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Phage Therapy for Antibiotic-Resistant Bacterial Infections

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Abstract

Antibiotic resistance in bacterial pathogens presents a substantial threat to the control of infectious diseases. Development of new classes of antibiotics has slowed in recent years due to pressures of cost and market profitability, and there is a strong need for new antimicrobial therapies. The therapeutic use of bacteriophages has long been considered, with numerous anecdotal reports of success. Interest in phage therapy has been renewed by recent clinical successes in case studies with personalized phage cocktails, and several clinical trials are in progress. We discuss recent progress in the therapeutic use of phages and contemplate the key factors influencing the opportunities and challenges. With strong safety profiles, the main challenges of phage therapeutics involve strain variation among clinical isolates of many pathogens, battling phage resistance, and the potential limitations of host immune responses. However, the opportunities are considerable, with the potential to enhance current antibiotic efficacy, protect newly developed antibiotics, and provide a last resort in response to complete antibiotic failure.

INTRODUCTION

Host range: the range of bacterial species and/or strains that a phage can infect

Bacteriophages were first described by Felix d'Herrelle and Frederick Twort a little over a century ago, and the therapeutic use of phages has been explored and debated with fluctuating levels of zeal for most of the time since (1). So-called phage therapy was extremely attractive to d'Herrelle and others in the early twentieth century as it offered the first plausible solution for treating bacterial infections. Such infections were common and often serious, and were major factors in determining average human life expectancy, which in 1900 in the United States was 47 years (46.3 for men and 48.3 for women) (2). However, the discovery of antibiotics and their widespread use in the 1940s and beyond proved a more powerful and broadly effective antimicrobial solution. Antibiotics contributed to a large increase in US life expectancy to over 70 years in 1970 (67.1 for men, 74.7 for women) (2). However, phage therapy continued in the Soviet Union during the mid-twentieth century, thanks to the work in Tbilisi, Georgia, pioneered by d'Herrelle (3, 4).

Renewed interest in the therapeutic use of phages often mirrors concerns about the emergence of antibiotic resistance and the prospects of a postantibiotic era (5). We find ourselves in one of those periods today, with antibiotic resistance declared by the World Health Organization to be one of the biggest threats to global health, food security, and development (6). The rapid growth of resistance to antibiotics is driven by their overuse and misuse, but it is not really a surprise, as the bacterial pathogens respond to the overwhelming selective pressures placed upon them. Unfortunately, the development of new antibiotics and new classes of drugs that act differently from extant ones is both expensive and slow, and such drugs are not as attractive for the pharmaceutical industry as they might once have seemed (7). The therapeutic use of phages would seem to be among the best of all potential alternatives to respond to this urgent global need.

If bacteriophages offer such promise for treating bacterial infections, why hasn't phage therapy become widespread? Are the impediments to its success terminally debilitating, or can they be addressed and overcome? Here we explore these questions and examine considerations for the effective therapeutic use of phages, as well as their prospects for further development.

WHAT ARE BACTERIOPHAGES? A BRIEF PRIMER

Bacteriophages (phages) are viruses that infect bacterial hosts. They share all the common characteristics of viruses. Phages cannot replicate on their own and require a bacterial host to reproduce; they are small (50–200 nm) and carry the genetic instructions for rapid and efficient replication. Like most viruses, they are typically specific for a particular bacterial host; any one phage may infect several different species within a genus and most or many strains within a species, but sometimes only one or very few individual isolates of a species. Few phages infect bacteria from different genera and typically can do so only when they are closely related phylogenetically. The bacterial preference of any particular phage is referred to as its host range, which may be very narrow (only a few bacterial isolates support replication) or broad (infecting many different species or even different genera). As we discuss below, host range or host "preference" is a key factor determining the therapeutic potential of phages.

Phages are ever-present in the environment and are abundant; ocean water, for example, contains $\sim 10^7$ phage particles per milliliter (8), and there is estimated to be a total of $\sim 10^{31}$ total phage particles in the biosphere, outnumbering bacteria by about a factor of ten (9, 10). The population is both old and highly dynamic, with constant infections leading to complete turnover of the phage population every few days (11). Not surprisingly, there is enormous genetic diversity among phages, and it is rare to isolate two individuals that are identical genetically, although the extent of variation differs depending on the host (12). However, the genomes can vary from each other in every imaginable way.

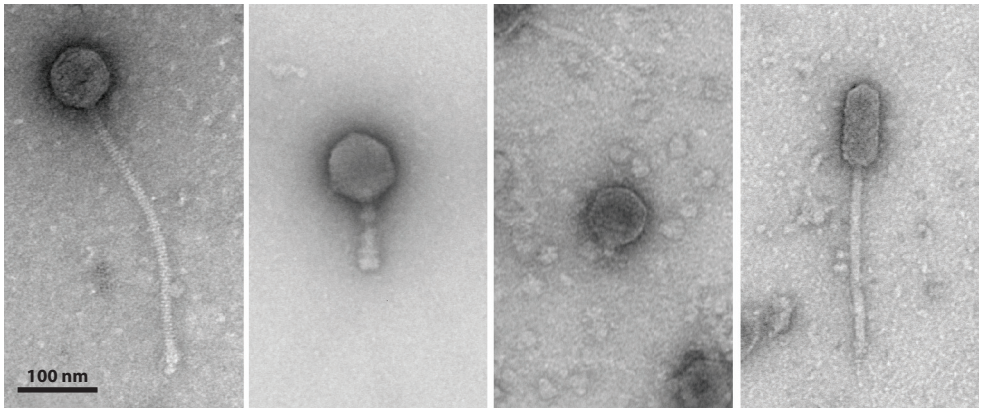

Figure 1

Electron micrographs of actinobacteriophages. All of the mycobacteriophages described to date are members of the *Caudovirales*, the double-stranded DNA (dsDNA) tailed phages. Although there are three morphotypes within the *Caudovirales* based on tail type, all of the mycobacteriophages have either siphoviridae or myoviridae virions; podoviridae have been described for phages of other actinobacteria but not *Mycobacterium*. The figure shows (*left to right*) mycobacteriophages Rich and Flabslab, *Microbacterium* phage Ashton, and mycobacteriophage Xula. The first three illustrate siphoviral, myoviral, and podoviral morphologies, respectively, all of which have isometric heads. At the right is mycobacteriophage Xula, which is siphoviral but has a prolate head.

Although small, phages can be readily visualized by electron microscopy, which reveals numerous and wondrously diverse shapes and sizes. However, the vast majority of phages in the environment are those in the order *Caudovirales*, which carry double-stranded DNA (dsDNA) and have tails. Morphologically, these phages all have a head containing the dsDNA and a tail that contains the receptor recognition functions at its tip. Generally, these phages can be divided into three morphotypes (**Figure 1**), depending on their types of tails: the siphoviridae (long, noncontractile tails), myoviridae (contractile tails), and podoviridae (short, stubby tails).

These dsDNA tailed phages—regardless of their morphology—can also be classified as either lytic or temperate, another distinction that is important for considering therapeutic use (**Figure 2**). Lytic phages are those for which there is only a single outcome of infection: phage replication, lysis (breaking open) of the bacterial cell, and the release of phage progeny. Temperate phages can grow lytically in just the same way, but it is not the sole outcome of infection. An alternative outcome is the establishment of lysogeny: The phage DNA, a prophage, is stably maintained within the bacterium (usually by integration into the host genome); the genes needed for lytic growth are switched off; and the lysogenic cell continues to grow, divide, and thrive. Both types of phages are prevalent in the environment, and it is a mistake to think of temperate phages as uncommon (13). This is illustrated by the hundreds of thousands of bacterial genomes that have been sequenced, many (perhaps most) of which carry at least one prophage in their genome; some can carry dozens, constituting a substantial proportion of their overall genome length. Furthermore, temperate phages and their cognate prophages often carry and express genes that influence bacterial physiology, pathogenicity, metabolism, and sensitivity to other phages (14, 15).

Lytic phage: a phage with a single outcome of infection—phage production and cell lysis

Temperate phage: a phage that can form stable lysogens carrying a prophage

EXPERIENCES IN PHAGE THERAPY

Over the past 100 years, there have been numerous examples of the therapeutic uses of phages, although many are anecdotal without records indicating outcomes or other clinical details.

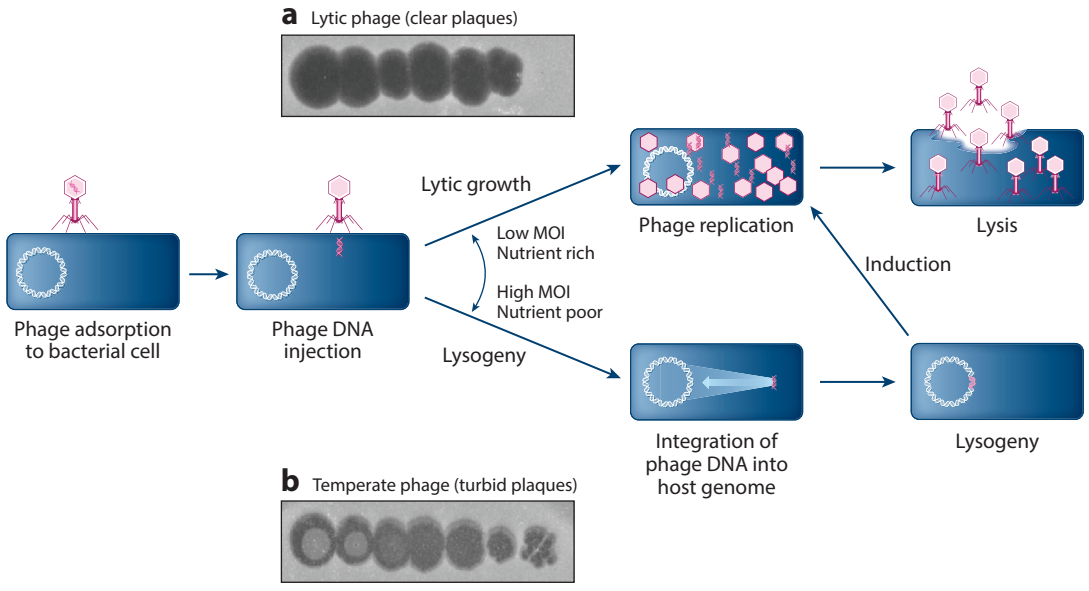

Figure 2

Bacteriophage life cycles. Lytic phages typically infect the host bacterium through adsorption, DNA injection, DNA replication, assembly of progeny phage particles, and lysis of the bacterial cell. The only outcome is phage growth and cell death, as illustrated by serial dilutions of a lytic phage stock on solid medium (*a*). The spots appear dark, compared to the surrounding lawn of cells, because all the cells in the spots are dead. Temperate phages similarly adsorb to the host bacterium and inject their DNA, but then "choose" to go through lytic growth or establish lysogeny in which the phage genome is integrated into the host chromosome (to form a prophage), and the lytic genes are switched off. This is illustrated by serial dilutions of a temperate phage stock on solid medium (*b*); turbid plaques form in which bacterial lysogens are growing. The path followed is influenced by many factors including the multiplicity of infection (MOI) and nutrient availability. Lysogens can be readily recovered from turbid plaques, purified, and stably propagated. However, the culture medium usually contains phage particles produced from low-level spontaneous induction of the prophage.

In general, there are two distinct types of applications. First is compassionate use, when an infection is life threatening or if a serious infection cannot be controlled using currently approved methods and drugs (16). Such interventions are usually personalized, with experimental demonstration that one or more phages can infect the specific bacterial strain causing the infection, at least in vitro. These interventions usually proceed on the assumption that efficient phage killing of the bacteria in the laboratory can be repeated in the patient. The prescreening of the specific infectious strains for phage sensitivity is critical because bacterial isolates can vary enormously in their phage infection profiles, and only rarely can it be confidently predicted that one or more phages will infect the bacterial clinical isolate without prior testing (17, 18).

The second approach is the use of randomized clinical trials that can inform about safety, efficacy, dosage, regimen, and other clinical parameters. Relatively few clinical trials have been conducted for phage therapy, although their successes and failures are extremely informative (19, 20). A substantial challenge in these trials is to assemble a phage preparation that is effective for the wide range of clinical isolates of the target pathogen. Below, we provide some examples of both approaches.

eIND: emergency Investigational New Drug

Personalized, Compassionate-Use Cases

Three seminal case reports of personalized phage therapy under the US Food and Drug Administration (FDA) emergency Investigational New Drug (eIND) mechanism appeared between

2017 and 2019. These cases played major roles in rekindling interest in phage therapy for serious bacterial infections in the United States (17, 21). In each case, traditional antimicrobial therapy had failed, and phages directed at specific infectious organisms utilized in combination with antibiotics were associated with successful microbiological and clinical responses. Critical lessons were learned from each case that have helped frame discussions about potential clinical niches for phage therapeutics and about the systematic knowledge base that would be required to develop phage therapeutics from a series of anecdotal experiences to a scientifically grounded area.

BRED: bacteriophage recombineering of electroporated DNA

Engineered phages: phages that have been genetically manipulated to remove or add genes

The first case was a man with a disseminated *Acinetobacter baumannii* infection who was near death after 5 months of failed conventional antibiotic therapy (17). This case demonstrated that multiple phages specifically active against a multi-drug-resistant organism could be identified and prepared at sufficient purity for parenteral administration on a timeline that was clinically relevant. The case also demonstrated that high doses of phages could be delivered to multiple disseminated sites of infection by repetitive intravenous infusion. In addition to the observed clinical response, the selection of phage-resistant *A. baumannii* made it clear that the phage exhibited a direct antimicrobial effect in vivo. Furthermore, in association with the development of phage resistance, clinical isolates of *A. baumannii* downmodulated capsular polysaccharide production and lost fitness in a silkworm invasion assay, demonstrating a key additional mechanism by which phage therapy might have a beneficial clinical effect. Finally, the investigative team demonstrated that a second-generation phage cocktail could be generated that was active against the *A. baumannii* that had been selected by the first round of phage therapy. This provides an avenue for multiple rounds of phage therapy should selection of phage-resistant mutants compromise clinical response.

The second seminal case was one that exploited two critical additional potential mechanisms by which phages might be used in conjunction with antibiotics in highly impactful ways (21). In this case, a man with an implanted Dacron aortic arch prosthesis had failed to respond to multiple courses of parenteral antibiotics over a 3-year period. After a prolonged period of antibiotic suppression without sterilization, the patient was left with an open sinus to the site of the graft that was continuously draining *Pseudomonas aeruginosa* despite prolonged parenteral ceftazidime therapy. The patient received a lytic phage (OMKO1) that bound to the outer membrane protein M of the mexAB- and mexXY-multidrug efflux systems of *P. aeruginosa* (22). Targeting these efflux pumps increased the susceptibility of this organism to ceftazidime by twofold and to ciprofloxacin by tenfold. In addition to the impact of OMKO1 on antibiotic susceptibility, the phage was shown to be highly disruptive to the biofilm deposited on the implanted device. This phage was instilled into the abscess cavity along the chronic aortocutaneous fistula. After an additional month of antibiotic therapy, antibiotics were discontinued, and the graft remained sterile.

The third case was a young woman with cystic fibrosis who had undergone bilateral orthotopic lung transplantation for a chronic *Mycobacterium abscessus* pulmonary infection that ultimately ceased responding to antimycobacterial agents (18). Her *M. abscessus* infection returned 5 weeks following lung transplantation and disseminated to her sternal wound, her lungs, her skin, and her porta hepatitis despite maximal combination antibiotic therapy. A phage cocktail directed at her *M. abscessus* infection was devised by screening a library of phages initially selected for activity against *M. smegmatis*. In this case, it was necessary to delete the repressor gene from two of the key phages in the regimen to convert them from temperate to lytic phages using bacteriophage recombineering of electroporated DNA (BRED) (23). This patient was treated parenterally with a combination of three lytic phages, and her disseminated atypical mycobacterial infection resolved. This case represented the first use of engineered phages and extended the reach of phage therapeutics to mycobacterial pathogens.

Since these three cases, a proliferation of cases of personalized phage therapeutics has occurred in the United States and in Europe (24). While not all of these cases have resulted in successful

outcomes, they have laid the groundwork for the clinical trials that have begun in earnest over the past 18 months.

Clinical Trials

For phage therapy to advance from individual case experiences to systematic use in clinical medicine, rigorous clinical trials modeled after those used to develop traditional small-molecule antimicrobial agents are needed. These trials will require a rich underpinning of translational endpoints that will better define key elements of phage therapy, including clinical conditions most likely to benefit from phage therapy, dosage, routes of administration, pharmacokinetics, pharmacodynamics, optimal valency for combination therapy, and optimal use in conjunction with antibiotics. At this writing, over 30 clinical trials of phage therapy have been recorded in the Cochrane register of controlled trials and/or ClinicalTrials.gov. These trials focus on multiple clinical indications including pneumonia, burns, urinary tract infections, atopic dermatitis, implanted prosthetic devices, otitis, and gastrointestinal infections.

Several recently completed clinical trials have provided key lessons in phage therapeutics. Perhaps the most illuminating clinical trial of the past decade was one directed at *P. aeruginosa* infection of burn wounds (19). This open-label, controlled trial compared topical treatment with a fixed cocktail of 12 antipseudomonas phages to 1% sulfadiazine silver emulsion cream. Though interpreted as a negative study when it was stopped for futility before reaching full enrollment, this study provided several key lessons for subsequent studies. The first is that it is critical that the phages in the treatment regimen are active against the organisms under treatment. In this study, all patients with topical isolation of *P. aeruginosa* from a burn were eligible to be enrolled in the trial whether or not their specific *P. aeruginosa* strain was susceptible to the phages in the cocktail used. A post hoc analysis demonstrated that the subset of patients with organisms that were susceptible to phages in the treatment regimen did see clinical benefit from the treatment regimen. Second, the study demonstrated that it was critical to evaluate phage–phage interactions in advance of combining them in a phage cocktail and to critically evaluate phage stability between the production line and the bedside. To the investigators' credit, these issues were detailed in the report of the clinical trial and now provide guidance to all studies that follow.

Another recently reported clinical trial of T4 phage in the oral treatment of acute bacterial diarrhea in Bangladesh demonstrated the central importance of understanding pharmacokinetic and pharmacodynamic issues prior to initiating large-scale clinical trials (20). In this study, the investigators sought to use T4 coliphages to treat infantile diarrhea hypothesized to be caused by enteropathogenic *Escherichia coli*. The study was halted after an interim analysis demonstrated no clinical benefit. At this interim analysis, the investigators determined that the multiplicity of infection achieved by the phage regimen failed to reliably trigger a self-sustaining replicative cycle in the gut lumen of treated individuals.

A final study of the use of a fixed phage cocktail directed at *Staphylococcus aureus* bacteremia demonstrated the challenges inherent in studying serious infections with binary outcomes. Thirteen patients with *S. aureus* infections were entered into a single-arm noncomparative trial and given a three-phage cocktail intravenously, twice daily, for 14 days (25). The cocktail was found to be safe and well tolerated by all patients in this study. Over half of the patients (62%) showed clinical improvement after phage therapy, with the other half exhibiting various issues seemingly unrelated to the phage. Although the study added to the body of data indicating that phages can be parenterally administered to critically ill patients, it also pointed out the challenges inherent in demonstrating benefits over current standard-of-care therapeutics.

These "failures" notwithstanding, the stage is now set for an era of clinical trials to determine phages' antimicrobial and clinical properties within a framework that builds on these early experiences and on the principles of antibiotic development that have provided the body of evidence underlying contemporary antimicrobial therapy. Phages are, after all is said and done, merely "living antibiotics" (26).

Endotoxins: toxic lipopolysaccharides in the cell wall of Gram-negative bacteria

PFU: plaque-forming units

KEY CONSIDERATIONS IN PHAGE THERAPY

Desirable Properties of Therapeutic Phages

In choosing phages for therapeutic use, there are several general properties to be considered. First, the phages should kill the specific bacterial pathogen efficiently in vitro, without significant levels of bacterial survival. Second, the phages should be easy to propagate, to produce in high-titer preparations, and to purify. Third, the phages should be stable over a range of concentrations, such that storage for extended time periods at refrigerator temperatures is not associated with substantial loss of infectivity. Third, the phage preparations must be sterile and free of endotoxins or other harmful contaminants. Fourth, the phage genomes should not include any genes known or suspected to be toxic. Fifth, the phages should not be able to act as generalized transducing phages. Some of these parameters can be deduced bioinformatically, but others typically require experimental validation.

It is also helpful to know the frequency and mechanisms of resistance to each phage, and most importantly, the coresistance patterns. For example, if a clinician is using two phages in a cocktail, it is advisable that they do not use the same cellular receptor, as receptor loss will confer resistance to both phages. Genetically distinct phages (i.e., with few if any shared genes) are less likely to use the same receptor and thus more acceptable as cocktail constituents. However, there are numerous mechanisms for resistance other than receptor loss, and some but not all may confer cross-resistance between phages. Experimental validation of the resistance patterns is desirable, but not commonly determined.

Finally, it should also be noted that for some phages, behaviors in vitro and in the patient may be quite different. In vivo, bacteria may form biofilms, and they may live in hypoxic or intracellular environments in which the phage particles either have poor access to their hosts or do not kill them efficiently. Further studies are needed to understand the circumstances in which differences between in vitro and in vivo behavior lead to poor clinical outcomes.

Phage Preparation, Dosage, and Administration

Most bacteriophages can be amplified to high titer ($>10^{10}$ PFU/ml) either in liquid or on solid media, concentrated, and purified to substantial homogeneity. A typical preparation at a research laboratory scale may yield $>10^{12}$ phage particles, sufficient for hundreds or even thousands of doses depending on dosage and stability. For compassionate-use cases, it is important that the preparations are sterile—usually by commercial certification—and have endotoxin [typically lipopolysaccharide (LPS)] levels below FDA-approved levels. For phages prepared on Gram-negative bacterial hosts, additional steps may be required to reduce endotoxin levels (17), whereas phages grown on non-LPS-containing hosts are typically endotoxin free (18). For clinical trials and commercial use, phages will need to be prepared using good manufacturing practices, a requirement that presents additional costs and challenges. Phage stability is an important factor, and it varies greatly for different phages and different concentrations. However, many phage preparations retain viability when stored cold (4–10°C) and concentrated. Diluted phage samples (e.g., 10^6 PFU/ml) may lose viability rapidly (19).

There is rather little clinical evidence that defines optimal dosages of phages or the pharmacokinetic parameters of therapy. Moreover, phage pharmacokinetics differs from that of antibiotics in that killing of the pathogen is associated with phage amplification, so phage levels in a patient may fluctuate substantially depending on when the phage infection occurs and where the bacterial infection is located. Intravenous administration has the advantage of delivering a defined dosage, and twice-daily administration at 10^9 PFU/dose has been used in several compassionate-use cases (17, 18, 27); however, different dosages or different regimens could be more or less effective in human patients. Similar dosages (10^9–10^{10} PFU) have been given by nebulization for pulmonary infections, especially for cystic fibrosis patients (28), although the choice of nebulizer is critical, as some damage the phage particles and can reduce the effective dose by up to a million-fold (29). Various dosages have been used for topical treatment of skin infections and direct injection into infected joints (30). Because the safety profile of phages is generally very good, complications from relatively high dosages are less concerning than they are for powerful small-molecule antibiotics.

Potential Impediments to Phage Therapy: Strain Variation and Resistance

The ability of phages to efficiently kill bacteria in vitro makes them attractive antimicrobials for clinical use, but two key issues commonly arise. First, for some bacterial pathogens, there is substantial variation among clinical isolates of strains belonging to the same species, and this variation extends to differences in the phage infection profiles. Thus, a phage that is effective for one patient may be inappropriate for another patient if the phage does not infect and kill both strains. This variation is substantial for some pathogens (e.g., *M. abscessus*, *S. aureus*) but less problematic for others (e.g., *Mycobacterium tuberculosis*) (31–33). The problem has two potential solutions: screening for and identifying phages that have the widest possible host ranges infecting as many different clinical isolates as possible, and developing cocktails containing sufficient phages to ensure high prospects that at least one will infect a high proportion of naturally occurring isolates. However, constructing, evaluating, and maintaining complex cocktails can become expensive and logistically complicated.

A second impediment is resistance to bacteriophages. In many different systems, phage resistance occurs quite frequently (e.g., 10^{-5}–10^{-7}), similarly to resistance to antibiotics, although there is of course substantial variation. Two approaches to this are either to use a cocktail with two or more phages for the target strain or to administer single phages sequentially. However, addressing both strain variation and resistance presents a challenge, as constructing a cocktail in which two or more phages will be active against most clinical isolates of a particular pathogen can be very challenging.

Do Immune Reactions Interfere with Phage Therapeutic Utility?

When phages are used topically or for short periods, immune reactions to the phages are not a substantial concern. However, when administered through other routes—especially intravenously—there is the possibility of immune reactions that interfere with phage function. For applications where the patient is immunosuppressed—such as following organ transplantation—immune reactions may be negated, at least temporarily, as was observed with treatment of a young cystic fibrosis patient with a bilateral lung transplant (18). In contrast, administering the same phage cocktail to an older immunocompetent patient with bronchiectasis resulted in treatment failure due to a robust IgG-mediated antibody response that was strongly neutralizing to at least two phages in the cocktail (34). Immune reactions against phages have been seen in both patients and animal model systems, although they are not necessarily neutralizing (35–38).

Although there is a clear imperative to learn more about the incidence, timing, and specificity of immune reactions following intravenous administration, several possibilities should be considered. First, if multiple phages are available for a given isolate, it may be helpful to administer these sequentially rather than together in a cocktail, so as to extend the potential treatment regimen. Second, it may be especially important to experimentally map the landscape of immunological cross-reactivity for phages of any particular pathogen, such as to determine whether a neutralizing reaction to one phage inactivates others. Finally, in some extreme clinical circumstances, administration of immunosuppressive drugs such as rituximab together with the phages could perhaps be contemplated.

CRISPR: clustered regularly interspaced short palindromic repeats

Are Temperate Phages Useful Therapeutically?

It is commonly assumed that phages that always replicate using the lytic life cycle are the only ones that are useful therapeutically, because the sole outcome of infection is lysis (death) of the host bacterial cell and release of progeny phages. Temperate phages are clearly undesirable as-is, as a high proportion of infected bacterial cells survive the infection and emerge as lysogens carrying the virus as a prophage (**Figure 2**). However, temperate phages can be engineered so that the lysogenic state is prevented by removing part or all of the repressor gene, and this has been achieved therapeutically with a positive outcome (18). Given a choice between a lytic and an engineered temperate phage, the simplest choice is to use the lytic phage. However, for some pathogens (e.g., *Mycobacterium* spp.), most of the phages with the desirable host preferences are temperate (12), and there simply is not a large suite of lytic phages available for use. In these cases, engineered temperate phages are helpful options.

Users of engineered temperate phages face several further considerations. First, removing the repressor gene may be insufficient, and it is desirable to also remove the integrase gene to prevent chromosomal integration. For example, we have observed instances where, after challenge with a repressor-defective but integration-proficient phage, survivors carry parts of the phage genome integrated at the normal chromosomal site (33). The phage segments must lack (or not express) genes toxic to the bacterium but plausibly express genes conferring resistance against superinfecting phage particles (33). Second, addition of genes to the phages makes them technically recombinants, so they may be categorized as genetically modified organisms and require additional regulatory approval. However, using engineering tools that are efficient and enable precise removal of genes can avoid this, and BRED and CRISPY-BRED (BRED combined with CRISPR technology) engineering strategies have been described that accomplish this (23, 39). Finally, it is plausible that some clinical isolates may naturally carry prophages that are related to the putative therapeutic phage, with the possibility that recombinants could be formed that reacquire repressor, integrase, or other functionalities, leading to production of phage types that are no longer therapeutically active. However, this scenario has yet to be observed clinically.

The Utilities of Phage Genomics

The availability of a well-annotated complete genome sequence is important for any phage to be used therapeutically. Although the genetic blueprint cannot readily inform about the phage's host range, it can provide other key insights. First, if a phage forms clear plaques (**Figure 2**) and efficiently kills its host, it cannot be assumed to be a true lytic phage, as it could be a naturally occurring lytic derivative of a temperate parent (40). Second, genome sequencing can help determine the system used for DNA packaging, which can predict the likelihood that the phage is capable of generalized transduction (transfer of bacterial genes) (41). Specifically, phages with terminally redundant, circularly permuted genomes are typically packaged using a headful

packaging system and are likely to be generalized transducers, an unpredictable and somewhat undesirable property for therapy. In contrast, in phages with defined short single-stranded DNA ends derived from cos-packaging systems, generalized transduction is predicted at only very low, even undetectable, levels. Third, the sequence can reveal the presence of undesirable genes such as those encoding toxins that could enhance pathogenicity. In practice, identifying such genes can be bioinformatically challenging because phage genomes are replete with genes of unknown functions. Well-studied toxin genes can be readily identified, but it is difficult to know with any certainty if any of the lesser-characterized genes might also have undesirable or toxic functions. Last, genomic data can suggest which phages are best combined into cocktails if no other data about resistance mechanisms are known. Phages are highly diverse genetically, so any two phages may share substantial sequence similarity or none at all. In general, phages that are unrelated genetically are less likely to share receptors and DNA entry mechanisms, and thus are less prone to the development of cross-resistance.

Phage Genome Engineering

Engineering of phage genomes may be necessary in some circumstances, but it also offers an array of possibilities for expanding phage utility. The simplest application is for deleting lysogeny genes, such as those encoding integrase and repressor, but adding genes to enhance killing or to alter host responses is feasible (42). Several technologies have been developed for phage genome engineering, including recombination between phage and plasmid recombinants (43), shuttle phasmid construction (44), direct cloning (45), recombineering (23), CRISPR-Cas selection (46), and a combination of recombineering and CRISPR-mediated counter-selection (39). Synthetic approaches building with oligonucleotides or yeast-mediated assembly have also been described (42). The keys to effective phage genome engineering largely revolve around efficient systems for generating recombinants and distinguishing desired recombinants from the parental phage. In the BRED strategy, phage genomic DNA and a synthetic DNA substrate are coelectroporated into bacterial cells expressing recombineering functions, and progeny plaques are recovered (23, 47). For constructing simple deletions, 5–50% of plaques contain the desired mutant allele and can be readily identified using polymerase chain reaction. The approach has been applied to phages of *Mycobacterium*, *Klebsiella*, *E. coli*, and *Salmonella* (23, 48–51) and has the advantage that no selection for recombinants is needed; precise unmarked deletions can thus be constructed. However, construction of more complex recombinants with gene insertions or replacements is less efficient. The derivative CRISPY-BRED approach couples BRED with CRISPR-mediated counterselection against the parent phage, such that only the desired recombinants are recovered (39). Because not all phage genomes can be efficiently transfected (i.e., electroporation into the bacterial host fails to efficiently produce plaques), the related CRISPY-BRIP (bacteriophage recombineering with infectious particles) approach can be used (39).

Phage–Antibiotic Interactions

In compassionate-use interventions, phages are typically added to a pre-existing antibiotic regimen, so phage–antibiotic interactions are likely, including synergism, antagonism, or additive effects. Interestingly, although antibiotics could interfere with phage replication, examples of antagonism are rare (52). However, in the first of the case studies described above, phage treatment of the *A. baumannii* infection may have selected for increased sensitivity to minocycline, which was also being administered (17). In the second compassionate-use study described above (21), the OMKO1 phage targeted a drug efflux pump, and phage treatment was associated with increased

sensitivity to both ceftazidime and ciprofloxacin, likely associated with the pressure for resistance to phage infection.

Interactions between *E. coli* phages and antibiotics were explored by means of a large matrix of antibiotic and phage concentrations using a synogram (53). Several instances were observed where phages provided an adjuvating effect by lowering the minimum inhibitory concentration for drug-resistant strains, although the effect was highly dependent on both the phage and the antibiotic. It is plausible that these interactions occur with other phages and pathogens and could be a major factor in phage efficacy.

Synogram: a representation of the combined effects of phages and antibiotics on bacterial killing

Phage Resistance

Resistance to phages is anticipated when they are heavily challenged, and strategies to address resistance are critical. However, the frequency and impact of resistance vary greatly in different bacteria (54). It is notable, for example, that resistance was observed after administration of the first series of phages for the *A. baumannii* infection discussed above, requiring switching to a different set of phages to which the resistant strain was sensitive (17). And for many bacteria, phage-resistant survivors can be recovered at frequencies of up to 10^{-5} from in vitro grown bacterial cultures. However, for many clinical isolates of *M. abscessus*, resistance in vitro appears to be less common and often below a limit of detection of about 5×10^{-7} (55). These variations can be accounted for in part by whether loss or variation in a phage receptor is tolerated without loss of viability. But while receptor variation is commonly involved in resistance, there are numerous other mechanisms, including restriction, CRISPR-Cas, abortive infection systems, and prophage-encoded defenses (56).

Frequencies of phage resistance may differ in vivo, where there are other pressures on the bacterial pathogen, especially for maintenance of virulence and antibiotic responsiveness, although resistance is not always observed (57). For example, some resistance mechanisms may not alter viability in vitro but could negatively impact survival in the pathogenic context. Resistance may also alter antibiotic susceptibility, and phage treatment of the *A. baumannii* case discussed above likely enhanced antibiotic sensitivity (17, 58). Isolation of *Pseudomonas* phages that specifically require a drug export pump for infection offers a powerful approach for ensuring tradeoffs between phage resistance and antibiotic susceptibility, as phage-resistant derivatives in which the pump is defective sensitize the pathogen to multiple drugs (22).

Regulatory Considerations

Regulatory approval for phage therapies varies by nation and whether a clinical trial or compassionate use is proposed. Compassionate use in the United States typically requires an application to the FDA for an eIND use from the supervising clinician, and approval of the local institutional review board. If the phage is prepared at another institution, then transfer of the materials typically requires a material transfer agreement. There may be additional requirements for consent forms and related paperwork. Clinical trials require an IND from the FDA.

SUMMARY AND FUTURE PROSPECTS

Interest in the therapeutic applications of phages has waxed and waned over the past 100 years, but the idea is enjoying renewed interest today, in part because of the alarming increase in antibiotic-resistant bacterial infections. It is unlikely that phages will ever replace antibiotics because of their relatively narrow specificity, as well as their large size, which may limit access to bacteria in some types of infections. However, it increasingly appears that phages are likely to be

important additions to the antimicrobial arsenal, and greater clarifications of their roles can be anticipated over the next few years.

SUMMARY POINTS

1. Bacteriophages have considerable potential for treating antibiotic-resistant bacterial infections.
2. Specificity of any particular phage may be restricted to only a small subset of clinical isolates of a pathogen.
3. Phages generally have very good safety features, and escalating dosage is not required.
4. Phage preparations for therapy must be sterile and endotoxin free.
5. For compassionate use, the patient strain should be shown to be sensitive to phages proposed for therapy.
6. Cocktails of more than one phage can help to minimize phage resistance.
7. Availability of therapeutically useful phages varies greatly from pathogen to pathogen.

FUTURE ISSUES

1. There is a strong need for clinical trials of phage efficacy for multiple pathogens, infections, and diseases.
2. Advancing our understanding of phage specificity will expand the phage repertoire and broaden therapeutic options.
3. Naturally occurring phages have great potential, but we know little about their biology, and synthetic phages are very attractive.

DISCLOSURE STATEMENT

G.F.H. is a paid consultant for Tessera Therapeutics and Janssen Inc. R.T.S. is a paid consultant to Vir Biotechnology and to LyseNTech and has stock options in Antiva Biosciences and CytoDyn. He previously served as an uncompensated member of the AmpliPhi Scientific Advisory Board.

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