The effects of bacteriophages on the expression of genes involved in antimicrobial immunity*

Wpływ bakteriofagów na ekspresję genów mających udział w odporności przeciwdrobnoustrojowej

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Summary

Introduction: Bacteriophages (viruses of bacteria) are used in the treatment of antibiotic-resistant infections. Moreover, they are an important component of the mucosal microbiota. The objective of this study was to investigate the effects of T4 and A5/80 bacteriophages on the expression of genes involved in antimicrobial immunity, including Toll-like receptors.

Material/Methods: The expression of genes was determined in the A549 cell line using RT^2 Profiler PCR Array.

Results: Purified T4 and A5/80 phage preparations significantly affected the expression of 7 and 10 out of 84 examined genes, respectively.

Discussion: Our results are important for phage therapy of bacterial infections and provide novel insights into the role of phages from the mucosal microbiota. They may also lead to novel applications of phages as antiviral and immunomodulatory agents.

Keywords: phage · gene expression · immunity · IL-2 · Toll-like receptor

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INTRODUCTION

Bacteriophages (phages) are viruses of bacteria. The antibacterial activity of phages has been studied in great detail and has enabled their use in the treatment of bacterial infections, including those caused by antibiotic-resistant strains [17]. Furthermore, bacteriophages present on mucous membranes may be involved in antibacterial immunity [5]. There are also reports showing that phages can exert other effects. For instance, our research revealed that phages can affect functions of some populations of immune cells [11]. Moreover, there are some data in the literature to suggest that phages, as well as some components of phage virions, may exert antiviral effects [27]. In this regard, our previous study showed that T4 bacteriophage can inhibit both the adsorption and replication of the adenovirus in the human lung carcinoma A549 cell line [30]. The main objective of the present study was to investigate whether phages can affect the expression of genes encoding proteins involved in antimicrobial immunity, including Toll-like receptors (TLRs) and proteins mediating TLR signaling pathways [35]. We hypothesized that the antiviral effects of phages found in our previous study [30] might be mediated at least in part by the induction of these genes.

MATERIAL AND METHODS

Purified phage preparations

Escherichia coli T4 phage [38] was obtained from the American Type Culture Collection (ATCC; USA). Staphylococcal A5/80 phage [25] was from the bacteriophage collection of the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS). Bacterial strains for phage propagation (*E. coli* B and *Staphylococcus aureus* 80 for T4 and A5/80, respectively) were from the Polish Collection of Microorganisms at the HIIET PAS. Crude bacterial lysates of both phages were prepared according to the modified method of Ślopek et al. [33]. Purified preparations of T4 and A5/80 phages were prepared according to the modified protocol developed at the HIIET PAS [6, 26]. The lipopolisaccharide (LPS) level in purified phage preparations was determined using a QLC1000 Endpoint Chromogenic LAL test kit (Lonza, Switzerland) according to the manufacturer's instructions. Phage titer was measured by the two-layer method of Adams [2]. Stock preparations of both phages (10^{10} plaque forming units (PFU)/ml) were diluted with a culture medium before they were added to the cell culture.

Preparation of LPS from *E. coli* B

E. coli B culture was grown for 24 h at 37°C in liquid LB medium, pH 7.0 in 10L BioFlo415 fermenter (Eppen-dorf, Germany) as described by Kaszowska et al. [21]. LPS was isolated by the PCP (phenol/chloroform/petroleum) method [8] for the extraction of R lipopolysaccharides and purified by ultracentrifugation (105.000 × *g*, 4 h, 4°C). It was then diluted with PBS and dispersed with the use of Sonopuls HD 2070 ultrasonic homogenizer connected to a UW 2070 HF-generator (both from Bandelin, Germany). Its activity in the stock suspension measured in the LAL test, as described earlier, was 5000 EU/ml. It was dispersed again just before it was diluted with a culture medium and added to the cell culture. LPS suspension was used as an additional control for phage preparations.

Cell line and cell culture

The human lung carcinoma cell line A549 (ATCC CCL-185) was obtained from LGC Promochem. A549 cells were grown in a culture medium composed of Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS, Thermo Scientific) and antibiotic-antimycotic solution (100 U/ml penicillin G, 100 mg/ml streptomycin and 0.25 µg/ml amphotericin B; Sigma-Aldrich) and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Before experiments, cell suspensions were seeded in 96-well microplates (Nunclon Surface, Nunc) at a density of 20.000 cells/200 µl per well and incubated for 24–36 h to form a confluent monolayer. Next, the cell culture medium was discarded and cells were treated for 2 h with one of the following additives at a volume of 200 µl: T4 preparation, A5/80 preparation, culture medium (untreated control), and *E. coli* B LPS at the final activity of 0.5 endotoxin units (EU)/ml, which corresponded to the final endotoxin activity in cultures incubated with phages (LPS control). The titers of both phages were adjusted so as to obtain the ratio of 100 phage particles per one cell. After incubation, the cell culture medium was discarded and cells were harvested for RNA isolation. Experiments were done in three independent replicates.

Evaluation of gene expression

RNA was isolated from A549 cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific). Expression of genes was determined with reverse-transcribed real-time PCR (RT-qPCR) using Toll-like receptor signaling pathway RT^2 Profiler PCR Array Format F (Qiagen) by Roche LightCycler 480 according to the manufacturer's protocol. This assay allows for the simultaneous evaluation of expression of 84 genes with the specificity and sensitivity of quantitative real-time PCR. We determined the expression of gene encoding for Toll-like receptors, proteins involved in pathogen-specific responses, Toll-like receptor sign-

aling, downstream signaling of Toll-like receptors, proteins involved in the regulation of adaptive immunity, Toll-like receptor interacting proteins and adaptors and downstream effectors of Toll-like receptor signaling. A complete list of these genes is available online as Supplementary Information. The level of genomic DNA contamination, the possibility of inhibition of the reverse-transcription reaction, the presence of PCR amplification inhibitors, the cycling conditions, and the relative sensitivity of the instrument were validated according to the RT^2 Profiler PCR Array manufacturer's guide.

DATA ANALYSIS

The relative transcript levels of genes of interest were analyzed using the $\Delta\Delta C_T$ method according to the RT^2 Profiler PCR Array manufacturer's guide. The average C_T of 5 housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*) was used for normalization. Difference in mRNA expression between T4, A5/80 or LPS treated groups and the untreated control was expressed as fold-change (f-c) values ($2^{-\Delta\Delta CT}$). We considered it significant when there was at least two-fold change: f-c ≤0.5 or f-c ≥2 (sample in triplicates) [8]. The range of f-c was indicated by $2^{-(\Delta\Delta C_T + SD)}$ and $2^{-(\Delta\Delta CT - SD)}$, where SD for $\Delta\Delta C_T$ was calculated based on standard deviations of ΔC_T values for treated and untreated groups [19]. To exclude potential bias due to averaging of data transformed through the $2^{-\Delta\Delta C_T}$ equation, statistical analysis of $\Delta\Delta C_T$ values obtained for each experiment was done with the use of a one-sample t-test with a 1.0 reference value [29]. The significance for this test was set at 5% (*p*-value <0.05). Analysis was performed using SigmaPlot 12.3 software (Systat Software, Germany).

RESULTS

The objective of this study was to investigate the effects of purified preparations of two bacteriophages – T4 and A5/80 – on the expression of genes encoding for proteins involved in the induction of antimicrobial immune responses. T4 is a classic *E. coli* phage that has been extensively characterized at the molecular and genetic level [38]. We used this phage because in our previous study it induced significant antiviral effects by blocking both the adsorption and replication of human adenovirus in the A549 cell line [30]. In addition, in the present study, to investigate whether antiviral effects can also be induced by other phages, we used staphylococcal A5/80 phage [25]. We hypothesized that antiviral effects of phages found in our previous study might be mediated (at least in part) by inducing some genes involved in antiviral immunity. To verify our hypothesis, we used an RT^2 Profiler PCR Array, which allows for the simultaneous evaluation of expression of 84 genes, including those encoding individual TLRs and proteins involved in TLR signaling. This assay determines the expression of the examined genes with the sensitivity and specificity of quantitative real-time PCR. Expression of genes in A549 cells was determined after 2-h incubation with

phage preparations. Compared with control cultures, T4 and A5/80 phage preparations significantly affected the expression of 7 and 10 genes, respectively (Table 1).

The most striking effect was high overexpression of the *HSPA1* gene in cells treated both with T4 and A5/80 compared with control cultures (5163.0 f-c and 7931.3 f-c, respectively). However, this effect was probably mediated by residual LPS present in both preparations, because control LPS suspension increased the expression of this gene to a comparable extent (5895.2 f-c).

Moreover, both T4 and A5/80 significantly increased the expression of the *CLEC4E* gene (6.3 f-c and 9.4 f-c, respectively); the effect of LPS on this gene was weaker (4.0 f-c; Table 1).

Of the 10 examined TLR genes (*TLR1-10*), only the expression of *TLR10* was significantly increased in A549 cells cultured with A5/80 phage preparation (2.8 f-c).

Other genes whose expression in cells cultured with phage preparations was significantly affected were some cytokine and colony-growth factor genes. Both phages influenced the expression of *TNF* and *IL12A* genes. Moreover, T4 affected the expression of the *IL1A* gene, while A5/80 changed the expression of the *IL2*, *CSF2* and *CSF3* genes.

Remarkably, a highly significant increase in *IL2* gene expression was observed following treatment with A5/80 (13.9 f-c; Table 1). Since LPS did not significantly affect the expression of any of these genes (except for *CSF2*), these changes were most likely caused by phage virions themselves.

Moreover, both phage preparations significantly affected the expression of a number of other genes. In particular, T4 preparation significantly increased the expression of the *CD86* gene (3.2 f-c compared with control cultures), while A5/80 preparation induced the *CD80* gene (3.8 f-c) and reduced the expression of the *LY96* gene.

In addition, there were several genes whose overexpression (fold change ≥ 2.0) or expression inhibition (fold change ≤ 0.5) in A549 cells cultured with phage preparations was observed, but fell short of statistical significance; these genes are also listed in Tab. 1. These include *TLR2*, *TLR4*, *IL6*, *IL10*, *IFNB1* and *BTK*.

DISCUSSION

The most striking effect found in our study was a very high overexpression of the *HSPA1* gene following treatment with either phage preparation. This gene encodes heat shock 70 kDa protein 1A (HSP1A), also called Hsp72, a major heat shock protein involved in a number of cellular activities, including protein synthesis, folding, and translocation into organelles as well as the assembly of multiprotein complexes. Generally, this protein is upreg-

Table 1. Genes whose overexpression (fold change ≥ 2.0) or expression inhibition (fold change ≤ 0.5) in A549 cells was observed following at least one of individual treatments (T4 phage preparation, A5/80 phage preparation, or *E. coli* B LPS)

No.	Gene	Fold-change value					
		T4 vs control		A5/80 vs control		LPS vs control	
1	**CD80**	1.4	(1.1-1.9)	**3.8**	(2.8-5.1) *	1.0	(0.9-1.1)
2	**CD86**	**3.2**	(3.0-3.4)***	1.3	(0.9-1.9)	0.9	(0.8-1.1)
3	**CLEC4E**	**6.3**	(6.0-6.5)***	**9.4**	(7.58-11.9)**	**4.0**	(1.1-14.0)
4	**HSPA1A**	**5163.0**	(1667.1-15990.0)***	**7931.3**	(2878.0-21856.8)**	**5895.2**	(2145.5-16198.6)**
5	**IFNB1**	**2.3**	(0.8-7.0)	1.5	(0.8-2.8)	1.0	(1.0-1.0)
6	**IL1A**	**0.1**	(0.0-0.4) *	1.2	(1.0-1.5)	**0.4**	(0.2-1.0)
7	**IL1B**	1.4	(0.7-2.9)	1.5	(0.7-3.1)	**0.2**	(0.1-0.5)
8	**IL2**	1.3	(1.2-1.3)**	**13.9**	(9.4-20.4)**	1.3	(0.9-2.0)
9	**IL6**	**3.0**	(0.3-29.0)	**3.0**	(0.5-17.5)	**2.5**	(0.4-15.1)
10	**IL10**	**2.2**	(0.8-6.1)	1.4	(0.9-2.1)	0.9	(0.8-1.1)
11	**IL12A**	**0.5**	(0.3-0.7)*	**0.5**	(0.4-0.7)**	0.7	(0.5-1.0)
12	**LTA**	1.0	(0.2-4.8)	**5.1**	(1.3-19.9)	0.9	(0.2-4.4)
13	**TNF**	**7.0**	(3.3-14.9)*	**2.3**	(1.7-3.1)*	**4.4**	(1.0-19.7)
14	**CSF2**	1.3	(0.5-3.3)	0.7	(0.4-1.1) *	**0.3**	(0.2-0.5)*
15	**CSF3**	0.7	(0.3-1.5)	**4.0**	(2.7-5.7)**	1.1	(0.3-3.9)
16	**TLR2**	**2.1**	(0.9-4.8)	1.3	(0.9-1.9)	1.1	(0.8-1.6)
17	**TLR4**	**0.2**	(0.0-1.4)	**0.4**	(0.0-3.5)	**0.4**	(0.2-1.3)
18	**TLR10**	**2.6**	(0.8-8.8)	**2.8**	(2.3-3.5)*	**3.8**	(1.2-11.8)
19	**LY96**	0.8	(0.6-1.2)	**0.4**	(0.4-0.5)**	**0.2**	(0.1-1.1)
20	**BTK**	1.5	(0.9-2.6)	**3.8**	(0.9-16.4)	0.9	(0.7-1.1)
21	**REL**	1.0	(0.7-1.6)	**2.1**	(1.5-2.8)	0.9	(0.7-13)
22	**FOS**	**0.4**	(0.3-0.7)	0.7	(0.6-0.9)	0.6	(0.4-0.9)

Legend: Shown are fold-changes (and their range) compared with untreated control cultures (values representing at least two-fold change are shown in bold). Significance of the difference in gene expression: *p <0.05; **p <0.01; ***p <0.001.

ulated in response to cellular stress and it has cytoprotective functions [23]. However, it needs to be stressed that in other experiments neither of the studied phage preparations nor LPS exerted any cytotoxic effects on A549 cells [30]. The mechanism and potential consequences of the induction of the *HSPA1A* gene by phage preparations need to be investigated in further studies.

An interesting finding was a significant increase in *TLR10* gene expression in cells treated with A5/80 phage preparation. TLR10 is unique among TLRs in that its activation can induce anti-inflammatory effects [20]. Thus, by increasing the expression of this gene some phages might exert anti-inflammatory activity. However, this needs to be verified by further studies. We also found that T4, but not A5/80 phage preparation, increased the

expression of the *TLR2* gene. This finding is of potential importance, because, according to a recent report, TLR2 is involved in the induction of initial antiviral immune response as a result of the recognition of the repeating protein subunit pattern common to many viral capsids [32]. Therefore, an increase in the expression of *TLR2* may have mediated (at least in part) antiviral activity of T4 phage found in our previous study [30]; however, this needs to be verified by future studies.

Another important result is a reduction in the expression of the *TLR4* gene induced by both phage preparations. TLR4 has been implicated in inflammatory reactions accompanying a number of diseases and pathological conditions, including sepsis. TLR4 signaling pathway modulators hold promise for use in sepsis [22]. Therefore, our finding may suggest a novel non-bactericidal mechanism that could contribute to the therapeutic effects of phage preparations in sepsis. In fact, as discussed in our recent paper, there are solid data in the literature to support the initiation of clinical trials to evaluate the safety and the efficacy of phages in sepsis [12]. Furthermore, phage-mediated interference with TLR signaling might promote beneficial therapeutic effects in autoimmune liver diseases, inflammatory bowel disease (IBD), allergies and graft-versus-host disease (GVHD) [13, 14, 15, 16].

Moreover, we found that both phage preparations significantly increased the expression of the *CLC4E* gene which encodes C-Type Lectin Domain Family 4 Member E, also referred to as Macrophage-inducible C-type lectin and Mincle. Like TLRs, it is an important pattern-recognition involved in the induction of antibacterial and antifungal immune responses [24]. Thus, it is possible that the induction of this gene might to some extent contribute to the antibacterial effects of phage preparations.

A5/80 significantly increased the expression of the *IL2* gene. This effect was likely mediated by some component(s) of A5/80 virions, especially capsid proteins. This data is in accord with what other authors have also found, namely, that phages can stimulate IL-2 production [7]. IL-2 drives NK cell proliferation and production of lytic molecules; their cytolytic potential is reduced in lung cancer. Recent clinical trials on NK based therapies, including IL-2 immunotherapy, showed promising results and fewer adverse effects in lung cancer and available data suggest that improving NK cell function may induce tumor regression [4]. Moreover, NK cells are important in immune defense against virus infections [36]. Moreover, NK cells have innate antibacterial activity against both intra- and extracellular pathogens and its augmentation may be considered to be a clinical intervention strategy [10]. Therefore, some experts believe that NK cells may become a tool to prevent or to combat bacterial infections [31]. Furthermore, NK cells play a vital role in IL-2-induced anticancer effects in A549-bearing nude mouse model [39]. In this context, our data showing that A5/80 phage induces significant upregulation of the *IL-2* gene in

A549 may suggest the existence of additional mechanisms of IL-2 – dependent NK cell mediated immune protection against bacterial and viral infections and cancer triggered by some phages.

We also noted that T4 phage preparation increased the expression of gene encoding interlukin-10 (IL-10), the major anti-inflammatory cytokine [40]. In our previous studies, we showed that during the treatment of bacterial infections phage preparations may exert anti-inflammatory activity that is independent of a reduction in the number of pathogenic bacteria [28]. Thus, it is possible that anti-inflammatory effects of some phage preparations might be mediated by an induction of IL-10 (perhaps along with a reduction in the expression of *TLR4* gene, as mentioned above). However, this hypothesis has to be verified by further experiments. Phage-mediated induction of IL-10 could open the way to phage application in treating some autoimmune diseases, inflammatory bowel disease, allergies and GVHD, where up-regulation of IL-10 production may promote beneficial therapeutic effects [13, 14, 15, 16]. Our results are in accord with the data of van Belleghem et al., [34] who showed that a *Pseudomonas* phage upregulates IL-10 expression in human mononuclear cells, while a *Staphyloccocus* phage does not.

Other genes whose expression in A549 cells was changed following phage treatment include *CD80*, *CD86* and *BTK*. All these genes encode proteins important for the immune system. CD80 and CD86 are proteins expressed largely by antigen presenting cells, which provide costimulatory signals essential for T cell activation [1]. The *BTK* gene encodes for Bruton's tyrosine kinase, which plays an important role in B cell development, differentiation and signaling [37]. Potential biological effects resulting from the induction of these genes should be investigated in further studies.

A number of studies have shown that bacteriophages are an important component of the mucous microflora [3]. It appears that these phages may be involved in antimicrobial immunity [5]. Data obtained in the present study seem to support this. In particular, the induction of the *TLR2* gene might account, at least in part, for antimicrobial effects of bacteriophages. The increase in the expression of IL-10 might contribute to anti-inflammatory activity of phages especially in the gut mucosa, which is in line with our earlier hypothesis that phages from the gut microbiota might contribute to the dampening of inflammatory reactions in the gut [18]. While our study is rather preliminary in nature, it provides a starting point for future investigations, which may generate new important data about the effects of phage preparations used for therapeutic purposes and phages being a natural component of the mucosal microbiota. Eventually, these studies may lead to novel applications of phage preparations as immunomodulatory and antiviral agents.

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