Experimental Protection of Mice against Lethal Staphylococcus aureus Infection by Novel Bacteriophage $\phi$MR11

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The protective effects of bacteriophages were assessed against experimental Staphylococcus aureus infection in mice. Of the S. aureus phages isolated in the study, $\phi$MR11 was representatively used for all testing, because its host range was the most broad and it carries no genes for known toxins or antibiotic resistance. Intraperitoneal injections ($8 \times 10^8$ cells) of S. aureus, including methicillin-resistant bacteria, caused bacteremia and eventual death in mice. In contrast, subsequent intraperitoneal administration of purified $\phi$MR11 (MOI $\geq 0.1$) suppressed S. aureus–induced lethality. This lifesaving effect coincided with the rapid appearance of $\phi$MR11 in the circulation, which remained at substantial levels until the bacteria were eradicated. Inoculation with high-dose $\phi$MR11 alone produced no adverse effects attributable to the phage. These results uphold the efficacy of phage therapy against pernicious S. aureus infections in humans and suggest that $\phi$MR11 may be a potential prototype for gene-modified, advanced therapeutic S. aureus phages.

The worldwide spread of pathogenic bacteria that are resistant to a variety of antibiotics and the significant problems involved with their control threaten to reduce modern medicine to a state reminiscent of the preantibiotic era. Although novel antibiotics directed against such drug-resistant bacteria can be developed when extensive funds are committed for research, the pathogens will ultimately become resistant to the new drugs. To break this vicious cycle, it will be necessary to adopt chemotherapy-independent remedial strategies to combat bacterial infections.

Bacteriophages (phages) are viruses that specifically infect and lyse bacteria. Phage therapy is a method of harnessing phages as bioagents for the treatment of bacterial infectious diseases. Phage therapy was originally introduced $\sim 80$ years ago by Felix d’Herelle, a discoverer of phages [1, 2]. Despite all his efforts, this therapeutic initiative was later abandoned in Western countries, in part because of the subsequent highly successful discovery and mass production of many kinds of effective antibiotics in the 1940s. Furthermore, most early research into the therapeutic use of phages was poorly organized or uncontrolled, and our understanding of the basis for phage biology was immature. These factors combined to produce a negative outcome for phage therapy [2–4]. Nevertheless, phages have been used for practical purposes in the former Soviet Union and eastern Europe to the present day [2–6]. The on-
going active bacterial evolution of multidrug resistance also has recently motivated the Western scientific community to reevaluate the therapeutic potential of phages for diverse bacterial infections that are virtually incurable by conventional chemotherapy [2–4]. Phage therapy may be an alternative to antibiotics, because it has proved to be medically superior to antibiotic therapy in many ways [7–10]. In financial terms, the developmental costs of phage therapy are expected to be much less than those involved in the development of novel antibiotics.

Growing evidence has suggested 4 different clinical applications for phages, as follows: (1) the administration of living bactericidal (virulent) phages or their natural mutants [11–18]; (2) the topical use of purified phage-encoded bacteriolytic peptidoglycan (cell wall) hydrolases (collectively referred to as endolysin or lysin), such as amidase and transglycosidase [19–22]; (3) the clinical use of phage structural proteins as a metabolic inhibitor to the key enzyme(s) of bacterial peptidoglycan synthesis [23]; and (4) the use of the phage-display system of the filamentous M13 phage, which expresses coat proteins fused to specific antibodies directed against bacterial antigens [24]. Besides these experimental achievements, clinical trials of phage therapy also may improve its prospects in the West [25].

*Staphylococcus aureus* is a pathogen of pyogenic inflammatory diseases, food poisoning, and toxic-shock syndrome; it is also a major causative agent for opportunistic and/or nosocomial infections, often with a high mortality rate [26]. According to one report, >50% of clinical *S. aureus* isolates in Japan today carry multidrug resistance, typically known as methicillin-resistant *S. aureus* (MRSA) [27]. Moreover, certain MRSA strains also have already acquired resistance to vancomycin (vancomycin-resistant *S. aureus* [VRSA]), a unique antibiotic previously considered to be effective against MRSA [28].

A study conducted in Poland has recently reported a summary of their 20-year experience of the clinical application of phages in humans, demonstrating their therapeutic efficacy, including *S. aureus* phages [5, 6]. Although these case-oriented reports are very attractive and valuable, additional basic information, such as details of the phages used and clinical data, should be presented, preferably in a publicized form, for the wider acceptance of phage therapy in the medical community. In the 1980s, Smith et al. [11–14] undertook rigorous investigations into phage therapy for pathogenic *Escherichia coli* infections in a veterinary context, thereby reopening this field of research in Western countries. Since Smith’s reevaluation, there has been, to the best of our knowledge, only 1 published report examining phage efficacy against experimental infections of *S. aureus* [15]. Using a mouse model, Soothill [15] examined the protective effects conferred by the *S. aureus* phage, φ-131, but, unfortunately, he failed to demonstrate the therapeutic efficacy of the phage (addressed again in Discussion). Therefore, the authenticity of phage therapy for *S. aureus*–induced diseases has not yet been confirmed, at least not on the animal experiment level.

Our goal was to establish an effective and advanced phage-therapy system against life-threatening infections caused by antibiotic-resistant bacteria. We first focused on and scrutinized the therapeutic potency of phages directed against *S. aureus* infections, including MRSA and VRSA, in a mouse model. We isolated a number of wild *S. aureus* phages, extensively examined their bacteriolytic activity, and identified some with a broad host range. Using one of these, designated φMR11, our study provides the first experimental evidence that the administration of phages can successfully protect mice against fatal *S. aureus* infection without any adverse effects, thereby implying the clinical applicability of phage to human *S. aureus*–induced diseases.

**MATERIALS AND METHODS**

**Culture media.** All reagents and constituents of culture media used in our study were purchased from Nacalai Tesque, unless otherwise stated. Tryptic soy broth (TSB) and heart infusion broth (HIB) were obtained from Difco Laboratories. TSBM is TSB medium supplemented with 20 mM MgCl₂, and HIBMC is HIB medium supplemented with 20 mM each MgCl₂ and CaCl₂. For phage-plaque formation and spot test, TSBM-based solid medium containing 1.5% or 0.5% agarose was used for the lower or upper layers, respectively.

*Measurements of bacterial growth and cell number.* Bacterial growth was monitored by measuring turbidity with a Klett-Summerson colorimeter (filter 54). One Klett unit was considered to be equivalent to 6.4 × 10⁶ *S. aureus* cells/mL. This conversion formula was based on a previously standardized correlation between turbidity and bacterial cell numbers counted directly with a Petroff-Hauser counting chamber (Hauser Scientific).

**Bacterial strains.** The bacterial strains used in our study included 43 methicillin-sensitive *S. aureus* ( MSSA) strains and 29 MRSA strains, denoted by SA or MR preceding each strain number, respectively. Most MSSA strains were derived from nasal swab samples obtained from 162 healthy individuals, and all MRSA samples were derived from clinical specimens obtained from patients in Kochi Medical School Hospital. Nasal swab samples and clinical specimens were spread on mannitol salt agar supplemented with egg yolk (Nissui Pharmaceutical), which is a selective and semispecific growth medium for *Staphylococcus*. All the isolated strains were confirmed as *S. aureus* by use of the API-STAPH identification kit (API Systems), together with the coagulase test and light-microscopic observations. Identification of MRSA strains was achieved by colony formation on a selective salt agar plate containing 6 μg/mL oxacillin (MSO medium; Nissui). Strains that yielded colonies on the MSO plates were tested further for the meca gene by use
of a polymerase chain reaction–based detection kit (MecA test Wakunaga; Wakunaga Pharmaceutical). Strains incapable of growing on MSO were judged to be MSSA. A standard MSSA strain, 209P (obtained from the Research Institute for Microbial Diseases of Osaka University), and 2 VRSA strains, Mu3 and Mu50 [28] (kindly provided by Professor K. Hiramatsu, Juntendo University), also were used.

In the present study, MSSA strain SA37 (mecA negative) was isolated from the nares of a healthy volunteer and served as the propagation host and/or experimental target of our particular S. aureus phage φMR11 (see below), unless otherwise stated, because SA37 is sensitive to most of the phages isolated, including φMR11. Two variants of SA37 were established for some animal experiments. For the examination of bacterial dynamics in vivo, a rifampicin-resistant mutant of SA37 (SA37-RIF2) was used to distinguish the injected strain from normal flora-derived strains. SA37-RIF2 was isolated by seeding the parental SA37 on selective TSB agar containing 2 μg/ml rifampicin. This mutant was confirmed as having the same growth properties and sensitivity to the fragment pattern of the phage released from SA37/MR11. This variant was derived from a rare bacterial colony that had appeared among φMR11-exposed SA37 cells spread on a TSBM plate. Preliminary confirmation of the lysogeny of φMR11 in SA37/φMR11 was made by HindIII- or XbaI-digestion analysis, which showed that the cleaved DNA fragment pattern of the phage released from SA37/φMR11 by mitomycin C (MMC) induction (see below) was identical to that of the original φMR11. The lysogen had the same biological properties as parental SA37 but was insensitive to lysis by φMR11.

Isolation of S. aureus bacteriophages. We attempted to induce the replication of possible lysogenic phages by treating 72 S. aureus strains (43 MSSAs and 29 MRSA) with MMC. In brief, bacterial cells were grown at 37°C until the late logarithmic phase (corresponding to 100 Klett units) in TSB medium. MMC was added to the cultures to a final concentration of 1 μg/ml, followed by further incubation for 30 min. After the cells were washed once with TSB, they were resuspended in the same culture medium and were incubated again at 37°C for 4 h. The cultures then were centrifuged to remove cell debris filtered through a 0.45-μm pore-sized membrane, and the resultant supernatants were subjected to spot-test screening for bacteriolytic activity by use of the 72 S. aureus wild strains and the 209P, Mu3, and Mu50 strains as indicators. If the supernatants contained phages, this screening also facilitated an estimation of their bacteriolytic host range. About 5 μL of each preparation was place directly onto lawns of the bacterial indicators with a disposable loop, and the production of lytic spots was assessed after incubation for 24 h at 37°C [29].

Lysis-positive supernatant samples sorted by the spot test then were subjected to single-plaque isolation using an appropriate host bacterium. The picked plaque was propagated in a larger volume of culture medium. The plaque-purified lytic agents then were examined by electron microscopy, regardless of whether they had the shape that is characteristic of phages. Of the identified phages, a phage designated φMR11 was representatively used for all subsequent experiments, because it was shown by the spot test to have the broadest host range (see Results).

Biological characterization of φMR11. The adsorption rate, latent period, and burst size of φMR11 were determined essentially according to the method of Adams [30]. All incubations for the analyses were carried out in TSBM medium at 37°C. In brief, for examination of the adsorption rate, φMR11 (1.5 × 10^3 pfu) were mixed with SA37 cells (10^7/mL), and the number of free infectious phage virions was measured in the phage-cell mixture after treatment with chloroform [31]. To determine the latent period and burst size, SA37 cells (5.8 × 10^7/mL) were exposed to φMR11 (2.4 × 10^5 pfu) for 5 min, washed thoroughly with cold TSBM medium to remove unbound phages, and then resuspended in fresh medium. An aliquot of the cell suspension was harvested regularly during incubation at 37°C to be titrated for newly produced phages, including both released and cell-associated phages, on a lawn of SA37.

Large-scale purification of phage particles. Phage φMR11 was purified essentially according to the procedure used for several T4-type phages [29, 32–36]. SA37 host cells suspended at 1.2 × 10^9 cells/mL in 500 mL TSBM medium were exposed to a crude preparation of φMR11 at an MOI of 0.01 and were vigorously shaken for ~4 h at 37°C, resulting in complete lysis of the bacteria. After 5 min of treatment with 1% chloroform at 37°C, the culture fluid was centrifuged at 8000 g for 10 min at 4°C to remove cell debris. Polyethylene glycol (average molecular weight, 6000) and NaCl were added to the supernatant to final concentrations of 10% and 0.5 M, respectively, and kept at 4°C for 3 days. The resultant precipitate containing the phage particles was collected by centrifugation at 8000 g for 20 min at 4°C, resuspended in 10 mM Tris–HCl and 5 mM MgCl2 (pH 7.2) buffer, and treated with 20 μg/mL DNase I (Type II; Sigma Chemical) and 10 μg/mL RNase A (Type 1A; Sigma) for 30 min at 37°C. The phage suspension then was placed on top of a discontinuous CsCl gradient (ρ = 1.3, 1.5, and 1.7) and centrifuged at 100,000 g for 1 h at 4°C. The phage band was collected and dialyzed against saline that contained 20 mM MgCl2 and 20 mM CaCl2 (SMC) for 2 h at 4°C. CsCl-gradient separation and dialysis (1 h) were repeated, and further dialysis against HIBMC medium was carried out for 1 h at 4°C. The purified phage suspension was filtered (0.45-μm pore size), divided into aliquots, and stored at 4°C until used. The samples were appropriately diluted with HIBMC just before use for infections. The titers (pfu/mL) of the purified samples were determined by inoculating them into bacterial strain SA37.
experiments that involved the inoculation of mice with MRSA strains, φMR11 was propagated once with each MRSA before its administration, to minimize any possible bacterial restriction-modification, which is known to inhibit phage replication [37], and then purified as described. Plaque-forming units of host-adapted φMR11s were each titrated with the corresponding MRSA.

**Bioassay for toxins of** *S. aureus*. Some bacterial strains used in the study were checked for the production of toxins known to be associated with *S. aureus*, using reversed passive latex agglutination (RPLA) [38]. Enterotoxin and toxic-shock syndrome toxin–1 (TSST-1) of *S. aureus* were measured by use of the bacterial culture supernatants harvested after incubation at 37°C for 18–20 h, strictly according to the protocols for the assay kits (SET–RPLA Seiken and TST–PRLA Seiken; Denka Seiken).

**Animal experiments.** For infection experiments, 6–8-week-old BALB/c female mice (body weight, ∼20 g) were used. *S. aureus* cells were grown in 100 mL TSB medium at 37°C and were centrifuged at 8000 × g for 5 min at the early stationary phase (∼250 Klett units). The cell pellet was washed with 100 mL saline, centrifuged again under the same conditions, and finally resuspended in 5 mL saline. In appropriate dilution, turbidity (in Klett units) was measured to determine bacterial cell numbers, as described above. Varying numbers of bacterial cells suspended in 0.5 mL saline were injected into the peritoneal cavities of mice through one side of the abdomen, and purified phage suspensions in 1 mL saline were injected into the other side. As controls, equal volumes of saline or HIBMC alone were injected intraperitoneally on all test occasions. The test animals were observed for between 1 week and 1 month.

Approximately 0.5 mL of blood was taken by puncturing the orbital plexus of test mice with a capillary tube and mixed immediately with 50 μL heparin (1000 U/mL; Aventis Pharma). After the heparinized blood was diluted with SMC, colony-forming units of SA37-RIF2 were measured on rifampicin–TSB plates, and plaque-forming units of φMR11 were measured by use of SA37 as the host on TSBM plates. Blood samples were also collected from untreated mice, to ensure that mice used in the experiments were free of naturally or accidentally contaminating phage or bacteria.

The data of survival rates of mice and dynamics of phage/host bacteria were analyzed statistically by use of Fisher’s exact test and a 2-way analysis of variance, respectively. The analyses were performed with SPSS 11.0j for Windows.

**Preparation of a mechanical lysate of** *S. aureus*. *S. aureus* SA37 was grown in 100 mL of TSB at 37°C to ∼250 Klett units, centrifuged at 12,000 × g for 5 min at 4°C, washed with saline, and resuspended in saline at the concentration of 2 × 10⁹ cells/mL. The cell pellet from 5 mL of the suspension was destroyed by grinding with a mortar and pestle at 4°C, and the volume was adjusted again to 5 mL with saline. Then the lysate was filtered through a membrane with a pore-size of 0.45 μm to remove residual living cells. The filtrate was used as a mechanical lysate of *S. aureus*.

**Electron microscopy.** Purified phage samples in SMC were fixed in 5% formalin and negatively stained with 2% uranyl acetate (pH 4.0). Electron micrographs were taken with a transmission electron microscope (Hitachi H-7100; Hitachi) at 75 kV.

**DNA sequencing.** Genome DNA of φMR11 was extracted, as described elsewhere [29]. HindIII-digested fragments of the genome were cloned into the pUC18 vector. Each cloned fragment was sequenced by PCR amplification (primer walking) by use of fluorescently labeled dideoxy chain-terminating nucleotides (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems), according to the specifications of the supplier. PCR products were purified by AutoSeq G-50 column (Amersham-Pharmacia Biotech) and were analyzed on an automated DNA sequencer (model 310; Applied Biosystems).

**RESULTS**

**Isolation of** *S. aureus*–specific phages with therapeutic potential. Cultures of 72 *S. aureus* strains (MSSA and MRSA) were treated with MMC, and the supernatants were harvested and then subjected to the spot test. The indicator panel for screening was composed of the same 72 *S. aureus* strains and strains 209P (MSSA), Mu3 (hetero-VRSA), and Mu50 (VRSA) [27]. Thirty-six of 72 supernatant samples clearly produced lytic spots on the lawn of at least 1 bacterial indicator, implying the possible induction of lysogenic phage replication by MMC. Of the 36 spot-positive supernatants, 8 formed lytic spots on lawns of most of the bacterial indicators and proved to contain phages at the time of testing, as ascertained by a subsequent plaque-formation assay and electron microscopy (see below). One of 8 phages identified as bacteriolytic for a broad host range, designated φMR11, formed spots on almost all *S. aureus* strains examined, indicating that this phage can adsorb efficiently to a wide range of *S. aureus* strains via the receptor molecule(s) ubiquitously expressed on the bacteria (see below). Moreover, φMR11 also produced large clear plaques on the greatest number of bacterial indicators (30/75 *S. aureus* strains tested). The discrepancy between the results for the spot test and the plaque-formation assay reflects the 2 different mechanisms underlying bacteriolysis by phage [20, 39]. Lytic spot formation represents a combination of both lysis from within associated with the phage replication (lytic) cycle and lysis from without caused by phage binding per se to bacteria [39]. Plaque formation, on the other hand, indicates the lysis from within mechanism only [39]. Therefore, a spot-positive but plaque-negative result implies the occurrence of lysis simply as the result of phage at-
attachment to the bacterial cell surface, with subsequent inhibition of any multiplicative process(es) of the progeny virus. Abortion of the fully lytic cycle is an unfavorable event for the practical application of therapeutic phage. However, we expect that it may be largely overcome by modifying the phage genome—for example, by introducing a mutation at the repressor-binding sites critical to lytic cycle initiation. To this end, phages with relatively small genomes, such as φMR11 (see below), will be an advantage because their genomes are easy to manipulate by standard genetic engineering techniques.

**Morphological, biological, and genetic characterizations of φMR11.** As shown in figure 1, electron microscopy demonstrated that φMR11 has an isometrically hexagonal head, 56 ± 2 nm in diameter, and a noncontractile tail of 175 ± 5 nm in length, with a knoblike structure at its distal end. Therefore, it should be assigned to the family Siphoviridae, morphology B1 [40]. Restriction-enzyme analysis revealed that the φMR11 genome is circularly permuted double-stranded DNA, with an expected size ~43 kbp. Sequencing the entire genome has been recently completed, and the exact genome size is 43,011 bp. φMR11 gene organization is unique (authors’ unpublished data) compared with 8 other S. aureus phages, the sequences of which have been registered in the nucleotide sequence databases. More important, the genomic sequence has verified that φMR11 carries neither antibiotic-resistance genes nor known toxin genes often associated with certain S. aureus phages, such as enterotoxin [41], leukocidin [42, 43], or exfoliative toxin [44, 45]. In this regard, the sequence data are consistent with the fact that the parental S. aureus strain, MR11, did not detectably produce enterotoxin (or TSST-1) on the RPLA assay (<1 ng/mL of bacterial culture fluid).

Biological studies clarified other features of φMR11 (figure 2), such as the following: (1) a rapid adsorption rate (when sensitive bacterial hosts were present in sufficient numbers, >90% of φMR11 particles bound to them within 1 min); (2) a short latent period (~25 min); and (3) a relatively large burst size (>100). The efficient host-cell lysis characteristics of φMR11 are reminiscent of typical virulent phages, such as the T-even coliphages [40]. Of interest, φMR11 even lysed sensitive bacteria efficiently during the stationary phase, at which stage phage replication generally declines in concert with the cessation of host cell growth (data not shown).

On the evidence cited above, φMR11 was considered to be a suitable candidate for the therapeutic phage, or its prototype, for the treatment of human S. aureus infections. Therefore, the following experiments were undertaken using φMR11 in an animal model.

**Mouse model of S. aureus–induced disease.** The dose of

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**Figure 1.** Electron micrograph of the *Staphylococcus aureus*–derived phage φMR11. Purified phage particles were negatively stained with 2% uranyl acetate. Bar, 100 nm.
Figure 2. Virological properties of the φMR11 phage. A, Adsorption rate. φMR11 phages were mixed with excess Staphylococcus aureus SA37 cells, and then nonadsorbed infectious phages were serially counted. Data are percentage of nonadsorbed φMR11 relative to the initial input dose of phages. B, Latent period and burst size. After a 5-min adsorption of φMR11 to a sufficient number of SA37 cells, the φMR11-exposed bacterial cells were thoroughly washed with tryptic soy broth medium supplemented with 20 mM MgCl₂ to remove free phages and then were resuspended in fresh medium. Newly synthesized phage virions, including both released and cell-associated virions, then were measured at regular intervals during the incubation, using harvested culture samples. In both panels, each black circle represents an average of 2 samples. SE bars are too small to be visible behind the circles.

Figure 3. Determination of the challenge dose of Staphylococcus aureus lethal to mice. Serially diluted suspensions of S. aureus SA37 cells were injected intraperitoneally into mice, and mouse fatalities were observed. White and black circles represent the survival rates 1 and 7 days after injection, respectively. Numerals associated with symbols indicate the nos. of mice tested. In the control experiment, denoted "0" on the horizontal axis, only 0.5 mL of saline was injected.

S. aureus lethal to mice was determined by injecting mice with varying numbers of strain SA37, ranging from \(2 \times 10^2\) to \(2 \times 10^9\) cells per dose (figure 3). Intraperitoneal injections of \(2 \times 10^2-10^8\) SA37 did not reduce the survival rate of mice during the subsequent 7-day observation period. By contrast, injections of \(3 \times 10^8-1 \times 10^9\) cells lowered the survival rate in a dose-dependent manner. Because the injection of \(8 \times 10^8\) SA37 cells was fatal in \(>80\%\) of mice within 24 h and in 100% within 7 days of injection, this level of challenge was considered to be optimal for observing the phage effect on bacterial lethality (figure 3). Therefore, the dose of S. aureus was fixed at \(8 \times 10^9\) cells throughout the following experiments. A more precise time-chase analysis showed that intraperitoneal injection of \(8 \times 10^8\) SA37 cells killed most mice between 6 and 7 h after injection, with associated preceding bacteremia (figure 4 and below). Dissection of mice that died from bacterial infection 6 h after injection revealed severe systemic congestion, with splenomegaly and acute ascites (data not shown).

Protective effects of phage administration against S. aureus infection in mice and associated safety issues. Purified φMR11 was injected into the mouse peritoneal cavity immediately after challenge with SA37, at various MOIs ranging from 0.01 to 200. Administration of φMR11 at an MOI of 0.1–200 significantly protected mice from SA37-induced death (figure 5). On the other hand, administration of a large amount (up to \(2.0 \times 10^{11}\) pfu) of φMR11 alone to 30 mice did not affect their physical condition or survival during the 1-month observation period. Consistent with the physical state of the mice, various tissues and organs of mice killed at 6 h and 1, 7, and 14 days after phage-only treatment did not differ in pathology from those of control mice untreated or injected with HIBMC medium only (data not shown). These findings indicate that φMR11 itself, at least when inoculated into the peritoneal cavity, does not give rise to any detectable adverse effects, despite its...
Figure 4. A precise time-chase analysis of *Staphylococcus aureus*-induced killing of mice. Ten mice were each inoculated with a fixed no. of *S. aureus* SA37 cells (8 x 10⁸), and their mortality was traced for up to 24 h. No. of surviving mice dropped suddenly 6 h after bacterial challenge, and death was preceded by obvious bacteremia (figure 7). These data are also relevant to the next experiment (see figure 6).

Figure 5. Dose-dependent lifesaving effects of phage MR11 against experimental *Staphylococcus aureus* infection in mice. After injection of *S. aureus* SA37 (8 x 10⁹) cells, MR11 were injected into the mouse peritoneal cavity at various MOIs, and the fate of the mouse was observed. Numerals adjacent to circles indicate the nos. of mice examined. Mice injected only with heart infusion broth supplemented with 20 mM MgCl₂ and 20 mM CaCl₂, which was used to prepare the phage suspensions, served as control mice and are represented by circles at an MOI of 0. There is a statistically significant difference in survival rates between mice treated with MR11 at an MOI ≥0.1 and untreated control mice (P<.012).

rapid entry into the circulation and its presumably systemic distribution in vivo (see below and figure 7).

**Possible mechanisms behind the lifesaving effects of MR11.** A previously published in vitro analysis using human materials showed that a crude lysate of *S. aureus* infected by phage provoked an immediate cell-mediated immune response [46]. Therefore, we investigated whether the direct bactericidal activity of MR11 was actually responsible for its protective effects. Intraperitoneal injections of mechanical lysate, an analogue of phage lysate, prepared from 1 x 10⁸ SA37 cells, was followed by the injection of an equivalent number of living SA37 cells. The mechanical lysate did not prevent death in the mice. Mice injected with mechanical lysate prepared only from twice the number of SA37 (2.0 x 10⁹) were not affected, indicating that the mouse-killing effect was not caused simply by the excessive bacterial burden imposed in this experiment. Therefore, it is unlikely that bacterial components, even those released by phage lysis, were involved in the protective effect. To study another critical possibility, that MR11 virions themselves stimulate an antibacterial immune response, a further experiment was conducted using the MR11-lysogen of SA37 (SA37/MR11) as the target bacterium. This SA37 derivative has biological properties and a genetic background identical to the parental SA37 strain, except for its nonsusceptibility to MR11-induced lysis. If the net lifesaving effect is mediated, not through MR11-induced bacteriolysis but via a virion-stimulated immune response (e.g., production of cytokines), the administration of phage should be effective against SA37/ MR11, as well as against SA37 infections. Injection of purified MR11 (MOI, 10) rescued mice challenged with SA37 from death but not mice challenged with SA37/MR11. These results support the conclusion that direct bactericidal activity exerted by MR11 is the prime and perhaps the sole determinant of the protective effect observed in the present study.

**Temporal sequence and in vivo distribution of MR11 and S. aureus.** Purified MR11 was administered intraperitoneally to mice at different times (up to 60 min) after SA37 injection (figure 6). Although MR11 administration proved to be effective at MOIs ranging widely from 1 to 200 (figure 5), the highest MOI of 200 was applied in this and subsequent investigations (see below), under the assumption that patients were in an extreme situation of systemic *S. aureus* infection such as sepsis. After 24 h, most mice treated with phages at any time point were still alive, whereas 80%–100% of mice not treated with phages were dead. The therapeutic efficacy of MR11 was even discernible in mice treated 60 min after injection with bacteria, when all the control mice injected with SA37-only already exhibited signs of physical deterioration, such as reduced activity and ruffled hair. Although a few MR11-treated mice died during
the following 6 days, the survival rates among mice treated with phages at any time point were always significantly higher than those of the untreated controls (figure 6).

On the basis of the above results, the in vivo dynamics of the bacteria and phages were investigated in detail using SA37-RIF2 as the target cell. Mice were injected intraperitoneally with \(8 \times 10^8\) SA37-RIF2 alone, with SA37-RIF2 plus \(\Phi MR11\) (\(1.6 \times 10^{11}\) pfu), or with \(\Phi MR11\) alone. Bacteremia occurred within 2 h and then persisted in the SA37-RIF2–injected mice, regardless of phage treatment (figure 7). However, the bacterial loads in the blood were significantly lower in \(\Phi MR11\)-treated mice than in untreated mice throughout our observations (figure 7). Compatible with the result shown in figure 4, all mice injected only with SA37-RIF2 died within 6 h, whereas \(\Phi MR11\)-treated mice were invariably saved, concomitantly with a subsiding of septicemia within 24 h. However, after \(\Phi MR11\) was injected into the peritoneal cavity, a significant number of infectious \(\Phi MR11\) was readily detected 2 h later in blood specimens from both the SA37-RIF2–infected and uninfected mice at titers of 1.3 and \(7.7 \times 10^7\) pfu/mL, respectively. This initial discrepancy between the titers of circulating phages probably represents an acute “consumption” of administered \(\Phi MR11\) by the inoculated target bacteria. It is noteworthy, however, that subsequent relative levels of circulating \(\Phi MR11\) were reversed in the 2 mouse groups: the titer rapidly decreased with time in mice not inoculated with SA37-RIF2, whereas it remained higher in SA37-RIF2–injected mice. Typical titers measured after 6 h were \(4.7 \times 10^7\) pfu/mL in the absence of SA37-RIF2 versus \(5.5 \times 10^8\) pfu/mL in the presence of SA37-RIF2 (figure 7). These results illustrate the in vivo kinetics, whereby intraperitoneal \(\Phi MR11\) was amplified by the coexisting target bacteria and migrated into the bloodstream, perhaps resulting in the systemic dissemination of the phages. Under these circumstances, the circulating \(\Phi MR11\) was sustained at a significant level until the target cells were eradicated, which must have counteracted the progression of bacteremia. The beneficial in vivo kinetics of \(\Phi MR11\) in curing the S. aureus infection also may be applicable in general to phages given therapeutically to counteract other bacterial infections [47].

**Efficacy of \(\Phi MR11\) for MRSA infection.** Finally, we investigated whether \(\Phi MR11\) is also efficacious against MRSA infections. One of 4 clinical MRSA strains—MR1, MR13, MR18, or MR28—was injected intraperitoneally (\(8 \times 10^6\) cells) into mice. For this experiment, \(\Phi MR11\) was propagated once beforehand with each corresponding MRSA strain to eradicate as much as possible any bacterial restriction-modification [36]. Phages then were purified through a CsCl gradient. As shown in figure 8, all mice treated with these “adapted” \(\Phi MR11s\) (MOI, 50) survived for 7 days after MRSA injection, in contrast to the \(90\%\) mortality in the phage-untreated mice, which suggests that \(\Phi MR11\) also is potentially useful for human MRSA infections.

**DISCUSSION**

The emergence of multidrug-resistant bacteria will have a serious impact on various aspects of medical practice. There are
of rod and between the dynamics of SA37-RIF2 in the presence and absence of rifampicin-resistant mutant of SA37 (SA37-RIF2; see text) was used as the target in this experiment. A peripheral blood sample was taken from 1 mouse from each group 2, 4, 6, and 24 h after injection and was titrated to estimate the nos. of phages and bacteria in the circulation. Black and white circles represent plaque-forming units of phage, respectively. All mice injected with only SA37-RIF2 died during the observation period within 6 h of injection [1]. Each circle and square represent an average of 2 samples. SE bars are too small to be visible behind the symbols. The 2-way analysis of variance revealed statistically significant differences between the in vivo dynamics of MR11 in the presence and absence of SA37-RIF2 [2], respectively. Black and white squares represent colony-forming units of the host bacteria with and without the administration of phage, respectively. All mice injected with only SA37-RIF2 died during the observation period within 6 h of injection [1]. Each circle and square represent an average of 2 samples. SE bars are too small to be visible behind the symbols. The 2-way analysis of variance revealed statistically significant differences between the in vivo dynamics of MR11 in the presence and absence of SA37-RIF2 [2] and between the dynamics of SA37-RIF2 in the presence and absence of MR11 [2].

several potential chemotherapy-independent schemes with which to address this clinical problem, one being the prevention of colonization by drug-resistant bacteria by using microbial interference [48, 49]. In the present study, using a newly isolated phage, we experimentally reevaluated the therapeutic potential of phages in mice infected with S. aureus, including MRSA.

A series of rigorous studies into phage therapy by Smith et al. [11–14] in the 1980s made a significant contribution that led to reevaluation of phage efficacy against infections of E. coli [16, 17], Acinetobacter baumanii [15], Pseudomonas aeruginosa [15], Klebsiella species [50, 51], Lactococcus garvieae [47], and Enterococcus faecium [18] in animal models or in natural animal targets of these virulent microbes. These, together with the present study, support the potential of phage therapy against various bacterial infectious diseases; in fact, successful treatment for humans has been reportedly achieved in eastern Europe and the former Soviet Union [2–6]. These reports are certainly noteworthy in the clinical context; however, basic features of the phages used for individual bacterial pathogens, including the pharmacokinetics involved, are still largely unclear. In addition, as described in the literature [5, 6], the phages were administered in a “made-to-order” manner, which may provide some clinical merit, but as crude preparations. For verification of the scientific as well as clinical validity of the therapy, well-characterized and purified phages will be desirable for therapeutic use.

The present study has shown that the selected phage φMR11 is effective in treating S. aureus infections in an animal model. There has been only one report published in 1992 on experimental phage therapy for S. aureus infection, by Soothill [15]. In that study, phage φ-131 (unpurified), which is commonly used in Poland, was administered to mice artificially infected with S. aureus strains 6409 or M60. However, this experiment failed to reproduce the efficacious effects reported in humans [5, 6], although therapeutic efficacy was successfully shown for phages specific to P. aeruginosa and A. baumanii. This negative result using S. aureus may be explained, in part, by the low dose of φ-131 administered: an MOI of 0.018 for strain 6409 and

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Figure 7. Rapid appearance of phage φMR11 in circulation. Target bacterium (8 × 10⁶ cells) and/or φMR11 (MOI, 200) were injected intraperitoneally into 4 groups of mice, each of which consisted of 5 mice. A peripheral blood sample was taken from 1 mouse from each group 2, 4, 6, and 24 h after injection and was titrated to estimate the nos. of phages and bacteria in the circulation. Black and white circles represent plaque-forming units of φMR11 in the presence and absence of SA37-RIF2, respectively. Black and white squares represent colony-forming units of the host bacteria with and without the administration of phage, respectively. All mice injected with only SA37-RIF2 died during the observation period within 6 h of injection [1]. Each circle and square represent an average of 2 samples. SE bars are too small to be visible behind the symbols. The 2-way analysis of variance revealed statistically significant differences between the in vivo dynamics of φMR11 in the presence and absence of SA37-RIF2 (P < .001) and between the dynamics of SA37-RIF2 in the presence and absence of φMR11 (P < .001).

Figure 8. Therapeutic efficacy of φMR11 for methicillin-resistant Staphylococcus aureus (MRSA) infections in mice. Four clinically isolated MRSA strains, MR1, MR13, MR18, and MR28, were used as host bacteria. φMR11 was propagated once with each MRSA, to minimize possible phage inactivation by the bacterial restriction-modification system, followed by CsCl purification. Each “adapted” φMR11 was administered (MOI, 50) intraperitoneally to mice immediately after challenge with the corresponding MRSA strain (8 × 10⁶ cells). Survival rates were determined 7 days after injection of the bacterium and/or phage. Shaded and hatched columns represent the phage-treated and -untreated mouse groups, respectively. Each group was composed of 10 mice. A statistically significant difference (P < .0002) in survival rates was observed between paired groups of phage-treated and -untreated mice for all MRSA, as indicated by horizontal brackets with asterisks.
0.086 for strain M60 [15]. Furthermore, the MOIs calculated in that study may have been overestimates because S. aureus tends to form cell clusters. Our data (figure 5), which are consistent in this context with Soothill’s findings [15], indicate that an MOI of 1 is the minimum required for φMR11 to yield a fully protective effect, at least in the mouse model. Taken together, these data imply that determination of the appropriate dose of phage is a prerequisite for successful phage therapy.

Some phage lysates of S. aureus have been reported to be capable of stimulating a cell-mediated immune response [46]. This raises the question of whether the protective effect induced by φMR11 is attributable to direct bacteriolysis by the phage or associated with vaccinellike immune activation by dispersed bacterial components. However, such a bacterial antigen-induced immune reaction can be discounted, at least as a principal mechanism, on the basis of our own results, which show that a “mechanical” lysozyme of S. aureus induced no antibacterial effect. The irrelevance of immune mediation in φMR11 treatment also is supported by the fact that bacterial antigens were completely removed from the φMR11 preparations during repeated CsCl-gradient centrifugation purification. Furthermore, any activation by phage particles themselves of an antibacterial immune response also was negligible, because φMR11 had no therapeutic effect against infections with the phage-lysogenic S. aureus strain SA37-φMR11.

Our study also demonstrated that φMR11 can rapidly enter the circulation (within 2 h), even when administered intraperitoneally, which implies rapid systemic distribution of the phage in vivo. In fact, infectious φMR11 were subsequently also detected in various tissues and organs such as the spleen, liver, kidney, brain, and skeletal muscle (10^5–10^6 pfu/g; authors’ unpublished data), which suggests an extension of the medical application of phage to systemic infections. Merril et al. [16] observed similar in vivo translocation of E. coli λ phage and Salmonella phage P22 administered in a mouse system. As an unfavorable consequence, however, these phages were rapidly eliminated from the blood, making the capture of circulating phages by the splenic reticuloendothelial system an anticipated problem of phage therapy [7, 16, 52]. Of interest, in the same article, Merril et al. also reported that a small population of mutant λ phages survived in the circulation, with a concomitant alteration to major head protein E. Therefore, the authors postulated that such “serially passaged” immune-escape mutants may facilitate improvements to the therapeutic efficacy of phages [16]. Our data, on the other hand, indicate that infectious φMR11 was maintained at high levels in the bloodstream in the presence of target bacteria, at up to 3 orders of magnitude above levels measured in the absence of target cells. φMR11 titers in the circulation were substantial, ~10^5 pfu/mL, even 24 h after injection (figure 7). As pointed out by Barrow and Soothill [9], our data and those of others [46] support the view that the immunologic elimination of certain phages in vivo may not be a serious issue for practical applications.

To establish an efficient phage-therapy system generally applicable against human S. aureus–induced diseases, several other obstacles must be overcome in the future. The occurrence of phage resistance is unquestionably a problem in phage therapy [53]. However, according to data described elsewhere [54] and our own data (unpublished), the incidence of bacteria that resist phage attack is ~10-fold lower than the incidence of bacteria insensitive to an antibiotic. Even if bacteria acquire phage resistance, new mutant phages that act lytically against these bacteria can be isolated quickly (usually in a few days). It will be necessary to create advanced therapeutic phages to circumvent other inevitable problems, at least at present, such as the lysogenicity of therapeutic phages and the restriction-modification systems of bacteria. We believe that it is possible to render prototype phages (i.e., with broad host ranges) nonlyogenic by artificial gene alteration, such as via the introduction of mutations into and/or deletion of genomic elements essential for phage integration into the host cell DNA. φMR11 will be an eligible candidate for a prototype with which to pursue these objectives, because its genome size is relatively small and allows for easy genetic manipulation. Of greater importance, φMR11 uses a binding receptor(s) commonly expressed on S. aureus, as indicated by its highly positive reactivity in the spot test. It also lacks toxin and drug-resistance genes. Finally, to minimize phage inactivation by the bacterial restriction-modification system [34], we are now constructing an alternative host bacterium for the production of φMR11 with methylated CG bases throughout the phage genome.

The data presented here, derived from using an animal model for S. aureus infections, support the therapeutic use of phages in humans. Phages are conceivably present in all kinds of bacteria. To search for and characterize further phages with therapeutic potential may cast new light on treating diverse bacterial infections that are uncontrollable by currently available antibiotics. Moreover, further scientific proof of the in vivo efficacy and safety of phage therapy will validate its clinical use in humans, thereby possibly greatly reducing antibiotic use, which is the major culprit in the recent increase in multidrug-resistant bacteria. Advanced therapeutic phages will provide, as d’Herelle hoped, a promising remedial resource to overcome the “modern plagues” on the condition that an appropriate phage strain and a relevant method for its administration are selected [15, 16, 55–57]. This revitalized therapy also may become a powerful weapon against bioterrorism based on pathogenic bacteria with intentionally introduced antibiotic resistance [22].
References


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