TRANSMISSION OF MYCOBACTERIUM ULCERANS
TO THE NINE-BANDED ARMADILLO

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Abstract. Animal models for Mycobacterium ulcerans infections (Buruli ulcer) include guinea pigs, rats, and mice, but each has limitations in replicating the spectrum of human disease. Here, 19 adult nine-banded armadillos were inoculated intradermally with M. ulcerans. Injection sites were examined and skin samples obtained for histologic and microbiology studies. Necropsies were conducted to assess systemic involvement. In group 1 (n = 4), 2 animals developed progressive skin ulcers with undernamed borders at the injection sites within 6–10 weeks. Biopsies showed features similar to human disease including extensive necrosis in the deep dermis and subcutaneous fat, mixed cellular infiltrates, and acid-fast bacilli (AFB). In group 2 (n = 15), 5 animals developed progressive skin ulcers, 3 had evanescent papulo-nodules, 3 died shortly after inoculation of unknown causes, and 4 showed no signs of infection. Lesion samples from 3 animals with progressive ulcers were culture positive for AFB. Our findings indicate that nine-banded armadillos are susceptible to M. ulcerans and may develop cutaneous lesions that closely mimic Buruli ulcer.

In tropical countries, skin ulcers caused by infectious agents are common. Mycobacterium ulcerans, the causative organism of Buruli ulcer first isolated in Australia, is considered a public health threat, especially in Africa. Recent observations suggest that environmental sources of M. ulcerans include swamps, with subsequent transmission by insects. The clinical features of Buruli ulcer are variable. In uncomplicated disease, painless skin ulcers, typically on the extremities, develop without fever or malaise. However, progressive lesions may result in large areas of ulceration, necrosis, and osteomyelitis. Many lesions tend to heal, but this may take years without medical intervention. Advanced disease requires surgical excision and skin grafts or amputation. Crippling contractures and lymphedema may accompany healing. Medical therapies include clofazimine, dapsone, rifampicin, phenytoin, cotrimoxazole, macrolide compounds, and heat. However, cure rates are generally low.

Understanding the pathogenesis of human M. ulcerans infections has been slowed by the lack of an animal model that develops features resembling human disease. Rats, mice, and guinea pigs are used as animal models for M. ulcerans infections, but each is limited in replicating the spectrum of features found in human disease. Rats and mice develop lesions after cutaneous inoculation with M. ulcerans, but lack the extensive ulceration characteristic of human disease. In the mouse footpad, the organisms multiply but necrosis without ulceration destroys the limb and causes death. Guinea pigs develop inflammatory lesions at the inoculation sites that usually resolve without ulcer formation.

The nine-banded armadillo is highly susceptible to M. leprae and has been a useful animal model for leprosy. Here, we report that armadillos are also susceptible to M. ulcerans and may develop cutaneous lesions closely approximating those of the human disease.

MATERIALS AND METHODS

Adult male and female nine-banded armadillos (Dasypus novemcinctus) at least 2 years old, captured in southwestern Louisiana, and adapted to captivity for a period of at least 3 months before infection were used in all experiments. The adaptation period also served as a quarantine to rule out the presence of other infections before inoculation with M. ulcerans. During the study, the animals were housed individually in standard armadillo caging and fed a standard diet of cat chow and water ad libitum. For all procedures, armadillos were anesthetized with ketamine hydrochloride (50 mg/kg).

The M. ulcerans inocula consisted of strains isolated on Lowenstein-Jensen media from Buruli ulcer patients in the Democratic Republic of Congo. Briefly, sterile cotton swabs were rubbed on the necrotic exudates of ulcer margins and then immersed in 5 ml of 5% oxalic acid at room temperature for 15 min. The swabs were removed, the contents were centrifuged, the supernatant was decanted, and 1 ml of saline and 1 drop of phenol red were then added to the sediment. This mixture was neutralized with 4% sodium hydroxide and then inoculated into Lowenstein-Jensen medium. Subsequently, the strains were passaged in Dubos media and then periodically injected into mouse footpads (inbred) to increase replication rates and maintain pathogenicity. Material obtained from mouse footpads was decontaminated in a similar fashion. Mycobacterium ulcerans organisms were suspended in phosphate-buffered saline, counted as previously described, and then administered intradermally on the medial thigh or lower abdomen. Injection sites were marked with a tattoo and observed every 2–4 weeks for up to 6 months, or until death. Periodically, samples were obtained from the ulcers for histologic and microbiologic studies. Specimens for histology were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μM, and then stained with hematoxylin and eosin or by the Fite-Faraco or Ziehl-Neelsen methods for mycobacterial detection. Complete histopathologic examinations were conducted on all animals after death.

A total of 19 armadillos were inoculated with M. ulcerans. In group 1, 4 armadillos were inoculated intradermally in the lower abdominal and medial thigh regions with a suspension containing approximately 3 × 10^8 M. ulcerans organisms (0.3 ml total volume) prepared from infected mouse footpad material. Later, a second group of 15 animals was inoculated in a similar fashion on the medial thighs. Approximate doses ranged from 3 × 10^4 to 3 × 10^6 organisms per inoculation site.
**RESULTS**

Of 19 armadillos inoculated with *M. ulcerans*, 7 developed progressive cutaneous infection, 3 had evanescent papulo-nodular lesions, 3 died within one week of inoculation from unknown causes, and 6 did not develop any clinical sign of infection. Of 16 evaluable animals, 10 (62%) developed cutaneous lesions. There was no discernible correlation between lesion development and the dose of *M. ulcerans* administered.

In group 1, 2 of 4 animals developed erythematous patches studded with papules and nodules at the sites of inoculation 4 weeks after inoculation (Figure 1). At 9 weeks after inoculation, 1 animal had ulcers at 3 of the 4 inoculation sites measuring 2–3 cm in diameter with necrotic, undermined borders and peripheral hyperpigmentation (Figure 2). Biopsy specimens of progressive lesions showed contiguous coagulation necrosis of the deep dermis and panniculus extending beyond the areas containing bacilli (Figure 3). Most specimens revealed variable amounts of edema and damaged blood vessels, but only sparse inflammatory infiltrates. Fat cells appeared enlarged but most retained ghost-like outlines. Mineralization or granuloma formation was not observed. Stains for acid fast bacilli (AFB) stains revealed numerous mycobacteria throughout the ulcer bed and surrounding necrotic fat, many in extracellular clumps (Figure 3). This animal died 16 weeks after inoculation. Necropsy revealed AFB only in necrotic skin lesions. Lowenstein-Jensen media were negative after 3 months. In the second animal, lesion development was slower but the clinical and histologic features were similar. Ulcers with a purulent exudate at 2 of the 4 sites, measuring 0.5–1 cm in diameter, were noted at 9 weeks post-inoculation. Biopsy samples showed subcutaneous fat necrosis and scattered AFB. This animal died 24 weeks after inoculation. Histopathologic examination of the skin lesions revealed AFB, but cultures on Lowenstein-Jensen media were negative after 3 months. There was no systemic spread. The third animal developed an erythematous patch at 1 of the 4 inoculation sites 9 weeks post-inoculation but died soon thereafter from bleeding beneath the carapace. Necropsy indicated no evidence for systemic spread of *M. ulcerans*. The fourth animal, examined for approximately 24 weeks post-inoculation, did not develop any skin lesions.

In group 2, 5 of 15 animals developed ulcerative skin lesions within 8–12 weeks that were clinically and histopathologically similar to the lesions observed in group 1. All 5 animals died within 6 months of inoculation. *Mycobacterium ulcerans* was cultivated from the skin lesions of 3 animals. In 3 other animals, evanescent papules or nodules were noted. At necropsy, there was no evidence of internal organ involvement in any animal, including underlying bone. Typically, the first sign of infection in all animals was an erythematous patch at the inoculation site, usually 4–6 weeks after inoculation. The patches ranged from 0.5 to 1.5 cm in diameter and either resolved or progressed into papules, nodules, or ulcers.

**DISCUSSION**

Of 16 armadillos that survived beyond 4 weeks post-inoculation with *M. ulcerans*, 7 developed cutaneous lesions with clinical features strikingly similar to Buruli ulcer in human lesions including undermined ulcers with shelf-like margins and extensive necrosis that extended to the surrounding subcutaneous fat. Three other animals developed...
evanescent papulo-nodular lesions that did not progress to ulceration. Interestingly, all animals that developed ulcers died between 4 and 6 months after inoculation. The cause of death was unclear but may have resulted from secondary infection, or toxin release from advanced *M. ulcerans* infections. Although systemic spread from cutaneous lesions has not been confirmed, some postulate metastatic spread to explain distant osteomyelitis found in some patients. However, there was no clinical or histopathologic evidence for systemic spread in any of the armadillos.

Histologic findings notably similar to those of human *M. ulcerans* infections included extensive coagulation necrosis of the dermis and subcutaneous tissue, sparse mixed cellular infiltrates (early lesions) and, in many samples, scattered clumps of extracellular AFB. Not unexpectedly, AFB were observed more frequently in animals with advanced lesions. Three skin samples collected during necropsy were culture positive for AFB on Lowenstein-Jensen media. The similarity between armadillo lesions and those observed in humans suggest that the armadillo may be a useful model to investigate the pathogenesis of *M. ulcerans*. It is generally accepted that the acute pathologic findings in Buruli ulcer are attributed to toxins released by *M. ulcerans*, including lipids and polyketides. This suggests that the pathogenesis of Buruli ulcer may be multifactorial, permitting a range of strategies for new treatments.

No single Buruli ulcer animal model completely mimics the range of clinical and histopathologic features of *M. ulcerans* infections in humans. The armadillo may be an ideal model for studying *M. ulcerans*. In addition to a well-established susceptibility to *M. leprae*, the armadillo has a low body temperature of approximately 30±35°C. Similar to *M. leprae*, *M. ulcerans* lesions are most commonly found on cooler parts of the body. In contrast, however, *M. ulcerans* will grow slowly on most types of mycobacterial media when incubated at temperatures of 32–33°C. At 37°C, the organisms eventually die. Here, we describe a spectrum of lesions in the armadillo typical of human disease, including erythematous patches, papules, nodules, and progressive ulcers.

Tropical skin ulcers are caused by many types of infec-
tious agents. In 1948, MacCallum and others implicated *M. ulcerans* as the cause of indolent, necrotizing skin ulcers in Australia. Belgian scientists in the Congo then began isolating the organism from patients with necrotizing skin ulcers, suggesting an endemic problem. In nearby Uganda, necrotizing skin ulcers containing *M. ulcerans* were seen in nearly epidemic proportions. Because the condition was especially common in Buruli County (Uganda), the term Buruli ulcer arose. Although widely present in west and central Africa, especially in Benin, Ghana, and Ivory Coast, other known endemic regions include Malaysia, Mexico, Bolivia, Peru, French Guyana, and Papua New Guinea. Some investigators suspect that *M. ulcerans* may be the cause of indolent skin ulcers in many other countries. Endemic ulcer arose. Although widely present in west and central Africa, especially in Benin, Ghana, and Ivory Coast, other known endemic regions include Malaysia, Mexico, Bolivia, Peru, French Guyana, and Papua New Guinea. Some investigators suspect that *M. ulcerans* may be the cause of indolent skin ulcers in many other countries.

In this regard, the experimental armadillo model may be a useful adjunct to provide further insight into the pathogenesis and assessment of new treatments for Buruli ulcer.

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