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Protective effect of a dewaxed whole-cell vaccine against *Mycobacterium ulcerans* infection in mice

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ABSTRACT

Mycobacterium ulcerans causes Buruli ulcer, a chronic and destructive necrotizing ulcer in humans. Effective vaccination should be one of the best methods for the prevention of this ulcer. However, no effective vaccines have been developed against *M. ulcerans* infection. In an effort to develop such a vaccine, we examined protective immunity against *M. ulcerans* infection in a mouse footpad-infection model. Prior infection of mice with a virulent strain of *M. ulcerans* or a mycolactone-deficient strain of *M. ulcerans* resulted in limited protection against subsequent challenge by a virulent strain of *M. ulcerans*. Protection was not induced in mice immunized with a formalin-treated killed whole-cell preparation of *M. ulcerans*. By contrast, a dewaxed whole-cell vaccine, prepared by dewaxing *M. ulcerans* with organic solvents that removed mycolactones and waxy cell walls from the cells, induced significant protection in mice. Our observations should facilitate development of effective vaccines against Buruli ulcer for control of this disease.

1. Introduction

Buruli ulcer, caused by *Mycobacterium ulcerans*, is a chronic and destructive necrotizing skin ulcer, and it is the third most common mycobacterial disease in humans after tuberculosis and leprosy [1–5]. The disease is found mainly in West Africa, but its incidence has increased in Asia, the Western Pacific, and Latin America [1,5]. Since initial symptoms of the disease are generally painless, many patients do not seek treatment until the disease has progressed to serious ulceration [5]. The infected ulcerative lesion is treated by surgical removal, sometimes with concomitant skin transplantation [1,3,4,6]. An extensive lesion can even require amputation of the affected limb. During the past decade, evidence of the efficacy of antibiotic treatment against Buruli ulcer has been established [1,7]. However, the standard protocol, with a combination of rifampin and streptomycin, requires oral and intramuscular administration for 8 weeks [1,7]. Unfortunately, for both financial and practical reasons, such treatment is often not available to patients in poor rural areas. Prevention appears to be preferable to control of Buruli ulcer in regions where it is endemic.

Buruli ulcer is usually associated with stagnant water in rural settings but the mode of transmission is not fully understood [1,4,8]. Moreover, avoiding contact with putative environmental sources of *M. ulcerans*, such as contaminated water, soil, or insects, is difficult. Effective vaccination against *M. ulcerans* infection should be straightforward strategy for the control of Buruli ulcer. However, little progress has been made in this field [5,6,8]. BCG vaccines have been studied for their protective activity against *M. ulcerans*. However, clinical studies suggest that BCG vaccines confer only limited and short-term protection against *M. ulcerans* [5,6,8–13]. Several vaccines are under investigation [5,6,8], for example, a preparation of mycolactone-deficient live *M. ulcerans* [14], mycolactone polyketide synthase-based vaccines [15], heat shock protein-based vaccines [16], and Ag85A-based vaccines [17,18]. However, no vaccine specific for *M. ulcerans* is available. In the present study, we prepared a dewaxed whole-cell vaccine from *M. ulcerans* and examined its protective effect, in a mouse model, against infection by *M. ulcerans*. Our results should contribute to the development of effective vaccines against infection of humans by *M. ulcerans*.

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2. Materials and methods

2.1. Mice

We chose BALB/C mice to evaluate protective immunity against *M. ulcerans* infection on the basis of the study by Converse et al. [19]. Specific-pathogen-free female BALB/C mice were obtained from Japan SLC (Hamamatsu, Japan). All mice were housed in an animal care facility at Kitasato University. Mice were allowed free access to water and a standard diet with a 12 h light/dark cycle, at a room temperature of 23 ± 2 °C with 55 ± 10% humidity. All cages were supplied filled with wood shavings. The study was approved by the Animal Research Committee of Kitasato University and conducted according to the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

2.2. Bacterial strains and culture conditions

M. ulcerans TMC1615 (a virulent strain producing mycolactone A/B) and ATCC19423 (a mycolactone-deficient attenuated strain) were used in this study. For routine propagation, bacteria were grown on Middlebrook 7H9 agar (BD Biosciences, Sparks, MD), supplemented with 0.05% (w/v) Tween 80 and 10% (v/v) Middlebrook OADC Enrichment (Middlebrook 7H9 agar) at 32 °C for three weeks.

2.3. Bacterial antigens

Killed whole cells of *M. ulcerans* were prepared by formalin treatment. In brief, *M. ulcerans* TMC1615 was used after three weeks of culture. Cells were collected by centrifugation (4700 × *g*, 30 min, 4 °C) and resuspended in ice-cold fresh Middlebrook 7H9 broth on a vortex mixer or by sonication (20 kHz, 300 W, 30 s on/off cycle, 10 min, on ice; BIORUPUTOR UCD-300, Cosmo Bio, Tokyo). The suspension of the cells was adjusted to an optical density at 650 nm (OD_{650}) of 1.0. Then formalin was added to a final concentration of 0.2% (v/v) and the mixture was incubated at 4 °C for three days. The resultant suspension was dialyzed against Dulbecco's modified phosphate-buffered saline without calcium and magnesium salts (DPBS) to remove formalin. Finally, 2-phenoxyethanol (0.5%, v/v) was added as a preservative.

Dewaxed whole-cell *M. ulcerans* antigen was prepared as follows. *M. ulcerans* TMC1615 was cultured in Middlebrook 7H9 broth and the culture was centrifuged for collection of cells. A mixture of chloroform and methanol (2:1, v/v) was added to the cell pellet and the mixture was stirred gently for 4 h at room temperature. The dewaxed cells were collected by centrifugation and washed three times with DPBS. The washed cells were suspended in DPBS with sonication (20 kHz, 300 W, 30 s on/off cycle, 10 min, on ice), and the sonicate was adjusted to an OD_{650} of 1.0. The suspension was supplemented with formalin (0.2%, v/v) and incubated at 4 °C for three days and then centrifuged (4700 × *g*, 30 min, 4 °C). After removal of the supernatant, a volume of DPBS equal to the volume of discarded supernatant was added to the cells, and 2-phenoxyethanol (0.5%, v/v) was added as a preservative.

Cell lysates of *M. ulcerans* TMC1615 were prepared as described by Mattow et al. [20]. In brief, *M. ulcerans* cells, cultured in Middlebrook 7H9 broth, were suspended in DPBS supplemented with 1% (v/v) Tween80 and 1% (v/v) Halt Protease Inhibitor Single-Use Cocktail (Thermo, Waltham, MA, U.S.A.), and disrupted by sonication (20 kHz, 300 W, 30 s on/off cycle, 30 min, on ice). The suspension was centrifuged (40,000 × *g*, 30 min, 4 °C) and the supernatant was stored at −80 °C prior to use.

2.4. Secondary antibodies

Alkaline phosphatase-conjugated goat antibodies against mouse immunoglobulin G (IgG), IgG_1, and IgG_{2a} were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.).

2.5. Infection with M. ulcerans

M. ulcerans was cultured in Middlebrook 7H9 broth at 30 to 32 °C for three to four weeks. The bacteria were then harvested in Middlebrook 7H9 broth at room temperature and suspended at 10^9 cells/ml in fresh Middlebrook 7H9 broth after measurement of OD_{650}. Mice were inoculated in the hind footpad with approximately 10^3 to10^6 cells of *M. ulcerans*, in a volume of 10 μl, under inhalation anesthesia (3% isoflurane in air). The thickness of the footpad was measured at weekly intervals to evaluate swelling. Three measurements for each footpad was done by one person in a blinded manner, and the mean was calculated. At 28 days after infection, mice were sacrificed and footpad tissue was removed for extraction of DNA and examination of histopathology. Five mice were included in each experimental group.

2.6. Quantitation of bacteria in hind footpads

After sacrifice, the hinds of mice were dissected out and incubated in 0.7 ml of lysis buffer (0.1 M Tris–HCl buffer containing 0.2 M NaCl, 5 mM EDTA, 0.2% SDS, and 200 μg/ml proteinase K, pH 8.0) at 56 °C for 18 h with horizontal shaking (200 cycle/min) [21]. Ribonuclease (DNase-free; Wako, Osaka, Japan) was added to 40 ng/ml, and then the mixture was incubated at 37 °C for 1 h with horizontal shaking. The mixture was then supplemented with 80 μl of 0.5 M NaOH and heated at 95 °C for 20 min. After the mixture had cooled to room temperature, total DNA was extracted by phenol and chloroform and purified by ethanol precipitation. Copy numbers of genomes of *M. ulcerans* in each hind were measured by *IS2404*-targeted real-time quantitative PCR (qPCR) described by Fyfe et al. [22].

2.7. Histopathological analysis

Samples of hind footpads were fixed in neutral buffered formalin, and then decalcified in 5% EDTA (pH 7.4) at 4 °C. Decalcified tissues were embedded in paraffin. Tissue sections (4-μm thickness) were stained with Ziehl-Neelsen/methylene blue (ZN stain) and examined by light microscopy.

2.8. Immunization

Specific-pathogen-free four-week-old female mice were immunized by subcutaneous injection into the subcutaneous tissue of the inguinal region. Three weeks after the first injection, they received a second injection (booster immunization). Blood was drawn for preparation of serum and/or mice were challenged with *M. ulcerans* for protection assays one week after the second injection.

2.9. Quantitation of antibodies by enzyme-linked immunosorbent assay (ELISA)

For the ELISA for quantitation of serum antibodies against a lysate of *M. ulcerans*, wells of 96-well microtiter plates were coated with 100 μl of lysate of *M. ulcerans* at a protein concentration of 1 μg/ml in DPBS, for 16 h at 4 °C. After three washes with DPBS