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Review

The potential role of endogenous bacteriophages in controlling invading pathogens

(article dedicated to Professor Ludwik Hirszfeld to commemorate the 50th anniversary of his death)

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Abstract. Bacteriophages (phages) are omnipresent in our environment, and recent studies highlight their potential impact on the microbial world. Phages can also be present in mammalian organisms, including man (intestines, oral cavity, urine, sputum and serum). Data are available which suggest that those endogenous phages could play an important role in eliminating bacteria and regulating the body ecosystem. Furthermore, our most recent findings suggest that phages can exert immunosuppressive action in the gut, helping control local inflammatory and autoimmune reactions, and demonstrate anticancer activity. We hypothesize that phages could act in concert with the immune system in immunosurveillance against bacteria, viruses and cancer.

Key words. Bacteriophage; phage therapy; infection; virus; immune response; immunomodulation; gut flora.

Introduction

Bacteriophages (phages) are bacterial viruses that infect bacterial cells, disrupt bacterial metabolism and cause the bacterium to lyse (lytic phages). Each kind of phage specifically targets only certain bacteria as its host. In contrast to lytic phages, lysogenic phages infect bacteria and integrate their own genome into the genome of the hosts (prophages). However, a lysogenic phage may retrieve its genome from the host and finally lyse the bacterium, thus becoming a lytic phage [1].
In the recent years, leading biomedical journals have published reviews on the current status and perspectives of bacteriophage (phage) therapy. Those articles emphasise the growing menace of the increasing prevalence of antibiotic-resistant bacterial infections and suggest that urgent action is needed to expand research and undertake all other necessary efforts to introduce phage therapy into clinical use [1–11].

Phage therapy: past, present and perspectives

Preclinical studies in animals have demonstrated a high efficacy of phages in preventing and treating bacterial infections. Probably the most fundamental data have been provided by Smith et al. [12], who showed that severe, experimentally induced *Escherichia coli* diarrhoea in calves could be cured by a single dose of 10^5 phage organisms. Phages multiplied rapidly and profusely in the *E. coli*-infected small intestine quickly reducing the offending bacteria to harmless numbers. Importantly, the disease could be prevented by phage doses as low as 10^2 and, interestingly, by spraying the litter in the calves' rooms with aqueous phage suspensions or even by keeping the calves in uncleaned rooms previously occupied by calves previ-

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ously treated with phages. Other authors essentially confirmed the effectiveness of phage therapy in laboratory animals, chickens and fish [4, 13–15].

Since human studies performed so far have not been done using current stringent criteria of clinical studies, including randomization and placebo controls, it has been argued that they offer little evidence to prove phage efficacy. However, such an opinion undermines the results achieved so far. First, it must be stressed that observational studies still have an important role in clinical research: '… observational studies have a record of extremely successful contributions to medicine. They are essential for our knowledge about causes and pathogenesis – e.g. … infectious causes of disease' [16]. Second, the studies performed so far have certainly provided support for the fundamental principle of 'primum non nocere' (first do no harm), as the data obtained from hundreds of patients who have been treated (including early initial trials involving parental phage administration in adults and children) have not provided any hint that the administration of 'therapeutic' phages could violate that principle, as there has been no proof of phage toxicity or significant side effects.

It has also been widely accepted that there are two major centers of human phage therapy: Georgian (Tbilisi) and Polish (Wroclaw). Regretfully, the Georgian center has published most of its reports in Russian, while our center has, according to *Science*, 'the most important data in the English literature' [17]. So far we have treated approx. 2000 patients with antibiotics-resistant bacterial infections, with an average success rate of some 80%: more data are available on our website:

http://surfer.iitd.pan.wroc.pl/phages/phages.html

Thus, there are sufficient data from preclinical and human studies to believe that phage therapy may provide a powerful weapon in combating bacterial infection, a conclusion which should be further strengthened by formal clinical trials.

The bionomics of phages

The greatly revived interest in the possible clinical application of phages has also resulted in significant advancements in our understanding of the occurrence and role of phages in our environment [18].

Recent progress in the bionomics of phages indicates that they should significantly impact upon microbial food webs. The abundance of phages in our environment has been greatly underestimated. In 1979, Torrella et al., using electron microscopy, reported high virus numbers in sea-water; those data have since been confirmed and extended [19]. As pointed out by Weinberger, total viral abundance across aquatic systems varies between 10^4 and more than 10^8/ml. In freshwater sediments it can range from 0.65 to 3×10^9/g (up to 12×10^9 in marine systems). In soil, phage abundance varies from 0.7 to 2.7×10^8/g, and the virus-to-bacteria ratio (VBR) may be as high as 100. In fact, the VBR can vary in different environments and be as low as 0.01, which probably reflects strong viral decay under unfavorable conditions [18]. Phages also occur in food, including such traditionally 'probiotic' products as yogurt and sauerkraut [20–22]. (It remains to be seen to what extent the suggested probiotic activity of those products is dependent on health – promoting bacteria or perhaps phages.)

The growth and survival of phages is controlled by a variety of factors (e.g. pH, water, temperature, presence of host bacteria). (The recent review by Weinbaurer addresses these issues in depth [18].) Of these factors, the metabolic status of the host is probably the most important factor regulating phage proliferation. Phage survival in the environment appears to be a more complex phenomenon, and phage decay rates vary greatly, from undetectable to 80%/h. Among the factors contributing to phage loss is the damaging effect of sunlight, although susceptibility to high-energy radiation may differ among phage species. Furthermore, variations in temperature can damage phage infectivity. On the other hand, inorganic and organic colloids and particles may increase phage survival.

While the fact that phages are omnipresent in our environment has been recognized in recent years, their possible presence and role in the human body is much less appreciated. D'Herelle already noted in 1922 frequent specificities of phages isolated from recuperating patients to their own disease bacteria [23]. This could suggest that phages present in human intestines may be responsible for gut defenses against harmful bacteria, their extensive spread, and the resulting local and generalized pathology, culminating in overt clinical disease. D'Herelle also believed in the superiority of phage over the immune system in fighting bacteria. This idea was, at least to some extent, shared by L. Hirszfeld, who presented the view that phages are much more efficient in eliminating bacteria than the immune system [24]. In fact, Kamme showed elevated anti-Staphylococcal phage antibodies in patients with *Staphylococcus aureus* infections: a fourfold rise of such anti phage antibodies may occur within 6–14 days in patients with acute infections. The author suggested that phages were released by infecting lysogenic *S. aureus* strains (the frequency of lysogeny in those strains may be as high as 95%) [25].

These data strongly suggest that *S. aureus* infections are associated with a concomitant increase in phages and that their anti-bacterial activity may determine the clinical picture of infection and the need for therapy.

The important role of intestinal phages in controlling local bacterial populations is suggested by Atterbury et al. [26], who found a correlation between the presence of

natural environmental phages and a reduction in the titer of campylobacters colonizing the chicken cecum. Furuse at al. [27] have described a marked increase in *Serratia* phage titer in feces of a patient who developed serious clinical symptoms caused by infection with that bacterium. However, D'Herelle was probably the only author so far who tested and formally proved the cidal activities of the phages against bacterial isolates from the same patients.

The intestinal phages

A number of reports describe the presence of phages in animal and human feces. In fact, Joshua Lederberg, a Nobel laureate, has stated that 'phages are universally present together with their bacterial host in the lower gut' [28]. Dhillon et al. have found somatic coliphage in the majority of fecal samples, with titers of up to $>10^7$ pfu/g. [29]. Osawa detected fecal coliphage in approx. 25% of samples [30], while Havelaar et al. in virtually all samples [31]. Gantzer et al. [32] confirmed that the majority (approx. 70%) of human fecal samples contain coliphage, with a mean concentration of 4×10^3 pfu/g. There was no correlation with either the age or sex of human subjects. In contrast, only 11% of samples were positive for *Bacterioides fragilis* phage, and their concentration was 60 times lower. The authors emphasize the geographical similarities and dissimilarities in phage detectability in feces: the frequency of samples positive for coliphage was similar in France, South Africa, Holland and the USA, but a higher value was reported in Japan. In addition, the mean concentration of the French samples was lower than that found in Holland and Japan, but similar to the values found in Spain. The incidence of *B. fragilis* phage was similar in Holland, Japan and South Africa. Interestingly, no such phage could be isolated from a total of 180 stools examined in the USA and Spain. Cornax et al. [33] have confirmed a high frequency of specific bacteriophage of *E. coli* (approx. 90% of fecal samples), whereas the detection percentages of other phages were lower. In addition, there was a positive relationship between the densities of phages and their hosts, but a linear regression could not be established. Furthermore, at a fecal coliform concentration under 10^6/g, the isolation frequency of phages was very low. This contrasts somewhat with the data of Havelaar et al. [31], who showed that in contrast to pig, chicken, cow, horse, sheep and calf, in dog and humans there was no positive relationship between average phage counts and average counts of coliforms. Interestingly, patients with functional gastrointestinal disturbances had lower frequencies of *E. coli* K12 phage than patients with infectious diarrhea [16.7% healthy subjects, 8.9 vs. 27.4%, respectively). The authors suggest that the phage can act as a biocontrol factor in the population dynamics of the intestinal bacteria in health and disease. However, other authors suggest that *E. coli* within the lumen of the colon may have altered metabolism and be non-replicating, and therefore a poor target for phages [34].

Coliphages and *Enterobacter* phages are resistant to acid up to pH 3; at pH 2.5 and lower their activity rapidly diminishes. Therefore, diet rich in alkali (milk) and other foodstuffs neutralizing gastric acid may have phage-protective effects, and it is thus believed that phages should be given with a meal or with alkali [12, 35]. Moreover, infection of *E. coli* by phages can be inhibited by the presence of certain bile salts and carbohydrates [36], so a diet influencing these parameters may also alter the activity of intestinal phages. However, it has to be kept in mind that different phages may have different susceptibility to both gastric acid and bile [37]. In addition, a high-fiber diet causes more rapid clearance of phage DNA from the gastrointestinal tract, while a high-fat diet was without such effect [38].

Even though the presence of phages in the human gut is so well documented, it is striking to note that many recent reviews, including ones published in *Nature* and the *Lancet* addressing the issue of gut flora in health and disease and the role of microbial interactions with the immune system in the strategic defenses of the intestinal mucosa do not even mention phages [39, 40]. There are more than 400 species of microbes in the human ileum and colon, and the factors which control this heterogeneity are poorly understood. In fact, most antigens enter the body through mucosal interfaces, and the microenvironment in these mucosal barriers has a marked influence on the resulting immune response. This is especially relevant for gut-associated lymphoid tissue (GALT), responsible for distinguishing harmless antigens present in food or commensal bacteria and pathogenic assault by microbes. Importantly, GALT contains more lymphocytes than all of the secondary lymphoid organs combined [41–43]. Various immune mechanisms have been suggested to understand the ways in which immune responses in the gut are induced or blocked, but how our immune system is able to deal with the massive antigen challenge provided by micobial flora remains a mystery. (It is estimated that around 60% of fecal solids consists of bacteria, so the average human intestine contains more than 1 kg of bacteria, which achieve concentrations of up to 10^{12} cells/g of luminal content – concentrations similar to those growing under optimal conditions on a laboratory plate [40, 43].)

Phage penetration and translocation

The presence of phages in the gastrointestinal tract suggests that they could, under certain circumstances, cross the epithelial barrier and enter extraintestinal sites:

mesenteric lymph nodes, spleen and liver (organs which are primarily responsible for phage clearance following their administration), and reach the bloodstream. This phenomenon could correspond to the passage of bacteria from the intestinal lumen, referred to as translocation. Studies in individuals undergoing laparotomy have revealed that in up to 5% of cases, mesenteric lymph nodes of otherwise healthy people could yield intestinal bacteria. This number may be as high as 40% in inflammatory gastrointestinal disorders, liver cirrhosis and intestinal obstruction, and may be associated with subsequent sepsis, multisystem organ failure and peritonitis [40]. Evidently, phages could also use those routes for their passage from the intestinal lumen to neighboring tissues, lymph nodes and, eventually, the peripheral blood ('phagemia'). Our group and others have demonstrated that orally administered phage can reach the peripheral blood and migrate to the infection sites [44, 45].

Furthermore, there are data suggesting that following oral administration, phages can also penetrate to normal tissues and organs, including spleen, liver and kidney. Moreover, such migration was also confirmed when phages were administered into the rectum and at other mucosal surfaces (trachea, vagina), while intranasal administration allows phages to migrate to the central nervous system [45, 46]. Thus it cannot be excluded that translocation of naturally occurring phage may indeed take place, similar to that of bacteria, and if so, phages could sometimes also be detectable in the peripheral blood. We have not found in the available literature any data on such migration of phages occurring in vivo. On the other hand, there have been numerous reports indicating that phages may be detected in bovine sera used for in vitro tissue culturing: their concentrations were low (in the majority of cases 5–10 pfu/ml). The reported level of contamination of sera with phages should be considered as minimal, since very limited bacterial targets were used in those studies [47–51]. In fact, when electron microscopy was applied, phage presence was documented in the majority (68%) of all sera examined [52]. No phages have been so far reported in commercially available human sera, but they may be present in licensed live-virus vaccines (such vaccines have been nevertheless approved for human use by the FDA) [53]. Although contamination during serum processing cannot be excluded as a source of phages in those sera, some authors believe that phage presence in sera reflects 'physiologic viremia' ('phagemia') [49].

Phage presence at sites outside the gastrointestinal tract

Recently, the presence of phages in the oral cavity has also been described. Fresh dental plaques may contain phages infecting *Actinomyces* [54]. Furthermore, phages against *Lactobacillus*, *Veilonella*, *Actinobacillus* and *Streptococcus mutans* have been obtained from oral material [55, 56]. Bachrach et al. [57] have found *Enterococcus faecalis* phages in human saliva and suggested that they can have a role in the oral ecosystem by restricting this bacterial population to the tooth root system (*Enterobacter faecalis* can frequently be isolated from root canals where endodonthic treatment has failed [58]). A recent report by Hitch et al. [59] suggests, however, that unlike other ecosystems, the composition of the oral cavity does not appear to be significantly influenced by phages. On the other hand, the most recent data of Paisano et al. [58] confirm the observation of Bachrach et al. [57] in that phages can penetrate the infected tooth tubules and eliminate dentin infection caused by *Ent. faecalis*.

Ojeniyi et al. [60] have found phages in the sputum samples of all 16 cystic fibrosis patients; this suggests that the sputum may be another ecosystem where phages can exert their action. Finally, phages have been also isolated from the urine of a patient with urinary tract infection [61].

We have not found in the available literature any reports on phage isolation from skin. However, *Borelia burgdorferi* isolated from infected human skin and exposed to ciprofloxacin release phages [62].

Phage cycle was also observed in diphtheroids in human skin [63]. Furthermore, phages may be highly efficacious in treating bacterial skin infections [64, 65]. Therefore, there exists indirect evidence that skin phages could provide some local anti-bacterial protection.

Phage occurrence in vivo, as already mentioned, can also be induced by their release from lysogenic bacteria. Such processes may be relevant in patients treated with agents which have been shown to cause such release of virulent phage: chemotherapeutic agents, some antibiotics, nitrofurantoin and cigarette smoke [66–68]. Furthermore, Broudy et al. have suggested that mammalian tissues may also induce phages [69]. However, the authors used only neoplastic cells for in vitro induction, and in vivo experiments in mice have only been partially successful; therefore further studies are needed to shed more light on this phenomenon.

Phages can be transmitted to further individuals. Touching door handles or shaking hands can transmit phages which have been applied to those primary contact points or hands of volunteers. Such transmission also takes place under everyday life conditions (sharing an apartment) [70].

Phage elimination by the immune system

Phages administered by the intravenous route may be rapidly eliminated from the circulatory system and are

primarily retained by spleen and liver, where they may persist for some days. However, phage penetration and presence in various tissues may vary depending upon their route of administration and the animal; this is presented in detail in our recent publication [45]. Merril et al. [71] used a serial passage technique in mice to obtain a phage mutant capable of evading the reticuloendothelial system and therefore capable of long circulation in the blood. Whether similar phenomena can also occur in vivo with endogenous phages is unclear, but given the well-known mutability of phages, cannot be excluded. On the other hand, it is also known that administered phages can multiply in vivo in the foci of bacterial infection, and such phages may be less sensitive to systemic anti-phage antibodies induced by intravenous administration during phage treatment. In addition, it should be emphasized that oral antigen administration may lead to tolerance rather than immunization [72]; therefore, one cannot exclude that such tolerizing effects may also occur following oral phage administration. Interestingly, *E. coli*, a host for coliphages, is important in oral tolerance induction [73]. Finally, although humoral anti-phage responses are quite well known, it is unclear whether T-cell-mediated anti-phage immunity also exists and, if so, what its actual role in phage elimination is. Thus, phage interactions with the immune system is a fascinating and completely novel field of research which warrants in-depth study.

The protective potential of endogenous phages ('natural phage therapy')

To summarize the above, naturally occurring phages can be detected in the body of animals and man, and their presence has been documented in feces, saliva, sputum, urine, as well as bovine and calf serum. An important question thus arises as to the biological role of phages in vivo. Are they merely 'innocent bystanders', or do they play some role in protecting our body against invading microbes, or could they perhaps also be harmful?

It has long been recognized that lysogenic (or temperate) phage (quiescent phages which integrated their genome into bacterial chromosome and do not lyse their host while present in this form) may encode factors enhancing bacterial pathogenesis and transfer those genes to other bacteria. However, one should realize that this phenomenon is 'a fact of life', as DNA transduction is going on throughout the environment anyway, and it is estimated that tons of DNA exchanged by phages at any one moment [1, 18]. Furthermore, this caveat does not apply to the lytic phages that have been used in animal and human phage therapy. In fact, no one has ever described any significant side effects of phage administration or therapy: the reported histological, biochemical and urinalysis data have all been normal [1–11, 74]. In addition, phages added in vitro have had no significant effect on eukaryotic cells [75].

One should also remember that antibody response to intravenously administered phages has been used for some 30 years to monitor immunity in immunocompromised patients [bone marrow recipients, human immunodeficiency virus (HIV)-infected patients, children with immunodeficiency syndrome] in leading US academic centers [76–78]. Thus, at the time of this writing one can assume that the phages present in vivo are no more harmful than the phages used in therapy, which is currently igniting the greatly revived interest and optimism.

In this situation, it would be important to highlight the possible advantages of harboring phages in our bodies and their potential participation in our defenses against invading agents. It is estimated that phages are responsible for up to 50% of the total bacterial mortality in surface waters [18, 79]. Goodridge believes that many pathogens that grow in plants are successfully controlled via phage-mediated biocontrol [80]. In a similar manner, phages could contribute to the biocontrol of bacteria, for example, by reducing the number of bacteria to levels that can be dealt with by our immune system. Indeed, experiments in mice have revealed that there is a threshold density of bacteria which causes mortality in untreated animals, and treatment with phage reduces the number of bacteria to a level within the range of this threshold [1].

Of particular interest is the potential role of phages present in the intestines, especially in view of our recent findings indicating that they may have immunosuppressive properties when administered in vivo, inhibiting both humoral and cell-mediated immunity [81, 82]. In this sense, intestinal phages may not only contribute to the elimination of harmful bacteria and reduction of commensal bacterial numbers, thus alleviating the burden of heavy bacterial load on local mucosa and GALT, but may also inhibit local immune reactions. Such reactions provoked by antigens of the enteric bacterial flora have been shown to play a role in some forms of colitis in mice (which can be transferred by T cells reactive with commensal flora) [41, 83]. Interestingly, our recent data suggest that phages can inhibit some dendritic cell functions as well as NF-kappaB activation [unpublished data]: those cells as well as NF-κB may be instrumental in immune-mediated gut injury [84–86]. Interestingly, dendritic cells have been shown to rapidly phagocytose T4 phage, and the efficacy of this process exceeded the phagocytosis of other markers [87]. The immune responses in the intestine remain in a state of controlled inflammation, so active suppression plays an important role in normal homeostasis [88]. Therefore, phages could play a protective role in the development of overt gut inflammation in healthy people, and breakdown of phage-medi-

ated tolerance (e.g. lack of specific phages or their low activity) may contribute to the development of inflammatory bowel diseases as well as disturbances caused by bacterial intestinal overgrowth.

Why do our phage defenses often fail?

If indeed our own phages play a protective role against invading bacteria, the question arises why such phage defenses often fail. As already mentioned, phage occurrence may vary in a population, and phage presence and activity in vivo may depend upon such variable factors as the use of drugs (especially antibiotics, which may reduce the number and viability of their bacterial hosts), diet, hygiene etc. Furthermore, it has been known for many years that serum can inactivate phage via its antibody dependence and other factors. In fact, the presence of naturally occurring anti-phage antibodies has already been reported by Niels Kaj Jerne, and those studies have led to the formation of his natural selection theory of antibody formation [89, 90]. Smith et al. have found that anti-coliphage neutralizing antibodies may be common in human, cattle, pig and bovine serum samples, and their incidence may vary in relation to a specific phage. It is clear that such neutralizing antibodies may adversely influence the ability of our phages to control bacteria (however, the presence of increased anti-phage antibodies in blood may also reflect their translocation rather than a phage defense response against bacteria). Furthermore, body temperature can influence phage activity: some phages are only virulent at the highest temperatures (43 °C), others being virulent at 37 °C and avirulent at higher temperatures etc. Since rectal temperature in mammals can vary between 37 and 39.5 °C, this phenomenon can also regulate phage functions in health and especially during infection with associated temperature elevations [91].

Another factor contributing to poor control of bacteria by endogenous phages may be the development of phage resistance by those bacteria. However, phages can also evolve and overcome this resistance. Moreover, phage-resistant bacteria may have reduced fitness and lower ability to colonize the host; in addition, phage receptors for bacteria may correspond to their virulence determinants, such that the phages might not be able to transfer virulence factor(s) to resistant bacteria which, in turn, will be no longer pathogenic. In other words, one could assume that in some circumstances the development of bacterial resistance to endogenous phages may be a positive phenomenon that could render the invading pathogen incapable of causing disease [1].

Phages as potential immunomodulators

Interestingly, phages may contain protein sequences that play an important role in human immunopathology. Thus, collagen sequences occur in a protein present in the heads of some coliphages [92]. In addition, the highly immunogenic outer capsid protein (Hoc) of the T4 phage head [93] contains an immunoglobulin superfamily domain (members of this superfamily include antibodies, major histocompatibility antigens, T-cell receptors and cell adhesion molecules) [94]. The functions of these phage proteins are unknown (they are not relevant for phage interactions with bacteria); thus, their possible role in potential phage interactions with the immune system cannot be excluded.

It has been recognized that viruses usually cause immunosuppression [95]. This could be relevant in viral immunopathology, as mammal genomes are highly infected with some potentially pathogenic viruses. In this sense, Villarreal believes that retrovirus-mediated immunosuppression may play an important role in suppressing mothers' immune reactivity to their own allogeneic embryo. The author also describes a similar system of virus-mediated suppression of host immunity in the reproduction of some insects [96]. In contrast to pathogenic viruses, phages do not appear to cause any evident harm to mammalian cells; therefore, our suggested theory of phage-mediated natural immunosuppression may be a more physiological mechanism for maintaining immune homeostasis and controlling the overgrowth of bacteria.

Our recent hypothesis suggests that phages may also contribute to our body's anti-viral and anti-cancer defenses [97], and our initial data provide some support for this [98]. Further studies are needed to shed more light on the phenomenon of phage occurrence in the human body, their interactions with the immune system and the possibility that phages contribute to our homeostasis.

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