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CHAPTER 1.1.20

Mycobacterium leprae

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Although *Mycobacterium leprae* was one of the first bacterial pathogens of humans to be described (Hansen, 1874), progress on understanding the basic biology and pathogenicity of this organism has been greatly hampered by the inability to find a conventional laboratory medium or tissue culture system that can support its growth. Consequently, the only means of propagating this organism at present is by using experimental animals. Furthermore, it has been found that the nine-banded armadillo can be used to produce large numbers of bacilli (Kirchheimer and Storrs, 1971; Storrs, 1971). Relatively little is known, therefore, about the taxonomy, genetics, and biochemistry of this species. A corollary of this is that much of what we do know about *M. leprae* has come from studies of the disease it causes (leprosy or Hansen's disease) and from the animal models. As such, this chapter emphasizes the characteristics and behavior of *M. leprae* in experimental animal model systems and in humans. Several excellent reviews on the clinical aspects, epidemiology, immunology, and pathology of leprosy and on the biochemistry and immunochemistry of *M. leprae* have been published recently, and the reader is referred to these for additional information (Bloom and Godal, 1983; Bloom and Mehra, 1984; Fine, 1982; Gaylord and Brennan, 1987; Hastings, 1986; Hastings and Franzblau, 1988; Jopling and McDougall, 1988; Kaplan and Cohn, 1986; Stewart-Tull, 1982).

Although *M. leprae* can occasionally be found in the body extracellularly, the bacillis appears to be able to replicate only within cells of the host, most commonly in macrophages and Schwann cells (Bloom and Godal, 1983; Kaplan and Cohn, 1986). Hence, *M. leprae* is considered to be an obligate intracellular pathogen. In host cells, the bacilli are found singly or in clumps referred to as *globi* (Cowdry, 1940). The bacilli are straight or slightly curved, Gram-positive, acid-fast, alcohol-fast, nonmotile rods ranging from 1 to 8 μm in length and 0.2 to 0.5 μm in width (Draper, 1983). Acid- and alcohol-fastness refers to the ability of the bacillus to retain the color of certain dyes, usually carbol fuchsin, following treatment with mild acid and alcohol, respectively.

M. leprae has been placed in the genus *Mycobacterium* in the family Actinomycetales based mainly on cell structure, staining properties and chemical composition as well as on the basis of the presence of mycolic acids, antigens characteristic of mycobacteria, and a lipid-rich cell envelope (Draper, 1976; Harboe et al., 1977; Stanford et al., 1975). For example, the *M. leprae* bacillus closely resembles *M. tuberculosis* bacilli in size, morphology, and staining characteristics, although it does stain a little more deeply with carbol fuchsin and the staining is somewhat less acid fast. Recently, analyses of the ribosomal RNA sequences of armadillo-grown *M. leprae* by nucleic acid hybridization techniques (Sela et al., 1989) and by ribosomal RNA sequence comparisons (Smida et al., 1988) revealed that *M. leprae* is closely related to the corynebacteria, nocardia, and mycobacteria, especially to the two slowly growing *Mycobacterium* species *M. avium* and *M. tuberculosis*. Although these observations indicate that *M. leprae* should be classified in the genus *Mycobacterium*, several features distinguish *M. leprae* from other members of the *Mycobacterium* genus. These are: 1) loss of acid-fastness upon extraction with pyridine, although *M. smegmatis*, *M. vaccae*, and *M. phlei* do lose acid fastness after prolonged exposure to pyridine (Fisher and Barksdale, 1971; McCormick and Sanchez, 1979; Skinsnes et al., 1975); 2) ability to oxidize 3,4-dihydroxyphenylalanine (Prabhakaran and Kirchheimer, 1966); 3) replacement of L-alanine with glycine in the linking peptide of peptidoglycan (Draper, 1976); 4) 56% GC content as compared with 65–70% for most *Mycobacterium* species (Clark-Curtiss et al., 1985; Imaeda et al., 1982; Wayne and Gross, 1968); and 5) lack of substantial genomic DNA homology with other *Mycobacterium* species (quite in contrast with the ribosomal rRNA results) (Athwal et al., 1984; Grosskinski et al., 1989). Although these differences are not sufficient to exclude *M. leprae* from the genus *Mycobacterium*, they are sufficient to render the final taxonomic position of *M. leprae* somewhat in doubt.

The Disease

Hansen's disease (leprosy) is a chronic infectious granulomatous disease that primarily affects the peripheral nervous system, skin, and mucous membranes, especially nasal mucosa. In advanced cases, other tissues—including muscle, testes, capillary endothelium, liver, spleen, and bone marrow—can be affected (Desikan and Job, 1968). Although leprosy is not usually fatal in and of itself, 20–30% of patients with untreated or neglected infections develop crippling deformities of hands and feet. A sequela of these deformities is the social stigma that has been historically associated with leprosy. An addition consequence is that suicide is a common cause of death among infected individuals, particularly during episodes of exacerbation of the lesions (erythema nodosum leprosum).

Leprosy has been estimated to afflict 10–12 million individuals world-wide, with most cases being found in tropical and subtropical regions (Sixth Report of the WHO Expert Committee

on Leprosy, 1988). Climatic factors do not seem to have a significant impact on the disease, however, since leprosy was widespread in Europe, particularly Norway, in the past centuries (Browne, 1975), and cases have been reported from above the Arctic Circle (Sansarricq, 1981). Within endemic areas, cases of leprosy appear to cluster geographically, with the prevalence of disease exceeding 10 cases per 1000 population in the high prevalence areas (Bloom and Godal, 1983). Although this disease has been cited as the "least infectious" of communicable diseases (McWhirter, 1981), epidemics of leprosy have occurred on some Pacific Islands in which as much as 35% of the population developed the disease over a 20-year period (Wade and Ledowski, 1952).

A feature of the disease that has intrigued clinicians and immunologists is the variety of clinical manifestations that can be found. These range from a single lesion with no detectable bacilli to multiple lesions containing large numbers of bacilli. Bacterial counts up to 5×10^9 organisms per gram of tissue have been found (Collaborative effort, 1975). The variety of disease symptoms is not related to the genetics of the bacterium but rather to the immune responsiveness of the host (Bloom and Mehra, 1984; Kaplan and Cohn, 1986). A clinically and experimentally useful categorization of this disease spectrum is the Ridley-Jopling classification scheme which is based on immunopathologic features of the disease (Ridley and Jopling, 1966). Tuberculoid leprosy (TT) is at one pole of the spectrum and is characterized by one or a few localized skin or nerve lesions, a strong cellular immune response, and a weak humoral immune response. Histologically, one finds well-organized epithelioid cell granulomas with multinucleated giant cells and abundant lymphocytes in TT lesions, but bacilli are usually absent ($<10^5$ bacilli/gram of tissue). At the other extreme is lepromatous leprosy (LL), characterized by numerous small, bilaterally symmetrical skin lesions, a weak or absent cellular immune response, and high antibody titers. Histologically, one observes a foamy macrophage granuloma with few lymphocytes in LL lesions, and the macrophages contain numerous bacilli ($>10^8$ bacilli/gram). Within these extremes are other conditions, including borderline lepromatous (BL; numerous skin lesions, numerous bacilli, granulomas with undifferentiated macrophages and histiocytes), borderline (BB; numerous skin lesions, few acid-fast bacilli, nerve involvement, epithelioid cell granulomas with no giant cells), and borderline tuberculoid (BT; multiple skin lesions, occasional bacilli, nerve involvement, diffuse epithelioid cell granulomas). One additional category, called indeterminant leprosy, often found when a patient first seeks treatment, usually consists of a single small hypopigmented plaque. Such a lesion may remain indeterminant, regress spontaneously, or progress into a lesion that falls into one of the above categories.

It is generally accepted that *Mycobacterium leprae* is the etiologic agent of leprosy. However, not all of Koch's postulates have been fulfilled for identifying *M. leprae* as the causative agent of human leprosy. That is, a pure culture of *M. leprae* has not been developed from a single bacillus and shown to cause disease. The inability to grow the organism outside of animal hosts is the major stumbling block to completing Koch's postulates. Nonetheless, much evidence has accumulated in support of the role of *M. leprae* as the agent of the disease. The evidence includes the following: 1) *M. leprae* is found in leprosy patients and not in nonleprosy patients; 2) *M. leprae* bacilli are invariably present in lepromatous lesions; 3) leprosy patients display characteristic immune responses to *M. leprae* antigens (Mitsuda and Fernandez reactions; see Jopling and McDougall; 1988); 4) antibodies and T-cells reactive with antigens or epitopes uniquely expressed by *M. leprae* can be isolated from leprosy patients (reviewed in Gaylord and Brennan, 1987); 5) a phenolic glycolipid (PGL-I; Hunter and Brennan, 1981; Hunter et al., 1982) uniquely found in *M. leprae* is also uniquely found in lesions, sera, and urines from leprosy patients (Cho et al., 1983; Cho et al., 1986; Koster et al., 1987; Vemuri et al., 1985; Young et al., 1985); 6) *M. leprae*-specific nucleotide sequences can be found in leprosy lesions (Clark-Curtiss and Docherty, 1989); and 7) drug susceptibility of *M. leprae* in animal models parallels drug efficacy in patients (reviewed in Shepard, 1986).

Several properties of the *M. leprae* bacillus contribute to the features of the disease:

1. *M. leprae* has a generation time of 11–13 days in experimental animals (Levy, 1976; Shepard and McRae, 1971b). Such slow growth might influence the length of time from infection to disease (median interval 2–8 years; Fine, 1982) and the chronic nature of the infection.
2. *M. leprae* grows best at 30°C, which may explain its affinity for cooler parts of the body including skin and nasal mucosa (Shepard, 1965).
3. *M. leprae* infects Schwann cells and is the only bacterial pathogen capable of entering peripheral nerves (Job, 1971; Ridley et al., 1987; Stoner, 1979). These facets play a role in the loss of nerve function and the generation of crippling deformities.
4. *M. leprae* can supress the cellular immune response, perhaps through induction of suppressor T-cells (Bloom and Mehra, 1984;

Mehra et al., 1984) and/or rendering the infected macrophage defective for activation or antigen processing (Desai et al., 1989; Prasad et al., 1987; Sibley and Krahenbuhl, 1988). This may allow the host to tolerate the large bacterial load seen in lepromatous leprosy patients (up to 10^{13} organisms).

5. The unique phenolic glycolipid of *M. leprae*, called PGL-I, is a major component of the cell envelope (2% of total bacterial mass; Hunter and Brennan, 1981), can scavenge hydroxyl radicals and superoxide anions in vitro (Chan et al., 1989) and can inhibit the oxidative response in macrophages that have ingested *M. leprae* (Vachula et al., 1989). Hence, PGL-I may play a role in the ability of the bacteria to survive within macrophages and may also play a role in immunosuppression (Mehra et al., 1984; Prasad et al., 1987).

6. *M. leprae* can survive and multiply within macrophages and Schwann cells. Three possible strategies of intracellular survival have been proposed for *M. leprae*. First, *M. leprae* bacilli might avoid the bactericidal activities of the phagocytic cells by escaping from the phagosome (phagolysosome?) and multiplying in the cytoplasm of the infected cells (Mor, 1983). Second, viable *M. leprae* has been reported to prevent the fusion of phagosomes and lysosomes in murine macrophages (Frehel and Rastogi, 1987; Sibley et al., 1987), but not in Schwann cells (Steinhoff et al., 1989). A similar prevention of phagosome-lysosome fusion is an intracellular survival strategy used by *M. tuberculosis* (d'Arcy Hart, 1982). Third, inside phagosomes *M. leprae* generates around the bacillus a characteristic electron microscope image called an "electron transparent zone (ETZ)" (Draper and Rees, 1970). The ETZ might represent a physical barrier to prevent degradative or bactericidal proteins of the lysosome from reaching the bacterial surface. Regardless of the precise strategy used, one result of evading the bactericidal and degradative activities of the phagocytic cells might be that the *M. leprae* antigens would be less likely to be processed and presented to the immune system. If so, the bacilli might thereby prevent or escape a protective immune response.

Habitat

Leprosy and *M. leprae* have historically been considered to be confined to humans. Indeed, humans are the major host and reservoir of the leprosy bacillus. Humans, however, are not the only possible habitat for *M. leprae*. Naturally acquired, leprosy-like infections have been observed in armadillos (*Dasypus novemcinctus*; Walsh et al., 1975), chimpanzees (*Pan troglodytes*; Donham and Leininger, 1977), and Mangabey monkeys (*Cercocebus torquatas atys*; Meyers et al., 1985). Since the bacilli infecting these animal species are identical to the human pathogen, based on a variety of DNA homology and biochemical studies (Athwal et al., 1984; Clark-Curtiss and Walsh, 1989; Meyers et al., 1985), leprosy should be considered a zoonotic disease (Walsh et al., 1981). Furthermore, the possibility of the transmission of *M. leprae* from animals to humans has been raised by the observation that five armadillo handlers in Texas developed leprosy in the absence of any known contact with a human source of *M. leprae* (Lumpkin et al., 1983).

The isolation of *M. leprae* from soil in Bombay and other environmental sources has also been reported (Kazda, 1981a; Kazda et al., 1986). It is unclear, however, if these reports identify an environmental niche for *M. leprae* or if the isolated bacilli represent "environmental contamination" from *M. leprae*-infected individuals. For example, one possible source of "environmental contamination" might be nasal secretions of lepromatous leprosy patients (see below). Finally, free-living amoebae have been suggested as a potential reservoir for *M. leprae* (Grange and Rowbotham, 1987), although no convincing evidence for the presence or multiplication of *M. leprae* in amoebae has been published.

The mode of transmission of the leprosy bacillus remains unknown. Evidence is accumulating that infection occurs predominantly by way of the respiratory route, although other routes may be responsible for some cases (reviewed in Pallen and McDermott, 1986). Some of the evidence for respiratory transmission includes: 1) the epidemiology of transmission is consistent with spread by a respiratory route (Barton, 1974; Davey and Rees, 1974). 2) The major portal of exit of the leprosy bacillus is the nose. Lepromatous leprosy patients can shed up to 10^8 bacilli per day in nasal discharges, and the bacilli can survive for several days in dried secretions (Davey and Rees, 1974; Shepard, 1962). 3) Bacilli can be aerosolized by coughing and sneezing. 4) The nose has been suggested as a possible site of initial infection in humans (Pallen and McDermott, 1986), and immunodeficient mice can be infected by exposing them to an aerosol containing *M. leprae* (Rees and McDougall, 1976; Chehl et al., 1985). 5) As originally discussed by Koch (1897) and Schaffer (1898), the similarities between tuberculosis and leprosy (e.g., the similarity between the large numbers of *M. leprae* in nasal secretions and the large numbers of *M. tuberculosis* in sputa) are suggestive of an analogous route of transmission.

At present, however, one can not exclude transmission by skin-to-skin contact or by insect vectors. For example, ulcerating skin lesions could be a source of bacilli that might enter a susceptible host through a skin abrasion. Similarly, broken skin might be a portal of entry for bacilli that were aerosolized or otherwise deposited in the environment. With respect to insect vectors, *M. leprae* can be found in flies that have fed on nasal secretions from lepromatous leprosy patients (Greater, 1975; Kirchheimer, 1976), and the transmission of *M. leprae* from humans to mouse footpad by a mosquito vector (*Aedes aegypti*) has been reported (Narayanan et al., 1977). Another possible route is via breast milk since *M. leprae* bacilli can be found in the breast milk of lepromatous patients (Pedley, 1968). The role, if any, of the gastrointestinal tract in the transmission of leprosy is not known. Finally, occasional cases of leprosy may result from accidental inoculation of susceptible individuals by needle prick (Wade, 1948).

Isolation and Propagation

The currently available methods for the isolation and propagation of *M. leprae* are greatly constrained by the lack of an axenic or cell-culture cultivation system. In the laboratory, *M. leprae* has been propagated in both immunocompetent and immunodeficient mice and rats (Colston and Hilson, 1976; Fieldsteel and Levy, 1976; Hilson, 1965; Rees, 1966; Shepard, 1960), hamsters (Binford, 1959), armadillos (Kirchheimer and Storrs, 1971), and in Mangabey, Rhesus, and African green monkeys (Wolf et al., 1985). The features of the growth and pathology of *M. leprae* in each species are somewhat different, and the choice of animal model often depends on exactly what experimental question is being asked (reviewed in Shepard, 1986).

Staining Methods

One consequence of having only animal systems in which to propagate *M. leprae* is that much of the work depends on the enumeration of acid-fast bacilli by direct counting in microscopic fields. The standard staining method is a modified Ziehl-Neelsen technique (e.g., see Jenkins et al., 1982, or Jopling and McDougall, 1988). Briefly, bacilli on a glass slide are covered with a carbol-fuchsin solution and left at room temperature for 20 minutes (cold Ziehl-Neelsen) or heated for 15 minutes over a boiling water bath (hot Ziehl-Neelsen). The slide is washed with water, destained with 1% hydrochloric acid in 70% ethanol, and washed again with water. The sample is then counterstained with 1% methylene blue, washed in water, and air dried. Several methods for the actual counting of bacilli in the microscope and for using the counts to determine the concentration of bacilli in a sample have been described (Hanks et al., 1964; Shepard and McRae, 1968). The uniformity of staining exhibited by a bacillus is used as an indication of the viability of the bacillus (Hansen and Looft, 1895; Shepard and McRae, 1965a; Waters and Rees, 1962). Rods showing uniform staining are considered to be viable, while bacilli displaying fragmented or granular staining are considered non viable. A "morphologic index" is calculated as the percentage of uniformly staining bacilli and has been used to follow the progress of chemotherapy (Waters and Rees, 1962).

Propagation in Mice

The most frequently used propagation system is growth in the footpad of immunocompetent mice, as was originally described in the landmark paper of Shepard (1960). In this animal model, one typically propagates *M. leprae* by injecting mice subcutaneously with about 5×10^3 bacilli in one of the hind footpads, although as few as 1–10 bacilli are sufficient to establish an infection (Shepard and McRae, 1965a). A typical growth curve displays an initial lag phase followed by logarithmic growth with a doubling time of 11–13 days and a stationary phase with a plateau level of about $1–2 \times 10^6$ bacilli per footpad. The plateau level is thought to be due to development of a cell-mediated immune response to the bacillus. As a consequence, the viability of the *M. leprae* in the footpad decreases dramatically during the plateau phase. Thus, to propagate a strain one should harvest bacilli prior to or early in the plateau phase.

A typical growth curve is accompanied by a typical histopathologic picture. Early in infection one finds single bacilli or small clumps of 2–5 bacilli aligned in a parallel fashion ("cigar packs") in tissue macrophages, muscle, and tendon cells. Later, a cellular infiltrate mainly of macrophages and a few lymphocytes appears, followed by development of a microscopic granuloma with a few large, "foamy" macrophages in the center surrounded by a loose collection of macrophages and lymphocytes. The large macrophages contain numerous bacilli. In late stages, one occasionally observes invasion of the nerves.

In the absence of a T-cell response, multiplication continues to much higher levels. Thus, immunodeficient animals such as thymectomized-irradiated mice (Rees, 1966), neonatally thymectomized Lewis rats (Fieldsteel and Levy; 1976), and nude mice (*nu/nu*) (Colston and Hilson, 1976; Chehl et al., 1983) have been used to propagate *M. leprae*. The minimum infec-

tious dose and generation time during logarithmic growth appear to be the same in the immunodeficient animals as in immunocompetent animals. The bacilli, however, can multiply to up to about 10^9 cells per foot pad in the neonatally thymectomized Lewis rat and about 5×10^{10} cells per footpad in the nude mouse. In the nude mouse one frequently observes spread of the *M. leprae* to other cool sites in the body (e.g., the ears) and invasion into nerves. Overall, the histopathology of the disease in the immunodeficient mice resembles that of human lepromatous leprosy. One drawback to the use of these animals on a routine basis is that they are particularly susceptible to a variety of other infections, and hence, special precautions need to be taken to ensure the survival of the animals for the duration of the experiment (often greater than 1 year).

Propagation in the Armadillo

A second major advance in leprosy research was the development of the nine-banded armadillo as an animal model system for the propagation of *M. leprae* (Kirchheimer and Storrs, 1971; Storrs, 1971). The key here is that in the armadillo the disease resembles human lepromatous leprosy and one can isolate very large numbers of bacilli from each infected armadillo. For example, the bacillary load in lesions can reach 10^{10} organisms per gram of tissue, and the total bacillary load can exceed 10^{12} bacilli per armadillo. Also, the liver and spleen are heavily infected and are easily manipulated sources of bacilli. The availability of the large amounts of *M. leprae* from armadillos opened the way for biochemical and molecular biologic analyses of the bacillus as well as provided a way to produce sufficient *M. leprae* for use in vaccine trials. One technical problem with the use of armadillos is that the *M. leprae* infection is fatal for the armadillo. Hence, to maximize bacillary yield and avoid postmortem contamination of tissues with other bacteria, care must be taken to sacrifice the animal and harvest bacilli just prior to the animal succumbing to the *M. leprae* infection itself. Finally, one must be aware that armadillos may harbor other naturally acquired, fastidious, acid-fast mycobacteria (Kazda, 1981b) and may harbor naturally acquired *M. leprae* (Walsh et al., 1975).

Primate Infection

Naturally occurring *M. leprae* infections have been observed in several primates, and *M. leprae* has been experimentally transmitted to three species of monkeys (Wolf et al., 1985). While the disease in these animals seems to parallel closely disease in humans, their use in the routine isolation and propagation of *M. leprae* is deterred by economic and humane concerns. Nonetheless, the primates may prove to be important model systems for studies on pathology, immunology, and vaccine development.

Maintenance of Viable Cells

Finally, a concern of those working with *M. leprae* is that the bacillus does not store well. Good viability of *M. leprae* in suspensions or in biopsy specimens is maintained for 7–10 days at 0–4°C (Shepard and McRae, 1965b). For long-term storage, one can store the bacilli in liquid nitrogen. Three important points here are: 1) the sample should contain 10% dimethyl sulphoxide or 10% glycerol; 2) slow freezing is required (<1°C/min); and 3) the bacilli rapidly lose viability upon repeated freezing and thawing (Colston and Hilson, 1979; Portaels et al., 1988). For routine laboratory work one usually maintains several stocks of *M. leprae* in continuous serial passage. Of course, one is concerned that serial passaging of *M. leprae*, which typically involves transfer of 10^3 to 10^4 bacilli from mouse to mouse, might lead to changes in the bacillus, e.g., outgrowth of variants. However, some strains have been serially passaged in the Hansen's Disease Laboratory at the Centers for Disease Control (CDC) for over 25 years without any detectable changes in growth pattern (rate and plateau level) or histopathology of infection. Furthermore, by restriction-fragment-length-polymorphism (RFLP) studies, Clark-Curtiss and Walsh (1989) have found that the *M. leprae* bacilli isolated from leprosy patients in India, armadillos in Louisiana, and a naturally infected Mangabey monkey were virtually identical. This exceptional conservation of nucleotide sequence might be related to the long generation time of *M. leprae* or to the presence of a very efficient DNA-damage-repair system. Overall, these observations suggest that *M. leprae* changes very slowly, if at all, during serial passaging.

Isolation from Clinical Specimens

With respect to the isolation of *M. leprae* from a clinical specimen (e.g., biopsy of an LL lesion), the first steps are usually to purify the bacilli and to inject them into the footpad of an immunocompetent mouse such as the BALB/c or CFW strain (reviewed in Shepard, 1986). To purify the bacilli, the usual procedure is to release the bacilli from their intracellular location within a biopsy of infected tissue by mincing the sample, suspending it in Hank's Balanced Salt solution containing 0.1% BSA, and homogenizing it in the presence of 2 to 3-mm-diameter glass beads. To reduce potential contamination with other

bacteria, the sample can be treated with 0.5M NaOH for 15 minutes and then neutralized with HCl. Following this treatment, the cells are usually harvested by centrifugation and resuspended in Hank's Balanced Salt solution containing 0.1% BSA. Samples of the bacterial suspension are stained and examined microscopically to determine the number of uniformly staining, acid-fast bacilli. One then injects portions of the samples containing 10^4 or fewer bacilli into the footpads of mice. Tissues from the mice are harvested periodically and examined for the presence and numbers of acid-fast bacilli and the histopathology of the site of infection.

These initial steps allow one to produce sufficient bacilli to begin the process of determining if the isolated organism is *M. leprae* (described below). In addition, the rate of growth in the footpad and the histopathology of the infection in the mouse footpad may be useful in distinguishing *M. leprae* from other mycobacteria (Shepard, 1986). That is, only *M. leprae*, *M. lepraemurium*, *M. marinum*, and *M. ulcerans* of the *Mycobacterium* species grow in the mouse footpad, and each displays a characteristic growth rate and histopathology. For example, *M. marinum* infections appear in about 2 weeks and often cause ulceration at the site of inoculation.

Identification

The lack of an in vitro system for the cultivation of *M. leprae* makes positive identification particularly difficult. Often a presumptive identification of an organism as *M. leprae* is made based simply on two observations—staining characteristics and lack of growth on conventional laboratory media. For example, with clinical specimens, any noncultivable, acid-fast, rod-shaped bacillus isolated from a site displaying a histopathology characteristic of a leprosy lesion is presumed to be *M. leprae*. Although such a definition is adequate for decisions on patient management, it is not particularly useful if one is trying to identify an organism isolated from a source devoid of histopathologic information, such as nasal secretions or the environment. Also, such a definition is clearly insufficient to prove an organism is *M. leprae*. Undoubtedly, the imprecise nature of the definition has contributed to the numerous erroneous claims for the cultivation of *M. leprae* in vitro.

Until recently, only a few rather labor-intensive, time-consuming, and technically difficult tests, such as growth in mouse footpads or presence of *M. leprae*-specific components, were available to confirm the presumptive identification. Fortunately, a variety of relatively simple biochemical, immunological, and molecular-biological tests is now becoming available that should allow a rapid and definitive identification of *M. leprae*. Some of the potentially useful identification tests are: 1) morphology and staining characteristics; 2) failure to grow on bacteriologic media; 3) growth and histopathology in animal models; 4) drug susceptibility patterns; 5) presence of unique biochemical components or activities; 6) presence of characteristic antigens; and 7) presence of specific nucleotide sequences.

The identification process still starts with an examination of the staining characteristics of the bacilli. *M. leprae* bacilli isolated from lesions are Gram-positive rods. The carbol fuchsin stain is not extractable with acid or alcohol but is extractable with pyridine (Fisher and Barksdale, 1971). Of the other mycobacteria, only *M. smegmatis*, *M. vaccae*, and *M. phlei* display somewhat similar pyridine-extractable acid-fast characteristics (McCormick and Sanchez, 1979; Skinsnes et al., 1975). An important caveat here is that absence of acid-fastness can not be taken as an indication that the organism is not *M. leprae*, since a variety of mycobacteria lose the acid-fast characteristic at various times during their growth.

The second routinely used test is a determination of growth on any of a variety of media used for the propagation of mycobacteria, such as Lowenstein-Jensen, Middlebrook 7H9, or Dubos medium. *M. leprae* is the only *Mycobacterium* species that does *not* grow on at least one type of axenic medium. Of course, one needs to include a control test, such as inoculation into mouse footpads, to ensure that the original preparation did indeed contain viable bacteria.

M. leprae grows in mice, rats, armadillos, and monkeys and produces a characteristic pattern of proliferation and histopathologic changes in each animal (for details, see Shepard, 1986). Hence, growth characteristics can be used for identification purposes. For practical and economic reasons, one usually starts by analyzing growth in mice and then proceeds onto other animal models only as warranted. A key step in this test is careful observation of histopathologic changes. For example, *M. leprae* is the only *Mycobacterium* species to invade peripheral nerves and such invasion is prominent in the later stages of an *M. leprae* infection in the thymectomized-irradiated mouse and in the armadillo. Hence, observation of acid-fast bacilli in nerves is a good indication that the organism is *M. leprae*.

Biochemical Tests

Among the mycobacteria, *M. leprae* has a unique pattern of drug susceptibilities (Shepard, 1971;

Shepard et al., 1983; Hastings and Franzblau, 1988). Thus, one could confirm a presumptive identification by measuring drug susceptibility in: 1) inhibition of in vivo growth in the mouse footpad (Shepard, 1971); 2) inhibition of growth in macrophage culture (Mittal et al., 1983; Ramasesh et al., 1987); or 3) effect of the drug on PGL-I synthesis, palmitate oxidation, or ATP generation in bacilli maintained in a synthetic medium (Franzblau, 1988; Franzblau and Hastings, 1987; Franzblau et al., 1987). Note that the bacilli do not multiply in the systems used by Franzblau and colleagues.

Several identification tests are based on the observation that *M. leprae* contains components, structures, enzymes, and antigens that apparently are not present in any of the other *Mycobacterium* species. For example, enzymatic activities that are apparently unique to *M. leprae* (among the mycobacteria) include the ability to oxidize 3,4-dihydroxyphenylalanine (DOPA) and the synthesis of phenolic glycolipid I (PGL-I). DOPA-oxidase activity can be measured in vitro by following the conversion of DOPA to the pigmented product indole-5,6-quinone (Prabhakaran and Kirchheimer, 1966). This test is not entirely specific for *M. leprae*, since some non-acid-fast bacteria and, more importantly, some mammalian tissues have similar oxidase (phenolase) activities (Prabhakaran, 1967). PGL-I biosynthesis can be followed by measuring the incorporation of ^{14}C-palmitate into PGL-I in an *M. leprae*/macrophage co-culture system (Ramasesh et al., 1987) or in bacilli maintained in a synthetic medium (Franzblau and Hastings, 1987). Since PGL-I is unique to *M. leprae*, this assay is particularly useful. Also, characterization of the susceptibility of the in vitro reaction to various drugs can help confirm the identification.

Characteristic biochemical components of *M. leprae* include: 1) peptidoglycan that contains glycine instead of L-alanine in the linking peptide (Draper, 1976); 2) a GC content of 56 mol% as opposed to GC contents of 65–70 mol% for the other *Mycobacterium* species (Clark-Curtiss et al., 1985; Imaeda et al., 1982; Wayne and Gross, 1968); 3) PGL-I. However, other mycobacteria (e.g., *M. tuberculosis*) have structurally similar phenol-phthiocerol triglycosides (Brennan, 1983; Chatterjee et al., 1989; Daffe et al., 1988; Hunter and Brennan; 1981); and 4) specific mycolic acids (Draper, 1976). Analysis of the mycolic acids may be particularly informative since the high pressure liquid chromatography (HPLC) pattern of mycolic acids produced following alkaline methanolysis seems to be unique for each species of *Mycobacterium*. Indeed, Butler and Kilburn (1988) have devised a relatively simple and rapid scheme based on such HPLC patterns for identifying many of the *Mycobacterium* species.

Immunological Tests

Several antigens (or epitopes?) also appear to be uniquely present in *M. leprae* (reviewed in Gaylord and Brennan, 1987). Particularly useful reagents are monoclonal antibodies directed against the *M. leprae*-specific oligosaccharide of PGL-I (Mehra et al., 1984; Young et al., 1984). Such antibodies have been shown to react only with *M. leprae* and have been used in the serodiagnosis of leprosy (Cho et al., 1986; Koster et al., 1987; Sanchez et al., 1986; Young et al., 1985). Several other monoclonal antibodies are also available that react with other targets that appear to be *M. leprae*-specific (reviewed in Gaylord and Brennan, 1987). One could also assay the reactivity of the unknown with T-cell clones that are directed against *M. leprae*-specific components. Other potential immunological tests include fluorescent-antibody stains (Abe et al., 1980), immunodiffusion assays (Payne et al., 1982), immunoprecipitation assays (Harboe et al., 1978), and the ability to induce a delayed type hypersensitivity reaction in infected individuals similar to that induced by authentic *M. leprae* preparations, e.g. lepromin (Shepard and Guinto, 1963). In any immunological test, one should be aware that *M. leprae* does contain many antigens and epitopes that are cross-reactive with antigens found in one or more of the other *Mycobacterium* species as well as antigens that are cross-reactive with organisms that are evolutionarily very distant, including humans.

Genetic Tests

One can measure the relatedness of two organisms by determining the degree of hybridization between genomic DNAs from the two organisms (Brenner, 1989). Cells from the same species have DNA homologies of greater than 70% and a difference in melting temperature of <5°C. Such genomic DNA:DNA hybridization studies have been reported for *M. leprae* and a variety of other *Mycobacterium* species. *M. leprae* shows little if any homology with the other mycobacteria (Athwal et al., 1984; Grosskinsky et al., 1989). Thus, this sort of test might be useful in identifying *M. leprae*. Unfortunately, such genomic DNA hybridization studies require fairly large amounts of DNA (10–100 µg, 10^9–10^{10} bacilli), not easily obtained for a nonaxenically cultivable bacterium.

One way to circumvent the need for such large amounts of DNA is to use a labelled (e.g., radioactive) nucleic acid probe to assess DNA homologies (reviewed in Tenover, 1989). Using

labelled probes, one can reduce the number of organisms needed for analysis to 10^5 to 10^6. Potential hybridization probes (nucleotide sequences that hybridize only with *M. leprae* nucleic acids) for *M. leprae* have been identified and cloned into *E. coli*, which allows easy production of large amounts of the probes. Of particular interest is the work of Clark-Curtiss and colleagues (Clark-Curtiss and Docherty, 1989; Grosskinsky et al., 1989), who have identified a sequence that is present in about 19 copies in the *M. leprae* genome and is not present in other mycobacteria. Using this sequence as a probe and purified DNA, they were able to detect as few as 4×10^3 genome equivalents of *M. leprae* DNA. Furthermore, they were able to detect homologous sequences in skin biopsy samples from lepromatous leprosy patients, which is of potential clinical importance. Incidentally, the *M. leprae* genome appears to be very stable with respect to this sequence in that all *M. leprae* isolates (from patients, armadillos, and monkeys) examined to date have identical hybridization patterns, with the exception of a single difference found in only one of the armadillo isolates (Clark-Curtiss and Walsh, 1989). This observation suggests that the probe should be capable of detecting all *M. leprae* isolates.

Several research groups are currently exploring the potential use of gene amplification techniques such as the polymerase chain reaction (PCR) to detect and identify *M. leprae*. In this technique, one uses oligonucleotide primers to direct the amplification of a particular nucleotide sequence via a bidirectional polymerase cascade reaction (Guatelli et al., 1989; Mullis and Faloona, 1987). The specificity of the reaction is determined by the choice of primers. In some preliminary studies, we have used oligonucleotide primers corresponding to various regions of the gene encoding the 65-kilodalton(kDa) antigen (B. B. Plikaytis and T. M. Shinnick, unpublished observations). Primers representing sequences unique to *M. leprae* direct the amplification of the gene from *M. leprae* but not from the other *Mycobacterium* species tested. The potential power of this technique can be demonstrated by two results. First, using these primers, we can specifically detect as few as 10 copies of the *M. leprae* 65-kDa antigen gene in a sample containing 10^6 copies of the *M. tuberculosis* 65-kDa antigen gene. Second, using crude lysates of *M. leprae*, grown in mouse footpads, we can detect a positive signal in a sample containing as few as 20 bacilli.

These results indicate that the gene amplification technology can be used to amplify specifically sequences from *M. leprae*. Importantly, without optimizing the system, we were able to detect the equivalent of as few as 20 bacilli in a sample—a sensitivity much better than that of the currently available clinical tests. However, much work needs to be done before we can truly assess the potential clinical impact of this technology for leprosy. For example, among other things the system must be optimized with respect to: 1) target sequence and primers; 2) recovery of bacilli and isolation of DNA; 3) reaction conditions; and 4) detection systems. Nonetheless, the degree of sensitivity and specificity displayed by the primers described above is quite encouraging for the potential use of this technology in the rapid detection of small numbers of mycobacteria in clinical specimens and the positive identification of the organism as *M. leprae*.

Summary

Although much has been learned about the structure and composition of the *M. leprae* bacillus, the challenge for the identification and characterization of *M. leprae* still lies with the inability to propagate the organism outside of animal hosts. Until a suitable axenic culture system is developed, one must rely on animal models, hence making identification and characterization quite laborious. Fortunately, there are methods and procedures on the horizon for the analysis of very small numbers of bacilli (PCR) and for the analysis of the metabolic activity of bacilli (Franzblau and Hastings, 1987). These advances will probably facilitate the identification of organisms as *M. leprae* and the characterization of relevant biochemical traits.

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