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EFFECT OF *B. SUBTILIS* IMV B-7724 LECTIN ON THE ACTIVITY OF EFFECTORS OF CELLULAR ANTITUMOR IMMUNITY OF MICE WITH LEWIS LUNG CARCINOMA

Aim. To evaluate the effect of *B. subtilis* IMV B-7724 lectin on the functional activity of macrophages (Mph), natural killer (NK) cells and cytotoxic lymphocytes (CTL) of mice bearing Lewis lung carcinoma (LLC). **Materials and Methods.** The studies were performed on C57Bl/6J mice; LLC was used as an experimental transplantable tumor. The lectin from *B. subtilis* IMV B-7724 was administered to LLC-bearing mice subcutaneously at a dose of 1 mg/kg of body weight for 10 days. The immunological testing was performed on days 14, 21, and 28 after tumor grafting. The cytotoxic activity of Mph, NK, and CTL was estimated in MTT-assay; the content of the stable metabolites of nitric oxide (NO) was measured by a standard Griess reaction; the arginase activity (Arg) was determined based on the measurement of urea. **Results.** The administration of the *B. subtilis* IMV B-7724 lectin to LLC-bearing mice exerted its antitumor and antimetastatic effects partially via a significant ($p < 0.05$) increase of Mph and NK activities after the completion of the treatment. In the group of animals injected with lectin, the NO/Arg ratio increased significantly, indicating the prevalence of Mph with proinflammatory and antitumor properties. The cytotoxic activity of Mph exceeded the indices of untreated mice and intact control by 1.8 times and 5.3 times respectively; of NK — by 2.8 and 1.3 times respectively. The effect of treatment on the CTL activity was less pronounced. **Conclusion.** Antitumor and antimetastatic activity of the lectin from *B. subtilis* IMV B-7724 ensured the preservation of the cytotoxic activity of the main effectors of antitumor immunity (Mph, NK, and CTL) throughout LLC growth.

Keywords: Lewis lung carcinoma, lectin from *B. subtilis* IMV B-7724, antimetastatic effect, macrophages, natural killer cells, cytotoxic lymphocytes.

Antitumor immunity is exerted through the combined action of the natural (immunologically non-specific) and adaptive (specific) mechanisms, which are aimed at maintaining the antigenic homeostasis of the body, including the recognition and destruction of foreign cells.

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Antitumor immunity encompasses both innate (nonspecific) and induced (specific) immune responses.

As known, tumor tissue is not homogeneous and contains both malignantly transformed and normal body cells that form the tumor stroma. The tumor microenvironment (TME) also includes cells of the immune system: macrophages (Mph), neutrophils, and natural killer (NK) cells. A significant role in the antitumor immune response belongs to such effectors of the innate immune system as Mph. Mph play a significant role in the development and restoration of tissues and homeostasis. Mph in the tumor focus regulate the interaction of tumor cells with the TME during all stages of its development.

Mph are characterized by significant phenotypic heterogeneity and functional diversity. They acquire different properties, depending on the tissue in which they function, as well as on its microenvironment. These cells have the ability to perceive signals from the tumor microenvironment and change the direction of their polarization from cells with antitumor properties (M1) to cells that promote tumor growth (M2), and *vice versa*.

M1 Mph perform the functions of immune surveillance and antigen presentation. Due to the production of a number of cytokines, Mph are able to enhance or inhibit the activity of effector cells of both innate (NK) and specific (T-helpers, cytotoxic lymphocytes (CTL)) immunity. M2 Mph inhibit the antitumor immune response due to the production of inhibitory cytokines, in particular IL-10 and/or TGF-β. The phenotype of the resident Mph regardless of their location is closely related to the dynamics of tumor growth and the prognosis of the disease. Usually, M2 Mph contribute to the formation of an immunosuppressive microenvironment *via* release of a number of cytokines, chemokines, and inflammatory mediators. The factors produced by M2 Mph stimulate angiogenesis, contribute to the migration of tumor cells and create a favorable environment for their further colonization, thereby promoting tumor growth and metastasis [1, 2].

It is known that Mph are characterized by high plasticity. That is, under the influence of appropriate external stimuli, these cells are able to change the direction of polarization M1↔M2. The ability of Mph to influence the tumor microenvironment through interaction with other cells of the immune system (NK, Th, CTL), as well as the changes in their activity and phenotype in response to external signals makes Mph a new promising target for antitumor therapy. [3, 4].

Today, the search for new means of immunotherapy continues. The major requirements for immunotherapeutic treatments comprise the ability to maintain immune homeostasis, nontoxicity for healthy cells and body tissues, minimal side effects, and accessibility for patients. To a greater extent, these requirements are met by preparations of natural origin, among which are lectins. The cytotoxic, antitumor, or immunomodulatory properties of lectins of various origins have been demonstrated [5, 6]. The antitumor activity of lectins can be realized by direct (directly against tumor cells) or indirect (through modulating immune anticancer reactions) cytotoxic action. The direct cytotoxic action is due to the binding of the lectin to the sugar-containing receptor on the cell surface and the induction of a cascade of intracellular processes that result in apoptosis, autophagy, cell cycle blocking, and inhibition of tumor cell proliferation. The mechanisms of the direct cytotoxic effects of lectins of the plant origin have been thoroughly studied and described in the scientific literature [7, 8]. Not less important is the ability of lectins to regulate the immune response due to the activation of the complement system, the enhancement of Mph activity, NK cells, and CTL. It was demonstrated, that lectins are triggers of proliferation and activation of Th1-lymphocytes and enhance the cytokine production, including IFN-γ, TNF-α, and IL-6 [9—11].

The aim of our study was to evaluate the effect of lectin from *B. subtilis* IMV B-7724 on the activity of peritoneal Mph, NK cells, and CTL in the growth kinetics of a metastasizing Lewis lung carcinoma (LLC), which is currently considered a reproduced syngeneic mouse model of human lung cancer [12].

Materials and Methods

Animals. The study was carried out on male C57Bl mice 2—2.5 months old weighting 19—20 g, bred at the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR). The use and care of the experimental animals were performed in accordance with the standard international rules on biological ethics and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [13] and approved by the Institutional Animal Care and Use Committee.

Experimental tumor. Standard metastasizing LLC strain was used in the experiment. Cancer cells were kindly granted by the Bank of Cell Lines from Human and Animal Tissues, IEPOR NASU.

Bacterial lectin. The *B. subtilis* IMV B-7724 strain of spore-forming, aerobic, gram-positive bacteria was used as a source of lectin. Lectin was isolated from the cultural fluid on day 4 of culturing as described in [14], freeze-dried at temperatures between –32 °C and +24 °C and stored as a powder at –20 °C.

Design of the experiment. Animals were distributed in 3 groups, 12 animals per group: intact control; untreated tumor-bearing mice injected with 0.9% NaCl solution; and the experimental group — tumor-bearing mice that were subcutaneously injected with *B. subtilis* IMV B-7724 lectin (at a dose of 1 mg/kg of body weight, for 10 days starting from day 2 after the tumor grafting). The immunological testing was performed on days 14, 21, and 28 after tumor grafting. The cytotoxic activity (Mph, NK, CTL), production of nitric oxide (NO), and arginase activity (Arg) were determined.

Peritoneal Mph and spleen lymphocytes were isolated by the standard methods. The mononuclear cells of the spleen were placed on a Ficoll-Verografin gradient (ρ = 1.077 kg/m^3) and centrifuged (550 g, 30 min). After two consecutive washings (medium 199, 550 g for 10 min), lymphocytes were resuspended in RPMI-1640 medium with 10% of bovine serum (Sigma, USA).

Cytotoxic activity (CTA) assay. CTA of the peritoneal Mph and spleen lymphocytes (CTL and NK) was determined by MTT-assay [15]. LLC cells were used as a target for peritoneal Mph and CTL; K562 cells were used as a target for NK. In brief, target cells (2×10^4 cells/well) in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics were placed in flat-bottom 96-well plates where Mph (4×10^5 cells/well) or lymphocytes (10^6 cells/well) were adhered beforehand and incubated for 18 h in a 100% humidity atmosphere with 5% CO_2 at 37 °C. The control wells contained target cells or effector cells only. Then 0.01 ml of MTT solution/well (5 mg/ml, Sigma, USA) was added, and incubation continued for 2 h. Then the plates were centrifuged (550 g for 15 min) and washed twice with 0.9% NaCl solution. After that, 0.12 ml of 2 M KOH and 0.14 ml of dimethyl sulfoxide (50% solution) were added into each well. Optical density was measured at λ = 545 nm *vs.* λ = 630 nm using a microplate ELISA reader (StatFax-2100, USA). Each sample was done in triplicate. The cytotoxic activity index (CTAI, %) was calculated by the formula:

$$CTAI = [1 - (OD_{effector+tc} - OD_{effector})/ /(OD_{tc} - OD_{blank})] \cdot 100\%,$$

where $OD_{effector}$ is the optical density of wells in which only effector cells were incubated; OD_{tc} is the optical density of wells in which only tumor cells were incubated; $OD_{effector+tc}$ is the optical

density of wells in which tumor cells and effector cells were incubated; OD_{blank} is the optical density of wells with the culture medium only.

Griess reaction. The content of stable NO metabolites was assessed by the standard Griess reaction [16]. In brief, peritoneal Mph suspensions (2 x 10^6 cells/well) were placed in a volume of 200 µl in 96-well flat-bottom tissue culture plates and cultured for 24 h. Each cell culture was investigated in duplicate. At the end of the incubation period, the supernatants were collected and NO production was assessed through the accumulation of nitrite (as a stable metabolite of NO) by the Griess reaction. The aliquot of culture supernatant (100 µl) was mixed with an equal volume of the Griess reagent (Acros Organics, Belgium) and incubated for 1 h at room temperature in the dark. The reaction products were quantified by colorimetry at λ = 550 nm. The standard curve plotted by the results of measurements of the solutions containing known concentration of $NaNO_2$ was used for converting the absorbance to micromolar concentrations of NO expressed in µM NO_2 per 10^6 cells.

Measurement of Arg activity. Arg activity was determined based on urea measurement [17]. Peritoneal Mph were lysed by double freezing and thawing. Then 50 µl of 50 mM Tris-HCl (pH 7.4) and 10 µl of 50 mM $MnCl_2$ were added to each sample. The samples were heated at 56 °C for 10 min and upon addition of 100 µl of 0.5 M L-arginine (pH 9.7) heated for further 30 min (37 °C). The reaction was stopped with 800 µl of acidic mixture (1:3:7, 96% H_2SO_4:85% H_3PO_4:H_2O). Then 40 µl of α-isonitrosopropriophenone (Sigma — Aldrich, USA) was added to the solution, which was heated for 30 min (95 °C) and incubated for 30 min at 4 °C. Urea concentration was measured by spectrophotometry at λ = 550 nm. The optical density values were converted to the mass of urea based on the calibration curve of the standard urea solution. One unit of Arg activity means the amount of the enzyme hydrolyzing 1 µM of arginine per 1 min. The results are expressed as units/10^6 cells.

Statistical analysis. Statistical significance was evaluated by nonparametric Mann — Whitney U-test using Prism software Version 8.0. The statistical significance of the differences between examined groups was assessed as $p < 0.05$.

Results and Discussion

The antitumor and antimetastatic efficiency of lectin from *B. subtilis* IMV B-7724 has been described in our previous publication [18]. We demonstrated that the administration of lectin from *B. subtilis* IMV B-7724 to mice bearing LLC without surgical removal of the primary tumor does not cause a significant antitumor effect but led to a significant decrease in the number and volume of lung metastases compared to the tumor-bearing control ($p < 0.05$). In the present study, we have assessed whether the antimetastatic effect of lectin could be exerted via its influence on the activity of peritoneal Mph, NK cells, and CTL. The evaluation of the polarization state of peritoneal Mph and detection of the subpopulation that prevails in a certain period of tumor growth were carried out by determining the characteristics of L-arginine metabolism (NO level, Arg activity).

Peritoneal Mph of intact C57Bl mice were characterized by a high level of NO production and low Arg activity (53.76 NO_2^-/10^6 cells and 2.1 units/10^6 cells, respectively). The NO/Arg ratio for peritoneal Mph was 25.6. In the untreated LLC-bearing group, the NO/Arg ratio on days 14 and 28 of tumor growth decreased significantly compared to the intact mice (8.8 and 9.1 a.u. *vs.* 25.6 a.u., respectively, $p < 0.05$), while on day 21, this ratio increased (28.4 a.u. *vs.* 25.6 a.u., $p < 0.05$). The data indicate a gradual change in Mph polarization from the M1 phenotype to the M2 phenotype at different stages of LLC growth. That is, at the late stages of tumor growth (28 days), cells with protumorigenic

properties prevailed in the population of peritoneal Mph of the untreated mice.

In the group of the LLC-bearing animals injected with lectin, on day 14, the NO/Arg ratio was 15.5 a.u., on day 21 — 38.8 a.u., and on day 28 — 30.0 a.u. ($p < 0.05$ compared to the untreated tumor-bearing mice at all mentioned terms). Therefore, during the entire period of the study, cells with the phenotype and functional properties of M1 prevailed in the population of peritoneal Mph in mice of the experimental group.

Analogous results were obtained when studying the cytotoxic activity of peritoneal Mph mice at different stages of LLC growth (Fig. 1). The cytotoxic activity of Mph of intact mice was 25.2 ± 2.7%. Starting from day 14 of tumor growth, in mice with LLC, these indicators were significantly ($p < 0.05$) lower than in intact animals; the maximum decrease was noted on day 28 (8.8 ± 2.2 *vs.* 25.2 ± 2.7). In the experimental group, a significant increase in the cytotoxic activity of peritoneal Mph was noted at the late stages of tumor growth compared to both untreated and intact animals. The highest cytotoxic activity was observed on day 21; subsequently, it decreased insignificantly (53.2 ± 2.5% and 46.7 ± 2.1%, respectively). Thus, the analysis of the functional activity of peritoneal Mph in the dynamics of LLC growth indicated a change in the polarization of Mph using lectin from *B. subtilis* IMV B-7724 toward the M1 phenotype at the late stages of tumor growth, which may be related to the demonstrated antimetastatic effect.

The data we obtained correlate with the results of experiments on determining the direction of Mph polarization and the possibility of their repolarization at various stages of growth and metastasis of malignant tumors of various origins (tumors of the lung, prostate, colon xenografts, and lung metastases) [19, 20]. In particular, when studying the composition of stromal cells of lung adenocarcinoma, induced in A/J mice with urethane, after 24-week exposure to the carcinogen, changes in the polarization state of alveolar Mph from unpolarized cells to M2 Mph were demonstrated. According to the authors, such changes in Mph polarization contribute to the increased tumor growth not only by reducing the direct cytotoxic effect (decrease in NO production). A significant role is played by the participation of these cells in the formation of immunosuppression by disrupting the antigen presentation to other cells of the immune system, cytokine-mediated enhancement of the ability of myeloid suppressor cells to inhibit the proliferation of T cells, and inhibition of Th1 activity [19].

There is a sufficient amount of data that confirm the key role of Mph in the implementation of various links of the antitumor response due to the cytokines and chemokines produced by these cells [1, 21, 22]. In particular, IL-12 and tumor necrosis factor-α are among the cytokines produced by Mph M1, which enhance the proliferation and activation of NK. Due to the effect of IL-12 on Th0, their polarization to Th1 occurs and, accordingly, the activation of a cellular specific immune response [23].

Taking into account the above, we conducted a study of the changes in the cytotoxic activity of NK and CTL at different terms of LLC growth and also assessed the possibility of enhancing such activity using lectin.

In the group of tumor-bearing mice, at all studied time points, the indicators of the cytotoxic activity of NK and CTL were significantly reduced compared to the intact control (Fig. 2, 3). In the experimental group, the cytotoxic activity of NK on day 28 of tumor growth was 2.8 times higher ($p < 0.05$) than that of the untreated mice and 1.3 times higher than that of the intact control. CTL activity at the late stages of tumor growth was maintained at the level of intact control.

Such dynamics of fluctuations in the cytotoxic activity of NK correspond to the changes in the activity of Mph. The significant increase in NK activity (on day 28) was preceded by the activa-

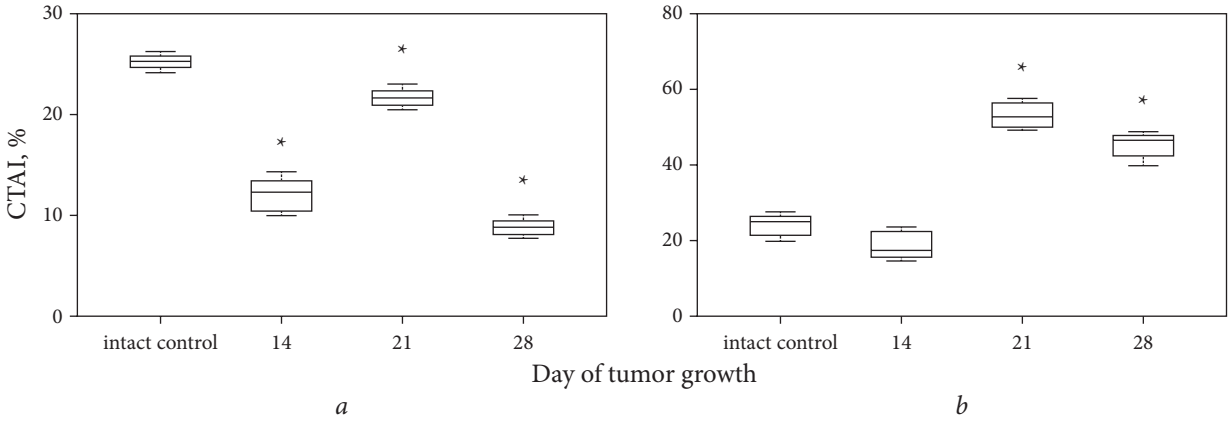

Fig. 1. Cytotoxic activity of peritoneal Mph in intact animals, untreated tumor-bearing animals (*a*), and experimental group (*b*). * $p < 0.05$ compared to the intact control group

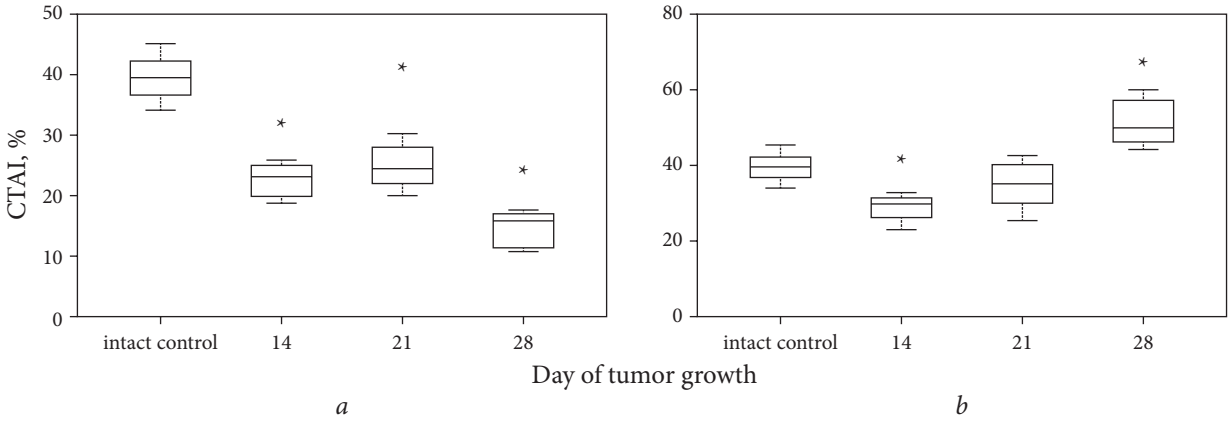

Fig. 2. Cytotoxic activity of NK in intact animals, untreated tumor-bearing animals (*a*), and experimental group (*b*). * $p < 0.05$ compared to the intact control group

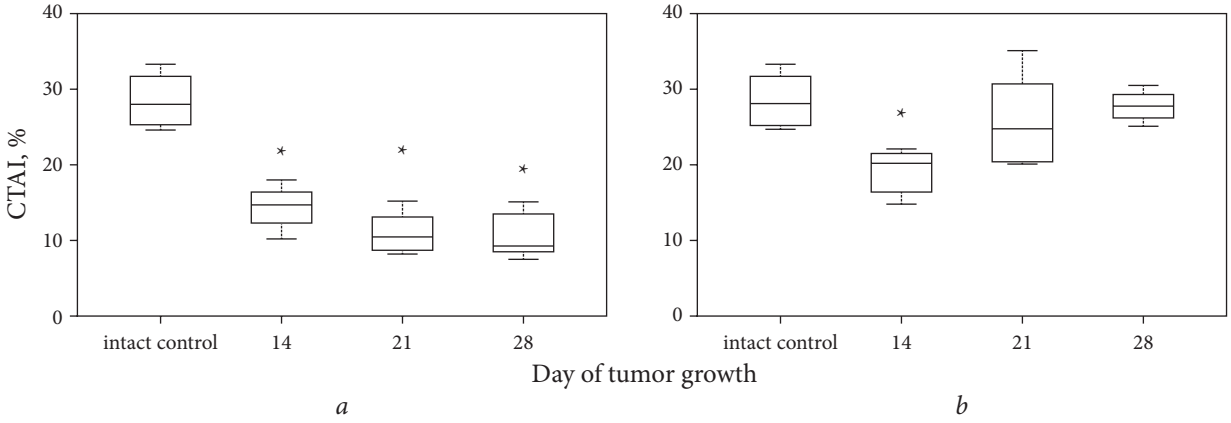

Fig. 3. Cytotoxic activity of CTL in intact animals, untreated tumor-bearing animals (*a*), and experimental group (*b*). * $p < 0.05$ compared to the intact control group

tion of Mph (day 21). Probably, the effect of the bacterial lectin on NK can be mediated through the soluble factors that are secreted by activated Mph. An increase in the cytotoxic activity may indicate the appearance of a number of cytokines in the systemic bloodstream (in particular, IL-1, IFN, TNF), which ensure not only the activation of the effectors of the innate immunity, but also support a specific immune response at the level of the intact animals. An important role in the implementation of the antitumor response is played by IFNγ, which is produced by T-lymphocytes, NK cells. This cytokine has a pronounced immunomodulatory effect due to its effect on almost all effector cells of the immune system [24—26]. Taking into account the information about the interferonogenic properties of lectins produced by related strains of *B. subtilis* [5], it can be assumed that the lectin from *B. subtilis* IMV B-7724 supports the activation of Mph, inducing the production of IFNγ by lymphocytes. That is, there is a mutual regulation and strengthening of the activity of the main effectors of the antitumor immunity. The effect of lectin on the CTL activity is moderately expressed and is probably mediated through the Th1 pathway.

Thus, the use of lectin from *B. subtilis* IMV B-7724 ensured the preservation of the cytotoxic activity of the main effectors of antitumor immunity (Mph, NK, CTL) during the entire period of LLC growth. Lectin had the least effect on the activity of CTL, that is, on the specific immune response. Its effect on the effectors of innate immunity was the most pronounced on days 21 (Mph) and 28 (NK). Probably, the maintenance of such a level of immune cell activities in the late term of tumor growth could in part determine the antimetastatic effect of the treatment.

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ВПЛИВ ЛЕКТИНУ B. SUBTILIS IMV B-7724 НА АКТИВНІСТЬ ЕФЕКТОРІВ КЛІТИННОЇ ЛАНКИ ПРОТИПУХЛИННОГО ІМУНІТЕТУ МИШЕЙ З КАРЦИНОМОЮ ЛЕГЕНІ ЛЬЮЇС

Мета. Оцінити вплив лектину *B.subtilis IMB B-7724* на активність макрофагів (Мф), природних кілерних клітин (ПКК) та цитотоксичних лімфоцитів (ЦТЛ) мишей з карциномою легені Льюїс (КЛЛ). **Матеріали та методи.** Дослідження проводили на мишах лінії C57Bl/6J; в якості експериментальної модельної пухлини використано карциному легені Льюїс. Лектин вводили мишам з пухлинами підшкірно, в разовій дозі 1 мг/кг ваги (10 введень). Імунологічні дослідження проводили на 14-, 21-, 28-ій добі пухлинного росту. Цитотоксичну активність Мф, ПКК, ЦТЛ оцінювали в МТТ-тесті; вміст стабільних метаболітів оксиду азоту (NO) вимірювали за допомогою стандартної реакції Гріса; активність аргінази (Arg) оцінювали за вмістом сечовини. **Результати.** Введення лектину *B. subtilis IMV B-7724* мишам з КЛЛ без видалення первинної пухлини супроводжувалось протипухлинним та антиметастатичним ефектом. Останній, вірогідно, був обумовлений суттєвим ($p < 0,05$) підвищенням активності Мф та ПКК після завершення повного курсу лікування. У групі тварин, яким вводили лектин, відмічали суттєве зростання співвідношення NO/Arg: на 21-шу добу — 38,8 ум.од., на 28-му — 30,0 ум.од. (проти 9,1 та 28,4 ум.од. у мишей, які не отримували лектину). Такі дані свідчать про превалювання у тварин, яким вводили лектин, Мф з прозапальними та протипухлинними властивостями. Цитотоксична активність Мф перевищувала показники в мишей, які не отримували лектину, в 1,8 раза та в групі інтактного контролю — у 5,3 раза; ПКК — у 2,8 та 1,3 раза відповідно. Активність ЦТЛ на віддалених термінах пухлинного росту зберігалась на рівні інтактного контролю. **Висновок.** Протипухлинна та антиметастатична активність лектину *B.subtilis IMB B-7724* обумовлена збереженням протягом усього терміну пухлинного росту функціональної активності основних ефекторів протипухлинного імунітету – Мф, ПКК, ЦТЛ. Найбільш вираженим був його вплив на ефектори неспецифіного імунітету Мф та ПКК. Найменшою мірою лектин впливав на активність ЦТЛ, тобто на специфічну імунну відповідь.

Ключові слова: карцинома легені Льюїс, лектин *B. subtilis IMB B-7724*, антиметастатичний ефект, макрофаги, природні кілерні клітини, цитотоксичні лімфоцити.