Clinical Microbiology Reviews, Apr. 2006, p. 338–381 Vol. 19, No. 2
0893-8512/06/$08.00+0 doi:10.1128/CMR.19.2.338–381.2006

The Continuing Challenges of Leprosy

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INTRODUCTION 339
- **Basic Clinical and Immunopathological Features of Leprosy** 339
- **Lepromin Test** 340
- **Leprosy in Immunocompromised Individuals** 340
- **Laboratory Tests for the Diagnosis of Leprosy** 342

***MYCOBACTERIUM LEPRAE*, THE ETIOLOGIC AGENT OF LEPROSY** 343
- **Basic Characteristics** 343
  - **Cellular morphology** 343
  - **Growth** 343
  - **Metabolism** 344
- **Genome, Transcriptome, and Proteome** 345
  - **Genome** 345
  - **Transcriptome** 345
  - **Proteome** 346
- **Molecular Identification by PCR** 347
- **Epidemiology and Strain Identification** 347

EXPERIMENTAL MODELS OF LEPROSY 348
- **Overcoming Obstacles to Leprosy Research** 348
- **Mouse Footpads and Nude Mice** 348
- **Gene Knockout Mice** 349
- **Nine-Banded Armadillo** 350

HOST RESPONSE TO *M. LEPRAE* 351
- **Genetic Influences on Leprosy in Humans** 351
  - **Genetic influences on innate resistance to *M. leprae*** 351
    - **(i) *PARK2/PACRG*** 351
    - **(ii) *NRAMP1*** 351
  - **Genetic influences on acquired immune responses in leprosy** 352
    - **(i) HLA** 352
    - **(ii) Chromosome 10p13** 352
    - **(iii) TAP** 352
    - **(iv) *TNFA*** 352
    - **(v) TLRs** 352
    - **(vi) *VDR*** 352
- **Development of the Immune Response** 352
  - **Innate immunity** 352
    - **(i) Antigen-presenting cells and dendritic cells** 352
    - **(ii) Pattern recognition receptors** 353
    - **(iii) C-type lectin receptors** 353
    - **(iv) Toll-like receptors** 353
    - **(v) C′ receptors** 354
  - **Adaptive immunity: development of cell-mediated immunity** 354
    - **(i) Protective and destructive effects of cell-mediated immunity** 354
    - **(ii) T-lymphocyte populations** 354
    - **(iii) Cytotoxic cells** 354
    - **(iv) Macrophages** 355
    - **(v) Cytokines in leprosy** 356

LEPROSY REACTIONS 357
- **Clinical Presentation, Diagnosis, and Histopathology** 357
- **Immunological Features of Reactions** 357

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Evidence regarding the mechanisms of leprosy reactions 357
(i) Type 1 (reversal) reactions 358
(ii) Type 2 leprosy reaction (erythema nodosum leprosum) 359
Evidence from therapeutic trials regarding mechanisms of reactions 359
MECHANISMS OF NERVE INJURY 360
Localization of *M. leprae* to Peripheral Nerves 361
***M. leprae* Interactions with Schwann Cells** 363
Adhesion to Schwann cells 363
Ingestion by SCs 363
Effects of SCs on *M. leprae* 363
Effects of *M. leprae* on SCs 363
Immune responses and SCs 364
CHEMOTHERAPY 364
Current Therapies and Drug Resistance 364
Molecular Mechanisms of Drug Resistance 365
Development of Drug Resistance in *M. leprae* 367
Detection of Drug-Resistant Leprosy 367
PCR-direct DNA sequencing 367
PCR-SSCP 368
PCR–solid-phase hybridization 368
PCR-heteroduplex analysis 368
PREVENTION: THE QUEST FOR A LEPROSY VACCINE 368
SUMMARY AND CONCLUSIONS 371
Future of Leprosy Treatment and Research 371
ACKNOWLEDGMENTS 372
REFERENCES 372

INTRODUCTION

Leprosy is best understood as two conjoined diseases. The first is a chronic mycobacterial infection that elicits an extraordinary range of cellular immune responses in humans. The second is a peripheral neuropathy that is initiated by the infection and its accompanying immunologic events, but whose course and sequelae often extend many years beyond the cure of the infection and may have severely debilitating physical, social, and psychological consequences. Both aspects must be considered by clinicians, researchers, and policymakers who deal with persons affected by this disease.

Leprosy is not going to disappear anytime soon. Effective multidrug regimens are now used worldwide, and the infection in individuals is curable. However, although the reported number of registered cases worldwide has declined in the last two decades, the reported number of new cases registered each year has remained the same (at 500,000 to 700,000) over the same interval (42, 237). In some countries where leprosy is endemic the number of new cases actually appears to be increasing, while in others decreasing trends are reported. Great caution must be used in reaching conclusions from these observations, however, because they are based entirely on operational data which reflect the intensity of ongoing work more than the extent of any given problem (112). Mathematical modeling of the potential decline in leprosy incidence and prevalence, using various premises regarding efficacy of treatment and prevention, suggests that the disease will remain a major public health problem for at least several decades (259).

The precise mechanism of transmission of *Mycobacterium leprae* is unknown. No highly effective vaccine has yet been developed, and extensive laboratory efforts have not yet produced any practical tools for early diagnosis of clinically unapparent disease.

The full genome of *M. leprae* was among the first to be sequenced, and this new knowledge is beginning to bear fruit. Molecular microbiology has begun to explain, for example, *M. leprae*'s fastidious nature and predilection for an intracellular lifestyle. Similarly, recent human genetic studies have been highly informative, indicating that immunity to *M. leprae* is controlled at two fundamental levels: first, genetic determinants of overall susceptibility and resistance to this organism have now been described, and second, a range of HLA-D-related immune responses have been demonstrated among individuals who are infected.

Only recently has the probable mechanism of intracellular killing of *M. leprae* been identified. The regulation of cell-mediated immunity to *M. leprae* by cellular and cytokine interactions continues to be unraveled. The major animal models available are the nine-banded armadillo and footpad infection of normal or immunologically crippled ($nu^{-/-}$) mice. These models, however, are seriously flawed in their ability to recapitulate many aspects of the human disease and are exceptionally slow, difficult, and expensive to employ. Leprosy therefore remains a medical and scientific challenge of the first order, even though support for research on this disease has declined substantially as other conditions have assumed greater global priority.

A great deal of important new information has been generated by recent research. Brief, authoritative overviews on progress in leprosy have been published in recent years, notably those of Jacobson and Krahenbuhl (167) and Britton and Lockwood (42). Specialized reviews of narrower scope are cited in the appropriate sections below. Here, we have attempted to provide a critical summary of current knowledge from basic and clinical research, focusing particularly on developments from 1990 to the present.

Basic Clinical and Immunopathological Features of Leprosy

Leprosy presents a wide range of clinical and histopathological manifestations. This great diversity puzzled and frustrated

clinicians and investigators until it was appreciated that this diversity was based on the ability of the host to develop a cellular immune response to *M. leprae*. The first full formulation of this concept was described by Skinsnes as an "immunopathological spectrum" in 1964 (384). Soon thereafter, a practical classification scheme based on the same principles was proposed by Ridley and Jopling (319), enabling a degree of global uniformity in clinical practice that gave renewed impetus to research on this disease. In the same decade, the discovery by immunologists of functionally and phenotypically distinct T- and B-lymphocyte subsets and their respective roles in cell-mediated and antibody-mediated immune responses revolutionized immunology. Scientists rapidly developed an entirely new set of tools and simultaneously discovered leprosy as a challenging human disease that appeared to be an ideal model with which to examine theories and methods related to cellular immunity in humans. The convergence of these and other factors prompted an extraordinary burst of research on leprosy during the last three decades of the 20th century (355).

The five-part Ridley-Jopling classification identifies, at one extreme, patients with a high degree of cell-mediated immunity and delayed hypersensitivity, presenting with a single, well-demarcated lesion with central hypopigmentation and hypoesthesia. Biopsies of these reveal well-developed granulomatous inflammation and rare acid-fast bacilli demonstrable in the tissues; this is termed the polar tuberculoid (TT) (Fig. 1). At the other extreme, patients have no apparent resistance to *M. leprae*. These patients present with numerous, poorly demarcated, raised or nodular lesions on all parts of the body, biopsies of which reveal sheets of foamy macrophages in the dermis containing very large numbers of bacilli and microcolonies called globi. This nonresistant, highly infected form of the disease is termed polar lepromatous (LL). The majority of patients, however, fall into a broad borderline category between these two polar forms; this is subdivided into borderline lepromatous (BL), mid-borderline (BB), and borderline tuberculoid (BT).

Very early lesions may present as relatively nonspecific perineural infiltrates in which rare acid-fast bacilli can be demonstrated, but without sufficient infiltrates to classify them; these are called indeterminate. This classification should be used only when the biopsy sample shows definite diagnostic evidence of leprosy (nerve involvement and acid-fast bacilli), since a diagnosis of leprosy may often have significant impact on a patient's family, employment, and psychological and social status.

In spite of nearly three decades of intensive research into the immunology of leprosy, the mechanism by which *M. leprae* is able to elicit the entire range of human cellular immune responses has still not been explained. Most clinical immunological inquiries have focused on the "immunologic defect" of lepromatous patients, i.e., their apparently specific anergy to *M. leprae*. The broad research efforts of recent years have, however, provided an increasingly detailed description of the immunological components in skin lesions across the leprosy spectrum, detailed below under Development of the Immune Response.

Lepromin Test

The lepromin test is often the cause of confusion and misplaced diagnostic expectations. The lepromin skin test is not diagnostic of leprosy or exposure to *M. leprae*. The test response is measured as induration (in mm) 4 weeks after injection and is ideally also evaluated by biopsy and histopathological examination of the test site. This test provides a measure of the individual's ability to mount a granulomatous response against the mixture of antigens present. Responses to lepromin are not leprosy specific; many individuals who have never been exposed to *M. leprae* will develop a positive lepromin reaction.

Leprosy bacilli, derived from different sources and subjected to different purification procedures, are the basis for different types of preparations used for intradermal skin testing (227). The most frequently used preparation, and the one for which the response is best characterized, is Mitsuda lepromin. This is a suspension of whole, autoclaved leprosy bacilli (357) that is injected intradermally. Early studies used bacilli isolated directly from human lepromatous lesions, but armadillo-derived organisms have been used exclusively since the 1970s. In recent years, Mitsuda lepromin has been distributed for research applications by the World Health Organization. This skin test material is not approved by the Food and Drug Administration and is not recommended or provided for diagnostic use in the United States by the National Hansen's Disease Programs. Studies are under way to try to identify defined protein antigens that might be useful as diagnostic reagents (37, 94), but none of these has yet been determined to be satisfactorily sensitive or specific for this purpose.

Although the response to Mitsuda lepromin is not leprosy specific, a negative response is associated with lepromatous types of leprosy, i.e., with an inability to respond to *M. leprae* and to eliminate the bacilli. A positive lepromin test (at 4 weeks) is associated with the ability to develop a granulomatous response, involving antigen-presenting cells and $CD4^+$ lymphocyte participation and, in leprosy patients, successful elimination of bacilli (121, 299).

Lepromin is probably the only widely studied skin test antigen that reflects the ability of an individual to generate a granulomatous response to mycobacterial antigens (as opposed to the 48- to 72-h delayed hypersensitivity response to tuberculin and other skin tests). For this reason, the possibility of genetic influences on lepromin responsiveness has been of interest to geneticists concerned with the inheritance of immunologic aspects of the granulomatous response (8, 30, 107).

Leprosy in Immunocompromised Individuals

Unlike tuberculosis, leprosy has not been observed to be more frequent in patients infected with human immunodeficiency virus (HIV) in regions where both diseases are endemic (162, 238, 303). It has been suggested that this may be due to the relatively low virulence of *M. leprae* or that HIV-infected individuals may die before leprosy (with its long incubation time) becomes clinically apparent (238). Nelson (286) has recently urged that investigators explore alternative explanations, however, since the apparent dissociation between the two diseases has continued even as the prevalence of AIDS has increased.

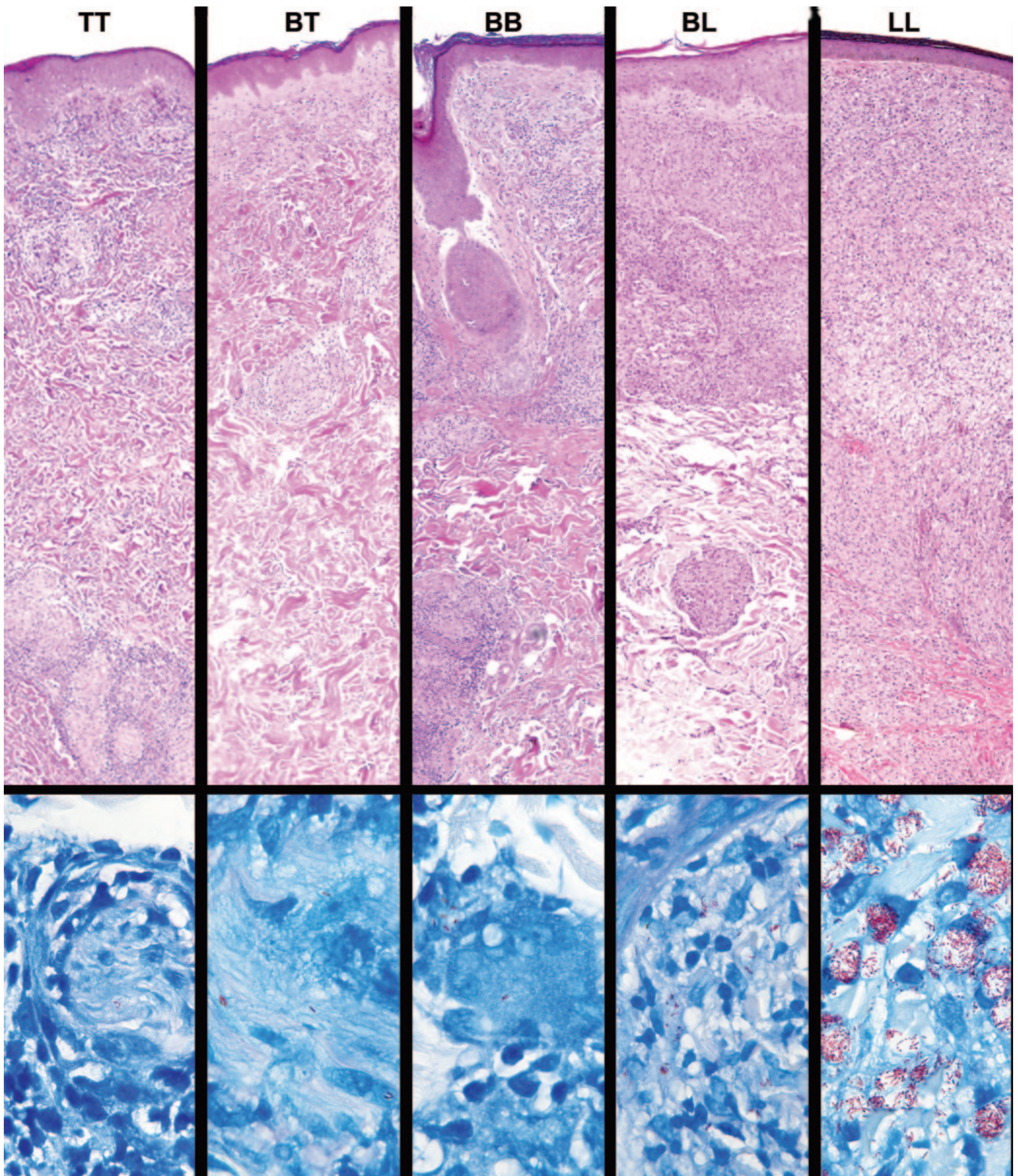

FIG. 1. Immunopathologic spectrum of leprosy. Representative fields from each of the histopathological types of leprosy in the Ridley-Jopling classification are presented in the upper panel, in hematoxylin- and eosin-stained sections (magnification, ×63). The well-formed epithelioid granulomatous infiltrates seen in polar tuberculoid (TT) lesions become increasingly disorganized in each successive increment in the scale until they become completely disorganized aggregates of foamy histiocytes, with only occasional lymphocytes, in polar lepromatous (LL) lesions. Representative fields of each classification are shown in Fite-stained sections in the lower panel (magnification, ×1,000). A search of more than 50 fields was required to find the two organisms shown in a cutaneous nerve in the TT sample, and organisms are often similarly difficult to find in BT lesions. This spectrum is the yardstick against which is measured each new hypothesis and discovery regarding immunological mechanisms proposed to be responsible for the wide range of human responses to *M. leprae*.

In contrast to all other experience with mycobacterial infections in HIV-positive individuals, coinfection with *M. leprae* and HIV appears to have minimal effect upon the course of either leprosy or HIV/AIDS. This is best illustrated by studies following cohorts of infected patients (162; reviewed in reference 221).

The occurrence of leprosy reactions in HIV-positive patients with leprosy has been the focus of several reports (18, 31, 34, 50, 230, 298, 300), but since leprosy reactions affect a high percentage of all leprosy patients (see Leprosy Reactions, below), it is not clear that they are actually more frequent or more severe in HIV-positive individuals. Studies of cell phenotypes and cytokines in leprosy lesions in patients with and without HIV infection have found no significant differences between the two groups with respect to these immunologic parameters (136, 298, 329). It is possible that the very slow growth of *M. leprae* allows the host immune response to keep pace with this infection to a much greater degree than is the case with *M. tuberculosis*, *M. avium*, and the other mycobacterial pathogens in AIDS. Notably, however, infection with *M. leprae* may elicit antibodies cross-reactive with HIV screening assays (13, 15, 205, 372).

Treatment of HIV infection with highly active antiretroviral therapy has resulted in the emergence of previously unsuspected leprosy in a small number of reported cases (77, 230) and notably, many of these individuals have also developed type 1 reactions. This suggests that infection with *M. leprae* may be more common than has been documented among HIV-positive individuals in leprosy-endemic regions of the world, but that early leprosy lesions are overlooked when these patients are confronted by the other major, life-threatening complications of AIDS.

Interestingly, immunosuppression with humanized monoclonal antibodies to tumor necrosis factor alpha (TNF-α) has resulted in the rapid development of lepromatous leprosy in at least two individuals who were being treated for severe arthritis (355a). These patients are presumed to have had minor, undetected leprosy lesions prior to the administration of immunosuppressive treatment. They responded promptly to antimicrobial treatment for *M. leprae* with a multidrug regimen (see Chemotherapy, below), but did develop type 1 reactions during their recovery, as noted above with AIDS patients receiving highly active antiretroviral therapy. Similarly, a small number of patients who have been immunosuppressed for renal or heart transplantation have developed leprosy (266; Scollard, unpublished observations), and these have also responded well to antileprosy treatment.

Together, the evidence indicates that broad therapeutic immunosuppression does render individuals highly susceptible to infection with *M. leprae*, but that in HIV-positive individuals a degree of host response to *M. leprae* is maintained, comparable to that of non-HIV-infected individuals, even as HIV infection progresses and circulating $CD4^{+}$ cell numbers decline. The experience with highly active antiretroviral therapy suggests that in leprosy-endemic areas, subclinical and early clinical infection with *M. leprae* may be more prevalent among HIV-positive individuals than has been generally recognized. In addition, it appears that the restoration of immune function after highly active antiretroviral therapy may be associated with the development of type 1 reactions.

Laboratory Tests for the Diagnosis of Leprosy

The "gold standard" for the diagnosis of leprosy is a full-thickness skin biopsy sample obtained from the advancing margin of an active lesion, fixed in neutral buffered formalin, embedded in paraffin, and examined by an experienced pathologist. The primary characteristics to be recognized are histological patterns of the host response in hematoxylin- and eosin-stained sections (described above), the involvement of cutaneous nerves, and the identification of acid-fast bacilli within nerves using the Fite-Faraco modification of the carbol fuchsin stain (70). In tuberculoid lesions, where bacilli may be rare and difficult to find, the differential diagnosis of the granulomatous response commonly includes cutaneous tuberculosis, sarcoidosis, and granuloma annulare. At the other extreme, bacilli are easily demonstrated in the infiltrates of polar lepromatous leprosy, but care must be exercised to identify bacilli within nerves because, in immunosuppressed individuals, cutaneous infections with other mycobacteria can mimic the florid infection of lepromatous leprosy.

An ancillary procedure, the slit-skin smear, can be used for the semiquantitative enumeration of acid-fast organisms in infected skin and is useful in follow-up of patients during and after treatment. This technique is reliable only when performed and interpreted by experienced technicians.

No serologic tests are available for the routine laboratory diagnosis of Hansen's disease, and no laboratories in the United States perform such assays routinely. Enzyme-linked immunosorbent assays and related immunoassays have been developed to detect antibodies to phenolic glycolipid 1 (PGL-1) of *M. leprae* (46, 97), and these have been used in epidemiological studies. Although they have some value in population follow-up studies, none of these assays has a satisfactory degree of sensitivity and specificity for diagnostic application. The greatest drawback to the serologic diagnosis of leprosy arises from the fact that patients in whom the diagnosis is most difficult, TT and BT patients with moderate to high-grade cellular immunity to *M. leprae* and only small numbers of organisms, do not reproducibly produce detectable, specific circulating antibodies.

Similarly, no skin test that enables the diagnosis of Hansen's disease has been developed. Intradermal injection of heat-killed *M. leprae* (the lepromin test, discussed above) reflects the ability of an individual to develop a granulomatous response to this organism, but it does not reflect infection by or even exposure to *M. leprae*. It has been used in epidemiological studies, but it has no diagnostic utility in individual cases, and it is not available in the United States.

M. leprae is not cultivable in vitro (see Basic Characteristics, below), and lack of growth on standard mycobacterial isolation media can be regarded as one laboratory criterion differentiating this organism from other mycobacterial pathogens. The major advance in the laboratory diagnosis of Hansen's disease in the last 15 years, however, has been the development of methods for the extraction, amplification, and identification of *M. leprae* DNA in clinical specimens using PCR and other molecular techniques. This is an invaluable addition to laboratory diagnosis and to studies of the basic microbiology of this uncultivable organism, although it is costly and has not yet been approved or become available as a routine clinical test. A

detailed discussion of PCR evaluation of specimens for *M. leprae* DNA is presented below (see Molecular Identification by PCR, below).

MYCOBACTERIUM LEPRAE, THE ETIOLOGIC AGENT OF LEPROSY

Basic Characteristics

Cellular morphology. *M. leprae* is a nonmotile, non-spore-forming, microaerophilic, acid-fast-staining bacterium that usually forms slightly curved or straight rods (Fig. 2). A great deal has been learned about the nature of the mycobacterial cell wall through biochemical and genetic manipulation of cultivable strains such as *M. tuberculosis*, *M. avium*, *M. smegmatis*, and *M. bovis* BCG. Similar approaches with *M. leprae* have been meager by comparison, but basic chemical studies have concluded that the cell wall is a covalently linked peptidoglycan-arabinogalactan-mycolic acid complex similar in composition to all mycobacterial cell walls (79, 98, 425) (Fig. 3).

The cell wall core contains peptidoglycan, composed of chains of alternating *N*-acetylglucosamine and *N*-glycolylmuramate linked by peptide cross-bridges, which is linked to the galactan layer by arabinogalactan. Three branched chains of arabinan are in turn linked to the galactan, forming, along with the peptidoglycan layer, an electron-dense zone around *M. leprae*. Mycolic acids are linked to the termini of arabinan chains to form the inner leaflet of a pseudolipid bilayer. The outer leaflet is composed of a rich array of intercalating mycolic acids of trehalose monomycolates and mycoserosoic acids of phthiocerol dimycocerosates as well as phenolic glycolipids (PGLs), forming the electron-transparent zone. It has been postulated that many of these same molecules together with phosphatidylinositol mannosides and phospholipids are released from the cell wall after synthesis, forming a capsule-like region. The dominant lipid in the cell wall which gives *M. leprae* immunological specificity is PGL-1. Recent studies suggest that PGL-1 is involved in the interaction of *M. leprae* with the laminin of Schwann cells, suggesting a role for PGL-1 in peripheral nerve-bacillus interactions (288).

Annotation of *M. leprae*'s genome and comparative genomic studies with other bacterial genomes have produced insight into the putative genes needed to direct the synthesis of this complex cell wall biopolymer (38). Most of the genes necessary to build the peptidoglycan-arabinogalactan-mycolate polymer appear to be present in the *M. leprae* genome and fit a reasonable strategy for its construction. A few exceptions are two genes involved in polyprenyl-phosphate synthesis (*dxs-II* and *idi*), a gene (*fabH*) involved in meromycolate synthesis, and a glycosyltransferase gene (*pimB*) involved in the biosynthesis of phosphatidylinositol, phosphatidylinositol mannosides, lipomannan, and lipoarabinomannan. It should be noted that much of this comparative work, while speculative, provides an important framework from which to investigate the authenticity of these putative pathways.

Growth. *M. leprae* has never been grown on artificial media but can be maintained in axenic cultures in what appears to be a stable metabolic state for a few weeks (414). As a result, propagation of *M. leprae* has been restricted to animal models, including the armadillo (415) and normal, athymic, and gene

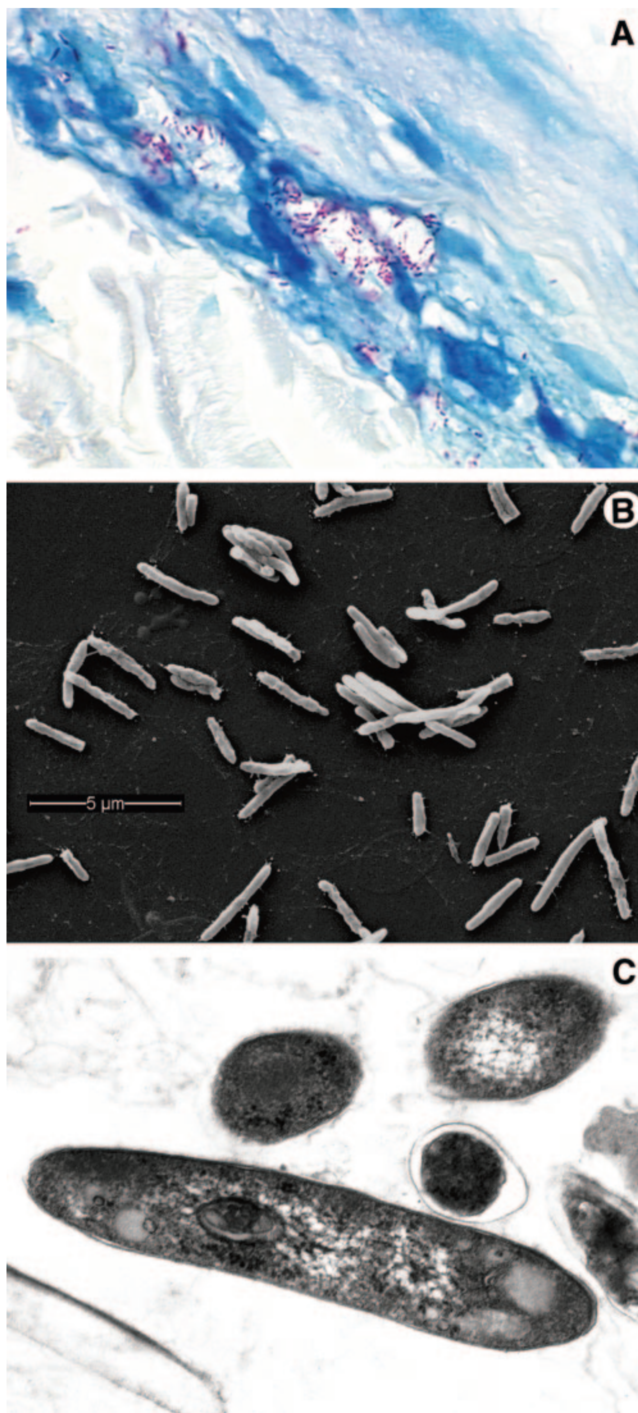

FIG. 2. Morphology of *M. leprae*. A. *M. leprae* is weakly acid fast but, when stained with the Fite-Faraco method, it appears as red, rod-shaped organisms; shorter beaded or granular shapes are observed when the bacilli are dead or dying. The organisms are seen here within a human nerve, counterstained with methylene blue. Magnification, approximately ×800. B. A suspension of nude-mouse footpad-derived *M. leprae* under the scanning electron microscope, which reveals the surface of the organisms. *M. leprae*, like other mycobacteria, tends to cluster. Magnification, approximately ×12,000. C. Internal features of *M. leprae* are observed in this ultrathin section of the bacilli under a transmission electron microscope. The round and oval images seen in the upper portion of this photograph are bacilli that have been cut in cross section. Magnification, ×29,000.

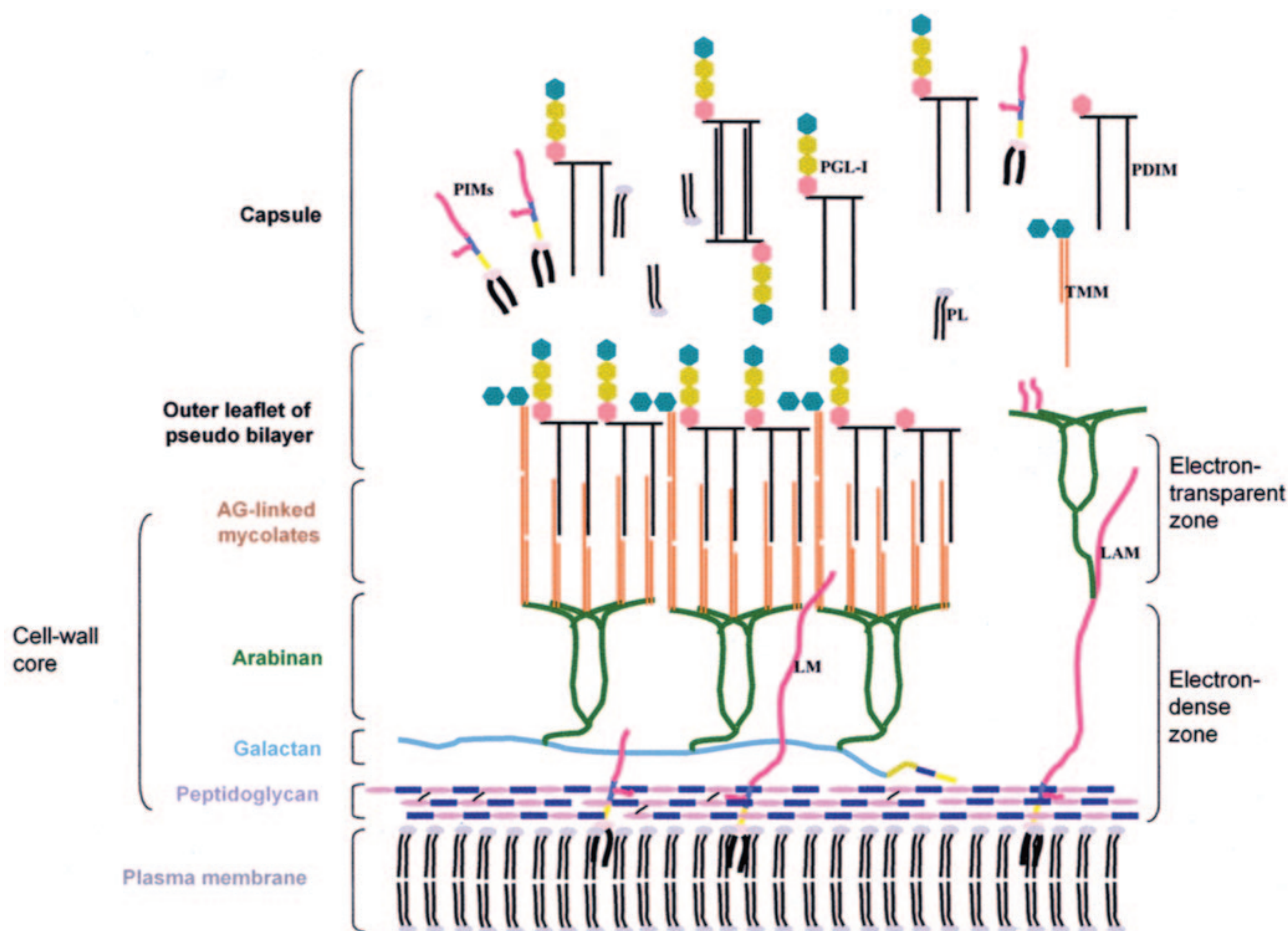

FIG. 3. Schematic model of the cell envelope of *M. leprae*. The plasma membrane is covered by a cell wall core made of peptidoglycan covalently linked to the galactan by a linker unit of arabinogalactan. Three branched chains of arabinan are in turn linked to the galactan. Mycolic acids are linked to the termini of the arabinan chains to form the inner leaflet of a pseudolipid bilayer. An outer leaflet is formed by the mycolic acids of trehalose monomycolates (TMM) and mycocerosoic acids of phthiocerol dimycocerosates (PDIMs) and PGLs as shown. A capsule presumably composed largely of PGLs and other molecules such as PDIMs, phosphatidylinositol mannosides, and phospholipids surrounds the bacterium. Lipoglycans such as phosphatidylinositol mannosides, lipomannan (LM), and lipoarabinomannan (LAM), known to be anchored in the plasma membrane, are also found in the capsular layer as shown. (Reprinted from reference 425 with permission of the publisher.)

knockout mice (222). These systems have provided the basic resources for genetic, metabolic, and antigenic studies of the bacillus. Growth of *M. leprae* in mouse footpads also provides a tool for assessing the viability of a preparation of bacteria and testing the drug susceptibility of clinical isolates (364, 414). The viability of *M. leprae* harvested from several different sources is now known to vary greatly, and many standard laboratory practices, such as incubation at 37°C, rapidly reduce the viability of this organism (414). However, *M. leprae* stored at 33°C in 7H12 medium has been shown to remain viable for weeks.

Metabolism. The primary reasons for investigating the metabolic aspects of *M. leprae* have been to determine whether special media could be formulated to support in vitro growth of the bacilli and to learn more about metabolic pathways that could potentially be exploited for developing new antileprosy drugs. Early work provided a picture of a bacterium with some basic anabolic and catabolic pathways needed for survival in the host, but a thorough assessment of *M. leprae*'s metabolic potential was still lacking. With the completed sequencing and annotation of *M. leprae*'s genome, an improved understanding of *M. leprae*'s metabolic capabilities now exists (43, 105, 432).

Annotation of the genome identified genes showing that *M. leprae* has the capacity to generate energy by oxidizing glucose to pyruvate through the Embden-Meyerhof-Parnas pathway, supporting earlier biochemical observations. Acetyl-coenzyme A from glycolysis enters the Krebs cycle, producing energy in the form of ATP. In addition to glycolysis for energy production, genome analysis as well as biochemical studies in *M. leprae* and *M. tuberculosis* suggest that these organisms rely heavily upon lipid degradation and the glyoxylate shunt for energy production. In this regard, *M. leprae* contains a full complement of genes for B oxidation but, compared to *M. tuberculosis*, very few genes capable of lipolysis. Acetate has been lost to *M. leprae* as a carbon source since only pseudogenes are present for acetate kinase, phosphate acetyltransferase, and acetyl-coenzyme A synthase.

Overall, *M. leprae* has many fewer enzymes involved in deg-

radative pathways for carbon and nitrogenous compounds than *M. tuberculosis*. This is reflected in the paucity of oxidoreductases, oxygenases, and short-chain alcohol dehydrogenases and their probable regulatory genes. In addition, other major problems associated with metabolism for *M. leprae* are that the bacilli have lost anaerobic and microaerophilic electron transfer systems and that the aerobic respiratory chain is severely curtailed, making it impossible for *M. leprae* to generate ATP from the oxidation of NADH. In contrast to the reduction in catabolic pathways, the anabolic capabilities of *M. leprae* appear relatively unharmed. For example, complete pathways are predicted for synthesis of purines, pyrimidines, most amino acids, nucleosides, nucleotides, and most vitamins and cofactors. The maintenance of these anabolic systems suggests that the intracellular niche that *M. leprae* has found for itself may not contain these compounds or transport systems.

Genome, Transcriptome, and Proteome

Genome. *M. leprae* that was originally purified from the skin lesions of a multibacillary leprosy patient from Tamil Nadu, India (TN strain), and subsequently expanded in and purified from the liver of a nine-banded armadillo provided the source of DNA for sequencing of the *M. leprae* genome (74). The genome sequence was generated by sequencing a combination of Lorist6 cosmid library inserts (103) and sixfold whole-genome shotgun sequencing of *M. leprae* insert DNA in pUC18 (74). Annotation of *M. leprae*'s genome has revived interest in basic investigations of its metabolic, biochemical, and pathogenic potential.

Comparison of *M. leprae*'s genome with that of its close relative *M. tuberculosis* (Table 1) suggests that *M. leprae* has undergone an extreme case of reductive evolution (reviewed in references 74 and 104). This is reflected by its smaller genome (3.3 Mb for *M. leprae* versus 4.4 Mb for *M. tuberculosis*) and a major reduction in G+C content (58% for *M. leprae* versus 66% for *M. tuberculosis*). *M. leprae*'s annotated genome contains only 1,614 open reading frames potentially encoding functional proteins, compared to 3,993 open reading frames predicted in *M. tuberculosis* (Table 1).

One of the most striking features of *M. leprae*'s genome is that it possesses 1,133 inactivated genes (genes lost through mutation, or pseudogenes), compared to six pseudogenes in *M. tuberculosis* (72). In addition, a large number of genes apparently have been entirely deleted from the genome. The result of this massive gene loss leaves *M. leprae* with less than 50% of its genome encoding functional genes, compared to *M. tuberculosis*, in which 90% of the genome encodes functional genes, and 34% of *M. leprae*'s proteins identified in silico appear to be the products of gene duplication events or share common domains (105).

Downsizing of the genome has resulted in the elimination of several metabolic pathways, leaving a pathogen with very specific growth requirements, as discussed above (Metabolism). The largest functional groups of genes in *M. leprae* are those involved in gene regulation, metabolism and modification of fatty acids and polyketides, cell envelope synthesis, and transport of metabolites (74, 104, 105). Defense against toxic radicals is severely degenerative, as neither *katG* nor the *narGHJI* cluster is functional. In addition, several other genes involved with detoxification are pseudogenes or are missing from the genome. Redundancy as seen in the *M. tuberculosis* genome is often lost in *M. leprae*, as most paralogues seen in *M. tuberculosis* are pseudogenes in *M. leprae*. None of the additional 142 genes found only in *M. leprae* appear to be associated with metabolic pathways.

TABLE 1. Comparative genomics of *M. leprae* and *M. tuberculosis*

Parameter	*M. leprae* (strain TN)[a]	*M. tuberculosis* (strain H37Rv)[b]
EMBL/GenBank/DDBJ accession no.	AL450380	AL123456
Genome size (bp)	3,268,203	4,411,532
No. of protein genes	1,614	3,993
No. of unknown genes	142	606
No. of pseudogenes	1,133	6
No. of tRNA genes	45	45
No. of rRNA genes	3	3
No. of stable RNA genes	2	2
Gene density (bases/gene)	2,024	1,106
Avg gene length (bases)	1,007	1,008
% Protein coding	49.5	91.2
% G+C	57.8	65.6
SNP[c] frequency	1 in 24,000 bp[d]	1 in 3,000 bp[e]

[a] Data obtained from the Current Data Release (17 October 2003) for the *M. tuberculosis* genome (http://genolist.pasteur.fr/TubercuList/).
[b] Data obtained from the Current Data Release (20 July 2005) for the *M. leprae* genome (http://genolist.pasteur.fr/Leproma/).
[c] SNP, single-nucleotide polymorphism.
[d] Data obtained from reference 272.
[e] Data obtained from reference 114.

M. leprae appears to have a major deficiency in its ability to acquire iron from its environment. The entire *mbt* operon is deleted, rendering it unable to make either the membrane-associated or excreted form of mycobactin T. While genes known to be involved in iron acquisition are not obvious in its genome, there is little doubt that *M. leprae* utilizes iron. Genes are present for cytochrome *c* (*ccsAB*), a ferredoxin (*fdxCD*), biosynthesis of the heme group (*hem* genes), a hemogloblin-like oxygen carrier (*glbO*), the iron storage bacterioferritin *bfrA*, and *ideR*, the iron regulation protein dependent on intracellular iron (432). There are no intact polymorphic G+C-rich sequence- or major polymorphic tandem repeat-related repetitive sequences in the genome of *M. leprae* and only a limited number of proline-proline-glutamic acid and proline-glutamic acid proteins (72). Annotation of *M. leprae*'s genome has thus provided insight into why the leprosy bacillus is an obligate intracellular parasite and has provided the basis for future experimentation to better understand its pathogenicity.

Transcriptome. While comparative genome analysis provides useful clues to identify deficits in general cellular metabolic potential and cellular composition, it offers only a starting point from which functional studies can proceed. Because of our inability to cultivate *M. leprae* axenically, extremely limited quantities of bacterial proteins can be purified for analysis. Transcriptional analysis of *M. leprae* genes provides a perspective that is complementary to protein analysis by identifying actively transcribed genes, thereby expanding our knowledge of genes expressed during infection that might otherwise be missed when examining *M. leprae*'s proteome.

With less than 50% coding capacity and 1,133 pseudogenes, those genes that are expressed help define the min-

imal gene set necessary for in vivo survival of this mycobacterial pathogen as well as genes potentially required for infection and pathogenesis as seen in leprosy. To identify genes transcribed during infection, gene transcripts from *M. leprae* growing in athymic nude mice have been surveyed using reverse transcription-PCR and cross-species DNA microarray technologies with an *M. tuberculosis* microarray (440). Transcripts were detected for 221 open reading frames, which include genes involved in DNA replication, cell division, SecA-dependent protein secretion, energy production, intermediary metabolism, and iron transport and storage and genes associated with virulence (see supplemental Table S1 at http://www.leprosy-ila.org/Mycobacterium.html).

These results support the view that *M. leprae* actively catabolizes fatty acids for energy, produces a wide array of secretory proteins, utilizes the limited array of sigma factors available, produces several proteins involved in iron transport, storage, and regulation (in the absence of recognizable genes encoding iron scavengers), and transcribes several genes associated with virulence in *M. tuberculosis*. Transcript levels of nine of these potential virulence genes (*aceA*, *esat6*, *fbpA*, *relA*, *sigA*, *sigE*, *soda*, *aphC*, and *mce1A*) were compared in *M. leprae* derived from the lesions of multibacillary leprosy patients and from infected nude mouse footpad tissue using quantitative real-time reverse transcription-PCR. Gene transcript levels were comparable in the two isolates for all but one of the genes studied (*esat6*), suggesting that profiling of *M. leprae* genes from animal models such as the nude mouse should be continued. Identifying genes associated with growth and survival during infection should lead to a more comprehensive understanding of *M. leprae*'s ability to cause disease.

Proteome. The functional complement of any genome is the proteome, which consists of the proteins expressed by a given organism under defined conditions. Understanding the basis of *M. leprae*'s growth, virulence, and immunogenicity has always been the focus of proteomic discovery, with the goal of developing strategies for improved treatment and control of leprosy. Early work was driven by efforts to produce vaccines and diagnostic reagents. Prior to the publication of the full DNA sequence and annotation of the *M. leprae* genome in 2001 (74) protein analysis of *M. leprae* relied primarily on traditional subcellular fractionation of purified bacilli and genomic library screening using immunologic reagents. Of the two strategies, immunologic screening of genomic libraries proved more fruitful and expanded the number of purified and characterized proteins to over 40 (41, 131, 338, 407). Immunologic screening took advantage of the fact that serum and T cells from leprosy patients or healthy individuals previously sensitized to *M. leprae* were abundant, providing powerful probes for antigenic proteins of *M. leprae* expressed in *Escherichia coli*.

A major drawback to immunologic screening was that nonimmunogenic proteins were missed. Clark-Curtiss used an alternative genomic screening approach in which *M. leprae* genes were examined for expression in *E. coli*. This led to the discovery of a 46-kDa protein from *M. leprae* capable of complementing a citrate synthase mutant of *E. coli* (165) and the demonstration that *M. leprae* promoter activity was possible in *E. coli* (356). Unfortunately, while genetic complementation in *E. coli* had been very successful for identifying genes in other enteric bacteria, it appeared that problems associated with either *M. leprae* gene expression in *E. coli* or the relatively large evolutionary distance between the two bacteria limited further application of the approach.

Brennan and coworkers have combined subcellular fractionation of *M. leprae* with immunologic screening and chemical analysis of purified proteins using microsequencing and mass spectrometry to expand our understanding of the cellular location and function of many *M. leprae* proteins. Proteins have been identified from major cellular compartments, including cytosol, membrane, and cell wall, and a comprehensive list of these proteins and their characteristics was published earlier (248).

Much of this work has now been assimilated into a larger framework established from the completion of the *M. leprae* genome sequence. Annotation of the *M. leprae* genome indicates that approximately 1,600 open reading frames are scattered among a decaying genome, including approximately 1,100 pseudogenes, with gene remnants making up the remaining 23.5% of the genome. By comparative genomic analysis with *M. tuberculosis* and other known gene sequences, it appears that approximately 50% of *M. leprae*'s open reading frames can be assigned putative functions. The other half of *M. leprae*'s genes are considered to encode hypothetical proteins, with a small percentage designated unknown genes.

The power of this new portrait of *M. leprae* helps integrate what the bacterium can and cannot do. For example, bioinformatic analyses of metabolic pathways indicated that *M. leprae* has lost many metabolic pathways, together with their regulatory circuits, particularly those involved with catabolic potential (104). Aided by this metabolic picture, researchers can now investigate gene expression as it relates to various metabolic conditions either in limited culture with *M. leprae* or by studying gene function in cultivable mycobacteria into which specific mutations have been introduced. In this way it may be possible to determine the conditions that enhance or suppress *M. leprae* viability when it is held in axenic culture.

Another important element of establishing the proteome of *M. leprae* impacts earlier work involving antigenic analysis of *M. leprae* with the purpose of identifying proteins useful for diagnostics and vaccines. For example, a group of *M. leprae* proteins that have gone understudied until recently is secreted proteins. Purified bacilli from armadillo, mouse, and human tissues by definition lack (or are significantly reduced in their concentration of) secreted proteins. Bioinformatic approaches have identified the basic genes necessary for a functional SecA-dependent secretory system in *M. leprae* (74). In addition, Williams and coworkers (440) have shown that all genes in the SecA-dependent pathway are transcribed during growth of *M. leprae* in nude mouse footpads and that some 25 proteins with potential for secretion are transcribed (see Transcriptome, above). Therefore, *M. leprae* has the potential to produce secreted proteins, most of which have not been studied for immunogenic potential. A full assessment of these proteins may give rise to important antigens useful for developing much-needed early diagnostic tests as well as therapeutic and prophylactic vaccines.

Finally, by virtue of its relatively small gene set and possibly smaller proteome, *M. leprae* has become an important model for conceptualizing the minimal gene set needed for obligate intracellular parasitism. It is unlikely that all of *M. leprae*'s

TABLE 2. Application of PCR for the detection of *M. leprae* in human specimens

Tissue sample	Reference(s)
Skin smears	194, 409
Nasal smears	32, 90, 194
Skin biopsies	68, 91, 150, 435
Paraffin-embedded skin biopsy samples	17, 109
Blood	334
Nerve lesions	62
Ocular (iris) lesions	54

TABLE 3. *M. leprae* genes used in the development of PCR assays

Template for assay	Reference(s)
Gene	
hsp18	435
ag36	150
groEL1	143, 301
16S rRNA	17, 297, 318
Noncoding sequences	
RLEP	68, 170
rRNA	
16S rRNA	146, 228

open reading frames are expressed during all phases of growth and parasitism. Teasing apart these intricate relationships should provide important insights into mechanisms of virulence, such as nerve infection, as well as identifying proteins expressed during early infection, which could give rise to better diagnostic reagents and potentially a vaccine to improve our chances of managing leprosy.

Molecular Identification by PCR

Definitive identification of *M. leprae* is sometimes problematic, since the organism is not cultivable. This problem is confounded today by the increased prevalence of other mycobacterial infections of the skin. Rapid molecular-type assays have been developed for detection of *M. leprae* directly from patient specimens using available genetic data (reviewed in references 132, 208, and 348).

These assays have been based primarily on the amplification of *M. leprae*-specific sequences using PCR and identification of the *M. leprae* DNA fragment. This technique has been applied not only to skin biopsy samples but also to several different types of specimens, as indicated in Table 2. However, one should not infer from this that any tissue or specimen is suitable for PCR-based detection of *M. leprae* (see Table 3).

Many different *M. leprae* genes have been utilized in the development of PCR assays for detection of *M. leprae* in clinical specimens, as summarized in Table 4. RNA analysis using 16S rRNA and reverse transcription-PCR has the added benefit of measuring viability posttreatment (146, 228). PCR has thus generated new approaches to the detection and identification of *M. leprae* and, coupled with mutation detection analyses, has the ability to provide rapid drug susceptibility results from specimens taken directly from the patient.

On the basis of extensive assessment of these tests in field studies, PCR-based and reverse transcription-PCR-based techniques have shown a specificity of 100% and a sensitivity ranging from 34 to 80% in patients with paucibacillary forms of the disease to greater than 90% in patients with multibacillary forms of the disease. Automation of PCR-based assays has allowed their implementation in many reference laboratories, chiefly in countries with endemic leprosy. Therefore, PCR can provide an excellent adjunct to clinical and histopathological diagnosis of leprosy.

Epidemiology and Strain Identification

Understanding the epidemiology of leprosy is a prerequisite for effective control of the disease. Since *M. leprae* cannot be cultured in vitro, it has been virtually impossible to assess exposure, onset of infection, and various aspects of disease progression. As a consequence, the sequence of events that must occur for successful transmission of leprosy is poorly understood. Genetic markers may hold the key to establishing species- and strain-specific markers for assessing exposure to *M. leprae* and tracing transmission patterns. These tools should be helpful for improving our understanding of the epidemiology of leprosy.

Over the last two decades, a wide range of molecular tests have been applied to reveal genotypic variation in *M. leprae*. The results of initial studies suggested that the genome of *M. leprae* was highly conserved. Restriction fragment length polymorphism analysis of *M. leprae* isolates using a combination of restriction enzymes and probes and sequencing of the internal transcribed spacer region of the 16S-23S rRNA operon yielded no polymorphic DNA sequences (69, 92, 436). A polymorphic structure in the *polA* gene (119) and variation in a GACATC repeat in the *rpoT* gene (252) have been described, but the value of these elements for differentiating possible *M. leprae* strains appears to be limited.

In completing the sequence of the *M. leprae* genome, Cole et al. (74) identified several tandem repeats that could prove useful for discriminating *M. leprae* strains. Recently, Shin et al. reported evidence for diversity among *M. leprae* isolates ob-

TABLE 4. Indications and suitable specimens for *M. leprae* PCR

Category	Description
PCR indicated	Identification of acid-fast organisms when bacilli are numerous but tissue site, clinical history, or other circumstances are questionable; bacilli are sparse and tissue site, clinical history, or other circumstances are questionable
PCR not indicated	To find bacilli that are not identifiable in good-quality Fite-stained sections
Biopsies suitable	Specimens from newly diagnosed, untreated, or relapsed leprosy patients not yet retreated
Suitable specimens	Freshly acquired and processed immediately; frozen at −80°C indefinitely; frozen in over-the-counter cryopreservative at −80°C; fixed in 10% formalin for ≤24 h; fixed in 10% formalin for ≤24 h and paraffin embedded; fixed in 70% ethanol and stored at room temperature for up to 2 yr
Unsuitable specimens	Biopsy samples from treated leprosy patients; refrigerated specimens; unfixed (and unfrozen) specimens

tained from several patient biopsy samples in the Philippines, based on the frequency of TTC repeats located downstream of a putative sugar transporter pseudogene (371). In addition to the TTC locus, in silico analysis of the genome sequence indicates that *M. leprae* has several other tandem repeat loci which may provide the genetic diversity necessary for creating a typing scheme capable of answering important questions related to the epidemiology of leprosy. Recent studies employing four different variable-number tandem repeat markers have successfully differentiated *M. leprae* strains used in the laboratory and successfully grouped identical samples and passage samples tested in blind panels (412).

Far less diversity is seen with regard to single-nucleotide polymorphisms within the genome. The *M. leprae* single-nucleotide polymorphism frequency (~1 per 28 kb) is among the lowest seen for a human pathogen, and only three informative single-nucleotide polymorphisms have been identified. Among the 64 possible permutations of different bases at each of these polymorphisms, only 4 are observed. The variation in single-nucleotide polymorphism genotype is highly correlated with the geographic origin of the strain, and analysis of strains from different continents has been useful in predicting the evolution and global spread of leprosy. The disease appears to have originated in eastern Africa or the Near East and spread with successive human immigrations (272). Europeans and North Africans appear to have introduced leprosy into West Africa and the Americas within the last 500 years.

EXPERIMENTAL MODELS OF LEPROSY

Overcoming Obstacles to Leprosy Research

Rees (315a) divided experimental leprosy research in animal models into two eras: 1874 to 1960, the dark ages, and post-1960, i.e., after the Shepard mouse footpad model. An exhaustive yet incomplete list of animal species tested as models for leprosy begins with rabbits infected by Hansen and includes dogs, cats, pigeons, chickens, paddy birds, canaries, parrots, lovebirds, eels, tadpoles, frogs, toads, pigs, turtles, snakes (including rattlesnakes), goldfish, rainbow perch, various saltwater fish, rats, black mice, white mice, "dancing" mice, chipmunks, golden hamsters, albino hamsters, gerbils, a variety of nonhuman primates, and guinea pigs (190). Experiments employed a confusing myriad of protocols with widely variant reports of "success," but these were usually abortive or at best inconclusive and unconfirmed findings. No serial passage was reported and, in hindsight, because of the relative indestructibility of even dead *M. leprae*, the minimal successes reported could have been due to local lepromin-like responses.

One obstacle to the development of an animal model for leprosy has been the poor quality of the *M. leprae* inoculum, which usually consisted of fresh or frozen homogenates of nodules and lesions from untreated human lepromas. An important research emphasis in the National Hansen's Disease Program laboratories over the past few years has been the production, characterization, and provision of viable *Mycobacterium leprae* for our own researchers and qualified investigators around the world. A large (>200 mice) colony of *M. leprae*-infected athymic *nu/nu* mice is maintained for this purpose. A protocol of rigorously programmed passage of *M. leprae*, with radiorespirometry (the oxidation of ^{14}C-labeled palmitic acid) as a measure of viability, has enabled the harvest, on a routine (weekly) schedule, of 4 to 6 billion bacilli that are 80 to 90% viable, a resource unprecedented in almost 130 years of leprosy research. With these organisms we have confirmed the preference of *M. leprae* for cooler temperatures (4°C for storage, 26°C to 33°C for metabolic activity) and the rapidly deleterious effects of incubation at 37°C or a single freezing-thawing cycle (414).

Whereas radiorespirometry measures the metabolic activity of a suspension of *M. leprae*, and this has been shown to be correlated with growth in the mouse footpad, a novel, two-color fluorescence viability staining assay (Molecular Probes BacLight bacterial viability kit) has recently been adapted to provide a rapid (~1-h), reliable, quantitative, direct-count viability assay that measures the cell wall integrity of individual bacilli (229). This confirms previous findings regarding biophysical optima for maintaining *M. leprae*.

The second impediment in the early attempts to develop a leprosy model in animals was failure to recognize the prolonged growth cycle of *M. leprae* and not acknowledging its preference for cooler body sites. A new era was entered with description of the mouse footpad model by Shepard in 1960 (363). Passage of *M. leprae* infection was achieved, drug evaluation and rudimentary immunology studies became feasible, and the basis for subsequent exploration of *M. leprae* infection in various immunocompromised, transgenic, and knockout murine models was established.

Mouse Footpads and Nude Mice

Shepard's demonstration of the multiplication of *M. leprae* in the footpads of Carworth Farms white mice (363) opened new opportunities for investigation into basic immunological mechanisms of host resistance as well as screening of antileprosy drugs and drug combinations and detection of drug-resistant strains of *M. leprae*.

The importance of the T lymphocyte in host resistance was revealed in experimental *M. leprae* infection of neonatally thymectomized or congenitally athymic mice and rats (75, 208, 315). In immunocompetent mice, an inoculum of a few thousand bacilli grows locally and plateaus at approximately 1 million organisms per footpad. There is virtually no disease in these footpads, and the histopathological changes are minor, consisting of small granulomas containing a few lymphocytes and very few bacilli. Furthermore, there is essentially no dissemination of infection. In athymic *nu/nu* mice, however, local footpad multiplication of *M. leprae* appears to be unimpeded, reaching 10^{10} or more bacilli per footpad.

Histopathologically, the infected footpad tissue becomes an enormous foreign body-type macrophage (Mϕ) granuloma, or leproma, and the cells are engorged with bacilli (61) (Fig. 4). Unlike the course in an immunologically intact mouse, some dissemination does occur, and if observed for a long enough interval, evidence of growth in the opposite hind footpad or forefeet is seen. Thus, development of this mouse model was a second major milestone in leprosy research for, in addition to its immunological significance, the athymic *nu/nu* mouse footpad allowed the routine culture of large numbers of highly viable *M. leprae* for experimental use (208, 229, 414) (see Basic

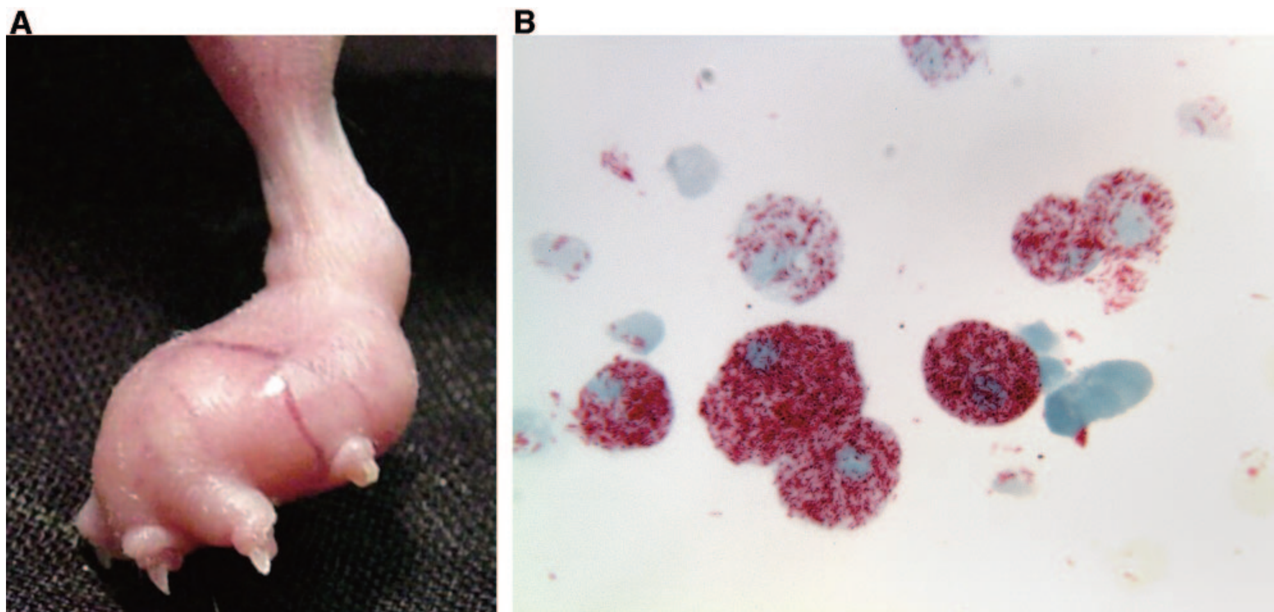

FIG. 4. Cultivation of *M. leprae* in mouse footpads. A. Enlarged nude-mouse footpad 6 months after infection with 5×10^7 live *M. leprae*. B. Heavily infected macrophages harvested from mouse footpad (magnification, ×1,000).

Characteristics, above). More recently, other immunosuppressed strains of mice and rats have been reported to allow enhanced growth of *M. leprae*, including severe combined immunodeficiency mice, which lack both T and B cells (22).

Gene Knockout Mice

The importance of the production of Th-1 cytokine responses in host resistance to intracellular infections, including *M. tuberculosis* and opportunistic mycobacteria, was confirmed with the recently identified human genetic deficiencies in cytokine receptors (420). Clinical evidence for an altered course of leprosy in such individuals has not been demonstrated, although this is most probably due to the protracted course of the disease and the relative avirulence of *M. leprae*. However, experimental *M. leprae* infections in cytokine knockout (KO) mice have substantiated the immunological importance of these cytokines across the leprosy spectrum and revealed compensatory mechanisms of host resistance to *M. leprae*.

An invaluable means for studying immunological parameters of host defense is via gene transfer technology in the murine system. Through a variety of mechanisms a specific gene can be rendered either totally or conditionally inactive (244). The resulting knockout models allow investigation at the level of the direct effects due to the loss of the functional gene as well as the compensatory mechanisms operational in its absence. Numerous targeted gene KO strains are now commercially available, including those unable to produce specific cytokines and chemokines, their receptors, immune modulators, or cell surface markers. In addition, new generations of mice, including tissue-specific KO, conditional KO, multiple KO, and knockin mutants, are continually being developed.

We have examined several strains of KO mice in our studies on *M. leprae* growth and granuloma development in experimental leprosy. Perhaps the best characterized is the inducible nitric oxide synthase (iNOS) KO ($NOS2^{-/-}$) strain. Mϕ isolated from $NOS2^{-/-}$ mice are incapable of producing reactive nitrogen products and they cannot inhibit *M. leprae* metabolic activity in vitro, although they are fully competent in producing reactive oxygen products (3). When inoculated into $NOS2^{-/-}$ mouse footpads, *M. leprae* initially grew to slightly higher levels than seen in wild-type controls, but thereafter the course of infection was similar. The granulomas formed in wild-type mice in response to *M. leprae* infection consisted of only small, focal collections of mononuclear cells; in contrast, the granulomas formed in $NOS2^{-/-}$ mice contained large, dense, organized collections of epithelioid cells and lymphocytes which infiltrated the perineurium and destroyed muscle bundles (6). In addition, Th-1-type cytokine expression was significantly augmented in $NOS2^{-/-}$ mice, and granulomas in liver tissue demonstrated a pattern similar to that seen in human leprosy lesions (268) with $CD4^+$ T cells distributed throughout the lesion, surrounded by $CD8^+$ T cells (D. A. Hagge et al., submitted for publication). Overall, the *M. leprae*-infected $NOS2^{-/-}$ mouse model exhibits findings similar to those of BT leprosy in humans. Interestingly, in contrast to $NOS2^{-/-}$ mice, macrophages from mice that are superoxide deficient due to a defective gp91 subunit of the phagocyte oxidase ($phox91^{-/-}$) (93) efficiently kill *M. leprae* in vitro, and infected $phox91^{-/-}$ mice develop a footpad induration similar to that of wild-type mice (Adams, Scollard, and Krahenbuhl, unpublished).

Gamma interferon (IFN-γ) KO (IFN-$\gamma^{-/-}$) mice also exhibited enlarged footpads and enhanced cellular infiltration upon infection with *M. leprae* (7). The footpad, however, contained epithelioid Mϕ and scattered lymphocytes that were not assembled into organized granulomas. Growth of *M. leprae* was enhanced approximately 1 log over that in wild-type mice and a Th-2-type cytokine profile was generated. Overall, the gen-

eral features exhibited by the IFN-$\gamma^{-/-}$ model were similar to those of BB-BL leprosy.

Studies in other KO mouse models are currently under way. Growth of *M. leprae* in mice deficient in interleukin-10 (IL-10) was similar to that seen in immunocompetent mice, whereas bacillary growth was augmented in mice deficient in IL-12, TNF-α, tumor necrosis factor receptor, lymphotoxin α, CD4, and CD8 (Adams, unpublished data). It is important to note, however, that *M. leprae* growth in these KO footpad models, including the IFN-$\gamma^{-/-}$ model, did not reach the enormous levels seen in the T-cell-deficient mouse models. Thus, even though these cytokines and cell types are important for the expression of cell-mediated immunity, compensatory mechanisms are able to limit bacillary growth. Immunoregulation in these mice is being studied further by additional "conditional" approaches, such as treatment with competitive inhibitors to create a second KO or restoration of the KO in infected mice. These immunoregulatory mechanisms may be crucial in the manifestation of the unstable borderline areas of the leprosy spectrum.

Nine-Banded Armadillo

Armadillos were originally adapted to captivity in order to study their unusual reproductive traits, which include both diapausic development and polyembrony (394). In 1968, Kirchheimer and Storrs began experimenting with armadillos to potentially exploit their cool body temperature (30 to 35°C) and showed that armadillos are uniquely susceptible to *M. leprae* (216, 217).

The nine-banded armadillo (*Dasypus novemcintus*) is the only immunologically intact animal that regularly develops fully disseminated *M. leprae* infections. Intravenous inoculation with 10^8 to 10^9 bacilli regularly results in 10,000-fold increases in the number of *M. leprae* over a span of about 18 months, and armadillos have been the hosts of choice for in vivo propagation of *M. leprae* for more than 30 years. With high burdens of bacilli in their reticuloendothelial tissues, armadillos can yield gram quantities of *M. leprae* (185, 413).

Approximately 65% of all armadillos experimentally inoculated with *M. leprae* will develop a fully disseminated infection. Infected armadillos exhibit few discernible clinical signs. Histopathological examination of infected animals typically reveals heavy infiltration of *M. leprae*-laden macrophages throughout the liver, spleen, and lymph nodes, as well as notable involvement of the lips, tongue, nose, nasal mucosa, skin, bone marrow, eyes, lungs, peripheral nervous system, gonads, and other tissues. Like humans, armadillos can upgrade and downgrade their response to *M. leprae* over the course of infection, but 90% of the animals that exhibit signs of systemic dissemination will eventually succumb to their leprosy (181). Intravenous inoculation promotes the most rapid and severe infections. However, respiratory instillation and percutaneous and intraperitoneal inoculation are also known to be effective (413).

Free-ranging armadillos are exposed to a number of atypical mycobacterial species in the environment and may develop nonspecific antibody responses cross-reactive with antigens shared by *M. leprae* (413). Armadillo immunoglobulin M (IgM) is highly cross-reactive with human IgM, and armadillo IgG reacts well with protein A or G.

The granulomatous response of armadillos to *M. leprae* is histopathologically identical to that seen in humans. Armadillo-derived lepromin (lepromin-A) can be used effectively to index the cell-mediated response of individual animals and to classify their type of leprosy according to the Ridley-Jopling scale. The reactions of individual animals can range from polar lepromatous to polar tuberculoid. Lepromatous armadillos develop the very large burdens of bacilli needed to obtain *M. leprae* from their tissues with high purity and are selected most commonly for propagation purposes (182–186), However, both lepromin-positive and lepromin-negative armadillos can resist challenge with *M. leprae*, and armadillos are useful models for studying both susceptibility and the variable resistance to leprosy that may result from treatment or vaccination (185, 187–189).

In 1973 Kirchheimer showed that 80% of the armadillos sensitized with heat-killed *M. leprae* could resist infectious challenge (213). Subsequent vaccination studies with BCG demonstrated effective protection of armadillos against *M. leprae*. The armadillo is the only animal model that can demonstrate effective protection against *M. leprae* with BCG, equivalent to rates observed for BCG in several human vaccination trials. Studies with these animals could potentially benefit our efforts to generate more efficacious antituberculosis and antileprosy vaccines (29, 188, 214, 215). Unfortunately, the number of armadillos and duration of time required (up to 1,140 days) for effective challenge studies with armadillos currently limit the utility of armadillos as vaccine models.

In addition to the armadillo's value as a source of organisms and an immunological model, the infection of peripheral nerves in the armadillo constitutes a unique model of lepromatous nerve involvement in humans (350, 351) (reviewed in Mechanisms of Nerve Injury, below).

Recently, the Human Genome Consortium completed the sequencing of the armadillo genome as part of a comparative genomics initiative. Armadillos are the most common modern representatives of *Xenathra*, a family of mammals that diverged from the rodent-primate tree in the Cretaceous period. The availability of extensive sequence information on the armadillo (http://www.ncbi.nlm.nih.gov/BLAST) is likely to rapidly expand the availability of new immunological probes and reagents for use with armadillos and will advance their use as models for resistance, vaccination, and nerve injury.

Wild nine-banded armadillos in the south central United States are highly susceptible natural hosts of *Mycobacterium leprae*. Surveys conducted over the last 30 years on more than 5,000 animals confirm that the infection is present among armadillos in Arkansas, Louisiana, Mississippi, and Texas. Little evidence for *M. leprae* infection is found among armadillos elsewhere in the U.S. range, and only a few reports relate finding the infection among animals in Central or South America. However, the issue has received only scant attention in other countries. Armadillos only recently expanded their range into the United States, and leprosy was present in Texas and Louisiana prior to the arrival of armadillos. The ecological relationship between humans and armadillos with *M. leprae* in this region remains unclear. However, infected armadillos constitute a large reservoir of *M. leprae*, and they may be a source

of infection for some humans in this country, and perhaps in other locations across the animal's range (415).

The impact of armadillo leprosy on humans is difficult to measure. A number of anecdotal reports have associated handling armadillos with individuals' developing leprosy, and case-control studies have yielded conflicting results (45, 239). Among Louisiana residents developing leprosy, no association with armadillo contact was found (110), but among Mexican-born patients who presented in Los Angeles and who had lived in areas where they could have been exposed to armadillos, an increased likelihood for lepromatous-type leprosy was reported (408). Leprosy remains rare in the United States, and the degree of risk attributable to armadillos is quite low. Nonetheless, armadillos are a large natural reservoir and can be an effective vehicle for exposure to large numbers of *M. leprae* (112). However, understanding the actual impact of armadillos on human infection will likely require the evolution of better molecular techniques that can track transmission.

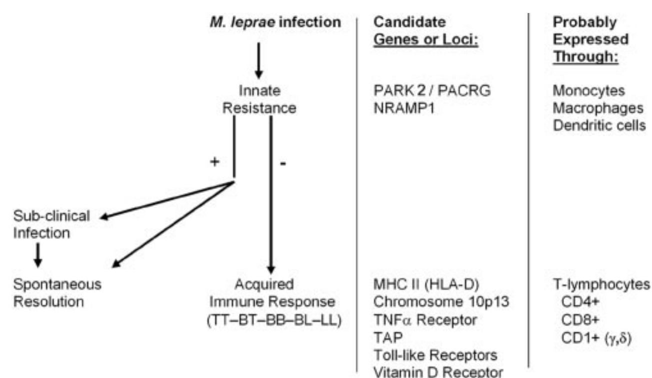

FIG. 5. Two-stage model of genetic influence on human immunity to *M. leprae*. Infection by *M. leprae* probably occurs through a skin or nasal route, by mechanisms that are not yet defined. The various genes and loci listed are discussed in the text under Genetic Influences, and the cells listed are discussed in the succeeding sections.

HOST RESPONSE TO *M. LEPRAE*

Genetic Influences on Leprosy in Humans

Before Hansen's discovery of the leprosy bacillus in 1874, leprosy was widely regarded as an inherited disease. Evidence from studies of twins with leprosy and of family clustering of cases continued to suggest that some inherited influence was a factor in susceptibility to this disease. An appreciation of the role of immunity in the different clinical and pathological manifestations of leprosy as well as subsequent advances in the field of immunology provided a foundation for focused inquiries concerning the genes that might influence susceptibility to *M. leprae*.

The idea that at least two different genes might control the human immune response to leprosy was proposed in the 1970s (89) and supported by subsequent investigations (1, 427). A convincing body of evidence now exists to indicate that different genes do influence the human immune response to *M. leprae*, operating at two levels (Fig. 5). The first level, overall susceptibility/resistance to the infection, is a manifestation of innate resistance mediated by cells of the monocyte lineage. If innate resistance is insufficient and infection becomes established, genetic influence is expressed at the second level, i.e., influencing the degree of specific cellular immunity and delayed hypersensitivity generated by the infected individual. Such acquired immunity is mediated primarily through the function of T lymphocytes, in cooperation with antigen-presenting cells (see Adaptive Immunity, below). The association of genes involved in both innate and acquired immunity to leprosy has recently been reviewed by Marquet and Schurr (249), and genetic defects in different components of the type 1 cytokine pathway that affect human resistance to mycobacteria have been reviewed by van de Vosse and colleagues (420).

Genetic influences on innate resistance to *M. leprae*. (i) *PARK2/PACRG*. One of the most extraordinary advances in the understanding of leprosy has been the identification by Mira and colleagues (262) of a locus within the gene *PARK2/PACRG* that is associated with overall susceptibility of human populations to *M. leprae*. This is the first example of the use of positional cloning to identify a human gene associated with susceptibility to an infectious disease (48). In their initial association scan of a linkage peak identified through a genome scan of a Vietnamese patient population, the investigators identified a locus within this gene that was highly associated with leprosy (leprosy per se), regardless of the subtype of this disease. These results were confirmed by a second analysis of Brazilian families with one or more persons affected by leprosy. The specific locus is a promoter region of *PARK2* and a coregulated gene, *PACRG*, located on chromosome 6q25-q27. *PARKIN* was named for its association with an early-onset form of Parkinson's disease, and the finding that a locus within this gene is also associated with susceptibility to leprosy is very unexpected.

Functionally, the specific locus identified codes for the synthesis of a ligase in the ubiquitin-proteasome pathway of intracellular protein degradation (454). Recent work has revealed some mechanisms by which this pathway regulates the processing of protein antigens within macrophages, thereby affecting antigen presentation to lymphocytes and the resulting immune response (278, 429). The exact mechanism by which this gene influences overall susceptibility to leprosy, however, remains to be determined.

(ii) *NRAMP1*. The first evidence of a genetic determinant of overall susceptibility or resistance that might relate to leprosy was the demonstration by Skamene and colleagues of a gene controlling susceptibility and resistance of mice to intracellular pathogens (383). Located at a single locus on mouse chromosome 1, this gene was initially designated *BCG*. Based on its function in mice it was termed natural resistance-associated macrophage protein 1 (*NRAMP1*) and is now designated *SLC11A1*. Functionally, murine NRAMP proteins influence pathogen viability and/or replication within macrophages by transporting iron and other divalent cations across the phagosomal membrane (139). Although the precise function of human *NRAMP1* has not been definitely established, the human gene, located on chromosome 2q35, is highly homologous with the mouse gene.

An association of this gene with overall susceptibility to leprosy was first reported in a study of families with multiple cases of leprosy (2). Subsequent studies have also suggested

that *NRAMP1* may be associated with different leprosy types in some populations, possibly through its influence on the expression of major histocompatibility complex (MHC) class II molecules, regulation of expression of *TNFA*, and induction of nitric oxide synthase (33). The ability (or inability) of an individual to develop a granulomatous response to an intradermal injection of killed *M. leprae* (the Mitsuda skin test) has been linked to NRAMP1 in some studies (8, 108) but not in others (152), possibly due to the frequency of different polymorphisms in different populations or to methodological differences in the studies.

Genetic influences on acquired immune responses in leprosy. (i) HLA. Early studies using serotyping techniques attempted to find associations between leprosy and human leukocyte antigens (HLAs) in major histocompatibility complex class II. These have been reviewed by Sergeantson (358) and Ottenhoff and de Vries (296). Overall, several of these serotyping studies suggested an association of HLA-DR2 and -DR3 with tuberculoid (paucibacillary) leprosy. Although some studies indicated an association of HLA-DR2 with both tuberculoid and lepromatous leprosy, no evidence convincingly demonstrated an association of the lepromatous response with any other HLA-D loci. Subsequent molecular genetic studies have borne out many of the early suggestions that the HLA region does play a determining role in the response to *M. leprae*. Advances in the technology of molecular genetics and in mathematical methods of interpretation of the data have extended these investigations far beyond the capabilities of the earlier techniques.

(ii) Chromosome 10p13. A genomewide linkage scan of 244 families in southern India revealed significant linkage of a series of microsatellite markers on chromosome 10p13 with susceptibility to leprosy (377). Most of the patients in these families had tuberculoid (paucibacillary) leprosy, and it is not clear whether the loci that were identified are associated with overall susceptibility to leprosy or only with the tuberculoid type of leprosy.

(iii) TAP. The transporter associated with antigen processing (TAP) is a protein composed of two polypeptides, TAP1 and TAP2. Their respective genes, located on chromosome 6p21, lie within the MHC class II region between HLA-DP and -DQ (388). Functionally, TAP proteins transport peptides to the endoplasmic reticulum in antigen-presenting cells, where they are joined to MHC class I molecules for antigen presentation. The *TAP2* gene has been associated with tuberculoid leprosy (306), but because this gene is located so close to other HLA genes, interpretation of the results has been difficult and the significance of this finding is uncertain and awaits more detailed studies.

(iv) *TNFA*. Tumor necrosis factor alpha, produced primarily by macrophages and causing activation of macrophages and T cells, plays a major role in nonspecific inflammation and innate resistance and is also one of the most powerful stimulants of cell-mediated immunity. In leprosy, TNF-α is generally associated with resistance to *M. leprae*. For example, serum levels of TNF-α are elevated in patients with resistant (tuberculoid) disease and with type 1 reactions, and expression of this cytokine is also increased locally in skin lesions in these manifestations of leprosy (see Leprosy Reactions, below).

The *TNFA* gene is located in the MHC class III region on chromosome 6p21. Several polymorphisms of this gene have been identified, especially in the promoter region. Because of the wide range of influence of TNF-α on cellular immunity, these promoter polymorphisms are of great interest as possible modulators of the degree of host response and therefore of clinical types of leprosy. Thus, several genetic studies have reported associations of *TNFA* alleles with different types of leprosy. In an Indian population, an association of one allele with lepromatous leprosy was observed (326); in a Brazilian population, another allele was associated with tuberculoid disease (335). The latter study also found this allele to be protective against leprosy per se, i.e., against the overall likelihood of acquiring leprosy of any type. Associations of some *TNFA* alleles with the strength of skin test responses to *M. leprae* (the Mitsuda test) have also been reported (108, 273). Together, the clinical, experimental, and genetic evidence suggests that the *TNFA* gene is involved in a complex manner in the regulation of human immune resistance to *M. leprae*.

(v) TLRs. Colorfully named after their counterpart in *Drosophila melanogaster*, human Toll-like receptors (TLRs) are cell surface molecules that play an important role in the recognition of pathogens. Because activation of TLRs results in the release of several chemical mediators of immunity, *TLR* genes also exert an important influence on the early events in specific immune responses (154). Evidence from studies of leprosy patients indicates that *TLR2* controls the production of cytokines, cell signaling, and other aspects of resistance to *M. leprae* (35, 195, 196, 223).

(vi) *VDR*. After earlier studies suggested that polymorphisms of the human vitamin D receptor gene (*VDR*) were associated with susceptibility to tuberculosis (323), a study of leprosy patients indicated that different alleles of this gene were associated with tuberculoid and lepromatous leprosy (325). The *VDR* gene, located on chromosome 12q12, encodes an intracellular receptor protein which binds the active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$. Binding to this receptor leads to the activation of monocytes and influences the function of both $CD4^+$ and $CD8^+$ T lymphocytes (153).

Development of the Immune Response

Innate immunity. The host defense events that operate early in infection during the indeterminate phase are perhaps the least understood aspects of the immunology of leprosy. An effective innate immune response in combination with the low virulence of the leprosy bacillus may underlie resistance to the development of clinical disease.

(i) Antigen-presenting cells and dendritic cells. Dendritic cells (DC) likely play a key role in modulating the early innate immune response to *M. leprae* (87). At the site of *M. leprae* invasion of the host, e.g., the nasal mucosa or skin abrasion, and in the absence of an adaptive immune response, the DC may be the first cell to encounter the bacilli. Uptake of the bacilli by DC and subsequent local production of cytokines and chemokines could regulate inflammation and manipulate the ensuing course of the adaptive cell-mediated immunity into a Th-1 or Th-2 response to *M. leprae*. DC have been found to be very effective presenters of *M. leprae* antigen (242, 265, 336). MHC class I and II expression was downregulated in monocyte-derived DC infected with *M. leprae* bacilli (151), but DC

stimulated with *M. leprae* membrane antigens upregulated MHC class II and CD40 ligand-associated IL-12 production (242). This suggests that whole bacilli may suppress the interaction of DC and T cells.

DC infected with *M. leprae* expressed PGL-1 on the cell surface. PGL-1 has exhibited immunosuppressive properties, and masking of DC-expressed PGL-1 with specific antibody upregulated both the proliferative response and IFN-γ production by T cells stimulated with *M. leprae*-infected DC (151).

Both IL-12 and IL-10 are produced by DC, and IL-10 and anti-IL-12 have been reported to inhibit the lymphoproliferative response following presentation of *M. leprae* by DC (336). Macrophage-derived DC have been shown to be even more efficient antigen-presenting cells; furthermore, they were highly susceptible to killing by *M. leprae* membrane-specific $CD8^+$ cytotoxic T cells (212). Higher levels of $CD1^+$ DC are found in TT lesions than in LL lesions (379).

Langerhans cells are a subset of DC that initiate immune responses in the skin. LL patients have significantly fewer Langerhans cells in the skin, regardless of whether the biopsy sample was taken from healthy skin or a lesion, compared to uninfected controls or TT patients (133). In contrast, patients with TT lesions have increased numbers of Langerhans cells in the lesions, suggesting an active infiltration of these cells to these sites. Langerhans cells found in the epidermis of leprosy lesions coexpress high levels of CD1a and langerin (161), and *M. leprae*-reactive, CD1a-restricted T-cell clones derived from leprosy patients responded to antigen presented by Langerhans cell-like DC. The antigen presented was likely arabinomycolate, a glycolipid component of the mycobacterial cell wall. Administration of recombinant cytokines such as granulocyte-macrophage colony-stimulating factor (201) and IL-2 (199) into LL lesions has been shown to induce an infiltration of Langerhans cells into the sites.

Examination of leprosy biopsy samples has revealed that monocytes and dendritic cells in tuberculoid lesions expressed Toll-like receptors TLR1 and TLR2 much more strongly than those in lepromatous lesions (223). In addition, in vitro studies showed that the *M. leprae* 19-kDa and 33-kDa lipoproteins could activate monocytes and monocyte-derived dendritic cells through TLR2. The cytokine profile present in the lesion also appeared to be correlated with TLR function: Th-1-type cytokines were generally associated with TLR1 and TLR2 activation, and Th-2-type cytokines were associated with inhibition of activation. Interestingly, specific cytokines could regulate the TLR through two independent mechanisms in vitro, via modulation of TLR expression or by affecting TLR activation.

(ii) Pattern recognition receptors. During the innate immune response, pathogen-associated molecular patterns displayed on many microorganisms are recognized by pattern recognition receptors expressed on immune cells at the sites of initial exposure. One class of pattern recognition receptor contains the calcium-dependent or C-type lectins, which bind specific carbohydrate moieties on pathogens and facilitate internalization for antigen processing and presentation. A second category of pattern recognition receptor is comprised of Toll-like receptors. Engagement of these receptors can trigger release of antimicrobial products which can exert a preliminary assault on the pathogen as well as signal expression of costimulatory molecules and production of cytokines which induce the adaptive immune system. A third class of receptor important for mycobacterial uptake contains the complement receptors.

(iii) C-type lectin receptors. The mannose receptor (also called CD206), a receptor belonging to the C-type lectin superfamily, binds carbohydrate moieties on a variety of pathogens (9). It is expressed primarily on cells of the myeloid lineage, especially mature Mϕ, although not on monocytes, and on some subsets of dendritic cells. The Mϕ has been shown to play a role in uptake of virulent mycobacteria (342), and a major mycobacterial ligand is likely lipoarabinomannan (304) which, on virulent strains of *M. tuberculosis* as well as *M. leprae*, contains terminal mannose caps on the arabinose side chains of the molecule (38). Mannose-capped lipoarabinomannan can modulate several effector functions of mononuclear phagocytes, including TNF-α, prostaglandin E_2, and nitrite production (5, 14, 24, 59), as well as Mϕ activation for microbicidal capacity (5). It has also been reported that uptake of mycobacteria via the mannose receptor does not elicit a respiratory burst (20).

Another C-type lectin is the dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN; also called CD209). DC-SIGN is expressed on dendritic cells and also recognizes pathogens via the binding of mannose-containing structures (422). In studies using *M. tuberculosis*, DC-SIGN was shown to be the major receptor on DC for the bacilli, with complement receptors and the mannose receptor playing a minor role (400). Again, the primary mycobacterial ligand for DC-SIGN was mannose-capped lipoarabinomannan (220, 242, 400, 422). Some investigators have proposed that virulent mycobacteria may subvert DC function via DC-SIGN by suppressing DC maturation, possibly through the inhibition of IL-12 production (290) and the induction of IL-10 (125). Engagement of DC-SIGN may also inhibit TLR signaling (422).

Langerin (CD207) is a C-type pattern recognition receptor expressed by Langerhans cells. Langerin oligomerizes as trimers at the cell surface and possesses a single calcium-dependent carbohydrate recognition domain with specificity for mannose, *N*-acetylglucosamine, and fucose (219).

Langerin is necessary for the formation of Birbeck granules, the pentalaminar endosomal structures found exclusively in Langerhans cells. Exogenous carbohydrate ligands are endocytosed via langerin and transported to the Birbeck granules for processing. Langerin may play a role in the uptake of nonpeptide mycobacterial antigens (161).

(iv) Toll-like receptors. Mammalian Toll-like receptors are crucial for the recognition of microbial pathogens by Mϕ and dendritic cells during innate immunity. TLRs are phylogenetically conserved transmembrane proteins that contain repeated leucine-rich motifs in their extracellular domains. The cytoplasmic signaling domain is linked to the IL-1 receptor-associated kinase, which activates transcription factors such as NF-κB to induce cytokine production. Ten TLRs have been identified, of which TLR2-TLR1 heterodimers, TLR2 homodimers, and TLR4 appear to be significant in the recognition of mycobacteria. TLRs have been found to be necessary for the optimal production of IL-12 (39), a proinflammatory cytokine responsible for the induction of Th-1-type immunity, as well as TNF-α (417), a cytokine important in cellular activation and granuloma formation but also implicated in the

tissue destruction associated with leprosy reactions.

Recent work has substantiated an important role for TLRs in the recognition and subsequent immune response to *M. leprae*, particularly through dendritic cells (discussed above). Kang and Chae (193) first noted the correlation of a C-to-T substitution at nucleotide 2029 of TLR2, which resulted in the change of Arg to Trp at amino acid residue 667, with the lepromatous form of leprosy. Subsequently, upon stimulation of cells expressing this mutation with *M. leprae* or *M. leprae* antigens, this mutation was shown to be associated with a defective activation of NF-κB (35) and decreased production of IL-12, IL-2, IFN-γ, and TNF-α but increased generation of IL-10 (195, 196) compared to wild-type cells.

(v) C′ receptors. Schlesinger and Horwitz (343) established that complement receptors 1 and 3 on the surface of monocytes and CR1, CR3, and CR4 on Mϕ are key mediators of phagocytosis of *M. leprae*. In addition, uptake of PGL-1, a major surface glycolipid of *M. leprae*, was facilitated by complement component C3 (343). Uptake via complement receptors does not elicit a respiratory burst (see below); thus, this is one mechanism whereby pathogenic mycobacteria can elude the toxic oxygen radicals which can be generated during phagocytosis.

Adaptive immunity: development of cell-mediated immunity. Cells of the T-cell lineage play an essential role in resistance to *M. leprae*, as evidenced by the prolific local footpad multiplication of the bacilli in neonatally thymectomized (315) and congenitally athymic (75) mice. However, LL patients are not immunocompromised hosts and are not prone to cancer or the opportunistic infections that afflict persons with immunodeficiency diseases. The immunological anergy associated with LL is specific for the antigens of *M. leprae*.

(i) Protective and destructive effects of cell-mediated immunity. It has been estimated that >95% of persons are resistant to leprosy. Upon exposure, protection probably occurs early, with no overt signs of disease. There is no test currently available to reliably detect exposure to *M. leprae* or to diagnose preclinical infection. Individuals with clinical leprosy, even those classified with paucibacillary disease and having high levels of cell-mediated immunity, possess living organisms in their tissues. The protective aspects of cell-mediated immunity in paucibacillary leprosy are largely defined as controlling the multiplication of organisms. The collateral damage to tissues caused by the granulomatous inflammation accompanying cell-mediated immunity may have serious, long-term consequences, such as injury to peripheral nerves.

(ii) T-lymphocyte populations. *(a) MHC-restricted CD4$^+$ and CD8$^+$ cells*. By immunohistological staining, TT lesions exhibited mostly CD4$^+$ helper cells with a CD4$^+$/CD8$^+$ ratio of 1.9:1 (269). Although the CD4$^+$/CD8$^+$ ratio in normal peripheral blood is also 2:1, there appeared to be a preferential migration into, proliferation in, or retention of selected cells in the various types of leprosy lesions in that cells of the T helper/memory phenotype outnumbered the naive phenotype 14-fold in TT lesions. T cytotoxic cells were numerous in TT lesions. These cells may play a role in mediating the Mϕ localization, activation, and maturation that lead to restriction or elimination of the pathogen. Interestingly, CD4$^+$ cells were distributed throughout the lesion, whereas CD8$^+$ cells were stationed at the periphery in the TT lesion (268).

LL lesions, in contrast, displayed a CD4$^+$/CD8$^+$ ratio of 0.6:1, and unlike TT lesions, the CD8$^+$ T cells were distributed throughout the lesion rather than at the periphery. Using monoclonal antibodies which could distinguish T-cell subsets, the authors found that the CD4$^+$ cells present were primarily of a naïve phenotype and the CD8$^+$ cells were predominantly of a suppressor subset; thus, they proposed that these CD8$^+$ suppressor cells may serve to downregulate Mϕ activation and suppress cell-mediated immunity. However, a role for the recently described Foxp3-expressing CD4$^+$ CD25$^+$ regulatory T cells (159) in the various forms of leprosy has yet to be determined but may prove critical in the development of LL.

(b) CD1-restricted T cells. CD1 molecules bind ligands via hydrophobic interactions in a structurally unique, deep antigen-binding pocket that is designed to accommodate the hydrocarbon chains of lipids. There are two distinct groups of CD1 molecules. Group I, comprised of CD1s a, b, c, and e, is found in human systems but not in rodents. Group II contains CD1d of humans and CD1 of rodents. All have evolved to present lipid and glycolipid antigens rather than peptides, and human CD1 molecules present nonpeptide components of mycobacteria to specific CD1-restricted T cells.

In vitro and in vivo studies have indicated an important role for the CD1 system of mycobacterial lipid antigen presentation in immunity to *M. leprae*. Mycobacterium-reactive double-negative T-cell lines derived from a skin lesion of a leprosy patient responded to subcellular fractions of mycobacteria in the presence of CD1-expressing antigen-presenting cells (378). Lipoarabinomannan-depleted soluble cell wall fraction did not induce detectable T-cell proliferation. Recognition of purified lipoarabinomannan from *M. leprae* was restricted by CD1b, and T cells lysed lipoarabinomannan-pulsed monocytes in a CD1b-restricted manner. Lipoarabinomannan also induced these T cells to secrete large amounts of IFN-γ. Upon examination of leprosy patients, few CD1$^+$ cells were found in LL leprosy lesions. In contrast, there was a strong upregulation of CD1$^+$ cells in the granulomatous lesions of patients with TT leprosy or reversal reaction (324). These cells were also CD83$^+$, a marker for dendritic cells, indicating a strong correlation between CD1 expression and cell-mediated immunity in leprosy. Interestingly, administration of granulocyte-macrophage colony-stimulating factor, a cytokine which can promote dendritic cell activation, to LL leprosy patients induced infiltration of CD1$^+$ cells into the lesions (332).

(iii) Cytotoxic cells. *(a) T cells*. CD8$^+$ and CD4$^+$ T cells can function as class I- and class II-restricted cytotoxic T cells, respectively, and both are capable of lysing *M. leprae*-infected Mϕ (65, 149, 192). Lysis of target cells by cytotoxic T lymphocytes is mediated by perforin and cytotoxic granules such as granzyme B, a serine protease located in cytotoxic T cells and NK cells (369). Upon contact with the target cell, perforin is released by cytotoxic T cells and forms pores in the target cell membrane, allowing granzyme B to enter the cell, where it activates caspases and leads to target cell death. Granulysin is another defensive antimicrobial protein used by T lymphocytes and is expressed in tuberculosis (393) and leprosy (293). In leprosy lesions the presence of granulysin correlated with the polar forms of the disease and was observed more frequently in TT skin lesions than in LL skin lesions. Perforin was equally distributed in cells across the spectrum. Neither NK cells, Mϕ,

nor dendritic cells expressed granulysin.

Lysis of *M. leprae*-infected Mϕ target cells may contribute to protection in leprosy as an adjunct to the ongoing attempts at intracellular killing or inhibition by IFN-γ-activated Mϕ. Ex vivo and in vitro data from mice have demonstrated that the long-term intracellular presence of live *M. leprae* impaired the afferent and efferent functions of the infected Mϕ, especially their ability to become activated upon stimulation with IFN-γ (373, 375, 376). *M. leprae* released from these heavily infected Mϕ after lysis by T cells would be phagocytized by newly arrived activated Mϕ, which are more able to cope with the bacilli than the previous host cells, and subjected to another round of attack by powerful antimicrobial mechanisms.

(b) Natural killer cells. NK cells exert spontaneous non-MHC-restricted cytotoxicity against a variety of neoplastic and pathogen-bearing target cells, and although $CD3^-$, they share many characteristics with cytotoxic $CD3^+$ T cells. While NK cell numbers in the blood were similar across the leprosy spectrum, a marked decrease in circulating NK has been reported when patients were undergoing erythema nodosum leprosum (ENL) reactions (see below) (160). This situation reversed when the ENL reaction subsided. NK cells appear to be recruited to LL lesions injected with IL-2, where they may be responsible for the subsequent local clearance of the bacilli (199). The cytotoxicity of NK cells and their more active IL-2-stimulated lymphokine-activated killer (LAK) cell lacks antigen specificity but is directed against *M. leprae*-infected macrophages (66) and Schwann cells (392).

(iv) Macrophages. The Mϕ is the primary host cell for *M. leprae*. In the absence of an effective adaptive immune response, these relatively nontoxic bacilli can multiply in Mϕ to over 100 organisms per cell (145). In vitro, *M. leprae* can be maintained in a metabolically active state for weeks in Mϕ supplemented with IL-10 and cultured at 33°C (122). The Mϕ also plays an important role in the host's defense against *M. leprae*, being a key player in both the afferent and efferent limbs of the immune response. Antigen processing and presentation and monokine secretion are three major functions of the Mϕ in the afferent stage. The primary efferent function of Mϕ is killing this intracellular pathogen.

TLRs have also recently been shown to be important in the differentiation of monocytes into macrophages capable of antimicrobial functions or dendritic cells having primarily antigen-presenting capabilities (224). Activation of TLR2, using mycobacterial antigen, on monocytes isolated from TT patients induced differentiation into both (DC-$SIGN^+$) Mϕ and $CD1b^+$ dendritic cells. In contrast, when peripheral blood monocytes from LL patients were stimulated in such a manner, the cells differentiated into DC-$SIGN^+$ Mϕ but not $CD1b^+$ dendritic cells. This pattern of expression was likewise observed in leprosy lesions. The implication of these findings is that upon initial *M. leprae* stimulation of monocytes via TLRs, both tuberculoid and lepromatous patients may generate similar innate responses to *M. leprae*, but lepromatous patients are unable to proceed to the adaptive response seen in tuberculoid patients. If confirmed and expanded, this may offer important insight into the mechanisms that underlie the broad spectrum of human immune response in this disease.

(a) Mechanisms of macrophage killing of M. leprae. Although the viability of *M. leprae* is supported in normal mouse Mϕ, IFN-γ-activated Mϕ can drastically inhibit or kill *M. leprae* in vitro (307). These findings confirmed the demonstration that in normal Mϕ, phagosome-lysosome fusion was blocked by live, but not dead, *M. leprae* (373) and, more importantly, that in activated Mϕ, phagosomes harboring *M. leprae* fused with secondary lysosomes. Two important antimicrobial pathways by which Mϕ can inhibit or kill invading pathogens are the generation of reactive oxygen intermediates and of reactive nitrogen intermediates.

Upon phagocytosis of microorganisms by Mϕ, a respiratory burst ensues in which there is a great increase in the consumption of oxygen catalyzed by NADPH oxidase and the production of superoxide. Other reactive oxygen intermediates, including hydrogen peroxide, hydroxyl radical, and singlet oxygen, are subsequently generated. These toxic oxygen products are an important antimicrobial defense mechanism of phagocyte cells, especially against extracellular pathogens. *M. leprae*, however, is only a weak stimulus of the Mϕ oxidative burst (155), possibly due to a downregulation of superoxide generation by PGL-1 (58). *M. leprae* also possesses a superoxide dismutase (406) and expresses both SodC and SodA by reverse transcription-PCR (440). Thus, leprosy bacilli appear to be well equipped to handle antimicrobial reactive oxygen intermediates generated by the host Mϕ.

Reactive nitrogen intermediates, primarily nitric oxide, are derived from the terminal guanidino nitrogen of L-arginine by a high-output, inducible form of nitric oxide synthase (iNOS) produced by activated Mϕ. The ability of activated murine Mϕ to inhibit *M. leprae* metabolic activity is dependent on the generation of such reactive nitrogen intermediates, as activated Mϕ cultured in the presence of competitive inhibitors of the enzyme, such as L-monomethylarginine or aminoguanidine, had no detrimental effect on bacterial metabolism (4). Furthermore, activated Mϕ from *NOS2* knockout mice could not kill *M. leprae* (6).

The role of reactive nitrogen intermediates as a Mϕ effector mechanism in humans is somewhat controversial as it has been difficult to get in vitro-cultured cells to generate high levels of nitrite in as consistent a manner as in murine cells (430). However, several studies have shown that iNOS is expressed, as detected by immunohistochemistry, at the site of disease in patients infected with intracellular pathogens, including *M. leprae*. Khanolkar-Young et al. (210), using antibodies against iNOS, found that iNOS was highly expressed in the lesions of tuberculoid leprosy patients and was increased to even higher levels during the reversal reaction (see below). Moreover, the levels of iNOS subsided over the course of prednisolone treatment (233).

Because detection of iNOS by immunohistochemistry does not necessarily indicate actual production of reactive nitrogen intermediates, lesions have also been stained for nitrotyrosine, the stable end product of the nitrosylation of tyrosine residues in proteins by peroxynitrite (345). It was found that iNOS and nitrotyrosine were expressed in borderline leprosy patients both with and without the reversal reaction. In addition, elevated levels of nitrates were measured in the urine of leprosy patients undergoing reversal reactions, and these levels decreased upon high-dose prednisolone treatment (344, 345).

Methods have recently been developed to isolate granuloma Mϕ from the footpads of *M. leprae*-infected mice (145, 373). This model, which enables the study of Mϕ from the actual site

TABLE 5. Cytokine gene expression in nonreactional leprosy[a]

Gene	Single lesion	T-cell clones		Skin lesions		PBMC	
		T	L	T	L	T	L
IL-1		+ (113, 381, 448)	+/− (113)				
IL-2		+ (113, 381, 448)	+/− (113)				
IL-4			+ (381, 448)	+/− (263)	+ (263, 285)	+/− (141)	+/− (281)
IL-5			+ (381, 448)			+/− (141)	
IL-8			+ (381, 448)				
IL-10	+ (391)				+/− (285)		
IL-12	+ (391)				+/− (285)		
TNF-α	+ (391)	+ (381, 448)				+ (141, 281)	
IFN-γ	+ (391)	+ (381, 448)		+ (263)	+ (263, 281, 285)	+ (141, 281)	+ (281)
TGF		+ (381, 448)					
GM-CSF		+ (381, 448)					
MIP	+ (391)						

[a] Specimens for reverse transcription-PCR were frozen biopsies (skin lesions), cultures of peripheral blood mononuclear cells stimulated in vitro (PBMC), or T-cell lines and clones established from PBMC of leprosy patients. Positive results were obtained for the cytokines indicated. Evidence from several reports is summarized to present a consensus of the findings, although the studies had different designs and different methods of quantification and used different conventions to express their results. Early, single-lesion leprosy was usually found histologically to be consistent with TT or BT disease in the one study of these lesions. Most studies classified patients only as tuberculoid (T) or lepromatous (L) and did not stratify results within the borderline portion of the leprosy spectrum. +, reported increase in expression (above controls); +/−, minimal or no increase in expression. Numbers in parentheses are references. TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

of infection in experimental leprosy, has allowed determination of culture characteristics, cytokine production, and cell surface phenotypic markers by flow cytometry of these granuloma-derived cells. Initial studies of footpad granuloma Mϕ from *M. leprae*-infected athymic *nu/nu* mice indicated that the Mϕ were phenotypically indistinguishable from normal peritoneal Mϕ except that they contained enormous numbers of *M. leprae* (374, 375). These *M. leprae*-infected footpad-derived granuloma Mϕ were also functionally similar to peritoneal Mϕ except for one notable defect: they were refractory to activation by IFN-γ for both microbicidal and tumoricidal activity. In addition, there was no IFN-γ-induced augmentation of class II MHC expression or phorbol myristate acetate-induced superoxide production in the *M. leprae*-engorged granuloma Mϕ. These results provide further support that *M. leprae* is a potent modulator of Mϕ effector functions and that its influence is largely restricted to the microenvironment of the granuloma. This work is now being extended to evaluation of *M. leprae*-infected gene knockout mice.

Recent evidence from our laboratory, using an in vitro system in which *M. leprae*-infected *nu/nu* mouse footpad granuloma target Mϕ are cocultured with fresh uninfected effector Mϕ, suggests that Mϕ may play a role in cell toxicity in leprosy lesions (145). When normal effector Mϕ were cocultured with target Mϕ, the effector Mϕ acquired the bacilli from the infected target Mϕ. Moreover, if the effector Mϕ were activated with IFN-γ, they could kill the target cell-derived *M. leprae*. Killing of the bacilli in this system was not a rapid process but required 3 to 5 days of coculture for optimal effect. Furthermore, it was dependent on cell-cell contact and production of reactive nitrogen intermediates, but did not require concomitant IFN-γ or TNF-α production. The exact mechanism by which the effector Mϕ acquired *M. leprae* from the target Mϕ is not yet known. However, in numerous systems, especially those involving tumor models, Mϕ have demonstrated cytotoxic capabilities using a variety of functions, including direct lysis, engulfment, and induction of apoptosis or necrosis. Current studies are aimed at determining which mechanism(s) a new Mϕ may employ to effect cellular turnover in a leprosy lesion.

(v) Cytokines in leprosy. The Th-1/Th-2 paradigm, based on functional discrimination of T-helper cells according to their pattern of cytokine production, asserts that Th-1 and Th-2 cells promote a cellular and humoral immune response, respectively (277). This functional differentiation has offered an attractive hypothesis to explain the differences between tuberculoid and lepromatous responses to *M. leprae*.

Several major studies of local immune responses in leprosy skin lesions have been published (Table 5). These are difficult to compare because they have used different designs, different methods of quantification, and different conventions to express their results. Overall, however, these studies have generally revealed a predominance of IL-2, TNF-α, and IFN-γ transcripts in tuberculoid lesions and IL-4 and IFN-γ in lepromatous ones, gene expression profiles consistent with Th-1 and Th-2 patterns, respectively (16, 113, 281, 380, 448). $CD4^+$ clones isolated from TT lesions secreted primarily IFN-γ, whereas a $CD4^+$ clone from an LL lesion produced predominantly IL-4 (381), and $CD8^+$ clones isolated from LL patients likewise generated large amounts of IL-4 (328).

Further studies have also indicated that IL-12 and IL-18 promote resistance to *M. leprae* and are highly expressed in tuberculoid lesions (123, 380). Most studies have grouped patients only into tuberculoid or lepromatous groupings, however, and it is not clear whether variations in cytokine production correspond well with the various degrees of cellular immunity represented in the borderline portion of the leprosy spectrum.

The studies noted above have examined biopsies from well-established lesions, usually present for at least 2 years. The immunologic activity within earlier lesions, in patients with only a single lesion, has recently been evaluated by Stefani et al. (391). These early lesions, histologically consistent with TT or BT disease, also displayed a Th-1-like pattern of cytokine gene expression (Table 5).

Circulating leukocytes and T-cell lines from tuberculoid pa-

tients stimulated by *M. leprae* in vitro have also generally been found to produce a Th-1 cytokine pattern (Table 5), while leukocytes and T-cell lines from lepromatous patients generally produce a Th-2 cytokine pattern (263, 285). However, leukocytes from approximately 40% of all patients in one such study produced a mixed Th-0 cytokine profile, i.e., IFN-γ, IL-2, and IL-4 (263). It is possible that some of the patients whose cells produced the Th-0 pattern were in the borderline portion of the leprosy spectrum (BL or BT); alternatively, the human immune response to *M. leprae* may not correspond entirely with the Th-1/Th-2 model. Fractionated *M. leprae* antigens have also been found to stimulate IFN-γ in vitro with leukocytes from tuberculoid patients (95).

Experimental immunotherapy with intradermal inoculation of cytokines has provided additional information about their roles in immunological events within leprosy skin lesions (197). Both short- and long-term intradermal administration of IFN-γ resulted in an influx of mononuclear cells and an increase in the CD4/CD8 ratio in the lesions, but did not reverse the specific nonresponsiveness of circulating leukocytes to *M. leprae* (200). *M. leprae* exposure in vitro did not elicit IFN-γ in circulating mononuclear cells of lepromatous patients; the addition of IL-2 reversed this in most (but not all) of these patients' peripheral blood mononuclear cells (291). In lepromatous patients, intradermal injections of IL-2 generated apparent increases in cell-mediated immunity within the skin lesions (199) and resulted in increased levels of antibodies to *M. leprae* antigens (198), but did not enhance systemic cell-mediated immunity to *M. leprae*.

In summary, studies of cytokine gene expression in leprosy lesions thus far have given us a more detailed description of the immunological parameters of the polar types of leprosy, confirming and supporting the original concept that tuberculoid lesions are manifestations of delayed hypersensitivity and cellular immunity and that lepromatous ones result when immune recognition occurs (as indicated by antibody production) but the host is unable to develop cellular immunity to *M. leprae*. However, these studies have not yet revealed the mechanisms by which the cellular immune response is so extraordinarily titrated to produce the entire leprosy spectrum. Research in this area continues on the premise that the answer will be found in an understanding of the complex immunoregulatory mechanisms of cytokine production and inhibition. Some of the answers to these questions may also be found in studies of the genetically inherited ability to respond to *M. leprae* and other pathogens (see Genetic Influences on Leprosy in Humans, above).

LEPROSY REACTIONS

Reactions are acute inflammatory complications often presenting as medical emergencies during the course of treated or untreated Hansen's disease. Two major clinical types of leprosy reactions occur; together they may affect 30 to 50% of all leprosy patients (28, 226, 352). Because *M. leprae* infects peripheral nerves, the inflammation associated with reactions is a medical emergency, as severe nerve injury may develop rapidly, with subsequent loss of sensation, paralysis, and deformity.

The cause(s), mechanisms, and treatment of these reactions remain highly problematic, for both clinicians and basic scientists. The different types of reactions appear to have different underlying immunologic mechanisms, but these are poorly understood in spite of a substantial body of detailed descriptive information, and the factors that initiate them are unknown.

Clinical Presentation, Diagnosis, and Histopathology

Type 1 reactions occur in patients in the borderline portion of the spectrum (i.e., BL, BB, and BT). They are also known as reversal reactions, because early observations suggested that after the reaction had subsided, clinical and histopathological evidence indicated that the immunity in the lesions had increased (upgrading) or decreased (downgrading) (320). Downgrading is rarely seen in the current era of antimicrobial treatment, and the evidence regarding type 1 reactions reviewed here is based on studies of upgrading reactions.

These reactions present as induration and erythema of existing lesions, frequently with prominent acral edema and often with progressive neuritis, causing sensory and motor neuropathy (Table 6). In severe reactions the lesions may ulcerate. Type 1 reactions usually develop gradually, and their natural course may last for many weeks.

Type 2 reactions, also known as erythema nodosum leprosum, occur in multibacillary patients (LL and BL). These patients experience an abrupt onset of crops of very tender, erythematous nodules that may develop on the face, extremities, or trunk, without predilection for existing lesions (Table 6). Systemically, these patients often also experience fever, malaise, and some degree of neuritis with sensory and motor neuropathy. Iridocyclitis and episcleritis, orchitis, arthritis, and myositis may also accompany this reaction. In severe type 2 reactions, some of the cutaneous lesions may ulcerate. The natural course of type 2 reactions is 1 to 2 weeks, but many patients experience multiple recurrences over several months.

The Lucio phenomenon is an acute, severe, necrotizing vasculitis occurring primarily in patients of Mexican ancestry (96). Fortunately, this complication is rare, because it is associated with high morbidity and mortality. These reactions have been associated with the presence of high levels of cryoglobulins, and *M. leprae* antigens have been associated with some of these (100, 305), but the role they might play in these reactions is unclear. Characteristically, endothelial cells are unusually heavily infected and may be seen in various stages of degeneration. Lucio reactions may be accompanied by a profound anemia, and severe cases may require intensive wound care and debridement comparable to that used in the management of severe burns. These reactions require intensive inpatient management, and in the United States, consultation with the National Hansen's Disease Program is recommended.

Immunological Features of Reactions

Evidence regarding the mechanisms of leprosy reactions. All types of leprosy reactions are believed to be immunologically mediated, but the mechanisms responsible for each type of reaction remain poorly understood. Although type 1 and 2 reactions together affect 40 to 50% of all patients at least once in the course of their disease, no clinical or laboratory tests can accurately predict who is most likely to develop a reaction or when it might occur. The factors precipitating reactions and

TABLE 6. Comparison of clinical features of type 1 and type 2 leprosy reactions

Parameter	Type 1	Type 2
Patients at risk	BL, BB, BT	LL, BL
Onset of reaction	Gradual, over a few weeks	Sudden, "overnight"
Cutaneous lesions	Increased erythema and induration of previously existing lesions	Numerous erythematous, tender nodules on face, extremities, or trunk, without relationship to prior lesions
Neuritis	Frequent, often severe	Frequent, often severe
Systemic symptoms	Malaise	Fever, malaise
Histopathological features	No specific findings	Polymorphonuclear cell infiltrates in lesions <24 h old
Course (untreated)	Weeks or months	Days to weeks
Treatment	Corticosteroids	Thalidomide, corticosteroids

the earliest events in the development of reactions are therefore unknown.

(i) Type 1 (reversal) reactions. Substantial evidence now indicates that type 1 reactions are the result of spontaneous enhancement of cellular immunity and delayed hypersensitivity to *M. leprae* antigens, but the causes and mechanisms of this enhancement remain poorly understood. Early functional studies of lymphocytes demonstrated increased lymphocyte proliferation in response to *M. leprae* antigens in vitro during type 1 reactions (26, 135). Subsequent immunophenotyping studies revealed that the number and percentage of $CD4^+$ T cells are increased in reacting skin lesions (267, 269, 353; reviewed in reference 270). Measurement of soluble IL-2 receptor levels in patient sera found high levels in type 1 reactions when the patients first presented for treatment and found that these levels declined steadily during treatment (416).

During type 1 reactions, increases in expression of the genes for several proinflammatory cytokines, including IL-1, IL-2, IL-12, IFN-γ, and TNF-α, have now been documented in several studies (Table 7). This activation is present both locally, in reacting skin lesions, and systemically, in serum and in circulating leukocytes. The pattern of cytokine expression has suggested to many investigators that type 1 leprosy reactions represent a spontaneously enhanced Th-1 response. However, these studies have not been able to clearly differentiate which changes observed are consequences of reaction and which (if any) reflect initiating events, and several of the reports have not clearly distinguished immunological from inflammatory phenomena.

Clinical studies have determined that the serum levels of some of these cytokines decline during the course of successful prednisolone treatment of type 1 reactions but show little or no reduction during the treatment of type 2 reactions. Such a decline has been documented, for example, for indicators of inflammation such as neopterin and iNOS (114, 233), as well as for cytokines and receptors more indicative of immunologic function such as soluble IL-2 receptor, IFN-γ, IL-6, IL-10, IL-12, and IL-13 (21, 233, 416). These observations suggest that in addition to the nonspecific anti-inflammatory effects of prednisolone, it may have some inhibitory effect on the underlying immunologic mechanisms of these reactions, although it is not certain that this is the case.

The events or conditions that trigger type 1 leprosy reactions are unknown; notably, only about 15 to 30% of the patients at risk (i.e., BL, BB, and BT) are affected. Type 1 reactions have been observed to follow immunization with other mycobacteria in some circumstances (433, 452), and various environmental factors have been considered but not confirmed to be associated with the onset of these reactions. The possibility that genetic factors might predispose some patients to develop type 1 reactions has not yet been examined (see Genetic Influences, above). Notably, experimental intradermal inoculation of IL-2 or of IFN-γ did not precipitate type 1 reactions (198, 332). In addition, although thalidomide was reported to inhibit TNF-α in several experimental conditions (410), it has no benefit in the treatment of type 1 reactions.

TABLE 7. Cytokine gene expression changes during type 1 and type 2 reactions[a]

Category and cytokine	Type 1 reaction compared to BT or BL (reference[s])	Type 2 reaction compared to BL or LL (reference[s])
Circulating		
TNF-α	↑ (337)	↑↑ (25, 115, 330, 337*)
IFN-γ	↑ (274*, 389*)	↑ (274*, 330, 389*)
IL-1	↑ (337)	↑ (337)
IL-2	↑ (274)	↑ (274*)
IL-2 receptor	↑ (416)	↑ (416)
IL-4	Not increased	↓ (284, 285*)
IL-6	↑ (274)	↑ (274)
IL-8	↑ (274*)	↑ (274*)
IL-10		↑ (389*) ↓ (284)
$IL-12^{p40}$	↑ (389*)	↑ (285*, 389*)
Cutaneous		
TNF-α	↑ (21, 274*, 449)	↑ (21, 274*, 449)
IFN-γ	↑ (233***, 274*, 389, 423**, 449)	↑ (274*, 389, 449)
IL-2	↑ (449)	↑ (449)
$IL-12^{p40}$	↑ (233***, 274*)	↑ (274*)
IL-4	Not increased	↑ (274*, 449)
IL-10	↑ (274*)	↑ (274*, 449)
IL-6	↑ (274*)	↑ (274*)
IL-8	↑ (218***, 274*)	↑ (274*, 449)
MCP-1	↑ (218***)	Not done
RANTES	↑ (218***)	Not done
iNOS	↑ (233)	Not done
Peripheral nerve		
TNF-α	↑ (21)	Not done

[a] This table summarizes the results of many studies that employed a variety of study designs, different methods of cytokine measurement, and different criteria for determination of an increase in gene expression. Most studies of skin lesions assessed mRNA by reverse transcription-PCR; some also measured protein levels of selected cytokines. The observation that expression or level of most of the proinflammatory cytokines is increased in both types of reactions highlights the difficulty in determining whether an increase in any cytokine reflects a causative immunologic event underlying the reaction or is a consequence of the intense inflammation occurring in the reaction. *, mRNA in peripheral blood mononuclear cells; **, mRNA in T-cell clones from lesions; ***, cytokines and chemokines assessed by immunostaining.

(ii) Type 2 leprosy reaction (erythema nodosum leprosum). Type 2 reactions occur in patients with poor cellular immunity to *M. leprae*, abundant bacilli (i.e., antigen) in cutaneous and peripheral nerve lesions, and a strong polyclonal antibody response with high levels of circulating immunoglobulins. The acute lesions are characterized by a neutrophilic infiltrate superimposed upon a chronic lepromatous pattern, observations that have long dominated thinking about this reaction. Based primarily on histological evidence, Wemambu and colleagues proposed that ENL represents an Arthus-like phenomenon mediated by immune complexes (431). Immunoglobulin and complement deposition have been demonstrated in the skin lesions, and serum complement is decreased in these patients, consistent with this hypothesis, and some mycobacterial constituents have been identified in some of these complexes (322). However, neither circulating nor fixed immune complexes have been reproducibly demonstrated in ENL lesions. The demonstration of immune complexes within clinical lesions in other diseases has also been difficult and problematic, however, and the immune complex theory of ENL is thus neither proved nor disproved.

Other studies have identified possible evidence of cellular immune activation in type 2 reactions, including increases in circulating IFN-γ, TNF-α, and IL-12 (Table 7) (449). Increases in mRNA levels for these cytokines have also been observed in biopsies of skin lesions, indicating that cellular immune activation is occurring locally. In contrast, increases in the expression of IL-6, IL-8, and IL-10 mRNAs and sustained expression of IL-4 and IL-5 mRNAs, all cytokines associated with neutrophil chemotaxis, antibody production, and reduced cell-mediated immunity, were observed in ENL lesions.

The factors that trigger type 2 reactions are even more poorly understood than the immunologic mechanisms of the reaction itself. Other infections or viral illness, fever, immunization, and psychological stress have all been invoked, but no convincing evidence has supported any of these. Pregnancy appears to have an inhibitory effect on type 2 reactions, whereas the reaction may recur severely in the postpartum period in women with lepromatous leprosy (101, 236). Anecdotal reports suggest that in some women, the severity of a type 2 reaction fluctuates during the menstrual cycle, and still other anecdotal evidence has suggested that the type 2 reaction is more frequent and severe among children who develop leprosy at the onset of puberty (81).

The only stimulus known to initiate a type 2 reaction is the intralesional injection of IFN-γ (332). In experimental immunotherapeutic trials, multiple intradermal injections of IFN-γ over 6 to 12 months elicited this reaction in 6 of 10 lepromatous patients studied; all 6 of these had polar LL disease. The four patients who did not develop the reaction had BL or subpolar LL types of leprosy, in which the ability to develop cell-mediated immunity to *M. leprae* is greatly reduced but is not altogether absent. The observation that type 2 reactions developed in patients who had the most profound inability to develop cell-mediated immunity to *M. leprae* may suggest that, in such patients, the effect of IFN is to activate immunologic mechanisms that, because they cannot generate cell-mediated immunity, are instead channeled into pathways that lead to enhanced humoral immune activity, i.e., Th-2 cytokine responses. Notably, intradermal injections of IL-2 in lepromatous patients did not elicit a type 2 reaction (198).

Evidence from therapeutic trials regarding mechanisms of reactions. Reactions are poorly understood, and their management is often difficult and perplexing. Corticosteroids are the mainstay of the treatment of type 1 reactions; high doses are often required, sometimes for prolonged periods of time (40), with the attendant risk of serious side effects (397). For type 2 reactions, thalidomide is the treatment of choice (405). (For decades, the type 2 reaction was the only clear indication for the use of thalidomide, and the drug might well have been forgotten entirely had it not been so valuable for the treatment of these reactions.) The anti-inflammatory properties of clofazimine are useful in treating type 2 reactions also. Corticosteroids are widely used for the treatment of these reactions (235, 349), sometimes because they do not respond well to thalidomide, and more often because the drug is highly restricted or unavailable. Antimicrobial therapy should be maintained throughout treatment for both types of reaction.

The great difficulties encountered in treating reactions provide an illuminating example of the importance of understanding the mechanisms of disease: i.e., since the cause(s) and mechanisms of reactions are poorly understood, treatment is largely empirical and is often suboptimal. A number of other immunosuppressive agents have therefore been tested for their effect on reactions in the search for additional regimens that might be effective individually or at least offer a steroid-sparing effect. Since these agents act at different points in the development of an immunological response and some information is available concerning their mechanisms of action, a review of these trials is of interest in trying to understand the immunological mechanisms causing reactions (Table 8).

Corticosteroids, due to their general anti-inflammatory effects, are highly effective clinically for both type 1 and type 2 reactions. It is not clear, however, that this treatment has a significant effect upon the underlying mechanisms of either type of reaction (282, 283). Reduced levels of proinflammatory cytokines have been observed in peripheral blood monocytes from patients treated with corticosteroids for type 1 reactions (245), but other studies have suggested that alterations in cytokine levels are not always observed in patients who do receive good clinical benefit from anti-inflammatory treatment (12, 275, 404).

Thalidomide was determined to be extraordinarily effective in the treatment of type 2 reactions before its teratogenic properties were recognized (367). The mechanism by which thalidomide exerts this strong anti-inflammatory effect in type 2 reactions is not clearly understood even today, however, in spite of the flurry of interest in the apparent inhibitory action of thalidomide on TNF-α (410). The effects of thalidomide are strikingly different over a wide range of concentrations, and at the concentrations used clinically it has also been observed to enhance the production of IL-2 (360). Early studies noted that thalidomide inhibited the IgM response (361), and it is also known to promote apoptosis in neutrophils (19), both of which are potentially significant effects in the context of type 2 reactions.

Cyclosporine has been used to treat type 2 reactions, with mixed results (260, 421, 418). Cyclosporine is a potent suppressor of cellular immunity, blocking the transcription of IL-2 and

TABLE 8. Effects of immunosuppressive agents on leprosy reactions[c]

Agent	Primary mechanism(s) of action (reference[s])	Effect on type 1 reaction[a] (reference[s])	Effect on type 2 reaction (reference[s])
Thalidomide	Multiple dose-related mechanisms: inhibits TNF-α, stimulates IL-2, inhibits IgM response (333, 362)	0 (166)	++++ (275, 276, 367, 404)
Methotrexate	Antimetabolite inhibiting lymphoid and myeloid proliferation (56)	ND	++ (202)
Cyclosporine	Inhibits IL-2 and other cytokines (27, 147, 211, 250)	++ (64, 116, 421)	+/− (225, 261, 283, 418, 421)
Azathioprine	Purine antagonist; inhibits lymphocyte proliferation; exact mechanism unknown (27, 147)	+[b] (246)	+[b] (243)
Pentoxifylline	Inhibits TNF-α and other cytokines (321, 395)	+/− (82, 275)	+/− (82, 276, 287, 331)
Mycophenolate mofetil	Blocks guanosine nucleotides, inhibiting proliferation of T and B cells (27, 147)	ND	0 (47)
Corticosteroids	Block transcription factors AP-1 and NF-κB; inhibit synthesis of many proinflammatory cytokines (203, 341)	++++ (283)	+++ (276)

[a] ND, no data available.
[b] Effect observed only in combination with prednisolone.
[c] 0, not beneficial; +/−, conflicting results; +, beneficial; ++, better results; +++, highly beneficial; ++++, treatment of choice.

several other cytokines by interfering with the calcineurin-dependent translocation of the nuclear factor of T-cell activation (NFAT) to the nucleus (27, 211, 250). The evidence that cyclosporine is beneficial in some severe cases of type 2 reaction may indicate that the mechanisms underlying these reactions are not homogeneous. T-cell-related mechanisms may be involved in the pathogenesis of severe or recurrent ENL, but the broader experience, in which cyclosporine was of little benefit, suggests that such T-cell functions may not be a major feature of most type 2 reactions.

Azathioprine is a purine antagonist and is well documented to inhibit lymphocyte proliferation, although its precise mechanism of immunosuppression is unknown (27). When followed by prednisolone, it has been found to provide results comparable to those with prednisolone alone in the treatment of type 1 reactions (246), thus possibly providing a steroid-sparing regimen in the treatment of this reaction. Azathioprine alone did not provide superior results in this study, however, suggesting that the broad immunosuppression associated with this agent did not interfere with some of the basic mechanisms underlying the reaction. Azathioprine alone has not been assessed in the treatment of type 2 reactions but has also been reported to be useful in combination with prednisolone in the management of intractable type 2 reactions that do not respond well to prednisolone alone (243). This has not been evaluated in a controlled study, however.

Methotrexate has recently been reported to be effective when used in combination with corticosteroids in patients whose type 2 reaction could not be controlled with corticosteroids alone (202). This remains to be confirmed in controlled studies.

Pentoxifylline, like thalidomide, has been observed to produce a reduction in circulating levels of TNF-α in patients with type 2 reactions as well as inhibit TNF-α mRNA in skin lesions (275, 331, 304). However, pentoxifylline has not provided a clinical benefit comparable to that of thalidomide (82, 276), and some evidence has suggested that the inhibition of TNF-α may not be the major beneficial effect of thalidomide in ENL (276).

Mycophenolate mofetil, an inhibitor of B- and T-cell proliferation that blocks the production of the guanosine nucleotides required for DNA synthesis (27), has shown no benefit in type 2 reactions in one small study (47), although the effect of higher doses has not yet been studied. This agent has not been tested in type 1 reactions.

In summary, inhibition of lymphocyte proliferation by several potent antimetabolites has had little or no consistent effect in the treatment of either type of leprosy reaction. Similarly, clinical inhibitors of TNF-α, IL-2, and other cytokines have had minimal effects on reactions in most cases. The mechanism of the remarkably beneficial effect of thalidomide on type 2 reactions remains unexplained. There is no convincing evidence to date that the anti-inflammatory treatments used actually interrupt the underlying immunological processes. The overall beneficial effects of corticosteroids on both types of reaction are probably due largely to their anti-inflammatory mechanisms. Although corticosteroid and thalidomide treatments greatly alleviate suffering and mitigate nerve injury, it is quite possible that the immunological events that fuel reactions simply run their course and abate independently of the anti-inflammatory treatment itself.

MECHANISMS OF NERVE INJURY

Among bacterial pathogens, infection of peripheral nerves is a unique property of *M. leprae*. Infection of peripheral nerves is the sine qua non of leprosy, but many clinical details regarding the frequency and extent of nerve injury have only recently been described, and the mechanism(s) underlying nerve injury in leprosy is very poorly understood (354).

Neuropathy in leprosy arises not only from the infection of peripheral nerves by *M. leprae* but also from the inflammatory and immunologic responses to this pathogen. This neuropathy is often devastating to the patient's health and well-being, through the development of anesthesia, paralysis, and potential crippling deformities of fingers and toes due to ulnar, median radial, peroneal, or posterior tibial neuritis or ocular damage in the case of facial nerve involvement. Studies of the pathogenesis of neuropathy in leprosy have been severely hampered by the lack of good experimental models and because

biopsy of the most actively inflamed sites of affected peripheral nerve trunks is not possible.

Although localized anesthesia is a serious and well-known consequence of leprosy, recent evidence also indicates that a large percentage of patients experience neuropathic pain (142, 396), sometimes long after they appear to be otherwise cured of infection. Little study has been done concerning the mechanisms of neuropathic pain in leprosy, and the remainder of this discussion of nerve injury will concentrate on mechanisms related primarily to injury resulting in anesthesia and, ultimately, paralysis.

Recent advances in the study of nerve injury in leprosy have been most prominent in five areas: improvements in clinical sensory testing with monofilaments, recognition that the mechanisms of localization of *M. leprae* to nerves may involve the vascular endothelium, direct examination of immunological parameters in biopsy samples of affected nerves in leprosy patients, identification of the molecular mechanisms of *M. leprae* binding to Schwann cells, and development of greatly improved Schwann cell culture models for in vitro studies of the consequences of *M. leprae* infection.

Accurate, reproducible measurement of sensory function using calibrated Semmes-Weinstein monofilaments has been a major advance in the study of nerve injury in leprosy (231). Such testing can clearly identify loss of protective sensation before it results in ulceration and other injury and can identify early neuropathy, with subtle functional impairment, that is otherwise often overlooked. This has contributed to advances in the prevention of disability in leprosy (419). Using this method, a 5-year follow-up study of nerve function impairment in over 200 patients has demonstrated that nerve injury continues to be a problem even after the infection is treated and cured (316).

Although neuritis and neuropathy have often been studied and discussed in the context of leprosy reactions, which may exacerbate neuritis, the more sensitive assessments of sensory loss have also demonstrated that nerve function impairment occurs independently of reactions (419). With these sensitive methods, it is now evident that early nerve function impairment occurs earlier in lepromatous than in tuberculoid patients (258, 316, 339). In addition, a study of prophylactic treatment with prednisolone has shown that it reduces nerve function impairment that is measurable at 4 months, but this improvement was not evident after 1 year (385). Thus, the ability to clinically evaluate different degrees of neuropathy and correlate this with responses to intervention has very important implications for both clinical and basic research on the mechanisms of nerve injury in leprosy.

Overt sensory and motor neuropathies that prompt patients to seek medical attention often occur earlier and more intensely in those patients whose lesions contain few bacilli (BT and TT types), most probably because the granulomatous inflammatory response to *M. leprae* in these patients leads to injury to adjacent nerves. In contrast, lepromatous patients often develop overt neuropathy more slowly, even though the Schwann cells and macrophages of their peripheral nerves are more heavily infected with *M. leprae*. After prolonged, untreated infection, however, the nerves of all types of patients are at risk of chronic inflammation and fibrosis that becomes the final common pathway of injury, potentially resulting in anesthesia with paralysis of intrinsic and extensor muscles of the hands and feet. The affected limbs are then at high risk of injury or mutilation, processes that accelerate the physiologic resorption of bone and result in loss of digits. Involvement of the facial nerve leaves patients at risk of corneal anesthesia, abrasion, and blindness.

Four related aspects of nerve injury in leprosy must be considered in understanding the pathogenesis of neuritis in leprosy: localization of *M. leprae* to peripheral nerves, infection of Schwann cells, immunologic responses, and inflammation. Of these, the infection of Schwann cells is the most obviously remarkable and has received by far the greatest experimental attention, and the other aspects have suffered especially from the inherent difficulties in obtaining material for investigation, since biopsy of affected nerves is seldom clinically indicated and is otherwise unethical. The armadillo appears to provide a model for some aspects of leprosy neuropathy in humans, as noted below, but also has posed major limitations as an experimental animal.

Localization of *M. leprae* to Peripheral Nerves

The first essential step in leprosy neuritis is the localization of *M. leprae* to peripheral nerves. Ever since autopsy dissections in the 1890s (85, 130), which followed affected peripheral nerves from the skin lesion to the spinal cord, the infection of peripheral nerves has been understood to be an ascending neuropathy originating in sensory cutaneous nerves and traveling proximally to involve larger nerve trunks carrying mixed sensory and motor fibers (327). This has been extrapolated to imply that *M. leprae* initially binds to exposed Schwann cells in the dermis and then moves proximally within the nerve, "swimming like fish up a stream" (209).

However, recent studies of peripheral nerves in experimentally infected armadillos have suggested that *M. leprae* infects nerves from the outside in, first aggregating in epineurial lymphatics and blood vessels and then entering the endoneurial compartment through its blood supply (347, 351).

A model illustrating this hypothesis of localization of *M. leprae* to peripheral nerves is presented in Fig. 6 (347). This view of the pathogenesis of infection of peripheral nerves raises significant implications with respect to both understanding the process and possible points of preventive or therapeutic intervention. If several steps are required for the ultimate entry of *M. leprae* into Schwann cells, then there are several potential sites of intervention, e.g., binding to endothelial cells, entry into the endothelium, exit from endothelial cells into the endoneurium, and binding to Schwann cells. If, on the other hand, *M. leprae* enters nerves exclusively via the single step of direct binding to exposed Schwann cells in the dermis, then this is the only opportunity for preventive or therapeutic intervention, and the likelihood of developing such interventions is correspondingly decreased.

The Schwann cell, the principal support cell in the peripheral nervous system, appears to be the major target of *M. leprae* in peripheral nerves. In patients with advanced leprosy, both myelinated and nonmyelinated Schwann cells are infected by *M. leprae* (163, 179, 180), although some reports have suggested some preference for nonmyelinating Schwann cells (311). In vitro, we have observed a similarly brisk and heavy

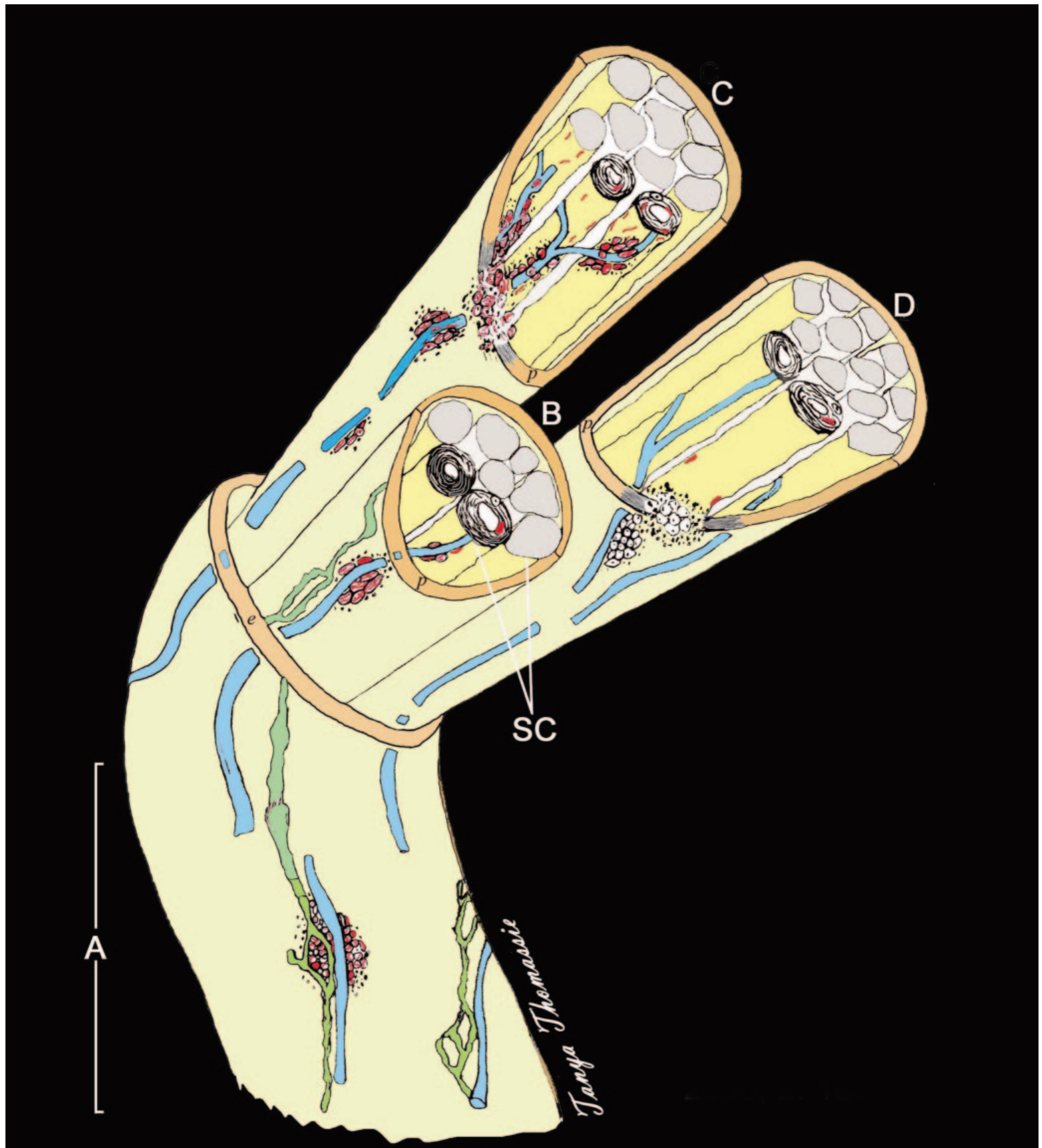

FIG. 6. Proposed model of infection of peripheral nerve by *M. leprae* via blood vessels. A cutaneous nerve with three fascicles is represented here to illustrate the proposed steps in the pathogenesis of infection of peripheral nerves by *M. leprae*. (A) Initially, colonization of the epineurium (e) occurs when bacilli (red) localize in cells in and around blood vessels (blue). It is possible that this is enhanced by drainage of bacilli through the lymphatics (green) that are intertwined with the blood vessels of the epineurium (lymphatics are here illustrated only at the lower end of the drawing). The resulting accumulation of bacilli within and around endothelial cells greatly increases the likelihood that bacilli will be available for circulation through the endoneurial vessels which branch off the epineurial ones. (B) Entry of *M. leprae* into the endoneurial compartment proceeds along blood vessels from foci on and within the perineurium (p), extending through it into the interior of the nerve. The mechanisms responsible for entry into the interstitial space of the endoneurium remain to be determined. Once inside, however, bacilli are available for phagocytosis by Schwann cells (SC), represented here with concentric layers of myelin surrounding axons. Although these initial events in the localization and entry of *M. leprae* into peripheral nerves are postulated to be unrelated to specific immune function, the subsequent pathogenesis of neuritis in leprosy probably depends in large part on the patient's immune response to *M. leprae*. (C) If no effective immune respose develops

infection of both cell types (144). Some investigators, however, have reported exclusive infection of nonmyelinating cells in vitro (309).

M. leprae Interactions with Schwann Cells

Adhesion to Schwann cells. Several potential mechanisms of binding of *M. leprae* to the Schwann cell (SC) have been elucidated (310, 308, 398). Antibodies directed against the polysaccharide and lipid components of *M. leprae* inhibited adhesion to SCs, while those directed against both surface and cytoplasmic protein epitopes did not show any such effect (67), indicating that the association of *M. leprae* with SCs may be mediated by more than one of its cell surface molecules. Recent studies have demonstrated that *M. leprae* specifically binds to α-dystroglycan in the presence of the G domain of the α2 chain of laminin-2 (310). Using α2 laminins as a probe, a major protein in the *M. leprae* cell wall fraction (ML-LBP21) that binds α2 laminins on the surface of SCs has been identified (370).

Phenolic glycolipid 1 of *M. leprae* has also been demonstrated to bind specifically to laminin-2 in the basal lamina of SC-axon units (288). PGL-1, therefore, appears to be involved in the invasion of SCs by *M. leprae* in a laminin-2-dependent pathway. Importantly, however, evidence clearly indicates that this mechanism of binding to the SC surface via α2-laminins is not unique to *M. leprae*. Other mycobacterial species, including *M. tuberculosis*, *M. chelonae*, and *M. smegmatis*, have been shown to express an α2-laminin-binding capacity (247) and these species readily interact with the ST88-14 Schwannoma cell line. This suggests that the ability to bind α2-laminins is conserved within the genus *Mycobacterium*. Other studies have also demonstrated the ability of myelin P_0 to bind *M. leprae* (398).

Ingestion by SCs. After *M. leprae* adheres to the SC surface, it is slowly ingested, as described in recent studies using primary denervated rat SC cultures and SC-neuron cocultures (144) (Fig. 7). After ingestion the SC appeared to be incapable of destroying this intracellular parasite when cultures were maintained at 33°C (optimal conditions for *M. leprae* viability and temperature of peripheral nerves) (414). In vitro studies of ingestion of *M. leprae* by a human Schwannoma cell line (ST88-14) found that several protein kinases were essential for ingestion but that cyclic AMP-dependent kinases were not (11). In these studies, acidification of vesicles containing irradiated *M. leprae* proceeded normally but was minimal when live *M. leprae* was used, suggesting that viable *M. leprae* interferes with normal endocytic maturation.

Effects of SCs on *M. leprae*. SCs apparently provide an environment suitable for the preservation and proliferation of *M. leprae*. Studies using highly viable suspensions of nude-mouse-derived *M. leprae* have demonstrated that the viability of the bacilli in rat SCs is comparable to that previously described for bacilli within macrophages in vitro and that survival of this organism within SCs is greater at 33°C than at 37°C (144). This survival within SCs in vitro is consistent with the long-standing histopathological observations that *M. leprae* appears to persist and grow within SCs in human nerves.

Effects of *M. leprae* on SCs. The effect of *M. leprae* on the SC has been the subject of many studies in vitro. Notably, however, optimal conditions (highly viable bacilli and cooler cultivation temperatures) have not been used in most studies of this interaction, possibly contributing to the variety of conflicting reports in the literature (264, 279, 382, 392). Infection of SCs with whole, viable *M. leprae* has not been observed to cause SC loss (144), and even appeared to favor SC survival rather than apoptosis (311). However, human SCs express Toll-like receptor 2 both in vitro and in vivo, and binding of an *M. leprae*-derived lipoprotein to TLR2 on SCs has been reported to result in apoptosis (294). These investigators also identified SCs that had undergone apoptosis in biopsies of human lesions. The significance of these observations with respect to clinical nerve injury remains uncertain.

M. leprae also appears to have no effect on intact, mature SC-axon units, but did alter SC expression of a small number of genes examined (those for glial fibrillary acidic protein, transforming growth factor β1, NCAM, ICAM, N-cadherin, and L1) (144). However, transcript levels for all but one of these genes, that encoding N-cadherin, varied less than twofold. Therefore, the functional significance of these alterations remains to be determined.

In contrast to these observations, Rambukkana and colleagues, also using a rat SC-axon coculture system, have described rapid demyelination following adherence of *M. leprae* to SCs in the absence of immune cells, interpreted to be a contact-dependent mechanism dependent on PGL-1, a component of the *M. leprae* cell wall (311). Similar findings in T- and B-cell-deficient (*Rag1*$^{-/-}$) mice led these authors to conclude that attachment of *M. leprae* to the myelinated SC surface is sufficient to induce rapid demyelination of these cells, thus suggesting a mechanism for demyelination of nerves in leprosy (for a review, see reference 309). These conclusions, however, are at considerable odds with well-documented clinical and histopathological observations. First, patients with untreated lepromatous leprosy may have billions of bacilli in their bodies but show little or no demyelination. Secondly, rapid

(e.g., lepromatous leprosy), bacilli proliferate within macrophages and Schwann cells. This results in perineurial inflammation and thickening (proliferation) and an increasing bacterial load both in the epineurium and in the endoneurium. Since *M. leprae* is an indolent, well-adapted intracellular pathogen, however, axons are not badly damaged for a long time, and a variable degree of nerve function is preserved until late in the course of the disease. (D) If effective cellular immunity and delayed hypersensitivity do develop (e.g., tuberculoid leprosy), a granulomatous response follows at sites of infection near epineurial and endoneurial vessels and Schwann cells. This immunologically elicited inflammation eliminates nearly all of the bacilli in the epi- and perineurium and also stimulates perineurial fibrosis and thickening. However, *M. leprae* organisms that have already been ingested by Schwann cells may be relatively protected from immunologically mediated destruction and able to maintain a persistent infection in these cells for a longer time. This is where most bacilli are found in diagnostic biopsies of tuberculoid lesions. Granulomatous inflammation is also potentially injurious to adjacent tissue. In *M. leprae*-infected nerves, this includes injury to axons in the vicinity of the granulomas, resulting in impaired nerve function. (Reprinted from reference 347.)

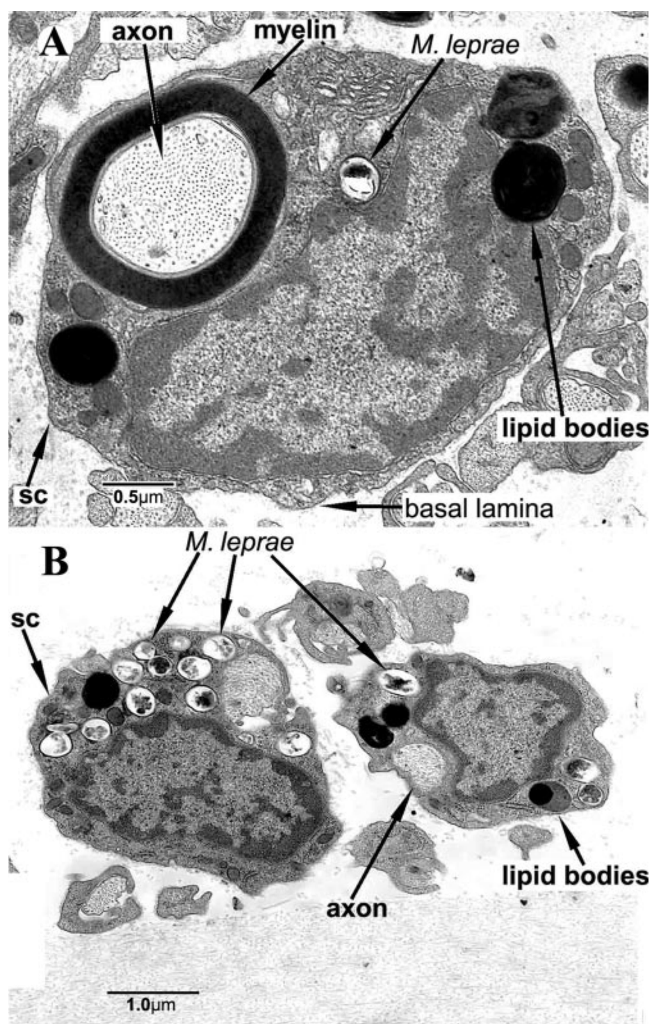

FIG. 7. Transmission electron micrographs of *Mycobacterium leprae*-infected rat Schwann cell (SC)-neuron cocultures. Infected cultures were obtained by exposure of primary Schwann cells to *M. leprae* for 48 h. After cultivation for 12 days at 33°C, they were seeded onto embryonic rat neurons. The infected Schwann cell cultures were induced to myelinate and cultured for 30 days at 33°C. A. Myelinating Schwann cells. B. Nonmyelinating Schwann cells. (Reprinted from reference 144 with permission. © 2002 by the Infectious Diseases Society of America. All rights reserved.)

demyelination (if it occurs in clinical leprosy) is a late manifestation of the disease rather than an early one (295).

Immune responses and SCs. Finally, the immune response may also be directed at *M. leprae*-infected SCs. Isolated human SC cultures appear to be able to process and present *M. leprae* antigens to $CD4^+$ T cells (387). The infected SCs were highly susceptible to killing by $CD4^+$ cytotoxic T-cell clones derived from leprosy patients. Long-term cultures of human SCs also express MHC class I and II, ICAM-1, and CD80 surface molecules involved in antigen presentation. These cells process and present *M. leprae* and some of its protein and peptide antigens to MHC class II-restricted $CD4^+$ T cells and are efficiently killed by these activated T cells.

As a long-term consequence of these and other, unknown mechanisms, SCs are ultimately functionally impaired or destroyed in infected nerves. The end result is a demyelination neuropathy (164, 399). Segmental demyelination is seen adjacent to areas of infection, as discussed above. Axonal atrophy and accompanying demyelination have also been described in leprous nerves, and this has been associated with abnormal phosphorylation of high- and medium-weight neurofilaments (340).

In summary, *M. leprae* has a unique ability to infect peripheral nerves, probably entering via their vascular endothelium by mechanisms not yet determined. Once they have gained access to the endoneurial compartment, *M. leprae* organisms bind to SCs via several binding molecules on the surface membrane. The bacilli are ingested by SCs, and viable organisms appear to be able to interfere with normal endocytic maturation and thus, probably, with potential killing mechanisms. Within SCs in vitro, *M. leprae* is able to survive for a limited time at 33°C. *M. leprae* does not appear to induce SC death, by apoptosis or otherwise, and infected SCs are able to associate normally with axons in vitro. Infection does, however, produce measurable changes in transcription of some of the genes that have been examined thus far.

Infected SCs are also able to process and present antigen to T cells, and thus may become targets of immune responses. Immunologically driven inflammation is probably responsible for much of the clinically apparent nerve injury, because nerve function impairment occurs more rapidly and more severely in patients with a strong cellular immune response (i.e., tuberculoid disease). The limited evidence available indicates that the immunological mechanisms operating within nerves are similar to those which have been described in much more detail in the skin. In lepromatous patients, with minimal immunological response to *M. leprae*, nerves may be heavily infected with only mild to moderate impairment of function. Ultimately, however, selected peripheral nerves in all forms of leprosy undergo demyelination. Without effective treatment, many such nerves will become completely nonfunctional, leaving the patient with an insensate, paralyzed hand or foot. Understanding the many undeciphered mechanisms underlying nerve injury and applying available clinical and rehabilitation tools to prevent and minimize nerve injury are among the greatest challenges and highest priorities in leprosy research today.

CHEMOTHERAPY

Current Therapies and Drug Resistance

In the 1950s, dapsone (diaminodimethyl sulfone) was introduced as standard chemotherapy for leprosy and was used worldwide for treatment of both multibacillary and paucibacillary forms of the disease. Long-term monotherapy with dapsone resulted in poor compliance in many areas, ultimately leading to the emergence of dapsone-resistant leprosy, resulting in treatment failures and resistance levels reported to be as high as 40% in some areas of the world (446, 365).

Fortunately, additional antimicrobial agents such as rifampin and clofazimine were developed and introduced for the treatment of leprosy (44, 232). Although rifampin proved to be a powerful antileprosy drug, use of rifampin alone or in combination with dapsone for the treatment of dapsone-resistant leprosy led to the rapid development of rifampin-resistant or-

TABLE 9. Antimicrobial agents for leprosy[a]

Agent	Routine dose (per day)	Antibacterial mechanism[b]	References
Dapsone	100 mg	Weakly bactericidal; competitive PABA antagonist	10, 225, 317, 359
Clofazimine	50 mg	Weakly bactericidal; unclear but binds DNA of mycobacteria	44, 169, 171, 175, 314
Rifampin	600 mg	Bactericidal; inhibits DNA-dependent RNA polymerase	156, 178, 232, 280, 403, 441
Minocycline	100 mg	Bactericidal; inhibits ribosomal protein synthesis	126, 171, 176, 402
Ofloxacin	400 g	Bactericidal; inhibits DNA gyrase	99, 120, 171, 172, 175, 176, 241, 401
Perfloxacin	800 mg	Bactericidal; inhibits DNA gyrase	106, 118, 129, 138
Clarithromycin	500 mg twice	Bactericidal; inhibits ribosomal protein synthesis	57, 174

[a] Modified from reference 349 with permission from Elsevier. Only dapsone is approved specifically for the treatment of leprosy by the Food and Drug Administration.
[b] PABA, *p*-aminobenzoate.

ganisms (137, 177). Other drugs with antileprosy activity were also evaluated. Clofazimine proved to be only weakly bactericidal against *M. leprae* and therefore was not suitable as monotherapy for leprosy (169, 177).

To overcome the problem of drug-resistant *M. leprae* and to improve treatment efficacy, the World Health Organization recommended multidrug therapy for leprosy in 1981. The initial recommendation for patients with multibacillary leprosy was to give daily dapsone and clofazimine with monthly rifampin and clofazimine for 2 years or until the skin smear was negative. These recommendations, as well as diagnostic criteria, have been modified several times since 1981. Currently the World Health Organization recommends counting lesions to distinguish paucibacillary from multibacillary disease, less than five lesions being classified as paucibacillary and five or more lesions as multibacillary. Since 1998 they have also recommended treating multibacillary patients for only 12 months and paucibacillary patients for only 6 months (reviewed in reference 349) (Table 9). In addition, a World Health Organization committee recommended that patients with a single lesion be treated with a single combination dose of rifampin (600 mg), ofloxacin (400 mg), and minocycline (100 mg) (312; www.who.int/lep/romfaq3.htm), but this regimen remains very controversial. These recommendations arise from efforts to reduce the resources allocated to leprosy in developing countries and are the subject of considerable debate. Optimal diagnostic evaluation employing skin smears or biopsies, classifying the lesions on the Ridley-Jopling scale, and conservative, longer duration of treatment with multiple antimicrobials are recommended in the United States and most developed countries (349).

Multidrug therapy has been very practical and successful for treatment of both multibacillary and paucibacillary leprosy (171, 444, 447), and the overall number of registered cases worldwide has fallen dramatically (171, 445). However, even with these powerful drug combinations, the number of newly registered cases has not fallen consistently, and drug resistance still occurs.

A recent report demonstrated that 19% of 265 *M. leprae* isolates from biopsied samples of leprosy patients were resistant to various concentrations of dapsone, rifampin, or clofazimine and 6.23% were resistant to more than one drug in the mouse footpad susceptibility assay (102). In addition, several investigators have identified multidrug-resistant strains of *M. leprae* (reviewed in reference 434). Ofloxacin and minocycline have been added to the drug arsenal for the treatment of leprosy (126, 172, 175, 176; see also http://www.who.int/lep/romfaq3.htm). Current treatment recommendations in the United States have been summarized elsewhere (276a, 349), and Lockwood has provided a rigorous evidence-based discussion of the treatment of leprosy (234).

With or without the development of drug resistance, relapse occurs in some cases even after multidrug therapy. The reported extent of relapse varies greatly, depending on several operational factors and on the duration of follow-up. Because of the very slow growth of this organism, follow-up of at least 10 years is necessary to obtain a reasonable assessment of relapse, and during the last 20 years recommendations concerning treatment duration and drug combinations have changed several times, further complicating this assessment. The largest study, although shorter than 10 years in duration, is a 6-year follow up of 47,276 patients in the Chinese national program (63), which revealed an overall relapse rate of 0.73/1,000 person-years, significantly greater for paucibacillary patients (1.04/1,000 person-years) than for multibacillary patients (0.61/1,000 person-years).

In southern India, a relapse rate equivalent to 20/1,000 person-years was observed among multibacillary patients given fixed-duration (2-year) multidrug therapy, reduced to 10/1,000 person-years in patients treated until smear negative (134). A 10-year prospective study in the Philippines (55) observed an overall relapse rate equivalent to 2.8/1,000 person-years. Significant differences were noted in the rates of relapse in multibacillary patients followed at a referral center (9%) versus field clinics (3%). Importantly, in both the southern India and Philippine studies, higher rates of relapse have been observed in patients with a high bacterial index (BI) ($\geq$4) at the time of diagnosis, underscoring the advice that such patients require longer treatment (128). In the longest study to date, a 16-year follow-up of patients in Karigiri, India (292), a relapse rate equivalent to 0.7/1,000 person-years was observed among multibacillary patients who had received multidrug therapy. Relapses occurred 14 to 15 years after release from treatment and again were more frequent in persons with a high initial BI.

Molecular Mechanisms of Drug Resistance

Dapsone (4,4-diaminodiphenyl sulfone) is a synthetic sulfone, is structurally and functionally related to the sulfonamide

TABLE 10. Mutations within drug target genes associated with drug resistance in *Mycobacterium leprae*

Drug/target gene	Drug susceptibility[a]	Mutation(s)	No. of isolates (%)[b]	Reference(s)
Rifampin/*rpoB*	R	Gly401Ser; His420Asp	1 (2)	51
	R	Gln407Val	1 (2)	51
	R	Phe408/Met409; LysPhe insertion	1 (2)	156
	NC	Asp410Asn	1 (2)	241
	NC	Asp410Asn; Leu427Pro	1 (2)	241
	R	Ser416Cys	1 (2)	158
	R	His420Asp	1 (2)	156
	R	His420Tyr	11 (20)	241
	R	Ser425Leu	33 (60)	51, 52, 156, 241, 251, 441
	R	Ser425Met	1 (2)	156
	R	Ser425Met; Leu427Val	1 (2)	51
	R	Ser425Phe	1 (2)	156
	NC	Ser425Trp	1 (2)	241
Dapsone/*folP1*	R	Thr53Ala	12 (40)	191, 241, 437
	NC	Thr53Ala; Pro55Leu	1 (3)	241
	R	Thr53Arg	2 (7)	437
	R	Thr53Ile	4 (13)	241, 439
	R	Pro55Arg	3 (10)	437, 439
	R	Pro55Leu	8 (27)	191, 241, 437
Ofloxacin/*gyrA*	NC	Gly89Cys	1 (14)	241
	R	Ala91Val	6 (86)	51, 241

[a] R, resistant phenotype, as determined by mouse footpad or radiorespirometry (Buddemeyer) drug susceptibility analysis; NC, not confirmed by either assay.
[b] Percentage (rounded to the nearest whole number) of isolates in each drug-resistant group that contain a specific mutation.

drugs, and targets dihydropteroate synthase, a key enzyme in the folate biosynthesis pathway in bacteria, by acting as a competitive inhibitor of *p*-aminobenzoic acid (317, 359). Dapsone has also been shown to target the folate biosynthetic pathway of *M. leprae* (225).

Specific mutations within the highly conserved *p*-aminobenzoic acid binding site of *E. coli* dihydropteroate synthase, encoded by *folP*, result in the development of dapsone resistance (80). New evidence from the *M. leprae* genome sequencing project indicated that *M. leprae* possesses two *folP* homologues (*folP1* and *folP2*) (74). Through surrogate genetic studies with *M. smegmatis*, the relationship between dapsone resistance and the dihydropteroate synthase of *M. leprae* has been established (439). Missense mutations within codons 53 and 55 of the sulfone resistance-determining region of *folP1* result in the development of high-level dapsone resistance in *M. leprae* (Table 10).

Rifampin (3-{[(4-methyl-1-piperazinyl)-imino]-methyl}rifamycin) is the key bactericidal component of all recommended antileprosy chemotherapeutic regimens. A single dose of 1,200 mg can reduce the number of viable bacilli in a patient's skin to undetectable levels within a few days (232). The target for rifampin in mycobacteria and *E. coli* is the β-subunit of the RNA polymerase encoded by *rpoB* (156, 178, 403, 441). Comparison of the deduced primary structures of β-subunit proteins from several bacteria to that of *M. leprae* demonstrated that *M. leprae* shares six highly conserved functional regions common to this enzyme in bacteria.

Mycobacterial resistance to rifampin correlates with changes in the structure of the β-subunit of the DNA-dependent RNA polymerase, primarily due to missense mutations within codons of a highly conserved region of the *rpoB* gene referred to as the rifampin resistance-determining region (280, 403, 441). Rifampin resistance in *M. leprae* also correlates with missense mutations within this region of *rpoB* (156). Substitutions within codon Ser425 have been shown to be the most frequent mutations associated with the development of the rifampin-resistant phenotype in *M. leprae* (Table 10).

Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine] is a substituted iminophenazine with antimycobacterial activity for which the mechanism has not been fully elucidated (175). Clofazimine attains high intracellular levels in mononuclear phagocytic cells, its metabolic elimination is slow, it has an anti-inflammatory effect, and the incidence of resistance to it in *M. leprae* is low. It is highly lipophilic and appears to bind preferentially to mycobacterial DNA (171). Binding of the drug to DNA appears to occur principally at base sequences containing guanine, explaining clofazimine's preference for the G+C-rich genomes of mycobacteria over human DNA.

Lysophospholipids appear to mediate the activity of clofazimine in some gram-positive bacteria (83). However, it is unclear whether this mechanism of action is operational in *M. leprae*. Since clofazimine may act through several different mechanisms, it is not difficult to understand why only a few cases of clofazimine-resistant leprosy have been reported over the years (84, 256, 368).

Clarithromycin is a semisynthetic macrolide that differs from erythromycin in its methyl substitution at the number 6 position of the macrolide ring (reviewed in reference 424). It displays significant bactericidal activity against *M. leprae* in humans (127, 173). In patients with lepromatous leprosy, daily administration of 500 mg of clarithromycin kills 99% of viable *M. leprae* within 28 days and >99.9% by 56 days. Although the mechanism of action of this antibiotic against *M. leprae* is unknown, it is thought to be similar to that of erythromycin,

which acts by inhibiting protein synthesis by binding to the ribosome.

Clarithromycin resistance in bacteria and mycobacteria appears to be due to a decrease in binding of the drug to ribosomes and is associated with missense mutations within the 23S rRNA gene (257, 424, 428). This has not yet been established to be the case with *M. leprae*, however, and in a recent study no mutations were observed within the 23S rRNA gene in clarithromycin-resistant *M. leprae* strains (450).

Minocycline (7-dimethylamino-6-demethyl-6-deoxytetracycline) is the only member of the tetracycline group of antibiotics to demonstrate significant activity against *M. leprae*, presumably due to its lipophilic property, which may enhance cell wall penetration (126, 176). Minocycline is bactericidal for *M. leprae*, and its activity is additive when it is combined with dapsone and rifampin. The mechanism of action of minocycline against *M. leprae* is unknown but is thought to be similar to that of all tetracyclines, which act by inhibiting protein synthesis. Tetracyclines bind reversibly to the 30S ribosomal subunit, blocking the binding of aminoacyl-tRNA to the mRNA-ribosome complex (402).

Resistance to tetracycline may be mediated by three different mechanisms: an energy-dependent efflux of tetracycline brought about by an integral membrane protein; ribosomal protection by a soluble protein; or enzymatic inactivation of tetracycline. The molecular mechanism of minocycline resistance has not been studied in *M. leprae* primarily because this drug has only recently been used widely (in the treatment of single-lesion, paucibacillary leprosy), and resistant strains have not been identified.

Ofloxacin (4-fluoroquinolone) is a fluorinated carboxyquinolone that has moderate anti-*M. leprae* activity (175, 176). The mechanism of action of ofloxacin on *M. leprae* is unknown, but in other bacteria it appears to inhibit DNA replication by inhibiting the DNA gyrase, a tetramer containing two A-subunits (GyrA) and two B-subunits (GyrB) (99).

Mutations within a highly conserved region of *gyrA*, the quinolone resistance-determining region, are associated with the development of ofloxacin resistance in most resistant strains of mycobacteria (53, 401). The quinolone resistance-determining region of *M. leprae gyrA* is highly homologous to that of *M. tuberculosis*, and missense mutations within this region have been found in ofloxacin-resistant strains of *M. leprae* (Table 10).

Development of Drug Resistance in *M. leprae*

Lacking direct evidence for the mechanisms of *M. leprae*'s resistance to most of the antileprosy drugs, our current understanding is based on studies carried out in *M. tuberculosis* (280) and other bacteria and limited studies with *M. leprae* genes in surrogate hosts. From these studies one can predict that drug resistance in *M. leprae* is attributable to chromosomal mutations in genes encoding drug targets, these mutations occur spontaneously as a result of errors in DNA replication, and these mutants are further enriched in a population by inappropriate or inadequate drug therapy.

Because *M. leprae* cannot be cultivated in vitro, the frequency of drug-resistant mutants in a population is primarily inferred from studies with *M. tuberculosis*. For example, the frequency of dapsone-resistant mutants in a population of *M. leprae* is estimated to be 10^{-6} and the frequency for rifampin or ofloxacin resistance is estimated at 10^{-7} to 10^{-8} (158, 280). Rates of clofazimine resistance in *M. leprae* are unknown but appear to be relatively low. Since untreated multibacillary patients can harbor large bacterial loads ($>10^{11}$ *M. leprae*), it is feasible that a patient could contain up to 10^5 dapsone-resistant organisms and thousands of rifampin- or ofloxacin-resistant organisms. Inappropriate therapy (noncompliance or inadequate therapy) for these patients has the potential to enrich the subpopulations of drug-resistant *M. leprae*, leading to the spread of one or more resistant phenotypes. Indeed, drug-resistant isolates of *M. leprae* have been found in many parts of the world (49, 51, 52, 84, 86, 102).

Detection of Drug-Resistant Leprosy

Leprosy presents a very special problem for the detection of resistance because of the inability to culture *M. leprae* axenically. Conventional drug susceptibility testing of *M. leprae* from clinical specimens relies on the ability to cultivate *M. leprae* in the hind footpads of mice by the method described by Shepard and Chang (364). This method requires the recovery of a sufficient number of viable organisms from a patient to inoculate the footpads of 20 to 40 mice (depending on the number of drugs to be tested), with each footpad receiving 5×10^3 organisms. Results are available after 6 months to 1 year. While this assay gives definitive information pertaining to the susceptibility of an *M. leprae* isolate to standard antileprosy drugs, it is cumbersome, very expensive, and very slow.

The first rapid drug-screening assays for *M. leprae* were developed based on radiorespirometry techniques (BACTEC and Buddemeyer) and have been used successfully to identify new antileprosy drugs (117). However, the use of these techniques for drug susceptibility testing in leprosy is limited by a stringent requirement for very large numbers ($\geq 10^7$) of viable organisms from each patient.

Molecular assays for resistance would simplify susceptibility testing and provide a means for monitoring resistance globally. To reduce the number of organisms needed and to minimize the time required for drug susceptibility testing of *M. leprae*, several protocols based on genotypic identification of drug-resistant mutants have been developed. These techniques are based on the amplification of specific DNA fragments from crude biological specimens (e.g., skin biopsy specimens from leprosy patients) using PCR amplification and detection of mutations associated with drug resistance within these DNA fragments by direct DNA sequencing, single-strand conformation polymorphism analysis, heteroduplex analysis, and solid-phase reverse hybridization analysis, similar to the line probe assay (Table 11).

PCR-direct DNA sequencing. Sequencing of the PCR amplicon is the most definitive of all of the nucleic acid-based mutation detection protocols because it detects the actual nucleotide changes in the target gene in which mutations associated with antibiotic resistance are found. In addition, the assay can be designed to be species specific, providing direct evidence for the presence of a particular pathogen in the specimen being tested. PCR-direct DNA sequencing has been used to identify rifampin-, dapsone-, and ofloxacin-resistant mutants

TABLE 11. Target genes for *M. leprae* drug resistance PCR-based assays

Assay	Target gene	Reference(s)
PCR-DNA sequencing	*gyrA*	51, 241
	folP	191, 241, 437, 439
	rpoB	51, 52, 157, 241, 251, 441
PCR-SSCP	*gyrA*	51
	rpoB	157
PCR-heteroduplex	*folP*	439
PCR UHG-heteroduplex	*folP*	437
PCR hybridization	*rpoB*	158

of *M. leprae* (Table 11). These assays are based on PCR amplification of the appropriate target DNA directly from skin biopsy specimens using oligonucleotide primers that are specific for the rifampin, sulfone, or quinolone resistance-determining region of *M. leprae*. The DNA sequence of these PCR products is then determined and examined for the presence of mutations previously associated with drug resistance. The Ser425Leu mutation in the B-subunit of the RNA polymerase is the most frequently detected mutation associated with rifampin resistance in *M. leprae* (Table 10). PCR-direct DNA sequencing can be performed in a well-equipped diagnostic laboratory with either manual or automated DNA sequencing systems and requires approximately 1 to 2 days to obtain drug susceptibility results directly from clinical specimens.

PCR-SSCP. A PCR-single-strand conformation polymorphism (SSCP) assay has been developed to detect rifampin-resistant *M. leprae* in human specimens (156, 157). To accomplish this, the rifampin resistance-determining region target was amplified by PCR and the double-stranded PCR products were heated to dissociate them into single strands and then separated by denaturing gel electrophoresis under stringently controlled temperature conditions. Gels were then stained to observe DNA fragment mobility patterns, called SSCP profiles. DNA strands from rifampin-susceptible organisms migrate at a rate proportional to their molecular weight and conformation and give a reproducible SSCP profile. The DNA fragment patterns observed with SSCP are highly reproducible and yield profiles unique to specific mutations.

PCR–solid-phase hybridization. A PCR–solid-phase hybridization assay has recently been developed for the detection of rifampin-resistant *M. leprae* (158). An initial PCR step with a biotinylated and an unlabeled primer produces an 83-bp, biotinylated fragment of the *M. leprae* rifampin resistance-determining region. The amplified PCR product is then hybridized to a set of DNA capture probes which have been immobilized at specific sites on a Biodyne C membrane. The immobilized capture probes are small DNA fragments that are homologous to short segments of the rifampin resistance-determining region of the *rpoB* gene from a rifampin-susceptible strain of *M. leprae* or specific mutant strains. The stringency of the hybridization reaction is designed so that the PCR product will only bind to probes with 100% sequence homology. The resultant hybrids are detected by chemiluminescence using a streptavidin-peroxidase conjugate. The genotype of the test organism is determined by the capture probes which hybridize.

PCR-heteroduplex analysis. A PCR heteroduplex-based assay was initially developed to detect the presence of drug-resistant *M. tuberculosis* from sputum specimens using a universal heteroduplex generator (UHG) (438). A similar approach has been used to develop a PCR-UHG assay to detect the presence of dapsone-resistant *M. leprae* in skin biopsy homogenates of lepromatous leprosy patients (437). The assay requires PCR amplification of the sulfone resistance-determining region of *folP1* and the mixing of these PCR products with a universal heteroduplex generator (UHG-DDS-141), a synthetic 141-bp sulfone resistance-determining region DNA fragment that contains several base pair mismatches flanking codons that are associated with dapsone resistance. When UHG-DDS-141 is denatured by heat and slowly annealed to denatured sulfone resistance-determining region PCR products from *M. leprae*, the resultant heteroduplexes form unique structures which, when analyzed by electrophoresis, provide enhanced mutation detection over standard heteroduplex detection. Enhanced mutation detection occurs because large areas of unmatched nucleotides (bubbles) in the newly formed duplexes greatly affect the mobility of the resultant DNA fragments. When the heteroduplexes are separated by electrophoresis on polyacrylamide minigels, unique heteroduplex profiles are observed for susceptible and resistant genotypes. PCR-UHG requires approximately 6 h to complete and uses 6% precast nondenaturing Tris-borate-EDTA minigels and a nonradioactive detection format.

PREVENTION: THE QUEST FOR A LEPROSY VACCINE

Vaccinology has grown from an empirical science with little in the way of biological understanding of events to a highly structured science drawing on detailed immunological studies at the cellular and molecular levels of both the host and the infectious agent. The cells, cytokines, and regulatory pathways active in the host's immune response to *M. leprae* and other mycobacterial pathogens continue to be elucidated, building a foundation for our understanding of the causes of immunopathogenesis and protective immunity. Annotation of the *M. leprae* genome and bioinformatic processing of the data have changed the way we investigate potential antigens for new vaccines. Given the potential for developing an effective vaccine for leprosy based on these new tools, the debate continues as to whether there is a need for a vaccine in the overall strategy to control or eradicate leprosy.

As discussed above, the current strategy for controlling leprosy is based on the implementation of effective drug regimens set forth by the World Health Organization. Unfortunately, recent epidemiological data suggest that this strategy appears to have had little effect on reducing the annual incidence of new cases of leprosy. Additionally, recent reports have shown that relapse rates of 16 to 39% among multiibacillary patients with high BIs are appearing 4 to 10 years after completion of 2-year multidrug therapy (128, 134, 168). Acknowledging these clinical realities requires an objective assessment of current control strategies and reminds us that other intervention strategies may be necessary to eradicate leprosy. Improved strategies may require new application of old tools, such as special

TABLE 12. Summary of estimates of efficacy of BCG and other vaccines against leprosy[a]

Country	Study population (incidence per 1,000 person-yr)	Placebo	Vaccination[b]	Randomized	% Efficacy (95% CI)[c]	Reference
Uganda	Child contact (3.1)	Unvaccinated	BCG Glaxo	Yes	80 (72–85)	390
Venezuela	Close contacts (3.1)	BCG without prior scar	BCG with prior scar(s)	No	56 (27–74)	76
Malawi	Total population (1.3)	No scar Same cohort with restrictions of no early lesion at start	BCG Glaxo, scar	No	54 (35–68) 65 (50–75)	302
Malawi	Total population (0.85)	Placebo with BCG scar	BCG Glaxo with prior BCG scar, with or without HK *M. leprae*	Yes	49 (1–74)	204
Papua New Guinea	Total population (5.4)	Saline	BCG Japan, 4 batches	Yes?	48 (34–59)	23
India	HHC[d] of active BB, BL, and LL patients (16.8)	Unvaccinated	HK *M. leprae*/BCG Japan/Mix BCG	No	42 (1–66)	60
India	General population (unknown)	Saline	BCG BCG + HK *M. leprae* M. w. ICRC bacillus	Yes Yes Yes Yes	34 (14–50) 71 (54–81) 31 (3–51) 65 (47–78)	140
India	Total population (unknown)	Placebo	2 BCG strains, French-Danish	Yes	30 (10–38)	411
Burma	Children ages 0–14 (5.5)	Unvaccinated	BCG Glaxo, 2 batches	Yes?	20 (12–28)	240

[a] Modified from reference 23a with permission of the publisher.
[b] HK, heat-killed.
[c] CI, confidence interval.
[d] HHC, household contacts.

programs designed to improve drug distribution and treatment compliance, but should also include basic research designed to develop tests for early diagnosis as well as pre- and postexposure vaccines for leprosy.

From the standpoint of disease control, vaccines are similar to drugs in that they may be applied as prophylactic (preexposure) or therapeutic (postexposure) measures. Vaccines, however, have a potential added advantage by producing a relatively long-lived immunological memory component. Accordingly, an effective prophylactic vaccine for leprosy could break transmission by conferring upon recipients immediate as well as extended protection from infection with *M. leprae*. A prophylactic vaccine should also protect against both drug-susceptible and drug-resistant strains, helping curb the emergence of drug resistance. Used as a therapeutic measure for leprosy control, a postexposure vaccine could improve a patient's response to multidrug therapy by hastening a cure and potentially reducing the incidence of relapse cases. While most of these concepts remain hypothetical, evidence is available that antileprosy vaccines can provide various levels of protection as well as limited beneficial therapeutic effects.

The vaccine studied most in leprosy is *M. bovis* BCG. Experience with BCG vaccination for leprosy remains enigmatic in that levels of protection vary from 20 to 80% (Table 12). These results are not unexpected considering BCG's variable efficacy against tuberculosis (71). Fine (111) has reviewed many of the issues surrounding the curious variability seen with BCG vaccination for tuberculosis. He speculates that factors such as biological differences in BCG strains, exposure to environmental mycobacteria, and ineffective boosting against reinfection with or reactivation of tuberculosis may give rise to the observed variability in protection seen with BCG vaccination. It is likely that some or all of these factors may play a role in the variability seen in BCG vaccine efficacy against leprosy.

Because leprosy is a relatively uncommon disease entity, even in countries with the highest prevalence, case-control studies have been particularly useful in obtaining information concerning vaccine efficacy. Two recent case-control studies, in India (455) and in Brazil (78), provide clear evidence that BCG protects against leprosy. In the Brazilian study, the investigators argue that their results suggest that neonatal BCG vaccination may have an important impact on transmission of leprosy and that environmental mycobacteria may not impact vaccine efficacy, at least in the Amazon region of Brazil. Zodpey and coworkers showed an overall vaccine efficacy of 54%, with the greatest protective effect seen for multibacillary leprosy (68%), suggesting that an effective vaccine for leprosy, like BCG, appears to have its greatest effect on the disease form (lepromatous) most likely to transmit *M. leprae* within the community.

Three relatively recent vaccine trials have studied the hypothesis that combining BCG with killed *M. leprae* improves vaccine efficacy. The primary reason for testing this hypothesis is to determine whether *M. leprae*-specific antigens are able to improve the efficacy of BCG. The earliest of the three studies (76) was conducted in Venezuela and concluded that the pro-

tective efficacy of BCG was proportional to the number of doses (i.e., the number of BCG scars) and that protection against leprosy was not improved when heat-killed *M. leprae* was combined with BCG. Similarly, in the double-blind, controlled trial done in Malawi (204), no advantage was observed by including heat-killed *M. leprae* (HKML) along with BCG. However, among scar-positive individuals, a second BCG vaccination gave further protection against leprosy (about 50%) over a first BCG vaccination.

In contrast, the vaccine trial done in southern India showed no positive effect of vaccinating BCG scar-positive individuals, but enhanced protection over that with BCG alone was seen in individuals receiving BCG plus HKML (140). Additional arms in the southern India vaccine trial included two atypical mycobacteria, *Mycobacterium* w (207) and ICRC bacillus (313). Both vaccines are nonliving preparations, and both were superior to BCG alone with respect to percent protection at the second and third resurveys of the trial. Interestingly, ICRC alone and BCG plus HKML gave approximately the same level of protection (64 and 65%, respectively), suggesting that a killed mycobacterial vaccine containing *M. leprae* cross-reactive antigens is as effective as a live, attenuated BCG vaccine in this population and setting. So, while the controversy continues over what elements of a leprosy vaccine are superior in a particular setting, there is little or no controversy over the positive effects of vaccination to reduce leprosy incidence.

Less is known about the application of leprosy vaccines for therapeutic purposes. A number of reports using *Mycobacterium* w as immunotherapy suggest that when given as adjunct therapy to multidrug therapy, significant clinical improvement does result (88, 206, 453). For example, De Sarkar et al. (88) observed significant improvement in both clinical and histopathological assessments of lesions in patients receiving *Mycobacterium* w plus multidrug therapy. In addition, patients vaccinated with *Mycobacterium* w demonstrated reduced bacillary indices over time compared to patients receiving only multidrug therapy for 12 months. An important finding in this study, corroborating findings from an earlier study (453), was that patients in the vaccine group experienced a higher percentage (30%) of type 1 reactions compared to the control group (10%). Fortunately, neuritis was not increased with vaccination in patients with or without reactions, a finding also reported in the earlier study (453). In contrast to the increased incidence of type 1 reactions following vaccination with *Mycobacterium* w, the incidence of type 2 reactions was similar in controls and vaccinated patients. This contrasts with the experience, noted above, that cytokine treatments using IFN-γ led to upgrading of LL and BL lesions and were accompanied by a high incidence of ENL (332). These findings underscore the challenge of producing an effective postexposure leprosy vaccine with the purpose of increasing cell-mediated immunity without inducing harmful sequelae.

Another important piece of the puzzle that is propelling investigators to search for new molecules with vaccine potential is the completion of the genome sequences of *M. leprae* (74) and *M. tuberculosis* (73). Prior to the complete sequencing of the *M. leprae* genome, the only reliable source of *M. leprae* antigens was highly purified bacilli originating from infected armadillo tissues. While many major compounds of *M. leprae* have been discovered and studied in detail using this material, purified proteins have been available in very limited quantities and of poor quality by contemporary standards, making them difficult to use for vaccine development. In addition, certain secreted proteins are almost surely lost upon purification of the bacilli from infected armadillo tissues.

New approaches to identifying genes from completed *M. leprae* genome sequences are being applied using standardized bioinformatics tools (253). These tools can identify proteins with special features, such as unique or shared amino acid sequence homologies with proteins of *M. tuberculosis* or other mycobacterial species, and the presence of specialized peptide signatures suggesting their cellular location and possible secretion across the cell membrane. Proteins of interest can be prioritized based on potential B-cell or T-cell epitopes, although strict associations between bioinformatics tools and antigenic epitopes remain underdeveloped. Finally, the proteins selected for further study can be purified as recombinant proteins for an endless supply of the protein for immunologic and vaccine studies (255, 289, 386, 442).

In addition to newly available search algorithms for genes of interest, new vehicles for delivery of protein antigens have been identified from research on recombinant DNA over the last 20 years. Much of this technology is being used to create vaccines to be administered in concert with BCG, either as recombinant BCG overexpressing one or more antigenic proteins or in a prime-boost scenario where antigen is given first in an adjuvant (priming) and then followed by BCG vaccination to boost the initial response. Since BCG is given at birth in many countries, the standard prime-boost schedule for leprosy and tuberculosis vaccines would likely be reversed. Supporting this reversal of priming and boosting with BCG are three studies using different protein vaccines for tuberculosis that have shown favorable protective responses in animal models (36, 254, 271). Application of this strategy for designing leprosy vaccines should not present any unique hurdles now that *M. leprae* antigens of interest can be cloned and expressed in large quantities.

Recalling the results of earlier vaccine trials with BCG reminds us that it is a fairly potent vaccine for leprosy in many settings. Newly designed vaccines for tuberculosis that may utilize BCG altered through genetic engineering or through a prime-boost strategy may or may not provide better protection against leprosy than is afforded by current BCG vaccines. Accordingly, newly configured vaccines for tuberculosis should be tested for efficacy against *M. leprae* challenge to show that benefits for leprosy control through vaccination for tuberculosis are not lost in the process.

While the immunogenicity of various candidate proteins can be tested in vitro using human cells, animal models in which to test the efficacy of a new vaccine are limited to the mouse and armadillo. Shepard's mouse footpad model is the gold standard for leprosy vaccine studies (366). The infection that develops in the mouse footpad may best be described as limited multiplication at the site of infection and may be somewhat analogous to indeterminate or tuberculoid leprosy in humans. Since many indeterminate cases self-heal and tuberculoid leprosy in humans involves nerve damage (not seen in mice), this model for defining vaccine efficacy has important limitations. The model does, however, enable screening out of vaccines

with little or no protective efficacy against a challenge with live *M. leprae*. Vaccines that are effective in the mouse model could then be tested in the armadillo, an animal that manifests most of the characteristics of human leprosy.

Finally, vaccine efficacy must be tested in humans following appropriate safety and potency trials. These trials must be large due to the relatively low incidence of leprosy in most settings, which continues to be an obstacle for assessing vaccines as disease management tools in leprosy. Therapeutic vaccine trials could be more focused and evaluated in smaller, defined groups of patients. With the advent of a better understanding of the molecular nature of *M. leprae* and the human response to infection, new vaccines will become a reality. The challenge for the research and public health systems is to unite the political will and financial resources to test one or more of the new vaccines for leprosy.

SUMMARY AND CONCLUSIONS

Based on operational data, with its inherent biases, the number of new cases of leprosy identified annually worldwide has probably not changed over the last two decades, although the number of registered cases has declined as a result of treatment and removal from registries. This disease poses major challenges to our understanding in microbiology, immunology, pathology, treatment, and prevention, requiring continued emphasis on basic research and clinical management in the field. Reviewed above are major advances that have been made in the basic understanding of leprosy since 1990, including the following.

The genome of *M. leprae* has been sequenced, and this organism has been shown to be able to synthesize far fewer proteins than the other major human-pathogenic mycobacterium, *M. tuberculosis*. Thus, although *M. leprae* still cannot be cultivated axenically, the new molecular ability to assess its ability to transcribe and synthesize various proteins in response to different environments and stresses will likely provide valuable information about its mechanisms of pathogenicity in the near future.

PCR analysis of tissues for *M. leprae* DNA now provides a valuable means for identifying this organism. Mutations in the *M. leprae* genome that are associated with resistance to several of the drugs used against this pathogen have been identified, and DNA analysis to detect these mutations is likely to replace the mouse footpad technique.

Major advances have been made in experimental models of leprosy. The availability of knockout mice deficient in selected immunologic abilities will enable dissection of the roles of different cytokines and T-cell subsets in the response to this infection. The armadillo genome has been partially sequenced and is being annotated; this will soon enable investigators to identify and synthesize immunologically relevant cytokines and probes for use in this animal model.

Within macrophages, *M. leprae* is now known to be killed by reactive nitrogen compounds. A variety of mechanisms of innate and adaptive immunity have been identified and postulated to play a role in the development of cellular immunity in leprosy. None of these can yet explain the remarkable spectrum of cellular immune responses to this organism in human subjects, however.

Genetic influences on immunity to *M. leprae* in humans appear to operate at two levels: some mechanisms act at the level of overall susceptibility, and others function at the level of acquired immunity. One leprosy susceptibility gene has been identified, and several genes possibly influencing adaptive immunity have also been described.

Reactions in leprosy remain poorly understood. Immunopathological studies have generally found that type 1 (reversal) reactions correspond to an up-regulation of Th-1-type immune responses, and that type 2 reactions (ENL) correspond to an enhancement of the Th-2 type of response. These findings are not yet very satisfying, however, since there are several unresolved discrepancies in these associations, and no information thus far indicates what triggers reactions or why they affect some patients but not others.

The mechanisms of nerve injury in leprosy remain poorly understood, although recent studies have shed light on the mechanisms of localization of *M. leprae* to nerves and on the molecular mechanisms of binding and ingestion of *M. leprae* by Schwann cells. A major frontier in clinical leprosy is the prevention of disability by active and persistent attention to nerve function impairment, since nerve injury may progress even after completion of chemotherapy.

Several effective antimicrobial agents are now available to treat leprosy, and this infection is curable. Molecular methods can now identify several mutations in *M. leprae* associated with antimicrobial resistance. The medically conservative approach to treatment recommends using multiple agents (multidrug therapy) for several months or years, depending on the classification of the disease. The World Health Organization has recommended much shorter treatment protocols, and these have been the subject of much controversy among physicians treating patients with leprosy.

BCG provides a low but measurable degree of protection against *M. leprae*, but no highly effective, specific vaccine has yet been developed. No infectious disease has been eliminated using drug treatment alone, without an effective vaccine, but the difficulties of implementation of a leprosy vaccine also pose profound challenges to the use of such a vaccine if one is developed.

Future of Leprosy Treatment and Research

Global elimination of leprosy has been the overarching goal of laboratory research and health policy for nearly 20 years. This was not accomplished by 2000 or 2005 and does not appear likely, probably due to a complex mixture of social, economic, and biological factors that cannot be resolved in the laboratory alone. Elimination of an infectious disease requires a highly effective vaccine; developing one was the central focus of leprosy research during the 1980s and early 1990s. This has not been successful thus far, although vaccine efforts continue.

With elimination still in mind, the current primary goal is early diagnosis, in order to try to interrupt transmission with treatment as early as possible. This is the major focus of the most concerted laboratory research efforts today, employing advanced molecular tools. Even if this should become technically feasible in the near future, this concept faces enormous challenges to verification before treatment of asymptomatic individuals can be recommended. Implementing treatment of

asymptomatic persons would be even more difficult; at this writing, in many areas with endemic leprosy, even patients with overt disease are finding that resources for diagnosis and treatment are being systematically reduced.

An alternative paradigm to elimination of leprosy is living with leprosy but rendering it harmless, an idea advanced by Yo Yuasa of the Sasakawa Memorial Health Foundation (451). Recognizing the high cost and apparent futility of elimination campaigns in the most highly leprosy-endemic regions of the world, this approach calls for improved tools for management of the infection and its complications and better methods for the prevention and treatment of nerve injury. Both of these paradigms, as well as the tension between them, reflect the continuing challenges of leprosy.

ACKNOWLEDGMENTS

The photographs in Fig. 2 were taken by G. McCormick, NHDP. Figure 6 was created by T. Thomassie, NHDP.

This work was supported in part by NIH grants AI050027 and AI045725 from the National Institute of Allergy and Infectious Diseases, NIAID contract Y1-AI-2646-01, and grants from the Heiser Foundation and from American Leprosy Missions.

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