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LEPROSY WORKSHOP

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Leprosy Research in the Post-Genome Era¹

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A workshop was held on 21–22 November 1999 in Washington, D.C., U.S.A., to review and discuss the current understanding of leprosy as a disease and *Mycobacterium leprae* as the causative agent from the points of view of epidemiological, clinical and basic research and, based on this review, to suggest areas of needed future research. This workshop was funded by the Heiser Program for Research in Leprosy and Tuberculosis and co-sponsored by the National Institute of Allergy and Infectious Diseases. Twenty-three scientists actively engaged in leprosy research, plus two additional individuals participating by telephone conference call, contributed to the proceedings their knowledge pertaining to leprosy as an infectious disease and *M. leprae* as the causative agent. A panel of six scientists with backgrounds in epidemiology, immunology, cell biology, molecular biology and genetics, dermatology and pharmacology, but not working in basic or clinical leprosy research, evaluated the presented and published information in regard to potential future research efforts that could aid in the further curtailment of this disease.

Topics reviewed included global epidemiology, detection and diagnosis, host susceptibility, host immune responses, *M. leprae* pathogenesis, disease pathology (especially nerve damage), molecular biology, genomics, genetics and physiology of *M. leprae*, availability of GLP (Good Laboratory Practice)-produced *M. leprae* products as diagnostic reagents, status of current efforts to develop new skin-test reagents for earlier diagnosis, and current status and prognosis of vaccine development to prevent and/or treat leprosy.

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HISTORICAL BACKGROUND

Leprosy as a disease has existed throughout recorded history and M. leprae was the first etiologic agent to be implicated as the causative agent of a human infectious disease by Hansen in 1873. Isolation of patients with the disease was the norm until the time of discovery of the first effective chemotherapeutic drug, dapsone (4,4'-diaminodiphenylsulfone), in treating the disease in the 1940s. Leprosy represents a spectrum of disease states depending upon the responsiveness or nonresponsiveness of various components in the cellular immune system. The disease was classified by Ridley and Jopling based on clinical, bacteriological, immunological and histopathological features. Tuberculoid leprosy, termed TT leprosy, is characterized by very low numbers of acid-fast bacilli (AFB) in skin, a few irregular nonsymmetrical anesthetic skin lesions, and an intact cellular immune response to M. leprae. Lepromatous leprosy, termed LL leprosy, is characterized by the presence of high numbers of AFB in skin, many regular, symmetrical skin lesions and a significant impairment in cellular immunity to M. leprae, but with high titers of circulating antibodies to M. leprae antigens. It is individuals with the latter disease state that are thought to be most highly infectious due to the large numbers of bacilli in secretions. The majority of cases are designated borderline (BL), which represents a disease state that shares clinical, histological and immunological features with one or the other polar forms of leprosy. This state can be unstable due to immunological perturbations occurring during active disease progression. Patients initially infected with M. leprae often present with an indeterminate response with a characteristic skin lesion with loss of sensation, but with a minimal inflammatory response and very few bacilli. In indeterminate leprosy, in the absence of drug therapy, the disease may self-cure or progress to borderline, tuberculoid or lepromatous leprosy. In recent years, the disease states have been defined more simply as paucibacillary (PB) and multibacillary (MB) based on the number of lesions or the level of AFB detected in skin biopsies to correlate with recommended drug treatment regimens.

With the emergence of dapsone resistance, first in individuals relapsing after apparent successful drug therapy and then later due to primary infection with dapsoneresistant M. leprae, the World Health Organization (WHO) established in 1976 the special program for research and training in tropical diseases (TDR). The same year, two WHO-TDR advisory bodies were established: IMMLEP to promote the development of vaccines and THELEP to deal with the problem of dapsone resistance. In the 1960s through to the 1980s, several trials were conducted to determine whether BCG vaccination could reduce M. leprae infection. Interpretation of results was complicated since some studies showed no protection with others yielding significant benefit (i.e., up to 50% protection in the Malawi trial and 20% to 60% protection of children in one trial in India). In 1982, WHO's study group on chemotherapy for leprosy recommended the establishment of a multidrug therapy (MDT) regimen for the treatment of individuals with leprosy. MDT involves use of rifampin (600 mg once per month), clofazamine (300 mg once per month, and 50 mg per day), and dapsone (100 mg per day). The treatment is carried out for 12 to 24 months in individuals with MB leprosy and for 6 months, usually with omission of clofazamine, in individuals with PB leprosy.

The research program to better understand M. leprae pathogenesis, to develop better drug therapies and to identify and test potential vaccine compositions was aided by a series of discoveries made over a number of years. A pioneering discovery by C. Shepard was the ability to infect the mouse hind foot pad with M. leprae which, because of the low temperature of the foot pad, grows 100- to 1000-fold over a period of 4 to 9 months. This system provided an opportunity to screen M. leprae for drug susceptibility. The discovery by E. Storrs and W. Kirchheimer of the susceptibility of the nine-banded armadillo (Dasypus nomencinctus) to infection by M. leprae provided a means to obtain large quantities of M. leprae from the spleen and liver after an 18- to 24-month infection period. Most recently, nude (nu/nu) mice have been used to obtain significant quantities of physiologically active M. leprae for detailed genetic and physiological studies. In these mice, hind foot pad infection yields $10^{10} M$. *leprae* in 9 to 12 months.

The WHO-supported MDT program was directed at the goal of reducing the prevalence of leprosy to less than I/I0,000 in endemic countries throughout the world. Toward this end, WHO recommended in 1997 an additional chemotherapeutic drug regimen called ROM, which includes the administration of a single dose of antibiotics to individuals with PB leprosy with a single lesion. The single dose contains 600 mg rifampin, 400 mg ofloxacin, and 100 mg minocycline. In 1999, the worldwide prevalence of leprosy still exceeds 1/10,000 individuals.

GLOBAL EPIDEMIOLOGY

In considering the epidemiology of M. leprae infection and leprosy as a disease, it is important to define the terminology currently used by investigators in this field. Prevalence, which is equal to the number of registered cases globally or by country, is the product of the incidence of disease, which is equivalent to the number of new cases or number of detected new cases during a given interval, such as within a year, and the duration of the disease in years. It should be emphasized that the completeness of case detection and reporting varies from country to country, hence the registered case prevalence often underrepresents true prevalence and new case detection underestimates true incidence. It should also be noted that individuals who have completed MDT or another drug treatment regimen and who are M. leprae slit-skin smear negative, but nevertheless have debilitating disease, are not counted among the number of registered cases. Thus, prevalence numbers begin to equal incidence numbers when the mean duration of disease approximates 1 year. This fits quite well with the use of MDT for 6 months to cure individuals with PB leprosy and 12 to 24 months to cure those with MB leprosy.

Since the inception of MDT in 1982, there has been an 85% reduction in global prevalence of leprosy, with the number of registered cases during the past 4 years or so being less than 1,000,000. In 1999, the number of registered cases was 795,000 for a global prevalence of 1.4 per 10,000 individuals, a number which is in excess of the WHO year 2000 goal for leprosy control (elimination) of less than 1 registered case per 10,000 population. The current plateau in prevalence during the past several years is quite likely due to the fact that the number of new cases detected each year has remained relatively static or, in fact, may be increasing slightly. Thus, the number of new cases detected was 550,000 in 1985 and 795,000 in 1999 (approximately equal to the global prevalence in 1999).

Although the prevalence of leprosy is well below 1/10,000 in the majority of countries, there are about 20 countries in which the prevalence is far in excess of this number, with India accounting for almost 80% of the registered cases globally and Brazil, Indonesia and Bangladesh having very significant numbers. It is thus of critical importance to maximize the effectiveness of identifying new cases in these highprevalence countries and enrolling these individuals in MDT programs in a timely manner.

In evaluating the significance of these numbers and the desired goal of eliminating, if not eradicating, leprosy as an infectious disease, the panel discussed a diversity of issues. In considering infection, it is generally acknowledged that many more individuals are infected with M. leprae than ever develop the disease. Thus, the majority probably undergoes self-cure prior to any disease symptoms being detected. In addition, some individuals who develop symptoms usually associated with the PB form of the disease also undergo self-cure even in the absence of MDT. The time between infection and the onset of disease symptoms has very rarely been documented within several months of birth in infants born from mothers infected with M. leprae. Leprosy is actually rare before 3 years of age for infants born into a family with another individual with clinical disease. At the other extreme, the onset of symptoms after infection can be as long as 30 years, as evidenced by the development of symptoms in military personnel who had been exposed many years previously in a leprosy-endemic area. Thus, with the time between infection and onset of disease varying from 3 to 30 years, it is evident that some of the new cases currently being identified may be due to infections that occurred years before the availability of effective MDT. Both the time for development of disease symptoms and the likelihood for development of overt disease depend on many factors, which include the route of infection, host genetic factors to be discussed below, malnutrition affecting the vitality of the immune system, and possible prior exposure to environmental mycobacteria. As with some other diseases, socioeconomic status is a significant factor. When the GNP per capita increases above \$500, the incidence of leprosy decreases. An additional important risk factor is the presence of infected household contacts. Estimates range from 10% to as high as 75% of new cases occurring in such households. Thus, 25% to 90% of all new cases arise in individuals who must have acquired the infection by contact with an infected individual outside of the household. Although the nasal route of infection is likely, it would appear that exposure of any mucosal surface to M. leprae organisms and infection through skin lesions or punctures are deserving of consideration. In terms of reservoirs, most consider that leprosy is uniquely a human disease. Even though the nine-banded armadillo in the central southern U.S.A. and Mexico is frequently infected or is at least seropositive for M. leprae, there appears to be a very low incidence of documented transmission of M. leprae from armadillos kept as pets to pet owners. Nevertheless, the nine-banded armadillo, which is restricted to North America [sic], is not likely a significant reservoir for M. leprae. More meaningful questions are how did the nine-banded armadillo become infected with M. leprae and do they or how might they transmit it to each other? Given their insectivorous attributes and burrowing ability, one has to question whether M. leprae might be a soil microorganism, at least in semitropical parts of the world where soil temperatures might be maintained at 30°C to 34°C, a temperature range known to be of critical importance for the metabolic activity and survivability of M. leprae in the laboratory. The inability to cultivate M. leprae in the laboratory has precluded an evaluation of such possibilities in the past, although the methods developed by Norman Pace (Science 276:734-740,1997) to look at microbial ecosystem diversity by ribotyping and other methods might be amenable to identification of organisms with *M. leprae*-specific gene sequences in soil samples.

In considering these possibilities and issues, it appears that MDT may not be implemented soon enough after clinical diagnosis of disease to preclude transmission of M. leprae to others. The issue of whether individuals without clinical symptoms, or those with just the paucibacillary disease, exhibit infectiousness for others is unanswered. Thus, developing better diagnostic methods to identify individuals who are infected but not yet diseased, so that MDT could be initiated in a more timely manner, is of paramount importance. Likewise, consideration of prophylactic drug therapy for family members in a household with disease might prove beneficial in eliminating new cases that occur in such households. In spite of the reduced prevalence, the steady or even increasing case detection caused the panel to be pessimistic about the capacity of MDT to reduce the prevalence of leprosy further in the global population, let alone to eradicate leprosy. Thus, continued research on many fronts is needed to acquire the information to improve upon MDT or seek alternative solutions to this global health problem.

SUSCEPTIBILITY

Although there are numerous factors that influence susceptibility to M. leprae infection or the likelihood that infection will lead to disease, there is evidence accruing that genetic factors may predispose individuals either to an increased likelihood of infection or influence their progression to overt disease. Thus, there is a highly significant association between the presence of the HLA-DR2 allele and leprosy in Asia and in Africa. There is also increasing evidence of leprosy susceptibility genes being present in certain families in India, although genetic loci associated with such susceptibility have yet to be identified. Recent linkage analysis data reveal a linkage between leprosy susceptibility and genetic markers on chromosome 10. This susceptibility to M. leprae is also shared with susceptibility to other infectious diseases. Although not adequately studied, there may be genetic differences that determine a relative propensity to the development of erythema nodosum leprosum (ENL) and other types of reactions associated with *M. leprae* infection. Results from the human genome project will contribute the framework upon which to base future studies to refine our understanding of the contribution of genetic susceptibility alleles to *M. leprae* infection and development of disease.

IMMUNOLOGY

The disease manifestations of leprosy represent a spectrum of immunologic responses that range from Th1 to Th2 type (Th1/Th2 paradigm) similar to leishmaniasis in the mouse. For this reason, studying M. leprae infection and causation of disease symptoms will contribute to better understanding of human immunology. M. leprae infect macrophages via mannose, CR1, CR3, and CR4 receptors and scavenger receptors. Whether M. leprae within the phagosome secrete antigens that traffic to the macrophage cytoplasm for class I presentation and/or stimulate class II presentation from within the endosome is as yet unknown. Recent studies of macrophage antigen processing makes it likely that uptake of M. leprae by these cells will lead to antigen presentation by both class I and class II pathways (A. Rodriguez, et al., Nature Cell Biology 6:362-368, 1999). On the other hand, the findings of Rodriguez, et al. suggest that uptake of M. leprae by dendritic cells might lead these cells to present antigen via both class I and II pathways. Both CD4+ and CD8+ T cells isolated from leprosy patients respond to M. leprae antigens, and these T cells may possess $\gamma\delta$ or $\alpha\beta$ receptors. T cells have been shown to respond to 30 or so M. leprae antigens, but there is no information as to whether these antigens have the potential to induce protective immunity or contribute to immune dysfunction and disease progression. CD1 antigenpresenting cells are important for presentation of lipid and glycolipid antigens, and play an as yet undefined role in the prevention or progression of disease. M. leprae infection initially promotes production of interleukin-12 (IL-12) and IL-18, the former potentiating the development of a Th1 response. In TT or PB leprosy, IL-2, gamma interferon (IFN-y) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced. Even though the Th1 response reflected by production of these cytokines is often referred to as protective and is associated with a reasonable frequency of eradication of *M. leprae* infections, individuals expressing a Th1-type immune response may still exhibit nerve involvement and disabling disease. In LL leprosy, IL-4, -5 and -10 are produced in abundance with high antibody titers to many M. leprae antigens. The most striking immunological feature in LL leprosy is the M. leprae antigenspecific anergy in cell-mediated immunity. In tuberculoid leprosy, there is a cytotoxic lymphocyte (CTL) response in which granules containing the antimicrobial protein granulysin are likely delivered by T cells to cells infected with M. leprae, thereby killing these cells. In this regard, granulysin is seen in CD4+ T cells in tuberculoid but not in lepromatous leprosy. This implies that the presence of granules and delivery of granulysin, whether by CD4+ or CD8+ T cells, is of importance in contending with M. leprae infection. On the other hand, there are CTL-type T cells that are doublenegative for CD4+ and CD8+ that secrete IFN- γ and are cytotoxic. It is unclear whether these double-negative T cells have any effect on M. leprae infections.

PATHOGENESIS

Nerve damage, and the consequences of nerve damage, and reactions set leprosy apart from other diseases. The irreversible motor and sensory impairments caused by leprosy lead to increasing secondary impairments long after the disease process has been arrested. Interventions that prevent, reverse, or limit nerve impairment due to leprosy are of the greatest priority. Much headway is being made in the fundamental understanding of *M. leprae* nerve-cell interactions.

Mononuclear phagocytes ingest *M. lep*rae via complement receptors (CR1 and CR3 on monocytes and CR1, CR3, and CR4 on macrophages) and fragments of complement component C3 fix to the bacterial surface. C3 binds selectively to phenolic glycolipid-I (PGL-I), a molecule on the surface of *M. leprae*. Thus, complement receptors on macrophages, complement component C3, and PGL-I comprise a three-component receptor-ligand-acceptor molecule system for mediating phagocytosis of *M. leprae*.

In addition to attaching to and infecting macrophages and a variety of nonphagocytic cells such as striated muscle, M. leprae attach to and invade Schwann cells, the glial cells of the peripheral nervous system. M. leprae possess a surface antigen that binds specifically to the G domain of the alpha-2 chain of laminin-2 which, in turn, binds to a laminin-2 receptor, alpha-dystroglycan, on Schwann cells. These receptors mediate the entry of M. leprae into Schwann cells. In LL leprosy, M. leprae proliferate extensively in Schwann cells of peripheral nerves, but whether M. leprae gene products or metabolites or the ensuing immune responses contribute to nerve damage within lesions is unknown. In any event, M. leprae elicit tumor necrosis factor-alpha (TNF- α) and IFN- γ production, both of which are associated with a strong inflammatory response.

It is important to identify specific adhesins on the surface of *M. leprae* that participate in one or more steps in the binding, entry and/or growth of *M. leprae* in macrophages and Schwann cells. Some progress has been made in this respect with the identification of the histone-like proteins and PGL-I as key adhesins. The question of the possible expression by *M. leprae* of invasins and the means by which *M. leprae* might use signal transduction mechanisms to stimulate cells to which they attach to endocytose them are also deserving of study.

A finding of considerable clinical importance is that many individuals 2 or 3 years after initiation of MDT, and lacking detectable viable *M. leprae*, nevertheless present with significant progressive nerve damage and persistent neuritis. Whether this is due to dead bacteria or the attempt to clear dead bacteria or their components is not clear. Steroid therapy administered prior to the onset of significant nerve damage is effective in about 60% of patients to prevent these permanent, disabling symptoms. Thus, other affordable immunosuppressants are currently being evaluated for improved performance in preventing disabling disease.

Immunologically mediated episodes of acute or subacute inflammation, known as "reactions," may occur in any type of leprosy except indeterminate leprosy, and can result in deformity and disability. Most reactions belong to two main types: type 2 (erythema nodosum leprosum) and type I (reversal reactions). The former occurs in LL and occasionally in BL cases; the latter occurs throughout the borderline spectrum. Thalidomide has been used to treat ENL since the early 1960s, and recent studies of its mode of action have provided useful insights into the pathogenesis and immunology of reactions. Patients with active ENL have elevated serum levels of TNF- α , and thalidomide treatment rapidly reduces these levels with improvement of clinical symptoms. Thalidomide also serves to inhibit monocyte activation and inhibit T-cell activation. Thus, present-day research focuses on identifying non-teratogenic thalidomide analogs for the alleviation and understanding of ENL. Cortocosteroids (prednisolone) are the mainstay of treatment for type I reversal reactions and apparently act by switching off the Th1 response associated with reactions. In important studies, it has been demonstrated that prednisolone had little effect on the initial cellular immune response and cytokine profiles but, by day 28, significant decreases were found in IFN-γ, IL-12, and inducible nitric oxide synthase (iNOS) in most patients with good clinical outcomes. These studies serve to better define the immunological basis of leprosy pathogenesis but also highlight the difficulty of modulating overactive immune responses.

GENOMICS AND MOLECULAR BIOLOGY

Sequencing of the M. leprae genome was initiated in 1991, and a fully sequenced and annotated genome became available in 2000. The M. leprae isolate sequenced came from an armadillo-passaged strain provided by the National Institute of Medical Research, Mill Hill, London. The M. leprae genome is circular and contains 3.3 Mb compared with 4.4 Mb for M. tuberculosis. The M. leprae genome possesses 1700 open reading frames (ORFs); whereas M. tuberculosis has 4000 open reading frames. Thus, functional gene density is considerably lower in the M. leprae genome than it is in the M. tuberculosis genome. The M. leprae genome contains a great deal of noncoding or pseudogene sequences, and it is surprising that this "junk DNA" has been retained. Another obligate intracellular parasite, Rickettsia, also has pseudogenes in its genome, although the 10% pseudogene content reported is less than that for M. leprae, where close to onehalf of the genome is comprised of noncoding pseudogene sequences. Also, there are fewer insertion sequences but more repetitive DNA sequences (RLEP, REPLEP, and LEPREP) in the M. leprae genome than in the M. tuberculosis genome. A comparison of coding sequences shows there are proteins that have 35% to 95% amino acid identity between M. leprae and M. tuberculosis or BCG. The latter observation probably explains why BCG vaccination is sometimes partially effective in preventing M. leprae infection.

There appear to be very few differences in M. leprae genomes from different strains, and this genome sequence conservation has hampered studies on transmission of specific strains within communities or countries. Only a single polymorphism has been found among M. leprae strains, and this is due to the presence of two copies of the repetitive sequence RLEP linked to the polA gene leading to inversion of polA in some strains as opposed to others. (There are at least 30 copies of the RLEP sequence scattered throughout the *M. leprae* genome.) Hopefully, the availability of the complete sequence of the M. leprae genome will facilitate the discovery of additional polymorphisms that will facilitate epidemiologic studies and enable tracing the chain of transmission of specific M. leprae strains in humans.

The constancy of the *M. leprae* genome and especially the maintenance of such an array of pseudogenes in the absence of any apparent benefit is most surprising. Quite possibly the exceedingly long generation time of 2 weeks or so and the ability to prosper in phagocytic cells designed to kill microorganisms by generation of free radicals and production of a diversity of antimicrobial compounds has selected for the inordinate ability of *M. leprae* to repair any and all genetic damage that may arise spontaneously or due to any of these insults. Such conjectures can be tested by using microarray technology and other means to identify genes expressed by *M. leprae* within specific cell types and by defining those gene products that enable *M. leprae* to attach to, invade and survive in macrophages and Schwann cells.

PHYSIOLOGY AND GENETICS

M. leprae frozen at -80°C and then thawed has greatly reduced viability when assayed for growth in mouse foot pads and greatly reduced metabolic activity as measured by CO, generated from oxidation of radioactive palmitate, a quantitative assay for measuring subtle changes in metabolism. Whether physiologic impairments consequent to freezing are repaired once M. leprae enters an animal host remains to be determined. Even so, for studies of organisms maintained at 32°C to 33°C, it seems preferable to use microorganisms freshly harvested from nude mice. In humans and mice, M. leprae prefer the cooler parts of the body, while being able to multiply systemically in the armadillo, whose body temperature is 33°C-34°C. In accord with these observations, metabolic activity is markedly impaired by incubation of M. leprae at 37°C. Storage at 4°C has the least adverse effect on M. leprae physiologic activity measured at 32°C to 33°C. Physiologically active M. leprae incubated under optimal physiologic conditions are able to take up and incorporate glucose, 6-phosphogluconate, glycerol (into lipids), amino acids into proteins and purines (hypoxanthine and adenine) and pyrimidines (cytosines and thymidine) into nucleic acids. Pyrimidines are incorporated into nucleic acids less efficiently than purines. Inorganic phosphate is also incorporated into macromolecules. Palmitate, in addition to its oxidation, is very efficiently incorporated into PGL-I and PDIM (phthiocerol dimycocerosate), but acetate and pyruvate are incorporated into macromolecular constituents very inefficiently. Unlike Rickettsia, M. leprae is unable to take up phosphorylated nucleotides. On the other hand, M. leprae take up and incorporate nucleosides more efficiently than purine and pyrimidine bases. Enzyme assays on metabolically active M. leprae indicate a functional tricarboxylic acid (TCA) cycle and glycolysis via the Embden-Meyerhof and hexose monophosphate shunt pathways.

Work to confirm and extend studies with armadillo-derived *M. leprae* is in progress using nude mouse-derived bacteria.

In terms of the very slow growth rate of *M. leprae*, there is some suggestion that it is defective in NADH metabolism. This defect, in combination with its content of only one copy of genes encoding ribosomal RNA, may contribute to a very long generation time. It is now possible to examine questions such as these by using *M. leprae* maintained under metabolically active conditions.

M. leprae cells can be infected with bacteriophage AE129 engineered to express luciferase. The finding that luciferase activity in these phage-infected cells can be inhibited by antileprosy drugs offers an opportunity to undertake new types of research. Efforts are in progress to introduce the green fluorescence protein gene (gfp), which will facilitate microscopic tracking of M. leprae in animal tissues or in cells in culture. Analysis of the M. leprae genome may suggest reasons for the inability of M. leprae to grow axenically and for its slow growth rate. The ability to introduce foreign DNA into M. leprae will allow investigators to test directly a variety of hypotheses by complementing M. leprae genes or by supplementing M. leprae with genes from other mycobacteria. Such introduced genetic information could be on a phage genome to be incorporated into the chromosome or more likely to replicate on a phasmid, cosmid or plasmid replicon within M. leprae cells. Thus, coupling new evolving gene transfer procedures with the array of data discernable from genomic analysis should accelerate the rate of progress in understanding M. leprae growth, infection, pathogenesis and distribution.

The availability of the sequence of all complete ORFs in the *M. leprae* genome, combined with the power of modern proteomics (two-dimensional polyacrylamide gel electrophoresis, selective ion monitoring and other forms of mass spectrometry), has resulted in the realistic expectation that the entire proteome will soon be defined. Already, specific cationic proteins involved in Schwann cell interaction and a modest complement (as compared to *M. tuberculosis*) of lipoproteins involved in Thl and Th2 immune responses have been defined.

ANTILEPROSY DRUGS AND DRUG RESISTANCE

With the commenced use of sulfa drugs for chemotherapy of bacterial diseases in the 1930s, a disubstituted sulfone (Promin) was tried for treating leprosy in Carville, Louisiana, U.S.A., in 1941, with encouraging results. Promin was relatively expensive and required intravenous administration and so other sulfone drugs were tried during the 1940s, including orally administered dapsone. Dapsone therapy was introduced in India, Nigeria and Brazil in the late 1940s, and was shown to be very effective in treating leprosy in all three countries. Since dapsone was at that time only useful for treating patients with leprosy, and since most of these patients were too poor to pay for drugs, there was effectively no market for it. Consequently, dapsone was available for governmental use at a very low cost. In this regard, dapsone is inexpensive (as cheap as aspirin), stable, and relatively nontoxic. Nonetheless, M. leprae infections with secondary and then primary dapsone resistance appeared, leading WHO to adopt the MDT program in 1982. This program was based on the premise that the use of three drugs (rifampin, clofazamine and dapsone) would preclude the development of resistance to any one of the component drugs used in MDT. It should be noted that rifampin was chosen as one of the three drugs because of its bactericidal effect on M. leprae but also because it was no longer under patent protection, thereby decreasing its cost. Clofazamine was also inexpensive. Thus, all three of the drugs used in MDT as well as the drugs for the ROM regimen are donated by drug manufacturers (especially Novartis) free of charge. The World Health Organization, Nipon Foundation and the International Federation of Anti-Leprosy Associations distribute these drugs. There are new macrolide and fluoroquinolone drugs that are highly active against M. leprae but because of cost are seldom used to treat leprosy in Africa, Asia and South America. These new drugs are likely to enter the treatment regimen only if they become inexpensive, or are proven effective after a very brief treatment regimen, or if drug resistance causes one or more of the existing drugs in the MDT repertoire to become

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ineffective. In this regard, it is known that rifampin resistance can arise quite readily if used alone, and ofloxacin-resistant *M. tuberculosis* has arisen in India. Dapsone resistance has long been known, but its mechanism has only recently been elucidated. In this last regard, it is not known whether use of MDT during the past 18 years has altered the frequency of dapsone-resistant *M. leprae*.

The availability of methods for research with metabolically active M. leprae along with the ability to use luciferase or other reporters should facilitate screening of new drugs for effectiveness in killing M. leprae. In this regard, the HIV epidemic has stimulated efforts to discover and evaluate new drugs for the control of M. tuberculosis and of the M. avium complex. Some of these new drugs are retained in tissues quite well and might be effective for short-term treatment regimens and in alleviating some of the problems associated with M. leprae's neurotropic propensity and adverse sequelae that arise as a consequence. Although the inclusion of three drugs in the MDT program is wise, it must be surmised that at some time multiple drug resistance will arise, especially if the incidence of new cases does not decline. Multidrug resistance would pose a significant impediment to the eradication of leprosy.

With the sequence of the M. leprae genome, it now becomes possible to use specific oligonucleotides and polymerase chain reaction (PCR) to recognize mutations for resistance to rifampin, ofloxacin, dapsone, etc. The problem with such PCR methods is that they must be done in an institution with the technical capabilities for such assays. Nevertheless, such tests can be done with small samples of materials sent to a central testing institution, and this would likely be as efficient an approach to monitor drug resistance as taking biopsy material, inoculating foot pads of nude mice, recovering sufficient cells and using a phage infection luciferase activity to screen for drug resistance or sensitivity.

DIAGNOSIS AND DIAGNOSTIC REAGENTS

The diagnosis of leprosy is still largely based on clinical symptomology and occasionally skin biopsies to verify the presence of acid-fast bacilli. It has been desired for many years, however, that some diagnostic method be developed that would detect infection and the presence of multiplying *M. leprae* as soon as possible before clinical signs are apparent. When PGL-I was identified as an *M. leprae*-specific antigen, efforts were directed at using the presence of antibodies to this molecule as a test for occult infection. Such antibodies can be detected prior to the onset of clinical symptoms. Anti-PGL antibodies, which are largely IgM, are quite prevalent in LL or MB patients but are not prevalent in TT or PB patients. Thus, the detection of PGL-I or antibodies to it became less attractive as an early diagnostic indicator.

Another immune reactivity test involves intradermal inoculation of an autoclaved extract of armadillo-derived M. leprae, the so-called lepromin test, and is read at 3 weeks. The lepromin test is not analogous to the tuberculin test, which is read as a delayed-type hypersensitivity (DTH) reaction to soluble M. tuberculosis purified protein derivative (PPD) 48 hours after injection, and has no value as a diagnostic test for subclinical leprosy. However, the lepromin test can be a prognosticator for the type of leprosy a person is likely to develop (i.e., borderline tuberculoid or tuberculoid if the test is positive and borderline lepromatous or lepromatous if the test is negative).

Two new approaches are being taken to develop improved skin-test antigens. In one such approach, armadillo-derived M. leprae are fractionated to generate a set of protein antigens associated with the cell wall in one case and from the cytoplasm in another. These protein preparations are purified to remove all nonspecific immunosuppressive lipoglycans and lipids, and these procedures are done under GLP conditions to satisfy U.S. Food and Drug Administration requirements for an IND (Investigative New Drug) to conduct a Phase I/II trial in a leprosy-endemic area. As this research progresses, there will be an integration between M. leprae genomics and proteomics wherein 2-D polyacrylamide gel electrophoresis is being used to identify M. leprae proteins encoded by the 80 or so genes unique to M. leprae and not present in M. tuberculosis. (Of course, these M. leprae-specific genes will have to be first evaluated for their absence in a diversity of other mycobacterial species, including those from soil.)

In another approach, peptide antigens representing M. leprae-specific epitopes have been suggested by comparison of M. leprae genome sequences with those from other mycobacteria. Some of these peptides seem likely to be M. leprae-specific by detection of T-cell responses to them in the blood of patients with leprosy, but not by T cells from individuals previously vaccinated with BCG or with tuberculosis. It is likely, however, that some of the epitopes on these peptides may be MHC-restricted and, thus, give rise to T-cell responses in only some patients. Nevertheless, the evaluation of many such peptides should enable the preparation of a cocktail that would be recognizable by one or more T-cell clones present in the blood of most patients with leprosy, provided they are capable of making a T-cell response to leprosy antigens. Whether the diagnostic methods under test will reveal infection long before clinical symptoms are recognizable is unknown. If so, then MDT can be initiated sooner with an increasing likelihood that both the prevalence and incidence of leprosy can be further reduced.

VACCINES AND VACCINATION

BCG vaccination in Malawi and India, at least in some studies, has resulted in a reduction in the incidence of leprosy. Now that the genetic relationship between the various BCG vaccine strains is known, the leprosy research community can evaluate the success or nonsuccess of various BCG vaccination trials, whether in controlling tuberculosis or leprosy, with the particular properties of the BCG strain used as the vaccine in that particular trial. This information would be useful for both M. tuberculosis and M. leprae control efforts, since those desiring to develop a vaccine against M. tuberculosis are endeavoring to specifically attenuate M. tuberculosis and M. bovis and would hopefully choose parent strains that would have the potential for inducing cross-protective immunity against both M. tuberculosis and M. leprae.

A prophylactic leprosy vaccine trial conducted in South India in the early 1990s, comparing four vaccines (BCG, BCG and killed *M. leprae*, and two cultivatable Indian mycobacterial strains, *Mycobacterium* w and ICRC) yielded promising results. Although it was possible to assess the overall protective efficacy of the candidate vaccines against leprosy in the study population, the observed incidence rates of leprosy were not sufficiently high to ascertain the protective efficacy of the candidate vaccines against progressive and serious forms of leprosy. Protection observed with the ICRC vaccine and the combination vaccine (BCG and killed M. leprae) meets the requirement of public health utility. However, the killed *M. leprae* vaccine is unlikely to be available in the future, but the ICRC vaccine is readily available and might be considered for more widespread implementation.

With the prevalence of leprosy approaching 1/10,000, it would seem unlikely that a prophylactic vaccine would be highly cost-effective in preventing leprosy. Thus, the community pressure to vaccinate would not be sufficiently large, except perhaps in localized highly endemic areas within countries with a high prevalence and incidence of leprosy. On the other hand, a vaccine that would prevent M. tuberculosis infection as well as M. leprae infection would likely enjoy a much higher use, and thus generate a more satisfactory outcome. By comparative analysis of the M. leprae and M. tuberculosis genomes, it may be possible to identify antigens that are highly cross-reactive and which might generate a protective immune response, specifically a CTL, Th1-dependent type of immunity. The induction of mucosal and systemic immune responses directed at relevant surface antigens of M. leprae and of M. tuberculosis might contribute significantly to decreasing the likelihood of infection, especially since M. tuberculosis is a respiratory pathogen, and there is considerable favor for this idea as a means of transmission of M. leprae.

BASIC AND CLINICAL LEPROSY RESEARCH COMMUNITY

The perceived success in eradicating leprosy coupled to the increasing global concern for tuberculosis, which has been very much augmented by the HIV epidemic and the development of drug-resistant *M. tuberculosis*, have caused many mycobacterial researchers previously working on leprosy to switch to working on tuberculosis. Thus, the U.S. National Institutes of Health is cur-

rently funding two contracts to provide much-needed *M. leprae* research resources and five investigator-originated research projects on various aspects of leprosy and M. leprae biology. The WHO TDR spends less than 2% of its total budget on leprosy and most of this is concerned with managing the MDT program. There has been a similar decline in the number of laboratories globally conducting basic research on leprosy, and this situation is not conducive to generating the types of knowledge needed to contend with this dreaded disease and to effect its eradication. It should be emphasized that the members of the small leprosy research community collaborate extensively with one another, sharing clinical materials, research reagents and, importantly, exchanging personnel, some of whom receive training in research laboratories and then return to clinical institutes. The Armauer Hansen Research Institute in Addis Ababa, Ethiopia; the Central JALMA Institute for Leprosy in Agra, India; the Aga Khan University in Karachi, Pakistan; the Leonard Wood Memorial Research Center in Cebu City, The Philippines; the Oswaldo Cruz Foundation with laboratories in Rio de Janeiro, Brazil, and the Anandaban Hospital in Kathmandu, Nepal, all located in countries with endemic disease, facilitate these ongoing research efforts. In addition, there are central leprosy research laboratories in many countries throughout the world that can be tapped for intellectual as well as clinical and research resources. Increased funding for leprosy research is needed and will be efficiently used by the cadre of welltrained and highly committed investigators currently working in this field.

CONCLUSIONS AND RECOMMENDATIONS

Based on the foregoing, the panel reached the following conclusions and recommendations.

1. It makes economic and human sense to eradicate leprosy as a global problem. This is an unobtainable goal with the current knowledge and tools and dependence entirely upon the use of multiple drug therapy as presently carried out. An intensification of a much better funded research enterprise on *M. leprae* and leprosy is therefore necessary. 2. Investigation of the epidemiology, natural history and transmission of leprosy should be given the highest priority. These studies will require development of improved DNA reagents, diagnostic materials and diagnostic tests suitable for use in endemic countries. The roles of animal, and even soil, reservoirs for *M. leprae* need to be determined since such information is essential to develop a plan for control and possible eradication of leprosy.

3. The materials and information required for modern molecular epidemiology and for M. leprae detection in potential reservoirs will likely soon be available from analysis of the M. leprae DNA genomic sequence. Further improvements of means for genetic manipulation of M. leprae coupled with studies of M. leprae under physiological conditions will allow identification of gene products required for pathogenicity. Identification of gene products expressed during infection will establish mechanisms for disease-associated pathogenesis and allow the development of better diagnostic reagents and vaccines. For these reasons, research on M. leprae genomics, molecular biology, genetics and physiology should have a high priority.

4. The continued availability of standardized, high-quality research materials including, but not limited to, armadillo- and nude mouse foot pad-derived *M. leprae*, purified and characterized cell constituents and proteins, both native and recombinant, antibodies and genetic constructs will be invaluable for future research progress.

5. Research on tuberculosis and *M. tuberculosis* is often relevant to the understanding of leprosy and *M. leprae.* For this reason, research on leprosy should take guidance from tuberculosis research in attempting to confirm important hypotheses without being merely duplicative. This will be a more cost-effective approach and focus leprosy research on issues that are unique to the disease and to the causative pathogen.

6. Since multiple drug therapy can eliminate the infection in the individual, but has little or no effect on subsequent pathology associated with reactional states and progressive nerve damage leading to deformity, it is imperative that further research be conducted on the basic mechanisms of immunological reactions and nerve damage in leprosy to develop interventions that prevent such damage in *M. leprae*-infected individuals. Collaborations between leprosy researchers and neuroscientists should therefore be fostered.

7. The current means to study physiologically active *M. leprae* should enable evaluative screens of new drugs for effectiveness in leprosy control, especially those now being developed and tested for efficacy against *M. tuberculosis*. These drugs and/or drug combinations can then be evaluated in the mouse foot pad model.

8. The ultimate goal of controlling and even eradicating leprosy is likely to be dependent on development of a safe efficacious vaccine that prevents infection, especially infection by both *M. tuberculosis* and *M. leprae*. Acquiring basic knowledge of the immune responses needed to confer protection and identifying the *M. leprae* antigens that elicit these responses is a high priority. In addition to a preventative vaccine, consideration should be given to the development and evaluation of a vaccine with therapeutic potential in treating patients with leprosy.

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