CHAPTER 2

# Phage as a Modulator of Immune Responses: Practical Implications for Phage Therapy

**Andrzej Górski,[*,†,#,1] Ryszard Międzybrodzki,[*,#] Jan Borysowski,[†] Krystyna Dąbrowska,[*] Piotr Wierzbicki,[†] Monika Ohams,[†] Grażyna Korczak-Kowalska,[†,‡] Natasza Olszowska-Zaremba,[†] Marzena Łusiak-Szelachowska,[*] Marlena Kłak,[*] Ewa Jończyk,[*] Ewelina Kaniuga,[†] Aneta Gołaś,[†] Sylwia Purchla,[†] Beata Weber-Dąbrowska,[*,#] Sławomir Letkiewicz,[#,§] Wojciech Fortuna,[*,#] Krzysztof Szufnarowski,[#,‖] Zdzisław Pawełczyk,[#] Paweł Rogóż,[#,¶]** and **Danuta Kłosowska[†]**

Contents



* Bacteriophage Laboratory, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland
# Phage Therapy Unit, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland
† Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland
‡ Department of Immunology, Warsaw University, Warsaw, Poland
§ Katowice School of Economics, Katowice, Poland
‖ Regional County Hospital and Research Center, Wrocław, Poland
¶ Department of Orthopedics, Wrocław Medical University, Wrocław, Poland
1 Corresponding author, E-mail: agorski@ikp.pl

Advances in Virus Research, Volume 83
ISSN 0065-3527, DOI: 10.1016/B978-0-12-394438-2.00002-5

**Abstract** Although the natural hosts for bacteriophages are bacteria, a growing body of data shows that phages can also interact with some populations of mammalian cells, especially with cells of the immune system. In general, these interactions include two main aspects. The first is the phage immunogenicity, that is, the capacity of phages to induce specific immune responses, in particular the generation of specific antibodies against phage antigens. The other aspect includes the immunomodulatory activity of phages, that is, the nonspecific effects of phages on different functions of major populations of immune cells involved in both innate and adaptive immune responses. These functions include, among others, phagocytosis and the respiratory burst of phagocytic cells, the production of cytokines, and the generation of antibodies against nonphage antigens. The aim of this chapter is to discuss the interactions between phages and cells of the immune system, along with their implications for phage therapy. These topics are presented based on the results of experimental studies and unique data on immunomodulatory effects found in patients with bacterial infections treated with phage preparations.

## ABBREVIATIONS:

| | |
|---|---|
| PPhs | purified phages |
| PhLs | phage lysates |
| Ig | Immunoglobulin |
| IFN | interferon |
| Il | interleukin |
| PT | phage therapy |
| RB | respiratory burst |
| ROS | reactive oxygen species |
| TLRs | Toll-like receptors |
| TCR | T-cell receptor |
| mAbs | monoclonal antibodies |
| SPL | Staphage Lysate |

## I. BACKGROUND

The vast majority of studies on phage biology have traditionally focused on the interactions of bacteriophages with bacterial cells. However, bacteriophages can also interact with some populations of mammalian cells, especially with immune cells. In fact, the first studies on the interactions between bacteriophages and immune cells were conducted by Felix d'Herelle shortly after the discovery of phages (d'Herelle, 1922). Since then, a considerable body of experimental data has accumulated to show that phages can substantially affect cells of the immune system both *in vitro* and *in vivo*.

Generally, the interactions between phages and immune cells include two main aspects. The first is phage immunogenicity, that is, the natural capacity of phages to induce specific immune responses, especially the production of antibodies against phage antigens (largely capsid proteins). The other aspect includes the immunomodulatory activity of phage preparations, that is, the nonspecific effects of phages on different functions of major populations of immune cells involved in both innate and adaptive immune responses. In fact, phages can affect immune functions as diverse as phagocytosis or the respiratory burst of phagocytic cells, the production of antibodies, and T-cell proliferation.

Studies on the effects of phages on immune cells are important for different medical applications of phages. For example, phage phi X174 has been used for over 30 years as an antigen to evaluate humoral immunity in patients with immunodeficiencies (Bearden *et al*., 2005). Furthermore, phage particles can be used as vehicles for vaccine antigens; promising results of experimental studies suggest that such vaccines can also be used in humans (Clark and March, 2004). Obviously, knowledge about phage immunogenicity is essential for the rational design of such vaccines. Moreover, studies on phage interactions with immune cells are very important for the rational use of phage therapy. For instance, specific antibodies are considered to be one of the most important factors limiting the therapeutic efficacy of phages *in vivo* (Sulakvelidze *et al*., 2001). Therefore, of paramount importance are data showing that the intensity of the anti-phage humoral response can vary depending on the route of administration of phage preparations. Research into the effects of phages on phagocytic cells may allow for better understanding of mechanisms involved in the elimination of bacteria by phages *in vivo*.

The main objective of this chapter is to present the current state of research on the interactions between bacteriophages and immune cells. It also discusses implications of those interactions for biomedical applications of bacteriophages, especially for phage therapy. This chapter

presents data regarding not only purified phages (PPhs), but also phage lysates (unpurified phage preparations; PhLs), because in fact it is Phls rather than PPhs that have been used at major centers of phage therapy, such as the Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland (Ślopek *et al*., 1987; Weber-Dąbrowska *et al*., 2000a).

## II. PHAGE IMMUNOGENICITY

### A. Anti-phage humoral responses

Antiviral antibodies are one of the main components of antiviral immune responses. In the case of pathogenic viruses, these antibodies can exhibit four main activities: virus neutralization, antibody-dependent cellular cytotoxicity, antibody-dependent cell-mediated virus inhibition, and phagocytosis (Forthal and Moog, 2009).

Antibodies that were examined in the vast majority of studies on phage immunogenicity are neutralizing antibodies. Essentially, these are defined as antibodies that bind epitopes within those parts of the virion essential for infecting the host cells (Forthal and Moog, 2009). In the case of phages, neutralizing antibodies inhibit the infection of the host bacterial cells by binding to the phage tails ( Jerne and Avegno, 1956). However, one must be aware that phage–antibody interactions do not necessarily mean phage inactivation. Usually, binding of antibodies to proteins engaged in infection may impede it and result in visible loss of phage antibacterial activity. Other proteins may be bound by antibodies without any visible effect on phage viability ( Jerne and Avegno, 1956).

Anti-phage-neutralizing antibodies are considered one of the most important factors potentially limiting the efficacy of phage therapy (Sulakvelidze *et al*., 2001). Indeed, a considerable body of experimental data indicates that these antibodies may reduce the therapeutic effectiveness of phages. First, many studies have shown that sera of nonimmunized humans and animals (or, more specifically, animals not immunized with phage during the experiment) in fact have a low level of phage-neutralizing antibodies (so-called ''natural antibodies'') ( Jerne 1956; Kamme 1973; Kucharewicz-Krukowska and Ślopek, 1987). Thus, in some individuals, phage-neutralizing antibodies can be present in the serum even before phage administration. It is believed that the presence of such antibodies can be explained by the well-documented omnipresence of phages in different environments, as well as in food and within normal microflora, where phages are present in very large numbers along with bacteria (Górski and Weber-Dąbrowska 2005). This implies constant natural ''immunization'' of humans (as well as animals) with phage antigens. Second, neutralizing antibodies are produced in high titers following the systemic administration of phages to animals ( Jerne 1956).

Third, the clearance of T7 phage from the blood of mice is slower in B-cell-deficient animals compared with wild-type mice (Srivastava *et al.*, 2004). Taken together, these data show that neutralizing antibodies indeed may decrease the efficacy of phage therapy substantially. However, the intensity of anti-phage humoral response may vary depending on the phage type; some phages are very weak immunogens and require repeated injection coupled with administration of adjuvant to induce detectable antibody titers (Sulakvelidze and Barrow 2005).

Our group has also examined anti-phage-neutralizing antibodies in sera of patients with bacterial infections treated with PhLs at the Phage Therapy Unit at Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland. Weak activities in sera were detected in patients prior to phage administration up to a serum dilution of 1:100. Antibody activity increased in some patients during the treatment up to a serum dilution of 1:1500. Interestingly, no significant increase in antibody activity was noted in patients who received PhLs orally (Figs. 1 and 2).

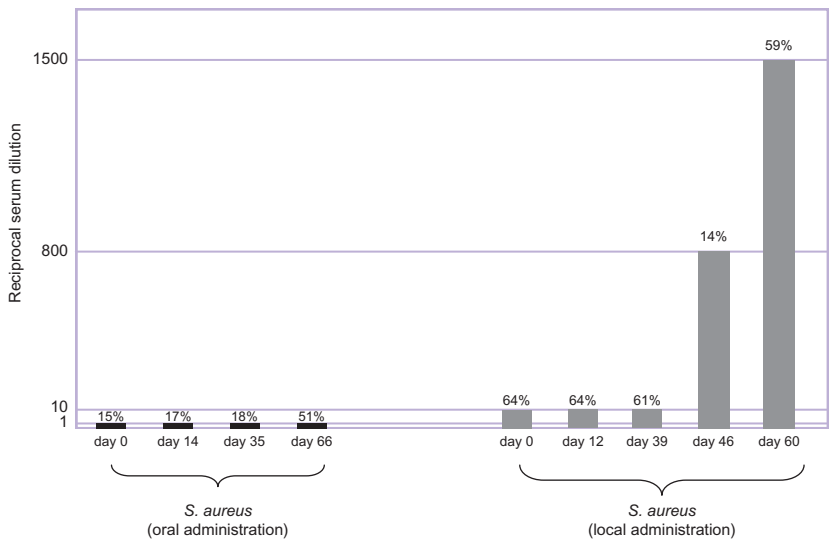



**FIGURE 1** Inactivation of phage by patients' sera during phage therapy (oral and local administration). Anti-phage activity of patients' sera was assayed as described in detail earlier (Kucharewicz-Krukowska and Ślopek, 1987). Briefly, 50 μl of phage ($10^6$ PFU/ml) was mixed with 450 μl of serum and incubated at 37 °C for 30 min. A sample was subsequently added to a bacterial strain on agar plates and incubated at 37 °C for 8 hr. The degree of phage neutralization by sera was determined in reference to the control (bacterial broth). Results obtained for two patients are shown: one patient treated orally (left) and another locally (right) with *S. aureus* phage. The inactivation of phage is presented as the highest reciprocal serum dilution that inactivated at least 10% of phages, and the exact percentage of neutralization of phages by patients' sera during phage therapy is shown over the bars.

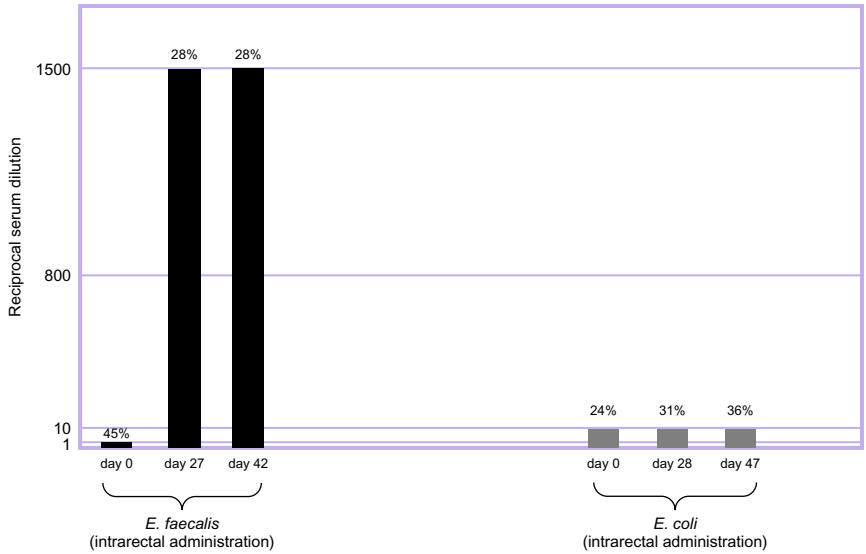



**FIGURE 2** Inactivation of phage by patients' sera (intrarectal administration). Anti-phage-neutralizing antibody responses in two patients are shown: one patient treated intrarectally with *Enterococcus faecalis* phage (left) and another intrarectally with *E. coli* phage (right). Data presented as in Figure 1.

Antibody responses to phages appear to be also dependent on the initial status of patients, who could be divided into ''responder'' and ''nonresponder'' groups (Łusiak-Szelachowska *et al.*, manuscript in preparation).

Immunogenicity of phages, coupled with ease of their quantification and lack of toxicity, has allowed for the use of phi X174 phage for the evaluation of humoral immunity in diagnosing and monitoring of patients with primary and secondary immunodeficiencies (Ochs *et al.*, 1971; Wedgwood *et al.*, 1975). This phage is a potent T-cell-dependent neoantigen that triggers typical primary, secondary, and tertiary humoral responses after intravenous injection to patients (Wedgwood *et al.*, 1975). It is currently considered one of the standard antigens used to evaluate humoral immunity in clinical medicine (Bearden *et al.*, 2005). However, in fact the generation of specific antibodies to phi X174 requires cooperation among antigen-presenting cells, B cells, and T cells. Therefore, its abnormalities can result not only from the dysfunction of B cells, but of T cells, complement, and adhesive molecules as well (Andrews *et al.*, 1997). So far, phi X174 has been employed for diagnosing and monitoring of patients with a wide range of both primary and secondary immunodeficiencies (Bearden *et al.*, 2005).

When interpreting the results of studies on anti-phage antibodies, one should take into account that their production can be stimulated not only by

phage particles themselves, but also by some components of bacterial cells present in phage preparations, especially lipopolysaccharides (LPS). This was shown in a study in which the production of anti-phage antibodies was induced by the systemic administration of T2 phage to mice (Michael and Kuwatch, 1969). The study revealed that systemic administration of 50 μg of LPS of *Escherichia coli* B (without preimmunizing animals with the phage) increased the level of anti-T2-neutralizing antibodies in the blood of the mice; a similar effect was found following the administration of Shigella LPS. However, the level of anti-phage antibodies was substantially lower than that found following the administration of T2 phage itself at the dose of $10^9$ plaque-forming units (PFU)/mouse. Moreover, the phage-neutralizing activity of the sera of mice was elevated for a shorter time following the administration of LPS compared with the T2 phage itself. Unfortunately, the phenomenon of LPS-induced generation of anti-phage antibodies was not examined in more detail. In particular, the authors did not attempt to determine the minimal dose of LPS capable of inducing anti-phage antibodies. Nevertheless, the results of this study are important because LPS is one of the components of bacterial cells known to be present in small concentrations, even in PPhs. Thus, its presence in phage preparations needs to be taken into account when designing (and interpreting the results of) studies on anti-phage antibodies, as well as in phage therapy. However, immunization with intact bacterial cells has no effect against phages and vice versa, as bacteriophages are distinct from bacteria in their antigenic characteristics (Kańtoch 1956).

## B. Anti-phage cellular responses

It is well known that T cells play an important role in the response to viral infections, with both virus-specific $CD8^+$ T cells and $CD4^+$ T cells being engaged in the cellular arm of adaptive immunity triggered during such infections (Amanna and Slifka 2011). Therefore, it could be expected that administered phage should also induce some form of cellular response. However, data on anti-phage cellular responses are very scanty compared with the knowledge about anti-phage humoral responses. In fact, to our knowledge, there is only one report in the literature clearly indicating that administration of phage can elicit cellular responses both *in vitro* and *in vivo*. In that study, Langbeheim *et al.* (1978) investigated the response of guinea pigs to MS-2 phage and a synthetic conjugate corresponding to a defined region of phage coat protein. The animals were sensitized by the antigens, and the sensitization *in vivo* was evaluated by intradermal injection of the test antigens, resulting in local erythema and induration. *In vitro* cellular sensitization was determined by measuring proliferative responses of lymph node cells to the test antigens. Strong *in vivo* reactions to injected phage were noted in all animals. Interestingly, whole phage

particles induced stronger sensitization than the conjugate. Lymphocytes from animals sensitized with phage gave brisk responses to the virus but no reaction was found to the conjugate. Accordingly, animals sensitized with the conjugate had lymphocytes that reacted only to the specific antigen. However, Srivastava *et al.* (2004) demonstrated that the kinetics of phages in the blood is similar in immunocompetent and T-cell-deficient mice. This result shows that T cells do not have any significant role in the inactivation of phages *in vivo*.

## C. Bacteriophage proteins' effects on mammalian immunity

Immunological effects of bacteriophages are fairly complex, as most bacteriophages are very complex structures, with a number of different capsid proteins exposed for external interactions. Therefore, the general effect of a phage is a resultant of multicomponent activity of capsid proteins. By breaking this effect down for particular proteins, we can gain a sensitive tool for dealing with anti-phage immunity.

The most spectacular effects of phages are antibodies induced by them (see Section II.A). At the early stage of phage investigations, phage proteins' antigenicity and antibody induction served as tools for characterization of evolutionary relatedness. The idea referred to serological cross-reactions, as the intensity of these reactions reflects the relatedness between phages, generally decreasing with growing evolutionary distance between species (Stent 1963). Permissible interactions of phage capsids with antisera reflect homologies and similarities among phages. Nowadays we know that the homologies of phage genomes usually accumulate in specific conserved regions (or even parts of a gene), whereas other regions are highly variable (Krisch and Comeau, 2008). Similarly, the arrangement of antigens on a capsid is not even, that is, potential similarities between phages may occur in selected parts only. Some capsid components are antigenically specific for a phage, whereas others can be shared by different phage species (Tikhonenko *et al.*, 1976).

Antibodies against selected phage gene products were also one of the main tools for recognition of gene function in phages. Structural proteins can be localized on capsids by means of specific antibodies, giving accurate and exact information on virus particle arrangement (Ishii and Yanagida, 1977; Yanagida, 1972).

Some interesting conclusions on phage proteins' immunogenicity can be drawn from investigations on engineered phages. They cannot always be simply transferred to wild-type phages, but these studies give important information about molecular details that probably determine phage effects on immunity. Studies of interactions of phage-displayed short peptides (T7 phage) made by Sokoloff *et al.* (2000) revealed that a dependency exists between the properties of phage surface peptides and the

innate immune system response. Natural antibodies, that is, those preexisting in mammalian serum, recognize phages and start complement activation. However, providing capsids with carboxy-terminal lysine or arginine residues protects the phage against complement-mediated inactivation by binding C-reactive protein in rats (Sokoloff *et al.*, 2000).

Another instance of molecular manipulations that change the effect of phage on innate immunity was described by Merril *et al.* (1996), who investigated phage λ derivatives in germ-free mice. The overall mechanism of phage clearance involves reticuloendothelial system filtration, which is usually proposed as the main path of phage removal from nonimmunized mammals. A single mutation in the major capsid protein gene (G → A transition) caused a substitution of the amino acid lysine for glutaminic acid, which determined a long-circulating phenotype. These long-circulating mutants were able to persist in the mice circulation much longer and also had better antibacterial activity in bacteriemic mice than the corresponding wild-type strain (Merrill *et al.*, 1996). Further studies have confirmed that indeed this single specific substitution confers the ''long circulating phenotype'' (Vitiello *et al.*, 2005), which emphasizes the importance of such subtle differences in phage antigenic structure for their potential use in therapy.

In the phage T4, an opposite phenomenon, that is, ''short circulating phage,'' was demonstrated by Dąbrowska *et al.* (2007). After T4 lost its nonessential decorating protein Hoc, its clearance from the mouse circulation occurred faster. This was not a change of a protein but its deletion, which strongly suggests that some proteins are able to mediate or moderate phage interactions with the immune system. The phage defective in the *hoc* gene was also described as more adhesive to mammalian cells than the wild-type phage (Dąbrowska *et al.*, 2004). It is not very likely that adhesion is really specific (see Section III.B); nevertheless, its intensity might influence phage susceptibility to filtration and removal from a higher organism. Elevated adhesion properties of Hoc-deprived phage are in line with the observations of Sathaliyawala *et al.* (2010), who reported that $hoc^{-}soc^{-}$ phages aggregate frequently, as visualized in cryoelectron microscopic images, apparently through interactions between capsids. Hoc is a prokaryotic protein that contains immunoglobulin-like domains in its structure (Bateman *et al.*, 1997). Possibly this makes it a modulator of T4 phage effects in mammals (Dąbrowska *et al.*, 2006, 2007), while it is still questionable what the exact role of Hoc on T4 capsid is. It was proposed that Hoc might prevent the aggregation of phage particles in the infected cell, where the concentration of newly assembled phage can be quite high or, oppositely, the exposed Ig-like domains may interact weakly and nonspecifically to surface carbohydrates and other molecules of bacteria. Adhesion to bacteria or biofilms may provide the phage with a survival advantage (Sathaliyawala *et al.*, 2010). Bioinformatics studies revealed that Ig-like domains are quite

common in phage genomes (Fraser *et al.*, 2006), which may suggest that a similar adhesion-modulating effect of some capsid proteins can be expected in other phage strains.

Receptor-targeted, that is, chemically modified, phage M13 was also investigated for its time of clearance. Molenaar *et al.* (2002) showed that conjugation of either galactose or succinic acid groups to phage coat proteins resulted in a substantially reduced plasma half-life of the phage. However, a phage display of random peptides (C-X7-C library) on the T7 phage coat was shown not to influence the clearance of phages in murine blood (Srivastava *et al.*, 2004). All these studies may play a crucial role in vaccine design and other molecular medicine approaches that use phages as a platform or delivery vector, but they obviously can also shed some light on phage effectiveness and side activities in phage therapy.

Of note, chemical modification of phages by conjugation of monomethoxy-polyethylene glycol (mPEG) to its proteins renders phages less immunogenic: PEGylated phages elicit diminished levels of cytokine [interferon (IFN)-γ and interleukin (IL)-6] and their circulation half-life is increased markedly, which suggests that this approach may be of interest for increasing efficacy of PT (Kim *et al.*, 2008).

## III. IMMUNOMODULATORY ACTIVITY OF PHAGE PREPARATIONS

### A. Effects of phages on phagocytic cells

The activity of phagocytic cells constitutes one of the essential functions of antibacterial immune responses (Silva 2010). Investigating phage interactions with major populations of phagocytic cells may thus verify whether phages could eliminate bacteria *in vivo* not only by direct bactericidal activity, but also by activating phagocytic cells. Furthermore, interactions between endogenous phages (known to be present in very large numbers within normal microflora; Górski and Weber-Dąbrowska 2005) and phagocytic cells may be relevant for understanding the complexity of the immune defenses against bacterial infections.

#### 1. Effects of phages on phagocytosis

The first description of the influence of phage on phagocytosis was a report by Felix d'Herelle, who studied the effects of a *Shigella* phage on phagocytosis of *Shigella* by guinea pig ''leukocytes'' (the author did not specify whether he was dealing with peritoneal cells, of which the majority are macrophages, or peripheral leukocytes, most of which are granulocytes) (d'Herelle 1922). After the coincubation of bacteria, phage, and leukocytes for 10 min, the phagocytic index of the cells increased

dramatically compared with the control (cells cultured with bacteria in the absence of phage). Another interesting finding was that the development of phage resistance by bacteria was associated with the development of their resistance to phagocytosis. d'Herelle suggests that phages act as specific opsonins markedly facilitating bacterial phagocytosis. This effect is mediated by a soluble factor(s) present in phage preparations.

Kańtoch *et al.* (1958) demonstrated that the T5 phage does not affect the phagocytosis of *E. coli* by guinea pig granulocytes. Furthermore, phages adsorbed to bacteria may remain biologically active upon their uptake by these cells. In contrast, after the completion of bacterial phagocytosis, phages are no longer able to lyse intracellular bacteria (Kańtoch and Szalaty, 1960). However, work by the same authors suggests that the T2 phage may diminish the ability of horse leukocytes to phagocytose bacteria from different species, including *Staphylococcus aureus*, *E. coli*, and *Mycobacterium tuberculosis* (Kańtoch *et al.*, 1958). This effect was found to be dose dependent—phage in a concentration of $10^{10}$/ml caused almost complete inhibition of phagocytosis, whereas lower concentrations of phage caused only a slight decrease of granulocyte activity. The process of inhibition was also time dependent (increasing incubation time and ingestion of phage particles decreased phagocyte ability to ingest bacteria) and was brought about by living as well as by heat- and serum-inactivated phage. Interestingly, the most efficient inhibition occurred when phages were inactivated by antibodies, which suggests that immune complexes consisting of phages and anti-phage antibodies may be especially active in diminishing granulocyte phagocytosis of bacteria.

Another study focused on the effects of two PPhs, T4 and F8 (a *Pseudomonas aeruginosa* phage), on the phagocytosis of *E. coli* (Przerwa *et al.*, 2006). *In vitro* experiments showed that both T4 and F8 inhibited the phagocytosis of bacteria in a dose-dependent manner when preincubated with phagocytic cells. However, preincubation of *E. coli* with T4 resulted in a small increase in the efficiency of phagocytosis. Coincubation of phages, bacteria, and phagocytic cells led to a reduction in the intensity of phagocytosis of *E. coli*. Similar results were obtained with neutrophils and monocytes. In the same study in experiments performed on mice inoculated with bacterial cells, T4 was found to stimulate the intensity of phagocytosis by neutrophils. However, in uninfected mice, T4 weakly reduced the phagocytosis by monocytes while having no effect on the intensity of phagocytosis by neutrophils.

*In vitro* monitoring of phagocytosis of *S. aureus* by neutrophils isolated from patients subjected to phage therapy has revealed that such therapy may further decrease this function in patients in whom phagocytic activity was lowered prior to the beginning of treatment. However, no correlation was found between alterations in phagocytosis and the outcome of therapy. Three months after completion of therapy, neutrophil functions

returned to normal. Moreover, phage therapy accelerated the turnover of neutrophils as manifested by an increase in the number of their immature forms and a concomitant decrease in the number of mature cells (Weber-Dąbrowska *et al.*, 2002).

## 2. Effects of phages on the respiratory burst

Respiratory burst (RB) is a rapid increase in the production of reactive oxygen species (ROS) during the phagocytosis of microbes. On the one hand, RB is an essential component of innate immunity enabling phagocytic cells to eliminate microbes. However, on the other hand, excessive production of ROS may result in the induction of oxidative stress in cells. Both pathogenic bacteria and viruses can induce oxidative stress in host cells during infection (Schwarz, 1996; Victor *et al.*, 2004). Therefore, examining the effects of phages on the production of ROS is essential in verifying the safety of phage therapy.

The first study to evaluate the influence of phages on ROS generation showed that purified T4 phage preparation induced a very weak RB compared with bacterial cells *in vitro* both in monocytes and in neutrophils (Przerwa *et al.*, 2006). It is noteworthy that the titer of phage used in these experiments ($10^9$ PFU/ml) was one order of magnitude higher than the titer of bacteria ($10^8$ colony-forming unit/ml). Furthermore, phages reduced, in a dose-dependent manner, *E. coli*-induced RB when preincubated with bacteria, the effect being significant with higher phage titers ($10^9$ and $10^{10}$ PFU/ml).

Another study showed that a purified T4 phage preparation can inhibit the RB induced by both LPS and bacterial cells (Międzybrodzki *et al.*, 2008). It was also shown that neither purified preparations nor lysates of staphylococcal A3/R phage induced a significant RB in monocytes or neutrophils *in vitro* (Borysowski *et al.*, 2010).

## 3. Effects of phages on other functions of phagocytic cells

Our group also examined the effects of phages on the migration of phagocytic cells, intracellular killing of bacteria, and expression of Toll-like receptors (TLRs). The majority of PPhs and PLs did not affect human granulocyte and mononuclear cell migration *in vitro* [the only exception was some inhibitory activity of T4 phage known to contain in its capsid Hoc protein, which, according to our hypothesis (Dąbrowska *et al.*, 2007), may have some immunomodulatory activities]. Furthermore, phage preparations did not influence intracellular killing of bacteria by these cells (Kurzepa, 2011).

Toll-like receptors are one of the most important classes of receptors of phagocytic cells involved in innate immune responses, including inflammatory reactions (Kumar *et al.*, 2009). In particular, the TLR-mediated activation of monocytes or macrophages by pathogenic bacteria and viruses results in an increase in the production of pro-inflammatory

cytokines, ROS, and nitric oxide. The natural ligand for TLR4 and TLR2 receptors is LPS. Our study showed that T4 phage preparations have no effect on the percentage of $TLR2^+$ and $TLR4^+$ cells in either unstimulated or LPS-activated monocytes *in vitro*. Furthermore, a purified preparation of T4 had no effect on the expression of either TLR2 or TLR4, but the T4 lysate slightly increased the expression of TLR4 on $CD14^+/CD16^-$ cells (G. Korczak-Kowalska *et al*., unpublished data). These findings are important for phage therapy, as they indicate that phage preparations are not likely to exert pro-inflammatory activity in a TLR-dependent mechanism.

## B. Phage interactions with T and B cells

Data show that phage preparations may also modulate immune functions by direct interactions with T and B cells. In general, PPhs seem to cause immunosuppressive effects; lysates of anti-staphylococcal phage may be stimulatory, while other preparations may mediate immunosuppression.

Mankiewicz *et al.* (1974) reported that tuberculous guinea pigs inoculated intraperitoneally with mycobacteriophages showed depression of their skin reactions to tuberculin. Furthermore, these authors demonstrated that mycobacteriophages could inhibit, in a dose-dependent manner, the phytohemagglutinin (PHA)-induced activation of lymphocytes when added to lymphocyte cultures. That phage may indeed exert immunosuppressive activity *in vitro* has been confirmed by studies showing that a purified T4 phage preparation inhibited human T-cell proliferation induced via the CD3–TCR complex (Górski *et al.*, 2006). However, Zimecki *et al.* (2003) showed that purified preparations of an *S. aureus* phage exert costimulatory effects on splenocytes activated by a suboptimal concentration of the mitogen concanavalin A (Con A).

Another line of research is the adhesion of phages to immune cells. Kniotek *et al.* (2004a) showed that human T cells exhibit adhesive interactions with immobilized T4 and HAP1 (a T4 mutant lacking the Hoc protein). Experiments using purified recombinant phage proteins have revealed that T cells adhere to gp24 (a T4 capsid protein) but not Hoc (Ohams *et al*., unpublished data). Monoclonal antibodies (mAbs) blocking beta1 and beta3 common chains of integrins diminish these interactions significantly, suggesting that this reactivity depends—at least in part—on the engagement of receptors belonging to both families of integrins (Fig. 3). This seems to be confirmed by the results of preliminary experiments with blocking mAbs to specific integrins, which revealed that VLA-5 appears to be the major integrin responsible for human T-cell adhesion to gp24 (Fig. 4), while the involvement of the beta3 family was confirmed in experiments showing that eptifibatide (a beta3 function inhibitor) was also functional in this system (Ohams *et al*., unpublished data). Moreover, phages could inhibit the interactions of platelets with their major ligand,

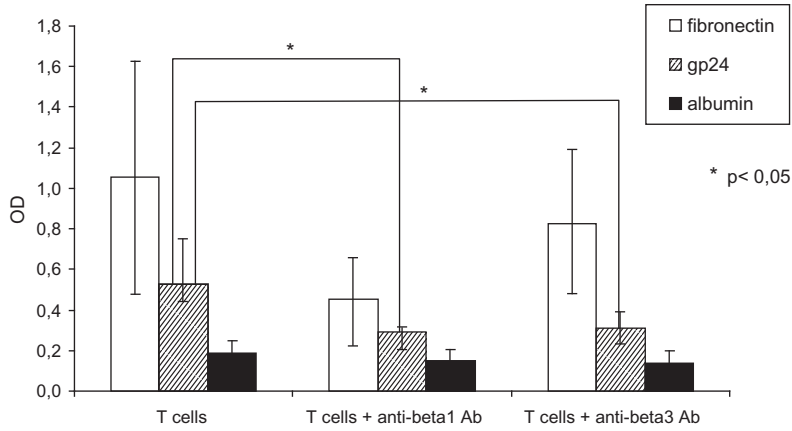



**FIGURE 3** Influence of anti-β1 and anti-β3 mAbs on T-cell adhesion to gp24 protein. The effects of anti-β1 and anti-β3 blocking monoclonal antibodies (mAbs) on human T-cell adhesion to gp24 (a T4 phage capsid protein) and fibronectin (FN) are shown. Experiments were conducted as described elsewhere ( Jerzak *et al*., 2002). Purified T cells were added ($2 \times 10^5$ per well) to microtiter plates coated with FN, gp24, or control protein (albumin, all 10 μg/ml) and incubated for 1 hr at 37 °C in a humidified atmosphere in the presence of phytohemagglutinin (50 μg/ml), as well as blocking mAbs to common chains of β1 and β3 integrins (Millipore). Controls received irrelevant antibody (mouse antihuman Ig, Daco). Following incubation, nonadherent cells were washed out with phosphate-buffered saline, the remaining cells stained with 0.1% crystal violet in alcohol, and color yields measured with an ELISA reader at 600 n*M*. Mean values of 10 experiments ± SD are shown. Both integrins appear to be involved in mediating the reactivity to gp24, while only β1 is functional in the case of FN.

fibrinogen, while their effects on T-cell adhesion to that ligand were weak (Kniotek *et al*., 2004a).

Phages may also diminish alloantigen-induced immunoglobulin production *in vitro* as well as specific antibody responses in mice (Kniotek *et al*., 2004b). Moreover, phages inhibit activation of NF-κB, a key transcription factor regulating the expression of many genes, including those encoding pro-inflammatory cytokines (Górski *et al*., 2006). These *in vitro* immunosuppressive effects of phage were confirmed by *in vivo* experiments, which have revealed an ability of phages to significantly extend the survival of allogeneic skin transplants in both normal and sensitized mice, as well as to diminish the development of an inflammatory infiltrate at the allograft site (Górski *et al*., 2006).

## C. Effects of phages on the production of cytokines

Several studies have shown that phages can substantially affect the production of various cytokines. One of the mechanisms mediating these effects could be inhibition of the activity of NF-κB (Górski *et al*., 2006).

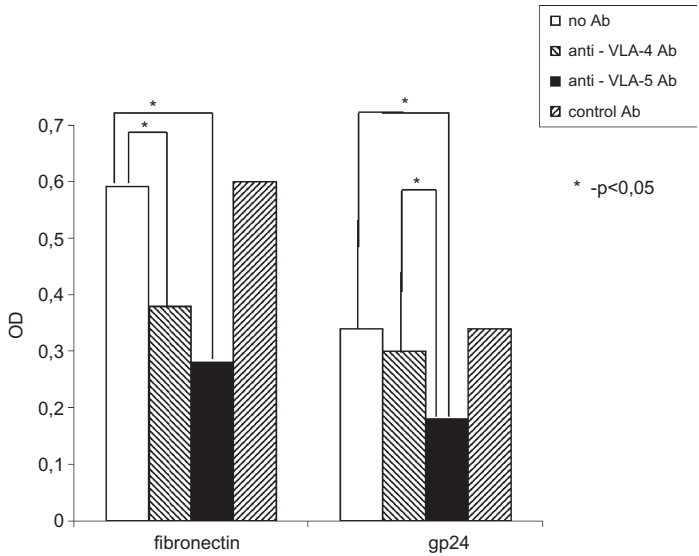



**FIGURE 4** Influence of anti-VLA-4 and anti-VLA-5 mAbs on T-cell adhesion to gp24 protein. To identify which specific integrins mediate T-cell adhesion to gp24, experiments identical to those shown in Figure 3 were performed using blocking mAbs to VLA-4 and VLA-5 integrin (Millipore). As an isotypic control, murine antihuman IgG3 mAb (BioLegend) was used. Results of one typical experiment out of four performed that gave similar results are shown. T-cell adhesion to gp24 is only inhibited by anti-VLA5 mAb, while the reactivity to fibronectin is inhibited by both VLA-4 and VLA-5.

Kleinschmidt *et al.* (1970) showed that a purified T4 phage preparation administered intravenously to mice increased the level of interferon in animal sera, which had strong inhibitory activity to vesicular stomatitis virus (this effect was unrelated to LPS). Zimecki *et al.* (2003) found that purified preparations of an *S. aureus* phage can induce IL-6 production in splenocyte cultures and enhance Con A-induced production of this cytokine. However, studies suggest that purified T4 does not induce significant intracellular interleukin (IL)-6 and tumor necrosis factor (TNF)-α synthesis in human monocytes, whereas T4 lysate causes a considerable increase in the production of IL-6 (P.Wierzbicki *et al.*, unpublished data). Furthermore, purified T4 inhibited mitogen-induced IL-2 production by human mononuclear cells (Przerwa *et al.*, 2005).

It was also shown that purified staphylococcal A3 phage does not induce the production of IL-6, whereas both purified preparations and lysates of this phage stimulate the synthesis of TNF-α (P. Wierzbicki *et al.*, unpublished data). In accord with the authors' data, Krishnan and Ganfield (1994) demonstrated that a PhL of *S. aureus*, called Staphage Lysate (SPL; see Section III.D), induces TNF-α, IL-1β, IFN-γ, and IL-10 synthesis by human mononuclear cells and the human monocytic cell line

THP-1. Data from experimental phage therapy of animals indicate that successful treatment corrects increased levels of pro-inflammatory cytokines associated with bacterial infections. Zimecki *et al.* (2009) described such effects for TNF-α and IL-6 in sera of mice infected with *S. aureus* and treated with phage. Similar results were obtained by Hung *et al.* (2011) in mice with *Klebsiella pneumoniae*-induced liver abscesses and bacteremia treated with phages. Furthermore, Kumari *et al.* (2010) showed that successful treatment of *K. pneumoniae*-induced burn wound infection complicated by bacteremia and organ injury was associated with lowering of IL-1β, TNF-α, and IL-10 in sera and lungs of phage-treated mice. Data from experimental phage therapy were confirmed by a clinical study by Weber-Dąbrowska *et al.* (2000b), who demonstrated that phage therapy may influence cytokine production in treated patients. Interestingly, these effects varied depending on the initial responsiveness of the patients found prior to treatment: in those with a low or moderate serum TNF-α level, phage therapy upregulated this cytokine production, lowering it in patients in whom TNF-α levels were initially high. Phage acted in a similar way on the LPS-induced cytokine production by patients' mononuclear cells *in vitro*.

## D. Immunomodulatory effects of Staphage Lysate

Staphage Lysate, also called SPL, is a PhL of *S. aureus* produced by Delmont Laboratories (www.delmont.com). Its immunomodulatory activity was studied in much detail and found some applications in experimental medicine. The majority of normal human subjects demonstrate a positive delayed-type reactivity to SPL (Dean *et al.*, 1975), which suggests that this reagent could be used for *in vitro* assessment of cell-mediated reactivity. These authors also performed more detailed studies to evaluate the effects of SPL on immune cells. SPL used contained approximately $2 \times 10^9$ *S. aureus* cells and $10^{10}$ phage particles per milliliter (7 mg/ml of protein). The authors showed that the optimal SPL concentration causing maximal lymphocyte proliferation ranged between 30 and 50 μg of protein/well and the proliferative response peaked on day 6 of culture. The mean stimulation index was 127 (approximately 50% of that caused by PHA, a well-known T-cell mitogen). Further studies have revealed that, in contrast to PHA, the pure T-cell mitogen SPL activates and induces the proliferation of both T and B cells, as well as causes inhibition of leukocyte migration in two out of three donors (another measure of cell-mediated immunity dependent on the production of migration inhibitory factor by T cells) (Dean *et al.*, 1975). These data suggest that some components of SPL can be recognized as specific antigens by lymphocytes of sensitized individuals but probably can also act as a nonspecific inducer of immunity. This assumption has been strengthened by data indicating that SPL is a

polyclonal activator of immunoglobulin production by mouse and human B cells (Lee *et al.*, 1985). SPL can also increase antibody titers up to 10-fold as well as susceptibility to phagocytosis (Mills, 1962). In addition, SPL was found to induce IL-1β, TNF-α, and IFN-γ production by human lymphocytes *in vitro* (Krishnan and Ganfield, 1994).

Cell reactivity to SPL measured as lymphocyte proliferation has been used as an *in vitro* measure of immunocompetence in patients with immunodeficiencies (Dean *et al.*, 1977). Studies in mice have revealed that SPL can enhance specific and nonspecific resistance to bacterial infections acting probably on T cells and macrophages (Esber *et al.*, 1981). The product has been licensed for the treatment of pyoderma caused by staphylococci in dogs with up to 77% efficacy (www.delmont.com).

Bogden and Esber (1978) advanced a model of immunotherapy referred to as ''induction and elicitation'' in which natural immunity to *S. aureus* or that caused by administration of these bacteria is associated with subsequent administration of SPL in order to boost the immune system. Using a metastasizing animal tumor model, the authors demonstrated that this form of immunotherapy was more effective than surgery. That the concept of induction and elicitation may be applied effectively as an immunotherapeutic modality in experimental cancer was confirmed using a similar experimental model by (Mathur *et al.*, 1988). The authors showed that rats receiving SPL treatment had significantly smaller lymph node involvement compared with rats from the control group. Moreover, the best results were achieved using induction (administration of dead staphylococcal cells with subsequent SPL treatment). However, SPL vaccination was not found to have a beneficial effect in a rabbit model of staphylococcal blepharitis and catarrhal infiltrates (Giese *et al.*, 1996).

Staphage Lysate has also been used in the treatment of patients with staphylococcal infections; published evidence dates back to the late 1970s. Mills (1956) used SPL as aerosol therapy of sinusitis, emphasizing the antiallergic effects of the lysate; this author suggested that polysaccharides and nucleoproteins present in the preparation may bring about a gradual hyposensitization to bacterial allergy present in the majority of sinusitis patients. In his subsequent work, the author extended these data, stressing SPL stimulatory effects at the level of local and systemic immunity. He also observed nonspecific positive effects related to SPL administration: infections caused by other bacteria and viruses seemed to heal quicker than formerly (Mills, 1962). In a study done by Baker *et al.* (1963), SPL was used to stimulate active immunity in patients exposed to or having chronic or recurrent staphylococcal infections. Notably, SPL was found to be more effective than any other staphylococcal vaccine. Salmon and Symonds *et al.* (1963) reported excellent results of SPL treatment of more than 600 patients with chronic staphylococcal infections. The authors believed that those beneficial effects were mediated by its vaccine-like action (SPL was used as

an antigen to stimulate production of antibodies, although no data on their actual levels were provided). Although some patients received intensive therapy (one cubic centimeter subcutaneously daily), no alarming or untoward effects were observed.

The concept of induction and elicitation has also been applied in the treatment of chronic osteomyelitis in children. It was demonstrated that the combination of anti-staphylococcal vaccine and SPL influenced the course of the disease positively and reduced the number of relapses (Pillich *et al.*, 1978). What is more, SPL is registered in the Czech Republic and Slovakia for the treatment of staphylococcal infections.

## E. Immunomodulatory effects of phage therapy

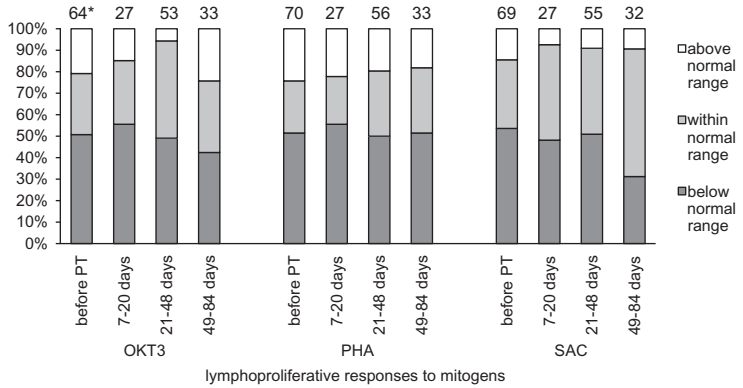
The authors' group evaluated selected immune parameters in 70 patients with antibiotic-resistant infections (44 males, 26 females, median age 46.5) treated with PhLs at the Phage Therapy Unit at the Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland, between 2007 and 2011 (Table I). Blood samples were collected from patients no earlier than 14 days before beginning phage therapy and no later than 14 days after suspending or ending the phage application (in some cases, one short break up to 14 days in the application of phages between days 21 and 84 was allowed). The following parameters were studied: T- and B-cell proliferation induced by the mitogens OKT3 (Muromonab CD3), PHA, and *Staphylococcus aureus* Cowan 1 (SAC), natural killer (NK) cell activity, T and NK cell percentage, phagocytosis of zymosan particles, and phorbol myristate acetate (PMA)-induced ROS production by phagocytes. Their changes after 7–20 days, 21–48 days, and 49–84 days of phage therapy compared to values before therapy were analyzed. The overall results of immunomonitoring of the patients are shown in Figure 5. Up to 50% of patients had signs of immunodeficiency prior to phage therapy, and on initial analysis (with the use of McNemar's test) of the number of patients below or above the lower limit of normal range, there were no clear effects of therapy except for a significant double increase (from 34 to 69%) in the number of patients with normal and heightened B-cell responses to SAC on days 49–84 of treatment. There were no significant changes when the numbers of patients below or above the upper limit of normal range were analyzed, which means that no significant overstimulation of the immune system by the phage lysates were observed.

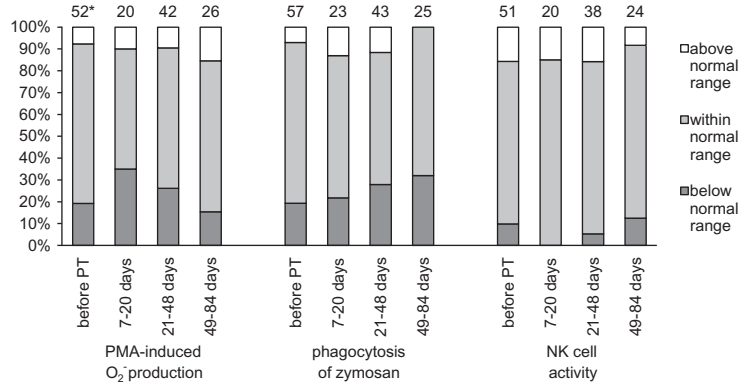
Further analysis was performed to determine whether the clinical outcome of phage therapy could be linked to alterations in patients' immune reactivity (changes in the values of the particular immunological parameter before and during therapy were compared using sign test or Wilcoxon matched-pairs test). A significant increase in phagocytosis

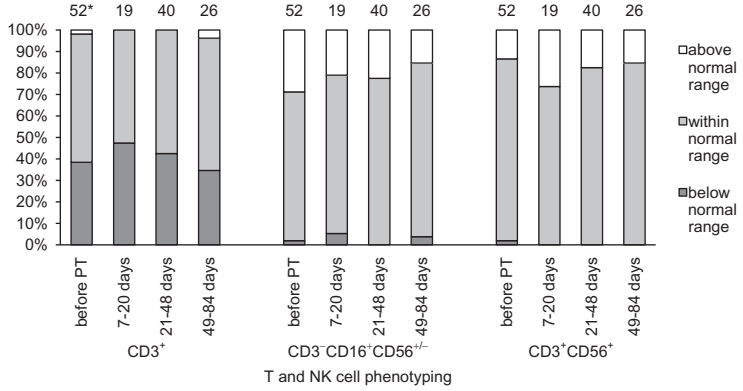
**TABLE I** Patient characteristics (total *n* = 70)

| | |
|---|---|
| Gender | Males: *n* = 44 |
| | Females: *n* = 26 |
| Median age | Males: 49.5 (23–79) years |
| | Females: 35.0 (20–82) years |
| | Total: 46.5 (20–82) years |
| Diagnosis | Urogenital infections: 31 cases (including 16 men with chronic bacterial prostatitis) |
| | Prosthetic joint infection: 6 cases |
| | Osteomyelitis: 8 cases |
| | Joint infection: 4 cases |
| | Skin and soft tissue infection: 13 cases |
| | Upper respiratory tract infection 6 cases (including 2 cases of chronic sinusitis) |
| | External ear infection: 2 cases |
| Pathogens causing infection | Gram-positive bacteria (*n* = 47): |
| | *S. aureus* (*n* = 31, including three MRSA cases) |
| | *E. faecalis* (*n* = 16) |
| | Gram-negative bacteria (*n* = 12): |
| | *E. coli* (*n* = 5) |
| | *P. aeruginosa* (*n* = 5) |
| | *P. putida* (*n* = 1) |
| | *K. pneumoniae* (*n* = 1) |
| | Mixed Gram-negative/Gram-positive infections (*n* = 11) |
| | *E. coli*/*E. faecalis* (*n* = 6) |
| | *E. faecalis*/*K. pneumoniae* (*n* = 1) |
| | *E. faecalis*/*K. oxytoca* (*n* = 1) |
| | *E. faecalis*/*P. aeruginosa* (*n* = 1) |
| | *S. aureus*/*E. cloace* (*n* = 1) |
| | *E. faecalis*/*E. coli*/*P. vulgaris* (*n* = 1) |
| Concomitant antibacterial treatment | Antibiotics/chemotherapeutics: *n* = 13 |
| | Disinfectants: *n* = 2 |
| Response to phage treatment[a] | Pathogen eradication and/or recovery: 9 cases |
| | Good clinical result: 11 cases |
| | Clinical improvement: 13 cases |
| | Questionable clinical improvement: 7 cases |
| | Transient clinical improvement observed only during phage therapy: 12 cases |
| | No response to treatment: 17 cases |
| | Clinical deterioration: 1 case |

[a] The response to phage treatment was established based on the results of control microbiological tests (bacterial cultures), assessment of the intensity of disease symptoms by the physician during patient's clinical examination, and evaluation of the results of other control relevant diagnostic tests, as well as the opinion of consulting medical specialists. Pathogen eradication was confirmed by bacterial cultures. Recovery means wound healing or complete subsidence of the symptoms of infection. A good clinical result means almost complete subsidence of some symptoms of infection (in some cases confirmed by the results of laboratory tests, such as normalization of inflammatory parameters) and significant improvement of general patient's condition. Clinical improvement means discernible decrease of the intensity of some symptoms of infection not observed during periods without treatment. Questionable clinical improvement means that the degree of reduction in the intensity of some symptoms of infection could have been observed in patient also during periods without treatment.


64* 27 53 33 70 27 56 33 69 27 55 32
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
above normal range
within normal range
below normal range
before PT
7-20 days
21-48 days
49-84 days
OKT3
PHA
SAC
lymphoproliferative responses to mitogens


52* 20 42 26 57 23 43 25 51 20 38 24
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
above normal range
within normal range
below normal range
before PT
7-20 days
21-48 days
49-84 days
PMA-induced $O_2^-$ production
phagocytosis of zymosan
NK cell activity


52* 19 40 26 52 19 40 26 52 19 40 26
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
above normal range
within normal range
below normal range
before PT
7-20 days
21-48 days
49-84 days
$CD3^+$
$CD3^-CD16^+CD56^{+/-}$
$CD3^+CD56^+$
T and NK cell phenotyping

evaluated on days 7–20 of phage therapy was associated with a good prognosis of the treatment (pathogen eradication and/or recovery, good clinical result, or clinical improvement according to Table I, was achieved in almost 50% of patients). Analysis of the relationship between phage type and immune reactivity revealed that patients treated with enterococcal phages or phages specific to Gram-negative bacterial species had diminished T-cell responses to OKT3. Patients who received only phages of Gram-negative bacteria presented a significant decrease in the number of $CD3^{+}CD56^{+}$ cells at days 21–48 of treatment. A group of patients receiving only enterococcal phages had a significantly increased median value of phagocytosis after 21–48 days of phage therapy (however, only six patients were available for this comparison). Interestingly, in patients receiving only staphylococcal phages, no significant changes were observed for any immunological parameter analyzed. Moreover, analysis of the influence of the routes of phage administration (phages were given by oral, intrarectal, or topical route) on immune parameters suggests that intrarectal administration (alone or combined with topical application) may reduce the number of circulating NK cells after 49–84 days of phage therapy. Finally, patients treated with phages may develop phage-neutralizing antibodies, although this effect is rather weak or absent following oral phage administration and probably depends on the initial responder or nonresponder status and phage type used (see Section II.A).

A separate study performed on 37 patients treated with PhLs at the Phage Therapy Unit at the Hirszfeld Institute (Miedzybrodzki *et al.*, 2009)

**FIGURE 5** Changes in different immunological parameters during phage treatment. Mitogen-induced proliferation of peripheral blood mononuclear cells (PBMCs) was measured by an [$^{3}$H]thymidine incorporation assay using the OKT3 monoclonal antibody (normal value: 22,832–51,111 cpm), phytohemagglutinin (normal value: 34,247–61,405 cpm), and *Staphylococcus aureus* Cowan 1 (normal value: 775–5692 cpm)(Górski *et al.*, 2006). Phorbol myristate acetate-induced ROS production by neutrophils was determined by the cytochrome *c* reduction assay as described elsewhere (Sustiel *et al.*, 1989) (normal value: 7.06–12.04 nmol $O_2^-$/2.5 min $\times 10^6$ cells). Neutrophil phagocytosis of zymosan particles (Invitrogen) was evaluated by flow cytometry according to the manufacturer's instructions (normal value: 75–95%). The percentages of $CD3^{+}$ (normal value: 65–81%), $CD3^{-}CD16^{+}CD56^{+/-}$ (normal value: 6–18%), and $CD3^{+}CD56^{+}$ cells (normal value: 2–10%) in PBMCs was determined by flow cytometry using anti-CD3, anti-CD16, and anti-CD56 mAbs from Becton-Dickinson according to the manufacturer's instructions. NK cell activity (normal value: 3–17% of dead target cells) was measured by flow cytometry as described elsewhere (Chang *et al.*, 1993). Bars show the cumulative percentage of patients below, above, or in a range of normal values before beginning phage therapy and in periods of 7–20 days, 21–48 days, and 49–84 days of the phage preparation application. *Values above bars show number of patients whose data were analyzed in individual subgroups.

showed that phage therapy results in a decrease in the C-reactive protein serum level and white blood cell count; however, administration of PhLs to patients had no significant effect on the erythrocyte sedimentation rate. Thus therapeutic effects of phage preparations may result not only from the elimination of bacteria, but also from normalization of some inflammatory markers associated with bacterial infections.

## IV. PHAGE VIRION INACTIVATION BY IMMUNE CELLS

Inchley (1969) showed that the majority of T4 phage particles are cleared rapidly from the circulation of mice by cells of the reticuloendothelial system of the liver and, to a lesser extent, of the spleen. However, experiments performed on nonimmune germ-free mice demonstrated that it is the spleen rather than the liver that clears most of the phage particles from the blood regardless of the route of phage administration (Geier *et al.*, 1973). Interestingly, both studies consistently showed that phage particles are inactivated by the spleen more slowly than by other organs, especially the liver. Although these classic studies clearly demonstrated that the majority of phage virions are eliminated *in vivo* by cells of the reticuloendothelial system of the spleen and the liver, data show that neutrophils and macrophages may also be involved to some extent in the degradation of phage virions.

Detailed studies on *in vivo* interactions between guinea pig peritoneal macrophages and T2 phage were carried out by Kańtoch (1961). Essentially, those interactions occur 30 min after phage administration. However, there was a great variation among animals in the amount of phage-positive cells (from 1:20 to 1:1630), which means that the majority of peritoneal leukocytes did not contain biologically active phage. *In vitro* studies revealed that phage uptake depended on virus concentration and did not occur below a phage concentration of $10^7$/ml. The process also depended on the presence of serum and was optimal at 2 °C. Studies of the uptake of phage by macrophages were also performed by Aronow *et al.* (1964) with the use of electron microscopy. These authors showed that the uptake of phage by rabbit peritoneal macrophages occurred after approximately 7 min of incubation and reached maximal intensity between 15 and 30 min. Intracellular destruction of ingested phages occurred some 2 hr after their uptake as no virus disintegration could be observed before that time. However, in neutrophils, disrupted phage particles were seen just after 15 min of incubation.

Ferrini *et al.* (1989) described the inactivation of λ phage vector particles *in vitro* when the phage was exposed to neutrophils stimulated with PMA. An important observation was that the presence of the phage did not stimulate neutrophils, and incubation of active phages with unstimulated

neutrophils did not cause any significant decrease in phage titer. However, incubation with stimulated neutrophils caused a decrease in phage titer, probably as a result of phage inactivation by hypochloric acid produced by the stimulated cells. Interestingly, rabbit macrophages previously ''immune'' (being able to make contact with a phage) remove the T1 phage faster than ''nonimmune'' cells *in vitro* (Nelstrop *et al.*, 1967).

Although the aforementioned studies showed that both neutrophils and macrophages can degrade phage virions (at least in some experimental settings), two studies demonstrated that neither of those populations is substantially involved in the clearance of phages from blood in mice following systemic administration of phages (Srivastava *et al.*, 2004, Uchiyama *et al.*, 2009).

It was also suggested that phages may retain their antibacterial activity after internalization to mammalian cells (Ivanekov *et al.*, 1999). Preliminary *in vitro* studies (E. Jończyk *et al.*, unpublished data) showed that T4 phage particles released from lysed human phagocytes after their incubation with phages *in vitro* retain their lytic activity. Moreover, when neutrophils or monocytes with phagocytosed *E. coli* B (cells initially prepared for the intracellular killing test) were incubated with the purified T4 phage, almost complete lysis (99%) of bacteria was observed compared to 63–82% in the control without phage. This may suggest that some phages possibly lyse intracellular bacteria and help phagocytic cells in the elimination of bacteria.

## V. CONCLUDING REMARKS

The aim of this chapter was to update and summarize existing knowledge on phage effects on the immune system. Evidently, such a report should be helpful in fully understanding the possible mechanisms of action of phage therapy and for appropriate planning of clinical trials, which would take into account different aspects of possible interactions of phages with the human organism. In this context, two questions are of paramount importance, namely: How does the immune system react to the administration of phages and how could these reactions, especially the production of antibodies, neutralize phage activities?

In general, when PPhs are studied *in vitro*, the prevailing effect is the suppression of lymphocyte activation coupled with anti-inflammatory effects (e.g., the reduction of ROS production and the inhibition of NF-κB activation). Furthermore, similar effects were observed *in vivo* (extension of skin allograft survival in mice). In contrast, SPL is a polyclonal lymphocyte activator and an inducer of immunoglobulin production, while its purified analogue does not have such effects (A. Górski *et al.*, unpublished data). Thus, SPL appears to be exceptional among phage

preparations used in phage therapy as it can cause both immune enhancement and pathogen eradication.

Current efforts to obtain PPhs that could meet the stringent requirements for parenteral administration in the clinic have been underway among centers involved in experimental and human phage therapy. However, it is believed that their eventual development in sufficient quantities should not entirely eliminate the need for PhLs currently applied. Patients with immunodeficiency syndrome need eradication of an offending pathogen, as well as enhancement of their immunity. Thus, when dealing with patients with difficult *S. aureus* infections and concurrent immunodeficiency, the treatment of choice would be SPL rather than its purified analogue.

Patients with chronic infections frequently have immune deficits that may predispose them to life-threatening infections. Furthermore, prolonged treatment with antibiotics (known to diminish immune responses) constitutes an additional risk factor perpetuating and aggravating their condition in a vicious circle. A significant proportion of the authors' patients had signs of immunodeficiency prior to therapy (e.g., approximately 50% for T and B lymphoproliferative responses). However, this condition does not constitute a contraindication for phage treatment, as pointed out in an earlier review (Borysowski and Górski, 2008).

There are no data in the literature to provide a definite answer to the important scientific and practical question of whether phage therapy significantly modifies immune functions in human and, if so, how such effects could contribute to the success or failure of treatment. Because current therapy involves PhLs rather than PPhs (at least in the majority of patients being treated), it could also be argued that phage preparations act in a similar manner to bacterial vaccines enhancing immunity and thereby contributing to the clearance of infection and improvement in patients' condition. However, data do not appear to support the notion that the major effect of phage therapy is to enhance immunity. In fact, the percentage of patients showing immune deficiencies at different stages of the treatment, as well as upon its completion, did not differ significantly from initial values (except for B-cell activation, which normalized in some patients, so their number was significantly higher upon completion of phage therapy). Because this does not exclude observed fluctuations in some immune parameters evaluated (e.g., T and B lymphoproliferative responses), it cannot be excluded that a more in-depth, detailed analysis performed at the level of individual patients would reveal more significant alterations of immunity, at least in some of them. This will be the subject of the authors' next report, including an enlarged cohort of treated patient. In fact, the authors previously described an increase in immunoglobulin production by B cells of patients treated with SPL (Kniotek *et al.*, 2004b), which corresponds to the trend toward enhanced B-cell proliferative responses currently reported.

It appears that the most interesting immunomodulatory phenomenon observed during the first weeks of therapy was the enhancement of granulocyte phagocytosis, which was indicative of concurrent patients' improvement. This observation is important for at least two reasons: (a) it has a prognostic value pointing to a potentially positive outcome of therapy, a factor of utmost clinical importance, and (b) it suggests that phage preparations may enhance granulocyte functions supporting and extending the assumptions suggesting that the mechanism of phage therapy is associated with an ability of an administered phage to reduce the number of offending bacteria to the threshold at which the immune system can successfully combat infection, a hypothesis originally put forward by d'Herelle (1922) and subsequently further developed by others (Górski and Weber-Dąbrowska, 2005).

When analyzing the effects of phage, one should keep in mind that *in vitro* data were obtained using PPhs that exerted suppressive effects on some immune functions (e.g., T-cell activation). However, similar effects could also be observed using these preparations *in vivo* in mice (an extension of allograft survival) and in patients treated with lysates of enterococcal phage or lysates of phage specific to Gram-negative bacteria (diminished T-cell responses to OKT3 at days 7–20 of phage therapy), as well as in some patients receiving PhLs intrarectally (a diminished level of NK-cell activity). Therefore, the final effect of phage preparations on the immune system may be regulated by a number of factors, especially initial immune status, the phage type, its form (PPhs vs PLs), route of phage administration, the length of treatment, and neutralizing antibody production.

Because patients received preparations that contained active phage particles and remnants of bacteria lysed by phage, the immunomodulatory effects may be mediated by both components of the preparations. As pointed out, the authors noted an increase in phagocytosis on days 7–20 of therapy in patients with a good prognosis. *In vitro* data obtained with purified phage suggest that phages inhibit human neutrophil phagocytosis in a dose-dependent manner, while they were without such effect when given in mice. Thus, the observed effect in patients was rather unrelated to the direct action of the phage on neutrophils and could have been caused by an improvement of phagocytic functions due to decreased bacterial load resulting from efficient phage therapy. Furthermore, the increased B-cell proliferative response observed on days 49–84 of the treatment could not be linked to the activity of purified phage proteins, as the authors' and other authors data cited in this chapter suggest that such an enhancement of immunity is caused by bacterial products present in phage preparations, especially in SPL (e.g., SPL but not its purified phage component causes human B-cell activation; see earlier discussion). At any rate, the reported immunomodulatory effects

of phage therapy appear to be so subtle that the issue of which component of the phage preparation is actually responsible for the phenomena observed is of a rather minor practical importance.

As far as the issue of antibody production is concerned, preliminary data contained in this chapter indicate that the problem of an antibody response to phage administered during therapy is more complex than has usually been presented in that phage therapy and neutralizing antibody formation do not necessarily parallel each other. Experimental animals injected with phage intravenously or intraperitoneally may indeed produce such antibodies, but it has been determined that using such efficient routes does not always produce positive results and some 10% of animals may not respond (Delmastro *et al.*, 1997). Some studies also demonstrated significant systemic and local (mucosal) humoral responses against phage coat proteins following oral administration of phage to rodents (Clark and March, 2004). Data suggest that neutralizing antibody responses may indeed occur in patients receiving phage locally and intrarectally, but oral administration may be less likely to induce such antibodies. This confirms the report of Kucharzewicz-Krukowska and Ślopek (1987) suggesting that the immunogenic effect of phage administered by the oral route in patients is very weak. Likewise, Bruttin and Brussow (2005) showed that oral administration of T4 phage to healthy volunteers does not result in the production of antibodies. In general, the anti-phage humoral response seems to be dependent on a number of factors, especially the phage type, the route of phage administration, and the responder/nonresponder status.

## ACKNOWLEDGMENTS

This work was supported by the European Regional Development Fund within the Operational Program Innovative Economy, 2007-2013, Priority axis 1. Research and Development of ModernTechnologies, Measure 1.3 Support for R&D projects for entrepreneurs carried out by scientific entities, Submeasure 1.3.1 Development projects as project No. POIG 01.03.01-02-003/08 entitled ''Optimization of the production and characterization of bacteriophage preparations for therapeutic use'', by statutory funds from the Medical University of Warsaw—1MG/N/2011, and by a Ministry of Science and Higher Education grant N N402 268036 entitled ''Involvement of bacteriophages T4 and A5 in the activation of human dendritic cells and macrophages'' (2009-2012).

## REFERENCES

Amanna, I. J., and Slifka, M. K. (2011). Contributions of humoral and cellular immunity to vaccine-induced protection in humans. *Virology* **15:**206–215.

Andrews, R. G., Winkler, A., Potter, J., Bryant, E., Knitter, G. H., Bernstein, J. D., and Och, H. D. (1997). Normal immunologic response to a neoantigen, bacteriophage phi X174 in baboons with long-term lymphohematopoietic reconstitution from highly purified CD34+ lin- allogeneic marrow cells. *Blood* **90:**1701–1708.

Aronow, R., Danon, D., Shahar, A., and Aronson, M. (1964). Electron microscopy of in vitro endocytosis of T2 phage by cells from rabbit peritoneal exudate. *J. Exp. Med.* **120:**943–954.

Baker, A. G. (1963). Staphylococcus bacteriophage lysate: Topical and parenteral use in allergic patients. *Pa Med. J.* **66:**25–28.

Bateman, A., Eddy, S. R., and Mesyanzhinov, V. V. (1997). A member of the immunoglobulin superfamily in bacteriophage T4. *Virus Genes* **14:**163–165.

Bearden, C. M., Agarwal, A., Book, B. K., Vieira, C. A., Sidner, R. A., Ochs, H. D., Young, M., and Pescovitz, M. D. (2005). Rituximab inhibits the in vivo primary and secondary antibody responses to a neoantigen, bacteriophage phi X174. *Am. J. Tranplant* **5:**50–57.

Bogden, A. E., and Esber, H. J. (1978). The concept of induction and elicitation as an immunotherapeutic approach. *Natl. Cancer Inst. Monogr.* **49:**365–367.

Borysowski, J., and Gorski, A. (2008). Is phage therapy acceptable in the immunocompromised host? *Int. J. Infect. Dis.* **12:**466–471.

Borysowski, J., Wierzbicki, P., Kłosowska, D., Korczak-Kowalska, G., Weber-Dabrowska, B., and Górski, A. (2010). The effects of T4 and A3/R phage preparations on whole-blood monocyte and neutrophil respiratory burst. *Viral Immunol.* **23:**541–544.

Bruttin, A., and Brussow, H. (2005). Human volunteers receiving *Escherichia coli* phage T4 orally: A safety test of phage therapy. *Antimicrob. Agents Chemother.* **49:**2874–2878.

Chang, L., Gusewitch, G. A., Chritton, D. B., Folz, J. C., Lebeck, L. K., and Nehlsen-Cannarellas, S. L. (1993). Rapid flow cytometric assay for the assessment of natural killer cell activity. *J. Immunol. Methods* **166:**45–54.

Clark, J. R., and March, J. B. (2004). Bacterial viruses as human vaccines? *Expert Rev Vaccines* **3:**463–476.

Dabrowska, K., Opolski, A., Wietrzyk, J., Switala-Jelen, K., Boratynski, J., Nasulewicz, A., Lipinska, L., Chybicka, A., Kujawa, M., Zabel, M., Dolinska-Krajewska, B., Piasecki, E., *et al.* (2004). Antitumor activity of bacteriophages in murine experimental cancer models caused possibly by inhibition of beta3 integrin signaling pathway. *Acta Virol.* **48:**241–248.

Dabrowska, K., Switała-Jeleń, K., Opolski, A., and Górski, A. (2006). Possible association between phages, Hoc protein, and the immune system. *Arch. Virol.* **15:**209–215.

Dabrowska, K., Zembala, M., Boratynski, J., Switala-Jelen, K., Wietrzyk, J., Opolski, A., Szczaurska, K., Kujawa, M., Godlewska, J., and Gorski, A. (2007). Hoc protein regulates the biological effects of T4 phage in mammals. *Arch. Microbiol.* **187:**489–498.

Dean, J. H., Connor, R., Herberman, R. B., Silva, J., McCoy, J. L., and Oldham, R. K. (1977). The relative proliferation index as a more sensitive parameter for evaluating lymphoproliferative responses of cancer patients to mitogens and alloantigens. *Int. J. Cancer* **20:**359–370.

Dean, J. H., Silva, J. S., McCoy, J. L., Chan, S. P., Baker, J. J., Leonard, C., and Herberman, R. B. (1975). *In vitro* human reactivity to staphylococcal phage lysate. *J. Immunol.* **115:**1060–1064.

Delmastro, P., Meola, A., Monaci, P., Cortese, R., and Galfre, G. (1997). Immunogenicity of filamentous phage displaying peptide mimotopes after oral administration. *Vaccine* **15:**1276–1285.

d'Herelle, F. (1992). Opsonic power of the lysins. *In* ''Bacteriophage: Its Role in Immunity'', p. 125. Williams & Wilkins, Baltimore.

Esber, H. J., DeCourcy, S. J., Jr., and Bogden, A. E. (1981). Specific and nonspecific immune resistance enhancing activity of staphage lysate. *J. Immunopharmacol.* **3:**79–92.

Ferrini, U., Mileo, M. M., Nista, A., Mattei, E., and Orofino, A. (1989). Polymorphonuclear leucocyte stimulation measured by phage inactivation. *Int. Arch. Allergy Appl. Immunol.* **90:**207–212.

Forthal, D. N., and Moog, C. (2009). Fc receptor-mediated antiviral antibodies. *Curr. Opin. HIV AIDS* **4:**388–393.

Fraser, J. S., Yu, Z., Maxwell, K. L., and Davidson, A. R. (2006). Ig-like domains on bacteriophages: A tale of promiscuity and deceit. *J. Mol. Biol.* **359:**496–507.

Geier, M. R., Trigg, M. E., and Merril, C. R. (1973). Fate of bacteriophage lambda in non-immune germ-free mice. *Nature* **246:**221–223.

Giese, M. J., Adamu, S. A., Pitchekian-Halabi, H., Ravindranath, R. M., and Mondino, B. J. (1996). The effect of *Staphylococcus aureus* phage lysate vaccine on a rabbit model of staphylococcal blepharitis, phlyctenulosis, and catarrhal infiltrates. *Am. J. Ophthalmol.* **122:**245–254.

Górski, A., and Weber-Dąbrowska, B. (2005). The potential role of endogenous bacteriophages in controlling invading pathogens. *Cell. Mol. Life Sci.* **62:**511–519.

Górski, A., Kniotek, M., Perkowska-Ptasińska, A., Mróz, A., Przerwa, A., Gorczyca, W., Dąbrowska, K., Weber-Dąbrowska, B., and Nowaczyk, M. (2006). Bacteriophages and transplantation tolerance. *Transplant Proc.* **38:**331–333.

Hung, C. H., Kuo, C. F., Wang, C. H., Wu, C. M., and Tsao, N. (2011). Experimental phage therapy in treating *Klebsiella pneumonia*-mediated liver abscesses and bacteremia in mice. *Antimicrob. Agents Chemother.* **55:**1358–1365.

Inchley, C. J. (1969). The activity of mouse Kupffer cells following intravenous injection of T4 bacteriophage. *Clin. Exp. Immunol.* **5:**173–187.

Ishii, T., and Yanagida, M. (1977). The two dispensable structural proteins (soc and hoc) of the T4 phage capsid; their purification and properties, isolation and characterization of the defective mutants, and their binding with the defective heads in vitro. *J. Mol. Biol.* **109:**487–514.

Ivanekov, V., Felici, F., and Menon, A. G. (1999). Uptake and intracellular fate of phage display vectors in mammalian cells. *Biochim. Biophys.* **1448:**450–462.

Jerne, N. K. (1956). The presence in normal serum of specific antibody against bacteriophage T4 and its increase during the earliest stages of immunization. *J. Immunol.* **76:**209–216.

Jerne, N. K., and Avegno, P. (1956). The development of the phage-inactivating properties of serum during the course of specific immunization of an animal: Reversible and irreversible inactivation. *J. Immunol.* **76:**200–208.

Jerzak, M., Baranowski, W., Rechberger, T., and Gorski, A. (2002). Enhanced T cells interactions with extracellular matrix proteins in infertile women with endometriosis. *Immunol. Lett.* **81:**65–70.

Kamme, C. (1973). Antibodies against staphylococcal bacteriophages in human sera. I. Assay of antibodies in healthy individuals and in patients with staphylococcal infections. *Acta Path. Microbiol. Scand. B* **81:**741–748.

Kańtoch, M. (1961). The role of phagocytes in virus infections. *Arch. Immunol. Ther. Exp.* **9:**261–268.

Kańtoch, M. (1956). Wybrane zagadnienia z nauki o bakteriofagach. *Post Hig Med Dośw* **10:**47–486.

Kańtoch, M., Skurski, A., and Wieczorek, Z. (1958). *In vitro* blockade of bacterial phagocytosis of leukocytes by means of bacterial viruses. *Schweiz Z Pathol. Mikrobiol.* **21:**1106–1119.

Kańtoch, M., and Szalaty, H. (1960). Lytic activity of bacteriophages toward phagocytized bacteria. *Arch. Immunol. Ther. Exp.* **8:**407–416.

Kim, K. P., Cha, J. D., Jang, E. H., Klumpp, J., Hagens, S., Hardt, W. D., Lee, K. Y., and Loessner, M. J. (2008). PEGylation of bacteriophages increases blood circulation time and reduces T-helper type 1 immune response. *Microb. Biotechnol.* **1:**247–257.

Kleinschmidt, W. J., Douthart, R. J., and Murphy, E. B. (1970). Interferon production by T4 coliphage. *Nature* **228:**27–30.

Kniotek, M., Ahmed, A. M. A., Dabrowska, K., Świtała-Jeleń, K., Weber-Dąbrowska, B., Boratyński, J., Nowaczyk, M., Opolski, A., and Górski, A. (2004a). Bacteriophage interactions with T cells and platelets. *In* ''Genomic Issues, Immune System Activation and Allergy'' (Immunology 2004a), Medimond International Proceedings (Bologna: Monduzzi Editors), pp. 189–193.

Kniotek, M., Weber-Dąbrowska, B., Dąbrowska, K., Świtała-Jeleń, K., Boratyński, J., Wiszniewski, W., Glinkowski, I., Babiak, I., Gorecki, M., and Nowaczyk, M. (2004b). Phages as immunomodulators of antibody production. *In* ''Genomic Issues, Immune System Activation and Allergy'' (Immunology 2004), Medimond International Proceedings (Bologna: Monduzzi Editors), pp. 33–36.

Krisch, H. M., and Comeau, A. M. (2008). The immense journey of bacteriophage T4: From d'Hérelle to Delbrück and then to Darwin and beyond. *Res. Microbiol.* **59:**314–324.

Krishnan, G., and Ganfield, D. J. (1994). ''Cytokines Produced by Staphage Lysate''. 12th Eur Immun Meeting Barcelona, Abstract Book, p. 395.

Kucharewicz-Krukowska, A., and Slopek, S. (1987). Immunogenic effect of bacteriophages in patients subjected to phage therapy. *Arch. Immunol. Ther. Exp.* **35:**553–561.

Kumar, H., Kawai, T., and Akira, S. (2009). Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.* **388:**621–625.

Kumari, S., Harjai, K., and Chhibber, S. (2010). Evidence to support the therapeutic potential of bacteriophage Kpn5 in burn wound infection caused by *Klebsiella pneumonia* in BALB/c mice. *J. Microbiol. Biotechnol.* **20:**935–941.

Kurzepa, A. (2011). ''The Influence of Bacteria on Migration and Intracellular Killing of Bacteria by Human Phagocytes''. Ph.D. thesis, Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland.

Langbeheim, H., Teitelbaum, D., and Arnon, R. (1978). Cellular immune responses toward MS-2 phage and a synthetic fragment of its coat protein. *Cell. Immunol.* **38:**193–197.

Lee, B., Murakami, M., Mizukoshi, M., Shinomiya, N., and Yada, J. (1985). Effects of Staphylococcal Phage Lysate (SPL) on immunoglobulin production in human peripheral lymphocyte cultures. *Yakugaki Zasshi* **105:**574–579.

Mankiewicz, E., Kurti, V., and Adomonis, H. (1974). The effect of mycobacteriophage particles on cell-mediated immune reactions. *Can. J. Microbiol.* **20:**1209–1218.

Mathur, A., Narayanan, K., Zerbe, A., Ganfield, D., Ramasastry, S. S., and Futrell, J. W. (1988). Immunomodulation of intradermal mammary carcinoma using staphage lysate in a rat model. *J. Invest. Surg.* **1:**117–123.

Merril, C. R., Biswas, B., Carlton, R., Jensen, N. C., Creed, G. J., Zullo, S., and Adhya, S. (1996). Long-circulating bacteriophage as antibacterial agents. *Proc. Natl. Acad. Sci. USA* **93:**3188–3192.

Michael, J. G., and Kuwatch, C. J. (1969). Contribution of endotoxin to antibody formation against T2 phage. *Nature* **222:**684–685.

Miedzybrodzki, R., Fortuna, W., Weber-Dabrowska, B., and Gorski, A. (2009). A retrospective analysis of changes in inflammatory markers in patients treated with bacterial viruses. *Clin. Exp. Med.* **9:**303–312.

Międzybrodzki, R., Switala-Jelen, K., Fortuna, W., Weber-Dabrowska, B., Przerwa, A., Lusiak-Szelachowska, M., Dabrowska, K., Kurzepa, A., Boratynski, J., Syper, D., Pozniak, G., Lugowski, C., and Gorski, A. (2008). Bacteriophage preparation inhibition of reactive oxygen species generation by endotoxin-stimulated polymorphonuclear leukocytes. *Virus Res.* **131:**233–242.

Mills, A. E. (1956). Staphylococcus bacteriophage lysate aerosol therapy of sinusitis. *Laryngoscope* **66:**846–858.

Mills, A. E. (1962). Staphylococcus phage lysates: An immune-biological therapy for the prevention and control of staphylococcal disease. *Laryngoscope* **72:**367–383.

Molenaar, T. J., Michon, I., de Haas, S. A., van Berkel, T. J., Kuiper, J., and Biessen, E. A. (2002). Uptake and processing of modified bacteriophage M13 in mice: Implications for phage display. *Virology* **293:**182–191.

Nelstrop, A., Taylor, G., and Collard, P. (1967). Studies on phagocytosis. *II. In vitro phagocytosis by macrophages. Immunology* **14:**339–346.

Ochs, H. D., Davis, S. D., and Wedgwood, R. J. (1971). Immunologic responses to bacteriophage phi X174 in immunodeficiency diseases. *J. Clin. Invest.* **50:**2559–2568.

Pillich, J., Tovarek, J., and Fait, M. (1978). Contribution to the treatment of acute haematogenous and chronic secondary osteomyelitis in children. *Z. Orthop. Ihre Grenzgeb.* **116:**40–46.

Przerwa, A., Kniotek, M., Nowaczyk, M., Weber-Dabrowska, B., Switala-Jelen, K., Dabrowska, K., and Gorski, A. (2005). Bacteriophages inhibit interleukin-2 production by human T lymphocytes. *In* ''12th Congress of the European Society for Organ Transplantation,'' Geneva, Switzerland.

Przerwa, A., Zimecki, M., Świtała-Jeleń, K., Dąbrowska, K., Krawczyk, E., Łuczak, M., Weber-Dąbrowska, B., Syper, D., Międzybrodzki, R., and Górski, A. (2006). Effects of bacteriophages on free radical production and phagocytic functions. *Med. Microbiol. Immunol.* **195:**143–150.

Salmon, G. G., Jr., and Symonds, M. (1963). Staphage lysate therapy in chronic staphylococcal infections. *J. Med. Soc. N. J.* **60:**188–193.

Sathaliyawala, T., Islam, M. Z., Li, Q., Fokine, A., Rossmann, M. G., and Rao, V. B. (2010). Functional analysis of the highly antigenic outer capsid protein, Hoc, a virus decoration protein from T4-like bacteriophages. *Mol. Microbiol.* **77:**444–455.

Schwarz, K. B. (1996). Oxidative stress during viral infection: A review. *Free Radic. Biol. Med.* **21:**641–649.

Silva, M. T. (2010). When two is better than one: Macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J. Leukocyte Biol.* **87:**93–106.

Ślopek, S., Weber-Dąbrowska, B., Dąbrowski, M., and Kucharewicz-Krukowska, A. (1987). Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. *Arch. Immunol. Ther. Exp. (Warsz)* **35:**569–583.

Sokoloff, A. V., Bock, I., Zhang, G., Sebestyén, M. G., and Wolff, J. A. (2000). The interactions of peptides with the innate immune system studied with use of T7 phage peptide display. *Mol. Ther.* **2:**131–139.

Srivastava, A. S., Kaido, T., and Carrier, E. (2004). Immunological factors that affect the in vivo fate of T7 phage in the mouse. *J. Virol Methods* **115:**99–104.

Stent, G. S. (1963). Molecular Biology of Bacterial Viruses. W.H Freeman and Company, San Francisco/London.

Sulakvelidze, A., Alavidze, Z., and Morris, J. G., Jr. (2001). Bacteriophage therapy. *Antimicrob. Agents Chemother.* **45:**649–659.

Sulakvelidze, A., and Barrow P. (2005). Phage therapy in animals and agribusiness. *In* ''Bacteriophages. Biology and Applications'' (E. Kutter and A. Sulakvelidze, eds.). CRC Press, Boca Raton, FL.

Sustiel, A. M., Joseph, B., Rocklin, R. E., and Borish, L. (1989). Asthmatic patients have neutrophils that exhibit diminished responsiveness to adenosine. *Am. Rev. Respir. Dis.* **140:**1556–1561.

Tikhonenko, A. S., Gachechiladze, K. K., Bespalova, I. A., Kretova, A. F., and Chanishvili, T. G. (1976). Electron-microscopic study of the serological affinity between the antigenic components of phages T4 and DDVI. *Mol. Biol. (Mosk)* **10:**667–673.

Uchiyama, J., Maeda, Y., Takemura, J., Chess-Williams, R., Wakiguchi, H., and Matsuzaki, M. (2009). Blood kinetics of four intraperitoneally administered therapeutic candidate bacteriophages in healthy and neutropenic mice. *Microbiol. Immunol.* **53:**301–304.

Victor, V. M., Rocha, M., and de la Fuente, M. (2004). Immune cells: Free radical and antioxidants in sepsis. *Int. Immunopharmacol.* **4:**327–347.

Vitiello, C. L., Merril, C. R., and Adhya, S. (2005). An amino acid substitution in a capsid protein enhances phage survival in mouse circulatory system more than a 1000-fold. *Virus Res.* **114:**101–103.

Weber-Dąbrowska, B., Mulczyk, M., and Górski, A. (2000a). Bacteriophage therapy of bacterial infections: An update of our institute's experience. *Arch. Immunol. Ther. Exp. (Warsz)* **48:**547–551.

Weber-Dąbrowska, B., Zimecki, M., and Mulczyk, M. (2000b). Effective phage therapy is associated with normalization of cytokine production by blood cell cultures. *Arch Immunol Ther Exp (Warsz)* **48:**31–37.

Weber-Dąbrowska, B., Zimecki, M., Mulczyk, M., and Górski, A. (2002). Effect of phage therapy on the turnover and function of peripheral neutrophils. *FEMS Immunol. Med. Microbiol.* **34:**135–138.

Wedgwood, R. J., Ochs, H. D., and Davis, S. D. (1975). The recognition and classification of immunodeficiency diseases with bacteriophage phi X174. *Birth Defects Orig. Artic Ser.* **11:**331–338.

Yanagida, M. (1972). Identification of some antigenic precursors of bacteriophage T4. *J. Mol. Biol.* **65:**501–517.

Zimecki, M., Artym, J., Kocieba, M., Weber-Dabrowska, B., Borysowski, J., and Górski, A. (2009). Effects of prophylactic administration of bacteriophages to immunosuppressed mice infected with *Staphylococcus aureus*. *BMC Microbiol.* **9:**169.

Zimecki, M., Weber-Dabrowska, B., Łusiak-Szelachowska, M., Mulczyk, M., Boratyński, J., Poźniak, G., Syper, D., and Górski, A. (2003). Bacteriophages provide regulatory signals in mitogen-induced murine splenocyte proliferation. *Cell. Mol. Biol. Lett.* **8:**699–711.