

REVIEW ARTICLE

Phage therapy—constraints and possibilities

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Abstract

The rise of antibiotic-resistant bacterial strains, causing intractable infections, has resulted in an increased interest in phage therapy. Phage therapy preceded antibiotic treatment against bacterial infections and involves the use of bacteriophages, bacterial viruses, to fight bacteria. Virulent phages are abundant and have proven to be very effective *in vitro*, where they in most cases lyse any bacteria within the hour. Clinical trials on animals and humans show promising results but also that the treatments are not completely effective. This is partly due to the studies being carried out with few phages, and with limited experimental groups, but also the fact that phage therapy has limitations *in vivo*. Phages are large compared with small antibiotic molecules, and each phage can only infect one or a few bacterial strains. A very large number of different phages are needed to treat infections as these are caused by genetically different strains of bacteria. Phages are effective only if enough of them can reach the bacteria and increase in number *in situ*. Taken together, this entails high demands on resources for the construction of phage libraries and the testing of individual phages. The effectiveness and host range must be characterized, and immunological risks must be assessed for every single phage.

Key words: *Antibiotic resistance, bacteriophage, phage, phage therapy*

Introduction

The possibility of using bacteriophages therapeutically has received renewed interest in recent years due to increased difficulties to cure infections caused by antibiotic-resistant bacterial strains, and by the increased knowledge of phages, including the possibility to characterize phages genetically by sequencing their entire genomes. Phage therapy involves the use of natural intact phages (bacterial viruses) for treatment of bacterial infections, and there are reasons to believe that this is a good idea; phages are abundant in nature, are easy to isolate, and they kill bacteria very effectively, at least in controlled laboratory experiments. Treating bacterial infections in humans with phages is, however, something completely different, which in addition does not function in the same way as treatment with antibiotics (1–4).

The scope of this paper is limited to a review of problems associated with phage therapy utilizing

unmodified selected phages, given their special nature and practical limitations. Other applications of phages or phage-derived products will not be considered here, but phages can also be used as vectors for delivery of materials into bacterial cells e.g. mediating genes coding for peptides that increase the sensitivity to a certain antibiotic (5), or to supply endolysins that can kill a bacterium by degrading the cell wall (6,7).

At a first glance, it seems that there are several advantages of phage therapy with virulent phages, and there are also many reports on successful treatments (see (8) for a historical background to phage therapy and a review of cases). A phage treatment is targeted and specific against just one bacterial clone, and does not affect the normal bacterial flora, which reduces the risk of secondary infections often associated with antibiotic treatment. It might also be more efficient since the phages thrive and multiply as long as the specific host is present, while antibiotics commonly are excreted and/or degraded. After an infection is

cured, phages are decomposed, whereas antibiotics may persist in nature (9). Moreover, phages are equally efficient killers of sensitive and antibiotic-resistant bacteria. Although phage therapy may have advantages, there are also major problems that must be addressed, and where the differences between applying phage therapy as opposed to treating an infection with antibiotics depend on the special nature of phages (9,10). Phage therapy requires specific knowledge of phage biology. Differences and problems that arise can be outlined within the following four areas: 1) the large natural variation of phages and target bacteria; 2) the emergence of resistance against phages; 3) the special pharmacology of phages during therapy; and 4) the conceivable immunologic responses. It is, however, important to stress that all of these problems need not to be considered in all cases where phages potentially could be applied, but it is only if the problems can be reasonably mastered that phage therapy can be applied on a larger scale.

Large variation

Approximately 90% of all phages infect, lyse, and kill bacteria in a way that might be considered as desirable, but not all are suitable for phage therapy (11). The phages to be used must be strictly virulent and reproduce effectively and preferably also rapidly. Phages that have long latency time, low burst size, or ability to lysogenize are unsuitable for use. The latter are defined as temperate phages, which, in addition to their ability to integrate into the genome of bacteria, pose a risk when used in that they potentially can transduce resistance genes from one bacterium to another, and thus paradoxically contribute to the spread of antibiotic resistance between bacteria. Among the other 10% are chronically infecting phages that are unsuitable for phage therapy too, as they do not lyse their host bacteria.

The infection of a virulent phage is initiated when it adsorbs to a bacterium's surface receptors. Some phages bind to one receptor only, others also need secondary receptors. These receptors are often the same as the antigens that constitute the serotypes of the bacteria, but they can also be transport channel proteins or pili, and there is generally substantial molecular variation within each type. The phage structures that bind to the bacterium, often tail fibres and/or base plate spikes, need to match strain-specific variants of surface receptors to be able to continue an infection, which has led to an equally large variation in phages, because of the continuous evolutionary arms race between phages and bacteria. A single phage can thus only infect one or a limited number of bacterial

strains. When 20 phages were isolated in an effort to isolate phages against different serotypes of multi-resistant (ESBL) *Klebsiella pneumoniae* clinical isolates, the average number of lysed target strains per isolated phage was four but the median was only two, indicating that most phages infect and lyse one or two bacterial strains. There was, however, a few phages that effectively lysed more than five strains, and one that lysed 20 of the multi-resistant strains (Eriksson H, unpublished observations). Similar results have been reported in *in vitro* efficacy tests of phages against pathogenic *Escherichia coli* strains causing diarrhoea in infants. A cocktail of 16 phages, selected from a phage bank of 140 phages, was effective against two-thirds of the 46 strains of one collection, and 21 of the 40 strains of another collection of these bacteria (12).

The problem with phage therapy is that even though there are phages exhibiting a broad host range, a complete coverage of pathogenic strains is difficult to achieve. The variation among bacteria may thus pose a great problem for putting phage therapy into practice, but given the simplicity of finding and isolating new phages it is practically feasible to assemble the large phage banks needed. It will, however, be necessary continuously to add phages to such a bank as new pathogenic bacteria evolve. It is fairly easy to find a phage that has the properties mentioned above—it can be done in a matter of days—but a new phage also needs to be characterized, produced in sufficient amounts, purified, validated, and finally approved for clinical use. All these steps are necessary, but also vastly time-consuming and overall very expensive (13). Consequently, phage therapy as a treatment option is likely to lag behind as new pathogenic strains appear.

Resistance

Phage and antibiotic therapy have one thing in common. Although the dynamics may differ, the evolution of bacterial resistance to a particular phage, just as to an antibiotic, is inevitable (14). The resistance to infection by a phage may typically involve just a single point mutation changing a bacterial surface antigen necessary for phage adhesion, an event that is quite frequent in a large population of bacteria and that is expected to happen during therapy (15), but can also be achieved via acquisition of the ability to degrade foreign DNA either by restriction endonucleases or mediated by the clustered regularly interspaced short palindromic repeats system (CRISPR) (16,17). Bacteria can also harbour phage abortive infection (abi) systems that cause cell death of infected cells before any progeny phages are released (18,19). Horizontal

transmission of often plasmid-borne resistance genes and natural selection will lead to spreading of resistance to other bacteria, be it against antibiotics or against phages. Notably, however, phages will themselves be under evolutionary selection to overcome the new resistance, contrary to antibiotics. Thus, new phage types evolve continuously, and, at any given moment under natural conditions, there will always be a few phages that have the ability to infect bacteria with resistance to the majority of phages. It has been reported that bacteriophages evolve resistance to all of the systems mentioned, including CRISPR (20,21).

There are several ways to tackle the problem with resistance. Firstly, it would be quite easy to switch to another phage, preferably to a phage that uses bacterial receptors that are more evolutionary conserved at the molecular level. In theory, phages with ability to use more universal surface receptors for adsorption, and thus showing a broader host range, are to be found in environments poor in nutrients. There is a bias of phages isolated from sewage water treatment works or garbage dumps because of the high variation of phages in these rich environments. Secondly, utilizing phages with fast adsorption rate and large burst size (the number of progeny phages released from one infected bacterial cell) could reduce the bacterial population size to such low numbers that the probability of resistance becomes very low and the remaining bacteria can be handled by the immune system (15). Thirdly, a cocktail of several phages each tested virulent on the target bacterial strain but binding to different surface receptors, will make it very difficult for the bacteria to develop resistance. A bacterium would need several mutations in different surface antigen genes to occur in the same cell simultaneously to survive. However, phage therapy with cocktails results in complex pharmacology (see below) and solves the problem of resistance only if all phages can persist in high titres throughout the treatment.

Special pharmacology

There are mainly three things that cause phage therapy to exhibit complex pharmacokinetics and pharmacodynamics. Firstly, phages are huge in comparison with simple antibiotic molecules. Secondly, the efficacy in the treatment of a bacterial infection is completely dependent on a sufficient number of phages infecting the bacteria in order to reduce the amount or at least prevent bacteria from multiplying up to a critical level. Thirdly, phages vary in virulence. The adsorption rate, as well as the latency time (the time from injection of DNA to the release of new phage particles), can be slow or fast, and the burst size

varies from a few to hundreds of progenies per cycle (15,22).

The large size of the phage particles limits phage therapy. For example, a common dose of penicillin V, 1 g a day during the course of treatment, is equivalent to approximately 0.03 moles (phenoxy-methylpenicillin, molecular mass 350.39 g/mole). If the entire drug dose is absorbed by the body, this dose results in 1.7×10^{21} circulating molecules. In order to reach the same number of phages in the body, 141 kg would have to be consumed (calculated from the mass of phage T7, a quite small phage (23)), and reach the infected tissue that is to be treated. The size of phages also means that they cannot be given in a sufficiently concentrated solution. Phage T7 belongs to a family of tailless phages, *Podoviridae*, and has a capsid with a diameter of 60 nm (24). It is just about possible to pack 5×10^{15} phages in 1 cm^3 , but solutions more concentrated than approximately 10^{13} phages per millilitre cannot be made since they become too viscous at higher concentrations. Consequently, only a small dose of phages can be given regardless of how it is administered, and phage therapy is thus completely dependent on achieving a productive infection, at which new phages are released from lysed bacteria, immediately attacking uninfected bacteria. Hence, the pharmacodynamics of phage therapy is tightly linked with the pharmacokinetics.

The probability of reaching a productive infection is density-dependent, it can only be reached if enough phages can reach the target bacteria, and in addition they also need to hit more or less directly since they diffuse very slowly, again because of their size (25). To reach the highest possible concentration of phages and to maximize the probability of achieving a productive infection, phages need to be applied at the very site of infection where the concentration of bacteria is highest. Given intravenously, phages that eventually arrive at the site of infection will be few, and phages in the bloodstream are also cleared from the body rather quickly (26). Several theoretical models have clearly delineated the importance of the initial dose of phages and, more significantly, the timing of the inoculation in successful phage infections of bacterial populations (15,27). It is obvious that it is better to employ a more virulent phage than a less virulent. A virulent phage with a large burst size might increase the dose of phages several hundred-fold in minutes, and partially compensate for the unavoidable low initial dose. It can be theoretically shown that treatment with a single phage clone might suppress bacterial growth to levels that would allow an immunocompetent host to manage the infecting

organism (15). This has also been confirmed in several *in vivo* experiments where phages have been applied directly into the infection centres (28,29).

There are two reasons for using a cocktail of different phages to treat an infection. If the serotype of the bacterium that causes the infection is unknown, one can hope that some of the phages in the cocktail can infect and kill it. An advantage is that it is possible to have such a cocktail pre-made. The downside is that there is a significant probability that no phage in the cocktail can infect and lyse bacteria, or perhaps more likely that it is just one phage in the cocktail that can infect and that the bacteria in such cases can develop resistance. The other reason to use a cocktail is to achieve synergy effects and radically reduce the likelihood of emergence of resistance. The bacterium's sensitivity to different phages must be known, and the phages in the cocktail must individually be able to infect the bacterium and also infect it via different receptors on the bacterial surface (30). The pharmacological problems arising from the use of a cocktail is that the concentration of each phage in the cocktail becomes lower, and that one of the phages may rapidly destroy the conditions for infection by other phages in the cocktail. A simple way to explain this is to start with just one virulence variable, e.g. the latency time. If the latency time differs between two phages with the same adsorption rate, a co-infection will result in the continuous loss of the 'slower' phage simply as a result of the density dependence requirement for productive infection to take place; as the faster phage lyses all co-infected cells, the slower would still be in the process of replicating its genome or assembling its structural components. Thus after this reproductive cycle, there will be too few free slow phages, and fewer bacterial cells to attack. This leads to a virtually zero probability of productive infection by the slow phage. The same argumentation can be adapted to other virulence parameters of the two phages, difference in adsorption rate or in burst size.

The most comprehensive study on the complex pharmacology resulting from simultaneous treatment with more than one phage tried to answer the question of whether two phages can coexist and together keep down bacterial density in a continuous culture model (31). This was also tested in chemostat experiments and, although one of the phages seems to be temperate, the results show dramatically lower bacterial densities when two phages are simultaneously present in the culture. In addition, this model also considered the dynamics that arise from bacteria migrating to and from crevices in the chemostat glass wall, comparable to the release of planktonic bacteria from biofilm in an *in vivo* infection.

Immunologic response

Treatment with phages can give rise to immunological reactions, but it all depends on where the infection is located and how the phages are added. Phages are present in our environment, in what we eat and drink, and regulate the composition of the bacterial flora in the intestinal tract (32). Phage preparations for therapy must, however, be purified and free from any toxic or allergenic substances emanating from the bacteria used for propagation of the phage. Consumption of large amounts of phages has not led to any immunological complications (33,34), and topical application has not shown any adverse effects (35,36). Other internal organs, including the bloodstream, are however not natural environments for phages, and it has been shown that phages activate both the innate and the adaptive immune system when administered intravenously (28). Each phage is unique; phage surfaces are covered with peptides that the body does not recognize. Phage titres fall rapidly after intravenous administration, mainly due to innate immunity and phagocytosis in the blood and liver and less due to the adaptive immune system (37). There is also an increase in non-neutralizing antibodies, IgM and later IgG, and an enhanced immune response after subsequent injections of certain phages (38). Previous clinical and animal trials have, however, not resulted in serious immunologic reactions (28,39), but the risk after intravenous phage therapy cannot be completely ruled out since all phages are different. It is therefore very important to test the immunological response of every single phage, particularly if intravenous therapy is being considered.

The great risk with phage therapy is more likely the rapid release of toxins that occurs when many bacteria lyse more or less simultaneously. Many pathogenic bacteria produce exotoxins e.g. Shiga-like toxins or cholera toxin, and the treatment causes the cells' supply of exotoxins to be quickly released and aggravate symptoms. Antibiotic treatment may pose a similar risk, while certain antibiotics up-regulate the expression of toxin genes (40). Interestingly, the toxin genes are often found in the genomes of temperate phages that can infect and reside in the genomes of bacteria as prophages. The possibility that the genomes of temperate phages may encode uncharacterized toxins is one of the reasons why they are unsuitable for phage therapy. The rapid release of lipopolysaccharides, which becomes the result when phages shred down the bacterial cell wall, also constitutes a risk since these substances are very allergenic. High concentrations in the body can lead to cytokine cascades and must be avoided.

Conclusions

There is probably a role for phage therapy in future medicine, but more research is needed in order to solve the problems, and the limitations of the method need to be accepted. There is reason to warn of both over-optimism and unfounded criticism. Phage therapy will most likely never totally replace conventional antibiotic treatment, but provide a complement in treating infections where it is theoretically and practically possible to apply large enough doses of phages, and where immunological complications are very unlikely. It would be a welcome addition to the treatment options for infections caused by antibiotic-resistant bacteria. Infections in the gastrointestinal tract, respiratory tract, urinary tracts, and wound infections should be treatable with phages. Several studies show good results, but are often not comprehensive enough and do not take sufficient account of the special pharmacology of phages. It ought to be possible to create phage libraries of highly virulent phages with the aim to cover all or at least a large part of the variation of a few bacteria, e.g. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and where the antibiotic resistance problem is extensive. It would also be possible to isolate phages with a broader host range or to alter virulent phages whose tail fibres bind to specific surface receptors to bind to a more general receptor of pathogenic bacteria. There are great advantages to treatment with phage cocktails, but it is important to develop theoretically well-founded treatment methods if the goal is to reduce the emergence of resistance and to gain synergies. It is also important to conduct clinical trials of phage therapy treatment of the types of infections where it can be expected that phages will be an effective method, where animal studies have already been implemented, and that these trials are carried out on sufficiently large groups.

There are many obstacles to an implementation of clinical trials of a limited phage therapy project, even if it is judged to have the best premises to show positive results. Who or what organizations should create the phage library of the pure well-characterized phages required, and what about funding of the clinical trials? There is no incentive for life science companies to invest money, since the risk that the investment does not generate profit is very large. The variation of phages is huge, and intellectual property protection is either not applicable or ineffective.

Bacteriophages for use in clinical applications in humans are not treated separately in the current regulations for permitted use of medical products. They are considered as biological medicinal products in Europe and must meet the same requirements for

extensive clinical trials prior to release for general use as any other medical product. Current rules thus imply that each treatment with phages carried out must have undergone full clinical trials. Phage therapy involves treatment with an individually selected phage, possibly a newly isolated one, or a specially adapted phage cocktail in which phages are replaced if resistance to target bacteria arises. This means that phage therapy will only be practically possible if either the rules or the definition of phages and phage therapy change (41). The increased interest in research on phage therapy and the realization that the rules preclude the introduction of phage therapy have initiated discussions in the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMA). The role of CHMP is to make an initial assessment of various medical products, and the committee will discuss and consider if there are scientific reasons to establish specific rules for phage therapy. This may lead to EMA adopting a new regulatory framework for phage therapy.

Of course, the very strict requirements for permitting use of a medical product not only inhibit the introduction of phage therapy; the effectiveness of research and development of new medical drugs has decreased worldwide. The number of drugs approved by the US food and drug administration per billion US dollars spent on research and development has been reduced by half every ninth year since the 1950s, now being down to approximately 0.8 per billion US dollars (42). The extensive and increasing regulation is one of the causes that have led to the development of a second track for the introduction of new drugs, adaptive licensing. In short that means a (in space and time as well as in patient numbers) limited authorization for clinical use, continuous collection of results of treatments followed by evaluation and feedback to reduce incrementally the uncertainty of a treatment (43). Currently, discussions are underway in many countries for the layout of national rules. It is thus possible that future policies regarding adaptive licensing will allow limited clinical use of phage therapy.

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