Molecular and immunological determinants for T4, A3R and 676Z phages presence in mice gut

Summary

Bacteriophages are an important component of human and animal microbiota, particularly in the gastrointestinal tract, where they constitute the dominant fraction of the virome and are involved in the regulation of bacterial gut microbiota. Recent studies of phage interactions with human and animal systems clearly indicate that the ways in which phages affect higher organisms go far beyond merely maintaining the balance of bacterial populations. Bacteriophages are also used for therapeutic purposes to treat patients suffering from bacterial infections and are considered one of the most important and promising alternatives to standard antibiotic therapy, especially in the face of the constant alarming spread of antibiotic resistance. Oral administration of therapeutic phage preparations is simple, relatively safe, and well-tolerated by patients and its major limitations include phage sensitivity to the highly acidic stomach environment. It is the dominant delivery route mostly in bacterial gastrointestinal infections, yet oral phage therapy has also been successfully applied to treat systemic infections, such as, e.g., sepsis. Moreover, oral administration of phage preparations can be used adjuvantly with other delivery routes. Regardless of the administration route, bacteriophages—being foreign particles—can induce the production of specific antibodies, which in turn may lead to the inactivation of phages, contributing to the diminished effectiveness of the treatment. However, based on the data reported to date, no clear correlation between the level of specific antibodies and the success of phage therapy has been demonstrated. The recent rapid development of novel techniques for precise, targeted modifications of bacteriophages opens up new prospects for phage therapy as it allows for the construction of phages with more desirable properties, e.g., removing genes involved in the process of lysogeny or modifying phage proteins involved in bacterial cell recognition and binding in order to shift or expand the lytic spectrum. In a wider context, similar targeted modifications could potentially be used in the future to manipulate the immunogenicity of phage virions or the susceptibility of phage particles to certain environmental factors, e.g., in the gastrointestinal tract, especially since medical applications of phages are not restricted to combating bacterial infections – whole phage particles and modified phage capsids have been proposed as drug carriers and vaccine platforms. This is particularly relevant in the case of phages that are thoroughly characterized in terms of their protein structure and phage particle properties, such as model bacteriophage T4. However, despite the important and potentially diverse roles of bacteriophages in human health—both administered therapeutically and naturally occurring as part of our microbiota—the factors involved in phage presence, persistence, and activity in the gastrointestinal tract are still underexplored. This includes both factors related to human and animal organisms as well as the molecular properties of phage virions themselves.

The objective of the doctoral dissertation was to identify selected immunological and molecular factors that affect the presence and activity of bacteriophages in the animal gastrointestinal tract – both in terms of the phage's ability to induce the production of specific antibodies and the potential impact of those antibodies on the bioavailability of phages and in the context of how individual structural proteins of the phage contribute to the

immunogenicity of the whole virion and also shape its resistance to environmental factors that characterize the gastrointestinal tract microenvironment, such as temperature, acidic and alkaline pH, and the presence of proteolytic digestive enzymes.

Three tailed bacteriophages—T4, A3R, and 676Z—were used in the research described herein, all representing the myovirus morphotype and previously classified as members of the *Myoviridae* family within the order *Caudovirales*. With the ongoing major changes to the bacteriophage taxonomy aiming to reflect actual phylogenetic relationships rather than morphological similarities, these phages are now placed in the class *Caudoviricetes* and the families *Straboviridae* (T4) and *Herelleviridae* (A3R and 676Z). Bacteriophage T4 is a model phage infecting *Escherichia coli* and is naturally present in the gastrointestinal tract of animals and humans as part of their normal microbiota. It is one of the most complex viruses, well-characterized in terms of its structural proteins, their organization, and interactions. Out of more than 40 proteins forming mature T4 phage virions, five are relevant in the context of this dissertation: four head proteins, namely major capsid protein (gp23), head vertex protein (gp24), Hoc, and Soc; and gp12, forming the short tail fibers of the phage baseplate and involved in the recognition and binding to the receptors on the surface of host bacterial cells. Bacteriophages A3R and 676Z both represent the genus *Kayvirus* and have a high degree of similarity. They are specific to *Staphylococcus aureus* and have been used as therapeutic phages at the Phage Therapy Unit of the Hirszfeld Institute of Immunology and Experimental Therapy. Their protein structure is less thoroughly characterized, yet in recent studies predicted structural function and localization within the virion were experimentally confirmed for several proteins with the use of immune-TEM.

The proposed doctoral dissertation comprises a series of three thematically related research articles. In the first publication (Viruses, 2015, 7(8):4783-99), in a first study of humoral response kinetics during a very long-term oral application of phage preparations, a mouse model of the induction of phage-specific antibodies by the model bacteriophage T4 administered in drinking water was investigated. The induction of humoral response in the blood (phage-specific IgG antibodies) and, much later, also in the gastrointestinal tract (phage-specific secretory IgA antibodies) was observed. The effect was dose-dependent, and the level of phage-specific antibodies in mice immunized *per os* with a relatively high T4 phage dose was significantly lower than in the case of subcutaneous immunization, indicating that in the model of oral administration, phage particles are weakly effective in inducing the humoral response. Importantly, a parallel assessment of the changes in phage-specific antibody levels and the evaluation of fecal samples regarding the presence of active phages and *E. coli* isolates resistant to T4 phage identified the production of secretory phage-specific IgA antibodies as a major factor limiting the activity of T4 phage in the gastrointestinal tract, far more important than the dominance of phage-resistant bacterial isolates. In the comparison of the levels of antibodies specific to five selected T4 structural proteins—gp23, gp24, Hoc, Soc, and gp12—a significant increase was detected only in the cases of Hoc and gp12, indicating their contribution to the induction of the humoral response in the investigated model. Finally, in the analogous model of oral administration of phage particles, an exemplary peptide antigen derived from the Ebola virus was used to demonstrate that induction of secretory IgA antibodies specific to a foreign antigen presented on the surface of the T4 phage capsid as a fusion with Hoc protein is also

possible. This observation may be relevant in the context of the potential application of phage particles as vaccine platforms or drug nanocarriers.

The second publication (Frontiers in Immunology, 2019, 10:2607) addressed the question of whether the observations previously made for the model bacteriophage T4 are universal and could be applied to other phages, particularly those representing the same morphotype but infecting a phylogenetically distant bacterial species far less abundantly than *E. coli* present within the normal microbiota of the gastrointestinal tract of animals and humans. Therefore, a mouse model of oral administration of therapeutic staphylococcal bacteriophages A3R and 676Z was established, analogous to that for the T4 phage. As both bacteriophages have been applied to treat staphylococcal infections at the Phage Therapy Unit, the results obtained in this study could have potential clinical implications. As in the case of bacteriophage T4, induction of phage-specific humoral response in the blood and in the gut was observed for phages A3R and 676Z, along with comparable antibody kinetics; however, a slight yet statistically significant increase of phage-specific IgM antibodies was detected this time. Importantly, changes in phage-specific IgA levels again correlated with the presence and absence of active phage particles in the fecal samples, which confirmed the role of secretory IgA as the major factor limiting phage activity in the gastrointestinal tract. Comparison of A3R and 676Z phage-specific antibody levels measured in the oral administration model and as a result of intraperitoneal immunization also showed a less prominent humoral response in the case of oral treatment, further supported by significantly weaker inactivation of phages after incubation with plasma collected from the mice immunized *per os.*

Interestingly, studies of the immunogenicity of three selected structural proteins—major capsid protein (Mcp) building the head of bacteriophages A3R and 676Z, tail morphogenetic protein H (TmpH), and gpORF096 located in the baseplate region and most likely involved in the bacterial cell surface recognition and binding—showed striking differences with regard to the results obtained for the T4 structural proteins. In the case of staphylococcal bacteriophages, a marked increase in the level of antibodies specific to Mcp was observed, indicating its immunogenic properties in the investigated model. Conversely, gpORF096 turned out to be nonimmunogenic. A significant increase in specific antibody levels was also observed for the tail sheath protein TmpH. These observations suggest that while the general schema of specific antibody induction following oral phage administration appears to be universal, the role of individual proteins in eliciting the humoral response may vary greatly between different phages.

Besides the antibody kinetics, phages A3R and 676Z were also investigated regarding their translocation from the gastrointestinal tract to the circulation in a mouse model. The results indicated poor and irregular translocation of active phage particles to the blood and a lack of clear correlation between detected phage titers in the blood and the administered phage dose or previous neutralization of the acidic contents of the stomach. Active phage titers in the blood were also significantly lower than the range expected based on the mathematical model proposed in the article.

The first two publications focused primarily on the interdependence of phages and the elements of the antiphage humoral response. However, factors affecting phage survival are not limited to immunology-related ones. Therefore, the third and final article of the series (Microbiology

Spectrum, 2023, in press) addressed the direct influence of factors typical for the gastrointestinal tract environment on phage activity using the model bacteriophage T4 as an example. In these studies, an evolutionary perspective was considered, in which the protein composition of the capsid could have been—at least partially—shaped in response to the selection pressure exerted by the specific conditions within the gastrointestinal tract of animals and humans: temperature, acidic and alkaline pH, and the presence of proteolytic digestive enzymes and bile. To identify the role that T4 phage head proteins gp24, Hoc, and Soc have in the resistance of phage particles to those factors, a panel of T4 phage variants deficient in one, two, or all three of these proteins was constructed. Homologous recombination followed by selection based on either microbiological techniques or PCR was applied to obtain the mutants, and their protein composition was verified using ELISA. The mutants lacking the specialized head vertex protein gp24 were more sensitive to low pH and the activity of proteolytic enzymes pepsin and α-chymotrypsin. Moreover, variants simultaneously deficient in both gp24 and Soc were less stable at 37°C. These results indicate the key role of gp24 in the T4 phage withstanding environmental conditions, including those present in the gastrointestinal tract, and support the previously postulated role of Soc as molecular glue that stabilizes the capsid. Additionally, recombinant proteins gp24, gp23, and two gp23 variants with single bypass-24 mutations (these mutations allow gp23 to form, besides the typical hexamers, also pentamers that replace gp24 pentamers at the capsid vertices, compensating for the lack of gp24, otherwise lethal for the phage) were compared in terms of thermal denaturation, revealing remarkable stability of gp24 and no significant differences between the wild-type gp23 and its two variants, which further supports the importance of gp24.

In conclusion, the objective of the doctoral dissertation has been achieved. Over the course of the presented studies, the kinetics of phage-specific antibody production in response to oral administration of phage preparations were determined in a mouse model. Secretory IgA antibodies were indicated as the major factor limiting the activity of bacteriophages in the gastrointestinal tract, much more important than the selection of bacteria with a phage-resistant phenotype. It was also demonstrated that phage structural proteins differ in terms of their contribution to the induction of the anti-phage humoral response, and the individual immunogenicity of proteins of identical or highly similar structural function (e.g., major capsid protein or baseplate proteins involved in the interactions with the host cell) could vary in different phages despite them representing the same morphotype. Additionally, with the example of the T4 phage, it was demonstrated that the complex protein composition of the capsid—in particular the presence of the specialized head vertex protein—determines the phage's resistance to external factors, including those related to gastrointestinal tract conditions, which could indicate the potential role of the gastrointestinal microenvironment in directing the evolution of phage virions.