SHORT REPORT: CLINICAL AND HISTOLOGIC FEATURES OF SKIN LESIONS IN A CYNOMOLGUS MONKEY EXPERIMENTALLY INFECTED WITH MYCOBACTERIUM ULCERANS (BURULI ULCER) BY INTRADERMAL INOCULATION

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Abstract. Buruli ulcer, caused by Mycobacterium ulcerans, is a destructive infection that most commonly affects the skin. Animal models for Buruli ulcer include guinea pigs, rats, mice, and armadillos, but each is limited in replicating the spectrum of human disease. Here, a cynomolgus monkey was infected with two concentrations of M. ulcerans (1.0 and 2.2 × 10⁶) by intradermal inoculation, 3 months apart. All injection sites developed papules that progressed to ulcers with undermined borders within 2–4 weeks. The rate of progression and size of the ulcers were proportional to the numbers of organisms inoculated. Biopsies from ulcer edges showed ulceration, robust inflammatory cell infiltrates, granulomatous-like responses, mild edema, and extracellular acid-fast bacilli. The ulcers healed spontaneously between Weeks 8 and 12, with no signs of systemic infection. This report, the first to describe a non-human primate experimentally infected with M. ulcerans, suggests that cynomolgus monkeys are modestly susceptible and develop some of the clinical and histologic features of Buruli ulcer.

Buruli ulcer, caused by Mycobacterium ulcerans, is the third most common mycobacterial infection in humans and an increasing public health threat, especially in Africa.¹ The clinical features of Buruli ulcer vary. In uncomplicated disease, painless skin ulcers, typically on the extremities, develop without fever or malaise.² However, progressive lesions may result in large areas of ulceration and necrosis and sometimes osteomyelitis. Many lesions tend to heal, but this may take years without medical intervention and may be accompanied by crippling contractures and lymphedema. Recommended medical therapies include rifampicin and an aminoglycoside, but cure rates are not well established.¹³ Advanced or progressive disease requires surgical excision and skin grafts or, occasionally, amputation of affected limbs.²

Understanding the pathogenesis and developing improved therapies for Buruli ulcer has been slowed by the lack of an experimental animal model that replicates the spectrum of features found in human disease. Laboratory rats and mice develop skin lesions after intradermal inoculation with M. ulcerans, but lack the extensive ulceration found in human disease. Multimammate rats (Mastomys natalensis) rapidly develop systemic infections and die.⁵ In the mouse footpad, M. ulcerans multiply, but necrosis without ulceration destroys the limb and causes death.⁶,⁷ Guinea pigs develop inflammatory lesions at the inoculation sites that usually resolve without ulcer formation.⁷,⁸ The nine-banded armadillo is susceptible to M. ulcerans and develops cutaneous lesions after experimental intradermal infection, approximating those of the human disease, but is phylogenetically distant.⁹ The cynomolgus monkey, routinely used in pre-clinical drug and vaccine studies, is susceptible to M. tuberculosis and, to a limited degree, M. leprae (G.P.W., unpublished data).¹⁰ Susceptibility of the cynomolgus monkey to M. ulcerans is unknown.

The animal use protocol for this work was approved by the Leonard Wood Memorial Animal Care and Use Committee. The study was conducted in compliance with the United States Animal Welfare Act and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals.

One male wild caught, healthy, Philippine cynomolgus monkey (Macaca fascicularis), estimated to be 5–7 years old and weighing 6.5 kg, was used in the experiment. There have been no reports of Buruli ulcer in the Philippines. For all procedures, the monkey was anesthetized with ketamine hydrochloride (20 mg/kg). No paralytics were used. The M. ulcerans inocula were prepared from subcultures of a single colony from a pure strain, originally isolated on Löwenstein-Jensen media from a patient with Buruli ulcer in the Democratic Republic of Congo in 1969. The strain, referred to as PORT, is well characterized and has maintained pathogenicity.¹¹ Mycobacterium ulcerans used to infect the cynomolgus monkey was suspended in sterile phosphate-buffered saline, counted, and administered intradermally to three adjacent sites as 0.1-mL injections on the medial thigh using a 1-mL syringe with a 27 gauge needle. First, we administered 1 × 10⁸ acid fast bacilli (AFB) per site to the right thigh. Three months later, an interval to allow for observation of systemic disease, we administered 2.2 × 10⁸ AFB per site to the left thigh.

The monkey was observed daily for changes in behavior, including appetite, and monitored for signs of pain or discomfort. Weights were recorded at least weekly. Injection sites were observed every other day for up to 12 weeks. Punch biopsies were obtained from inoculation site lesions between 1 and 6 weeks after infection. Specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm/L, and stained with hematoxylin and eosin for routine histology or by Ziehl-Neelsen for the detection of AFB. Periodically, swabs of ulcer material were plated on Löwenstein-Jensen media slants to assess for viable AFB. Stained paraffin sections on some glass slides were removed, deparaffinized, and analyzed by an M. ulcerans–specific IS2404 polymerase chain reaction (PCR).¹²

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After the first series of inoculations (1 × 10^8 M. ulcerans per site), erythematous papules (2–3 mm) began to develop at the three inoculation sites ~4 weeks after infection. By Week 6, the papules had progressed to ulcers measuring 0.5–1 cm, with necrotic undermined borders. Regional inguinal lymphadenopathy ipsilateral to the inoculation was noted. A biopsy from an ulcer edge at Week 6 after infection showed ulceration, a mixed dermal inflammatory cellular infiltrate, an organizing granulomatous response in some areas, and numerous extracellular AFB, some in small clumps (Figure 1A and B). Mild edema was noted, but coagulation necrosis and vessel damage were not observed. M. ulcerans–specific IS2404 PCR analyses on paraffin-embedded tissue sections were inconclusive. Ulcer material obtained by swab at 6 weeks after infection plated on Löwenstein-Jensen media grew scattered colonies that contained gram-positive cocci, suggestive of staphylococci, but no AFB. Between 9 and 12 weeks after infection, the ulcers had healed without sequelae. There was no evidence of systemic disease, including osteomyelitis. The monkey’s weight remained stable, and there were no signs of pain or discomfort.

During the second series of inoculations (2.2 × 10^8 M. ulcerans per site), erythematous papules developed into ulcers over a period of 1–2 weeks. Ulcers at the three adjacent inoculation sites coalesced into a single lesion (1.6 × 2.8 cm), with necrotic, undermined borders (Figure 2). Inguinal lymphadenopathy ipsilateral to the inoculation site was noted. Biopsies from the ulcer edge obtained at 1 and 3 weeks after infection showed ulceration, mixed dermal inflammatory cellular infiltrates, an organizing granulomatous response, focal areas of necrosis, and mild edema. Numerous AFB, most extracellular, were scattered or in small clumps. M. ulcerans–specific IS2404 PCR analyses on paraffin-embedded tissue sections were inconclusive. Ulcer material obtained by swab at 1 week after infection plated on Löwenstein-Jensen media grew scattered colonies that contained AFB and gram-positive cocci, suggestive of staphylococci. By Week 12, the lesion healed without sequelae. There was no evidence of systemic disease, including osteomyelitis. The monkey’s weight remained stable, and there were no signs of pain or discomfort.

One cynomolgus monkey experimentally infected with M. ulcerans by intradermal injection on the thigh developed lesions at the inoculation site that progressed from erythematous papules to ulcers with necrotic, undermined borders extending to the subcutaneous fat. These features approximated Buruli ulcer in humans. The rate of ulcer development and lesion sizes paralleled the number of organisms inoculated. Biopsies from ulcer edges showed heavy mixed inflammatory...
cellular infiltrates, granulomatous-like responses, mild edema, focal areas of necrosis, and extracellular AFB, scattered or in small clumps. However, the lesions lacked some of the classic histologic features of human Buruli ulcer such as coagulation necrosis and vessel damage. These clinical and histologic observations, the first ever in a non-human primate experimentally infected with *M. ulcerans*, suggest that the cynomolgus monkey is modestly susceptible to *M. ulcerans* and may develop some of the features of Buruli ulcer.

Buruli ulcer in humans is sometimes associated with osteomyelitis of the bone underlying the skin ulcers or at a distant site. Visceral disease has not been reported. In the cynomolgus monkey, despite administering an amount of *M. ulcerans* that is probably far above what is inoculated when humans are naturally infected, there were no signs of systemic disease, including osteomyelitis. The 3-month period between *M. ulcerans* inoculations was to allow evaluation for systemic disease. Regional lymphadenopathy ipsilateral to the ulcers, not a common feature of Buruli ulcer, was attributed to colonization of the ulcer by bacterial skin flora, most likely staphylococci.

Infection of the cynomolgus monkey with *M. ulcerans* resulted in lesion development within 4 weeks after the first inoculation and within 1–2 weeks after the second inoculation. It is unclear if the rapid onset of lesions after the second inoculation was related to the slightly higher number of organisms or whether the second *M. ulcerans* exposure triggered an anamnestic immune response, affecting lesion development. In Buruli ulcer–endemic areas of Africa, low-grade exposure to *M. ulcerans* before disease onset may alter or even enhance the ulcerative process. In humans, mature Buruli ulcer lesions contain remarkably small amounts of cellular exudates, whereas experimental lesions in mice have persistent large cellular infiltrates, suggesting wide species-specific variability in immune responsiveness.

The extensive ulceration in the cynomolgus monkey at the *M. ulcerans* inoculation sites, seemingly disproportionate to the amount of AFB present on biopsies, was likely caused by the release of mycolactone, a well-characterized *M. ulcerans*–specific exotoxin that mediates tissue necrosis, at least partially through apoptosis. *M. ulcerans* strains found in Africa, including the PORT strain used here, are thought to be the most clinically virulent, likely related to their mycolactone structure. 

Swab material from one of two ulcers placed on Löwenstein-Jensen media was culture positive for AFB, and biopsy samples stained by Ziehl-Neelsen contained numerous AFB, consistent with *M. ulcerans* infection. However, PCR analyses of paraffin-embedded ulcer sections for *M. ulcerans* were inconclusive. We attributed this to tissue fixation in formalin, not optimal for PCR, as well as an assay not optimized for paraffin-embedded samples.

The cynomolgus monkey, a non-human primate used in pre-clinical vaccine and drug studies, is susceptible to *M. tuberculosis* and is an established animal model for human tuberculosis. For the first time, the susceptibility of the cynomolgus monkey to *M. ulcerans* has been assessed. Our findings suggest that additional testing using *M. ulcerans* strains from different geographic locations and a wider range of inocula sizes may be warranted to better define the cynomolgus model.

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