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ANTITUMOR AND ADJUVANT EFFECTS OF PHAGELYSATES OF *E.COLI* IN MICE WITH EHRLICH CARCINOMA

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***Aim*: To augment anti-tumor host response and overcome the tumor-induced immunosuppression is of paramount importance especially when patient is subjected to radio-/chemotherapy and immune system suffers significantly. Various immunological methods have been employed as supplemental antitumor therapies. We were aimed to investigate the antitumor potential of phagelysates of gram-negative bacteria and their adjuvant effects for conventional chemotherapy in experiment. *Methods*: Bacterial phagelysates of *E.coli* and purified suspensions of corresponding Un bacteriophage were obtained by standard methods of phage research. Experiments were carried out on BL57C/6J mice bearing transplanted Ehrlich carcinoma. Different regimens of phagelysate administration (0,5 ml *E. coli* phagelysate, 3/8 times with 5 day intervals) and conventional chemotherapy (combination of Doxorubicin 60 mg/m^2, Cyclophosphan 800 mg/m^2, Ftoruracil 600 mg/m^2, 3 times with 21 day intervals) were tested. Treatment efficacy was evaluated by tumor growth inhibition percent, index of malignant growth, lifespan and survival percent. *Results*: Experiments have shown that application of optimal doses of *E. coli* phagelysate can be well tolerated in mice. No stimulation or support of malignant growth was observed. *E. coli* phagelysate exhibited significant anticancer effect and adjuvant efficacy. Cancer development was delayed in 65% of inoculated animals in the test group. *E. coli* phagelysate inhibited tumor growth by 80–90% without apparent side effects. The mice survival was prolonged twice and more. On 65th-69th days of tumor growth in 13% animals complete regression of neoplasms was registered. Application of phagelysates in combination with chemotherapy significantly increased antitumor efficacy of conventional chemotherapeutic drugs. *Conclusion*: Application of bacterial phagelysates can be considered as promising novel strategy in cancer therapeutics.**

***Key Words*: cancer, immunotherapy, phagelysates, *E. coli*, chemotherapy.**

World Cancer Report provides clear evidence that cancer incidence rates could further increase by 50% to 15 million new cases in 2020. Contemporary treatment of malignant tumors is mainly through surgical intervention, chemo- and radiotherapy, but side effects of conventional chemo- and radiotherapy such as myelodepression, nephro- and hepatotoxicity decreases efficacy of treatment and as a consequence, prognosis is poor [1, 2]. Chemo- and radiotherapy-induced immunosuppression is extremely dangerous in cancer patients, because cancer growth in turn creates immunosuppressive environment. Progressing tumor uses the immunosuppressive cells, such as M2-regulatory macrophages, mast cells, myeloid-derived suppressor cells (MDSC), CD8 T-suppressor, T-reg lymphocytes and suppressive cytokines (IL-10 and TGF-beta) promoting the malignant growth [3–5]. At the same time immune system of organism is severely affected that gives rise to infections, reactivation of existing focal inflammations and decrease in quantitative and functional indices of cellular immunity (T cells, NK cells, monocyte/macrophages, LAK cells etc.). Thus, to augment the host response against malignant growth is of paramount importance especially when patient is subjected to radio- or chemotherapy.

During the last decade, immunological methods recruiting the immune system to activate the natural defenses of the organism have been employed as supplemental antitumor therapies. Vaccination is one of the most prospective ways to prevent and control several diseases and there is still a great need to develop a new generation of safe vaccines that can be efficiently administered by simple and economical immunization procedures [6, 7]. Application of bacterial preparations for stimulation of organism's nonspecific immune reaction is a promising and cost-effective way of immunotherapy. One of the earliest approaches for the immunotherapy against malignant tumors was implemented by W. Coley [8] and after that there were a numbers of attempts of nonspecific stimulation of immune system for cancer treatment. However, significant results were obtained only in the last decade by application of immune modulators such as bacteria, or bacterial components (Bacillus Calmette-Guerin — BCG), also synthetic preparations (levamisole), interferon and interleukins [9, 10].

Microbial lysates contain pathogen associated molecular patterns (PAMP) which are recognized by specific toll-like receptors (TLRs), NOD-like receptors (NLR) and other pattern recognition receptors (PRRs) expressed on immunocompetent cells: dendritic cells, monocyte/macrophages, mast cells, neutrophils and NK-cells. Bacterial lipopolysaccharides (LPS) are considered to be the

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Abbreviations used: BCG – Bacille Calmette-Guerin (antituberculosis vaccine); exp. – exponent; LAK – lymphokine-activated killer cell; LPS – bacterial lipopolysaccharide; Math – mathematical; MDSC – myeloid-derived suppressor cells; NLR – NOD-like receptors; NK – natural killer cells; PAMP – pathogen associated molecular patterns; Ph.L. – Phagelysate; PRRs – pattern recognition receptors; TLRs – Toll-like receptors.

prototypical PAMPs. Many commercial as well as experimental, vaccines incorporate ligands for TLR that may not only protect against infectious diseases but also play important role in therapeutic immunization against noninfectious diseases, such as cancer. TLRs triggering a cascade of inflammatory and regulatory events make them an interesting targets for immuno- or conventional drug therapy against various infectious, autoimmune, and cancer diseases [11–14]. Although bacterial preparations express anti-tumor activity they have not been introduced into clinical practice due to toxic/pyrogenic symptoms (fever, pain, infiltrations at the site of injection etc) and even endotoxic shock at overdoses.

Our previous studies [15] have shown that administration of bacterial lysates of *Staphylococcus, Proteus, Klebsiella, E. coli* in experimental animals is safe, well tolerated and does not stimulate cancer growth. However, supposedly, vaccination with bacterial phage lysates, obtained in the course of phage lysis of host bacterial cells, could have better therapeutic effects.

Phage lysates have been shown earlier [16–18] to have high immunogenicity and minimal side effects compared to bacterial lysates obtained by other methods. Presumably, the lysis/destruction of bacterial walls occurs in such a manner that released pattern-containing conglomerates do not result in hyper-stimulation of immune system, and manifestation of reactogenic adverse effects may be reduced to a minimal level. There is an assumption that clinical effect of commercial preparations of bacteriophages for treatment of chronic infections also may results of certain immunomodulatory effect in parallel with obvious anti-infection action of phages. Proceeding from the aforesaid, we aimed to study the antitumor potential of gram-negative bacterial phagelysates and their adjuvant effects at conventional chemotherapy in experiment.

MATERIAL AND METHODS

The phagelysates were obtained in the synthetic M9 medium enriched with 0.1% yeast extract, inoculated with overnight *E. coli* (C) 600 suspension with the final concentration of $1–5x10^7$cfu/ml and incubated at 37°C with shaking [19, 20]. The Un phage [21] was inoculated at the exponential growth phase (OD = 0.5 at 660 nm) with phage: bacteria ratio 1:10 and shaking was continued 4–5 h at 32°C. The obtained lysate after kept overnight at 4°C was centrifuged at 4000 g for 20 min and filtered through 0.8 μm pore size Cellulose Nitrate membrane filters (Whatman International, UK). The filtrate was characterized by phage titer, optical density at 660 nm and protein concentration by Bradford method [22]. Content of endotoxins in the all series of phagelysates was estimated by Gel-clot test, Limulus amebocyte lysate (LAL) assay (Associates of Cape cod, Inc, MA, USA) according to manufacturer's instructions. The endotoxins were detected in all tested preparations with different levels exceeding 0.03 EU. The phagelysates were also tested for safety and pyrogenic effect on the healthy white and black mice. The mild pyrogenic effect (slight increase of temperature) within 2 h after inoculation of phagelysates and also some behavioral change (mainly dizziness) was registered with normalization in few hours. No death cases were reported. For animal trials *E. coli C-Un* phagelysates with average values for phage titer $1x10^{10}$ pfu/ml and protein concentration 0.12 mg/ml were used.

Animal studies were carried out on 75 BL57C/6J male mice 2–3 months old and with body weight of 18–20 g. All animals were fed standard laboratory chow and given free access to water. The care and use of the animals complied with the Georgian regulations on protection of animals, with Guidelines prepared by the Ethics Committee of the Institutional Animal Care and with the National Institutes of Health Guide for the Care and Use of Laboratory animals.

For creation of cancer model, all experimental animals were subjected to subcutaneous inoculations with Ehrlich carcinoma ($1x10^6$ tumor cells). Thereafter, they were randomly divided into 5 groups. Each group consisted of 15 mice. The group I (control) included inoculated (tumor bearing) animals with no anti-cancer treatment. In the group II animals were under conventional chemotherapy with combination of Doxorubicin 60 mg/m^2 (EBV, Austria), Cyclophosphan 800 mg/m^2 (Baxter, Germany), Ftoruracil 600 mg/m^2 (EBV, Austria), 3 times with 21 day intervals. Only at first chemotherapy the total dose was divided and injected twice with one week interval (half dose on 15th day and remaining — on 21st day after carcinoma inoculation). In the group III, all animals from the second day after Ehrlich carcinoma inoculation were subjected to the permanent intraperitoneal administration of *E. coli* phagelysate (0,5 ml) with 5 day intervals. The IV group animals were subjected to combined treatment: standard course of chemotherapy on the background of intraperitoneal injections of 0,5 ml *E. coli* phagelysate on the 2nd, 6th and 11th day after Ehrlich carcinoma inoculation. In the group V, animals were also under combined treatment, but chemotherapy was carried out on the background of permanent vaccination (intraperitoneal injections of 0,5 ml *E. coli* phagelysate with 5 day intervals).

Treatment efficacy was evaluated by calculating: i) Index of malignant tumor growth using semi-empirical mathematical model V=Vo{exponent[(t-to)/T]-1} describing tumor volume variations in relation to time passed after tumor transplantation; ii) tumor growth inhibition percent; iii) life-span and percent of survivors. Indices of cancer growth were measured every 3rd day of tumor growth. The volume of tumor tissue was calculated with the use of formula $V = \pi/6$ (AxBxC), where π=3,14, (A) is the length, (B) is the width, and (C) is the height of the tumor tissue. Obtained data were analyzed statistically with the use of SPSS 16.0 for Windows. Differences between tumor control and treated animals were determined by using the Student's *t*-test. The criterion for significance was set to $p < 0.05$.

RESULTS AND DISCUSSION

The experiments have been carried out during 135 days in total. On the basis of the results of experi-

ments the diagrams/charts have been constructed describing correlation between variation in tumor volume and time passed after tumor transplantation for all animal groups (Fig. 1).

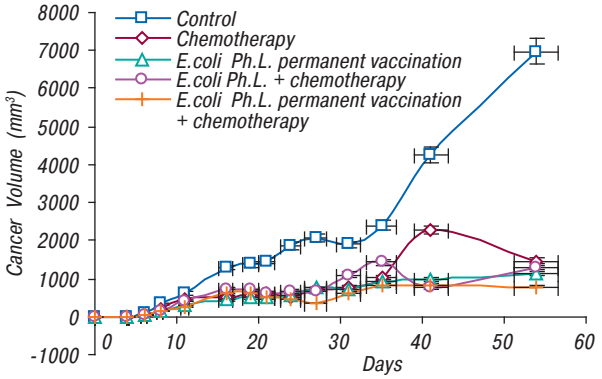

Fig. 1. Tumor growth dynamics in mice with Ehrlich carcinoma at different treatment methods

As it has been shown (Fig. 1), all treatment methods used for suppression of cancer have led to significant delay in tumor development. However, it appeared to be quite hard and disputable to talk about the treatment efficacy and relay on absolute sizes of tumor tissue. Evaluation only by tumor size wasn't sufficient because differences in tumor volumes of mice in experimental groups often were not significantly distinguishable especially at the early stages of cancer growth.

Proceeding from the aforesaid, we decided to describe the process of cancer growth with the use of semi-empirical mathematical model. Analyzing charts created on the basis of experimental data we concluded that development of cancer tissue could be preciously described with the use of formula providing exact and reliable information about cancer volume variations in relation to time passed after carcinoma inoculation:

$$V(t) = Vo\,[exp\,(t/T) - 1]$$.

V(t) — is the tumor volume at the moment of time ***t*;**

V_o — parameter characteristic for exact tumor line (here Ehrlich carcinoma);

T — is the key parameter. The more the value of it, the slow is the rate of cancer growth. Comparative analysis of the ***T*** parameter at different methods of treatment provides valuable information and perfect estimation of treatment efficacy.

Correlation between cancer volume variations and time passed after carcinoma inoculation for animals of group I (control) is presented on the Fig. 2. This chart also provides calculable curve, which in turn well coincides with experimental curve, giving us the confidence to say that above-used formula sufficiently describes the process of cancer growth, namely the increase in volume of cancer tissue in relation to time. Thus, in the group I animals the parameter V_0 amounted 3000 mm^3, and T = 53 days.

The Fig. 3 provides correlation between cancer volume variations and time passed after carcinoma inoculation in the group II animals subjected to conventional chemotherapy (Doxorubicin 60 mg/m^2, Cyclophosphan 800 mg/m^2, Phtoruracil 600 mg/m^2) and days of injections. As it has shown, cancer volume was decreasing after the each chemotherapy. However, cancer growth tendency, albeit at low level, was still maintained. In this group of experimental animals V_0 consisted 3000 mm^3, and parameter T — 117 days.

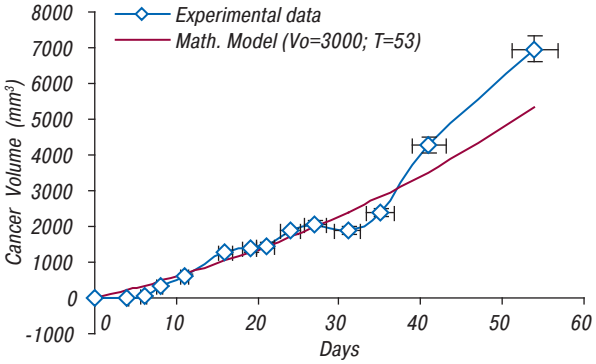

Fig. 2. Tumor growth dynamics in mice with Ehrlich carcinoma (Group I — Control)

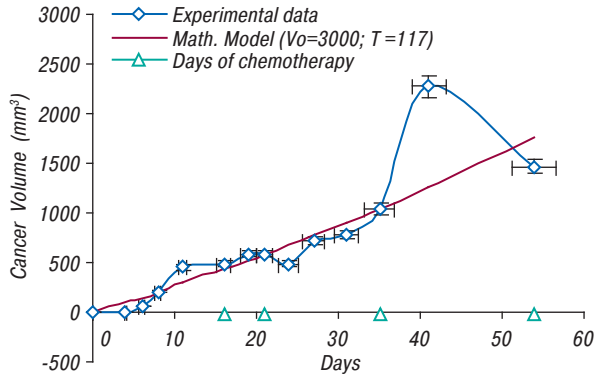

Fig. 3. Tumor growth dynamics in mice with Ehrlich carcinoma (Group II — Chemotherapy)

The Fig. 4 shows correlation between cancer volume variations and time passed after carcinoma inoculation in the group III animals treated permanently with *E. coli* phagelysate with 5 day intervals, also days of injections. Results have shown that V_0 was 3000 mm^3, and parameter T — 141 days.

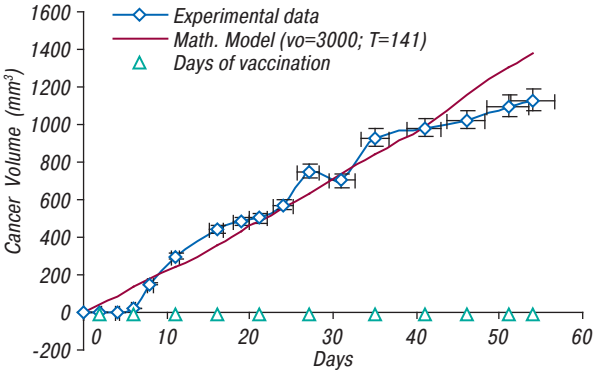

Fig. 4. Tumor growth dynamics in mice with Ehrlich carcinoma (Group III — *E. coli* Ph.L. permanent vaccination)

Results of investigations obtained from the IV group where animals were subjected to combined treatment — chemotherapy on the background of administration of *E. coli* phage lysate (intraperitoneal injections of 0,5 ml on the 2nd, 6th and 11th day after Ehrlich carcinoma inoculation) and days of injection

are presented on the Fig. 5. At such regimen of treatment V_0 was 3000 mm^3, and parameter T — 135 days.

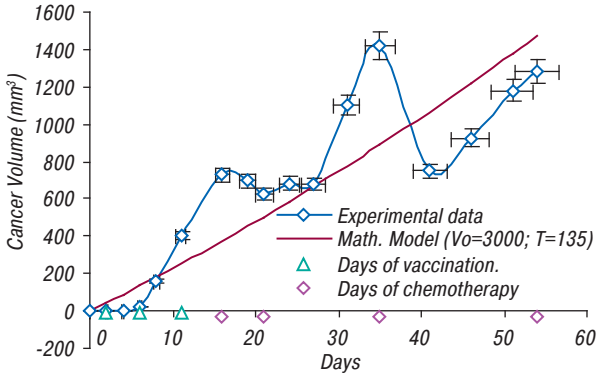

Fig. 5. Tumor growth dynamics in mice with Ehrlich carcinoma (Group IV — *E. coli* Ph.L. + chemotherapy)

The Fig. 6 provides correlation between tumor volume variations and time passed after tumor transplantation in the group V animals where animals were subjected also to combined treatment — chemotherapy on the background of permanent administration of *E. coli* phage lysate (intraperitoneal injections of 0,5 ml *E. coli* phagelysate) and days of injection. In the group V animals V_0 was 3000 mm^3, and parameter T — 169 days.

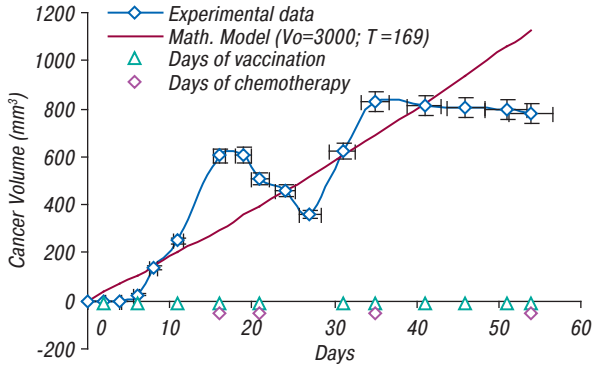

Fig. 6. Tumor growth dynamics in mice with Ehrlich carcinoma (Group V — *E. coli* Ph.L. permanent vaccination + chemotherapy)

It must be mentioned that in all groups of experimental animals (treated with different regimens, or untreated — control), the parameter V_0 always consisted of 3000 mm^3. Thus, could be said that parameter V_0 is common for the given model of cancer (in this case for the Ehrlich carcinoma). As for parameter T, it is variable and depends on the method/regimen selected for treatment purposes. According to the parameter T treatment efficacy of the different methods can be evaluated with sufficient reliability. As it was mentioned, the more is the parameter T, the less is the rate of tumor growth. Significance of parameter T for all experimental groups of animals treated with different methods and regimens are shown in Table 1.

As we can see, parameter T is the highest in case of combined treatment: administration of permanent *E. coli* phagelysate in parallel with conventional chemotherapy.

For estimation of treatment efficacy of different methods, it is convenient to calculate the time T_0, during which tumor volume V(t) reaches its characteristic volume V_0 (in this case V_0 = 3000 mm^3). Calculations have shown that time T_0 = T ln 2. Significances of parameter To for all experimental groups of animals treated with different methods and regimens are shown in Table 2.

Table 1. Significance of T in BL57C/6J mice with Ehrlich carcinoma at different treatment method

Experimental Group	I	II	III	IV	V
Treatment method	Control Untreated, cancer bearing mice	Chemotherapy	*E. coli* phagelysate (permanent vaccination)	*E. coli* phagelysate (vaccination 3x) + Chemotherapy	*E. coli* phagelysate (permanent vaccination) + Chemotherapy
T (Day)	53	117	141	135	169

T – parameter, indicating the rate of cancer growth

Table 2. Significance of To in BL57C/6J mice with Ehrlich carcinoma at different treatment methods

Experimental Group	I	II	III	IV	V
Treatment method	Control Untreated, cancer bearing mice	Chemotherapy	*E. coli* phagelysate (permanent vaccination)	*E. coli* phagelysate (vaccination 3x) + Chemotherapy	*E. coli* phagelysate (permanent vaccination) + Chemotherapy
To (Day)	37	81	98	94	117

Note: T_0 – time (number of days) during which cancer volume V(t) reaches 3000 mm^3

Comparative analysis of the results has shown that in case of chemotherapy the rate of cancer growth was inhibited by 2.2 times, in case of permanent application of *E. coli* phagelysate — 2.6 times; in case of the combined treatment (administration of phagelysates 3 times with 5 day intervals in combination with chemotherapy), more specifically, at the early period of cancer growth 2.5 fold inhibition of tumor growth rate was registered; another treatment regimen — permanent administration of phagelysate with 5 day intervals and chemotherapy, resulted in the inhibition of cancer growth by 3,2 times compared to the control group respectively.

Summarizing the obtained data, it could be concluded that permanent administration of *E. coli* phagelysate (group III) and combined treatment (group IV) have almost the similar treatment outcomes only at the beginning of the treatment, but both treatment methods (groups III and IV) have demonstrated higher efficacy than that of chemotherapy only (group II). The significant inhibition of tumor growth was registered for the combined treatment at the regimen of permanent application of *E. coli* phagelysate and chemotherapy (group V).

The life-span of experimental animals is another important parameter for assessment of anticancer treatment efficacy. For this purpose, in our investigations the number of deaths and percent of survived mice were calculated. According to the obtained results (Fig. 7), during the first 16 days of cancer development animals in all experimental groups were alive. Comparing the mortality rate in control group I (untreated animals) and the animals treated only with chemotherapy (group II), we found that although chemotherapy results in inhibition of tumor growth, the percent of animal death rates is nearly the same in the both groups, and maximal lifespan of mice was about 60 days after Ehrlich carcinoma inoculation.

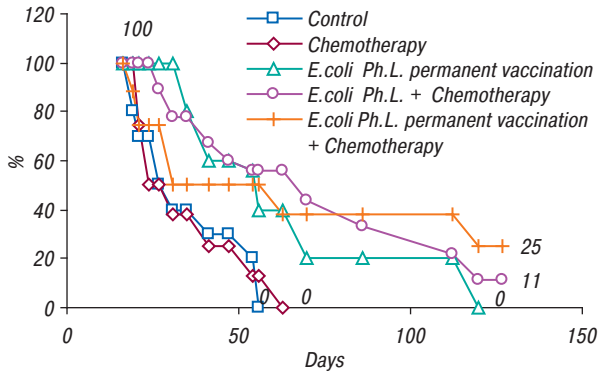

Fig. 7. Survival of Ehrlich carcinoma bearing mice treated by different methods

In experimental group III (permanent administration of *E. coli* phagelysates), during up to 30 days of cancer growth, all animals were alive and the maximal lifespan was close to 120 days. In groups IV and V, where animals underwent combined treatment, the maximal lifespan of experimental animals was significantly higher, in particular, in group V, 25% of animals were alive even on the 130th day of tumor growth.

Summarizing the results of our investigation we can suggest that bacterial phagelysates holds particular promise as a novel strategy for cancer therapeutics.

In conclusion, application of optimal doses of phagelysates is well tolerated in mice and did not support, or stimulate malignant growth. Although chemotherapy (group II) inhibited cancer growth, no significant increase in lifespan was observed that could be explained by immunosuppressive and toxic effects of chemopreparations. Application of *E. coli* phagelysates (group III) decreased tumor growth rate and elevated the lifespan and percent of survived experimental animals. Supposedly, it could be related to immunomodulatory effects of *E. coli* phagelysate increasing anticancer immune response. Combination of two methods of treatment (vaccination with *E. coli* phagelysates plus chemotherapy, groups IV and V) increased efficacy of chemotherapy. *E. coli* phagelysates exhibited significant efficacy as an anticancer treatment with adjuvant properties: tumor development was delayed in 65% cases, tumor growth was inhibited by 80–90%, and in 13% of mice complete regression of tumors (with average volume of 250 mm^3) was detected. No apparent side effects were registered.

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