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SYSTEMATIC STUDENT REVIEW

A Question of Attire: Dressing Up Bacteriophage Therapy for the Battle Against Antibiotic-Resistant Intracellular Bacteria

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Abstract More and more bacteria are developing severe antibiotic resistance. Among them are important intracellular pathogens such as *Mycobacterium tuberculosis*. Alternatives to classical antibiotics are urgently needed and bacteriophage therapy is a promising candidate for alternative or supplemental treatment. Until now, bacteriophages have been thought to be non-suitable for therapy against intracellular pathogens. Still, a few studies have been carried out to assess the efficacy of bacteriophage therapy against intracellular pathogens both in vitro and in vivo, with variable results. Recently, some successful studies have been conducted, in which bacteriophages were carried into infected cells by different bacterial vectors and killed intracellular pathogens. In this review, we aim to recapitulate the existing literature on bacteriophage therapy of intracellular pathogens and discuss possible ways of bacteriophage entry into infected cells, including different Trojan horse strategies and the question of whether free bacteriophages are able to enter mammalian cells. Finally, we sum up attempts of bacteriophage microencapsulation and speculate about the advantages of artificial vectorization for efficient and targeted intracellular delivery.

Keywords Bacteriophage therapy · Intracellular bacteria · Tuberculosis · Antibiotics · Trojan horse approaches · Microencapsulation

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Introduction

Bacteriophages are viruses that specifically infect bacteria. They were discovered in the early 20th century for their ability to effectively destroy pathogenic bacteria [11]. Since then they have been studied as potential therapeutic agents [53] and up until the 1940s, bacteriophages were regarded as powerful new drugs in defending infectious diseases. Therapeutic bacteriophage products were commercially available at that time, but with the advent of chemical antibiotics such as sulfa drugs and later on penicillin, their therapeutic potential has fallen into oblivion. However, in the former Soviet Republics bacteriophage therapy has been pursued continuously and has been well established for several decades [16, 51].

It seems likely that we are now entering a post-antibiotic era: chemical antibiotics are losing a lot of their power because more and more bacterial species are developing antibiotic-resistant strains. The most important examples are multi-resistant strains like, for example, methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-

resistant Gram-negative bacteria. Often, infections with these multi-resistant bacteria are hospital-acquired. According to the World Health Organization (WHO), they are "an increasingly serious threat to global public health that requires action across all government sectors and society" [56]. Consequently, the interest in alternatives to chemical antibiotics has risen dramatically and bacteriophage therapy has been proposed as a strong candidate [52]. More and more studies as well as clinical trials are being carried out in order to test the efficacy and safety of therapeutic bacteriophages [14, 33, 51]. Recent studies with a clinical focus include bacteriophage therapy against *Pseudomonas aeruginosa* [19, 34, 48], *Staphylococcus aureus* [42], *Escherichia coli* [21, 32], and *Salmonella enteritidis* [30].

Bacteriophages have several advantages as therapeutic agents against infectious diseases. Their high specificity for the bacterial host largely avoids deleterious side effects on the commensal microbiota [16]. Other severe side effects have not been observed so far. Also, their self-replication within their bacterial targets should theoretically keep them highly concentrated at the site of infection. Clearance of the infection automatically leads to a replication stop due to the absence of the bacterial host. Finally, their mode of action is so different from that of chemical antibiotics that they are highly effective even against multi-resistant bacterial strains [37].

However, some bacteria that are able to cause severe illness are leading an intracellular life-style, meaning that they invade mammalian host cells and survive and even multiply within them. Perhaps, the most important example of such intracellular pathogens is *Mycobacterium tuberculosis*, the causative agent of tuberculosis. With 1.3 million deaths annually, it is, according to the WHO, the second greatest cause of mortality worldwide by a single infectious agent. Multiple (MDR) and Extensively (XDR) Drug Resistant mycobacterial strains are a serious problem for the already difficult antibiotic treatment of this disease [55]. Hence, it is fairly relevant for the development of phagotherapy to address the treatment of intracellular pathogens as well.

Still, the effectiveness of phages against diseases caused by intracellular bacteria is to date unclear. Bacteriophages are able to reach many different compartments of the human body, even the brain [8, 12], but seemingly they cannot diffuse across eukaryotic cellular membranes, which would be a prerequisite in order to reach and infect intracellular pathogens. This inability of bacteriophages is received wisdom rather than a scientific finding, but as a consequence, phagotherapy is generally considered as non-suitable against bacteria that are inside eukaryotic cells [34, 51]. However, data about it are scarce and in some cases controversial, and despite the inability of bacteriophages to diffuse across membranes, a few studies imply that phages can gain access to intracellular bacteria. This review is meant to provide an overview of the existing literature on phagotherapy against intracellular bacterial pathogens as well as to speculate about future implementation of phagotherapy against such pathogens. Special focus will be directed toward strategies for intracellular delivery of therapeutic bacteriophages.

Two main mechanisms, not necessarily exclusive, have been proposed so far to explain the ability of some bacteriophages to gain access to intracellular bacteria. Some publications suggest that phages are carried into cells by infected bacteria that invade eukaryotic cells before being destroyed by the phage. In this way, phage particles would be liberated within eukaryotic cells and could thus reach other bacteria within the same cells. Another possibility is direct uptake of free bacteriophages. This has also been proposed for some phage species, e.g., the mycobacteriophage D29 [39] or the *Brucella*-directed Tbilisi phage (Corbel and Morris 1980, reviewed in [13]).

In the following sections, we will review attempts to cure intracellular mycobacterial, staphylococcal, and chlamydial infections as well as infections with *Brucella abortus* and *Burkholderia cenocepacia* with the help of bacteriophages. A brief summary of the studies discussed is given in Table 1. Furthermore, we will develop a scheme of possible ways that bacteriophages could gain access to intracellular pathogens (Fig. 1) and discuss the potential and technical possibilities of artificial vectorization.

Mycobacteria and Mycobacteriophages

The main causative agent of tuberculosis, *Mycobacterium tuberculosis*, is a facultative intracellular pathogen. In the lungs, the main site of tuberculosis infection, the bacterium is taken up by alveolar macrophages. Inside, instead of being degraded by lysosomal enzymes, these pathogens can block endosomal maturation and hence produce the formation of a so-called mycobacterial phagosome [1, 40]. Within this organelle, they are able to persist over long periods of time, shielded from the immune system.

Because tuberculosis, and especially MDR- and XDR-tuberculosis, is a serious problem worldwide, it would be a great breakthrough to implement phagotherapy against this disease. Since 1947, several hundreds of mycobacteriophages have been identified [33] and many of them have been sequenced [17, 18]. However, only a limited number of published studies assessed the efficacy of phage administrations to cure mycobacterial infections.

During early in vivo experiments, treatments of infected rodents with mycobacteriophages were unsuccessful. When Hauduroy and Rosset tried to cure *M. tuberculosis*-infected

Table 1 Studies addressing bacteriophage therapy against intracellular pathogens

Study	Target bacteria	Bacteriophage	Animal model	Success
Cater and Redmond (1960), reviewed in [13]	*M. tuberculosis*	DS-6A, prior to infection	Guinea pig	Protective effect
Hauduroy and Rosset (1960), reviewed in [13]	*M. tuberculosis*	"Lytic mycobacteriophages"	Guinea pig	No
Hauduroy and Rosset (1963), reviewed in [13]	Bacille Calmette Guérin (BCG)	"Lytic mycobacteriophages"	Hamster	No
Mankiewicz and Beland (1964), reviewed in [13]	*M. tuberculosis* H37Rv	DS-6A, single dose	Guinea pig	No
Sula et al. [50]	*M. tuberculosis* H37Rv	DS-6A, GR-21 T, My-327 repeated doses	Guinea pig	Yes (DS-6A), comparable to isoniazid
Zemskova and Dorozhkova (1991), reviewed in [33]	*M. tuberculosis*	DS-6A	Guinea pig, disseminated infection	Beneficial effect
Broxmeyer et al. [3]	*M. tuberculosis* H37Rv, *M. avium* 109	TM4, *M. smegmatis* as vehicle	Cell line: RAW264.7	Yes
Danelishvili et al. [9]	*M. avium* 109	TM4, *M. smegmatis* as vehicle	Mouse	Limited
Peng et al. [39]	*M. tuberculosis* H37Rv	D29	Primary cells: mouse peritoneal macrophages	Yes
Capparelli et al. [5]	*S. aureus*	M^{sa}	Mouse	Yes
Hsia et al. [20]	*C. psittaci*	ϕCPG1	Cell line: HeLa	Yes
Corbel and Morris (1980), reviewed in [52]	*B. abortus*	Tbilisi phage	Guinea pig	Yes, for fresh infections
				No, for chronic infections
Carmody et al. [6]	*B. cenocepacia*	BcepIL02	Mouse	Yes, when phage was injected intraperitoneally no, when phage was applied intranasally

guinea pigs (0.01 mg/animal of the human pathogenic strain n° 3447 injected subcutaneously, Hauduroy and Rosset 1960, reviewed in [13]) and BCG-infected hamsters (Hauduroy and Rosset 1963, reviewed in [13]), repeated injections of lytic mycobacteriophages had an even negative impact on their survival.

In 1964, Mankiewcz and Beland assessed the effect of the bacteriophage DS-6A on *M. tuberculosis* H37Rv-infected guinea pigs. They evidenced no beneficial effect of a single 10^5 plaque-forming units (PFU) dose given subcutaneously and simultaneously with the mycobacterial inoculation (14/20 treated animals died after 10 weeks, against 10/20 within the control group). Yet interestingly, the differences at the level of tuberculosis lesions were observed between the treated and the control groups (Mankiewcz and Beland 1964, reviewed in [13]).

More encouraging results were obtained later. In 1981, Sula et al. published a study where guinea pigs had been infected with approx. 5000 live germs of *M. tuberculosis* H37Rv and treated with bacteriophages DS-6A, GR-21 T, or My-327 by subcutaneous injection of 10^6 PFU in 1 ml twice weekly for 10 weeks. Phage DS-6A administration reduced the relative weights of the spleen and associated lymph nodes of treated animals and reduced lesions in their spleen, lungs, and livers. The efficacy of this treatment was close to that of isoniazid [50]. This efficacy has been suggested to be due to the infection of *M. tuberculosis* bacilli by the phage while extracellular, which might then have carried the phage inside macrophages [4].

Finally, in 1991, Zemskova and Dorozhkova evidenced beneficial effects of a mycobacteriophage DS-6A therapy in guinea pigs with disseminated tuberculosis, even though this treatment was less effective than an isoniazid therapy (Zemskova and Dorozhkova 1991, reviewed in [33]). However, the article was published in Russian and there is no detailed information in the abstract that would allow judging the standards under which the study was performed.

In spite of a few relative successes, early attempts to cure mycobacterial diseases with mycobacteriophages were generally not successful. One of the key reasons for this failure was probably the fact that free bacteriophages did not reach intracellular mycobacteria.

More recent in vitro [3] and in vivo [9] studies relied on a different approach and thereby showed the potential of the mycobacteriophage TM4 to kill intracellular

Mycobacterium tuberculosis H37Rv and *Mycobacterium avium* 109. The mycobacteriophage was carried by an infected non-virulent but invasive mycobacterium, *Mycobacterium smegmatis* mc^{2}155. During in vitro trials, cultured mouse macrophages (RAW 264.7) were infected with *M. avium* or *M. tuberculosis* and subsequently incubated with either TM4 alone, TM4-infected, or non-infected *M. smegmatis*. Results showed that TM4 alone did not significantly reduce the number of viable *M. avium* or *M. tuberculosis* inside macrophages but when delivered by *M. smegmatis*, TM4 caused a significant time- and titer-dependent reduction in the number of viable intracellular bacilli. *M. avium* and *M. smegmatis*-containing vacuoles were shown to fuse with each other in infected macrophages, bringing together both mycobacterial species and the lytic bacteriophages. This route of intracellular bacteriophage delivery is depicted in section 3 of Fig. 1.

Later on, the same team showed that splenic bacterial loads of *M. avium*-infected mice (3×10^7 colony-forming units (CFU)/mouse, injected intravenously) were significantly decreased when 4×10^7 CFU of TM4-infected *M. smegmatis* (but not TM4 or *M. smegmatis* alone) were injected intravenously [9]. However, a second injection did not lead to a further decrease and subsequent assays revealed that more than a fifth of mycobacteria recovered from the spleen were resistant to the phage. In addition, not all *M. avium*-containing vacuoles did fuse with a vacuole harboring TM4-infected *M. smegmatis*. In order to reach infection levels high enough for every *M. avium*-vacuole to be hit by a *M. smegmatis*-vacuole, a much higher number of infecting *M. smegmatis* would have been needed. The authors state that this dose, in turn, might be harmful because of the high concentration of mycobacterial antigens the animals would get exposed to.

Hence, the above described experiments are an important landmark for the development of an intracellular bacteriophage treatment, but the development of other means of intracellular delivery seems to be needed for the clinical success of such a therapy.

Bacteriophages Against Intracellular *Staphylococcus aureus* Infections

The phenomenon of bacteriophages being carried into bacteria-infected cells by phage-infected bacteria has also been illustrated with *Staphylococcus aureus*. Even though this bacterium has long been considered as an extracellular pathogen, many recent studies suggest that it can invade and survive within various non-professional phagocytes like epithelial cells, endothelial cells, or osteoblasts [2, 25]. This could play an important role in the pathogenesis and persistence of diseases caused by *S. aureus* [25, 47]. It has been shown that it is also able to survive engulfment by macrophages. Bacteria can survive for several days in vacuoles within macrophages without affecting cell viability, before reaching the cytosol and lysing their host. Macrophages can consequently act as bacterial reservoirs and are suspected to play a role in *S. aureus* dissemination [26].

Capparelli et al. [5] studied the effect of bacteriophage administration to *S. aureus*-infected mice and showed that, when given concurrently with a lethal dose of *S. aureus* (10^8 CFU/mouse, injected intravenously) or after 10 days of infection with a smaller dose of bacteria (5×10^6 CFU/mouse, injected intravenously), intravenous injection of 10^9 PFU/mouse of an *S. aureus*-specific, lytic bacteriophage (M^{sa}) led to a complete clearance of the infection. Given the efficacy of this treatment in spite of the ability of *S. aureus* to adopt an intracellular life-style, they investigated the phage's ability to lyse bacteria intracellularly. They demonstrated that phage M^{sa} alone could not reduce the number of bacteria recovered from infected mouse peritoneal macrophages, presumably because phage particles did not reach bacteria. However, treatment of these infected macrophages with *S. aureus* previously infected with M^{sa} efficiently reduced the number of intracellular bacteria.

The principle of bacteria-delivered bacteriophages leading to intracellular clearance of pathogens that we know from studies with mycobacteria-infected cells also applies to this study. There is one important difference, though: *S. aureus* is a fast-growing pathogen that is acting both extra- and intracellularly and it is dividing and infecting cells at a high rate. Extracellular bacteriophages can infect bacteria that are released from host cells and they will quickly re-infect other cells where they can release their bactericidal cargo (section 2 of Fig. 1). For a slow-growing and preferentially intracellular bacterium like *M. tuberculosis,* this approach would not work as easily.

Bacteriophage Therapy Against *Chlamydia* Infections

Results from a study conducted by Hsia et al. [20] also support the possibility that bacteriophages can be carried into bacteria-infected cells by phage-infected bacteria. They studied the infectious cycle of ϕCPG1, a *Chlamydia*-infecting bacteriophage, within *C. psittaci*-infected HeLa cells. *Chlamydia* spp. are obligate intracellular pathogens with a particular developmental cycle. They assume two main forms: elementary bodies and reticulate bodies. Elementary bodies are the extracellular infectious form, biologically inactive and resistant to various environmental stresses. They cannot replicate by themselves but need to invade host cells in which they transform into reticulate bodies, the intracellular replicative form. After replication,

the host cell is lysed and new elementary bodies are released into the environment. Because of this special life cycle, *Chlamydia* spp. were long considered as viruses.

Since elementary bodies are metabolically inert, they cannot sustain phage replication. Chlamydia phages must consequently gain access to the intracellular reticulate bodies.

Hsia et al. infected HeLa cells with *C. psittaci* previously infected with ϕCPG1. Using transmission electron microscopy, they observed phages associated with elementary bodies within intracellular vacuoles during the first hours following infection. After differentiation, phages were not observed to be attached to early reticulate bodies, presumably because bacteria were infected when differentiation occurred. From 32 h post-infection, lysed *C. psittaci* and phage progeny were found inside infected cells. Disruption of the vacuolar membrane was readily found close to lysed bacteria, promoting phage release to the cytosolic compartment. The authors hypothesized that ϕCPG1 attaches to elementary bodies extracellularly but infects them intracellularly, as soon as they differentiate into initial reticulate bodies. This would lead to bacterial lysis, abundant release of phage progeny, and disruption of the inclusion membrane. Phage particles could then bind to elementary bodies and/or infect differentiated forms. Even though the authors observed phage particles bound to elementary bodies during bacterial entry in cells, they did not test *Chlamydia*-free phage preparations, and therefore, the possibility that phages alone can enter mammalian cells cannot be excluded.

In earlier experiments, Richmond et al. [43] had shown that Chp1, another Chlamydia-infecting phage sharing genome similarities with ϕCPG1, bound poorly to reticulate bodies but extensively to elementary bodies. Regarding the phage infectious cycle, Richmond et al. did not suggest attachment and infection to take place at different stages of the Chlamydia life cycle. They rather hypothesized that both attachment and infection take place at reticulate bodies within the mammalian cell and that elementary bodies derived from infected reticulate bodies would produce new phage particles when the next mammalian cell is infected.

Trojan Horse Approaches for the Intracellular Delivery of Bacteriophages

Based on the above discussed studies, it seems that at least in some cases, bacteriophages can lead to the destruction of intracellular bacteria. In these successful cases, bacteria were infected by the phage extracellularly and did release phage particles upon invasion into already infected eukaryotic cells.

The term "Trojan horse" approach was coined for this type of intracellular delivery [23, 4]. When phages are administered without carrier, the importance of this mechanism presumably depends on the proportions of intra- and extracellular life-style of a bacterial species as well as the propagation rate and frequency of cell infection. When the extracellular life is extensive and the intracellular life transient, as in the case of the experimental infection of mice with *S. aureus* [5], it is plausible that the phenomenon occurs quite frequently given the high number of extracellular bacteria available for phage infection. This is illustrated in section 2 of Fig. 1. However, when bacteria reside mainly in intracellular compartments and only a few extracellular bacteria could serve as vehicles, as in many cases of mycobacterial infections, the described phenomenon would probably have a much lower frequency. In these cases, an external vehicle would be needed, as, for example, depicted in section 3 of Fig. 1. This is consistent with the failure of the sole TM4-treatment of *M. avium*-infected mice [9]. The dependence on the intra- and extracellular characteristics also implies that the efficacy of free bacteriophages can be influenced by the state of infection.

The results obtained by Corbel and Morris (1980) with a lytic bacteriophage (Tbilisi phage, Tb) in *Brucella abortus* infections also support this theory. After successfully reducing the number of viable *B. abortus* within cultured bovine monocytes using the Tb phage, the authors assessed the efficacy of this phage in vivo (Corbel and Morris 1980, reviewed in [13]). First, guinea pigs infected with 2×10^9 CFU of *B. abortus* were injected with a single dose of 10^9 PFU of lytic Tb phage. This treatment greatly reduced splenic bacterial counts after 7 days. However, when guinea pigs were infected with 3000 CFU of *B. abortus* and subsequently treated with seven phage inocula between 14 and 26 days after infection, phages had no significant effect on splenic bacterial counts after 8 weeks of infection. These latter results indicate a lower efficacy of the treatment against more established or chronic infections.

Although bacteria recovered from treated animals were not tested for phage resistance, which could also have contributed to the above findings, the results suggest that free bacteriophages had a therapeutic effect only while most bacteria were extracellular. In that case, free phages could be used before the infection becomes mainly intracellular and even before the infection occurs. Consistent with this, phage treatment was reported to be effective in preventing salmonellosis in children (Kiknadze et al. 1986, reviewed in [51]). Earlier, three guinea pigs infected with 0.1 mg *M. tuberculosis* after immunization with phage DS-6A did not show gross lesions 7–9 weeks later (Cater and Redmond 1960, reviewed in [13]).

For cases where bacteriophage delivery by extracellularly infected bacteria is unlikely or even impossible, other solutions have to be found. The use of infected bacteria as carriers has been shown to have a significant effect and seems very elegant at first glance. At a second glance, this natural system has some inherent difficulties due to the bacterial nature of the vector. These are (a) from a technical point of view, the need for a non-pathogenic but invasive bacterium sensitive to the phage of choice, which becomes even more difficult when bacteriophage cocktails are to be used in order to prevent or overcome resistance of the bacterial pathogens [7], (b) targeted delivery to infected and/or non-phagocytic cells, (c) from an immunological point of view, the heavy exposure of the already infected and hence weakened host to bacterial antigens from the vehicle [9], and (d) the risk of the vector acquiring pathogenicity from the related pathogenic strains that are actually to be fought. Against this background, the administration of artificially vectorized bacteriophages (section 4 of Fig. 1) is an appealing approach.

Does Cellular Uptake of Free Phages Occur?

Another theoretically possible way of bacteriophage delivery to intracellular pathogens is free uptake by phagocytic cells (illustrated in section 1 of Fig. 1). Since some bacteria of major medical interest are able or even obliged to survive and multiply within phagocytic cells [38], this aspect is of great relevance for the development of bacteriophage therapy against intracellular pathogens. However, so far, the literature dealing with bacteriophage therapy contains only very little information about this issue and, in addition, is controversial.

The studies involving the mycobacteriophage TM4 and the staphylococcal phage M^{sa} described above indicate that at least some bacteriophages cannot be internalized by macrophages without carrier. Also a study by Shaw et al. [46] argues against it. They assessed the ability of mouse peritoneal macrophages and of the 407 line of human intestinal epithelial cells to take up bacteriophage T6 derivatives ($T6^{+}$ and $T6h^{yp}$). This was carried out in the

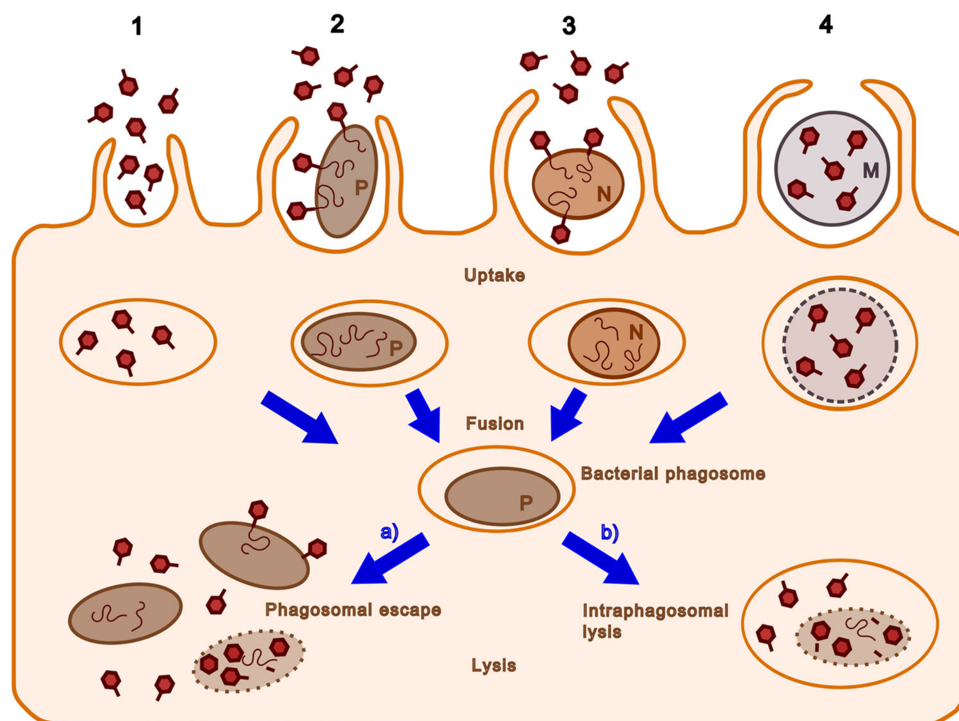

Fig. 1 Pathways of bacteriophage entry into infected phagocytic cells for intracellular bacterial lysis. Depicted are four main possible ways how bacteriophages can get access to intracellular pathogens: *1* endocytic uptake of free bacteriophages (under debate), *2* entry of bacteriophages via infected pathogenic bacteria (*P*) of the same species, *3* entry of bacteriophages via infected, susceptible, and invasive non-pathogenic bacteria (*N*), or *4* entry of bacteriophages artificially vectorized by microencapsulation (*M*). All four possible entry routes produce endosomes that need to fuse with the bacterial phagosome for bacteriophage access to the intracellular pathogen. From there, two scenarios are possible that depend on the nature of the pathogen to be eliminated: *a* phagosomal escape of the infected pathogens and further lysis in the cytosol. This is, for example, applicable for pathogens like *S. aureus* as described by Capparelli et al. [5]. Scenario *b* describes a situation where lysis of the pathogen takes place within the bacterial phagosome. This applies to bacteria that do not escape into the cytosol as, for example, *M. tuberculosis* and *M. avium* phage-infected via *M. smegmatis* as described by Broxmeyer et al. [3]. Legend: *dark red hexagons* bacteriophage particles, *brown filled ovals* bacteria, *P* pathogenic, *N* non-pathogenic, *curly lines within bacteria* bacteriophage nucleic acids marking infected bacteria, *orange ovals* endosomal vesicles, *bluish circle M* microcapsule, *dotted lines* dissolving/degrading structures

context of a study where UV-inactivated (253.7 nm, 300 J/m^2) bacteriophages were used to kill extracellular *Yersinia pestis* KIM strains and *Shigella flexneri* 2a by premature lysis, or 'lysis from without' [10], when studying interactions between bacteria and eukaryotic cells. This kind of lysis may occur independently of phage replication at very high phage-to-bacteria ratios, when the number of adsorbed phages is above a threshold limit [10].

When cultured mouse peritoneal macrophages were incubated with non-inactivated phage T6h^{yp}, more than 98 % of the bacteriophage titer was recovered in culture supernatants. Most of bacteriophage particles had consequently not been taken up. Inactivated T6 killed more than 99.9 % *Y. pestis* and *S. flexneri* in the absence of eukaryotic cells, presumably by premature lysis, but did not significantly alter the survival of *Y. pestis* within cultured mouse peritoneal macrophages. This is not surprising given the high phage-to-bacteria ratio which is needed for premature lysis.

Shigella flexneri-infected Henle cell monolayers were incubated with 10^{10} PFU/ml of non-inactivated T6$^+$, and the number of intracellular bacteria was examined at 30 min intervals. During 150 min of incubation, the number of bacteria per infected cell increased about 18 times in spite of the presence of T6$^+$ in the medium. In addition, the percent infection as well as the number of bacteria per infected cell was similar after 150 min of incubation with and without T6$^+$. The authors conclude that T6 does not affect the ability of *S. flexneri* 2a to replicate intracellularly [46].

On the other hand, a few authors drew different conclusions from studies with other bacteriophages. First, when working on the response of liver and spleen to a single, intravenous dose of 5×10^8 PFU of T4 bacteriophages, Inchley [22] showed in mice that T4 particles were rapidly phagocytosed by Kupffer cells and, to a lesser extent and with four times the inactivation time, by splenic macrophages.

However, the fact that bacteriophages can be taken up by these cells does not mean that they could reach and destroy bacteria residing inside. Experiments conducted by other teams have been designed to assess the ability of phage particles to penetrate inside some eukaryotic cells and also to eliminate intracellular bacteria.

In 1980, a Tbilisi phage was shown to reduce by more than 95 % the number of viable *Brucella abortus* within cultured bovine monocytes when added either an hour before or after the bacteria (Corbel and Morris 1980, reviewed in [13]). Emery and Whittington wrote in 2004 that it was then the only example where intracellular proliferation was reduced by a phage treatment administered after bacterial phagocytosis. However, no heated, crude phage lysate was used as a control, and the possibility that proteins within it enhanced the antibacterial activity of monocytes can consequently not be excluded [13].

Following the study in which TM4 was shown to be effective against intracellular mycobacteria only if delivered by infected bacteria Broxmeyer et al. [3] and Peng et al. [39] assessed the ability of mycobacteriophage D29 to eliminate *M. tuberculosis* H37Rv within mouse peritoneal macrophages. They did not use any delivery system. Their results seem to indicate that, in contrary to TM4, D29 alone can decrease the number of intracellular mycobacteria in a titer-dependent way. Yet, the electron microscopic image intended to show intracellular phage propagation is unsatisfying.

Another study supporting the hypothesis that phages alone can be taken up by macrophages was carried out by Carmody et al. [6], who assessed the therapeutic potential of phages in a mouse model of acute *Burkholderia cenocepacia* pulmonary infection. *B. cenocepacia* is belonging to the *Burkholderia cepacia* complex. They are ubiquitous bacteria present, e.g., in soil and they are naturally resistant to many antibiotics. Normally they are plant pathogens, but they can also cause heavy opportunistic infections in humans with a compromised immune system, mostly affecting the lungs. *B. cenocepacia* is found extracellularly but is also able to persist and survive intracellularly in so-called *B. cepacia*-containing vacuoles. These vacuoles have been shown to be less acidic than lysosomes and lysosome-associated membrane protein (LAMP-1) is accumulated in a delayed fashion [27].

In the study by Carmody et al. [6], the authors showed that the lung bacterial density was significantly reduced in mice infected with 10^7 or 10^8 CFU *B. cenocepacia* by tracheotomy and treated with single intra-peritoneal injections of 10^9 or 10^{10} PFU bacteriophage BcepIL02, but not in mice treated with the same dose of bacteriophage by intranasal inhalation. Among other hypotheses, they suggest that in the latter case, phages could not cross the respiratory epithelium because of their inability to readily penetrate eukaryotic cells and could consequently not reach bacteria located within the lung interstitium. Using polarized 16HBE14o human bronchial epithelial cells, they observed in vitro that BcepIL02 did not penetrate the intact epithelium.

Interestingly, they showed that phages administered via intranasal inhalation were co-localized with alveolar macrophages after 48 h, both in *B. cenocepacia*-infected and mock-infected mice. This suggests that phage BcepIL02 alone can be taken up by alveolar macrophages. In addition, phage titers in lungs of *B. cenocepacia*-infected mice were greater than in those of mock-infected mice, showing that phages had probably gained access to and replicated within some bacteria. The authors suggested that this high titer may indicate that a significant proportion of phages

remain viable after uptake by macrophages. However, no mechanism by which BcepIL02 could reach bacteria within alveolar macrophages was evidenced.

In summary, there are some results that support the uptake of free bacteriophages by certain cell types. In a few cases, they also seemed to effectively reduce bacterial proliferation within cells. A schematic illustration of this process is depicted in section 1 of Fig. 1. However, very little information is currently available. It would be useful to generate more data by testing different combinations of bacteriophages, cell types, and bacteria, and to see whether the uptake of free bacteriophages is a rare or a common event and in which cases it is effective.

Artificially Vectorized Bacteriophages

From the above sections, we can conclude that in some rare cases, free bacteriophages can reach and defend intracellular pathogens (Fig. 1, section 1). In other cases, related but non-virulent bacteria serve as a carrier and in others, in turn, the pathogens themselves help carrying the bacteriophages into cells (Fig. 1, sections 2, 3). These so-called Trojan horse approaches bring some disadvantages linked to the bacterial nature of the carrier, but another relevant disadvantage is the low controllability of bacteriophage transport and release. More control could be achieved by vectoring bacteriophages artificially (Fig. 1, section 4).

In the context of food safety, for example, bacteriophages are used to prevent bacterial contamination of fresh foods like meat or dairy products. Here, some research has been conducted in order to encapsulate bacteriophages into electrospun biopolymer fibers for controlled liberation at the desired site of action [24, 29, 45]).

For pulmonary infections, some efforts have been made to develop bacteriophage formulations that can be inhaled. Golshahi et al. [15] managed to aerosolize lyophilized bacteriophages KS4-M (active against a *Burkholderia cepacia* complex strain) and ϕKZ (active against *Pseudomonas aeruginosa*) with the help of dry powder inhalers. They noted a tolerable loss of titer during the lyophilization procedure (from about 10^{10} PFU/ml to 10^{8} PFU/100 mg powder for KS4-M and from about 10^{9} PFU/ml to ca. 8×10^{8} PFU/100 mg powder for ϕKZ) and a good fine particle fraction (FPF), allowing the particles to reach the lungs. In an idealized mouth–throat replica, they managed to show that such aerosolized bacteriophages reached the lungs with a negligible titer drop. However, the minimal titer for the treatment of lung infections is unclear.

Puapermpoonsiri et al. [41] experimented with freeze-dried bacteriophages encapsulated in biodegradable microspheres made from poly(DL-lactic-co-glycolic acid) (PLGA). Bacteriophages selective for *S. aureus* maintained their lytic activity during encapsulation into PLGA microspheres. Although the stability of bacteriophages within this preparation was very poor with no bioactivity left after 7 days of storage at 4 °C or 22 °C, it seems to be a good principle to encapsulate bacteriophages for a controlled release at the site of infection.

Other groups have worked on the microencapsulation of viruses for oral administration. Different materials such as Chitosan-bile salt [28], spermine-alginate [35, 36], or also PLGA [49] have been tested. Ma et al. [31] used a chitosan-alginate-$CaCl_2$ system for microencapsulation of the *Salmonella*-specific bacteriophage Felix O1. Thus, microencapsulated bacteriophages were relatively stable in the presence of simulated gastric fluid or bile salts and were liberated in the presence of simulated intestinal fluid. Even better results were obtained by Dini et al. (2012) for the encapsulation of a bacteriophage specific for enterohemorrhagic *E. coli* (EHEC) by emulsified pectin with oleic acid.

Another well-established way to "dress up" therapeutically active compounds for a more efficient and better targeted release at the site of infection is their encapsulation into liposomes. Liposomal delivery of small molecule drugs such as the anticancer drugs Paclitaxel (Lipusu®, Luye Pharma, China) or Doxorubicin (Caelyx®, Janssen Pharmaceutica N.V.) is applied regularly in clinical practice. Liposomes possess good cell penetration characteristics and hence would be a suitable dress for bacteriophages employed against intracellular pathogens. Liposomes furthermore possess a large "wardrobe" of lipids with different properties in terms of internalization behavior or possibilities for functionalization with, e.g., "accessories" like carbohydrate or peptide/protein moieties or even with ligands for specific cell targeting.

Thinking about compartments, artificially vectorized therapeutic bacteriophages have to reach the correct intracellular compartment in order to get direct access to the targeted pathogens. There is evidence that liposomes are taken up by endocytosis and are found within early endosomes after uptake [54]. Pathogenic mycobacteria reside within mycobacterial phagosomes that prevent phago-lysosomal fusion but allow fusion with early endosomes [44]. Hence, liposomal-delivered drugs can theoretically gain direct access to intracellular mycobacterial pathogens, rendering liposomes a suitable vector for the intracellular bacteriophage therapy of mycobacterial diseases.

Certainly, it will be technically more difficult to entrap relatively large, spiky bacteriophages into liposomes than small molecules like the above-mentioned anticancer drugs. But the benefits of such a targeted delivery might outweigh the difficulties of development.

Conclusions

In the preceding paragraphs, we attempted to sum up the existing literature on the use of bacteriophage therapy against intracellular pathogenic bacteria. However, this literature has to be read with caution. While the recent studies treating this topic were performed at current standards, some were published decades ago and standards for clinical trials, especially concerning controls, have changed since then. Also, a lot of the literature is published in Russian, Georgian, or Polish, and we are reliant on overview articles summing up this part of the literature. Based on the available studies, we have tried to shed some light on the question of free bacteriophage uptake into infected cells versus vectorized intracellular delivery. Although there are controversial results regarding the uptake of free bacteriophages, the bulk of studies underpin a scenario in which bacteriophages are dependent on some kind of mediation for intracellular uptake. The strongest argument for this direction is the fact that most reports of successful intracellular killing of bacterial pathogens by bacteriophages involved a bacterial vector that was infected by bacteriophages before entry into cells. In some cases, like the studies regarding mycobacteria from the group of Luiz E. Bermudez [3, 9], this vectorization took place willingly via a non-pathogenic *Mycobacterium*. In other studies regarding *S. aureus* [5], *C. psittaci* [20, 43], *B. abortus* (Corbel and Morris 1980, reviewed in [13]), or *Salmonella* species (Kiknadze et al. 1986, reviewed in [51]), the infecting bacteria themselves may have served as vectors for intracellular delivery. The clearest argument against this hypothesis and for a direct uptake of free therapeutic bacteriophages comes from Peng et al. [39]. This is a single report but it clearly demonstrates the need for more systematic data collection.

So while the question of intracellular delivery is still controversial, the scientific community has already started to work on more sophisticated ways of bacteriophage delivery, be it for oral, pulmonary, or intracellular administration. Several principles for the microencapsulation of bacteriophages, in order to achieve a higher level of administration control, have been developed and tested. For intracellular delivery, the yet untested method of liposomal delivery might be a versatile alternative, offering the possibility to choose from many different lipid species and options for modification and functionalization. These four methods of cellular entry and two scenarios of intracellular pathogen lysis are depicted schematically in Fig. 1.

The development of encapsulated therapeutic bacteriophages that (a) are efficiently delivered into infected cells and (b) destroy pathogenic bacteria within them would be a breakthrough in the struggle to find alternative treatments against antibiotic-resistant pathogens like MDR- or XDR-*M. tuberculosis*.

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