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Short communication

Experimental phage therapy against lethal lung-derived septicemia caused by *Staphylococcus aureus* in mice

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Abstract

Nosocomial respiratory infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) can progress to lethal systemic infections. Bacteriophage (phage) therapy is expected to be effective against these critical infections. Previously, phage S13′ was proposed as a potential therapeutic phage. We here examined phage treatment in a mouse model of lung-derived septicemia using phage S13′. Intraperitoneal phage administration at 6 h postinfection reduced the severity of infection and rescued the infected mice. Phage S13′ can efficiently lyse hospital-acquired MRSA strains causing pneumonia-associated bacteremia *in vitro*. Thus, phage therapy may be a possible therapeutic intervention in staphylococcal lung-derived septicemia.

Keywords: Bacteriophage; Mouse; *Podoviridae*; Therapeutic; Therapy

1. Introduction

Staphylococcus aureus is a Gram-positive coccus and an opportunistic pathogen that causes a variety of diseases. The prevalence of methicillin-resistant *S. aureus* (MRSA) threatens immunocompromised patients in hospitals and healthy individuals in the community [1]. Respiratory infections caused by MRSA are difficult to treat using conventional chemotherapy and they have a high risk of progression to fatal systemic infections [1,2]. Patients who contract healthcare-associated, hospital-acquired, and ventilator-associated staphylococcal pneumonia, which are caused mainly by hospital-acquired MRSA (HA-MRSA), have a mortality rate of almost 50% despite the application of therapy [3,4].

Bacteriophage (phage) therapy, where phages are applied as a bioagent to destroy targeted bacteria, was proposed for the treatment of human bacterial infections after the discovery of phages in 1915 and 1917 [5]. However, after a short period of phage therapy development, the developmental focus of antimicrobial therapy shifted from phage therapy to chemotherapy, except in Eastern Europe where phage therapy has been used clinically [5]. However, because of the growing problems with drug-resistant bacteria, the limited choice of effective treatments, and the declining development of novel antibiotics, research into phage therapy has recommenced recently [5,6]. The effectiveness of experimental phage therapy against

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pneumonia caused by Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Klebsiella pneumoniae*, has been demonstrated recently [7–9]. However, to our knowledge, phage therapy has not been tested against staphylococcal respiratory infection with severe septicemia.

Previously, we isolated a virulent *S. aureus* phage S13′, which belongs to the virulent phage family *Podoviridae*, genus AHJD-like viruses [10]. Phage S13′ has an *ex vivo* lytic activity and its effectiveness and safety have been demonstrated in an *in vivo* silkworm larval infection model [10]. In this study, we constructed a mouse model of staphylococcal lung-derived lethal septicemia and examined the therapeutic effects of phage S13′.

2. Materials and methods

2.1. Phage, bacteria, reagents, culture media, and culture condition

Phage S13′ was isolated from a water sample derived from local sewage, as described previously [10]. *S. aureus* strain SA27, which was isolated from a healthy individual at Kochi University, Japan, was used for phage amplification and in the animal experiments [10]. Clinical MRSA strains were used in the *in vitro* phage infection experiments, as described in Supplementary Table S1. Multiple locus sequencing typing was conducted to characterize the bacterial isolates, as described elsewhere (www.mlst.net) [11]. PCR targeted at the Panton–Valentine leukocidin gene (*pvl*) was also conducted [12]. All of the primers used in this study are listed in Supplementary Table S2.

Both the phage and *S. aureus* were cultured at 37 °C. Tryptic soy broth (TSB) was used to culture *S. aureus* and its phage. Mannitol salt agar was used to enumerate the *S. aureus* concentrations as colony-forming units (CFU) [13]. The phage concentrations were measured on strain SA27 as plaque-forming units (PFU) using the double-layer agar method with TSB medium.

The culture media were purchased from Becton, Dickinson, and Company (Sparks, MD, USA), and the reagents were purchased from Sigma–Aldrich (St Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan), unless stated otherwise.

2.2. Large-scale phage culture and phage purification

Phage S13′ was cultured with *S. aureus* strain SA27 in 300 mL of liquid medium and purified by iodixanol density-gradient ultracentrifugation, as described previously [10]. Briefly, the phage lysate was supplemented with polyethylene glycol 6000 (10%) and 0.5 M NaCl, and the phage pellet was obtained by centrifugation (10,000 × *g*, 10 min, 4 °C). The phage pellet was incubated with 50 μg/mL of DNase I and RNase A (37 °C for 30 min) in TM buffer (10 mM Tris–HCl, pH 7.2; 5 mM $MgCl_2$). The phage suspension was then placed on top of a discontinuous iodixanol (OptiPrep; Axis-Shield PoC, Oslo, Norway) density-gradient (40%, 35%, and 30%), and centrifuged (200,000 × *g*, 2 h, 4 °C). The phage bands were collected and subjected to iodixanol density-gradient ultracentrifugation once more. The phage band was collected and stored at 4 °C until use. The concentration of the purified phage was measured before use.

2.3. Construction of the lung-derived septicemic animal model

2.3.1. Animals

Female four-week-old ICR mice (weight = 22.5 ± 1.3 g [mean ± standard deviation]) were obtained from Japan SLC (Shizuoka, Japan). The mice were anesthetized before the invasive procedures or euthanized if required, via intraperitoneal injection with pentobarbital sodium (6.5 mg/kg) (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan). The mice experiments were approved by the Animal Experiment Committee of Kochi University (permission no. E-00042).

2.3.2. Neutropenic mouse model

The neutropenic mice were generated by intraperitoneal administration of 200 mg/kg and 150 mg/kg of cyclophosphamide on day 1 and day 4, respectively. The neutropenic conditions were maintained for 4 days after the final administration of cyclophosphamide (Supplementary Fig. S1A).

2.3.3. Lung-derived septicemia in mice

Different concentrations of *S. aureus* were suspended in cold TSB and inoculated intranasally into the neutropenic mice on the day after the final administration of cyclophosphamide. To prepare the inoculum, *S. aureus* strain SA27 at the mid-log phase was washed three times with saline and the bacterial concentration (bacterial cells/mL) was determined based on the turbidity, as described previously [10,14]. Twenty microliters of *S. aureus* cells were suspended in cold TSB and inoculated intranasally into the mice immediately after anesthesia. Some mice died because of suffocation during the intranasal application of bacteria and only the surviving mice were used in the experiments. Their survival rates were recorded daily. An inoculum of 6.4×10^8 bacterial cells/mL was determined to be optimal and was used in the following experiments (Supplementary Fig. S1B).

The mice were sacrificed to examine the *S. aureus*-inoculated mice. After isolating the organs, two small pieces of the organ tissues were homogenized in saline. The bacterial concentrations in the blood and tissue homogenates were measured.

2.4. Histology

The tissues were also prepared for histological examination, where they were fixed with 10% formalin and embedded in paraffin, before cutting 4 μm sections, which were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Two sections of each tissue obtained from a mouse were prepared. Four mice were sacrificed in each experimental group. Microscopic tissue observations were performed by two laboratory members under the supervision of a pathologist.

2.5. Phage therapy of lung-derived septicemia in mice

To validate the safety of phage administration, separate injections of 0.2 mL of heart infusion broth (HIB), 30% iodixanol diluted with saline, and the phage (1.0×10^{10} PFU)