EDITORIAL

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Current Status and Perspectives of Phage Therapy*

Terapia fagowa – stan obecny i perspektywy

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A dramatic rise in antibiotic resistance in many clinically significant bacterial species [1] coupled with a shortage of novel classes of antibiotics [2] has created an urgent need for the development of alternative antibacterial agents [3, 4]. One of the most interesting and promising of such therapeutic modalities are currently lytic bacteriophages (phages), i.e. highly specialized viruses that infect and kill solely bacterial cells [4, 5]. These were discovered by Frederick Twort in 1915 and, independently, by Felix d'Herelle in 1917. Although at the beginning of the twentieth century knowledge about bacteriophages was very scant, some researchers even then realized phages' antibacterial activity and attempts were made to exploit it for therapeutic purposes, the first clinical trial taking place in Paris in 1919. Thus the history of phage treatment of bacterial infections encompasses an impressive 87 years – almost 30 years more than the antibiotic era. However, the therapeutic and prophylactic trials that were conducted in the early part of the twentieth century were often unsuccessful and, accordingly, the first phage researchers failed to inculcate the idea of bacteriophage therapy in Western medicine. Basically, the major reasons for this failure were inadequate knowledge of phage biology and a poor standard of scientific research. Thus, not surprisingly, interest in the therapeutic use of bacteriophages dwindled in the West following the introduction of antibiotics for the treatment of infectious diseases. However, bacteriophages continued to be used in Eastern Europe, especially in Poland and the former Soviet Union (Georgia and Russia), the two leading centers being the Institute of Immunology and Experimental Therapy (IITD) in Wrocław and the Eliava Institute in Tbilisi [6, 7].

Bacteriophages as Antibacterial Agents

One of the most significant features of bacteriophages as potential antibacterial agents is their narrow antibacterial range. Essentially, lytic bacteriophages infect bacteria in a sub-species-specific manner, i.e. they are capable of killing only certain strains within a given bacterial species [3]. For example, the enterococcal phage ENB6, studied with a view to potential therapeutic use, was found to kill 57% of different clinical isolates of VRE tested and to inhibit the growth of an additional 22% of the isolates [8]. On one hand, this feature appears to be advantageous, as phages, unlike antibiotics, can clear pathogenic bacteria without disturbing the balance of the indigenous bacterial microflora [3]. On the other hand, however, a narrow antibacterial range may be deemed a drawback, because it requires determining whether the bacteria causing infection in a given patient are sensitive to phages *in vitro* [9]. Alternative approaches include administration of a phage "cocktail", i.e. a mixture of a few different phages, collectively providing a wider antibacterial range

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[9], or the use of a single phage with a broader antibacterial spectrum [10].

With respect to the major mode of the antibacterial action of bacteriophages, it is rather far more complicated than the mechanisms of action of typical small-molecule antibiotics and is part of the complex interaction between phage virions and the host bacterial cell. Basically, over the course of infection of a susceptible bacterial cell, lytic bacteriophages divert various essential bacterial metabolic pathways (e.g. protein biosynthesis or ATP generation) from their normal functions and direct them towards phage progeny production [11]. A growing body of experimental findings clearly shows that it is indeed direct killing of bacteria by phage virions that forms the basis of the therapeutic effect of bacteriophages [8, 12–15]. The other potential mechanism, i.e. the induction of an immune response by some component(s) of the bacteriophage preparation, plays a minor role, if any, in that regard. It was shown in elegant experiments that only functional phage particles, i.e. those having the capacity to kill bacteria *in vitro,* were capable of curing mice from a potentially lethal infection, whereas preparations containing functionally inactivated phage failed [8, 13, 14].

A therapeutic strategy was recently reported that apparently may constitute a viable alternative to the use of lytic bacteriophages. This method relies on using a nonlytic filamentous phage (e.g. M13), which basically replicates in the host bacterial cell without killing it. In this case, the virions act as vehicles delivering DNA encoding bactericidal proteins, e.g. addiction toxins or restriction endonucleases, to bacterial cells. The antibacterial activity of such recombinant phages does not result from phage virions themselves killing bacterial cells, but from the bactericidal activity of the proteins, products of genes which were introduced into the bacteria. The effectiveness of this novel therapeutic approach was shown both *in vitro* and *in vivo* [16].

In view of the growing menace of multi-drug-resistant bacteria, of paramount importance is the fact that the mode of antibacterial action of bacteriophages is entirely distinct from those employed by traditional antibiotics. This feature seems to render phages particularly useful in the treatment of infections caused by antibiotic-resistant bacteria [17]. Indeed, in experimental studies bacteriophages proved highly effective in that regard, being capable of rescuing mice challenged with a lethal dose of vancomycin-resistant enterococci [8], imipenem-resistant *P. aeruginosa* [13], *E. coli* ESBL [14], as well as methicillin-resistant *S. aureus* [15].

For many years, antibiotics have been, and still are, the unquestionable standard in the treatment of bacterial infections. Thus, the obvious question arises whether the therapeutic efficacy of bacteriophages can exceed that of traditional small-molecule antibacterial agents. This fundamental issue was addressed in a classical study by Smith and Huggins, who showed that one dose of coliphage was more effective than multiple doses of four different antibiotics in curing mice of a potentially lethal *E. coli* infection. Apparently, the superiority of bacteriophage over antibiotics resulted from the phage-unique capacity for exponential growth, a phenomenon dependent upon replication within bacterial cells [12]. Thus, bacteriophages are the only known class of antibacterial agents whose titer (i.e. an equivalent of the concentration of an antibiotic) grows over the course of treatment, thereby increasing its efficacy.

Contemporary phage therapy has benefited considerably not only from a better understanding of phage biology, but also from substantial progress in molecular biology and chemistry. For example, thanks to major advances in the knowledge of bacteriophage life cycles in bacterial cells, it is currently known that only lytic phages may be used for therapeutic purposes, whereas temperate ones should be excluded from treatment (the latter do not kill the host cell upon entering it, but rather integrate their genome into the host chromosome) [9]. Moreover, the genomes of phages to be used in treatment may be sequenced to determine if any of the putative phage proteins have any homologies to potentially deleterious bacterial proteins, including toxins, pathogenicity factors, or antibiotic-resistance determinants [18]. Another factor substantially contributing to improvement in the safety of phage therapy is the development of novel purification protocols of crude bacteriophage suspensions. At the IITD, for instance, a method was developed that enables one to obtain highly purified preparations of bacteriophages specific to different Gram-negative bacterial species. Such preparations formerly contained considerable amounts of endotoxin. It is now possible to obtain preparations contaminated with a mere 0.4–7 EU of endotoxin per milliliter, a value allowing even intravenous administration [19].

The results of well-controlled preclinical studies clearly point to the high efficacy of phage therapy. However, these studies have also revealed that bacteriophages, as all other classes of antibacterial agents, do have some inherent drawbacks which may considerably diminish their therapeutic effectiveness. The importance of a narrow antibacterial range – a classical problem associated with phage therapy – was mentioned above. Another major problem which definitely diminishes the antibacterial activity of bacteriophages *in vivo* is their rapid clearance, determined largely by the non-specific

entrapment of phage particles by cells of the reticulo-endothelial system (RES) of the liver and spleen. However, it is possible to isolate, relatively easily, phage mutants featuring a considerably prolonged serum half-life. Predictably, such phages exert a more potent antibacterial activity *in vivo* [20]. Another factor that is believed to diminish the therapeutic effectiveness of bacteriophages is specific humoral immunity, i.e. the generation of neutralizing anti-phage antibodies. Such antibodies are very likely to disturb the interaction between phage virions and bacterial cells [3, 9]. To the best of our knowledge, no reliable solution to this problem has been reported as yet. While the immunogenicity of protein pharmaceuticals can be considerably reduced by means of conjugation to polyethylene glycol (PEG) [21], bacteriophages have a far more complicated structure, their particles being made up of many different proteins. Thus, the pegylation of phage virions to reduce their immunogenicity is apparently not feasible. Perhaps increasing the dose of bacteriophage or administering a phage with a different antigenic specificity following the generation of neutralizing antibodies could be helpful in this regard.

One of the major problems in antimicrobial therapy is the development of resistance. In fact, it is the sharp rise in the prevalence of antibiotic-resistant bacterial strains that has prompted a resurgence of interest in phage therapy in Western medicine over the past years [3, 4]. Thus the question arises whether or not bacteriophages will meet the fate of the traditional, small-molecule antibacterial agents, i.e. an increasing frequency of treatment failures owing to the emergence of multi-resistant bacterial strains. Apparently they will not. First, over the very long co-evolution with their host cells, bacteriophages have developed some very effective means of dealing with bacteria [22], an example being the activity of the highly evolved endolysins (see below). Accordingly, it is believed that resistance to phages develops ten times more rarely than resistance to antibiotics [9]. Furthermore, the development of resistance can be considerably delayed by using a phage "cocktail" [23]. Importantly, phage-resistant bacterial mutants were found to feature a lowered virulence *in vivo* [12]. Thus we have reason to believe that the development of resistance – an apparent and inevitable consequence of using any antibacterial agent – will in fact not have as dramatic an effect on phage therapy as it had on antibiotics.

Phage Therapy in Humans

Over the past few years, the history, current status, and future prospects of phage therapy in humans have been commented and reviewed extensively [5–7, 9, 24–27]. Of special importance are the papers published in top-tier journals, such as The Lancet [28, 29], JAMA [30], Science [31], Nature Biotechnology [4], and Nature Reviews [3, 32], as they reflect the great significance of the topic. As pointed out in Science [31] and in an excellent review in Antimicrobial Agents and Chemotherapy [7], the most detailed series of studies were conducted in Poland using phage preparations developed at the IITD. The major conclusions arising from these studies may be summarized as follows: 1) Bacteriophages are very effective in many different kinds of infections caused by both Gram-positive and Gram-negative antibiotic-resistant bacterial strains, with an overall therapeutic success rate exceeding 85%. 2) Bacteriophages are efficacious in both non-invasive and invasive infections (e.g. septicemia). 3) Phages can be used in both mono- and polyinfections. 4) Bacteriophages can be administered along with antibiotics, though in this case they are significantly less effective. 5) The treatment is apparently safe, with no side-effects occurring in patients following phage administration [33] (more data are available on our website: http://surfer.iitd.pan.wroc.pl/phages/phages.html). However, it should be kept in mind that these studies were not controlled studies, as were practically all of the others regarding phage therapy in humans (several hundred papers in all). Accordingly, they do not meet current rigorous standards for clinical trials and cannot provide ultimate confirmation of either the effectiveness or the safety of phage therapy. Thus one of the greatest challenges to be met in the nearest future is to conduct a controlled clinical trial using a well-characterized phage preparation. The first step towards achieving this goal was recently made with the carrying out of the first well-controlled safety test of phage therapy, during which no side-effects occurred in 15 healthy volunteers following oral administration of T4 coliphage [34].

Other Activities of Bacteriophages

Since the discovery of bacteriophages at the beginning of the twentieth century, their major medical application has been the treatment of bacterial infections. However, we believe that phages should not be pigeonholed as merely "viruses of bacteria", as they are also capable of exerting other and sometimes unexpected activities, some of which have already been exploited in medicine. For instance, a considerable body of experimental evidence indicates that phages may also be used for the treatment of viral infections [for a review, see Ref. 35]. Basically, there are two major mech-

anisms by which bacteriophages may diminish the infectivity of pathogenic viruses. First, phage nucleic acids can induce the synthesis of interferons, cytokines known to exert a potent anti-viral activity. The other mechanism could be direct competition of phage particles and pathogenic virions for cellular surface receptors. More recently, a third possible mechanism has been reported, i.e. direct binding of viral proteins by bacteriophage proteins, leading to an inhibition of their natural activity [36]. Moreover, phages can be exploited for the development of anti-viral vaccines in which phage particles constitute delivery vehicles for either vaccine antigens themselves or their corresponding DNA sequences ("DNA vaccines") [37]. Although the majority of the relevant findings come from preclinical studies, there are some encouraging data in the literature suggesting that the anti-viral activity of bacteriophages can also be successfully employed in the treatment of viral infections in humans [35].

Another interesting (and potentially very important) activity of bacteriophages is their effect on the immune system. For example, our group showed that at least some phages specific to different species of Gram-negative bacteria can exert immunosuppressive activity, an example of such a phage being T4 coliphage, which was found to inhibit human T-cell proliferation, mouse antibody production, as well as NF-kappaB activation *in vitro*. These findings gain special significance in the context of the postulated use of bacteriophages in the treatment of bacterial infections in allograft recipients. In such patients, an immunostimulative effect of phage could accelerate allograft rejection, whereas the immunosuppressive activity could be beneficial. Here it is worth pointing out that T4 was found to actually extend skin allograft survival in mice and to diminish cellular infiltration of the graft [38, 39].

Endolysins

In 2001, a very interesting alternative to the classical phage therapy was reported, i.e. recombinant endolysins, or lysins [40]. These are dsDNA bacteriophage-encoded enzymes that are produced in phage-infected bacterial cells during the later stages of the lytic cycle, their major function being the cleavage of peptidoglycan covalent bonds, which results in lysis of the host bacterial cell and ensures the successful release of progeny virions [41]. Over the course of phage infection of a bacterial cell, endolysin molecules are synthesized in the cytoplasm and reach their substrate, peptidoglycan, from within the cell. The feasibility of using lysins as antibacterial agents results from the fact that they are capable of cleaving peptidoglycan also when applied exogenously (as purified recombinant proteins) to the bacterial cell wall. In this case, their lytic effect is very rapid and potent, especially in Gram-positive bacteria, whereas Gram-negative bacteria are generally considered to be resistant owing to the presence of the outer membrane, which blocks the access of lysin molecules to peptidoglycan [41, 42]. Accordingly, one of the biggest challenges endolysin researchers must now face is finding some means of enabling lysin molecules to penetrate through the outer membrane. In fact, some findings indicate that at least some lytic enzymes can also be successful in killing Gram-negative bacteria when acting on them from outside [42].

The most important features of endolysins as potential antibacterial agents include: 1) a very rapid and potent antibacterial activity against Gram-positive bacteria both *in vitro* and *in vivo*, 2) a novel mode of antibacterial action associated with enzymatic cleavage of peptidoglycan covalent bonds, 3) the capability to kill bacteria regardless of their antibiotic sensitivity, 4) a narrow, species-specific antibacterial range, 5) a very low probability of the development of resistance, 6) apparent safety, and 7) relatively easy modifications using genetic engineering. These features clearly set them apart from traditional antibiotics and create a truly novel and unique class of antibacterial agents [42]. Lytic enzymes were originally developed with a view to killing Gram-positive bacteria colonizing mucous membranes. This colonization is of great importance to medicine, as it provides a potential starting point for infection and contributes to the horizontal spread of bacteria within the community. Hence, owing to their rapid killing of bacteria in a basically species-specific manner, lysins provide a unique means of selective prophylaxis of infections without disturbing the balance of the indigenous microflora [43]. In fact, several studies have clearly shown a great capacity of lytic enzymes for killing bacteria colonizing mucous membranes of mice following topical administration [40, 44, 45]. The other potential application of lysins may be the treatment of bacterial infections, the results of the first relevant studies being very encouraging [46, 47]. Interestingly, it has been shown that antibodies, contrary to expectations, do not neutralize, but rather slightly decrease the antibacterial activity of lytic enzymes *in vivo* [46, 47]. This finding is very important, as it provides an additional argument in favor of the possibility of using lysins for the treatment of systemic bacterial infections.

Endolysins and bacteriophages have a few sig-

nificant features in common. These include: a novel (as compared with antibiotics) mode of action, the capability to kill bacteria regardless of their antibiotic-sensitivity, and a narrow antibacterial range. On the other hand, there are also some major differences between them, as endolysins, unlike bacteriophages, do not have the capacity for exponential growth and are less likely to be used for the treatment of Gram-negative bacterial infections. However, owing to the lack of reports directly comparing the antibacterial activity of lysins and phages, no general conclusions can be drawn regarding the superiority of either modality.

Over past years we have been witnessing a great resurgence of interest in phage therapy. Bacteriophages possess several important features which collectively set them clearly apart from traditional antibiotics and render them a unique class of antibacterial agents. Judging from the results of preclinical studies and clinical trials conducted hitherto, phage therapy appears to be very effective and safe. However, as impressive as some of these results are, they must be ultimately verified by controlled clinical trials. We believe that further research will confirm both the high effectiveness and the safety of phage therapy and that this therapeutic modality will soon become a widely accepted way of treating infections caused by antibiotic-resistant bacteria – a great challenge of modern medicine.

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Conflict of interest: The Institute has filed a patent application covering therapeutic use of phages in bacterial infections.

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