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REVIEW ARTICLE

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Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases

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Abstract Bacteriophage (phage) therapy involves using phages or their products as bioagents for the treatment or prophylaxis of bacterial infectious diseases. Much evidence in support of the effectiveness of phage therapy against bacterial infectious diseases has accumulated since 1980 from animal model studies conducted in Western countries. Reports indicate that appropriate administration of living phages can be used to treat lethal infectious diseases caused by gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Vibrio vulnificus*, and *Salmonella* spp., and gram-positive bacteria, such as *Enterococcus faecium* and *Staphylococcus aureus*. The phage display system and genetically modified nonreplicating phages are also effective for treatment of *Helicobacter pylori* and *P. aeruginosa*, respectively. In addition to phage particles per se, purified phage-encoded peptidoglycan hydrolase (lysin) is also reported to be effective for the treatment of bacterial infectious diseases caused by gram-positive bacteria such as *Streptococcus pyogenes*, *S. pneumoniae*, *Bacillus anthracis*, and group B streptococci. All phage lysins that have been studied to date exhibit immediate and strong bacteriolytic activity when applied exogenously. Furthermore, phage-coded inhibitors of peptidoglycan synthesis (protein antibiotics), search methods for novel antibacterial agents using phage genome informatics, and vaccines utilizing phages or their products are being developed. Phage therapy will compensate for unavoidable complications of chemotherapy such as the appearance of multidrug resistance or substituted microbism.

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Introduction

The worldwide spread of pathogenic bacteria that are resistant to a variety of antibiotics threatens to reduce modern medicine to a state reminiscent of the preantibiotic era. Even though novel antibiotics directed against such drug-resistant bacteria can be developed when extensive funds are committed for research, the pathogens ultimately become resistant to such 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, a method using phages for the treatment of bacterial infectious diseases, was introduced by Félix d'Herelle, who codiscovered phages in about 1920.[1] This discovery occurred about 20 years before practical application of penicillin, the first antibiotic. At the time of its discovery, phage therapy was regarded as a possible treatment method against bacterial infectious diseases.[2,3] Although phage therapy was used to treat and prevent bacterial infectious diseases in the former Soviet Union and Eastern Europe,[4–7] it was abandoned by the West in the 1940s with the arrival of the antibiotic era. However, the ongoing evolution of bacterial multidrug-resistance has recently motivated the Western scientific community to reevaluate phage therapy for bacterial infections that are incurable by conventional chemotherapy.[8–20]

Phage therapy has many advantages over chemotherapy: (1) it is effective against multidrug-resistant pathogenic bacteria because the mechanisms by which it induces bacteriolysis differ completely from those of antibiotics; (2) substituted microbism does not occur because it has high specificity for target bacteria; (3) it can respond rapidly to the appearance of phage-resistant mutants because the phages themselves are able to mutate; (4) the cost of developing a phage system is cheaper than that of developing a new

Table 1. Classification of phages

Order	Family	Morphology	Nucleic acid
Caudovirales	*Myoviridae*		Double-stranded DNA
	Siphoviridae		
	Podoviridae		
	Tectiviridae[a]		
	Corticoviridae[a]		
	Lipothrixviridae[b]		
	Plasmaviridae[b]		
	Rudiviridae		
	Fuselloviridae		
	Inoviridae		Single-stranded DNA
	Microviridae		
	Leviviridae		Single-stranded RNA
	Cytoviridae[b]		Segmented, double-stranded RNA

[a] Lipid containing
[b] Enveloped

antibiotic; and (5) because phages or their products (e.g., lysin, see below) do not affect eukaryotic cells, side effects from phages per se are uncommon. This review summarizes the current state of phage therapy.

Classification of phages

Phages were independently discovered by Twort (1915) and d'Herelle (1917) as factors that could lyse *Micrococcus* (now known as *Staphylococcus*) and dysentery bacillus,[21] and about 5100 phages had been reported by the end of the 20th century.[22] They are classified into 13 families according to morphology, type of nucleic acid, and presence or absence of an envelope or lipid (Table 1). About 96% of reported phages are "tailed phages" composed of an icosahedral head and tail, and all of them have double-stranded DNA as the genome. Tailed phages are classified into three families according to the morphological features of the tail: *Myoviridae* (contractile tail; e.g., KVP20,[23] KVP40,[24–31] KVP241,[32] and T-even phages), *Siphoviridae* (long noncontractile tail; e.g., ϕMR11[33] and λ), and *Podoviridae* (extremely short tail; e.g., T7) (Fig. 1). These three families comprise the order *Caudovirales*.[34] The other phages, which are classified into ten families although they only constitute 4% of the total, are cubic, filamentous, or pleomorphic. They contain double-stranded or single-stranded DNA or RNA as the genome. Although most therapeutic phages are tailed, some cubic phages (ϕX174 and Qβ)[35–37] or filamentous phages (M13 and Pf3) [38,39] have also been used (see later in this article).

Life cycle of phages

Aside from the morphological classification system, phages can be divided into roughly two groups according to their life cycle (Fig. 2): "the lytic phage," which repeats a cycle in which self-proliferation is synchronous with destruction of bacteria (lytic cycle) (e.g., KVP20, KVP40, KVP241, and T-even phages), and "the lysogenic phage," which has a lysogenic cycle in addition to a lytic cycle. In the lysogenic cycle, the phage genome is integrated into the bacterial genome, and the phage genome multiplies cooperatively with the host bacteria without destroying it (e.g., ϕMR11 and λ). Bacterial strains that integrate the phage genome into their genome are known as lysogens, and they are resistant to infection by phages that are genetically related to previously lysogenized phages. Some lysogenic phages have toxic genes in their genome.[16,40–44] For these reasons, the lytic phages are thought to be more suitable therapeutic candidates than lysogenic phages. However, it may be possible to overcome the disadvantages of lysogenic phages by genetic modifications that inactivate genes responsible for lysogenicity and toxin production. In fact, a lysogenization-deficient mutant that we constructed from a parent staphylococcal phage showed higher therapeutic efficacy than the parent (manuscript in preparation). Such a genetic alteration is also presumed to avoid picking up bacterial toxic genes, thereby considerably minimizing a possible disadvantage of lysogenic phages (also see "Problems to overcome").

Mechanism of bacteriolysis by phages

Figure 2 illustrates the general mechanism of bacteriolysis by tailed phages.[45,46] The first step of phage infection is adsorption to the receptor, usually a protein or sugar on the bacterial surface. Phages are able to adsorb to specific bacterial species or to specific strains; phages capable of infecting across bacterial species or genera (so-called polyvalent phages) are few in number. Phage therapy can therefore eradicate target bacteria without disturbing the normal flora. After adsorption, phage DNA is injected into the bacterial cytoplasm, the DNA is replicated, and synthesized multiple copies of DNAs are then taken into the capsid, which is constructed de novo during the late stage of phage infection. Descendant phage particles are completed by the attachment of a tail to the DNA-filled head. Finally, the progeny phages are liberated by the coordinated action of

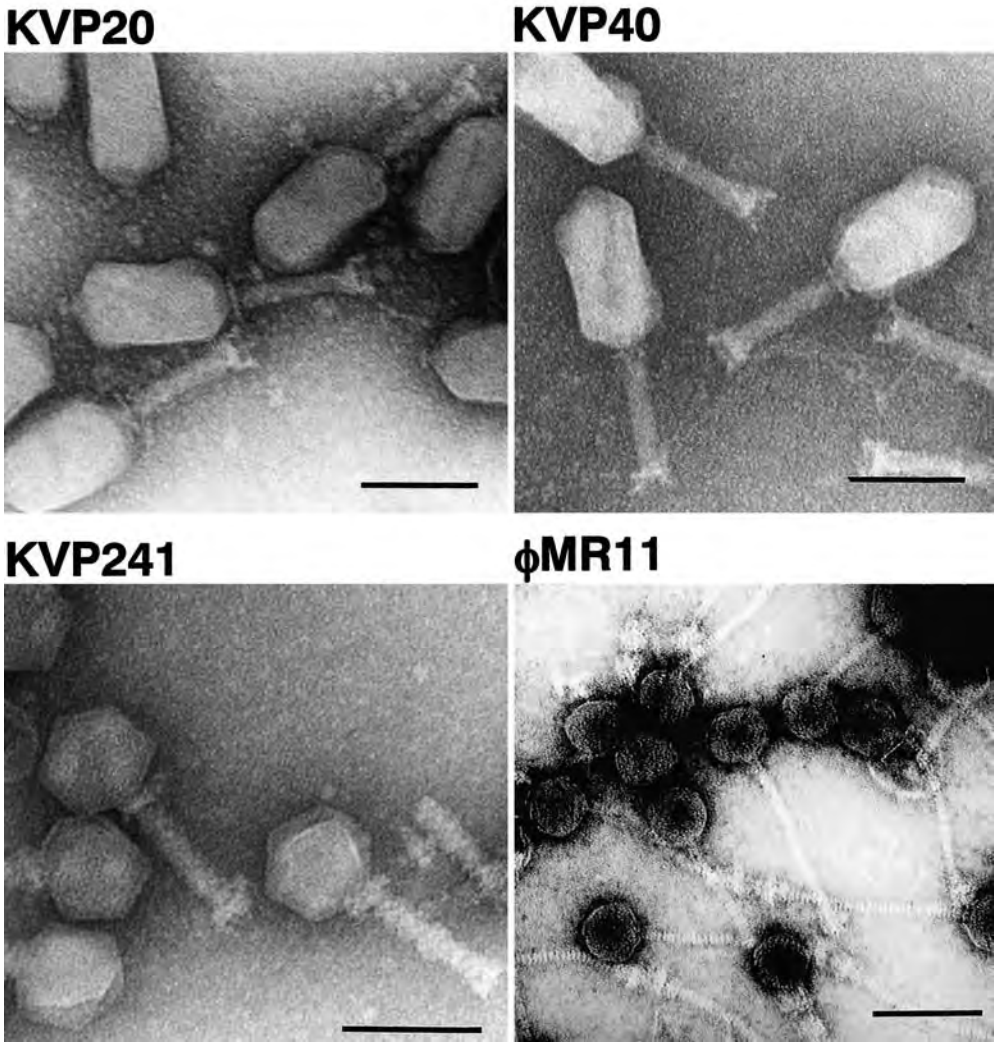

Fig. 1. Electron micrographs of tailed phages. *KVP20*, *KVP40*, and *KVP241* are *Vibrio* phages belonging to family *Myoviridae*; ϕ*MR11* is a *Staphylococccus* phage belonging to family *Siphoviridae*. Phages were negatively stained with 1% ammonium molybdate, pH 7.2 (KVP20, KVP40, and KVP241) or 2% uranyl acetate, pH 4.0 (ϕMR11). *Bars* 100 nm

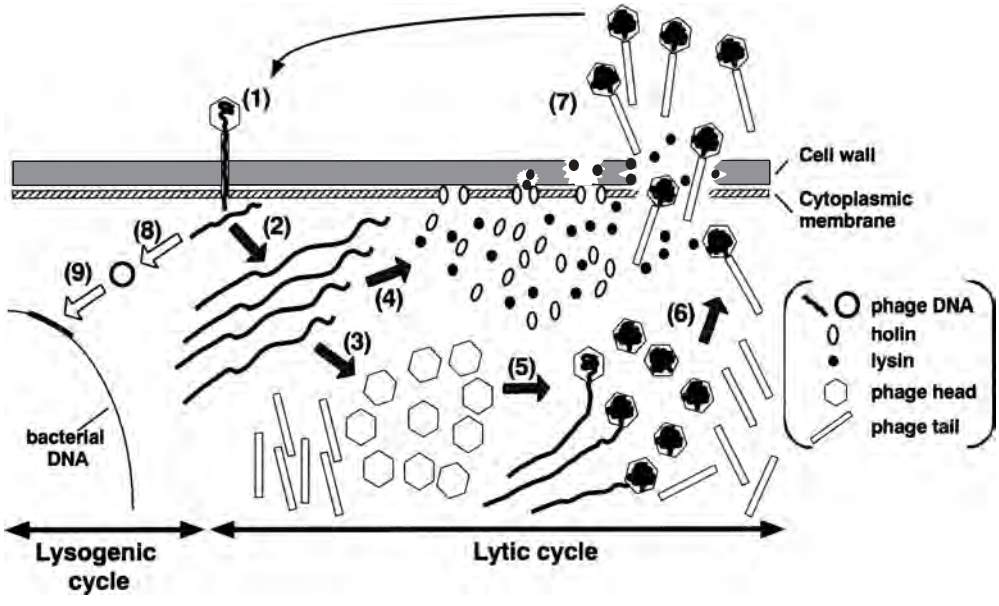

Fig. 2. Schematic illustration of phage-induced bacteriolysis. (*1*) Adsorption and DNA injection; (*2*) DNA replication; (*3*) production of head and tail; (*4*) synthesis of holin and lysin; (*5*) DNA packaging; (*6*) completion of phage particle; (*7*) disruption of the cell wall and release of the progeny; (*8*) circularization of phage DNA; (*9*) integration of the phage DNA into the host genome

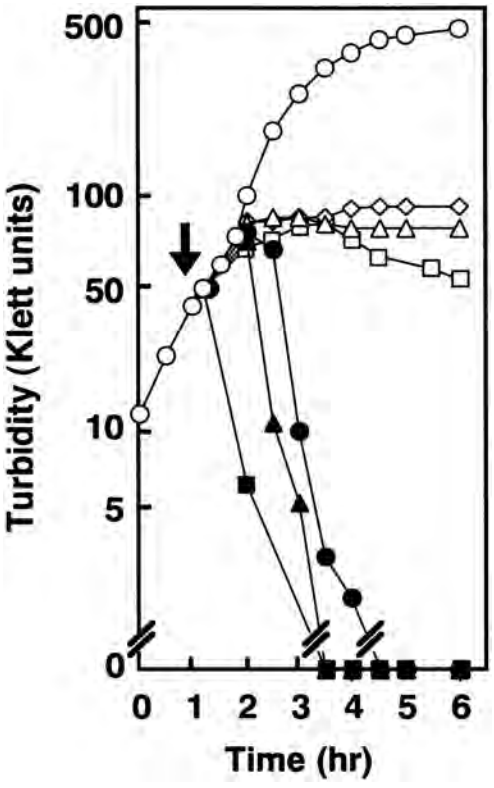

Fig. 3. Effect of addition of phage ϕMR11 or antibiotics (vancomycin, oxacillin, and rifampicin) on *Staphylococcus aureus* growth. *S. aureus* SA37 was cultured in TSBM at 37°C. Bacterial growth was monitored by measuring turbidity with a Klett–Summerson colorimeter (filter #54). *S. aureus* SA37, phage ϕMR11, and TSBM medium were described previously.[61] At the time point indicated by the *arrow*, the phage was added to the culture at multiplicity of infection (MOI) = 0.1 (*closed circle*), 1 (*closed triangle*), or 10 (*closed square*). Vancomycin (*open triangle*), oxacillin (*open square*), or rifampicin (*open rhomboid*) was added to the culture at concentrations of 10 μg/ml at the same time point. An *open circle* indicates the growth of SA37 in the absence of treatments

two proteins, holin and endolysin (lysin), coded by the phage genome. Lysin is a peptidoglycan-degrading enzyme (peptidoglycan hydrolase). Holin proteins form a "hole" in the cell membrane, enabling lysin to reach the outer peptidoglycan layers.[47] As described later, phage lysin is also thought to be a candidate therapeutic agent against bacterial infectious diseases. The released descendant phages infect neighboring bacteria in quick succession. Even if the initial number of phages is less than that of bacteria, the number of phages will exceed that of bacteria after several generations, and the entire bacterial population will eventually lyse (Fig. 3). The bacteriolytic activity of phages seems to be stronger than that of bactericidal antibiotics such as vancomycin, oxacillin, and rifampicin (Fig. 3).

Therapy using living phages

In the 1980s, Smith et al. undertook rigorous investigations into phage therapy for pathogenic *Escherichia coli* infections in a veterinary context,[48–51] thereby reopening this field of research in Western countries. Smith et al. showed that a single intramuscular dose of one anti-K1 phage is more effective for treating mice challenged with *E. coli* intramuscularly or intracerebrally than multiple intramuscular doses of tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulfafurazole. Since Smith's reevaluation, there have been many published reports examining phage efficacy against experimental infections by *E. coli*,[52–54] *Pseudomonas aeruginosa*,[55–57] *Acinetobacter baumanii*,[55] *Klebsiella pneumoniae*,[58,59] *Enterococcus faecium* (vancomycin-resistant strain, VRE),[60] *Vibrio vulnificus*,[61] and *Salmonella* spp.[62] in animal models.

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 and often results in high mortality rates.[63] More than 50% of clinical *S. aureus* isolates in Japan today carry multidrug resistance and are generally referred to as methicillin-resistant *S. aureus* (MRSA).[64,65] Moreover, certain MRSA strains have already acquired low sensitivity or resistance to vancomycin, a unique antibiotic previously considered effective against MRSA, e.g., vancomycin-intermediate *S. aureus* (VISA),[66] or vancomycin-resistant *S. aureus* (VRSA).[67,68] Furthermore, *S. aureus* strains resistant to linezolid, a recently developed novel synthetic antibiotic, are already reported to be present in the United States and Europe.[69–71] We therefore examined the possibility of phage therapy for *S. aureus* infectious disease.

In our previous study,[33] some *S. aureus* phages with theraputic potential were selected, and one of the phages, designated ϕMR11, was representatively used for the following examinations. Intraperitoneal injections (8×10^8 cells) of *S. aureus*, including MRSA, caused bacteremia and eventual death of mice. However, the subsequent intraperitoneal administration of purified phage ϕMR11 (multiplicity of infection ≥0.1) suppressed *S. aureus*-induced lethality. Moreover, inoculation with a high dose of ϕMR11 did not have adverse effects on the host animals. The therapeutic efficacy of ϕMR11 was discernible even when mice were treated 60 min after injection of the bacteria, at which time they were already exhibiting signs of physical deterioration such as reduced activity and ruffled fur. These results suggest that phage therapy against *S. aureus* infection is effective and safe.[33]

Recently, a staphylococcal phage (2×10^9) was shown to prevent abscess formation in a rabbit model of wound infection in which it was injected simultaneously with 8×10^7 *S. aureus* cells into a subcutaneous site. This result indicates that phages might be a valuable prophylaxis against staphylococcal infection.[72] Furthermore, in hand-wash studies in situ, a phage-enriched wash solution resulted in a 100-fold reduction in staphylococcal numbers on human skin compared with a phage-free wash solution.[73] These results provide strong evidence for the usefulness of living staphylococcal phages as agents for therapy, prophylaxis, and disinfection of *S. aureus* infection.

For phage therapy, trapping of phages by the reticuloendothelial system in the spleen was thought to be a major problem,[9,74] but Merril et al. developed an ingenious

method to solve the problem.[52] They succeeded in isolating the mutants, whose stability in the blood increased, by repeating the following procedure eight to ten times: (1) administration of λ (*E. coli* phage) or P22 (*Salmonella typhimurium* phage) into the peritoneal cavity of the mouse, (2) recovery of phages from the blood 7–18h after the injection, (3) multiplication of the recovered phages in vitro, and (4) readministration of the proliferated phages to mice. Interestingly, the long-circulating mutants derived from λ phages had an altered capsid protein (gpE).

The effectiveness of phage administration for the control of fish diseases and for food disinfection has also been documented. Nakai et al. succeeded in saving the lives of cultured fish challenged by *Lactococcus garvieae* and *Pseudomonas plecoglossicida*, which are fish pathogens.[75–78] Phages were also shown to be effective for the elimination of food poisoning pathogens such as *Listeria monocytogenes*,[79,80] *Campylobacter jejuni*,[81–83] and *Salmonella* spp.[83,84] from the surface of foods.

Research on theoretical aspects of phage therapy has also advanced. Unlike antibiotics, the pharmacokinetics of phages in vivo is complicated by their self-replicating nature. It is thus difficult to construct a mathematical model to explain phage–host interaction in vivo. Several theoretical studies have been carried out to address this question.[85–90] Whether a replication threshold density of the host cells in phage therapy exists is a major point of controversy in this field.

Phage therapy using nonreplicating genetically modified phages

A method of eliminating *Helicobacter pylori* using a phage-display technique has been described. A modified filamentous phage, M13, which expressed a coat protein fused with part of an antibody specific to an antigen on the cell surface, was constructed. The modified M13 did not multiply on *H. pylori*, but suppressed its growth in vitro. Furthermore, oral administration of the phage decreased the number of bacteria colonies in the stomachs of mice.[38]

The release of endotoxin (lipopolysaccharide), a component of the outer membrane in gram-negative bacteria, by phage infection is thought to be an important problem in phage therapy. Recently, a unique method to minimize the release of endotoxin in phage therapy against *P. aeruginosa* disease was reported.[39] Hagens et al. constructed a recombinant phage derived from the *P. aeruginosa* filamentous phage, Pf3.[39] In this phage, the export protein gene of the genome was replaced with a restriction endonuclease gene. Although the mutant phage could not multiply in *P. aeruginosa* cells, the restriction endonuclease expressed by the injected phage DNA digested the host genomic DNA and consequentially killed the bacteria with minimal release of endotoxin in vitro. This modified phage reduced mortality rate to a greater extent than the wild type in mice challenged with *P. aeruginosa*.[39]

Utilization of phage lysin

As described earlier in this article, most tailed phages produce peptidoglycan hydrolase (endolysin or lysin) to release their progeny at the final stage of multiplication. Amidase (*N*-acetyl-muramyl-L-alanine bond), endopeptidase (cross-linking peptide bond), or muramidase or glucosaminidase (sugar chain) may be released, depending on the cutting site (Fig. 4).[45,91,92] Lysin is able to degrade peptidoglycan even if it is made to react from outside the cell wall.[92–99] Although penicillin and cephalosporin antibiotics inhibit peptidoglycan synthesis, lysing the bacterial cell upon cell division, phage lysin destroys the peptidoglycan directly, exerting a bacteriolytic effect within several seconds of administration. It can also destroy the cell walls of nongrowing bacte-

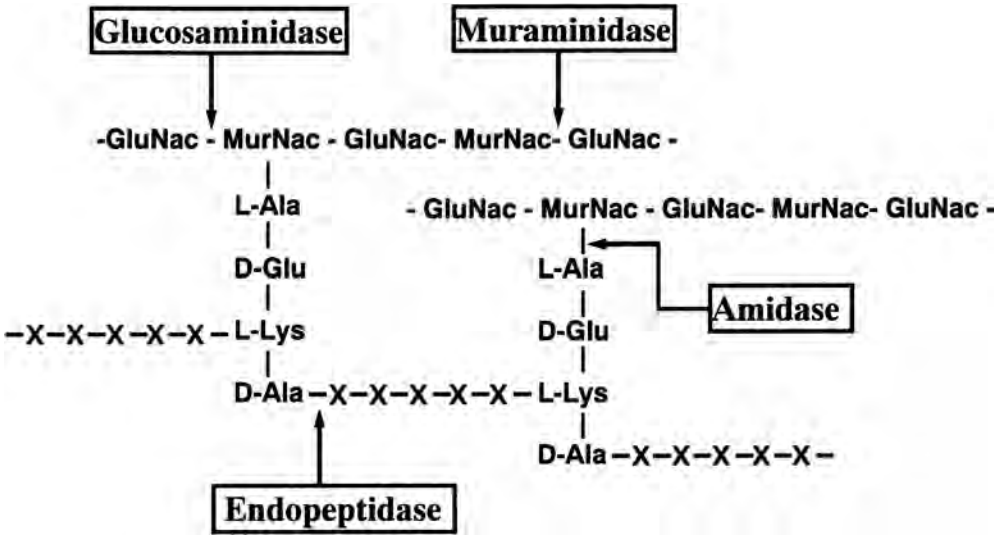

Fig. 4. Attack points of phage-encoded lysins on the peptidoglycan of gram-positive bacteria. GlcNac and MurNac indicate *N*-acetylglucosamine and *N*-acetylmuramic acid, respectively. *X* shows the amino acid composing the interpeptide bridge of the peptidoglycan. The number and type of amino acids formed differ according to the bacterial species

ria, which are insensitive to many antibiotics. The simultaneous administration of two lysins that have different peptidoglycan cutting sites has a synergistic effect.[92,96]

Interestingly, except for the lysin of an enterococcal phage,[99] lysin is fairly specific for bacterial species as well as phages themselves, indicating that phage lysin can very likely eliminate the targeted bacteria without disturbing the normal flora.

In vivo efficacy of lysin treatment has been examined using mice challenged by *Streptococcus pyogenes*,[93] *S. pneumoniae*,[92,94–96] *Bacillus anthracis*,[97] and group B streptococcus.[98] Lysin treatment was shown to be effective not only against localized infections in the nasal cavity or vagina, but also against systemic infections. We obtained similar results using a staphylococcal phage lysin (manuscript in preparation).

Utilization of phages to identify an antibacterial substance

Liu et al. developed a procedure to search for antibacterial agents using phage genomic informatics.[100] They first identified several phage genes coding small peptides that inhibited the growth of *Staphylococcus aureus* and then identified host factors (e.g., components of DNA polymerase or RNA polymerase) targeted by the peptides. Finally, 125000 compounds were screened in vitro for small molecules that interacted with the host factors in a fluorescence polarization assay which used the Oregon Green 488-labeled small peptide. Using this method, they succeeded in discovering new potent antibacterial substances that inhibit the growth of *S. aureus*. The compounds were shown to inhibit DNA or RNA synthesis in *S. aureus*.

Protein antibiotics

Some small phages such as ϕX174 or Qβ, which have single-stranded DNA or RNA, respectively, do not have the genes for holin or lysin proteins, which are expressed by tailed phages to degrade peptidoglycan as described earlier in this article.[35–37] Instead, they produce a protein that inhibits a step in murein monomer synthesis. The ϕX174 gene product, gpE, inhibits MraY, which catalyzes the formation of the first lipid-linked murein precursor, and Qβ gpA2 inhibits MurA, which catalyzes the first step in the murein biosynthesis pathway. Inhibition of synthesis of the cell wall is thought to be a general strategy in small phages that do not produce holin or lysin; their inhibitory gene products are known as "protein antibiotics."[101] If a method can be developed to transport them efficiently into the host cytoplasm through the cell membrane, they would be useful as antibacterial agents.

Vaccine construction

When a plasmid carrying the ϕX174 gene *E* was introduced into an *H. pylori* strain and the gene induced, the *Helicobacter pylori* cells were destroyed, changing into so-called ghosts without cytoplasm.[102] Prophylactic oral vaccination experiments using these *H. pylori* ghosts in the mouse model resulted in a significant reduction in the number of colonized bacteria. This method of vaccine construction may be applicable to other gram-negative bacteria. On the other hand, it was reported that *E. coli* phage λ is a suitable vector for DNA vaccine.[103–105] Animals (mice and rabbits) vaccinated with whole λ particles containing a DNA vaccine-expression cassette that expressed the hepatitis B virus surface antigen (HBs) gene under the control of the cytomegalovirus (CMV) promoter produced antibodies specific for HBs.

Problems to overcome

In phage therapy, the following problems remain to be solved: (i) inactivation of administered phages or lysin by a neutralizing antibody and allergic reactions to them, (ii) appearance of mutants resistant to phages, and (iii) capture and transfer of bacterial toxin genes by phages.

Regarding the first problem, decreases in the therapeutic effect with multiple administrations have not been shown, nor have side effects such as allergies been observed for phages or lysin, although antibodies against them have been detected in mouse blood[98,99] (and our data, not shown). To circumvent this problem, nevertheless, phages or lysins with different antigenicities or with low immunogenicities could be prepared.

Resistance of bacteria to phages is often caused by changes in the phage-receptor molecules in gram-negative bacteria. In phages of gram-negative bacteria, host-range mutant phages, which restore the ability to adsorb to the host, are easily isolated from the original phage population.[106–108] For example, a T-even type phage, Ox2, recognizes OmpA (an outer membrane protein) of *E. coli* as receptor. When OmpA was changed by a mutation, most wild-type Ox2 phages could not adsorb to the mutant bacteria, but some mutant phages that did adsorb were isolated. The mutant phage may use OmpC, another outer membrane protein, as an alternative receptor. When both OmpA and OmpC were deficient, the phage changed to recognize OmpX as receptor. Surprisingly, in the absence of OmpA, OmpC, and OmpX, the phage changed further to recognize lipopolysaccharide as receptor. The fact represents well the coevolution of phages with bacteria, which heghtens the therapeutical value of live (self-multiplicative) phages. On the other hand, there are very few studies on interactions between gram-positive bacteria and their phages, and more research is required for further development of phage therapy.

The problem of capture of bacterial toxin or antibiotic-resistant genes by phages may be overcome by selection

of suitable phages that do not have natural generalized or specialized transduction abilities, or by construction of genetically modified mutant phages against such phages.[109]

Conclusion

Much of the evidence presented in this review strongly shows that appropriately administered phage therapy is very effective for treatment and prevention of many kinds of bacterial infectious diseases, especially those caused by multidrug-resistant bacteria. Currently, many pathogenic bacteria have acquired multiple drug resistance, which is a serious clinical problem. Although some problems remain to be solved, many experts are of the opinion that phage therapy will find a niche in modern Western medicine in the future.[14]

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