DOI 10.1007/s10517-018-3986-0

Evaluation of the Efficiency of Lytic Mycobacteriophage D29 on the Model of *M. tuberculosis*-Infected Macrophage RAW 264 Cell Line

M. B. Lapenkova[1], N. S. Smirnova[1], P. N. Rutkevich[2], and M. A. Vladimirsky[1]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 164, No. 9, pp. 326-329, September, 2017
Original article submitted April 26, 2017

Culture of mouse macrophages (RAW 264.7 ATCC strain) in wells of a 6-well plate was infected with *M. tuberculosis* in proportion of 15 mycobacteria per one macrophage and then treated with a lytic strain of mycobacteriophage D29. Antibacterial efficacy of mycobacteriophages was studied using D29 phage (activity 10^8 plaque-forming units/ml) previously purified by ion exchange chromatography. After single and double 24-h treatment, the lysed cultures of macrophages were inoculated onto Middlebrook 7H10 agar medium. The number of mycobacterial colonies in control and test wells (at least 3 wells in each group) was 300.178±12.500 and 36.0±5.4, respectively (p<0.01).

Key Words: *mycobacterium tuberculosis; mycobacteriophages; macrophages; cell culture; phage DNA*

Intensive spreading of drug-resistant tuberculosis and isolation of *Mycobacterium tuberculosis* (MBT) resistant to the main first line drugs isoniazid and rifampicin (multidrug-resistant strains) and to the second and third line drugs (extensively drug-resistant strains) attracted much interest to mycobacteriophages. This interest, in addition to fundamental studies of the evolution and diversity of a large number of different types of mycobacteriophages with the possibility of their DNA sequencing [3,5], is also based on the possibilities of therapeutic application of mycobacteriophages with lytic properties for virulent MTB species against tuberculosis, in particular, for the development of rapid phenotypic methods for determining the drug sensitivity of MBT, as well as for the search for new ways of treating or preventing active forms of tuberculosis.

In this context, the most studied are virulent mycobacteriophages TM4 and D29 with double-stranded DNA (~50,000 nucleotides) infecting both fast-growing non-tuberculosis species and slow growing species of mycobacteria [13]. The ability of phage D29, in contrast to TM4, to penetrate into macrophages and kill MBT that infected macrophages [11] was explained by the presence of protein receptors of phage D29 that recognize macrophages, thus accelerating phagocytosis of mycobacteriophages.

Mycobacteriophage D29 is rapidly reproduced in non-tuberculosis fast-growing mycobacteria, especially in *M. smegmatis* mc^2 155, and can accumulate in these cultures attaining high titers (up to $10^{11—12}$ plaque-forming units per 1 ml in *M. smegmatis* culture). In some studies, this phage was used to develop phenotypic methods for rapid detection of drug sensitivity of MBT [2,12,13], *e.g.* by quantitative assay of phage DNA in culture samples after 2-day incubation with an antibiotic. Inhibition of MBT metabolism correlated with a decrease in the content of phage DNA [12].

Lytic activity of phage D29 is determined by two kinds of endolysins (lysin A and B) that destroy the thick lipid membrane of mycobacteria and also by transmembrane proteolytic protein choline that perforates the cytoplasmic membrane [6,9,10]. These phage

[1]Research Institute of Phthisiopulmonology, I. M. Sechenov First Moscow State Medical University, Ministry of Health of the Russian Federation; [2]Research Institute of Experimental Cardiology, Russian Cardiology Research and Production Complex, Ministry of Health of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** mvladimirskij@mail.ru. M. A. Vladimirsky

products destroy the cell membrane and provide the release of the mycobacteriophage particles. Lytic activity of mycobacteriophage D29 against replicating mycobacteria manifested rapidly, because doubling time of phage particles is measured in minutes, which leads to effective lysis of mycobacterial cell membranes by the complex of endolysins. However, the main problem is the access of mycobacteriophages into zones of MBT reproduction surrounded by activated macrophages whose enzyme systems can eliminate phage particles. In light of this, study of the activity of phage D29 against MBT that reproduced within macrophages seems to be an adequate experimental model.

Here we studied the efficacy of mycobacteriophage D29 in inhibiting MBT reproduction in cultured macrophage of RAW 264.7 line.

MATERIALS AND METHODS

Culture of mouse macrophages RAW 264.7 ATCC from the bank of cell cultures was used as the model. The cells after thawing were inoculated into 75 cm^2-flasks in RPMI-1640 medium (10% fetal calf serum, 2 mM L-glutamine, PenStrep, MEM, MEM Vitamins, 1 mM sodium pyruvate) and cultured in a CO_2 incubator at 5% CO_2 at 37°C. After short-term adaptation and pre-cultivation for 7 days, the macrophage cells were counted with a Scepter cell counter and inoculated into wells of a 6-well Eppendorf plate in 500 µl (1.5-1.7×10^5/ml). After 24-h incubation, the cells occupy most of the well surface were infected with multiplying MBT of virulent strain H37Rv; the number of microbial bodies was preliminarily determined by quantitative analysis of MBT DNA with a single-copy *RegX3* gene at a ratio of 15 mycobacteria per macrophage.

Mycobacteriophage D29 was previously expanded in *M. smegmatis* culture and its biological activity was evaluated by the number of plaque-forming units (lysis zones) on plates with *M. smegmatis*. Then, mycobacteriophage was purified from the mycobacteria lysate that consisted of lipases, proteases, and fragments of DNA molecules by IEC-HPLC on a DEAE-cellulose column [1]. The phage binds to the column at low ionic strength (0.1 M NaCl) and is eluted after increasing NaCl concentration to 1 M. The mycobacteriophage preparation was dialysed against phage buffer (in mM: 10 Tris (pH 7.5), 1 $CaCl_2$, 70 NaCl), sterilized by filtration (Millex GP 0.22 µM; Millipore), and stored at 4°C. Biological activity of the phage preparation remained unchanged for a long time (at least 6 months).

On the next day after infection, the wells with cultured macrophages were washed once with RPMI-1640 medium and 100 µl of mycobacteriophage D29 with biological activity 10^8 plaque-forming units per 1 ml phage buffer was added to the test wells (3 wells for each group). In 24 h, the medium was changed and mycobacteriophage preparation was repeatedly added to some experimental wells for 24 h; then, the medium was changed. In control wells infected with MBT, the medium was replaced, but mycobacteriophage preparation was not added.

After 2-3-day incubation of macrophages, the cells in experimental and control wells were scraped from the surface in a volume of 200 µl and twice frozen at -20°C and thawed at 37°C; a 100-µl aliquot was seeded in plates with Middlebrook 7H10 agar and cultured in CO_2 incubator for 3 weeks; the number of MBT colonies was determined. The rest portion (100 µl) was used for isolation and quantitative analysis of DNA MBT with measuring of single copy fragments of *RegX3* gene (Amplitub-RV test-system). Quantitative analysis of phage D29 DNA was performed by RT-PCR using a calibration curve constructed by titration of phage D29 plasmid DNA. The kits for quantitative analysis of phage DNA were provided by Syntol company.

RESULTS

The preparation of mycobacteriophage D29 after chromatographic purification, in contrast to crude phage lysate produced no toxic effect on macrophage RAW 264.7 culture. The amount of phage DNA in wells with MBT-infected macrophages at single and double application of the mycobacteriophage was 4.3×10^6 and 1.36×10^7 phage DNA molecules, respectively. Thus, the content of phage DNA in wells after double application was 3-fold higher.

The number of MBT colonies in control plates (without phage application) was about 300 in each plate. In plates with phage-infected MBT, the number of colonies was 178.0±12.5 (single application) and 36.0±5.4 (double application; $p<0.01$) (Fig. 1).

Despite very low efficiency of penetration of mycobacteriophage D29 into cultured non-infected macrophages (~0.1% according to preliminary experiments), it produced a significant bactericidal effect after 24-h incubation with MBT-infected macrophages that depended on the proportion of administered phage particles. Hence, the MBT-inhibiting effect of mycobacteriophage correlated with intracellular content of phage particles that multiply against the background of metabolic activity of MBT.

The use of bacteriophages for therapeutic purposes in tuberculosis under conditions of global spread of drug resistance to antibacterial drugs is associated with great problems: sequestration of phages in the liver and spleen and poor accessibility of infected tissues, zones of specific inflammation, for phage particles. Nevertheless, this possibility is discussed in the

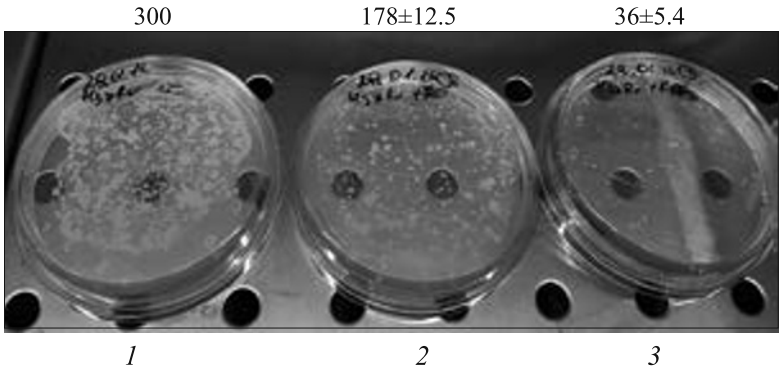

Fig. 1. Bactericidal effect of D29 phage. *1*) Control (MBT), *2*) MBT+D29 phage, once, *3*) MBT+D29 phage, twice.

context of selective transport of phage and also as a method of analysis of metabolism of clinical MBT cultures during antibacterial therapy of tuberculosis [15].

It was previously shown [14], that the intensity of phage D29 phagocytosis in MBT-infected macrophages was higher than in non-infected cells. The number of D29 phages in macrophages dynamically increased within 1 h, after which most of the phage particles were eliminated by macrophages. It should be also noted, that the immune function of macrophages assessed by induction of NO and IL-12 production remained changed.

The improvement of antimycobacterial efficacy of lytic mycobacteriophage depends on the development of means for delivery or penetration of phage particles into macrophages, including macrophages in the inflammatory infiltrate.

Aerosol therapy [7] and the use of large liposomes for mycobacteriophage delivery to zones of granulomatous inflammation [8] are most promising and actively developed methods. A very interesting approach is construction of a virulent recombinant mycobacteriophage against MBT capable of producing resuscitation promoting factor (Rpf) in the host cells (proteins of mycobacteria that overcome the state of bacterial persistence), which will increase the effectiveness of antibacterial therapy of tuberculosis [4].

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