BACTERIOPHAGES PROVIDE REGULATORY SIGNALS IN MITOGEN-INDUCED MURINE SPLENOCYTE PROLIFERATION

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Abstract: The aim of this investigation was to reveal the regulatory properties of bacteriophage preparations in a model of mitogen-induced splenocyte proliferation in mice. We showed that sepharose 4B-purified preparations of the Staphylococcus aureus phage A20/R exhibited costimulatory activity in splenocyte proliferation induced by suboptimal (0.25 µg/ml) concentrations of ConA. On the other hand, the purified phage fraction was regulatory with regard to splenocyte proliferation induced by the optimal (2.5 µg/ml) ConA concentration. We also showed that the phage preparation can elicit IL-6 production in splenocyte cultures and enhance ConA-induced production of that cytokine. Furthermore, the phages preferentially induced IL-6 production in adherent splenocytes and increased levels of that cytokine in cultures of peritoneal cells from mice and rats. This phenomenon may explain the costimulatory activity of phages in the model described.

Key Words: Bacteriophages, Splenocyte Proliferation, Concanavalin A, Regulation, Interleukin 6

INTRODUCTION

Although there has been enormous progress in health care over the last decades, mortality rates in hospitals and intensive care units remain high. This
phenomenon predominantly results from the increasing resistance of pathogenic bacteria to antibiotics [1, 2]. Among the new preventive and therapeutical strategies, the treatment of patients with bacteriophages has attracted increasing attention [3].

Bacteriophages selected for specific bacteria strains have proved to be very effective in combating infections otherwise resistant to antibiotic therapy [4, 5, 6]. Our recent study revealed that effective phage therapy was associated with a normalization of cytokine production by peripheral blood mononuclear cells [7]. In addition, phage therapy resulted in a stimulation of the neutrophil turnover in the peripheral blood [8]. These findings suggest that the phages and/or components of the bacterial cell walls contained in the lyzates administered orally to patients may interact with cells of the immune system.

Knowledge of the interdependence between bacteriophages and eukariotic cells is scant. In 1940, Bloch et al. [9] found that bacteriophages can enter and remain in malignant tumors for a much longer period than they can in the normal tissue of the respective animal. In addition, the Ehrlich carcinoma lost a high percentage of its transplantation ability upon the addition of bacteriophages. Even more interesting, a similar phenomenon of preferential accumulation of phages was observed in rapidly growing testicle tissue [9]. Other early studies confirmed the in vitro binding of bacteriophages (type T2 specific for E. coli strain B, and type A acting on S. typhi Vi) to Ehrlich carcinoma and Amytal sarcoma cells/tissues [10]. Two decades later, data became available on phage interaction with the plasma membranes of lymphocytes, resulting in a reduction of cell division [11]. It also became evident that T cells were involved in the immune response to phages [12].

However, not until very recently have new reports appeared suggesting a role for selected bacteriophage clones in the regulation of T-cell proliferation [13] and signal transduction [14, 15]. There are no data available with regard to the regulatory action of bacteriophages used in the therapy of bacterial infections on mitogen-induced lymphocyte proliferation. In this report, we present for the first time information on the interesting costimulatory and regulatory actions of the phage A20/R in concanavalin A- and lipopolysaccharide-induced splenocyte proliferation. Moreover, we demonstrate that a purified phage preparation can induce interleukin 6 production in adherent splenocyte cultures and peritoneal macrophages in the absence of other stimuli, and enhance IL-6 production in ConA-stimulated cell cultures.

**MATERIALS AND METHODS**

**Preparation of bacteriophages and their purification**

Crude bacteriophage preparations (bacteriophage No A20/R from our phage collection) were obtained as described in detail previously [16]. Briefly, a 5 volume percent of phage lyzate was added to a 4h culture of *Staphylococcus aureus* in a broth containing glucose (logarithmic phase growth), and the bottles
were shaken in an incubator for 3h at 37°C. When lysis occurred, the cultures were left overnight at 4°C. The residual bacterial cells were removed by membrane filtration (0.2-0.4 µm). The density of phage particles was determined using the two-layer Gratia method. As a control, a bacterial culture incubated without phages was used.

Phage lyzates (2 liters) were concentrated by ultrafiltration to a volume of 6-8 ml. The preparation was centrifuged at 8000 rpm to remove bacterial debris, and the titer of the bacteriophages was determined in the supernatant. The supernatant was applied onto a column containing sepharose 4B (Pharmacia, volume 500 ml) [17]. Fractions of 5 ml each were collected (phosphate buffer, 0.068M, pH 7.2). A 50-ml phage-containing batch was collected by combing 10 fractions (Fig. 1). The preparation was passed through a membrane filter (0.22 µm) and the phage titer was determined (10^{12} phage particles/ml, on average).

Fig. 1. Separation of A 20/R phage from the lyzate on a sepharose 4B column. The figure shows an elution profile at a wavelength of 260 nm. The first peak represents purified phages – 10^{12} phage particles/ml. The second peak contains bacteria-derived material.

**Membrane formation and properties**

Porous asymmetric ultrafiltration membranes were formed due to the phase inversion. The DMF solutions (NN-dimethylformamide, POCH, Gliwice, Poland) of PSU (polysulfone – UDEL-P-1700 from Amoco Performance Products, Atlanta, GA, USA) were cast on a glass plate, formed into a 0.25 mm film using the doctor-blade technique and immediately immersed in water. The membrane porosity and average pore diameter were determined gravimetrically and calculated based on the Elford-Ferry equation as described by Bodzek [18].

The hydraulic flux of the water was determined in a dead-end type cell Amicon 4200. The membrane surface was 19.6 cm² and the transmembrane pressure was adjusted to 0.1 Mpa. The characteristics of the membrane used for phage
isolation were as follows: concentration of PSU (weight) – 14.5, water flux (dcm$^3$/cm$^2$/h) – 107, porosity (%) – 73, average pore diameter (nm) – 16.

**Separation of bacteriophages on membranes**

The purification of bacteriophages was conducted on an American apparatus using a polysulphonic membrane described above. 200 ml of the bacterial lysate containing bacteriophages was filtered through a bacteriological filter. The lysate was subsequently concentrated in an Amicon apparatus (0.7 bar pressure) to a volume of 5 ml. The concentrated fraction was diluted with PBS to a volume of 50 ml and concentrated again. This procedure was repeated eleven times to remove bacteriophages present in the lysate. The final number of phages per ml was $10^{12}$. Peptidoglycans were not determined in the lysate due to the lack of available commercial (commercially viable) tests.

**Mice**

3-month old CBA mice of both sexes and male Wistar rats (150-200 g) and were obtained from the Animal Facility of the Institute of Immunology, Wroclaw. The animals were fed a commercial pellet food diet and provided with filtered tap water *ad libidum*.

**Separation of spleen cells and isolation of peritoneal macrophages**

Aseptically removed spleens were pressed through plastic screens into precooled Hanks’ medium, centrifuged, and treated for 5 min at room temperature with 0.83% ammonium chloride to lyse erythrocytes. The cells were then washed twice with Hanks’ medium, resuspended in a culture medium of RPMI 1640, 10% FCS, 2 mM glutamine, sodium pyruvate, 2 mercaptoethanol and antibiotics (penicillin and streptomycin, 100 µg/ml) at a concentration of $10^7$/ml and placed in 24-well flat-bottomed culture plates (Nunc) (1 ml/well) for 2 h at 37°C. Following incubation, the nonadherent cells were removed by gentle washing with a Pasteur pipette.

Isolation of peritoneal macrophages was performed by lavage of the peritoneal cavities with 5-8 ml (mice) or 10-15 ml (rats) of Hanks’ medium using a syringe. The cells were washed twice with Hanks’ medium, resuspended in the culture medium at a density of $2 \times 10^6$/ml, placed in 24-well culture plates (1 ml/well) and incubated for 2h at 37°C. Following incubation, the nonadherent cells were washed off using Hanks’ medium and gentle pipetting.

**Proliferation tests**

The spleens were aseptically removed and placed in precooled Hanks’ medium. A single cell suspension was prepared by pressing the organs through a plastic screen. The cells were washed twice in Hanks’ medium, counted and resuspended in culture medium at a density 2 x $10^6$/ml. The cells were then distributed to 96-well flat-bottomed plates (100 µl/2 x $10^5$ cells). The phage preparations studied were applied at 2-fold dilutions (16-512 final dilution in the assay).
Concanavalin A (Sigma) was used at optimal (2.5 µg/ml) or suboptimal (0.25-0.5 µg/ml) concentrations. After a 4-day incubation, the degree of cell proliferation was determined via the MTT colorimetric method [19]. The effects of the phage preparation were evaluated relative to appropriate controls. In the case of the crude lyzate, this was the mean optical density (OD) value from a 16-512 dilution of the broth medium, while with the purified phage preparation it was the mean OD value from a 16-512 dilution of the phosphate buffer used for column fractionation.

**Induction of IL-6 and determination of IL-6 activity**

IL-6 activity was determined in the 24-hour supernatants from the splenocyte cultures treated with the phage preparations in the absence or presence of 0.5 µg ConA/ml using the identical culture conditions as described for the proliferation assay.

The assay was performed according to Van Snick [20]. Briefly, 7TD1 indicator cells were washed 3 times with Hanks’ medium and resuspended in Iscove’s medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics, with a density of 2 x 10^4 cells/ml. Then the cells were distributed in 100 µl aliquots into 96-well flat-bottomed plates containing 100 µl of serially diluted supernatant in triplicate. After 72 hours of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method [19]. The results of the IL-6 activity are expressed as picograms per ml, which is the concentration of IL-6 corresponding to the activity of IL-6 expressed in units/ml [20]. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

**Colorimetric method of determination of cell proliferation**

The assay was performed according to Hansen [19]. Briefly, MTT solution, 5 mg/ml in 0.9% NaCl, was added at a volume of 25 µl/well and incubated for 2-4 hours. Then, 100 µl of a lysing buffer was added (20% SDS, 50% DMF, pH 4.7). After an overnight incubation at 37°C, the optical density (OD) was measured using the Dynatech 5000 ELISA reader at a wavelength of 550 nm and a reference wavelength of 630 nm.

**Statistics**

The t-student’s test was applied for the statistical evaluation of the data. Results are presented as mean values ± standard error. The differences were regarded as significant when p<0.5.

**RESULTS**

**The costimulatory activity of the phage preparation in splenocyte proliferation induced by suboptimal doses of ConA**

Preliminary experiments revealed that the phage lyzates or purified phage preparations did not significantly change the degree of lymphocyte proliferation
induced by the optimal dose of ConA (2.5 µg/ml). Assuming that the preparations could provide costimulatory signals in mitogen-induced T-cell proliferation, we applied significantly lower (0.25 and 0.5 µg/ml) doses of ConA. Effects of the addition of a crude phage lyzate and the purified preparations (Fig. 1) on splenocyte proliferation induced by suboptimal doses of ConA are shown in Fig. 2A, B (two selected experiments).

Fig. 2. The effects of phage preparations on splenocyte proliferation induced by a suboptimal (0.25 µg/ml) ConA dose. The data are presented as mean OD values from triplicate wells ± standard error. ◼ crude lyzate; ■ purified phage fraction; ----- broth control; --- phosphate buffer control. The asterisks indicate the significance between OD in respective phage dilutions as compared with controls (broth or buffer). *p<0.05, **p<0.02, ***p< 0.01, ****p< 0.001

In these experiments, the stimulation indices varied from 1.13 to 3.35 with the application of 0.25 µg/ml ConA and from 1.70 to 4.94 when 0.5 µg/ml ConA was used. At 0.25 µg/ml ConA, the crude lyzates did not affect the proliferative response of the control cultures, whereas the purified preparation significantly enhanced the control response at all dilutions. At 0.5 µg/ml ConA, though the crude lyzates were stimulatory in some experiments, a better stimulation was achieved with the purified phage fraction (data not shown).

The regulatory activity of the phage preparations in the splenocyte proliferation induced by the optimal dose of ConA

Quite a different action of the phage preparation on splenocyte proliferation was observed when the optimal dose of ConA (2.5 µg/ml) was used, resulting in stimulation indices ranging from 5.1 to 13.3 (results of representative experiments shown in Fig. 3A-C). The crude phage lyzate was only stimulatory in one experiment (Fig. 3B). The effects of the purified phage preparation, were, on the other hand, neutral (Fig. 3A), stimulatory (Fig. 3B), or regulatory (Fig.
3C). In another series of experiments, we also found that the phage preparation stimulated lipopolysaccharide-induced splenocyte proliferation, involving B cells (data not presented).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 3. The effects of phage preparations on splenocyte proliferation induced by the optimal (2.5 µg/ml) ConA dose. The data are presented as mean OD values from triplicate wells ± standard error. □ crude lyzate, ■ purified phage fraction, − − − − broth control, − − phosphate buffer control. The asterisks indicate the significance between OD in respective phage dilutions as compared with controls (broth or buffer). *p<0.05, ** p<0.02, *** p< 0.01, **** p<0.001

The purified phage preparation is able to induce IL-6 production in unstimulated splenocyte and peritoneal macrophage cultures and to enhance ConA-induced cytokine production

The costimulatory signals in mitogen-induced T-cell proliferation have been identified as cytokines provided by accessory cells [21, 22, 23]. IL-6 is regarded
as the most important cytokine in this process. Therefore, we decided to check whether the phages could induce IL-6 production in unstimulated splenocyte cultures. Fig. 4A, B shows that the purified phage preparation was able to induce significant IL-6 activity in a dose-dependent manner. The phages also substantially elevated ConA-induced IL-6 production. The phage preparations produced similar results in 3 independent experiments (not shown).

Fig. 4. The induction of interleukin 6 activity in whole splenocyte cultures by a purified phage preparation. The data are expressed as IL-6 levels, expressed in pg/ml. A – unstimulated splenocyte cultures; B – splenocyte cultures induced with 0.5 µg/ml of ConA. ■ purified phage fraction; — phosphate buffer

Fig. 5. The induction of interleukin 6 activity in nonadherent and adherent splenocyte cultures by a purified phage preparation. The data are expressed as IL-6 levels, expressed in pg/ml. A – nonadherent cells; B – adherent cells. Presentation of data as in Fig. 4.
In order to identify the cell types which are the targets of the phage action and the source of IL-6, we removed adherent cells as described in the Materials and Methods. Fig. 4 shows that the ability of the purified phage fraction to induce IL-6 in adherent splenocytes was much higher than that in the cell population deprived of adherent cells. In addition, the ability of the phage preparation to elevate IL-6 activity in the peritoneal macrophage cultures, above the values seen in unstimulated cultures was also strong (4x, 2.5x and 2x at the preparation dilution: 16, 64 and 256, respectively) – data not shown.

**The membrane-separated phage fraction induces IL-6 production in rat macrophages**

The phage fraction was prepared by the alternative method, as described above, to eliminate the possibility of contamination with bacterial products. Tab. 1 gives the details of the IL-6 producion induction ability of the crude A20/R phage lyzate, the filtrate (possibly containing bacterial products) and the membrane-separated A5 phage fraction in 24 h cultures of rat peritoneal macrophages. The spontaneous IL-6 production was 1065 pg/ml and the LPS-induced IL-6 level was 3558 pg/ml. The results show that the purified phage fraction, at the dilution 248-8192, markedly enhanced spontaneous IL-6 production (almost 2-fold). High stimulatory activity was also exhibited by the filtrate at 128 and 9192 dilution. The crude lyzate was moderately stimulatory.

Tab. 1. The levels of IL-6 induced by purified A20/R bacteriophage fraction in the rat peritoneal cell cultures.

<table>
<thead>
<tr>
<th>Supernatant dilution</th>
<th>IL-6 (pg/ml)</th>
<th>A20/R lysate (5 x 10⁸ phage particles per culture)</th>
<th>A20/R filtrate</th>
<th>A20/R phage (5 x 10⁸ phage particles per culture)</th>
</tr>
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<tbody>
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<td>1383</td>
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controls: medium only – 1065, 5 µg LPS/ml – 3558 pg/ml

**DISCUSSION**

In this report, we showed that a phage preparation (No A20/R) used for the therapy of infections can deliver costimulatory signals in the mitogen-induced splenocyte proliferation in the mouse model. The phages strongly stimulated ConA-induced proliferation at suboptimal doses of the mitogen and regulated cell proliferation at the optimal dose of ConA. The demonstration that phages
can induce IL-6 production in the splenocyte cultures indicates a possible mechanism of this costimulatory action.

Our finding that phage preparations stimulate spleen cell activity confirms previous data from studies using other models [12, 13], and supports the notion that both T and B cells are involved in in vitro stimulation with phages [12]. Our results are, however, contradictory to the demonstration that phages suppress DNA synthesis [11].

A report suggesting that bacteriophages may encode products resembling proteins involved in signal transduction in eukariotic cells is very relevant to this study [14]. The authors showed that the structure of the N-terminal domain of the g 3p-D1 protein from the bacteriophage fd was similar to the DTB and PD2 domains involved in signal transduction of eukariotic cells. In another study [15], a phage 933W gene stk encoded a product bearing similarity to eukariotic serine and threonine protein kinases. The authors suggested that such a product could interfere with the signal transduction of the mammalian host. The above-cited studies provide a possible explanation for the observed regulatory effects of phages in lymphocytes.

In another investigation [13], some phage clones and their products were shown to costimulate the proliferative response of splenocytes derived from mice sensitized with a wild-type phage. These phages or their products (peptides) mimicked the CTLA-4 binding domain. The authors hypothesized that the peptides, by a selective block of the negative signal delivered by the CTLA-4/CD80 interaction, could facilitate a positive signal delivered through CD28/CD86 interaction. However, although the molecular relationship between the phage product and the CTLA-4 binding domain was identified, the mechanism of the costimulatory effect was purely hypothetical. In any case, the costimulatory activity of phage products in the model described was only demonstrated in the phage-specific immune response. Our results, in turn, revealed the costimulatory activity of phages in mitogen-induced splenocyte proliferation.

The results of our study represent a more definite step towards an explanation of the costimulatory activity of phages. We have provided evidence that phages can induce the production of IL-6 in splenocyte cultures. IL-6 is regarded as a main costimulatory signal in the process of the mitogen-induced proliferation of T cells [21, 22]. Other cytokines, such as IL-1 [22] and TNF-α [23], also contribute to this process. In fact, we also identified induction of TNF-α in splenocyte cultures (not shown here). More importantly, phage preparations enhanced IL-6 production induced by suboptimal doses of ConA; this effect directly correlated with its costimulatory activity in the proliferation assay. Interestingly, the phages induced cytokine synthesis in resting cells, though it is not known whether this was associated with the entering of phages into the cells. On the other hand, early studies showed that phages preferentially interacted with malignant or quickly dividing cells [9, 10].
It seems unlikely that bacterial products were responsible for the described phenomena for the following reasons: 1) the activity of crude phage lyzates was much weaker or absent; 2) the cytokine-inducing activity of Gram+ bacterial products is several times weaker than that of lipopolysaccharide [24]; and 3) bacterial impurities (components) should be lost during the purification procedure (Fig. 1). In addition, phages separated on membranes and washed also induced IL-6 production in peritoneal cell cultures. Moreover, the above-cited literature data support the notion that purified phages may be solely responsible for signal transduction in eukariotic cells. However, careful biochemical analysis of the phage preparation will be required to exclude the possibility that traces of bacterial product may be responsible for cell stimulation.

The regulatory nature of phages is reflected by the fact that the optimal proliferation of the splenocytes was subjected to only limited changes or was not affected at all by phages. On the other hand, the proliferative response of lymphocytes to suboptimal ConA concentrations was significantly upregulated. These findings may, therefore, provide some additional explanation with regard to the regulatory effects on several immunological parameters seen in patients treated orally with phage lyzates. We recently showed that the ability to produce spontaneous and mitogen-induced TNF-α in peripheral blood mononuclear cell cultures was particularly regulated following phage treatment [21]. In addition, other indices, such as the phagocytosis ability and the levels of endogenous lactoferrin in patients’ plasma, were also regulated (to be published).

In conclusion, this study represents an important step in the clarification of phage-eukariotic cell interaction. In addition, it suggests that the therapeutic action of phage preparations not only results from their direct bacteriolytic property, but it also appears that phages may stimulate cells of the immune system, as reflected by cytokine production and an increased ability to respond to mitogens.

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REFERENCES


