Exp Oncol 2010
32, 4, 254–257

Experimental ONCOLOGY

INFLUENCE OF BACTERIAL LECTINS ON SOME REACTIONS OF NONSPECIFIC IMMUNITY IN SARCOMA 37 TRANSPLANTED MICE

O.A. Tanasienko[1], M.P. Rudyk[2], V.V. Pozur[2], G.P. Potebnya[1]*
[1]*R.E.Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Vasylkivska 45, Kyiv 01601, Ukraine*
[2]*T.G. Shevchenko Kyiv National University, Volodymirska 64, Kyiv 01033, Ukraine*

***Aim*: To study preventive effect of cytotoxic lectin from *Bacillus subtilis* B-7025 on the tumor growth and nonspecific immunity in sarcoma 37 transplanted mice. *Methods*: Sarcoma 37 cells were transplanted in Balb/c mice. Cytotoxic lectin (CL) was isolated from cultural fluid of *B.subtilis* strain B-7025 and inoculated at a dose of 0.2 mg per animal in prophylactic or combined schedule. Functional activity of macrophages was evaluated by NBT-test and the level of cytotoxicity, cytotoxic activity of splenocytes was assayed against K562 cells. *Results*: CL administration in prophylactic or combined schedule results in inhibition of sarcoma 37 growth in mice. Stimulating effect of CL on peritoneal macrophages of sarcoma 37-bearing mice, especially at early stages of tumor growth, has been observed. At the late stage of tumor growth, the effect of lectin on cytotoxic activity of spleen mononuclear cells has been registered. *Conclusion*: Upon the use of lectin from *B. subtilis* B-7025, positive shifts of antitumor immunity reactions leading to tumor growth inhibition and elevation of average life span of tumor-bearing mice, have been detected.**
***Key Words*: cytotoxic lectin, sarcoma 37, peritoneal macrophages, splenocytes, cytotoxic activity.**

Lectins are the proteins widely distributed in nature and isolated from animal and human tissues as well as from plants, viruses and bacteria. The characteristic feature of these biopolymers consists in their ability to recognize specifically and bind reversibly to glycoside structures in the complexes that contain carbohydrates. Lectins are highly stable and multifunctional molecules and may be isolated from various sources as well as produced by recombinant technology. It is important also that their reactions with soluble compounds and cells may be reversible in the presence of simple sugars.

The majority of lectins studied up to date possesses hemagglutinating activity, and some of them cause agglutination of malignant, embryonic cells, yeast and bacteria [1], and demonstrate antitumor activity and immunomodulating effects [2–4]. For example, the mitogenic effect of lectins toward T-lymphocytes, their ability to stimulate the production of various cytokines, in particular TNF-α, IFN-γ, IFN-α, proinflammatory interleukins (IL-1α, IL-1β, IL-6, IL-12), have been revealed. There are some reports on stimulating effects of lectins on macrophages and granulocytes [5, 6]. Such biologic properties of lectins are considered as potentially important ones for the therapy of various pathologies related to immune system disorders and cancer.

Immunomodulating effects of lectins [7, 8] may be promising in cancer treatment, in particular for the prevention of metastasizing after the surgical removal of tumor. Therefore, the cytotoxic lectins as unique immunomodulators with targeted action [9–11] are advantageous for the development of novel preparations for vector therapy of malignant tumors. The complex of means for elevation of immunoreactivity in combination with immunotherapy may significantly elevate an efficacy of treatment and improve the quality of life of cancer patients.

It is known that anticancer action of many lectins of plant and fungal origin is realized via their immunomodulating properties. Recently, the study of the lectins of bacterial origin has been initiated. In this regard, of special interest is the group of extracellular lectins of saprophytic bacteria from genus *Bacillus* with a rare specificity toward acidic carbohydrates, in particular glucuronic, N-acetyl- and N-glycolylneuraminic acids, fructose-1,6-diphosphate [12], which is unusual for lectins of plant and animal origin.

We have earlier shown that the binding of cytotoxic lectins (CL) from *B.subtilis* B-7025 to cell surface receptors modifies tumor-associated antigens elevating their immunogenicity, which may be advantageous for the development and generation of anticancer vaccines [13, 14]. However, antitumor and immunomodulating properties CL from *B.subtilis* B-7025 in itself, have not been yet studied.

An established preventive anticancer effect of CL from *B.subtilis* B-7025 in experimental tumors of different genesis [15] has forced us to study immunomodulating properties of the preparation using experimental tumor models. The present work was aimed at studying preventive antitumor affect of CL from *B.subtilis* B-7025 on the tumor growth and nonspecific immunity in sarcoma-37 transplanted mice.

MATERIALS AND METHODS

The cytotoxic lectins were isolated from cultural fluid of *B. subtilis* strain B-7025 registered in the collection of Institute of Microbiology and Virology NAS of Ukraine [16] by the method developed by us ear-

Received: April 25, 2010.
*Correspondence: E-mail: iris@onconet.kiev.ua, rosiente@gmail.com;
Fax: +380442581656

Abbreviations used : ALS – average life span; CL – cytotoxic lectin; IFN – interferon; IL – interleukin; MI – modulation index; NBT – nitroblue tetrazolium; PMp – peritoneal macrophage; PS – physiologic saline; TCT – tumor cell transplantation; TGI – tumor growth inhibition; TNF – tumor necrosis factor.

lier [12]. Isolated and purified cytotoxic lectins are thermostable glycoproteins with isoelectric points of 3.6 and 4.4 units possessing molecular mass polymorphism caused by formation of multiple forms of the biopolymer [17].

2-2.5-months old female Balb/c mice were used in the study. The animals were injected subcutaneously with CL (0.2 mg per animal) 4 times with 1–2 days intervals. The next day after the last CL administration, 0.5 x 10^6 sarcoma 37 cells were grafted in the thigh muscle of both experimental and control animals. For prophylactic schedule of treatment with CL, animals were housed in 2 groups: mice from 1st group were further treated with CL (0.05 mg per animal) twice a week for 3 weeks (combined scheme), while mice from 2nd experimental group (prophylactic scheme) and control animals received physiologic solution. Using a caliper, the tumor diameters were measured daily, and tumor volumes were calculated using the following formula: $V = D \times d^2 \times 0.52$ (V, tumor volume; D, the largest dimension; d, the smallest dimension). All experiments were approved by Ethical Committee of the institute.

The functional activity of the effectors of immunologic response was studied in experimental animals one day prior to tumor transplantation and on days 6 and 34 after tumor cell transplantation (TCT) (as well as in control animals).

The functional activity of peritoneal macrophages (PMp) was evaluated by the level of oxygen-dependent metabolism and cytotoxicity. Oxygen-dependent metabolism was determined by reduction of tetrazolium nitroblue (NBT) [18]. Optical density of reduced NBT was analyzed using microplate photometer "Reader"(Labotech, Latvia) at the wavelength of 630 nm.

The cytotoxic activity of PMp was evaluated by MTT colorimetric test [19, 20]. The cells of allogenic tumor served as target cells. Optical density was measured using microplate photometer "Reader" (Labotech, Latvia) at the wavelength of 545 nm. Specific killing of target cells was calculated by formula:

$CA = (1 - (E_m - E_e)/(E_t - E_c)) \times 100\%$, where E is mean absorbance of the wells: E_m — the wells with mixture of the target and effector cells; E_e — the wells with the effector cells; E_t — the wells with the target cells; E_c — control of medium [20].

The cytotoxic activity of spleen mononuclear cells was evaluated by MTT colorimetric test [20, 21]. Mononuclear cells were isolated by standard method from spleen by centrifugation of cell suspension (obtained after homogenisation of spleen tissue in Potter's homogenizer) in the Ficoll-verografin density gradient ($\rho = 1.077$). Cell suspensions with viability higher then 80% were used [22]. K562 cells were used as target cells, sensitive to natural killer cells (NK). The ratio between effector cells and target cells was 1/50; duration of co-incubation was 18 h. Then, 20 µl of MTT solution (5 mg/ml MTT in PBS) were added to each well and the plates were incubated for 4 h at 37 °C. In order to stop the reaction, 100 µl DMSO were then added with vigorous mixing. Optical density of incubation mixture in the wells was measured using microplate photometer "Reader" (Labotech, Latvia) at the wavelength of 545 nm. Cytotoxic activity of spleen mononuclear cells was calculated by the foresaid formula.

Anticancer efficacy of lectin was evaluated by analysis of tumor growth dynamics and average life span (ALS) of experimental animals using tumor growth inhibition index (TGI) and ALS modulation index (MI) [23, 24]. The indices were calculated using the formulas:

$TGI = ((V - V_k)/V_k) \times 100\%$, where V is mean tumor volume calculated for experimental groups treated by CL; Vk is mean tumor volume in the control group [23].

$MI = ((E - K)/K) \times 100\%$, where E is mean ALS of groups treated by CL; K is mean ALS in control group [24].

Statistical analysis of the data was performed by standard methods using Student's t-test.

RESULTS AND DISCUSSION

The inhibition of sarcoma 37 growth was assessed in the animals treated with CL isolated from metabolic products of *B. subtilis* B-7025 in prophylactic or combined schedule. As seen in Fig. 1, such treatment resulted in tumor growth inhibition. On day 28 after tumor cell transplantation (TCT), TGI in mice from experimental group I was 66%, while in mice preventively treated with lectin (experimental group II) TGI amounted to 50.5%. The average life span (ALS) of experimental animals relative to untreated controls increased (Table).

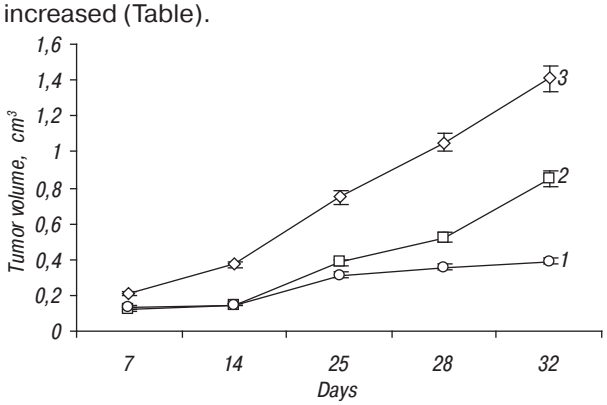

Fig. 1. Effect of prophylactic and combined use of lectin on the growth of sarcoma 37 in tumor bearing mice. 1 — administration of CL before and after tumor cell transplantation, 2 — administration of CL before tumor cell transplantation, 3 — control

Table. Average life span of mice with transplanted sarcoma 37 upon prophylactic and combined treatment with bacterial lectin

Group	Number of mice	Dosage and method of administration of lectin, mg/animal	ALS, M ± m	t	MI,%
CL→TCT→CL	8	0.2 before and 0.05 after TCT, s/c	85.1 ± 9.3*	3.6	69.5
CL→TCT→PS	7	0.2, s/c	71.1 ± 4.5*	4.1	41.6
Control	8	-	50.2 ± 2.3	-	-

Notes: *– $p < 0.05$ compared to control group.

The effects of CL on tumor growth in mice may be caused by two reasons: activation of antitumor defense and direct cytotoxic action of lectin on tumor cells. We have attempted to analyze CL effects on the activity of

PMp in tumor-bearing animals. Macrophages represent an important component of antitumor resistance. According to the literature data [25, 26], the lectins of different origin activate both peripheral blood monocytes and resident macrophages. In our experiment four injections of bacterial lectins to intact animals at a dose of 0.2 mg have led to activation of these effector cells. Sarcoma 37 transplantation additionally stimulated their activity registered on day 6 after TCT. This trend persists even on day 34 after TCT in the case of the combined schedule of the treatment, although the differences were not significant (data are not shown).

On day 6 after tumor transplantation, in both experimental groups the index of cytotoxic activity of PMp increased more than twice compared to that in tumor-bearing control (Fig. 2). On day 34, cytotoxicity of macrophages was similar to control values. These results allow us to suppose that at the late stages of tumor growth macrophages-suppressors that inhibit macrophage-mediated cytotoxicity and promote tumor growth, began to be involved in the process.

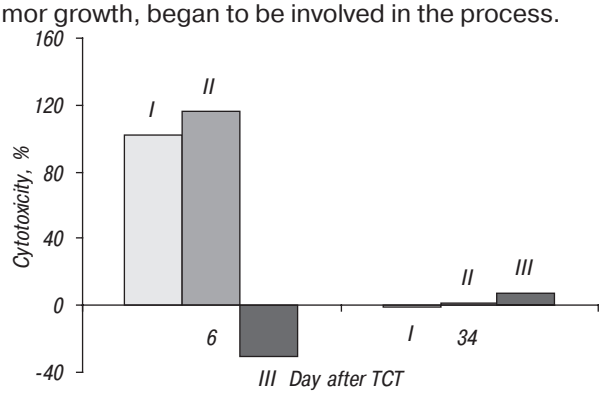

Fig. 2. Effect of prophylactic and combined use of lectin on cytotoxic activity of peritoneal macrophages from mice bearing sarcoma 37. I — administration of CL before and after TCT, II — administration of CL before TCT, III — tumor-bearing control

It is known that the development of tumor growth suppresses the functional activity of splenocytes, including natural killer cells. As one may see in Fig. 3, in tumor-bearing mice an immunosuppressive effect of tumor toward these effector cells of natural immunity could be observed manifesting by the decreased ability of spleen mononuclear cells from control animals to cause lysis of target cells.

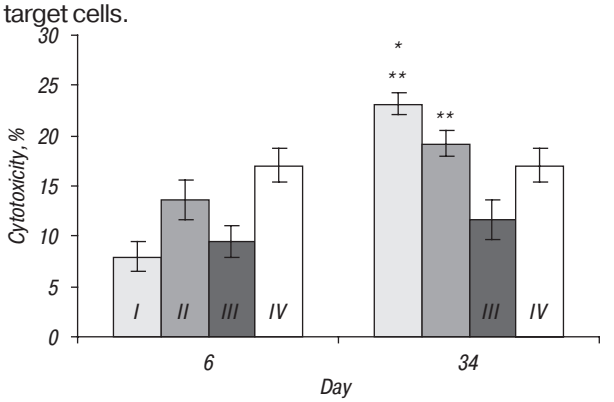

Fig. 3. Effect of prophylactic and combined use of lectin on cytotoxic activity of mononuclear splenocytes from mice bearing sarcoma 37. I — administration of CL before and after TCT, II — administration of CL before TCT, III — tumor bearing control, IV — intact control

In prophylactic use of lectin, on day 6 after TCT elevated cytotoxicity of splenocytes against sensitive K562 cells was evident. However, the most potent stimulating effect of prophylactic and combined use of lectin on cytotoxic activity of spleen cells was revealed on day 34 after TCT: the cytotoxicity of macrophages of experimental animals was more than twice higher than those of control tumor-bearing animals. Since NK-sensitive K562 cells were used as target cells, we suppose that NK cells were predominantly responsible for the observed cytotoxic effect of spleen mononuclear cells. Nevertheless, other subpopulations of spleen lymphoid cells can also be responsible for negligible part of cytotoxic effect of spleen cells against target cells used in the study.

Therefore, antitumor effect of lectin administration is accompanied by activation of immunologic reactivity of mice. Stimulating influence of lectins on tumor-bearing animals at the early stage of tumor growth has been registered for functional activity of peritoneal macrophages, and at the late stage — for spleen mononuclear cells.

In oncological practice bacterial lectins are used as the components of antitumor vaccines and as adjuvants but not as an individual anticancer or immunomodulating preparations. Nevertheless, high cytotoxicity against malignant cells has been already shown for several lectins of plant and fungal origin [3–5, 27, 28]. Antiproliferative activity of lectins is often combined with their ability to stimulate distinct reactions of immune system [2, 3, 6–8, 26]. It has been previously shown that *B. subtilis B-7025* lectins are highly cytotoxic *in vitro* against tumor cells of different genesis [17]. We also have described [15] significant inhibitory preventive influence of lectins from *B. subtilis B-7025 in vivo* on tumor growth and metastasizing in mice. This antitumor effect of bacterial lectin is very similar to that revealed for Aloe vera lectin [29] and mistletoe lectins [5].

In present work immunomodulating properties of cytotoxic lectin in sarcoma 37 bearing mice in dynamics were displayed. This allows us to assume that antitumor effect of CLs like some other lectins is realized via not only its cytotoxic action against tumor cells but also via enhancing impact on innate immunity. According to our data, the combined schedule of *B. subtilis* lectins administration seems to be more effective than prophylactic one to provide complete realization of all beneficial potentials of lectins.

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