The Armadillo Model for Leprosy

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Summary

Nine-banded armadillos are well suited as hosts for in vivo propagation of *M. leprae,* and they are used as laboratory and population models for leprosy. Other than humans, armadillos are the only animals that become infected with *M. leprae* in nature, and many functional, physiological, and structural aspects of leprosy in armadillos closely replicate those seen in human leprosy. Armadillos are immunologically intact and can manifest the full spectrum of histopathological responses to *M. leprae* that are seen in humans. Most importantly, armadillos are the only animal that reliably develops extensive neurological involvement with *M. leprae*, thereby providing an abundant source of rare research materials for studying the neuropathogenesis of leprosy.

Because of their unique capability to establish infection with *M. leprae,* armadillos can be used to aid the development of new assays for the early diagnosis and monitoring of leprosy. A principal limitation in the development of armadillos as models for leprosy has been the paucity of armadillo-specific immunological reagents and assays. However, with the recent completion of the armadillo whole genomic sequence, a variety of armadillo proteins can now be expressed in vitro and used for the production of monoclonal antibodies. A wide array of genetic markers or gene expression profiling systems can also be explored to better understand the molecular basis of the disease in the armadillo.

Armadillos are the only proven non-human reservoir of *M. leprae.* They are found only in the Americas, and there is no known equivalent animal reservoir in other regions of the world. However, free-ranging armadillos in the southern U.S. are known to sustain a high level of natural infection with *M. leprae* and, along with laboratory cohort studies, these populations may be useful for modeling the efficiency of new diagnostic techniques for the early detection of leprosy.

New evidence suggests that the zoonotic transmission of *M. leprae* from armadillos in the southern U.S. contributes to a high percentage of the endemic human leprosy infections that present in the region each year. The degree of involvement that armadillos in other parts of the Americas may have with *M. leprae* has not been well documented, but merits additional investigation. Understanding the role that these animals may play in helping to perpetuate leprosy in this hemisphere could have a significant impact on our abilities to ultimately eliminate leprosy.

Introduction

In addition to humans, nine-banded armadillos (*Dasypus novemcinctus*) are the only other natural host of *M. leprae*. Free ranging armadillos in the southern U.S. are known to harbor high rates of *M. leprae* infection, and zoonotic transmission of *M. leprae* from armadillos to humans has been established (65, 86). Retrospective analysis has shown that armadillos harbored *M. leprae* for decades before they were ever used in leprosy research, and that the infection in wild armadillos originated by natural means (74, 83).

M. leprae infection in the armadillo closely replicates many of the structural, physiological, and functional aspects of leprosy seen in humans and, most importantly, armadillos are the only non-human hosts that develop extensive neurological involvement with *M. leprae*. Because of the heavy burdens of bacilli they harbor (up to 10^{12} *M. leprae* per animal), nine-banded armadillos have become the hosts of choice for propagating large quantities of *M. leprae*, and they are advancing now as important models for the pathogenesis of nerve injury in leprosy.

Although they are not typically used as laboratory animals, the recently completed whole genome sequence for the nine-banded armadillo has enabled researchers to undertake more sophisticated molecular studies and to develop an array of armadillo-specific reagents. The availability of these reagents will aid in piloting new therapies and diagnostic regimens as well as provide new insights into this infectious neurodegenerative disorder.

Husbandry and Physiology of the Armadillo

Armadillos are exotic looking mammals about the size of housecats (Figure 1). As members of the super-order Xenarthra, order Cingulata, they are evolutionarily related to sloths and anteaters. Armadillos lack full dentition and have short limbs with strong claws and a hard but flexible banded carapace protecting their body. The term "armadillo" can be applied to 20 different species in nine different genera (18). However, the armadillo of greatest importance in leprosy research is *Dasypus novemcinctus* (i.e., the long-nosed southern or nine-banded armadillo), although *Dasypus septemcinctus* (seven-banded armadillo) and *Euphractus sexcinctus* (six-banded armadillo) may also be partially susceptible to *M. leprae* (10).

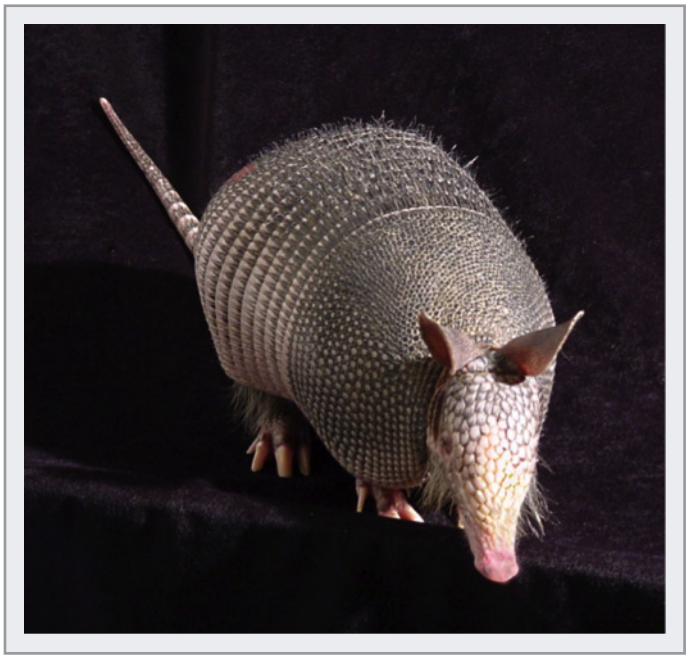

FIG 1 Nine banded armadillo (*Dasypus novemcinctus*).

The armadillo's long life span (12 years) and cool body temperature (32°–35°C, optimal for *M. leprae*) are the main physiological traits that first attracted the attention of leprosy researchers. The reproductive cycle of the armadillo is characterized by diapausic development and polyembryony (70). Females typically mate in the summer, but the embryos do not implant in the uterine wall until late fall, when they divide into identical quadruplicates sharing the same hemochorial placenta. The genetically identical quadruplets show a heritable component in the armadillo's response to *M. leprae* that can be seen among litter-mates (69). Siblings experimentally infected with *M. leprae* show similar susceptibility and manifest similar numbers of bacilli at the time of harvest. High responder siblings of an individual litter routinely yield more than 10^9 *M. leprae*/gram of reticuloendothelial (RES) tissue; low responders exhibit non-productive infections with low bacillary counts (<10^9 *M. leprae*/gram); and 15–20% of the armadillos readily resist the infection. Observations of genetically identical human twins with leprosy also suggest an innate predisposition for the infection and mirror the similarity in responses seen among armadillo siblings (4). Although armadillos are not reliably bred in captivity and must be obtained from the wild for investigative purposes, gravid females taken from the wild will litter in captivity and the genetically identical offspring can be ideal models to study the role of host genetics and

genomic factors on disease susceptibility (50). Contrary to what is seen among humans, the vast majority of armadillos appear to be susceptible to infection with *M. leprae*. However, a reliable percentage (~15–20%) of the animals readily resists challenge (82). There is a clear genetic association for susceptibility to *M. leprae* among humans, and a number of gene sequence polymorphisms have been identified that appear to be associated with susceptibility to leprosy as well as with the type of leprosy that an individual might manifest (1, 4, 13, 49). Studies also show that some *M. leprae*-resistant armadillos have single nucleotide polymorphisms in toll-like receptors (TLR) at locations similar to those that have been reportedly associated with resistance to leprosy in some human studies (3).

Armadillos can be maintained by any laboratory capable of housing rabbits or other medium-sized mammals. Modified rabbit cages with soft plastic flooring inserts are used to house armadillos and the animals adapt to using cat litter pans. Individual units can be ganged together with a tunnel to separate the sleeping and feeding areas from the litter pan side. A small plastic trash can with shredded paper functions as a sham burrow and enriches their environment (Figure 2).

FIG 2 Modified rabbit cages are used to house armadillos.

Infection and Clinical Manifestations of Disease

In humans, leprosy lesions manifest mainly in the cooler body regions, including the skin and mucous membranes of the upper respiratory tract. Soon after Hansen discovered the leprosy bacillus, investigators speculated about the predilection of *M. leprae* for cooler regions of the human body, and began experimenting with animal hosts whose body temperatures were lower than that of humans in order to propagate the organism. In most instances, the bacilli inoculated in the animals were rapidly cleared, but in a few they seemed to sustain for some period (16). It was not until the 1960s that Charles Shepard (66), adapting techniques previously applied with *M. ulcerans* (24), inoculated the footpads of conventional mice and demonstrated quantifiable growth of *M. leprae* outside of humans. Obviously, a cool body temperature alone is not sufficient to facilitate the growth of *M. leprae,* but Shepard's discovery prompted several additional investigations of hypothermic and poikilothermic hosts (25), including the nine-banded armadillo.

The armadillo's cool core temperature, large body size, long life span, and ability to yield quadruplicate offspring recommended them as experimental hosts for *M. leprae.* Intravenous infection of the armadillo could result in a 10,000 fold increase in the number of bacilli, and armadillos were shown to be susceptible to experimental infection by a variety of routes, including intravenous, intradermal, percutaneus, and respiratory instillation (39, 68). As few as 1×10^3 *M. leprae* are sufficient to establish infection in the armadillo (34), but incubation periods are shortest when high doses of bacilli ($0.1–4\times10^9$) are delivered intravenously (56). Following the experimental infection of the armadillo, *M. leprae* become localized in the peripheral nerves and cells of the reticuloendothelial system. Intermittent low-level bacteremia then leads to a generalized dissemination of bacilli without the significant impairment of most vital organ systems. No organ system is spared, but cooler body regions such as ears, nose, tongue, footpads, bronchi, and lungs tend to exhibit greater involvement; adrenal glands, bladder, heart, intestine, kidneys, ovaries, and testes (which are internal in armadillos) are less commonly involved (30, 34). Most animals develop heavy infections, with up to 10^{12} recoverable bacilli in the liver, spleen, and lymph nodes within 18–24 months of experimentally induced infection (29, 34, 78).

Armadillos exhibit few overt signs of clinical disease. A large portion of the armadillo's body is occluded from view by its carapace (73). Abrasions around the eyes, nose, and feet are the most common signs evidencing an evolving insensitivity in the skin, but are also somewhat non-specific. In the laboratory, plantar ulceration is common in the later stages of infection and the involvement of neural tissues is demonstrable at even the earliest stages of disease. Classical foot drop or malformation is not commonly observed in armadillos, and the animals probably best represent the early clinical stages of active leprosy uncomplicated by secondary injury or long-term therapy.

Although *M. leprae* has no toxins and is not life threatening in man, infected armadillos develop a severe hypochromic microcytic anemia following the invasion of their bone marrow. Through

extra-vascular hemolysis of red blood cells (RBC), along with compromised liver and renal functions, they will eventually succumb to the secondary complications of persistent bacteremia if not humanely sacrificed (81). Serum lactate dehydrogenase (LDH) concentrations >2000 IU are highly correlated with finding >1×10^9 bacteria per gram of liver or spleen. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are similarly altered.

Immunological and Histopathological Spectrum

In both humans and armadillos, leprosy exhibits a wide immunological and histopathological spectrum, classifiable over a range from the polar extremes of tuberculoid (TT) to lepromatous (LL), with the indistinct borderline forms in between. The majority of armadillos (70%) exhibit a lepromatous-type of response to *M. leprae,* but the full spectrum of disease can be observed among individuals within the overall population (33, 36, 41, 51). The type of leprosy that each animal might manifest seems to have no relationship to their differing environmental exposures or geographic origins; the response appears to be innate. Lepromatous-type armadillos can manifest the largest numbers of leprosy bacilli in their reticuloendothelial tissues (>= 1×10^9 per gram). Therefore, they are the ones more commonly selected for in vivo propagation of *M. leprae* and have been the most studied.

The development of early diagnostic methods is a major priority in leprosy research. However, efforts to generate new assays are complicated by the low frequency of susceptibility to leprosy in most human populations, the extraordinarily long incubation period of leprosy, and the inability to discern which individuals in a population may be incubating sub-clinical infections. The compressed nature of the *M. leprae* infection in armadillos allows both cross-sectional and prospective cohort studies, as well as a clear understanding about the actual susceptibility of the individuals under study based on known challenge results.

Early serological studies using the *M. leprae*-specific PGL-1 antigen confirmed its high specificity among armadillos and showed that experimentally infected armadillos produce a detectable IgM response beginning only about six months post experimental infection. These antibodies remain detectable over the course of disease and the titer generally correlates with the load of *M. leprae* in the animal's reticuloendothelial tissues (31, 32, 79, 80, 84, 85). The first detection is generally associated with a 1+ BI (Bacteriological Index, approximating 10^4 bacilli/gram) in some RES tissues (31, 32). The levels of IgM antibodies to PGL-1 increase with increasing bacterial load in the animal and persist over the course of the disease (80). More recent studies using the new Leprosy IDRI Diagnostic (LID)-1 antigen have also confirmed excellent specificity in reaction and found mainly an IgG-type response among armadillos, suggesting that a combination of the two antigens might enhance the serological detection of *M. leprae* infections (23). Similarly, IFN-γ re-

lease assays have been piloted in experimentally infected armadillos. The *M. leprae*-specific proteins ML0009, ML1601, ML2478, and ML2531 antigens induced significant IFN-γ levels in PBMCs from *M. leprae*-infected armadillos when compared to naïve controls (55). These findings suggest that, in addition to serological responses, even lepromatous hosts with significant exposure to *M. leprae* may have discernible cell-mediated immune responses that can be exploited for diagnostic purposes. Experimentally infected armadillos are challenged with very high numbers of bacilli for in-vivo propagation of *M. leprae.* These artificially high challenge doses may skew some immunological responses, generating immune profiles that may not be entirely representative of a natural infection. However, in addition to experimentally infected armadillos in the laboratory, free-ranging armadillos in some regions are also known to harbor a natural infection with *M. leprae* and they too may be exploited as population models for diagnostic development.

Antibodies against molecular markers are essential for in vivo and in vitro studies in order to help delineate immunological responses to infection with *M. leprae.* A major hurdle in studying the immune response of armadillos has been the limited availability of armadillo-specific reagents and assays. However, the recent completion of the armadillo whole genomic sequence is aiding the creation of new reagents through the expression of functional proteins in vitro and the development of custom monoclonal antibodies. In addition, a variety of molecular probes can now be constructed to facilitate gene expression profiling in armadillos (2, 64). The ability to better dissect the immunological response of armadillos could benefit our understanding of leprosy pathogenesis and aid the evolution of new diagnostic techniques.

Neurological Involvement and Pathogenesis

Beyond sharing a unique susceptibility to *M. leprae*, the most important feature of leprosy common to both humans and armadillos is that they develop extensive neurological involvement with *M. leprae*. Although the bacterium has been known as the causative agent of leprosy for more than a century, little is known about the pathophysiology of the underlying nerve damage, which likely involves a complicated interplay of both host inflammatory and bacterial-mediated events (60, 90). A major impediment to our understanding is obtaining suitable tissues for study. Leprotic lesions are highly focal and usually distributed asymmetrically over the body (52, 57). Ethical and practical limitations make it almost impossible to biopsy affected human nerves. Furthermore, human nerves derived from amputated limbs reflect only the end-stages of pathogenesis and are generally not suitable for detailed molecular analysis or intervention studies (6). An effective animal model would greatly facilitate progress in this area, but most common laboratory animals (i.e., rat, rabbit, guinea pig, etc.) are naturally resistant to *M. leprae*. Although *M. leprae* does replicate when inoculated into the foot pads of mice, the infection exhibits no nerve involvement. Only the nine-banded armadillo reliably exhibits extensive neurological involvement upon infec-

tion with *M. leprae*. Considering the practical and ethical restrictions regarding the sampling or study of human nerves, armadillos are likely the most abundant source of leprotic neural fibers for studying leprosy neuropathology. No other common laboratory animals such as guinea pigs, mice, rabbits, or rats develop neurological involvement with *M. leprae,* and the pathology and functional deficits seen in *M. leprae*-infected armadillo nerves closely replicate those seen in human leprosy.

M. leprae manifests in armadillos with structural and pathological changes similar to those observed in the skin and nerves of human leprosy patients. There is marked inflammation, with bacilli attached to the progressively demyelinating Schwann cells (SCs), and a functional neural deficit can be demonstrated in leprotic nerves using electrophysiology (6, 60). Armadillo peripheral nerves are infected early in the disease process. Histopathologocial examination reveals characteristic interstitial neuritis, with the infiltration of inflammatory cells such as macrophages, and bacilli in the perineurium, epineurium, and endoneurium (Figure 3). Given the unique susceptibility of armadillos to neurological involvement with *M. leprae,* the same granulomas that form against *M. leprae* in skin or other tissues can also be found in their peripheral nerves. The quantitative estimation of bacilli in infected nerves shows higher bacterial loads and increased involvement distally. Estimates of more than 1×10^6 *M. leprae/*cm of some major nerves are not uncommon, and both sensory and motor neurons are involved (60, 61, 62, 63). Leprosy in humans can only be diagnosed clinically, and all patients already exhibit some degree of nerve damage by the time their disease is first discovered. A notable advantage of the armadillo is the opportunity to examine the pathogenesis of the infection at pre-clinical stages that have never been observed in humans and that are more likely to be effective targets for therapeutic intervention.

Following the experimental infection of armadillos, *M. leprae* populates the peripheral nerves and reticuloendothelial tissues and slowly but systemically disseminates from these early foci. The armadillo posterior tibial nerve runs for approximately 5 cm just beneath the skin surface of the medial hind limb. This nerve has a high frequency of involvement in both armadillo and human infections. It is easily accessible in the armadillo and a useful target for studies of armadillos. Although the duration of an experimental infection in armadillos (4–24 months) is highly compressed when compared to the many years involved in the human disease, bacillary loads of $\geq10^6$ *M. leprae*/cm are common in armadillo post-tibial nerves even during this short time period.

Once *M. leprae* populates the nerve, the number of bacilli can escalate to high numbers and spread to adjacent nerve trunks. Anti-leprosy drug therapies must have good neural penetration in order to kill bacilli sequestered in nerves. However, even when effectively killed by the anti-microbial drugs, the bacilli in humans and armadillos only slowly clear the nerves and skin lesions. In one study, we infected armadillos and allowed them to incubate their infections for 12 months before treating them with 10mg/kg rifampin once monthly for an additional 12 months, then sacrificing them at 24 months post infection. Although the animals showed clinical improvement in skin lesions and ulcers as a result of the anti-microbial therapy, an examination of their posterior tibial nerves showed the continued presence of *M. leprae*. A molecular assessment of the *M. leprae* viability suggested that the organisms had been effectively killed by the rifampin

therapy. However, bacterial counts averaging 10^4–10^5 bacilli per cm of posterior tibial nerve were still observed, even after a full year of treatment. This heavy burden of (dead) bacilli provides a rich substrate for continued immunological interaction with the host and suggests that there is insidious chronic injury to nerves involved with *M. leprae* (64).

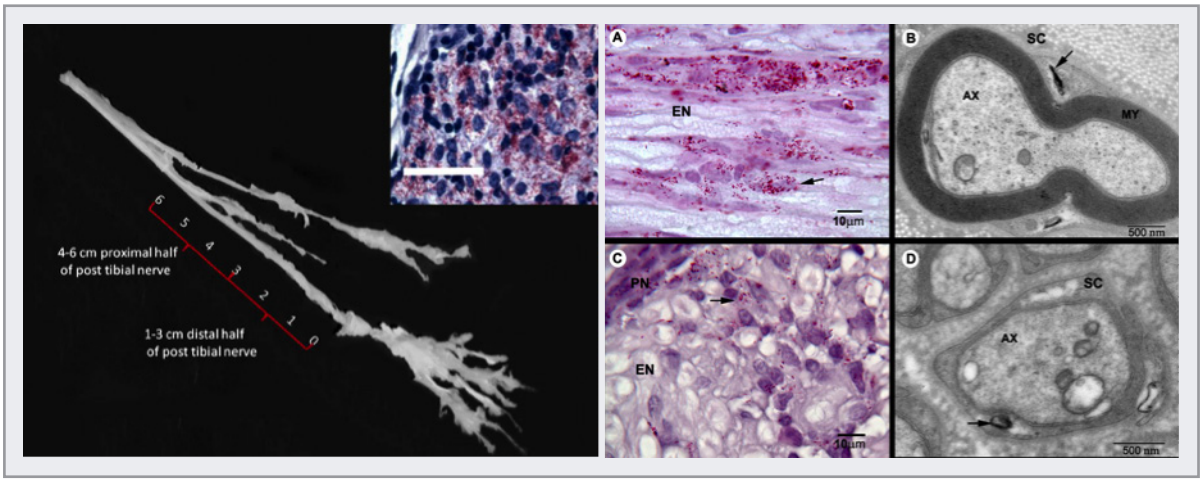

FIG 3 Inflammation, *M. leprae* infiltration, and demyelination in an infected armadillo PT nerve.

Left panel: armadillo post-tibial nerve infiltrated with *M. leprae*. Right panel: (A) Longitudinal section showing a large number of acid-fast bacilli (AFB; arrow) in endoneurium (EN) of nerve. (B) Electron micrograph of a myelinated Schwann cell (SC) infected with *M. leprae* (arrow). AX, axon; MY, myelin sheath. (C) Cross-section of an infected nerve showing infiltration of *M. leprae* (arrow) in EN and perinurium (PN), as well as an infiltrate of mononuclear cells at the site of infection. (D) *M. leprae* (arrow) infecting non-myelinated Schwann cells in an infected armadillo nerve.

Although there are no comparable human studies, armadillo nerve segments can be used effectively for gene expression profiling and analyzing cell signaling pathways. Gene expression profiles have shown ongoing degeneration and regeneration processes among infected animals when compared to naïve controls, along with evidence of persistent inflammation with an enhanced expression of both IFN-γ and TNF-α (Figure 4). The gene expression profiles of nerve segments from rifampin-treated animals more closely resembled those of the untreated animals over the naïve controls (Figure 4). The slow clearance of killed bacilli can be problematic for nerve injury. The molecular markers for neurodegeneration and regeneration, the gene expression profile of inflammatory genes, and the enumeration of the bacterial load of *M. leprae* in the nerve are useful therapeutic endpoints for laboratory studies. These endpoints highlight the importance of developing new therapies to enhance the clearance of bacilli from the host, in conjunction with anti-bacterial treatment to limit the progress of insidious neuropathy.

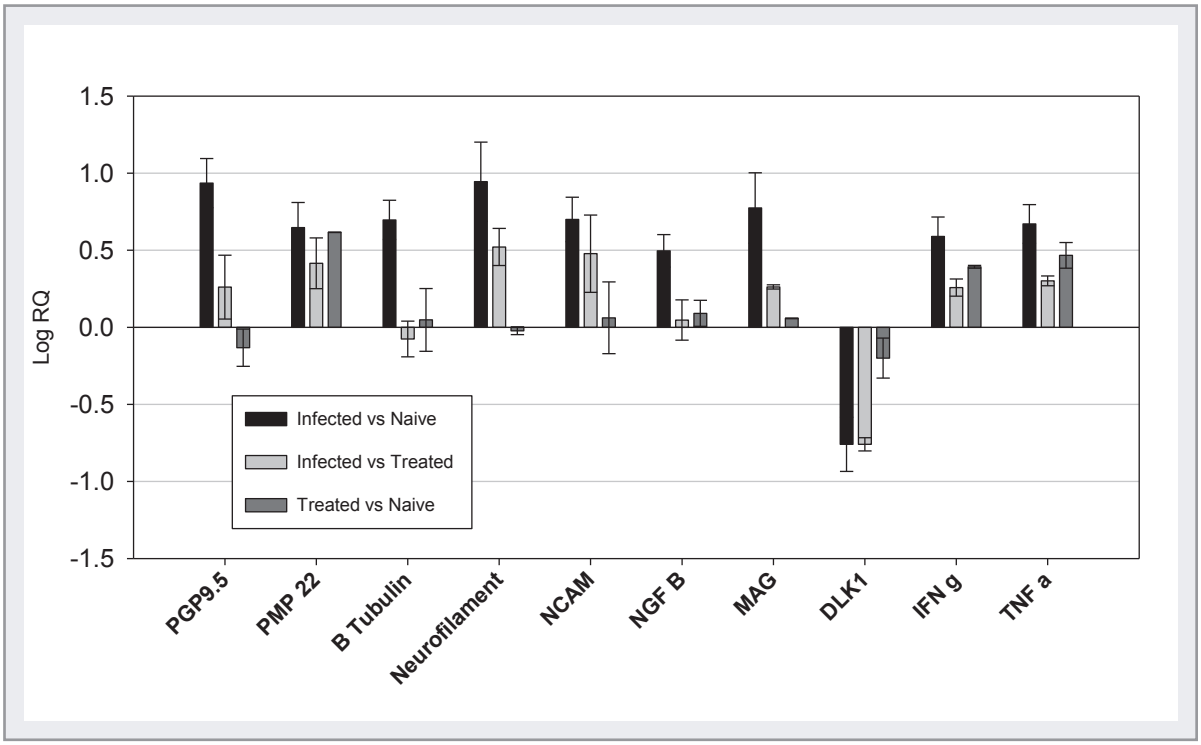

FIG 4 Relative expression of molecular markers for neurodegeneration, regeneration, and inflammation, compared between uninfected-normal nerves (Naïve), infected-untreated (Infected), and treated with rifampin for one year (Treated) armadillo post-tibial nerves. Results represent mean ± SD from duplicate experiments on five animals.

Electrophysiological Studies

Defective nociceptive perception, especially hot and cold stimulation, is recognized as an early indicator of neuropathy in leprosy. Although such techniques are not suitable for armadillos, the morphological and quantitative study of nerves in a skin biopsy can offer an effective alternative tool for assessing the thin nerve fiber structure related to the thermal sensitivity function (described in Chapter 2.5; see also Chapter 9.2).

Although the hard carapace and thick skin of armadillos limit the number of nerves that can be examined, techniques have been adapted to permit the assessment of conduction in both hind limbs along the posterior tibial nerve. This nerve lies just beneath the skin surface between the ankle and knee and innervates the small lumbrical and flexor muscles of each foot. Nerve con-

duction studies are noninvasive and are ideal for repeated or prospective assessments of the onset and progress of peripheral neuropathy over time in the same subject. Therefore, this type of study has been adapted for use with armadillos (28, 64) (Figure 5). Infection with *M. leprae* induces the demyelination of axons that results in decreased nerve conduction velocity (NCV), measured in m/sec. The loss of axons and muscular atrophy leads to a decrease in the Compound Motor Action Potential (CMAP), measured in mV (26). Conduction deficit is observed in the posterior tibial nerves of 75% of all experimentally infected armadillos, with onset occurring as early as 90 days post infection and progressing over time. Naïve (non-infected) armadillos exhibit conduction profiles similar to humans (mean NCV 62.09 ± 10.72 m/sec, mean CMAP 1.55 ± 0.33 mV). Also similar to humans, depressed CMAP amplitude (<0.9mV, mean ± 2 SD) is the most common presentation, but abnormal nerve conduction velocity (NCV <40m/s, mean ±2SD) can also be observed (Figure 5). Most armadillos progress from normal conduction to a total conduction block in the later stages of their experimentally induced infections with *M. leprae*. Nearly all of the animals that develop conduction deficit also eventually exhibit other clinical signs of neuropathy (64). The onset of a conduction abnormality generally coincides with the evolution of a detectable immune response to *M. leprae* (e.g., detectable PGL1 IgM antibodies) and is a significant predictor of other non-specific symptoms of neuropathy, such as foot ulcers and nail avulsion or hypertrophy (64). Of more than 175 different armadillos, nearly all of the animals that developed a conduction deficit also eventually exhibited signs of clinical neuropathy in their foot pads. An increased PGL1 antibody level and decreased CMAP were also highly correlated with the clinical appearance of wounds under heavy calluses, hypertrophic nails ($p<0.03$), and nail avulsion ($p<0.008$, $r=0.2–0.26$).

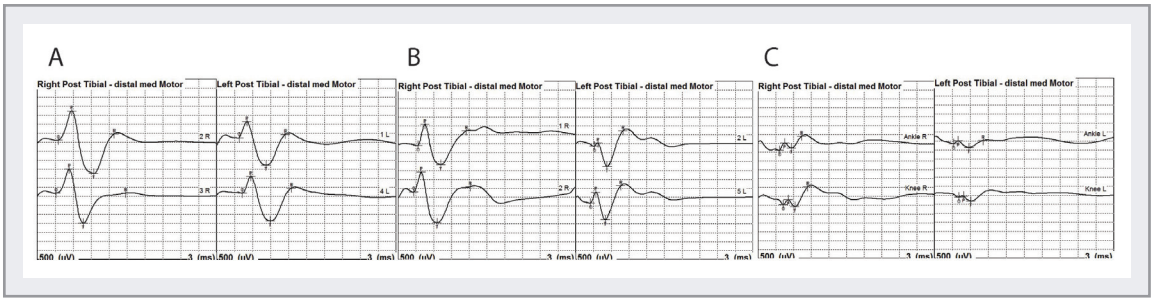

FIG 5 Armadillo post-tibial nerve conduction impairment in early and late leprosy stages. Motor nerve conduction velocity (MNCV) and compound muscle action potential (CMAP) in naïve and infected armadillos were measured by percutaneous stimulation of the posterior tibial nerve, distally at the ankle and proximally at the knee. A: naïve armadillo that shows both right and left normal MNCV, B: infected armadillo showing abnormal CMAP on left nerve, and C: infected armadillo showing both right and left nerves impaired.

Impairment of Muscle Architecture and Function in Infected Armadillos

A common pathological hallmark of human leprosy and *M. leprae*-infected armadillos is the involvement of the extremities. In the foot, the lumbrical muscles are innervated by the medial and lateral plantar nerves, nerves that are similarly involved in some leprosy patients. Muscle paralysis can result from injury to these nerves, and the pathological status of lumbrical muscles from the armadillo hind limb has been examined. The organization of intact muscle architecture can be evaluated by labeling muscle tissues with antibodies to adult myosin, a family of ATP-dependent, actin-binding, and highly conserved muscle motor protein. The labeling of transverse sections of control armadillo muscles has shown a highly organized architecture of muscle fibers with a clear endomysium and perimysium, similar to the skeletal muscle of humans and rodents (75). In contrast, the lumbrical muscles from infected armadillos can display a markedly disorganized pattern of muscle fibers with a disorganized endomysium and perimysium. The analysis of the transverse sections of the lumbrical muscles with antibodies specific for basal lamina components, laminin and collagen, also may show markedly disrupted and abnormal extracellular matrix expression in infected muscles as compared to control animals. Nuclear labeling has revealed an increased accumulation of cells in the muscle, most likely mononuclear inflammatory cells or macrophages, and the distribution of *M. leprae* within the lumbrical muscles in infected animals can be seen using the antibody to *M. leprae*, PGL-1, that specifically detects whole *M. leprae* (47). These data revealed that *M. leprae* are predominantly localized to cells in the interstitial tissues in the perimysium, most likely within the infiltrated cells.

Similarly, Brand (11) showed that the PCSA (cross sectional area/mass) of muscles in the hands of leprosy patients could be used as a surrogate measure of grip strength and index muscle atrophy. Examining the PCSA of the small (intrinsic) lumbrical and flexor muscles of an armadillo also shows a qualitative reduction of muscle mass and atrophy among infected armadillos, with PGL1 IgM positive animals having an average of 20% less muscle mass than naïve normal animals (75).

Detailed histopathological studies have shown that long-term infection in the armadillo also has a discernible effect on the morphological and molecular composition of skeletal muscle fibers. These features in the skeletal muscles of infected armadillos resemble the muscle pathology and function impairment documented in patients with LL leprosy (89) and suggest the potential of using the armadillo model not only for the neuropathies but also for the myopathies associated with human leprosy.

Zoonotic Leprosy

In 1975, Walsh et al. (88) discovered that free-ranging armadillos in Louisiana harbored a natural infection with *M. leprae*, and subsequent investigations confirmed that the disease is widespread among North American armadillos (72, 83, 87). Leprosy was not present in the New World during pre-Colombian times, but the disease was already well established among settlers in the vicinity of New Orleans by 1750, as well as in other countries around the Gulf Coast of Mexico (8). Armadillos must have acquired *M. leprae* from humans sometime in the last 300 years, and if they did so in North America, then it seems possible that armadillos also could have repeated the event at many times and in many locations over the years. The specific origins of *M. leprae* infections among wild armadillos, the geographic range of those infections, and the risks that infection in these animals might present to humans have remained topics of interest.

Armadillos are found only in the Americas, ranging from northern Argentina to the central U.S. They can adapt to many diverse habitats but are most abundant in low-lying and coastal areas. Their population has undergone extensive geographic expansion in recent years (71); however, they do not hibernate and a poor tolerance of cold temperatures is the main factor limiting their ultimate range.

Numerous surveys have now confirmed that armadillos are a large, natural reservoir of *M. leprae*. Though originally thought to be localized to western Gulf of Mexico areas, the infection is now recognized to occur among armadillos throughout the extended northern range. The highest prevalence rates in the U.S. are usually reported among animals inhabiting low-lying, poorly drained areas, especially those in the southern Mississippi River valley and other bottomlands (74). These areas have high carrying capacities for insects, which might be a vector for the transmission of leprosy among armadillos. In contrast, disease prevalence rates tend to be lower in better-drained locales or areas with lower animal densities.

Leprosy appears to be an emerging infection among armadillos in North America. The detectable prevalence of the disease is associated with increasing population densities of the newly arrived animal species. Early reports hypothesizing that geographic trends in point prevalence rates of *M. leprae* infections between different armadillo groups might indicate a particular nidus for the infection were probably confused by the different population structures of the groups examined (5, 67, 87). As leprosy is a slow chronic infection, variations in its point prevalence rates probably reflect the differential crowding rates of armadillos in diverse habitats and other population-based factors that can influence the intensity of disease transmission or detection in different locales (72).

Recent studies confirm that leprosy is a zoonosis in the southern U.S. and that armadillos can be involved in transmitting *M. leprae* to humans. Using a combination of single nucleotide polymorphism analysis and variable number tandem repeat genotyping, a single unique *M. leprae* genotypic-strain (3I-2-v1) has been found to occur among 88% of the wild armadillos sampled in a 400,000 square mile area in the southern U.S. Analysis of the 3I-2-v1 genotype showed that it

is highly unique, having less than a 1:10,000 predicted probability of random occurrence. Most of the animals are infected with a single predominant *M. leprae* strain-type (3I-2-v1), which already has been associated with probable zoonotic transmission of leprosy to humans (86). In addition, presentation with the 3I-2-v1 strain has been significantly associated with patients' histories of residence in areas where *M. leprae*-infected armadillos are found, and several patients have reported using armadillos for food or having other direct contact with the animals (86).

Multiple *M. leprae* genotypic-strains have been identified to be circulating among human patients in U.S., an observation consistent with the hypothesis that leprosy has been introduced from a variety of regions. However, the homogeneity of the *M. leprae* strain-types seen among armadillos in the U.S. suggests that interspecies transfer of *M. leprae* from humans to armadillos has been relatively infrequent. The armadillo-associated 3I-2-v1 *M. leprae* genotype has not been reported in other countries (77); however, in the U.S., the armadillos have markedly amplified the 3I-2-v1 strain and helped spread it over a large geographic area. A second zoonotic strain (3I-2-v15) of *M. leprae* has been identified among armadillos in southern Florida and is shared by several autochthonous patients from the area (65). This new armadillo-associated genotype strain has multiple allele changes at three variable number of tandem repeats (VNTR) loci, differentiating it from the 3I-2-v1 strain, and whole genome sequencing of 3I-2-v15 has identified nine additional single nucleotide polymorphisms (SNPs) unique to this second zoonotic strain. Animals from this area were free from the infection in earlier surveys, and this strain has never been reported elsewhere.

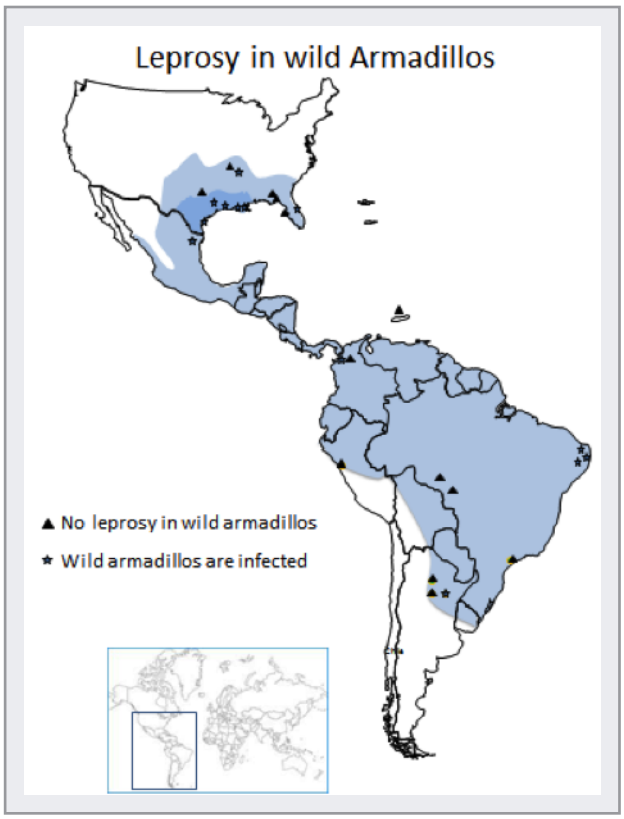

FIG 6 Natural habitat range of armadillos indicating the areas where biomarkers of natural infection are presented. Range of armadillos is presented by shaded area in the map and areas with higher density (>50 animals/km^2) are presented with darker shade.

There have been relatively few studies concerning leprosy in South American armadillos. The techniques used to detect the infection have not been comparable, and the findings with regards to the detection of the infection or risks associated with armadillos have been inconsistent (38, 54, 59, 74). However, biomarkers of *M. leprae* have been reported among armadillos in Argentina (46, 91), Brazil (20, 21, 22, 27), Colombia (12), and Mexico (5) (Figure 6). In addition, epidemiological studies in Brazil have implicated environmental sources for some cases of human infection (37), and

another Brazilian study found contact with armadillos to be a significant risk factor for leprosy (19). Where enzootic infection can be confirmed, education about armadillos as a potential risk factor for leprosy could have a tremendous impact on the health of many individuals.

The ecology of armadillos and the infections they carry may differ in North and South America. Armadillos only began expanding their range from Mexico to the U.S. in the 1880s. They continue to colonize new areas and are expected to eventually roam over more than 50% of the entire geographic area of the U.S. The specific factors that promoted this dramatic expansion of armadillos are not certain, but the expansion probably benefited from the active removal of predators by agricultural interests in the northern range and the high carrying capacity and low competition of the animals found in the northern bottom-lands (71). Armadillos in parts of Texas have been reported to reach densities of up to 50/Km^2, but much lower densities have been reported for South American armadillos (17, 42, 43, 45, 48, 72) (Figure 6). Pioneering armadillo populations show less genetic diversity than their counterparts elsewhere in Central and South America (7, 44); however, genetic diversity is thought to benefit a population's ability to resist disease. Thus, South American armadillos may not exhibit the high prevalence rates of *M. leprae* as seen in the U.S., with many showing advanced stages of the disease, owing to their lower population densities and greater genetic diversity. On the other hand, Brazilian armadillos show similar reactivity to heat-killed *M. leprae* as their North American counterparts (3, 58). In addition, armadillos in Brazil (53, 58) and Venezuela (14, 15) have been shown to be susceptible to experimental infection with *M. leprae*. Furthermore, other species of South American armadillos that are not present in North America also appear to be susceptible to experimental infection with *M. leprae* (9, 14). Large-scale systematic surveys are needed to better understand the role that armadillos may play in the ecology of leprosy throughout the Americas (76).

Many leprosy patients are often confused about how they may have acquired leprosy and unable to relate ever having come in contact with someone who had the disease. They frequently suffer profound anxiety following the diagnosis, so understanding that armadillos are a plausible biological source of their infection may provide relief. In addition, leprosy is a rare disease, the diagnosis of which is often delayed for months or years while other possibilities are exhausted. Physicians treating patients who may have had exposure to armadillos should retain leprosy in their differential diagnosis, especially when dealing with skin lesions that do not easily resolve with normal therapies (86).

The Use of Armadillos as Vaccine Models

Since armadillos so closely replicate leprosy in humans, there has been interest in using them to test specific interventions, especially anti-leprosy vaccines. The earliest vaccination studies in

armadillos used increased lymphoblast transformation to assess effective vaccine sensitization and then monitored animal survival as endpoints. In those studies, heat killed preparations of *M. leprae* alone, or *M. leprae* potentiated with BCG, increased the survival of challenged animals. However, more than three years were required for the studies to culminate (40). Later, Job et al. (35) vaccinated armadillos with BCG and examined the animal's histopathological response to lepromin as an early indicator of effective vaccination. He found that 20–40% of the animals had an intensified lepromin reaction in their skin following vaccination, but his results also showed poor correlation with long-term survival rates.

Since leprosy is an infectious neurological disorder in humans—not a life-threatening disease—a more appropriate endpoint for clinical interventions is the onset and progress of neuropathy rather than the survival rate of animals. *M. leprae* can be found localized in peripheral armadillo nerves within days of experimental inoculation, and motor nerve injury can be demonstrated with electrophysiology within just a few months. In a recent study using the BCG vaccine and a standard high-dose *M. leprae* challenge, we saw that both vaccinated and sham-vaccinated control armadillos showed nerve involvement as early as 6–8 months post-challenge. In addition, about 70% of the armadillos in both groups had detectable PGL-1 IgM antibodies levels by seven months after the challenge. In this study, BCG was seen as partially protective in armadillos with regards to survival, but ineffective at preventing or delaying the nerve injury caused by an *M. leprae* infection. Importantly, nerve injury, which might better reflect the most salient features of leprosy, can be used as an effective early endpoint in vaccine and other intervention studies.

Conclusions

Effective animal models can help provide pivotal new understandings about the mechanisms involved in complex disease processes such as those manifested in leprosy. The armadillo provides large numbers of highly viable *M. leprae* for experimental use from a controlled and known infection status. With armadillos, comparative pathological studies have shown that many of the physiological and structural aspects of human leprosy are closely replicated in a highly compressed disease duration that exhibits equivalent functional defects in the animals. Other than humans, the nine-banded armadillo is the only animal that develops extensive neurological involvement with *M. leprae*, and they are an abundant source of leprotic neurological fibers for basic science investigations. Rare neurological events in both normal and leprotic tissues, from time periods and in material quantities that cannot be obtained from human subjects, can only be studied with these animals. Developing techniques to effectively detect and monitor the onset and progress of leprosy neuropathy could provide significant benefits to leprosy patients. The use of the armadillo to study leprosy can provide new insights for developing effective intervention strategies and ameliorating the human suffering caused by leprosy.

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