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ANTIMETASTATIC EFFECT OF *B. SUBTILIS IMV B-7724* LECTIN OBSERVED IN LEWIS LUNG CARCINOMA MODEL

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Aim: **To study the antitumor and antimetastatic effects of *B. subtilis IMV B-7724* lectin used in neoadjuvant and adjuvant settings *in vivo*. *Materials and Methods:* Studies were performed on C57Bl/6J mice; Lewis lung carcinoma (LLC) was used as an experimental tumor. *B. subtilis IMV B-7724* lectin was administered to tumor-bearing mice or to mice which underwent surgical resection of the primary tumor. The lectin was injected subcutaneously, 10 times, at a single dose of 5 or 1 mg/kg of body weight. The standard indicators of tumor growth and metastasis were evaluated. *Results:* Independently of the application settings, the lectin at a dose of 1 mg/kg of b.w. caused more pronounced effect than at a dose of 5 mg/kg of b.w. The administration of *B. subtilis IMV B-7724* lectin to the mice with LLC in neoadjuvant setting did not cause notable antitumor effect but led to a significant decrease in the number and volume of lung metastases. The lectin administration in adjuvant setting significantly inhibited metastasis: the metastasis inhibition index reached 63.0% and 100% in the mice treated with the lectin at a dose of 5 mg/kg and 1 mg/kg respectively. The mean survival time of the treated animals significantly increased. *Conclusion:* A pronounced antimetastatic effect of *B. subtilis IMV B-7724* lectin administered in an adjuvant setting was demonstrated.**

Key Words: **Lewis lung carcinoma, *B. subtilis IMV B-7724* lectin, antitumor effect, antimetastatic effect.**

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The development of new means of cancer therapy remains a compelling challenge for modern researchers. The list of substances with an antitumor activity is expanding thanks to the drugs of natural origin, which not only possess cytotoxic activity but are able to optimize the patient's immune response too. It is known that immune antitumor reactions (primarily of natural immunity) play a significant role in preventing the relapses and metastatic spread of the primary tumor. Therefore, in the complex treatment of patients, it can be effective to apply agents preventing the suppression of immune response imposed by the progressing tumor process or side effects of chemo- and radiation therapy. As substances possessing immunomodulatory and antitumor properties, lectins — glycoproteins selectively binding carbohydrates and carbohydrate components of glycoconjugates of various nature — draw the attention of researchers. Due to their strong affinity for certain carbohydrates on the cell surface, lectins take part in a wide range of biological processes, in particular intercellular interaction, embryogenesis, pathogens recognition, etc. Experimental studies have shown that lectins may possess antiviral, antibacterial, fungicidal, immunomodulatory, and antitumor properties [1, 2].

The mechanisms of antitumor activity of lectins are based on both a direct cytotoxic effect on tumor cells and an indirect effect due to the modulation of immune reactions. To date, the properties of plant lectins have been described in more detail, demonstrating that all investigated substances of this group are quite toxic, their cytotoxic effect extends not only to tumor cells, but also to the cells of healthy tissues [3, 4]. The information regarding the properties and use of microbial lectins in medical practice (in particular, oncological) is much less present in the scientific literature [5]. From the biotechnological point of view, the process of obtaining extracellular bacterial lectins is much easier and more suitable for standardization. The high rate of microbial biomass growth ensures easier obtaining of a larger amount of active substance compared to plant lectins. Considering this as well as the information about the low toxicity of bacterial lectins they can be regarded as prospective antitumor and immunomodulatory agents.

Given that malignant cells express excessive amounts of sialic acids of different types in the surface glycoconjugates, sialo-specific lectins can be considered as potential antitumor agents. To date, lectins produced by bacteria of the *Bacillus* genus (in particular, *B. polymyxa 102, B. subtilis 316M, B. subtilis 7025*) possessing high specificity for sialic acids have been demonstrated to exert antitumor activity [6]. Theantitumor effect examination of the extracellular lectin obtained from another strain of the *Bacillus* genus — *B. subtilis IMV B-7724* — seems to be promising. Its physico-chemical and biological properties have been studied and cytotoxic activity towards tumor cells of various lines was shown *in vitro* [7]. Lectin's antitumor activity was shown to differ when applied in mice transplanted with tumors of different histogenesis [8]. In order to examine more precisely the antitumor and antimetastatic properties of *B. subtilis IMV B-7724* lectin, it is necessary to study its activities *in vivo* in different settings. Considering all above mentioned, the aim of this work was to study the antitumor and

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Abbreviations used: LLC – Lewis lung carcinoma; MII – metastases inhibition index; TII – tumor inhibition index.

antimetastatic activity of *B. subtilis IMV B-7724* lectin used in neoadjuvant and adjuvant settings in mice with transplanted Lewis lung carcinoma.

MATERIALS AND METHODS

Experimental animals and tumor strain. The study has been carried out on male C57Bl/6J mice (2–2.5-month-old weighting 19–22 g), bred at the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of the National Academy of Science of Ukraine. The animals were housed under the standard vivarium conditions with natural lighting and standard cereal-based diet. The use and care of experimental animals have been performed in accordance with standard international rules on biologic ethics and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [9].

Lewis lung carcinoma (LLC) cells obtained from the Bank of cell lines from human and animal tissues of the R.E. Kavetsky IEPOR of the National Academy of Sciences of Ukraine were used to induce tumors.

Bacterial lectin. Strain *B. subtilis IMV B-7724* of spore forming, aerobic, gram-positive bacteria was used as a source of the lectin. The lectin was isolated from the cultural fluid on day 4 of the culture as described in [7]. The isolated lectin was freeze-dried at temperatures between –32 °C and +24 °C and stored as a powder at –20 °C.

Lectin dosage. Two doses of the lectin were examined to select a more effective one: 5 mg/kg or 1 mg/kg of body weight per one administration. The complete course consisted of 10 administrations of the lectin; the cumulative doses reached 50 mg/kg and 10 mg/kg of body weight, respectively.

Experimental schedules. Two series of the experiment were held differing in the administration settings. The bacterial lectin was applied either to tumor-bearing mice (neoadjuvant setting) or after the surgical removal of the primary tumor (adjuvant mode).

In the neoadjuvant settings of lectin administration, LLC cells ($4 \cdot 10^5$ cells/mouse) were injected intramuscularly in the thigh. The experimental animals were divided into 3 groups: untreated mice with tumors, referred as tumor-bearing control (n = 10); "Lectin 5 mg/kg" (n = 10) and "Lectin 1 mg/kg" (n = 10) – tumor-bearing mice, which were injected s.c. with the lectin, respectively, at a dose of 5 or 1 mg/kg of body weight per 1 administration. Independently of the dose, lectin injections started on day 2 after tumor transplantation.

In the adjuvant settings of lectin administration, tumor cells were injected into the foot at a dose of $2.5 \cdot 10^5$ cells/mouse. On day 16 after tumor transplantation, the primary nodule was surgically removed. Lectin administration started on day 2 after the surgery. The division of experimental mice into groups and the scheme of lectin administration were the same as is described above. The experiment lasted for 90 days. Metastasis volume and number were evaluated during the experiment and on day 90 after the tumor transplantation in survived animals.

Assessment of antitumor and antimetastatic effects. The course of the tumor process was characterized by the standard indicators [10]: the latent period (day) and frequency (%) of tumor occurrence; the frequency of metastasis (%), number (n) and volume of metastases (mm^3); survival rate (%) and mean survival time (days) of experimental animals. Additionally, the metastasis inhibition index (MII) and the tumor inhibition index (TII) were calculated. The MII was calculated as following:

$$MII = (A_c \cdot B_c - A \cdot B / A_c \cdot B_c) \cdot 100\%,$$

where A_c and A stand for the number of mice bearing lung metastases in the groups of control and the treated mice respectively. B_c and B stand for the mean number of lung metastases in the groups of control and the treated mice respectively.

TII was calculated according to the formula:

$$TII = ((V_c - V_t) / V_c) \cdot 100\%,$$

where V_c and V_t stand for mean tumor volume in the groups of control and the treated mice respectively.

Statistical analysis of the data was performed using Student's *t*-test. Values $p < 0.05$ were considered as statistically significant [11].

RESULTS AND DISCUSSION

Tumors developed in all experimental animals 8–11 days after the transplantation of LLC cells. Applied in tumor-bearing mice (neoadjuvant settings), the lectin, regardless of the dose used, did not elicit a significant effect on the latent period of tumor development, the dynamics of primary tumor growth, survival rate and survival time of the experimental animals (Table 1).

Table 1. Effect of *B. subtilis* IMV B-7724 lectin on growth of LLC

Index	Tumor-bearing control (n = 10)	Lectin-treated mice 5 mg/kg (n = 10)	Lectin-treated mice 1 mg/kg (n = 10)
Tumor yield, %	91.7 ± 7.7 (10/10)	91.7 ± 7.7 (10/10)	91.7 ± 7.7 (10/10)
Latent period, days	9.3 ± 1.2	10.2 ± 1.4	9.5 ± 1.3
Tumor volume, mm^3			
Day 8	123.8 ± 27.2	125.3 ± 31.2	146.6 ± 26.0
Day 14	200.3 ± 47.4	262.0 ± 40.5	253.0 ± 40.3
Day 22	1415.5 ± 259.2	1216.0 ± 259.6	1195.9 ± 150.2
Day 29	3161.4 ± 329.2	2810.0 ± 305.6	2614.0 ± 288.7
Day 35	3472.9 ± 270.6	3105.6 ± 295.6	2721.0 ± 307.5
Day 42	–	3415.3 ± 154.2	3891.2 ± 298.8
Day 49	–	–	4190.0 ± 40.4
TII, %	–	14.9 ± 2.1	18.2 ± 1.8
Mean survival time, days	39.6 ± 2.7	40.5 ± 2.9	44.9 ± 4.2

At the same time, there was noticed an antimetastatic effect in the group of mice injected with the lectin at a dose of 1 mg/kg: the number and volume of lung metastases were significantly lower comparing to both the control tumor-bearing animals (1.6 and 10.5 times respectively) and the mice receiving the lectin at a dose of 5 mg/kg (1.3 and 1.5 times respectively) (Table 2). MII in this group reached 36.0%.

Therefore, the application of *B. subtilis IMV B-7724* lectin to the tumor-bearing mice (without sur-

gical removal of the tumor) did not affect the growth of the primary tumor node but had a significant antimetastatic effect. Administration of lectin in a lower dose (1 mg/kg) was more effective.

Table 2. Metastasis in LLC bearing mice treated with different doses of *B. subtilis* IMV B-7724 lectin (neoadjuvant setting)

Index	Tumor-bearing control (n = 10)	Lectin-treated mice	
		5 mg/kg (n = 10)	1 mg/kg (n = 10)
Frequency of metastasis development, %	91.7 ± 7.7 (10/10)	91.7 ± 7.7 (10/10)	91.7 ± 7.7 (10/10)
MII, %	–	19.1	36.0
Mean metastasis number, n	44.4 ± 3.4	35.9 ± 1.3	28.4 ± 1.3[1, 2]
Mean metastasis volume, mm^3	604.2 ± 50.3	85.8 ± 1.7[1]	57.3 ± 2.9[1, 2]

Note: [1]$p < 0.05$ as compared to the tumor-bearing control; [2]$p < 0.05$ as compared to the group "Lectin 5 mg/kg".

When antimetastatic effects of *B. subtilis IMV B-7724* lectin was assessed after the surgical removal of the primary tumor, a significant antimetastatic effect of the lectin in both doses was demonstrated (Table 3). Although, the antimetastatic effect was more pronounced in the group of mice receiving the lectin at a dose of 1 mg/kg of body weight. In this group, metastases did not develop in any mouse during the entire observation period (90 days).

Table 3. Metastases in mice treated with different doses of *B. subtilis* IMV B-7724 lectin after the surgical resection of LLC tumor (adjuvant setting)

Index	Control mice (n = 10)	Lectin-treated mice	
		5 mg/kg (n = 10)	1 mg/kg (n = 10)
Frequency of metastasis development, %	80.0 ± 9.7 (8/10)	50.0 ± 17.7 (5/10)	5.0 ± 4.0[1, 2] (0/10)
MII, %	–	63.0	100
Mean metastasis number, n	26.8 ± 1.5	15.9 ± 1.4	0[1, 2]
Mean metastasis volume, mm^3	627.3 ± 78.6	18.4 ± 2.0[1]	0[1, 2]
Mean survival time, days	56.1 ± 3.3	74.6 ± 5.5[1]	90.0 ± 0.0[1,2]

Note: [1]$p < 0.05$ as compared to the control mice, which underwent tumor resection only; [2]$p < 0.05$ as compared to the group "Lectin 5 mg/kg".

The obtained results demonstrate that *B. subtilis IMV B-7724* lectin at a dose of 1 mg/kg (total dose of 10 mg/kg of body weight) applied after surgical removal of the primary tumor completely prevents the development of metastases. At the end of the experiment (day 90 after tumor transplantation), all animals in the group remained alive (the Figure). The application of the lectin in a larger dose (5 mg/kg of body weight) had a less pronounced effect (see the Figure). In this group, however, the survival time exceeded that of the animals, which underwent only tumor removal (74.6 ± 5.5 *vs* 56.1 ± 3.3 days in the control group, $p < 0.05$).

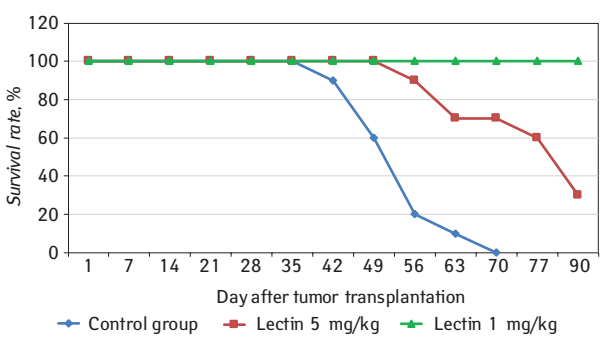

Figure. Survival rate of mice treated with *B. subtilis IMV B-7724* lectin after the surgical resection of LLC tumor

Our data are in line with other reports on the effectiveness of different immunotherapeutic agents of natural origin on the LLC model and provide evidence to support for the prospects of adjuvant treatment [12–14].

It is well known that the antitumor effects of lectins of various origins may be due not only to a direct cytotoxic effect toward transformed cells, but also to their ability to activate antitumor immune reactions [15]. The same mode of action cannot be excluded for *B. subtilis IMV B-7724* lectin. This assumption is supported by the results of our previous *in vitro* studies demonstrating the dose-dependent effect of the lectin on the peritoneal macrophages of intact Balb/c mice. In particular, it was shown that in concentrations ⩾ 0.1 mg/ml it exerts cytotoxic effect while in concentration 0.02 mg/ml it stimulates the functional activity of macrophages [16]. Added to a culture medium, 0.02 mg/ml of the lectin led to a significant increase in NO production and a decrease in arginase activity which is characteristic of macrophages with the M1 phenotype. M1 macrophages are known to play a significant role in antitumor defense. Therefore, it is tempting to conclude that higher antimetastatic activity of the lectin applied in a lower dose is based on its activating effects on macrophages and/or possibly other immune cells that, as a result, caused a pronounced anticancer effect of the lectin. Though, this assumption needs to be experimentally explored.

To sum up, the application of *B. subtilis IMV B-7724* lectin to the mice bearing metastatic LLC tumor did not affect the primary tumor growth but led to a significant decrease in the number and volume of lung metastases in treated mice. As the tumor volume limits the antitumor effect of most immunotherapy remedies of natural origin, the most notable effect can be achieved when biotherapy is used in an adjuvant setting aiming to prevent tumor relapse and metastasis. From this point of view, the revealed potent antimetastatic effect of *B. subtilis IMV B-7724* lectin applied after surgical removal of the primary tumor seems quite explicable. On the other hand, it cannot be excluded that the high antimetastatic activity of the lectin may be related to differences in the surface glycosylation of the primary tumor and metastatic cells.

An increase in the number of glycans containing sialic acids — N-glycolylneuraminic (Neu5Gc) or N-acetylneuraminic (Neu5Ac) — is known to be a common form of aberrant glycosylation of human malignant cells. For example, it has been shown that an increase in the level of Neu5Gc and Neu5Ac is associated with breast, ovarian, prostate, colon, and lung cancer [17, 18]. Considering the high affinity of the studied lectin to sialic acids, it can be assumed that the detected antimetastatic effect may be due to the interaction of the lectin with the cell membrane glycoconjugates leading to the subsequent activation of the tumor cell death. Nevertheless, the precise

mechanisms resulting in malignant cells elimination caused by the lectin remain to be elucidated.

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АНТИМЕТАСТАТИЧНА ДІЯ ЛЕКТИНУ *B. SUBTILIS IMB B-7724* НА МОДЕЛІ КАРЦИНОМИ ЛЕГЕНІ ЛЬЮЇС

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Мета: Експериментальне дослідження антиметастатичної ефективності лектину *B. subtilis IMB B-7724* за умови його застосування в неоад'ювантному та ад'ювантному режимах. ***Матеріали та методи:*** Дослідження проведено на мишах лінії C57Bl/6J, у якості експериментальної модельної пухлини використана карцинома легені Льюїс. Досліджено 2 режими введення лектину *B. subtilis IMB B-7724*: на тлі пухлинного росту та після видалення первинної пухлини. Лектин вводили підшкірно, 10-разово, разова доза становила 5 або 1 мг/кг маси тіла. Оцінювали стандартні показники пухлинного росту та метастазування. ***Результати:*** Застосування лектину *B. subtilis IMB B-7724* у мишей з карциномою легені Льюїс без оперативного видалення первинної пухлини не супроводжувалося достовірним протипухлинним ефектом, проте призводило до суттєвого зменшення кількості та об'єму легеневих метастазів. Введення бактеріального лектину мишам після оперативного видалення первинної пухлини достовірно пригнічувало процес метастазування: індекс інгібіції метастазування становив 63,0% (5 мг/кг) та 100% (1 мг/кг). Відмічали також суттєве збільшення середньої тривалості життя пролікованих тварин. Більш виражений антиметастатичний ефект виявлено при застосуванні лектину в дозі 1 мг/кг маси тіла. ***Висновки:*** На моделі карциноми легені Льюїс виражений антиметастатичний ефект лектину *B. subtilis IMB B-7724* виявлено за умови його застосування в ад'ювантному режимі.

Ключові слова: карцинома легені Льюїс, лектин *B. subtilis IMB B-7724*, протипухлинний ефект, антиметастатичний ефект.