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Anticancer Activity of Bacteriophage T4 and its Mutant HAP1 in Mouse Experimental Tumour Models

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Abstract. *Background: Previously, we have shown the ability of the bacteriophage T4 and its substrain HAP1 (selected for a higher affinity to melanoma cells) to reveal antimetastatic activity in a mouse melanoma model. Here, we investigated the potential phage anticancer activity in primary tumour models. Materials and Methods: Mice were inoculated subcutaneously with B16 or LLC cells (collected from in vitro culture). Bacteriophages T4 and HAP1 were injected intraperitoneally daily ($8x10^8$pfu/mouse, except the experiment concerning the dose-dependence). Results: Treatment with purified preparations of bacteriophage T4 resulted in significant reduction of tumour size, the effect being dose-dependent. HAP1 was more effective than T4 and its activity was also dose-dependent. Parallel experiments with non-purified bacteriophage lysates resulted in significant stimulation of tumour growth. Conclusion: These data suggest that purified bacteriophages may inhibit tumour growth, a phenomenon with potentially important clinical implications in oncology.*

Bacteriophages are viruses that infect, multiply within, and cause lysis of bacterial cells. They are known to be effective in the treatment of bacterial infections, especially those resistant to antibiotics (1). Nevertheless, the biology of bacteriophages seems to be more complicated. Previously, we have shown the ability of bacteriophage T4 and its substrain HAP1 (selected for a higher affinity to melanoma cells) to bind to cancer cells and to reveal antimetastatic activity in a mouse B16 melanoma model (experimental metastasis model - intravenous injection of tumour cells). Both bacteriophages significantly reduced the number of murine melanoma metastases in the lung. This effect was significantly stronger for HAP1 compared to the parental strain. We also observed binding of bacteriophages to cancer cells membrane, in confocal and electron microscopy. This binding was inhibited by β3 integrins ligands and anti-β3 antibodies. Therefore, we proposed a molecular mechanism of phage antimetastatic activity based on interactions of T4 capsid proteins with β3 integrins on target cells. The probable T4 phage proteins that interact with β3 integrins are gp24 that contain KGD-aminoacid motifs, *i.e.* RGD homologs, able to bind β3 receptors. Ligands binding these cell targets are able to block the integrin functions and to diminish tumorigenicity of the cells (2-4).

With these data in mind, we decided to investigate potential phage anticancer activity in primary tumour models. Here, we describe this activity, its dose-dependence, and the role of bacterial residues in the observed effects.

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Materials and Methods

Bacteriophages. The T4 phage was purchased from ATCC (Rockville, Maryland, USA). The HAP1 (T4 substrain with a high affinity to melanoma cells) was selected in our Institute (Institute of Immunology and Experimental Therapy, IIET, Wroclaw, Poland) (2). The material applied was either the lysate: the culture with *Escherichia coli B* (IIET Collection of Microorganisms), filtered through Millipore 0.22-mm filters, or highly purified preparations. Usually, T4 bacteriophage cultures from *Escherichia coli* contain 2000-2500 U/ml endotoxin. Bacteriophages T4 and HAP1 were purified by filtration through polysulfone membranes and by two chromatographic techniques: gel filtration on Sepharose 4B followed by cellulofine sulfate (Millipore, Billerica, USA) chromatography (5). The purification procedure afforded preparations of phages containing 3-7 U/ml endotoxin for 10^9pfu/ml, as determined by chromogenic Limulus Amebocyte Lysate (QLC-1000 Chromogenic Endpoint LAL, Bio Whittaker, Rockland, USA).

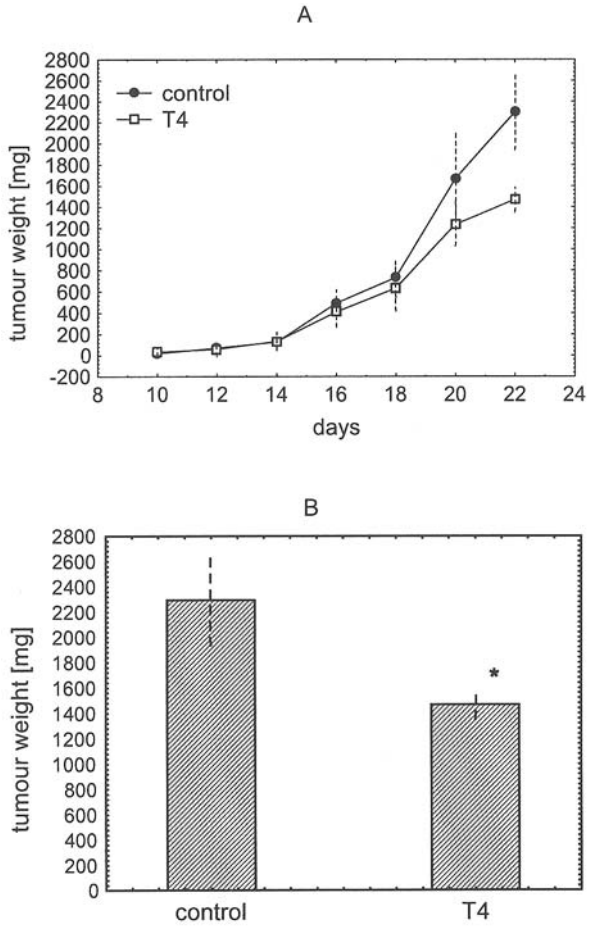

Figure 1. *Antitumour activity of bacteriophage T4 in mouse B16 melanoma model. Mice were inoculated subcutaneously with 3x10^5 B16 cells, collected from in vitro culture, in 0.2 ml of physiological saline; purified bacteriophages (T4) were injected intraperitoneally every consecutive day, the concentration of bacteriophages was 8x10^8/mouse. Control mice were injected intraperitoneally with PBS every consecutive day (control). (A) Kinetics of tumour growth, (B) mean tumour weight at day 22.*
** – statistically significant as compared to control (Student's t-test, p<0.05).*

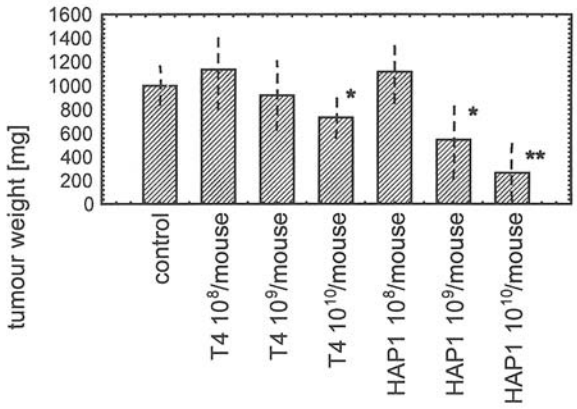

Figure 2. *Antitumour activity of bacteriophage T4 and HAP1 in mouse B16 melanoma model, dose-dependence of the effect. Mice were inoculated subcutaneously with 3x10^5 B16 cells, collected from in vitro culture, in 0.2 ml of physiological saline; purified bacteriophages (T4, HAP1) were injected intraperitoneally every consecutive day. Control mice were injected intraperitoneally with PBS every consecutive day (control). Mean tumour weight at day 16.*
** – statistically significant as compared to control (Student's t-test, p<0.05)*
*** – statistically significant as compared to control and to the T4 10^{10}pfu/mouse group (Student's t-test, p<0.05)*

Phage concentration in the lysates and purified preparations was measured by the two-layer method of Adams (6). The *Escherichia coli B* culture disrupted by ultrasound (control for bacteriophage lysates) was filtered through Millipore 0.22-mm filters and the LPS concentration was comparable to the lysates of phages (measured by Gas Chromatography Mass Spectrometry and chromogenic Limulus Amebocyte Lysate, QLC-1000 Chromogenic Endpoint LAL, Bio Whittaker).

Tumour cells. The B16 mouse melanoma cancer cell line was obtained from ATCC, while the mouse Lewis lung carcinoma line LLC was received as a gift from Dr. I. Wodinsky, National Cancer Institute, Bethesda, USA. Both lines are maintained in the Cell Culture Collection of the IIET.

In vivo anticancer assay. Six- to twelve-week-old C57Bl/6/IiW female mice were bred in the Animal Breeding Centre of the IIET and kept in standard minimal disease (MD) conditions.

All experiments were performed according to *Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education* issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

Mice were inoculated subcutaneously (*s.c.*) with 3x10^5 B16 cells or 2x10^5 LLC cells (collected from *in vitro* culture) in 0.2 ml of PBS. When tumours became palpable, their maximum length and width were measured and the tumour weights, calculated as a^2 x b/2 (a = shorter diameter, b = longer diameter), were determined until completion of the study. Tumour growth inhibition (TGI) was calculated taking values of control (injected with PBS) as 100%. The experiments were terminated 21 days after tumour cells inoculation. Bacteriophages were injected intraperitoneally (*i.p.*) daily, the first dose being applied 1h before the inoculation with cancer cells. Apart from the experiment concerning the dose-dependence of the investigated effects, the concentration of bacteriophages was 8x10^8/mouse.

Statistical methods. The Student's *t*-test and the Statistica 5.0 and Statistica 6.0 software packages were applied.

Results

Anticancer activity of purified bacteriophage T4. Daily treatment of mice bearing subcutaneous melanoma tumour

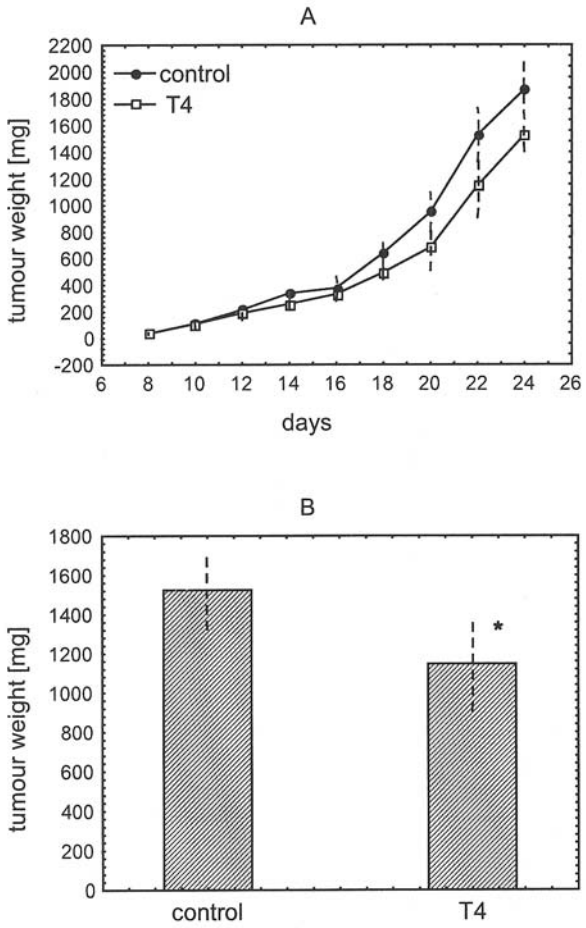

Figure 3. *Antitumour activity of bacteriophage T4 in mouse lung carcinoma (LLC) model. Mice were inoculated subcutaneously with 2x10^5 LLC cells, collected from in vitro culture, in 0.2 ml of physiological saline; purified bacteriophages (T4) were injected intraperitoneally every consecutive day, the concentration of bacteriophages was 8x10^8/mouse. Control mice were injected intraperitoneally with PBS every consecutive day (control). (A) Kinetics of tumour growth, (B) mean tumour weight at day 22.*

** – statistically significant as compared to control (Student's t-test, $p<0.05$)*

with purified preparations of bacteriophage T4 resulted in significant reduction of tumour size. The differences between the average weight of tumour in phage-treated and control mice increased during the experiment, reaching 36.1% (in comparison to the control) on day 22 (Figure 1). Afterwards, we investigated the dose-dependence of T4 treatment effects. In these experiments, we applied the highest technically available concentrations of purified bacteriophages. The results indicate that the effect of treatment with T4 is dose-dependent. At the 16th day of experiment, the tumours weight in mice was decreased by 26.5% (as compared to control) after the treatment with 10^{10}pfu/mouse, 8% after treatment with 10^9pfu/mouse, while the treatment with 10^8pfu/mouse was ineffective (Figure 2, bars 1-4). Also, treatment with lower (10^5-10^7) bacteriophages concentrations revealed no significant effect (data not shown).

Bacteriophage T4 was also shown to be effective in the treatment of mice bearing subcutaneous lung carcinoma. Significant inhibition of tumour growth was observed. The reduction of tumour weight reached 24.8% (in comparison to the control) on day 22 (Figure 3).

HAP1- the highly anticancer active mutant of T4 phage. In this work, we also investigated the activity of HAP1 bacteriophage, which is a substrain of T4, selected for its higher (than T4) affinity to B16 melanoma cells and which was previously demonstrated to have significantly stronger (than T4) antimetastatic activity (2, 3). The results clearly indicate that HAP1 is more effective in the treatment of mice bearing melanoma tumours than T4 and that its activity is also dose-dependent (Figure 2). On the 16th day of experiment, tumours volume in mice treated with 10^{10}pfu/mouse of HAP1 was decreased by 73%. The difference was significant not only in comparison to the control mice, but also to mice treated with the same dose of T4 phage (26.5%). HAP1 was also more effective in the concentration 10^9pfu/mose: 45% of inhibition *versus* 8% for T4. Interestingly, both T4 and HAP1 were ineffective at the dose of 10^8pfu/mouse (Figure 2).

Bacteriophage lysates stimulate tumour growth. Parallel experiments were conducted with non-purified (raw) bacteriophage lysates. These were previously shown to have substantial antimetastatic activity (2, 3).

Daily treatment of subcutaneous melanoma tumour with bacteriophage lysates resulted in significant stimulation of tumour growth. On day 19, tumours in mice treated with lysates were 2.3(HAP1) and 3.1(T4) times larger than that in control mice (Figure 4). In the experiments with non-purified lysates of bacteriophages, we included an additional control group of animals. Mice in this group were treated with the *Escherichia coli B* culture disrupted by ultrasound (with no bacteriophages) in which the LPS concentration was comparable to the phage lysates. This was aimed at elucidating the role of bacterial impurities in the activity of bacteriophage lysates. This *E. coli* preparation also revealed strong stimulative activity: tumours were 2.9 times bigger than in the control group. All differences were statistically significant (Figure 4).

Similar results were obtained in a lung cancer model. Tumour stimulation achieved 45% (T4), 51% (HAP1) and 44% (*E. coli* preparation) on the 17th day of the treatment, although the differences were not statistically significant when compared with control (Figure 5).

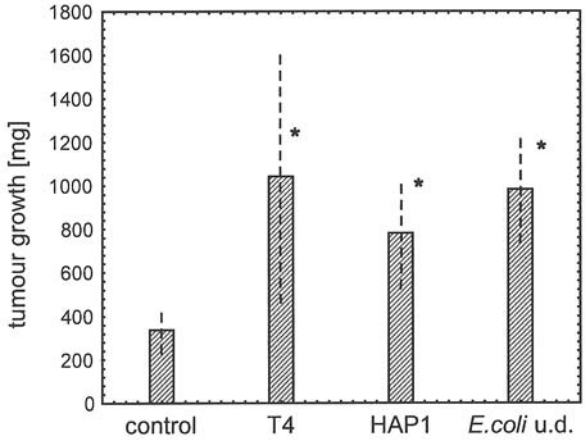

Figure 4. *Stimulation of primary tumour growth by non-purified bacteriophage lysates in mouse melanoma B16 model. Mice were inoculated subcutaneously with 3x10^5 B16 cells, collected from in vitro culture, in 0.2 ml of physiological saline; bacteriophages as raw lysates (T4, HAP1) and Escherichia coli culture disrupted with ultrasounds (E.coli u.d.) were injected intraperitoneally every consecutive day, the concentration of bacteriophages was 8x10^8/mouse. Control mice were injected intraperitoneally with PBS every consecutive day (control). Mean tumour weight at day 19.*
** – statistically significant as compared to control (Student's t-test, p<0.05)*

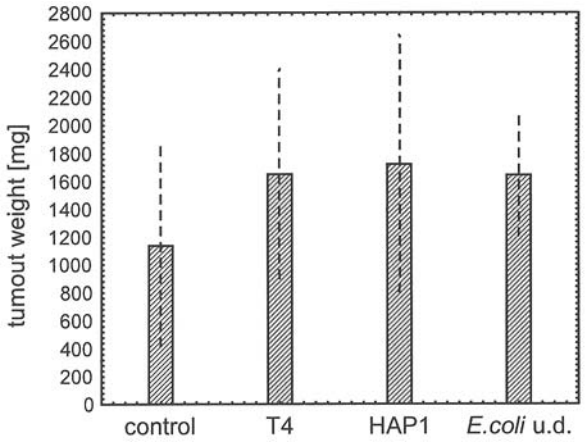

Figure 5. *Stimulation of primary tumour growth by non-purified bacteriophage lysates in mouse lung carcinoma (LLC) model. Mice were inoculated subcutaneously with 2x10^5 LLC cells, collected from in vitro culture, in 0.2 ml of physiological saline; bacteriophages as raw lysates (T4, HAP1) and Escherichia coli culture disrupted with ultrasounds (E.coli u.d.) were injected intraperitoneally every consecutive day, the concentration of bacteriophages was 8x10^8/mouse. Control mice were injected intraperitoneally with PBS every consecutive day (control). Mean tumour weight at day 17.*

Discussion

The presented results demonstrate the antitumour activity of bacteriophages. Bacteriophage T4 was effective in two murine tumour models: melanoma and lung cancer. The anticancer effect was much stronger for the mutant HAP1 than for the parental strain T4. These results correlate with our previous data showing antimetastatic activity of both bacteriophages and a significant advantage of HAP1 (2, 3). We also showed the dose-dependence of the antitumour effect of the phages. It should be stressed that the purification procedure limits the maximum technically available dose of purified bacteriophages to 10^{10}/pfu/mouse. As the effect of HAP1 is strong (73% of tumour weight reduction), we think that higher concentrations should be obtained and applied in further investigations. This indicates the importance of further improvement in the purification process in order to obtain higher, potentially more effective, doses of bacteriophages.

The importance of the purification of bacteriophages is evident when the results are compared with those obtained with non-purified bacteriophage lysates. Our lysates stimulated solid tumour growth even 3 times over the control. It is known that LPS can stimulate tumour growth (7, 8). Given also the results of the treatment with purified phage preparations and with *E. coli* cultures (disrupted with ultrasounds), we conclude that the stimulation effect of phage lysates is due to the activity of bacterial impurities and not the bacteriophages themselves. This should certainly be considered in any studies on bacteriophage treatment and stimulate extensive studies on the biotechnology of phage production and purification.

Previously, we described binding of bacteriophages to cancer cells and presented a hypothesis of its molecular basis: interactions of bacteriophage proteins with cellular receptors. We also concluded that these interactions cause the antimetastatic activity of bacteriophages (2, 4).

We believe that these observations are of great interest and importance for potential clinical applications of bacteriophages in that bacteriophages and/or phage capsid proteins should be regarded as potential anticancer and cancer-prevention modalities.

Acknowledgements

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References

1 Stone R: Stalin's forgotten cure. Science *298*: 728-731, 2002.

2 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, Weber-Dabrowska B, Rybka J, Salwa J, Wojdat E, Nowaczyk M and Gorski A: Antitumour activity of bacteriophages in murine experimental cancer models caused possibly by inhibition of β3 integrin signaling pathway. Acta Virol *48*: 237-244, 2004.

3 Gorski A, Dabrowska K, Wietrzyk J, Nasulewicz A and Opolski A: Bacteriophage inhibits metastasis in mouse transplantable melanoma model. American Association for Cancer Research: An AACR Special Conference in Cancer Research "Proteases, Extracellular Matrix, and Cancer" 9-13.10.2002 Hilton Head Island, USA, Conference Proceedings: Abstracts of Poster Presentation n. B33.

4 Gorski A, Dabrowska K, Switala-Jelen K, Nowaczyk M, Weber-Dabrowska B, Boratynski J, Wietrzyk J and Opolski A: New insights into the possible role of bacteriophages in host defence and disease. Med Immunol *2*: 2, 2003.

5 Boratynski J, Syper D, Weber-Dabrowska B, Lusiak- Szelachowska M, Pozniak G and Gorski A: Preparation of endotoxin-free bacteriophages. Cell Mol Biol Lett *9*: 253-259, 2004.

6 Adams MH: Bacteriophages. New York, Inter Science Publ 1959.

7 Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, Bouchier-Hayes D and Dong Z: Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. Int J Cancer *10*: 415-422, 2002.

8 Wang JH, Manning BJ, Wu QD, Blankson S, Bouchier-Hayes D and Redmond HP: Endotoxin/lipopolysaccharide activates NF-kappa B and enhances tumor cell adhesion and invasion through a beta1 integrin-dependent mechanism. J Immunol *170*: 795-804, 2003.

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