Clinical and Histopathological Presentation of Buruli Ulcer in Experimentally Infected Grasscutters (Thryonomys swinderianus)

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Citation

Abstract
Buruli ulcer (BU) is a skin disease caused by Mycobacterium ulcerans, a toxin-producing mycobacterium. BU manifests as papule, plaque, nodule, oedema and undermined ulcer with accompanying complications such as contracture deformities and osteomyelitis. Its mode of transmission and pathogenesis are unclear and effective treatment is not available. Therefore animal modelling, among other strategies is being undertaken to elucidate the problems. This study investigated the grasscutter (Thryonomys swinderianus), a hystricomorph rodent as a BU animal model. Grasscutters were inoculated subcutaneously with Mycobacterium ulcerans and developed progressive skin lesions: erythema, papule, nodule, oedema, undermined ulcer and contracture. The lesions were accompanied by histopathological changes, specifically: coagulative necrosis, mixed inflammatory reactions, myolisis, perineuritis, neurogenic muscular atrophy and osteomyelitis; some of which were accompanied by extracellular and intravascular acid fast bacilli (AFBs). Our findings show that grasscutters are susceptible to Mycobacterium ulcerans; they develop clinical, microbiological and histopathological lesions that mimic BU in humans and therefore are potential BU animal models.

STUDY LOCATION
Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research

SOURCE OF FUNDING
Noguchi Memorial Institute for Medical Research

INTRODUCTION
Buruli ulcer (BU) is a poorly understood disease caused by Mycobacterium ulcerans, a toxin-producing mycobacterium (Krieg et al, 1974; Read et al, 1974) with predilection for the skin and its deeper tissues. It is the third most common mycobacteriosis of immunocompetent hosts after tuberculosis and leprosy (Meyers et al, 1996). BU is a disease of public health importance because the exact mode of its transmission is unclear, and its treatment with antimicrobials has not been very successful, thus resulting in its prevalence being higher than that of tuberculosis and leprosy in some communities (Amofah et al, 2002). BU manifests as papule, plaque, nodule, oedema and undermined ulcer with accompanying complications such as contracture deformities, osteomyelitis, amputation of limbs and involvement of the eye, breast and genitalia (Barneston, 1993; WHO, 2001a; WHO, 2001b). progresses slowly, is usually painless and patients are systemically well (Walsh et al, 1999; Johnson et al, 2005); these factors contribute to the late presentation of patients at hospitals with resultant massive ulceration of the skin, accompanying complications, fever and pain due to the secondary infection of the lesions (Thangaraj et al, 1999; Johnson et al, 2005). Furthermore, the absence of effective treatment worsens the prognosis because the complications lead to impairment of body functions with long-term socio-economic impact (WHO, 2001a; 2001b). Though some BU patients heal spontaneously (Walsh et al, 1999; Thangaraj et al, 1999; WHO, 2001; Johnson et al, 2005) and recent data also suggests that rifampicin, streptomycin and amikacin are effective in combination with surgery (WHO, 2004; Etuaful et al, 2005), 16-47% of the spontaneously and antibiotic healed patients relapse (WHO, 2001a; WHO, 2001b; Teelken et al, 2003), underscoring the difficulty associated with the control of BU.
In the face of the enigma that BU presents, the World Health Organization (WHO) has recommended that animal studies should be undertaken worldwide to elucidate the pathogenesis and mode of transmission of the disease and to provide valuable new approaches for its diagnosis, treatment, management and general control (WHO, 2001c). Though mice, rats, guinea pigs and the nine-banded armadillo are BU animal models (Walsh et al, 1999), each of them has limitations in replicating the whole spectrum of features presented in humans, thus leaving a lot of gaps in knowledge on the pathogenesis, transmission, diagnosis, treatment and general control of BU (Pattyn & Royacker, 1965; Reed et al, 1974; Krieg et al, 1974). Despite the limitations of the available animal models, the mouse, (which is the most frequently used model) has provided a lot of useful information on both the pathogenesis (Pattyn & Royacker, 1965; Reed et al, 1974; Addo et al, 2005; Goto et al, 2005) and treatment of BU (Bentoucha et al, 2001; Dega et al, 2000 & 2002). This study is a follow-up to a BU mouse study undertaken at the Noguchi Memorial Institute for Medical Research (NMIMR). In the said study, though the mouse presented a plethora of BU lesions (Addo et al, 2005), confirming its usefulness as a BU animal model, it did not manifest undermined ulcer which is the hallmark of BU. The mouse was also too small to facilitate proper clinical evaluation of the sores that developed, which also unfortunately progressed rapidly to necrosis, resulting in the destruction of the limb. These findings gave support to the assertion that better BU animal models need to be found (Walsh et al, 1999; Addo et al, 2005). This paper reports on an attempt to identify another animal that would present in addition to other BU lesions, undermined ulcer, the hallmark of BU.

The grasscutter (Thryonomys swinderianus) is a hystricomorph rodent. It was chosen for the study because (i) it is widely distributed in Sub-Saharan Africa, where BU is most prevalent; (ii) it has been established as a laboratory animal in Ghana (Addo, 1997; Asibey & Addo, 2000) and therefore could be maintained in other animal facilities and laboratories outside Africa, and above all (iii) it has a large body size (adult weighs between 1.5-5kg), which would facilitate the proper assessment of BU lesions. This paper reports that the grasscutter is experimentally susceptible to Mycobacterium ulcerans and develops BU lesions, including undermined ulcers, the hallmark of BU disease. The clinical and histopathological presentation of BU in the grasscutter closely mimicked those in humans, suggesting that the grasscutter could be a useful BU animal model.

MATERIALS AND METHODS

BACTERIAL STRAIN

The Mycobacterium ulcerans strain that was used for the study was obtained from biopsy of a Ghanaian BU patient. The M. ulcerans isolate was authenticated by standard biochemical tests (WHO, 2001b) and polymerise chain reaction (PCR) (WHO, 2001b).

EXPERIMENTAL ANIMALS

Twenty-six, 4-month old grasscutters produced in the conventional animal facility of the Department of Animal Experimentation were used for the study. They had been born and bred in an animal facility in which all conventional animal colonies are microbiologically monitored quarterly, specific pathogen free colonies are monitored monthly, and the animal facilities (conventional, barrier) are environmentally monitored monthly. A month before the inception of the study the microbiological status of the selected animals was ascertained by use of non-invasive monitoring methods namely; swabbing of eyes, nostrils and ears; sampling of faeces, blood and urine for bacteria, mycoplasma, fungi and ecto and endoparasites. Thereafter they were transferred to the animal experimentation facility (level 2 containment) to acclimatize. The animals were separated into two experimental groups and one control group. Each of the experimental groups consisted of 5 males and 5 females while the control group consisted of 3 males and 3 females. The animals were maintained and manipulated in accordance with the Institutional animal care and use guidelines.

RESEARCH CLEARANCE AND BIOSAFETY CONSIDERATIONS

Institutional research clearance was obtained before the study was undertaken. The study was carried out in a level 2 biosafety laboratory. All microbiological manipulations were conducted in a class II/IB3 biosafety cabinet and the technical team observed all institutional biosafety guidelines for protection of personnel and laboratory.

METHODS

ISOLATION OF FROM TISSUE

The tissue was decontaminated and inoculated onto Lowenstein-Jensen (L-J) medium within 24 hours of its collection from the hospital. Briefly, the tissue was divided into two portions; one portion was homogenized for primary
culture of M. ulcerans, while the other portion was kept in the department’s tissue bank. Portions of the homogenized tissue were spread out into smears, stained with Ziehl-Neeelsen (ZN) stain and examined for the presence of acid fast bacilli (AFBs). The rest of the tissue homogenate was decontaminated by the Petroff (Petroff, 1915), modified Petroff (WHO, 2001b) and oxalic acid (Kent & Kubica, 1985) methods, as well as that described by Douglas Walsh et al (1999). These different methods were employed to enhance recovery of viable M. ulcerans, since the microbe is susceptible to decontamination methods (Palomino & Portaels, 1998). A sample of the decontaminated homogenate was spread out into smears, ZN stained and examined for the presence of AFBs. The rest of the decontaminated homogenate was inoculated onto L-J slants and incubated at 32 C for 8-12 weeks, during which period they were examined for growth and contamination.

PREPARATION OF INOCULUM.

The primary isolate was subcultured on L-J slants at 32 C for eight weeks, during which period they were examined for growth and contamination. The subcultured M. ulcerans was suspended in phosphate-buffered saline (PBS) and standardized by the McFarland nephelometric standard. Briefly, a drop of sterile PBS was added to a test tube containing 15-20 sterilized glass beads to wet them. A loopful of M. ulcerans subculture was added to the beads and drops of the sterile PBS were added intermittently and vortexed to break up the M. ulcerans colonies and to adjust the turbidity of the suspension to that of a 1.0 and 5.0 McFarland nephelometric standard. Since M. ulcerans in culture is very waxy and not easily dispersible in water, all manipulations were done on ice. Smears of the resultant M. ulcerans suspensions were ZN and Gram stained for detection of AFBs and assessment of microbiological purity respectively.

EXPERIMENTAL INFECTION OF GRASSCUTTERS

The two experimental groups were inoculated subcutaneously with M. ulcerans suspensions equivalent to 1.0 (Group 1) and 5.0 (Group 2) McFarland standards respectively. Each animal was inoculated with 200 l of the inoculum into a circled area on its shaved right thigh. The control group (Group 3) was similarly inoculated in the right thigh with 200 l sterile PBS (the diluent used in the preparation of the inoculum). The remainder of the M. ulcerans suspensions which were used for the inoculation of the two experimental groups of grasscutters was inoculated onto L-J slants and incubated at 32 C for 8 weeks to check for viability of the used inoculum.

MANAGEMENT OF GRASSCUTTERS AFTER EXPERIMENTAL INFECTION

The experimental and control groups of grasscutters were managed in the same manner. In brief, the three groups of animals were kept in the same containment facility (under negative pressure) but in three separate negative pressure air racks. The animals were provided with HEPA-filtered air, maintained at 24-25°C ambient temperature, 55-65% relative humidity and a 12-12 hour light-dark cycle (by means of an automatic lighting system). Each grasscutter was kept in a metal cage (H: 50cm, W: 40cm, L: 40cm) provided with a floor mesh, feeder and drinking bottle. All three groups of grasscutters were fed daily with autoclaved commercial rodent feed pellets (Ghana Agro Food Complex), sanitized Guinea grass (Panicum maximum) and autoclaved water, ad libitum. They were also provided thrice a week with sugar cane (Saccharum spp.), a grasscutter delicacy. The animal room, clean air racks and cage tops were cleaned and disinfected daily with 1% Antec Longlife 250S (a synergistic blend of organic acids, organic biocides and surfactants; effective against bacteria, fungi, mycoplasma and viruses. It is produced by Antec International Ltd, England). The feeders, drinking bottles, floor meshes and sawdust bedding were changed twice weekly and replaced with autoclaved ones, while the cages were replaced monthly.

EVALUATION OF GRASSCUTTERS AFTER INOCULATION

CLINICAL AND MICROBIOLOGICAL EVALUATION

The experimental and control groups of grasscutters were observed for post-inoculation changes (erythema, papule, plaque, oedema and ulcer) at the points of inoculation in particular and shaved thighs in general. The animals were initially observed daily for one month and thereafter thrice weekly (Mondays, Wednesdays and Fridays). The faeces, urine and the blood of the animals in Groups 1 and 2 were examined monthly for AFBs after ZN staining. Whenever any discharge (exudate, pus) or scab was available they were ZN-stained for the detection of AFBs. The discharge was also cultured on different types of media (blood agar, MacConkey agar, Mannitol salt agar, Pseudomonas agar, Bacillus cereus agar and Sabouraud agar) for detection of fast-growing microbes that may be implicated in the disease...
process.

GROSS/HISTOPATHOLOGICAL AND MICROBIOLOGICAL EVALUATION

Each grasscutter was euthanized with diethyl ether on developing an undermined ulcer. The carcass was exsanguinated by cardiac puncture and examined for gross and histopathological changes, presence of AFBs, M. ulcerans and fast-growing microbes as follows: (i) the inoculated limbs were examined for evidence of the causative agent by ZN staining for AFBs and by culture on L-J medium for M. ulcerans; (ii) the blood and lymph nodes (popliteal, inguinal, mesenteric, axillary, mandibular, cervical and suprascapular) were examined for AFBs by ZN staining to determine the mode of dissemination; (iii) the brain, heart, lung, liver, spleen, pancreas, kidney, urinary bladder, testis, scrotum, uteri, fallopian tubes, ovaries, caecum, contralateral limbs (non-inoculated limbs) and sciatic nerves were examined for AFBs by ZN staining for evidence of systemic spread of the infection and (iv) the inoculated limbs, non-inoculated limbs and all other tissues were cultured on various types of media for identification of other microorganisms that may be implicated in the pathology of the disease. Briefly, each tissue was divided into two portions; one portion was homogenised for microbiological evaluation, while the other portion was fixed as such in 10% PBS for histopathological evaluation. The homogenised portions were spread out into smears and ZN-stained for AFBs and also cultured on different types of media (blood agar, MacConkey agar, Mannitol salt agar, Pseudomonas agar, Bacillus cereus agar and Sabouraud agar) for fast-growing microbes. A portion of each inoculated limb was decontaminated by the Petroff [18], modified Petroff, (WHO, 2001b), oxalic acid (Kent & Kubica, 1985) and ‘Douglas Walsh’ (Douglas Walsh et al, 1999) methods. The decontaminated limb homogenates were inoculated on L-J slants and incubated at 32 C for eight weeks for the isolation of M. ulcerans. The tissues that were fixed in 10% phosphate buffered formalin were processed using standard histochemical methods; the processed tissues were stained with haematoxylin eosin (H&E) and ZN for histopathological evaluation and detection of AFBs respectively. The portions of the limbs that were fixed in 10% phosphate buffered formalin, decalcified and processed similarly for histological and microbiological comparison.

STATISTICAL ANALYSES

Statistical analysis was conducted with the Statistical Package for the Social Sciences (SPSS), Standard version, Release 12.0.1. (SPSS Inc. 1989-2003). The case summaries procedure was used to determine the mean, standard deviation, minimum and maximum values of the times of onset of lesions for each inoculum dose (experimental group). After conducting the above-mentioned determinations the data were screened for normality and homogeneity of variance. Most of the data violated the assumptions of the normal distribution and therefore were analysed with nonparametric tests. The Mann-Whitney test was used to determine if the time of lesion onset varied with inoculum dose (McFarland 1 and 5). Spearman’s rho was used to (i) determine if there was any association between the inoculum dose and time of lesion onset and (ii) to determine the direction, strength and significance of the associations. A P value less than 0.05 was considered statistically significant.

RESULTS

CLINICAL PRESENTATION

All the animals in groups 1 (McFarland 1) and 2 (McFarland 5) developed similar clinical lesions regardless of the inoculum dose. However, the higher the inoculum dose, the earlier the onset of the lesions and the more severe their presentation. Each of the grasscutters in groups 1 and 2 presented a pinpointed or broad erythematous lesion at the site of inoculation 24-48 hours after inoculation however, no lesion was observed in any of the animals in group 3 after inoculation (hereafter ‘after inoculation’ is abbreviated to ai). In both experimental groups the erythematous lesion was replaced by a scale (4-7 days ai), then one or two papules (9-19 days ai), which were sited 2.2-3.7.cm from the point of inoculation. Thereafter the infection progressed a little differently with each group. In group 1 the papule disappeared and was replaced by a blister-like lesion (Figure 1) (33-47 days ai), represented by a small skin elevation without any palpable content. The ‘blister’ widened, hardened and became a nodule (Figure 2) (83-91 days ai), which disappeared and was replaced by a scratch (133-151 days ai). Exudate from the scratch hardened into a crust, followed by the formation of a small scab (135-154 days ai). In group 2, the papule developed into a nodule (29-37 days ai), devoid of the ‘blister’ stage. A superficial triangular sore
(Figure 2) developed (35-43 days ai) on the central zone of the nodule, healed spontaneously within 5-7 days and resulted in the development of a scab (Figure 3) (40-49 days ai). The scabs from both experimental groups progressed similarly; the scabs adhered tightly to the skin, hardened and became brittle with time, which upon examination were found to contain AFBs. With the development of the scab the infection stalled, during this phase the scabs either persisted or disappeared intermittently such that both groups of animals often remained without any visible sign of infection for long periods. On the whole, this indeterminate or inactive infection phase was of a longer duration in the animals in group 1 (269-371 days) than those in group 2 (265-283 days); the differences were however not statistically significant (P>0.05). Surprisingly, with 2 of the animals in group 1 the infection did not progress beyond the scab stage (inactive infection) during the course of the study. The rest of the animals in both experimental groups relapsed after the long inactive infection phase and experienced a fulminant progression of the infection. Briefly, the inactive infection ended with sudden congestion of the thigh and portions of the pelvis as follows: (i) with reference to the animals in Group 1, the congestion initially localized around the scabs or the lower half of the thigh (in the absence of a scab) and subsequently engulfed the entire inoculated thigh. The congested area swelled slightly and was accompanied by gradual elevation of the scabs or the softening of certain congested areas (in the absence of a scab). The scabs and the softened areas eroded from underneath with the onset of purulent inflammation and eventually culminated in the formation of an undermined ulcer (441-540 days ai) with abundant fatty tissue on its floor. In the case of one of the animals in group 1, the entire pelvis, scrotum and contralateral limb became congested and oedematous (pitting oedema); the animal died a day after the onset of the oedema; (ii) regarding the animals in Group 2, the entire inoculated limb (i.e. thigh to the footpad) became slightly or intensely congested and oedematous ((Figure 4) and in some cases extended to the thigh of the contralateral limb. With 3 of the animals (2 females and 1 male) the congestion and oedema were extensive such that the entire pelvis and contralateral limb were severely affected within 48 hours, necessitating their immediate euthanasia (although they had not reached the research endpoint). Regarding the rest of the animals in group 2, the oedematous limbs (inoculated and non-inoculated) lacerated spontaneously with the subsequent development of a large scab. The scab eroded from underneath (Figure 5) with the onset of purulent inflammation, which culminated in the formation of an undermined ulcer (Figure 6) (368-379 days ai).

Unexpectedly, 2 of the grasscutters with undermined ulcers (one presented the undermined ulcer on the inoculated limb while the other presented it on the contralateral limb) showed signs of spontaneous healing before the scheduled date of euthanasia, and therefore were left for further observation. Strangely the ulcers on both animals healed completely within 28-36 days without any evidence that the animals had previously presented undermined ulcers or any other BU lesion. None of the animals in the control group developed any visible or palpable lesion throughout the period of observation.

**Figure 1**
Figure 1: Clinical presentation of the blister-like lesion (arrowed)
Clinical and Histopathological Presentation of Buruli Ulcer in Experimentally Infected Grasscutters (Thryonomys swinderianus)

Figure 2
Figure 2: Clinical presentation of a congested nodule with a triangular superficial sore on its central surface.

Figure 3
Figure 3: Clinical presentation of a partly broken scab on a superficial sore undergoing healing.

Figure 4
Figure 4: Clinical presentation of an oedema. The infection has descended from the right thigh to the right foot/footpad and expressed itself as an oedema and ulcer. The inception of the undermined ulcer (arrowed) is seen on the lateral aspect of the footpad towards the heel.

Figure 5
Figure 5: An undermined Buruli ulcer formed from a pus-undermined scab. Original site of inoculation is slightly discoloured (arrowed).
The differences in the onset of the lesions between the two experimental groups were statistically significant with respect to the development of scale (P<0.01), papule (P<0.01), nodule (P<0.01), superficial sore/scratch (P<0.01), scab (P<0.01), relapse (P<0.05) and undermined ulcer (P<0.05). There was negative correlation between the inoculum dose and time of lesion development: erythema (-.229, P>0.05), scale (-.872, P<0.01), papule (-.873, P<0.01), nodule (-.872, P<0.01), superficial sore/scratch (-.869, P<0.01), scab (-.869, P<0.01), inactive infection (-.252, P>0.05), relapse (-.521, P<0.05) and undermined ulcer (-.521, P<0.05). The descriptive statistics are presented in table 1.

**Table 1: Onset and duration of Buruli ulcer lesions in relation to inoculum dose**

<table>
<thead>
<tr>
<th>Inoculum dose</th>
<th>Description/Statistics</th>
<th>Lesions</th>
<th>Erythema</th>
<th>papule</th>
<th>Superficial sore/scratch</th>
<th>Scab</th>
<th>Relapse</th>
<th>Inactivated infection</th>
<th>Unundermined ulcer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninfected 1</td>
<td>Minimum</td>
<td>0.02</td>
<td>5.00</td>
<td>11.89</td>
<td>53.39</td>
<td>0.02</td>
<td>12.00</td>
<td>116.00</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>6.00</td>
<td>19.00</td>
<td>31.30</td>
<td>91.10</td>
<td>0.02</td>
<td>12.00</td>
<td>116.00</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>1.93</td>
<td>9.30</td>
<td>13.19</td>
<td>34.49</td>
<td>0.02</td>
<td>12.00</td>
<td>116.00</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>1.32</td>
<td>0.95</td>
<td>2.39</td>
<td>0.46</td>
<td>1.05</td>
<td>6.00</td>
<td>6.44</td>
<td>12.17</td>
</tr>
</tbody>
</table>

* Lesions not manifested

**GROSS/HISTOPATHOLOGICAL PRESENTATION**

**SKIN**

Grossly, the skin and subcutaneous tissue on the rump, thighs and lower limbs of all the grasscutters from the two experimental groups looked slightly or intensely congested. However that of the 4 grasscutters (from groups 1 and 2) with pronounced oedema looked cooked (coagulative necrosis) and the epidermis was tightly bound to the subcutaneous tissue, which was also calcified. In view of the pronounced oedema, fluid oozed from the subcutaneous tissues during dissection. Surprisingly, though the fluid from the inoculated/diseased limbs was AFB positive in all four grasscutters, the fluid from their non-inoculated/diseased limbs was AFB negative. The exudate from both limbs in all four grasscutters also contained Staphylococcus aureus and Pseudomonas aeruginosa and though Streptococcus spp., Staphylococcus spp., Klebsiella spp, Bacillus spp and a few yeast cells were also isolated they were not consistently present in both limbs.

Histologically, the epidermis in both the inoculated/diseased and non-inoculated/diseased sites of all the experimental animals was generally intact but the dermis and subcutaneous tissue were oedematous, inflamed and necrotized (Figure 7a), and occasionally contained inflamed and occluded blood vessels. The skin at the original sites of inoculation were without AFBs, however, the skin where the infection eventually manifested in the inoculated limb was laden with clusters of extracellular AFBs (Figure 7b); while the skin of the non-inoculated/diseased limb was AFB negative. A few AFBs were detected in blood vessels of detached epidermises (Figure 8a) and necrotized areas of the dermis (Figure 8b). The scabs covering the ulcers were in

**MICROBIOLOGICAL FINDINGS**

The microbiological findings were similar in the two experimental groups. The blood, urine and faeces were consistently AFB negative. The exudate and pus from the inoculated/diseased limbs were consistently AFB positive while similar samples from their non-inoculated/diseased limbs were consistently AFB negative. Staphylococcus aureus and Pseudomonas aeruginosa were consistently isolated from the exudate, pus and undermined ulcers of both the inoculated/diseased and non-inoculated/diseased limbs, while Streptococcus spp., Staphylococcus spp., Klebsiella spp., and Bacillus spp were only occasionally isolated from them.
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Various stages of repair represented by vascular granulation tissue, fibrous granulation tissue and fibrous scar tissue. The young granulation tissue was laden with AFBs (Figure 9a) while the fibrous scar tissue was sparingly embedded with AFBs (Figure 9b).

Figure 8
Figure 7: a) Integument with extracellular AFBs: Intact epidermis with necrotized and oedematous dermis and subcutaneous tissue with accompanying cellular infiltration [H&E x330]; b) Clusters of extracellular AFBs in necrotic skin tissue [ZN x1320]

Figure 9
Figure 8: a) Integument with intravascular AFBs: AFBs in blood vessels of a partly detached epidermis [ZN x660]; b) AFBs (arrowed) in blood vessel of necrotized dermis [ZN x1320]

Figure 10
Figure 9: a) Integument undergoing healing: Site of skin lesion undergoing spontaneous healing; represented by various stages of scarring in the skin with clusters of extracellular AFBs [ZN x330]; b) Virtually healed skin lesion with AFBs embedded in scar [ZN x660].

One other animal in Group 1 presented a large granulomatous lymph node (weighed 1.57g while normal lymph nodes weighed 0.04g – 0.06g) in the subcutaneous
tissue on the spinal column (Figure 10a). Histologically, the granulomatous lymph node consisted of peripheral scar tissue, blood vessels, granulomas (Figure 10b), mixture of lymphocytes, neutrophils, epithelioid and giant cells, and several extracellular AFBs. The rest of the animals also presented a few chronically inflamed and necrotized subcutaneous lymph nodes (0.25g-0.28g) that contained AFBs.

**GRANULOMA**

*Figure 11*

Figure 10: a). Granuloma: Gross appearance of the subcutaneous granuloma (arrowed); b) Microphotograph of the granulomatous subcutaneous lymph node [H&E x132]

**MUSCLES**

At necropsy it was detected that all the animals had contracture (Figure 11a) in one or both hind limbs. The affected limbs were occasionally smaller in volume by 2.1-3.5cm and shorter in length by 1.6-2.0cm than the unaffected limbs. The sciatic nerves in the inoculated limbs were thicker than the sciatic nerves in the non-inoculated limb by 0.35-0.37 cm. The popliteal lymph nodes in the inoculated/diseased limbs were also larger than the popliteal lymph nodes in the non-inoculated/healthy limbs by 0.51-0.52g. Though the popliteal lymph nodes were inflamed and necrotized they contained no AFBs. Histologically the muscle fibres in parts of the contracted limbs were curvilinear (Figure 11b) but other findings such as myolysis, myositis and neurogenic muscular atrophy were common to all the other limbs.

*Figure 12*

Figure 11: a) Gross appearance of contracture of inoculated right limb discovered at post mortem. b) Microphotograph of contracted limb showing curvilinear muscle fibres (top; blue arrow), neurogenic atrophied muscle fibres (down, right corner, green arrow) and necrotized and inflamed fascia (down, pink arrow) [H&E x132]

Histologically, the muscles of both the inoculated/diseased and non-inoculated/diseased limbs were similarly affected and the most prominent and diffuse lesions were neurogenic muscular atrophy, coagulative necrosis and myositis. Neurogenic muscular atrophy (Figure 12a) was represented by groups of angular atrophied muscle fibres, sometimes accompanied by perineuritis and perivasculitis in the underlying connective tissue (Figure 12b). The muscle fibres were extensively necrotized (coagulative necrosis) in some areas resulting in the dissolution of the fibres, thereby giving them a lacy or moth-eaten appearance (Figure 13a); the vacant areas were taken up by scarring tissue and occasionally by fibrofatty tissues. Some areas in the muscles were diffusely oedematous; other areas, including the interstitium were chronically inflamed and contained several epithelioid cells and a few giant cells, including Langhans giant cells. The underlying connective tissue was invaded with AFBs (Figure 13b) but no AFBs were detected in the muscle or nerve fibres.
Figure 13
Figure 12: a) Neurogenic atrophy: Extensive neurogenic atrophy represented by groups of angular atrophied muscle fibres (on the right) adjacent to somewhat normal-looking fibres (on the left) [H&E x 660]. b) Diffuse perineuritis and perivasculitis in the fascia [H&E x660]

Figure 14
Figure 13: a) Myolysis and Myositis: Extensive myolysis and myositis represented by necrotized muscle fibres with lace-like appearance. Necrotic areas are replaced by scar tissue and accompanied by diffuse chronic inflammation. [H&E x 132]. b) AFBs in a connective tissue adjacent to a muscle fibre [ZN x X1300]

BONE
The bones in both the inoculated/diseased and non-inoculated/diseased limbs were diffusely and chronically inflamed and necrotized. Osteomyelitis (Figure 14a) was advanced and was represented by chronically inflamed and necrotized bone marrow accompanied by thinned, broken, eroded and in some cases deformed trabeculae. Though the affected bones were filled with pus cells and several giant cells (Figure 14b) they were unexpectedly completely devoid of AFBs.
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Figure 15
Figure 14: a) Osteomyelitis: Severe osteomyelitis represented by extensive necrosis of bone marrow with diffuse cellular infiltration and extensive rarefication and deformation of the trabeculae (H&E x 660); b. Necrotized and inflamed bone marrow with giant cells [H&E X1320]

OTHER TISSUES
A few (2-5) AFBs were detected in smears of the small intestines and caeca of all the animals in the two experimental groups; however none were detected in the histological slides. All other tissues (brain, heart, lung, liver, spleen, pancreas, kidney, urinary bladder, testis, scrotum, uterus, fallopian tubes, ovaries, sciatic nerves), which were examined by smear, culture and histology were without AFBs and fast-growing organisms.

DISCUSSION
The animals in the two experimental groups presented similar clinical lesions, but with obvious differences in the incubation periods and severity of lesions; the higher the inoculum dose, the earlier the onset of the lesions, and the more severe their presentation. These findings confirm observations of a mouse study (Addo et al, 2005) but contradict those of Walsh et al (1999). The findings of our study suggest that the infective dose, among other factors may account for the differences observed in the clinical presentation of BU in humans.

Clinically, BU in humans manifests as papule, plaque, nodule, osteomyelitis with diffuse cellular infiltration and extensive rarefication and deformation of the trabeculae (H&E x 660); b. Necrotized and inflamed bone marrow with giant cells [H&E X1320].

One obvious clinical difference between the grasscutter model and BU patients was with the presentation of the 'blister'-like lesion. A 'blister' has never been reported as a BU lesion therefore we have difficulty explaining its development in the grasscutter. That notwithstanding, its presentation by only animals in group 1 (group of animals that received the lower inoculum dose) and its unequivocal absence in animals in group 2 (group of animals that received the higher inoculum dose) suggests that probably most BU patients are infected with higher doses and as such do not develop 'blisters' in the course of their infection.

BU oedema is usually non-pitting (WHO, 2001a & 2001b), whilst that in the grasscutter was usually the pitting type, suggesting that the grasscutter model may only offer limited information on the pathogenesis of oedematous BU lesions.

Oedema in the grasscutter was not only confined to the inoculated limb but extended to the contralateral limb, rump, tail and scrotum (hydrocel). Notably, these non-inoculated areas were conspicuously devoid of AFBs, suggesting that the oedema might be attributable to the systemic effect of the toxin; confirming similar findings and assertion in a mouse BU study (Addo et al, 2005) and also lending support to the suggestion that oedematous BU lesions involving large body areas are attributable to a systemic effect of the toxin (WHO, 2001b).
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It is also reported that sometimes the oedematous form of the disease progresses rapidly and is associated with a worse prognosis (Thangaraj et al., 1999; WHO, 2001b). This was confirmed in the grasscutter study, given that the grasscutters progressed from an inactive infection to a fulminant form of the disease with the onset of diffuse oedema which in some of the cases resulted in the death of one grasscutter and necessitated the euthanasia of three others.

Clinically, all the grasscutters healed spontaneously in the course of their infection, confirming reports that some BU patients at some stage of the infection heal spontaneously by an unknown mechanism (Thangaraj et al., 1999; Walsh et al., 1999; WHO, 2001b; Johnson, 2005). Healing in BU patients is represented histologically by granulomatous infiltration, granulation tissue, fibrosis and a depressed scar with very few or no AFBs (WHO, 2001b). Similar histological findings were associated with the scabs that covered the undermined ulcers; however, unlike the case with BU patients, AFBs were consistently present in the scab. The presence of AFBs in the friable scabs during the period of inactive infection was unexpected and worrying because the finding suggests that ‘healed’ lesions of BU patients should be handled with circumspection since they may be infectious. Notwithstanding that the AFB-laden scabs were not cultured to ascertain the viability of the organisms, it would be safer to err on the side of caution, than to be infected for being over optimistic, especially since the mode of transmission is unknown.

Recurrence of infection occurs in about 16-47% of healed BU patients (WHO, 2001a, 2001b; Teelken et al., 2003). In our study, 90% of the spontaneously healed grasscutters relapsed and it was always a prelude to a more severe infection, which always culminated in the development of the undermined ulcer. This finding suggests that the microbes were actually disseminating during the ‘healed’ phase, therefore BU patients have to be maintained on systemic antibiotic treatment during the ‘healed’ phase to prevent a recurrence. In view of the non-availability of an effective antibiotic, the currently recommended WHO drugs could be tried in the interim; the duration of the treatment would also have to be determined. Among the animals that relapsed, 2 healed spontaneously again after having developed undermined ulcers. The reason for this occurrence may not be immediately evident; however, in view of the previous healing incidents and relapses the possibility exists that the animals could relapse again. As observed in this and the mouse study (Addo et al., 2005), the grasscutters and mice that relapsed with concomitant metastasis had earlier presented clean healed scars. This situation goes to underscore the urgency of identifying antimicrobials with bactericidal effect.

For the most part, all the BU lesions in the grasscutters developed away from the site of inoculation, specifically towards the posterior (rump, tail, scrotum) and lower extremities (distal portion of thigh, entire hindlimb to the level of the footpad and entire contralateral limb) confirming the organism’s preference for cooler areas (Thangaraj et al., 1999) and also providing evidence that by virtue of its toxin it has the ability to disseminate widely. The findings of our study also suggest that dissemination probably occurred by lymphatic and haematogenous spread because AFBs were detected in both the lymph nodes and blood vessels. These findings in the grasscutters further suggest that the site(s) at which BU lesions manifest in humans may not necessarily be the site of entry for the bacterium but may rather be the preferred site(s) of the bacterium and its toxin. This assertion is plausible because there are several diseases that manifest at sites far removed from their points of entry (Cotran et al., 1999). Chicken pox and measles viruses for example enter through the airways but manifests themselves first as skin rashes (Cotran et al., 1999). If this possibility is borne in mind in our quest to unravel the mode of M. ulcerans transmission we may be more successful. In view of the microbe’s preference for cooler body areas, the heat therapy option being advocated for the treatment of BU (Glynn, 1972) and the traditional treatment of sores with hot water in Ghana should be upheld. However, the former has drawbacks (Thangaraj et al., 1999) and should be improved to make it user-friendlier, while the traditional use of heat therapy should not be discouraged. It could also be gathered from this study that probably some of the lesions referred to as new, on BU patients (either at the same or different sites) may not necessarily be new infections but rather the exteriorization of an on-going dissemination from a previous infection.

It is reported that BU lesions rarely extend into the underlying muscle (WHO 2001B) because it is speculated that muscles slow down the disease process, though not immune from attack by M. ulcerans (Mork & Mensah-Quainoo, 2005). However, in the grasscutters the lesions consistently extended into the muscles of the inoculated limb.
as well as that of the contralateral limb, and though no AFBs were found in the muscle, a lot were found in the connective tissue and intermuscular septae of the inoculated limb. This confirms the finding that BU lesions spread more along the deep fascia covering the muscle and along the intermuscular septae resulting in the destruction of the deep fascia and muscle (Mork & Mensah-Quainoo, 2005). The findings of this study further demonstrate that the muscle fibres are not immune to the toxin(s) though they may be to the microbe.

Though coagulative necrosis and inflammation are not unusual BU findings; the consistent detection of neurogenic atrophy and perineuritis are enormously important. Clinically the pathology was manifested as a decrease in muscle volume and contracture; symptoms that are also present in BU patients. The manifestation of neurogenic atrophy suggests that BU affects the peripheral nerves, which in turn lead to the destruction of the muscle fibres (microscopic atrophy, clinical contracture) and could be the reason for the absence of pain at some stages of the disease. This finding also goes to confirm that as BU progresses all elements of the skin are affected, including the nerves (Thangaraj et al, 1999). In view of the nerve involvement BU should be studied in the light of what is known about leprosy. Rather frighteningly, these neurogenic lesions extended to the contraleteral limb in the absence of M. ulcerans suggesting that the toxin(s) are responsible for this diffuse damage. Above all, the lesions in the muscle were very pronounced to the extent that muscle fibres disappeared and were replaced by scar tissue and fibrofatty material. In view of the far reaching negative effects of the toxins, research efforts must be intensified to produce antitoxin to effectively manage the toxin-related features of the disease.

The lesions in the bones were similar in all respects to those of BU patients. However, a surprising and important difference was the unequivocal absence of AFBs in the grasscutter lesions. AFBs are present in human BU lesions (WHO, 2001b) and were also observed in a mouse BU study (Addo et al, 2005). It has been assumed that BU bone lesions are the result of haematogenous spread of M. ulcerans and by direct extension from an overlying BU lesion (WHO, 2001b). Therefore with the absence of M. ulcerans the lesion was due to M. ulcerans toxin(s).

In BU patients, 50% of the osteomyelitic lesions are co-infected by pyogenic organisms such as streptococci, staphylococci and corynebacterium sp. (WHO, 2001b). The osteomyelitic lesions in the grasscutters were similarly infected by a number of fast-growing organisms; a finding that sheds further light on why BU is difficult to treat. The involvement of these microbes further complicates the treatment of BU because it implies that anti-BU drugs should have broadspectrum activity to be really effective or other antibiotics have to be administered alongside the anti-BU drugs in order to effectively treat the disease.

The lesions in the lymph nodes were similar to those found in BU patients. Similarly, AFBs were only detected in lymph nodes in the vicinity of the skin lesions but never in the regional nodes. Granulomatous changes are also not usually seen in BU patients (WHO, 2001b); similarly, only one granulomatous lymph node was detected in the study.

The presentation of hydrocel by the grasscutters confirms similar findings in BU mice (Addo et al, 2005) and reports of genital involvement (WHO, 2001a). The hydrocel was devoid of AFBs suggesting that it was due to the systemic effect of M. ulcerans toxin(s) and therefore goes to underscore the urgency of developing antitoxin to effectively manage the disease.

In conclusion, the clinical and histopathological presentations of Mycobacterium ulcerans infection in the experimentally infected grasscutters were in most respects similar to those of BU patients, suggesting that grasscutters could be good BU animal models. In view of the long incubation period associated with infection in the grasscutter, the grasscutter model may best serve as a template for investigating the pathology of the disease, while the mouse would be more appropriate for drug susceptibility studies, in view of its ability to manifest the disease in a much shorter time.

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