Nerve damage induced by *Mycobacterium ulcerans* - Role of mycolactone -

Masamichi Goto¹, Junichiro En¹, Kazue Nakanaga², Norihisa Ishii², Suguru Yonezawa¹, Hajime Saito³, Pamela Small⁴

¹Department of Human Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan (masagoto@m2.kufm.kagoshima-u.ac.jp)

²Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

³Hiroshima Environment and Health Association, Hiroshima, Japan

⁴Department of Microbiology, University of Tennessee, U.S.A.

Almost nothing was known why large and deep Buruli ulcers remain *painless*.

Last year, we first reported histopathology of nerve invasion and nerve damage in mouse footpad inoculated with *M. ulcerans*.

This year, we tried to answer two questions raised last year.

1. Are the mouse lesions really painless?
2. What is the role of mycolactone in the development of nerve damage?
Summary of our previous works

BALB/c mice inoculated with *M. ulcerans*

Mouse foot pad on day 33

Mouse foot pad on day 55

Non-ulcerative edema

Ulcercative edema

Ulcer and edema on day 55

Sparse inflammatory cells and HEV on day 55
BALB/c mouse 55 days after inoculation of *M. ulcerans* (Fite)
Massive nerve damage by *M. ulcerans* (Epon 1um)
Recently published study of human Buruli ulcer

Simona Rondini, Catherine Horsfield, Ernestina Mensah-Quainoo, Thomas Junghanss, Sebastian Lucas and Gerd Pluschke
(Molecular Immunology, Swiss Tropical Institute, Basel, Switzerland)

Contiguous spread of *Mycobacterium ulcerans* in Buruli ulcer lesions analysed by histopathology and real-time PCR quantification of mycobacterial DNA, *J Pathol* 2006; 208: 119–128

Patient II. Contiguous dissemination and development of a satellite lesion

"Granulomas were present within the central zones, as well as neuritis (Figure 6h), venulitis and calcification. No AFBs were seen within the inflamed nerves."
Are the mouse lesions really painless?
Pain tests (Nociceptive Reflex)

**Mechanical stimulus**

- von Frey test (nylon filaments)

**Thermal stimulus**

- Hot plate

**Chemical stimulus**

- Formalin test
MATERIALS AND METHODS (von Frey test)

ANIMALS
Sex-matched control mice without inoculation (11w, n=11)
Mice inoculated with *M. ulcerans* (day 52, n=10)
Low-dose *M. ulcerans* inoculated mice (day 94, n=5)
von Frey sensory test of mouse footpad

Touch-Test Sensory Evaluator
Instruments, North Coast Medical, Inc, CA
There was decreased sensitivity ($P<0.001$) against the stimuli in day 52 mice, when their footpads were moderately swollen and eroded. In the day 94 mice having marked swelling and ulceration, threshold of nociceptive reflex was not significantly changed from the control.
DISCUSSION (sensory test)

From the sensory test of footpads, two findings were obtained.

• First, there was decreased sensitivity ($p<0.001$) against the stimuli in day 52 mice, when their footpads were moderately swollen and eroded. This suggests that pain sensation is decreased even though the lesion is inflamed, which may reflect the painlessness of the human ulcer.

• Second, the day 94 mice having more advanced lesions where marked swelling and ulceration are prominent, threshold of nociceptive reflex was not significantly changed, suggesting that these animals recovered sensation or become painful by other factors such as secondary infection.
What is the role of mycolactone in the development of nerve damage?
MATERIALS AND METHODS (mycolactone)

• Mycolactone A/B was isolated from M. ulcerans 1615. It was dissolved by small amount of ethanol, diluted by 7H9 broth, and inoculated to left footpad of female BALB/c mice aged 6 weeks old.

Animals: 1, 3, 10 ug (n=4 each)

30, 100, 200 ug (n=5 each)

• On day 4 and 7, footpad sensory disturbance was examined by von Frey microfilaments.

• On day 7, perfusion fixation was done by 4% paraformaldehyde (PFA) or 4% PFA + 1% glutaraldehyde. Footpad, spleen, thymus, lung, liver, small intestine and kidney were examined by H&E staining. Epon sections of footpads were also examined.
Figure 1. Swelling of mycolactone-injected foot (100ug injection to left footpad, day 7).  A. Swelling and redness of left footpad (arrow).  B. Swelling of back of left foot (arrow).
Figure 2a. **Footpad thickness** of mycolactone-injected footpads on day 4. Dose-dependent footpad swelling is noted.
Figure 2b. **Footpad thickness** of mycolactone-injected footpads on day 7. Dose-dependent footpad swelling is noted.
Figure 3a. von Frey sensory test of mycolactone-injected footpads on day 4. No significant change of sensory response was observed.
Figure 3b. **von Frey sensory test** of mycolactone-injected footpads on day 7. Loss of sensory response (shift to right side) was not observed, instead, there was increased sensory response (Mann-Whitney U-test; control vs. 30ug, $P=0.02$; control vs. 100ug, $P=0.07$; control vs. 200ug, $P=0.16$; control vs. all (30-200), $P=0.02$).
Figure 4A&B. Histopathological changes in the footpad of mycolactone-injected mice (200ug, day 7). **A.** Low magnification shows stromal edema (*) and epidermal erosion (arrow). **B.** Close up view shows mild neutrophilic infiltration and high endothelial venules (HEV) (Hematoxylin-Eosin staining)
Figure 4C. Footpad of mycolactone-injected mice (100ug, day 7). Stromal edema and mild neutrophilic infiltration (Hematoxylin-Eosin staining)
Figure 5. Nerve damage in the footpads of mycolactone-injected mice. Mycolactone was injected to BALB/c mice and examined on day 7, when feet became swollen and erosive. A. Control shows preserved nerves (arrow), but mycolactone-injected mice (B-D) show loss of Schwann cell nuclei (arrowheads), which indicate degeneration of nerves (B, 30ug; C and D, 200ug). (Hematoxylin-Eosin staining)
Vacuolar change of Schwann cells by *M. ulcerans* (1um and EM)
Figure 6. **No vacuolar change of Schwann cells** in the footpads of mycolactone-injected mice. Mycolactone was injected to BALB/c mice and examined on day 7, when foots became swollen and erosive. **A.** Control. **B.** 30ug. **C.** 100ug. **D.** 200ug. (Epon 1um, Toluidine-blue)
Schwann cell apoptosis in *M. ulcerans*-injected mice by TUNEL method. Arrows show apoptosis of Schwann cell nuclei. (A and B, H&E; C and D, TUNEL staining)
Figure 7. Apoptosis in the nerves in the footpads of mycolactone-injected mice. Mycolactone was injected to BALB/c mice and examined on day 7, when foots became swollen and erosive.  

**ADDITIONAL RESULTS**

Spleens of 100 and 200 ug inoculation showed mild swelling, but thymus, lung, liver, small intestine and kidneys showed no significant changes.

---

**Table 1. Comparison of M.ulcerans and mycolactone injection**

<table>
<thead>
<tr>
<th></th>
<th><em>M. ulcerans</em></th>
<th>Mycolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal erosion</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stromal edema</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>sparse</td>
<td>sparse</td>
</tr>
<tr>
<td>HEV</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nerve lesion</td>
<td>Invasion, vacuolar change</td>
<td>Hemorrhage, loss of nuclei</td>
</tr>
<tr>
<td>Nerve apoptosis</td>
<td>+</td>
<td>(under study)</td>
</tr>
</tbody>
</table>
**Non-ulcerative form**

a. Papule
   - Painless, sometimes itchy, non tender, palpable intradermal lesion (seen in Australia but rare in Africa)

b. Nodule
   - Painless palpable, firm lesion 1-2cm in diameter, situated in the subcutaneous tissue and usually attached to the skin (nodules are uncommon in Australia).

c. Plaque
   - Painless, well demarcated, elevated, dry, indurated lesion more than 2cm.

d. Edematous
   - Diffuse, extensive, non-pitting swelling, ill-defined margin, firm, usually painful with or without colour change over the affected skin.

**Ulcerative form**

- Painless lesion, characterised by necrotic center, undermined edges and edematous skin. In the absence of superinfections, ulcers are painless or minimally painful.

(from WHO website)
Our study clarified that lesions similar to Buruli ulcer can be induced by mycolactone, associated with nerve degeneration.

However, von Frey sensory test did not show paralysis of the lesion, instead, hyperesthesia was observed. This result was not consistent with our previous study of *M. ulcerans* inoculation to mice, where loss of sensation was evident by the same sensory test (Goto et al. Am J Pathol 168:805-811, 2006).

Further study to evaluate the short- and long-term neurotoxic effects of mycolactone is required.