into saliva without the production of circulating IgA antibodies. The deficiency of salivary IgA antibodies in lepromatous leprosy patients was not due to a lowered secretion of IgA in saliva, because the average levels of IgG and IgA did not show significant differences between the IgA antibody-positive and IgA antibody-negative groups of patients, irrespective of the classification of leprosy. The immunological significance of these find-

ings is discussed with reference to recent knowledge on local defense mechanisms in mucous membranes.—[National Institute for Leprosy Research, Higashimurayama-shi, Tokyo; National Leprosarium Okinawa Airaku-en, Nago-shi, Okinawa, Japan]

Buchanan, T. M., Dissanayake, S., Young, D. B., Miller, R. A., Acedo, J. R., Harnish, J. P., Khanolkar, S. R. and Estrada-Parra, S. Evaluation of the significance of antibodies to phenolic glycolipid of *Mycobacterium leprae* in leprosy patients and their contacts.

The phenolic glycolipid (PG) of *Mycobacterium leprae* contains core lipid and phenol ring moieties that are shared with other mycobacteria, and a terminal trisaccharide that has been found only on the leprosy bacillus. Since this molecule is produced in quantity by *M. leprae*, an immunoassay that measures antibody predominantly to the unique trisaccharide might prove useful for the detection of subclinical infection, and for the early diagnosis of leprosy. To test this possibility, the ELISA employing deacylated PG as described by Young and Buchanan was used to evaluate more than 3000 sera from 2179 individuals. These persons included 578 patients with leprosy treated from 0–20 years, 342 "normal" controls from Seattle (123), Sri Lanka (118), and Culiacan, Mexico (101), and 1261 household contacts of leprosy patients in Sri Lanka (688) and Mexico (573). Approximately 500 separate families have been evaluated in these studies with the intent of selecting in each country 100 families that contain household contacts with elevated antibody levels to PG, and 100 families without elevated antibodies to PG except for the patient. These families will then be studied in greater detail over a period of several years to define the risk factors associated with the subsequent development of leprosy.

For 200 patients in Sri Lanka who were carefully characterized according to Ridley-Jopling criteria, there was a direct correlation between the level of antibody to PG and the class of disease. Specifically, the mean A_{492} for 66 patients with LL or BL disease was 0.62, and that for 63 BT patients and 71 TT patients was 0.33 and 0.11, respectively. Furthermore, in the Seattle and Sri Lanka patients from whom serial serum samples were available, the antibody levels to PG consistently declined with chemotherapy. Our observation of an overall rate of seropositivity of 63% (363/578) for the total patient group must therefore reflect both the type of leprosy and the duration of treatment of the patients studied. Irrespective of treatment duration, using a definition of seropositive as an A_{492} of >0.09 at a serum dilution of 1:20, the seropositivity rate by type of leprosy for the 200 well-characterized Sri Lanka patients was 39% of 71 TT cases, 56% of 63 BT, 76% of 25 BB, 82% of 22 BL, and 89% of 44 LL cases.

Elevated antibody levels to PG were found in a small percentage of individuals with no known direct exposure to leprosy. Specifically, 5% of the "normal" controls from Seattle (6/123), 5% of those from Culiacan, Mexico (5/101), and 9% of those from Sri Lanka (11/118) were seropositive. It is likely that the Seattle controls represent "false positives" for the test. Their antibodies reacting with the *M. leprae* PG may have been formed initially against the phenol and lipid moieties shared with other mycobacteria, and/or oligosaccharides of other as yet unidentified microorganisms antigenically similar to the trisaccharide portion of the PG. Antibodies in sera from some of these reactive "normal" control sera recognize the PG of *M. kansasii*, which contains the same phenol and lipid moieties in its structure as found in the PG of *M. leprae*. Whether the higher reactivity rate in "normal" controls from Sri Lanka reflects both false positivity and some previously undetected exposure to the leprosy bacillus is as yet undetermined. Perhaps the monoclonal antibodies specific for the terminal saccharide or trisaccharide portion of the PG of *M. leprae* will help to answer this question.

The overall seropositivity rates to PG in household contacts of leprosy patients was 23% (133/573) of those from Culiacan, Mexico, and 33% (230/688) of those from Sri Lanka. It is unlikely that all of these persons will develop leprosy, and further criteria are needed to distinguish those who

become immune to continuation of the infection from those who will subsequently develop the disease. At least three serum samples, collected 6-12 months apart over a period of 2½ years, were available from 112 household contacts of leprosy patients in Sri Lanka. Fifty (45%) of these contacts showed no evidence of elevated antibodies to PG during the entire period. Forty-four (39%) had one or two serum samples with elevated antibodies, and in 18 (16%) of the contacts all of the serum samples were seropositive. For the entire contact group, when the index case had elevated antibodies to PG, 22% (13/59) of the contacts were in the consistently seropositive group. In contrast, when the index case was seronegative, only 9% (5/53) of the contacts were in the group with all seropositive samples. All of the 112 contacts were examined on at least three occasions, and two of these contacts were diagnosed as TT leprosy by two different physicians during December 1982 and March 1983. Both of these patients were within the group of 18 contacts with consistently elevated antibody levels to the *M. leprae* PG, and each had been seropositive at least 1½ years before the onset of clinical disease. One maintained ELISA A_{492} levels of 0.14–0.19 and the other maintained levels of 0.38–0.43 throughout the followup period. Biopsies have been obtained from these individuals and will be studied for the presence of epithelioid granulomas, acid-fast bacilli, and PG antigen. These two new presumptive cases were among 39 seropositive contacts on the first evaluation. Twenty-five of these seropositive contacts remained seropositive in their second serum sample, and 18 of these were seropositive in their third sample. With further evaluation, the ELISA to PG may permit identification of those at greatest risk of developing leprosy as a subgroup of only 16%–35% of the original household contacts studied. If the above example is representative of leprosy development over a 2½-year period in 112 household contacts in Sri Lanka, one might expect that 12 new cases developed during the same period in the total group of 688 contacts, and that all of these new cases were within a subset of 16% of the total group whose sera contained elevated antibodies to the PG of *M. leprae*. The ELISA with defined criteria of high predictive value might have allowed identification of these 12 new cases while concentrating on only 110 of the total group of 688 contacts. ELISA criteria that will be studied for their predictive value will include consistent seropositivity, very high antibody levels ($A_{492} > 0.5$), and antibody levels that increase during the followup period. The test may ultimately prove of the greatest value to detect persons who will subsequently develop lepromatous leprosy, since the incubation period from first symptoms to first diagnosis for lepromatous disease is commonly eight years, and there is likely to be an extended period when the contact has elevated antibody levels to PG and leprosy bacilli in their skin at concentrations of $>10^5$ per gram of tissue.—[Immunology Research Laboratory, Seattle Public Health Hospital, University of Washington, Seattle, Washington, U.S.A.; Department of Biochemistry, Faculty and School of Medicine, University of Peradeniya, Peradeniya, Sri Lanka; Salud Publica, Culiacan, State of Sinaloa, Mexico; Department of Dermatology, University of Washington, Seattle, Washington, U.S.A.; Department of Immunology, National School of Biological Sciences, IPN, Mexico D.F., Mexico; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

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Cho, S.-N., Yanagihara, D. L., Hunter, S. W., Gelber, R. H. and Brennan, P. J. Serological specificity of phenolic glycolipid-I from *Mycobacterium leprae* and use of serodiagnosis in leprosy.

The serological activity of the specific phenolic glycolipid-I from *Mycobacterium leprae,* its dissected parts, and related glycolipids from other mycobacteria was examined by enzyme-linked immunosorbent

assay against hyperimmune anti-*M. leprae* rabbit antiserum and sera from patients with leprosy and other mycobacterial diseases. High anti-phenolic glycolipid-I immunoglobulin M antibodies were found in 23 of 24 (96%) lepromatous leprosy patients on short-term chemotherapy, and in 8 of 13 (62%) tuberculoid leprosy patients. Sera from patients with tuberculosis or atypical mycobacterial infections were devoid of anti-phenolic glycolipid-I activity. The structurally related phenolic glycolipids from *M. kansasii* and *M. bovis* and the aglycone segments of the *M. leprae* product showed no significant activity. Thus, the trisaccharide determinant of phenolic glycolipid-I is specific in its structure, serological activity and, to a lesser extent, the antibody class it evokes.—[Colorado State University, Fort Collins, Colorado 80523; U.S. Public Health Service, Hansen's Disease Program, Seton Medical Center, Daly City, California 94015, U.S.A.]

Young, D. B. and Buchanan, T. M. Development of an enzyme-linked immunosorbent assay (ELISA) to measure antibodies to the phenolic glycolipid of *Mycobacterium leprae.*

A phenolic glycolipid derived from *Mycobacterium leprae* has been found in large amounts in leprosy-infected tissues of human and armadillo origin. Its core lipid and phenol ring moieties have been biochemically characterized as related to similar glycolipids of the mycoside A group as represented by *M. kansasii,* or the mycoside B group as represented by *M. bovis.* Hunter and Brennan, and Hunter, *et al.* have determined the composition and sequence structure of the trisaccharide attached to the phenol and lipid moieties of *M. leprae* as a terminal 3,6-di-O-methyl glucose linked beta 1-4 to a 2,3-di-O-methyl rhamnose which is linked alpha 1-2 to a 3-O-methyl rhamnose which is, in turn, linked to the phenol ring through an alpha linkage. This structure has been termed phenolic glycolipid-I by these authors and no compound of this structure has been previously described, suggesting the possibility that the trisaccharide might be an antigen found only on the leprosy bacillus. An immunoassay that could accurately quantitate the specific trisaccharide antigen and not the shared antigens on the molecule might prove useful to quantitate exposure to *M. leprae.* We have developed an ELISA procedure that measures predominantly the trisaccharide antigen(s) of the phenolic glycolipid (PG), and the development of the assay and its characteristics are described below.

The *M. leprae* phenolic glycolipid (PG) was purified from supernatant material derived from homogenization of infected armadillo liver using the Bligh-Dyer monophasic extraction method followed by elution from a silica gel-celite column with 2% (v/v) methanol in chloroform and preparative thin-layer chromatography on silica gel G plates developed with chloroform : methanol (15:1, v/v). The phenolic glycolipid from *M. kansasii* was purified in the same way from bacteria grown on Middlebrook's 7H11 medium. The identities of the glycolipids were established by combined gas-liquid chromatography and mass spectroscopy of the alditol acetate derivatives generated by trifluoracetic acid hydrolysis followed by borohydride reduction and acetylation with acetic anhydride. Comparison of mass spectrograms of derivatives from the two glycolipids with those of standard partially methylated alditol acetates confirmed the carbohydrate composition of the *M. leprae* glycolipid as 2,3-di-O-methyl rhamnose, 3-O-methyl rhamnose, 3,6-di-O-methyl glucose, and that of the *M. kansasii* glycolipid as 2,4-di-O-methyl rhamnose, 2-O-methyl rhamnose, 2-O-methyl fucose.

Our initial attempts to utilize the native PG of *M. leprae* were discouraging. The native molecule was insoluble in aqueous environments normally utilized for the ELISA methodology, and organic solvents created slight damage to the polystyrene or polyvinyl microtiter plates resulting in nonspecific binding of immunoglobulins. Detergents such as deoxycholate or Tween did not produce adequate exposure of the trisaccharide moiety, or resulted in excessive removal of the PG antigen coated to the plates.

In order to decrease the hydrophobicity of the native PG molecule and facilitate its use in ELISA tests it was deacylated under alkaline conditions. The two long chain fat-

ty acids from the phthiocerol core were hydrolyzed from the native molecule in 10% (w/v) methanolic NaOH at 100°C for 16 hr. The deacylated PG was recovered by preparative thin-layer chromatography (TLC). The TLC migration characteristics of the deacylated and native PG molecules demonstrated that deacylation had occurred. For both the *M. leprae* and *M. kansasii* glycolipids, the more polar deacylated molecules migrated less rapidly than the native molecule in the organic solvents used for TLC, and no native PG remained after the deacylation treatment.

To test whether the trisaccharide antigen was preserved following the deacylation conditions, equal concentrations of the native and deacylated PG molecules (based upon carbohydrate content using rhamnose as a standard) were compared in the ELISA. A concentration of 32 micrograms/ml of the purified native PG produced an A_{492} of 0.24 as compared to an A_{492} of 0.32 produced by only 1 microgram/ml of the purified deacylated PG. Thus, once separated from its natural configuration in the cell wall of the leprosy bacillus, the purified deacylated molecule had approximately 40-fold greater antigenicity than the purified native PG when used in aqueous systems such as the ELISA. In addition, only 4 micrograms/ml concentrations of the deacylated molecule were required to produce A_{492} of 1.2, an absorbance level not possible with practical concentrations of the purified native PG. These differences in ELISA performance of the purified native and deacylated PG molecules presumably relate to the hydrophobicity of the native molecule and its insolubility in aqueous immunoassay systems, resulting in concealment of the unique trisaccharide antigenic moiety by the excess lipid. When the molecule is deacylated, approximately one half of the total lipid of the native PG is removed, and 50 microgram/ml concentrations of the deacylated molecule form a clear solution, in contrast to the visibly turbid suspension character of the same concentrations of the native PG. With the deacylated PG, coating concentrations of 5 micrograms/ml or 0.5 micrograms per well produced maximal absorbances with serum specimens from leprosy patients tested at a 1:20 dilution.

To demonstrate that the ELISA employing deacylated *M. leprae* PG recognized the trisaccharide component of the molecule, the ELISA was inhibited by native and deacylated PG from *M. leprae* and *M. kansasii*. Preincubation of leprosy patients sera with the glycolipids in 1 microgram/ml concentrations prior to adding the mixture to the ELISA plates coated with deacylated PG produced 70% inhibition of subsequent absorbance with *M. leprae* but only 10% inhibition with *M. kansasii* glycolipids.

The ELISA employed utilized peroxidase enzyme conjugated to goat IgG immunoglobulin directed at human IgG, IgM, and IgA. We reasoned that this might be important if the dominant human immune response to the PG antigen was IgM, similar to the human immune response to other glycolipids. Conjugate capable of detecting IgM antibodies has not been employed by other investigators who have developed quantitative immunoassays for *M. leprae* antigens. To test the importance of this concept, we evaluated the human antibody levels to PG in sera from 24 leprosy patients with peroxidase conjugates capable of detecting all three immunoglobulin classes, or specific for IgM, IgG, or IgA. The mean A_{492} for the 24 sera with the conjugate capable of detecting all three immunoglobulin classes was 0.89. In contrast, the mean A_{492} for the class-specific enzyme conjugates was 0.98 (IgM), 0.22 (IgG), and 0.28 (IgA). In addition, all 24 patients had detectably elevated antibody levels to PG when the enzyme conjugates to IgM, or to IgM, IgA and IgG were used, but only 14 patients and 17 patients were detected with IgG and IgA specific conjugates, respectively. Thus it is very important to utilize enzyme conjugates capable of measuring IgM antibodies in ELISA tests designed to measure antibodies to the PG antigen.

In our laboratory's previous experience, IgM antibodies from human and other sources frequently stick to plastic surfaces more nonspecifically than IgG or IgA immunoglobulins. For this reason, the ELISA for measuring antibodies to PG utilizes microtiter plate wells that are not coated with any antigen, in addition to wells coated with deacylated PG. The absorbance values are then expressed as the A_{492} developed in PG

coated wells minus the A_{492} developed in microtiter plate wells containing no antigen. Using this method, the usual A_{492} range for normal human sera at a dilution of 1:20 was −0.05 to 0.05. An A_{492} of greater than or equal to 0.1 was considered positive.

The specificity of the PG ELISA was further evaluated using sera from groups having different exposure to mycobacteria. These were: a) normal individuals from the Seattle area having no known mycobacterial infections; b) volunteers who had recently been vaccinated with BCG; c) patients from Mexico with highly advanced tuberculosis infection; these patients all had high levels of antibodies against *M. tuberculosis* antigens as judged by counter-immunoelectrophoresis; d) patients with mycobacterial infections other than leprosy and tuberculosis (including infection with *M. kansasii, M. chelonei, M. avium-intracellulare* and *M. fortuitum*) all of whom had elevated titers of anti-mycobacterial antibodies as judged by an ELISA technique employing mycobacterial arabinomannan as antigen; e) leprosy patients from Seattle and Sri Lanka who were clinically and histologically characterized according to the Ridley-Jopling criteria. While the control groups were uniformly negative by the PG ELISA ($A_{492} < 0.1$), 73% of the sera from 60 leprosy patients were positive. Also, the mean antibody levels directly correlated with the clinical classification of the leprosy patients, with the highest mean A_{492} observed in lepromatous patients (.82) and progressively lower levels in patients with lower bacterial loads—BL (.73), BB (.67), BT (.32), and TT (.12).

The results reported here suggest that the ELISA employing deacylated PG is a potentially specific assay for detection of a humoral response to infection with *M. leprae*. It will be of particular interest to apply such a test to detection of the very early stages of leprosy infection. The test is quantitative and objective, and requires no pre-adsorption of the sera to be tested. It is easy to perform, and should prove suitable for large scale epidemiologic studies.—[Immunology Research Laboratory, Seattle Public Health Service Hospital, University of Washington, Seattle, Washington; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Acknowledgments. This research was supported in part by the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and by the Rockefeller Foundation Program for Research on Great Neglected Diseases.

Khanolkar, S. R., Young, D. B., Barg, L. L. and Buchanan, T. M. Generation and characterization of monoclonal antibodies to the *Mycobacterium leprae* phenolic glycolipid.

The phenolic glycolipid (PG) of *Mycobacterium leprae* is an important and useful antigen apparently unique to the leprosy bacillus. In order to further explore the immunochemical properties of this molecule, we have generated a set of monoclonal antibodies to the glycolipid.

BALB/c mice were immunized by intraperitoneal injection of the phenolic glycolipid complexed with methylated BSA. Three days after a final intravenous injection of antigen, mice were sacrificed and their spleen cells fused with the myeloma cell line NS1. Hybrid cells producing antibodies directed against the glycolipid were identified by the ELISA technique using deacylated PG as antigen, and clones of such cells were established by the standard monoclonal antibody techniques used in our laboratory. The cloned cell lines were injected into the peritoneal cavity of pristane-primed mice, and the resulting ascitic fluid was used as a source of monoclonal antibodies. All of the antibodies produced were found to belong to the IgM class of immunoglobulins when tested by immunodiffusion using class-specific, anti-mouse antibody reagents.

Of the nine cloned cell lines, one produced antibodies which reacted in an ELISA assay with the deacylated phenolic glycolipid from *M. kansasii* in addition to *M. leprae*; whereas the remaining eight recognized only the *M. leprae* glycolipid. One of the *M. leprae*-specific antibodies was purified by gel filtration and labelled with ^{125}I. Its binding to deacylated PG was susceptible to competitive inhibition by each of the other antibodies and by serum from a lepromatous leprosy patient. The specificity of the antibody was investigated by direct in-

teraction of the ^{125}I-labelled antibody with TLC plates on which a variety of lipids had been chromatographed. Subsequent autoradiography showed a clear interaction between the monoclonal antibody and the *M. leprae* PG in its native and deacylated forms with no detectable binding to the *M. kansasii* phenolic glycolipid. Removal of the terminal sugar residue from the *M. leprae* glycolipid by mild acid hydrolysis resulted in loss of recognition of the molecule by the labelled antibody. This demonstrates the key role of the terminal sugar (3,6-di-O-methyl glucose) in the antibody-antigen interaction.

Each antibody was tested against a panel of 20 mycobacteria in an ELISA assay in which whole organisms are bound to microtiter plates. All of the antibodies bound strongly to *M. leprae* in an interaction which could be inhibited by preincubation of the antibody with deacylated glycolipid prior to addition to the ELISA plate. Similarly, binding of the antibodies in the deacylated PG ELISA was inhibited by preincubation with whole *M. leprae*. One of the antibodies reacted with all of the mycobacteria tested, while the remainder showed strong specificity for *M. leprae* with some weak reactions being evident only in the case of *M. terrae*, *M. nonchromogenicum* and one strain of *M. bovis*. These three strains were also able to inhibit binding of monoclonal antibodies to deacylated PG, although only when added at concentrations 5–10-fold higher than that required for inhibition by *M. leprae*.

The monoclonal antibodies showed a strong reaction with *M. leprae* in immunofluorescence experiments, generating a fluorescent zone around the bacilli which was particularly marked in bacterial clumps. These experiments provide convincing visual evidence to support the proposed role of the phenolic glycolipid in the formation of a glycolipid capsule surrounding *M. leprae*.

Potential uses of monoclonal antibodies to the phenolic glycolipid in the further study of the pathology of leprosy and for quantitative detection of small amounts of antigen in infected tissues were discussed.—[Immunology Research Laboratory, Seattle Public Health Hospital, University of Washington, Seattle, Washington; National Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.]

Acknowledgments. This research was supported in part by the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and by the Rockefeller Foundation Program for Research on Great Neglected Diseases.

Smith, J. H., Folse, D. S., Long, E. G., Barksdale, L. S., Imaeda, T. and Meier, J. L. Sylvatic leprosy in wild armadillos of the Texas Gulf Coast.

Armadillo-human contact in Texas is increased because of local mores. For this reason, it was imperative to survey Texas armadillos for the "naturally occurring leprosy-like disease of wild armadillos." Efforts were concentrated in counties along the Gulf Coast where human leprosy remains endemic; 451 armadillos were screened by physical examination, ear-snip and slit-scrape technique. After ketamine HCl anesthesia, armadillos which had been positive on screening were scrubbed with detergent for 5+ min, then scrubbed with Povidone iodine solution for 5+ min, rinsed in 70% ethanol, and sacrificed by cardiac puncture and exsanguination. Aseptic autopsy was performed and specimens for mycobacterial culture were taken from liver, spleen, bilateral axillary-cervical lymph nodes, bilateral superficial inguinal lymph nodes, and any gross lesions. Specimens from the same sites and a wide variety of other tissues were fixed in 10% neutral-buffered formalin, sectioned and stained with hematoxylin and eosin and Fite-Faraco stains for histologic analysis. Multiple 1 mm^3 blocks of liver and spleen from heavily infected armadillos were fixed in half-strength Karnovsky's solution, embedded in Polybed 812 and examined electron microscopically. Residual aseptic tissue from the liver, spleen and large lesions were frozen at −70°C and aliquots of these were used for DNA homology studies. Additionally, residual tissue from the ear-snip screening procedure was fixed in 10% formalin, sectioned, and stained with Fite-Faraco stain, and examined light microscopically. Tissues for culture were homogenized and con-

centrated; concentrates were inoculated onto Middlebrook 7H10 and Löwenstein-Jensen media and incubated at 25°C, 30°C and 35°C for 12 weeks with weekly inspection. Isolated mycobacteria were identified by standard techniques.

Thirty-four armadillos were sacrificed; 17 of these proved to have leprosy-like disease by light microscopy; an additional four cases were discovered upon examination of earsnip residues. Guanosine and cytosine percentage and genome size of organisms, extracted from the liver of two armadillos and a lesion from one of the armadillos, were identical to organisms extracted from a lesion in a Florida armadillo inoculated with lepromatous tissue from a human from Thailand; DNA homologies of the organism from wild armadillos and the experimental infection were 100%. Thus, the disease in armadillos in Texas is caused by *Mycobacterium leprae* and is sylvatic leprosy.

Sylvatic leprosy was found in 4.66% of wild armadillos of the Texas Gulf Coast with local variations between 0%–15.4%. Prevalence increased from east Texas southwest along the Gulf Coast. In ten leprous armadillos, other mycobacteria grew from one or more sites; these included *M. scrofulaceum, M. avium-intracellulare* and *M. gordonae.* The significance of these other organisms, the histologic and ultrastructural findings, and the significance of zoonotic leprosy were discussed.—[Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77550; Department of Microbiology, New York University School of Medicine, New York, New York 10016; Department of Microbiology; New Jersey Medical School, Newark, New Jersey, 07103, U.S.A.]

Wolf, R. H., Martin, L. N., Gormus, B. J., Baskin, G. B., Gerone, P. J., Walsh, G. P., Meyers, W. M., Brown, H. L. and Binford, C. H. Experimental transmission of leprosy in African green monkeys (*Cercopithecus aethiops*) and the rhesus monkey (*Macaca mulatta*).

The transmission of leprosy to *Cercocebus atys atys* has been described by our laboratories. Both mangabeys which received mangabey-origin *Mycobactaerium leprae* 36 months ago are now in the advanced stages of LL-type leprosy. The two that received armadillo-derived *M. leprae* 27 months ago now have large but localized lesions that continue to spread at various inoculation sites. It appears that, by all criteria, the mangabey can serve as an excellent nonhuman primate model for leprosy. Unfortunately, the mangabey monkey is not widely available in research laboratories. It was therefore considered important to explore the potential host range of susceptibility.

Approximately 25 months ago, we inoculated 2 juvenile rhesus monkeys (*Macaca mulatta*), 3 African green monkeys (*Cercopithecus aethiops*), and 3 squirrel monkeys (*Saimiri sciureus*) with mangabey-derived *M. leprae.* Each was inoculated by both the intravenous and intradermal route except for one juvenile female rhesus monkey which received the organisms only by the intradermal route. The female rhesus monkey and the three squirrel monkeys have not yet developed signs of disease. The second rhesus monkey, a juvenile male, developed the disease at several inoculation sites with dissemination to uninoculated sites by 14 months after inoculation. Massive infiltration of the uninoculated scrotum occurred and lepromas up to 2 cm developed. Enlargement and spread of the lesions continued until about 19 months after inoculation. A biopsy showed a histopathologic pattern in the lepromatous area (LLs) of the scale. Rapid regression began at this time, and the severity and numbers of lesions decreased dramatically, although nasal secretions continued to contain normal-appearing *M. leprae.* Biopsies taken during this time showed histopathologic features consistent with the borderline (BB) area of the spectrum. By 23 months most of the major lesions had been reduced to small erythematous or depigmented foci which lacked dermal thickening.

The process then began to exacerbate markedly and has continued to progress through the 25th month. The reappearance of lesions at sites originally involved and their development at previously uninvolved sites occurred quickly. Current biopsies showed histopathologic patterns consistent with polar lepromatous leprosy.

Approximately nine months ago, 12 additional rhesus monkeys were inoculated with human-derived *M. leprae,* using grad-

ed doses and varied combinations of intravenous and intradermal routes. Additionally, four rhesus monkeys were inoculated with mangabey-derived *M. leprae*. It is too soon to evaluate the possible course of leprosy in these animals.

A small 4 mm nodule developed at one injection site on the ear of one of three African green monkeys three months after inoculation with mangabey-origin *M. leprae*. The lesion remained unchanged for 18 months. Histopathologic examination of the nodule revealed a histiocytic infiltrate containing many acid-fast bacilli (AFB) and small nerve involvement consistent with lepromatous leprosy. No other lesions were observed on this or the other two African green monkeys during this period. AFB were found in nasal smears 20 months after inoculation, and by 23 months the ears of all three monkeys were slightly thickened and erythematous. Small coalescing nodular lesions had developed on the ears of all animals by the 25th month after inoculation. These lesions were histologically compatible with a diagnosis of lepromatous leprosy.

Ten additional African green monkeys inoculated by the intravenous and intradermal route with human origin *M. leprae* 18 months ago have not as yet shown signs of infection.

Our results show that the rhesus monkey and the African green monkey both show significant potential as models for the study of leprosy. The outcome of our more recent inoculation studies are of critical importance in establishing the extent to which these species will be susceptible. Information obtained during the next two years should allow us to determine the usefulness of both the African green monkey and rhesus monkey in leprosy research.—[Delta Regional Primate Research Center, Tulane University, Covington, Louisiana 70433; Armed Forces Institute of Pathology, Washington, D.C. 20306, U.S.A.]

Martin, L. N., Gormus, B. J., Wolf, R. H., Baskin, G. B., Gerone, P. J., Meyers, W. M., Walsh, G. P., Brown, H. L., Binford, C. H., Schlagel, C. J. and Hadfield, T. L. Immunological effects of experimental leprosy in the mangabey (*Cercocebus atys*).

We have previously reported on naturally acquired and experimental leprosy in mangabey monkeys (*Cercocebus atys*). Clinical, bacteriological, and histopathological studies have demonstrated that the disease in mangabeys is similar to human leprosy in the borderline to near-lepromatous forms. The characterizations of the acid-fast bacteria (AFB) from the mangabey with naturally acquired disease have shown that they are identical to human *Mycobacterium leprae* regarding: noncultivability, enhanced staining with the Fite-Faraco technique, extraction of acid fastness with pyridine, oxidation of D-Dopa, antigenicity in skin tests in tuberculoid and lepromatous patients, and DNA homology.

The transmissibility of leprosy in the mangabey has been demonstrated with two different inocula. Two mangabeys were inoculated in March 1980 with a suspension of AFB from biopsies of lesions from the naturally infected mangabey. Two mangabeys were inoculated in December 1980 with a suspension of *M. leprae* from armadillos that had been inoculated with *M. leprae* of human origin. The inoculation method was similar for both preparations. Each animal received an intravenous inoculation of 1.2×10^9 AFB and intracutaneous inoculations of 3×10^8 AFB at each of five sites.

Monkeys inoculated with mangabey *M. leprae* developed lesions at inoculation sites that were histopathologically similar to human borderline to borderline lepromatous leprosy by 4–11 months postinoculation. Disseminated lesions in uninoculated sites developed by 11–20 months. Both animals had AFB in smears of nasal secretions by 29 months when this test was first performed. The two monkeys inoculated with human *M. leprae* had nodular lesions at inoculation sites by 4–11 months but dissemination has been slower. At 24 months postinoculation one of these monkeys had lesions on extremities and AFB in nasal secretions. The second animal had lesions restricted to inoculation sites on the nose and ears. In both animals, lesions at inoculation sites continue to enlarge and extend.

Alterations of the immune system appear to occur in mangabeys with disseminated progressive disease. Lymphocytes from the mangabeys inoculated with mangabey *M.*

leprae became progressively less responsive to phytohemagglutinin, concanavalin A, and pokeweed mitogens during the second and third years postinoculation, when the disease became extensively disseminated. In contrast, progressive depression of mitogen responsiveness was not apparent in lymphocytes from mangabeys inoculated with human *M. leprae*, in which the disease has not disseminated extensively. Mitogen responses by lymphocytes from the naturally infected mangabey were relatively low for one year prior to treatment as lesions became extensively disseminated. Treatment resulted in dramatic clinical improvement with decreased Morphological Indexes, but mitogen responses remained low during 15 months of treatment. However, lymphocyte mitogen responses were increased dramatically 19 months and 24 months after treatment. These results suggest that mitogen responses may be depressed in disseminated leprosy, but that prolonged treatment may allow a subsequent increase in mitogen responsiveness.

The formation of immunoglobulin-producing, plaque-forming cells (PFC) by lymphocytes stimulated with PWM was studied in control and infected mangabeys. The PFC response was significantly reduced in the experimentally infected monkeys. In contrast, the PFC response in the treated naturally infected monkey was in the high normal range. The percentages of "helper" (OKT 4^+ or Leu $3a^+$) and "suppressor" (OKT 8^+) lymphocytes were comparable in infected and control monkeys as has been reported for human patients. Other markers of lymphocyte subpopulations were also similar.

The numbers of blood lymphocytes and certain subpopulations in control and infected mangabeys appeared to vary with a circannual periodicity. Mitogen responsiveness also varied with yearly peaks and depressions. Circannual physiological rhythms must be considered in evaluating longitudinal data in this model.

The depression of lymphocyte blastogenic and PFC responses to mitogens in experimentally infected mangabeys indicates that this model should be useful for studies of mechanisms involved in immunological alterations of leprosy.—[Delta Regional Primate Research Center, Tulane University, Covington, Louisiana 70433; Armed Forces Institute of Pathology, Washington, D.C. 20306, U.S.A.]

Acknowledgments. This work was supported in part by U.S.P.H.S. grants AI19302 and RR00164. We thank Drs. Frederick King and Harold McClure of the Yerkes Regional Primate Research Center, Atlanta, Georgia, U.S.A. for generous provision of mangabeys for transmission experiments.

Fukunishi, Y., Meyers, W. M., Binford, C. H., Walsh, G. P., Johnson, F. B., Gerone, P. J., Wolf, R. H., Gormus, B. J. and Martin, L. N. Electron microscopic study of leprosy in a mangabey monkey (naturally acquired infection).

Our objective is to report ultrastructural features of the growth of *Mycobacterium leprae* in a mangabey monkey. The mangabey monkey was imported from West Africa in 1975 and housed at Gulf South Research Institute, Louisiana, U.S.A. In 1979, a diagnosis of multibacillary leprosy was made on the basis of clinical and histopathological findings. After transfer to the Delta Regional Primate Research Center, Covington, Louisiana, detailed clinical, histopathologic, immunologic, and bacteriologic studies established that the disease in this mangabey monkey was caused by *M. leprae*. The animal had never been inoculated with *M. leprae*, thus all evidence suggests that the disease was acquired naturally.

MATERIALS AND METHODS

Leproma of the naturally infected mangabey monkey and lepromata and livers of *Dasypus novemcinctus*; leproma of a mangabey monkey, a rhesus monkey and a green monkey inoculated with *M. leprae* isolated from the lesions of the first mangabey monkey were studied.

Portions of the lepromata and livers were fixed with 3% glutaraldehyde in 0.06 M phosphate buffer at pH 7.4 for 24–48 hr at 4°C for ultrathin-section study. The tissues were post-fixed with 2% OsO_4 in distilled water for 24 hr at 4°C. After dehydration, the tissues were embedded in Spurr low viscosity medium, cut by an ultramicrotome with glass knives, and then stained with uranyl acetate and lead nitrate.

The tissues for study by freeze-etching techniques were fixed with 3% glutaraldehyde under the same conditions as those for ultrathin sectioning, and then immersed in 20% glycerol for 24–48 hr at 4°C. Other procedures were the same, as described by Nishiura, *et al.* (Int. J. Lepr. **45**:248, 1977).

RESULTS

Leprosy bacilli multiply in phagolysosomes of macrophages of all these specimens in essentially the same fashion as they do in humans and in armadillos and nude mice inoculated with *M. leprae* isolated from human lepromas.

In ultrathin sections, electron transparent zones were seen around the growing leprosy bacilli. The changes seen in freeze-etched specimens were strikingly similar to those in human and in armadillo and nude mouse lepra cells of animals inoculated with *M. leprae*. The bacilli in these specimens were long and thin and had band structures on smooth cell wall surfaces. Distinct accumulations of small spherical droplets were observed around leprosy bacilli in phagolysosomes.

DISCUSSION

The small spherical droplets in lepra cells observed by the freeze-etching technique are believed to be unique for *M. leprae* infection. These droplets appear as peribacillary substance and are believed to contain specific phenolic glycolipids of *M. leprae*.

All the observations made on these specimens provide further evidence that leprosy in the naturally infected mangabey monkey is caused by *M. leprae* and that the disease is like that in humans with lepromatous leprosy.—[Armed Forces Institute of Pathology, Washington, D.C. 20306; Delta Regional Primate Center, Tulane University, Covington, Louisiana 70433, U.S.A.]

Acknowledgments. This study was supported in part by the Sasakawa Memorial Health Foundation; the Immunology of Leprosy (IMMLEP) component of the UDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases; Grant #1R22AI19302-01 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health; the American Leprosy Missions, Inc.; the Damien-Dutton Society for Leprosy Aid, Inc.; and the Victor Heiser Program for Research in Leprosy.

Jacobs, W. R., Clark-Curtiss, J. E., Ritchie, L. R. and Curtiss, R., III. Construction and partial characterization of *Mycobacterium leprae* genomic libraries using an *in vivo* cosmid cloning system.

Recombinant DNA technology has made possible a means to study the genetics and physiology of organisms, such as *Mycobacterium leprae*, that heretofore have not been amenable to these studies. We have used recombinant DNA techniques to construct genomic libraries of *M. leprae* in cosmid cloning vectors and have introduced the recombinant molecules into strains of *Escherichia coli* K-12 for further characterization.

Cosmid cloning vectors are plasmids that contain a part of the bacteriophage lambda chromosome (the *cos* site) and thus are able to be packaged within bacteriophage lambda heads in a system which contains all of the proteins necessary to assemble the heads, which occurs spontaneously. Since cosmids are small [5.6–13.5 kilobases (kb)] molecules, large pieces (34–50 kb) of inserted (*M. leprae*) DNA must be ligated to the vector so that the recombinant cosmids can be packaged. After the recombinant cosmids are in the lambda heads, they can be introduced into *E. coli* strains via the lambda transducing mechanism. Once inside the bacteria, the recombinant cosmids behave as plasmids. Because cosmid vectors can accept such large pieces of DNA, each recombinant molecule can potentially code for 25–35 medium-sized proteins, specified by the inserted DNA, which is four to five times as many proteins as can be coded for by inserted DNA in plasmid or lambda cloning vectors.

Recombinant cosmids are usually packaged in an *in vitro* system. We have developed an *in vivo* packaging system as well. Strains of *E. coli* were lysogenized with a lambda bacteriophage that has mutations which a) greatly reduce the frequency with which the prophage can excise from the *E. coli* chromosome, b) preclude recombination between the prophage and any other DNA, and c) allow synthesis of phage head and tail proteins only at certain tempera-

tures. Thus, the prophage DNA cannot be packaged but if there is a recombinant cosmid molecule in the cytoplasm, this will be packaged if the head and tail proteins are present. When the bacterial cell is lysed, a lysate of packaged recombinant cosmids is produced.

We have prepared four genomic libraries of *M. leprae* DNA ligated to two cosmid vectors, pHC79 (a 5.6 kb cosmid possessing genes conferring ampicillin and tetracycline resistance) and pMMB34 (a 13.5 kb cosmid possessing a gene conferring kanamycin resistance). The *M. leprae* DNA and the cosmid DNA were digested with different restriction endonucleases (*Hind*III, *Pst*I and *Sau*3A) to prepare the libraries. Thus, if one restriction endonuclease inactivated an *M. leprae* gene by cutting within the gene, the library prepared from DNA restricted by the other endonuclease should contain that gene intact. Two types of libraries were contructed with the *Pst*I-digested *M. leprae* DNA: one using large fragments of *M. leprae* DNA, in which the genes present on each fragment should be in the same order as they are on the intact chromosome and the other using small fragments of *M. leprae* DNA, in which several small fragments would randomly ligate together to form a sufficiently large insert to ligate to the cosmid. In this latter type of library, the genes within the large fragments would probably not be in the same order as they are on the intact chromosome.

We have analyzed approximately 100 different *M. leprae*::cosmid recombinant molecules by agarose gel electrophoresis and have determined that they are all approximately 50 kb in size. We have also looked for complementation of *dapD, asd, araC, lacZ, proA, trpE* and *thyA* mutations in *E. coli* by *M. leprae* genes on approximately 300 recombinant molecules but have not detected complementation. We are beginning to look for production of recombinant cosmid-specified proteins using the *E. coli* minicell and maxicell systems.—[Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294, U.S.A.]

Fukunishi, Y., Kearney, G. P., Whiting, J., Jr., Manders, W. W., Walsh, G. P., Binford, C. H., Meyers, W. M. and Johnson, F. B. Biochemical investigation of the peribacillary substance of *Mycobacterium leprae.*

The objective of this study was to define the chemical composition of the peribacillary substance (small spherical droplets) of *Mycobacterium leprae.* This peribacillary substance has been observed around leprosy bacilli in phagolysosomes of macrophages of freeze-etched specimens of tissues from humans, nude mice, and armadillos infected with leprosy. There is a corresponding electron transparent zone around bacilli in ultra-thin sections. These electron microscopic findings suggest that lipids constitute the major portion of the peribacillary substance. We have reported that the small spherical droplets of the peribacillary substance were peptidoglycolipid based on the results of histochemical tests (i.e., Sudan Black B, Oil Red O, Van Gieson and colloidal iron).

Our present findings on the chemical composition of the small droplets were obtained by the following methods: high-performance liquid chromatography (HPLC) in the normal phase, reversed phase, and gel-permeation mode (GPC); gas-liquid chromatography; UV/Vis spectrophotometry, infrared spectroscopy, and mass spectroscopy.

MATERIALS AND METHODS

Lepromas (15 g) were obtained from a nine-banded armadillo infected with *M. leprae* and from a mangabey monkey with naturally acquired leprosy.

Purified mycoside A from *M. kansasii* was supplied by Dr. Patrick Brennan of Colorado State University.

We obtained polystyrene calibration standards of known molecular weight from Waters Associates, Millipore Corporation, Milford, Massachusetts, U.S.A.

Samples were minced and extracted with a series of chloroform-methanol solvents (1:1 v/v followed by 2:1 v/v). Extracts were taken to dryness in a rotary evaporator. Acetone soluble lipids were obtained from the resulting residue and the solvent was removed under reduced pressure. The lipids were taken up in tetrahydrofuran (THF) for further analysis.

Spectrophotometric analyses were made

with a Perkin-Elmer model 283 infrared spectrophotometer and Hewlett-Packard 845A UV/Vis spectrophotometer.

HPLC studies were made as follows: Solvent delivery was by Waters' 6000× system and injection was by a Waters' U6K injector. The columns employed were: Gel permeation-Shimadzu Ltd. HSG 15 (75 cm) and HSG 20 (25 cm), normal phase-Dupont-Zorbax SIL (25 cm), reverse phase-DuPont Zorbax ODS (25 cm) or Perkin-Elmer Hx5 C_8 (10 cm). Detection was with Perkin-Elmer model LC 15 refractive index detector, or Waters' R 401 differential refractometer and Waters' model 440 dual wavelength absorbance detector. Integration was accomplished with a Hewlett-Packard 3390A digital integrator.

Gas liquid chromatographic analysis was performed with a Hewlett-Packard 5880A microprocessor controlled instrument equipped with FID detector. Mass spectral analysis was done on a Hewlett-Packard 5985 GC/MS.

RESULTS

HPLC-GPC chromatography with RI detection yielded two peaks peculiar to infected tissue. The peaks were of relatively high molecular weight (approximately 2000 and 1600). The 1600 molecular weight (MW) peak was not detectable at wavelengths of 254 nm or 280 nm, and a full spectrum of the UV/Vis region from 200 to 800 nm was indistinguishable from the neat solvent.

Purified mycoside A analyzed under identical conditions yielded a single peak of approximately 2000 MW.

Reversed phase chromatography of material derived from the 2000 MW peak yielded three peaks on the C_8 column. Purified mycoside A coeluted with the peak of lowest retention volume.

Normal phase chromatography of the 2000 MW peak redissolved in hexane showed several peaks. The major constituent had a retention volume of 28.5 ml in hexane/THF (60:40) and was resolved from all other materials.

When purified mycoside A was analyzed, an elution volume of 10.5 ml was observed. Both the 2000/MW constituent from *M. leprae* and mycoside A from *M. kansasii* showed similar UV/Vis and IR spectra. The methyl esters of fatty acid hydrolysis products from the 28.5 ml peak of *M. leprae* and the 10.5 ml volume peak of purified mycoside A from *M. kansasii* by normal phase HPLC showed very similar patterns by GLC and GC/MS.

CONCLUSIONS

a) The unknown lipid of molecular weight 2000 is a glycolipid with characteristics similar to mycoside A on HPLC by GPC and reversed phase column analysis. Absorbances of the two materials in the UV/Vis and IR regions of the spectrum are also similar. HPLC in the normal phase mode reveals distinct differences between the two molecules.

b) The unknown lipid of 1600 MW is devoid of a phenolic functional group. The molecule possesses no absorbance properties in the region from 210 nm–300 nm.

c) The peak of 1000 MW contains material with spectral qualities similar to the 2000 MW peak. The peak contains many substances, and one of the components may represent either a precursor to the 2000 MW material or a degradation product. Further analysis is needed to clarify this point.—[Armed Forces Institute of Pathology, Washington, D.C. 20306, U.S.A.]

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Nath, I., Sathish, M., Bhutani, L. K. and Sharma, A. K. Monocyte suppressor factor(s) and release of *in vitro* suppression in lepromatous leprosy.

Earlier studies from our laboratory had shown an association of suppressor T cell activity with tuberculoid leprosy and not with lepromatous leprosy. The present results indicate that supernatants from adherent cells of peripheral blood mononu-

clear cells of 15 lepromatous leprosy (LL) patients suppress antigen-induced lymphoproliferation of tuberculoid patients by 60%–80%. Suppression was observed with *de novo* supernatants and was not significantly affected by the addition of antigen. However, similar *de novo* supernatants from six treated bacillary negative LL patients showed markedly less suppression (4%) which increased to 30%–40% when monocytes had been treated with heat-killed *Mycobacterium leprae*. Supernatants from adherent cells treated with or without *M. leprae* antigens from seven tuberculoid leprosy patients failed to suppress lymphoproliferation. Suppressive activity of lepromatous monocytes was not abrogated by indomethacin, was heat stable, and resided in the >25,000 dalton fraction.

Preliminary evidence was obtained to indicate that lepromatous monocytes (Mo) pulsed with antigen for 18 hr, washed, and reconstituted (5%–10%) with autologous T-enriched fractions from nylon wool columns enhanced antigen-induced lymphoproliferation 2–5 fold over the proliferation seen with unfractionated peripheral blood lymphocytes. Of the 16 patients tested, 8 patients showed enhanced responses with T + Mo combinations; 2 patients showed marginal responses, and 6 showed no effects. Thus it would appear that the unresponsiveness observed in some lepromatous leprosy patients may be relieved by modulation of monocyte functions *in vitro*. However, the degree of responsiveness obtained by such *in vitro* manipulation was of a low order and ranged from 1000 cpm–2000 cpm with T + Mo combinations as compared to 85 cpm–300 cpm with unfractionated peripheral blood lymphocytes.—[All India Institute of Medical Sciences and Safdarjung Hospital, New Delhi 110029, India]

Convit, J., Ulrich, M., Aranzazu, N., Zúñiga, M., Castellazzi, Z. and Aragón, M. E. A model of vaccination with two microorganisms in infectious disease.

Two tropical diseases, leprosy and American cutaneous leishmaniasis, share common clinical, histopathological and immunological features which suggest that a similar approach might be used in the immunotherapy and immunoprophylaxis of both diseases. These features can be summarized as follows: 1) Both are characterized by a spectrum of clinical and histopathological manifestations which reflect the nature of the host's immunological response to the infection. 2) The immunological defect in cell-mediated reactivity which is most fully expressed in the severe, progressive forms of both leprosy and leishmaniasis appears to be highly specific for the causative microorganism. 3) In both diseases, the defective immunological response in the severe form of the infection is associated with the presence of massive numbers of microorganisms within granulomata composed of nondifferentiated macrophages; digestion of the parasites by these macrophages is clearly deficient.

The immunological defect in lepromatous, borderline lepromatous, and Mitsuda-negative indeterminate leprosy, as well as in persistently Mitsuda-negative contacts, can be visualized by the intracutaneous injection of heat-killed *Mycobacterium leprae*. Nonresponsive individuals develop a granulomatous lesion formed by undifferentiated macrophages; intact bacilli persist for months, and lymphocytes do not constitute a significant organized element in the cellular reaction [Bull. WHO **46** (1972) 821]. This type of lesion contrasts sharply with the immune type of granuloma formed by epithelioid cells, giant cells, and organized lymphoid elements that is observed when heat-killed *M. leprae* is injected into responsive individuals, or BCG is injected into lepromatous patients. In subsequent studies, we demonstrated that a mixture of heat-killed *M. leprae* and viable BCG produced the immune type of granuloma in the nonresponsive group mentioned previously, with rapid clearance of both mycobacteria [Clin. Exp. Immunol. **17** (1974) 261]. The macrophages in these individuals could be activated by BCG in the presence of *M. leprae*, and this activation coincided with the digestion of the latter.

The assumption that the reaction induced by the mixture of heat-killed *M. leprae* and viable BCG could lead to the development of systemic sensitization to *M. leprae*, through the liberation of specific immunogens in the immune granuloma, led to the testing of this mixture as an immunother-

apeutic procedure in Mitsuda-negative patients with lepromatous, borderline lepromatous, and indeterminate leprosy [Clin. Exp. Immunol. **36** (1979) 214; Int. J. Lepr. **50** (1982) 415–424]. Significant clinical and immunological changes were induced in a substantial number of these patients, as were immunological changes in Mitsuda-negative contacts, who received the vaccine as an immunoprophylactic measure. More than 85% of a group of serial biopsies taken from 60 lepromatous and borderline lepromatous patients showed histopathological changes consistent with reversal reactions of variable intensity, sufficient to permit reclassification toward groups with a more evident component of cell-mediated immunity to *M. leprae*.

Preliminary field trials have been initiated in Venezuela to evaluate the use of the same mixture as an immunoprophylactic vaccine in healthy contacts who are unresponsive to *M. leprae*. Several years will be required to evaluate these trials in terms of the incidence of new cases of leprosy, but preliminary followup has demonstrated a high level of conversion of skin test reactivity to soluble antigens of *M. leprae* after a single dose of vaccine. Comparing the reactivity to soluble antigen of groups of nonresponsive contacts vaccinated with BCG or with the *M. leprae*-BCG mixture eight months earlier, 58 persons (47.9%) of the BCG vaccinated group gave reactions of 9 mm or less, while only 13.5% (28 persons) of the group vaccinated with the mixture gave similar weak or negative reactions. In contrast, 64% of the group vaccinated with the mixture gave reactions of 14 mm or more at 48 hr, while only 15% of the BCG group gave reactions of this intensity.

The two-microorganism model for immunotherapy has also been evaluated in a limited number of patients with American cutaneous leishmaniasis. A mixture of 1.5×10^8 killed promastigotes or amastigotes of *Leishmania brasiliensis* and a variable quantity of BCG, depending upon previous sensitivity to PPD, has been used to treat three patients with diffuse cutaneous leishmaniasis, the progressive form of the disease which shares many features with lepromatous leprosy. These patients have shown a favorable clinical response, with regression of lesions and positivization of the cutaneous response to leishmanin. In addition, three patients with an intermediate form of leishmaniasis characterized by persistent lesions, frequent relapses, and unusually strong responses of delayed hypersensitivity to *L. brasiliensis* have been treated with the same mixture. In a period of 4–8 months, these patients showed regression of their lesions, and they have been completely free from relapses for more than two years. The characteristics of this intermediate form of leishmaniasis clearly suggest an imbalance between the hypersensitivity and protective components of cell-mediated immunity; the use of BCG together with inactivated *L. brasiliensis* may represent a favorable form of antigen presentation to correct this imbalance.

Human tuberculosis presents some of the characteristics of the intermediate form of leishmaniasis described above, including persistence of microorganisms in spite of strong hypersensitivity and a tendency to relapse after apparently effective chemotherapy. The history of preventive vaccination with BCG in human beings has been characterized by difficulty in demonstrating a clear protective effect. Many details of a two microorganism vaccine for this disease, including the most appropriate form of presentation of the antigens of *M. tuberculosis*, definition of the secondary nonspecific component of the mixture, and evaluation of the immunotherapeutic value of the mixture as well as its protective value, could be defined in the experimental model of infection by *M. tuberculosis* in the guinea pig.

In summary, a new model for preventive vaccination and immunotherapy in infectious disease is described, which employs a microorganism toward which the individual is able to develop a normal immune response, and an inactivated form of the parasite toward which the host is unreactive or shows an aberrant response. This model has been successfully applied to nonreactors or weak reactors to *M. leprae*, including patients and healthy contacts and to nonreactive patients suffering from diffuse cutaneous leishmaniasis, as well as patients who show an imbalance between hypersensitivity and protective phenomena to *Leishmania*. Modifications of this model may be applicable to other diseases, such as deep mycoses with defective responses in cell-

mediated immunity, and infections such as tuberculosis, in which imbalance between protective and hypersensitivity mechanisms may exacerbate the pathological manifestations of the disease. Both the immunotherapeutic and immunoprophylactic potentials of this model offer areas for active study.—[Instituto Nacional de Dermatologia, Caracas, Venezuela]

CLOSING REMARKS

Ladies and Gentlemen:

It has been a great honor and a pleasure to participate again in the Joint Leprosy and Tuberculosis Symposium. Since the Twelfth Joint Conference in Boston in 1977, both leprosy and tuberculosis panels have had no opportunity to hold a subsequent joint symposium. This six-year void was time enough to accumulate several topics suitable for common discussion, and this is clearly shown by the successful and fruitful results of this symposium. In my impression, however, it was very difficult within a short time to understand a lot of new knowledge and to discuss many things without preliminary knowledge on recent advances in leprosy and tuberculosis research. There might be many other presentations in the respective conferences to be discussed at this symposium. This may be due to the simple reason that the numbers of such presentations exceeded the capacity of this symposium which had been scheduled for the last half-day. Therefore, I would like to propose that the next joint symposium be planned to run for a longer time if more presentations of common interest are anticipated.

Lastly, on behalf of the Japanese participants, I express my hearty thanks to Dr. Hastings, Dr. Goren, and to all the United States' participants for the splendid arrangements and warm hospitality extended during our stay in Bethesda. We look forward to welcoming you to the Nineteenth Joint Conference in Japan next year, and we also look forward to holding another fruitful Joint Leprosy and Tuberculosis Symposium.

Thank you.

—Masahide Abe, *Chairman*
Japanese Leprosy Panel

Dr. Masahide Abe, Chairman, Japanese Leprosy Panel and Dr. Robert C. Hastings, Chairman, U.S. Leprosy Panel.