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THE INFLUENCE OF *BIFIDOBACTERIUM ANIMALIS* AND LECTIN OF *B. SUBTILIS* IMV B-7724 ON THE ANTITUMOR IMMUNE RESPONSE OF MICE WITH EHRLICH ADENOCARCINOMA

Aim. To investigate the effect of bacteria of the genus *Bifidobacterium* and the extracellular metabolite of *B. subtilis* IMV B-7724 on the antitumor immune response of mice with a model tumor. **Materials and Methods**. The study was conducted on Balb/c mice with transplanted solid Ehrlich adenocarcinoma (ACE). Starting from the 2nd day after the transplantation of tumor cells, the animals of the experimental groups were treated with lectin of *B. subtilis* IMV B-7724 (s/c, 1 mg/kg of weight), *Bifidobacterium animalis* (*per os*, 7×10^5 CFU/mouse) or their combination. The immunological studies were performed on the 21st and 28th days of tumor growth. The functional activity of natural killer cells (NK), cytotoxic T-lymphocytes (CTL), as well as the ability of lymphocytes from the peripheral lymph nodes (PLN) to transform into blast cells under the influence of T- (Con A) and B-cell (LPS) mitogens were determined. **Results.** Administration of probiotic components to the mice with ACE led to the activation of innate immune responses, that is, to a significant increase in the cytotoxic activity of NK, especially in the case of their combined use. The NK cytotoxicity index was higher than that in the non-treated ACE-bearing mice and the intact control by 3.7 and 2.1 times, respectively ($p < 0.05$). Similarly, the highest specific cytotoxic activity of spleen lymphocytes was observed upon the combined use of the microbial preparations: the CTL cytotoxicity index was nearly 2.5-fold higher than in the non-treated ACE-bearing mice. The data on the ability of PLN lymphocytes to transform into blast cells under the influence of Con A and LPS indicated the preservation of the functional activity of lymphocytes in the animals of the experimental groups during ACE growth. **Conclusion**. Both *B. animalis* and lectin of *B. subtilis* IMV B-7724 have a significant influence on the effectors of the natural and adaptive immunity of mice with ACE. Their combined use was found to be the most effective.

Keywords: Ehrlich adenocarcinoma, lectin of *B. subtilis* IMV B-7724, *Bifidobacterium animalis*, antitumor resistance.

In recent years, more and more data on the role of endogenous human microflora in maintaining homeostasis and the formation of a number of athophysiological conditions, including cancer [1—3], have been published. The positive effect of microbiota on the human body is due to the

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formation of useful microbial products (enzymes, vitamins, etc.) and the metabolism of the substances that prevent or slow down the growth of pathogens. The interaction of microbiota with stromal and epithelial cells of the intestine is a necessary component for launching a number of the key regulatory functions such as control of the overgrowth of pathogens, regulation of metabolism, strengthening of the barrier function of the mucous membrane, and maintenance of immune homeostasis. However, the human microbiota is constantly changing under the influence of various internal and external factors. Any significant change in the quantitative and qualitative composition of the intestinal microbiota can lead to serious consequences including the development of diseases, such as irritable bowel syndrome, celiac disease, diabetes, obesity, neurological disorders, and cancer. To date, the role of bacteria in the occurrence of cancer of the oral cavity, esophagus, stomach, pancreas, liver, colon, and rectum has been demonstrated [4]. An imbalance of intestinal microbiota can not only contribute to the development of many local and systemic diseases but also affect the outcome of their treatment [2—4].

Endogenous microflora is also involved in the carcinogenesis of the mammary gland, which has its unique microbiota closely related to the physiological properties of this organ. The reports on the differences in the microbial composition between normal and malignantly transformed tissues, as well as between benign and malignant breast tumors, support the importance of the microbiota in tumor progression. In the course of the malignant growth, the representatives of the intestinal microbiota are also involved in a number of metabolic transformations, which significantly affect the physiological and pathological processes in the mammary gland, in particular, the metabolism of estrogens, bile acids, and short-chain fatty acids. The mechanisms of the influence of intestinal microbiota on the occurrence and progression of breast cancer include the direct effect of bacterial metabolites on tumor cells and their microenvironment, as well as the modulation of the activity of the immune and endocrine systems [5, 6].

The composition of the intestinal microbiota can be adjusted using probiotics, prebiotics, and synbiotics. Modulation of the intestinal microflora with the help of probiotics can prevent the development and progression of cancer, and can also be useful to support its effective treatment [7]. Intestinal probiotics affect the state of the host's immune system through the interaction with the lymphoid tissues of the intestinal lining, regulating both innate and adaptive immunity [8, 9]. Probiotics with protective and anticarcinogenic properties that can enhance the effectiveness of anticancer therapy include, in particular, the representatives of the genus *Bifidobacterium*. Bifidobacteria have numerous therapeutic properties (antioxidant, proapoptotic, and antiproliferative), which may determine the antitumor effect. These microorganisms regulate intestinal homeostasis using such mechanisms as strengthening the barrier function of the mucous membrane, inhibiting the growth and adhesion of pathogens, regulating the activity of immune cells, and modulating the immune system [10, 11]. Although the positive properties of *Bifidobacterium spp*. are well known, their role in cancer is still controversial, and the specific mechanism by which these bacteria stimulate the antitumor immune response has not yet been well understood.

Anticarcinogenic properties are also demonstrated by other saprophytic microorganisms — bacteria of the genus *Bacillus*. These bacteria are characterized by the production of metabolites, in particular exogenous lectins, which exert cytotoxic activity against tumor cells or immunomodulatory activity [12—14].

The relationship between the effect of bifidobacteria on the body and the action of immunotherapy agents is considered an important factor affecting the therapeutic effectiveness of the preparations. However, information on the role of bifidobacteria in carcinogenesis and their effectiveness in the treatment of patients with cancer of different types is contradictory. The question of the possibility of strengthening the anti-carcinogenic/immunomodulatory effect through the simultaneous use of different types of bacteria (or their metabolites) is also insufficiently studied. Therefore, the aim of this work was to investigate the effect of bacteria *Bifidobacterium animalis* and the extracellular metabolite of *Bacillus subtilis* IMV B-7724 on the antitumor immune response in mice with experimental tumors.

Materials and Methods

Animals and tumor strain. The study was carried out on female Balb/c mice (2—2.5 months old, weighting 22—25 g) raised in the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR NASU). The use and care of experimental animals have been performed in accordance with standard international rules on biological ethics and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [15] and were approved by the Institute Animal Care and Use Committee.

A solid variant of Ehrlich adenocarcinoma (ACE) was used as a model of tumor growth. Ehrlich tumor is a spontaneous adenocarcinoma of the murine mammary gland and is widely used as a syngeneic model of human breast cancer [16]. Cancer cells were kindly granted by the Bank of Cell Lines from Human and Animal Tissues, IEPOR NASU. Tumor cells were injected intramuscularly into the hind limb (5×10^5 cells/mouse).

Bacterial lectin was isolated from the cultural fluid of *B. subtilis IMV B-7724* and stored as a powder at −20 °C. This lectin is resistant to pH changes in the environment and has high thermal stability and good solubility in water and buffer solutions (in particular, in physiological solution) [17]. Bacterial lectin was subcutaneously administered to tumor-bearing mice at a dose of 1 mg/kg body weight.

In the experiment, lyophilized cells of *B. animalis* subsp. *lactis* BB-12 (Lek Pharmaceuticals, Ljubljana, Slovenia) were used as a probiotic. *B. animalis* cells were administered *per os* to the tumor-bearing mice (7×10^5 CFU/mouse per injection).

The scheme of the experiment. Animals were divided into 5 groups: IC — intact control (n = 8); ACE — control of tumor growth, tumor-bearing mice that were injected with 0.9% NaCl solution (n = 8); Lectin — tumor-bearing mice that were injected with lectin of *B. subtilis* IMV B-7724 (n = 8); *B. animalis* — tumor-bearing mice that were administered with *Bifidobacterium* (n = 8); Lectin + *B. animalis* — tumor-bearing mice that were administered with both bacterial lectin and *B. animalis* (n = 8).

The immunological testing was performed on days 21 and 28 after tumor grafting. The indicators of weight and cellularity of immunocompetent organs, cytotoxic activity of spleen lymphocytes (natural killer cells (NK) and cytotoxic lymphocytes (CTL)), and ability of lymphocytes of peripheral lymph nodes (PLN) to respond to mitogenic stimuli were determined.

Lymphocytes were isolated by standard methods. The mononuclear cells were placed on a Ficoll-verografin gradient ($\rho = 1.077$ 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 (FBS) (all reagents from Sigma, USA).

The indexes of weight and cellularity of immunocompetent organs were determined by the standard method of supravital staining with trypan blue [18].

Cytotoxic activity (CTA) assay. CTA was determined by MTT-assay [19]. ACE cells were used as a target for CTL; K562 cells were used as a target for NK. In brief, the target cells (2×10^4 cells/well) in RPMI medium supplemented with 10% FBS and antibiotics, were placed in flat-bottom 96-well plates, where lymphocytes (1×10^6 cells/well) had been adhered beforehand, and incubated for 18 h in a 100% humidity atmosphere with 5% CO_2 at 37 °C. 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 $\lambda = 545$ nm *vs.* $\lambda = 630$ nm using a microplate ELISA reader (StatFax-2100, USA). Each sample was done in triplicate. Cytotoxic activity index (CTAI, %) was calculated by the formula:

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

where $OD_{effector}$ — optical density of wells in which only effector cells were incubated; OD_{tc} — optical density of wells in which only tumor cells were incubated; $OD_{effector+tc}$ — optical density of wells in which tumor cells and effector cells were incubated; OD_{blank} — optical density of wells with the culture medium only.

Lymphocyte blast transformation assay was carried out as described in [20]. Lymphocytes were obtained from aseptically removed lymph nodes by

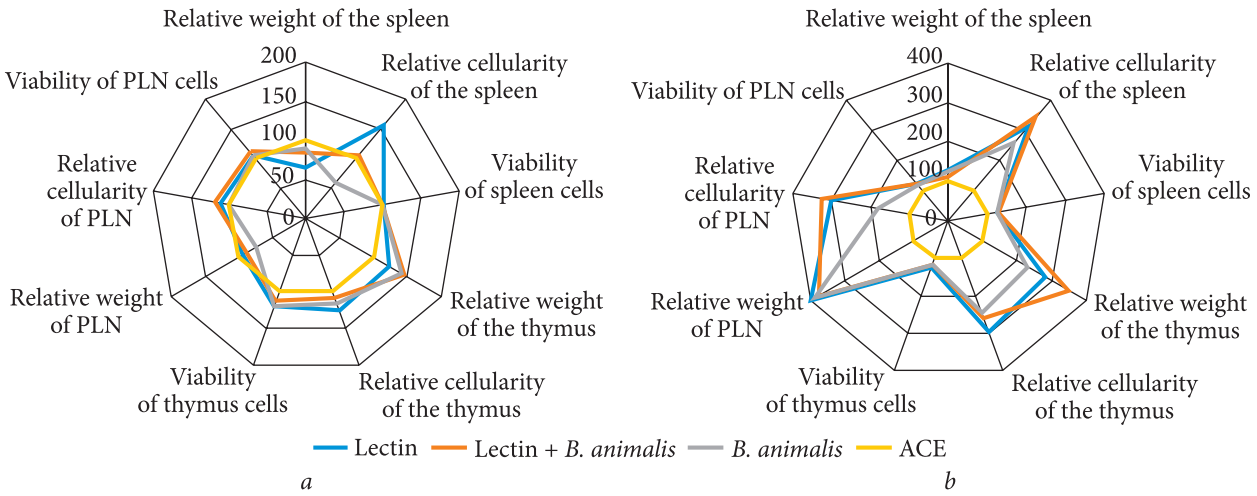

Fig. 1. Effect of *B. animalis* and lectin of *B. subtilis* IMV B-7724 on the weight and cellularity of immunocompetent organs of mice on the 21st (*a*) and 28th (*b*) days of Ehrlich adenocarcinoma growth. The parameters are given as percentages of the indicators of the ACE group, the levels of which are taken as 100%

homogenizing in a Potter homogenizer. Aliquots of lymphocytes (2×10^6 cells/mL) in RPMI-1640 medium supplemented with 10% FBS and 100 μg/mL gentamicin were transferred to flat-bottom plates (200 μL/well) and stimulated by 10 μg/well of concanavalin A (ConA, Sigma, USA) or 15 μL/well lipopolysaccharide (LPS, Sigma, USA) or left without stimulation (spontaneous reaction). The plates were incubated for 2 days at 37 °C in a 5% CO_2 atmosphere. The reaction was evaluated as described above.

Stimulation index, % (SI, %) was calculated by the formula:

$$SI = (OD_{mitogen} / OD_{spontaneous}) \times 100\%,$$

where $OD_{mitogen}$ — optical density of wells in which lymphocytes and mitogen were incubated; $OD_{spontaneous}$ — optical density of wells with only lymphocytes.

Statistical analysis. Statistical significance was evaluated by the nonparametric Mann — Whitney U-test, and correlation analysis was conducted according to Spearman`s correlation using Prism software Version 8.0. Statistical significance between examined groups was assessed as $p < 0.05$.

Results and Discussion

In our previous study [21], we have shown that administration of lectin of *B. subtilis* IMV B-7724 to animals with ACE, alone and in combination with *B. animalis*, resulted in significant inhibition of tumor growth (>50%) and an increase in the lifespan of mice with tumors (>50%). The use of *B. animalis* separately did not exert a pronounced effect.

The next stage of the study included an assessment of the effect of these microbial agents on the main innate and adaptive antitumor immune responses. Determining the indicators of weight and cellularity of the immunocompetent organs made it possible to assess both the presence of immunotoxic effects of the treatment and the development or suppression of the immune response. Thus, in untreated mice on the 28th day of ACE growth, we observed a significant decrease in the relative weight of the thymus and peripheral lymph nodes (PLN) compared to the indicators of intact animals (0.52 ± 0.08 *vs.* $1.86 \pm 0.27 \times 10^{-3}$; 0.49 ± 0.07 *vs.* $1.23 \pm 0.06 \times 10^{-3}$, respectively) ($p < 0.05$). A statistically significant decrease in the relative cellularity of the spleen (by 6.6 times, $p < 0.05$), thymus (by 2.7 times, $p < 0.05$), PLN (by 2.3 times, $p < 0.05$), as well as the number of living cells in these organs were recorded. Therefore, the growth of the tumor was accompanied by the involution of the thymus and a decrease in the number of viable lymphocytes in the PLN.

Fig. 1 shows the results of the analysis of the changes in the weight and cellularity of immunocompetent organs after administration of the lectin, *B. animalis*, or their combination to mice with ACE. The parameters are given as percentages of the indicators of the ACE group, the levels of which are taken as 100%. On the next day after the treat-

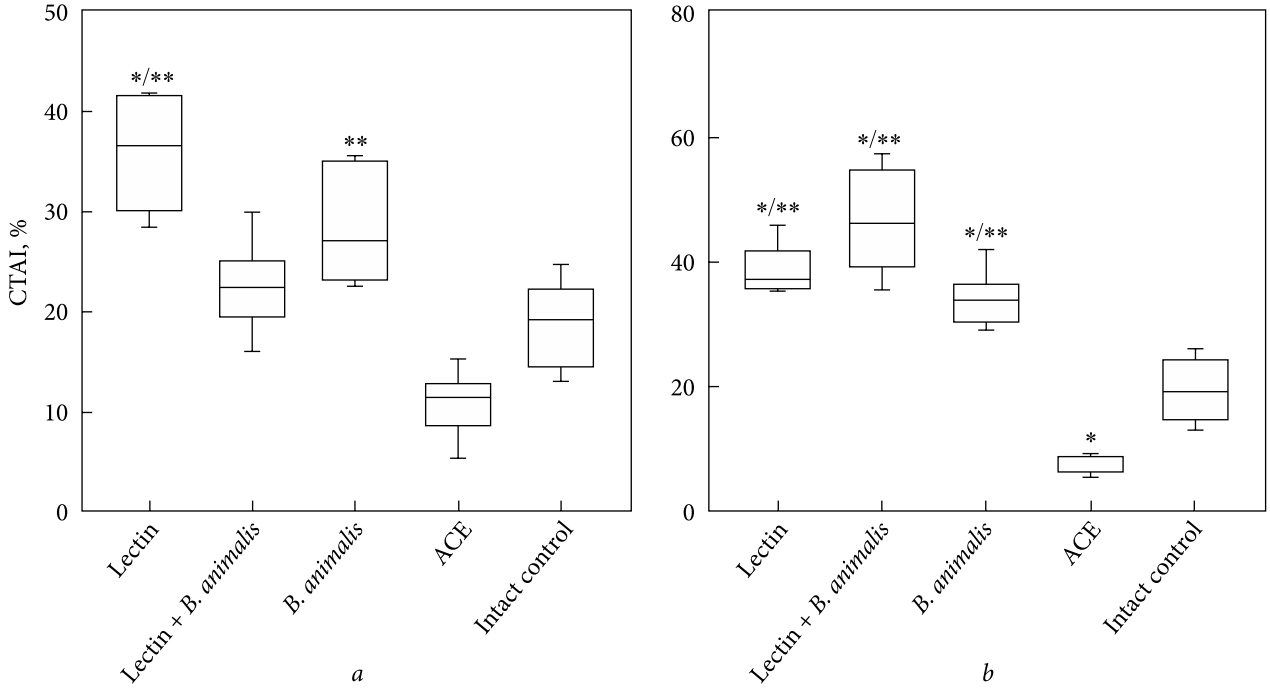

Fig. 2. Effect of administration of *B. animalis* and lectin of *B. subtilis* IMV B-7724 on the cytotoxic activity of NK cells of mice on the 21st (*a*) and 28th (*b*) days of Ehrlich adenocarcinoma growth. *$p < 0.05$ compared to the indicator of the intact control; **$p < 0.05$ compared to the indicator of the ACE group

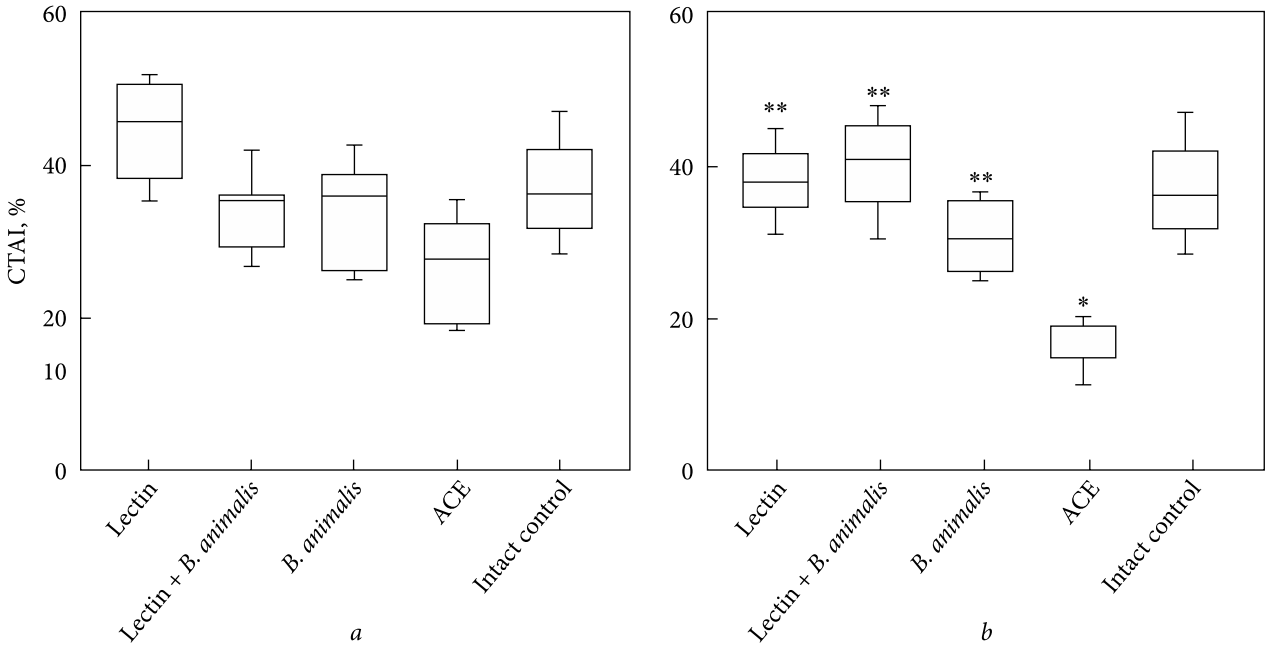

Fig. 3. Effect of administration of *B. animalis* and lectin of *B. subtilis* IMV B-7724 on the cytotoxic activity of CTL on the 21st (*a*) and 28th (*b*) days of Ehrlich adenocarcinoma growth. *$p < 0.05$ compared to the intact control; **$p < 0.05$ compared to the ACE group

ment course (the 21st day of tumor growth), almost all studied parameters did not significantly differ from the ones of the untreated animals with tumors. Just an increase ($p < 0.05$) in the number of cells in the spleen and thymus was noted in response to the lectin administration (Fig. 1, *a*). However, on the 7th day from the end of the treatment course (the 28th day of ACE growth), the

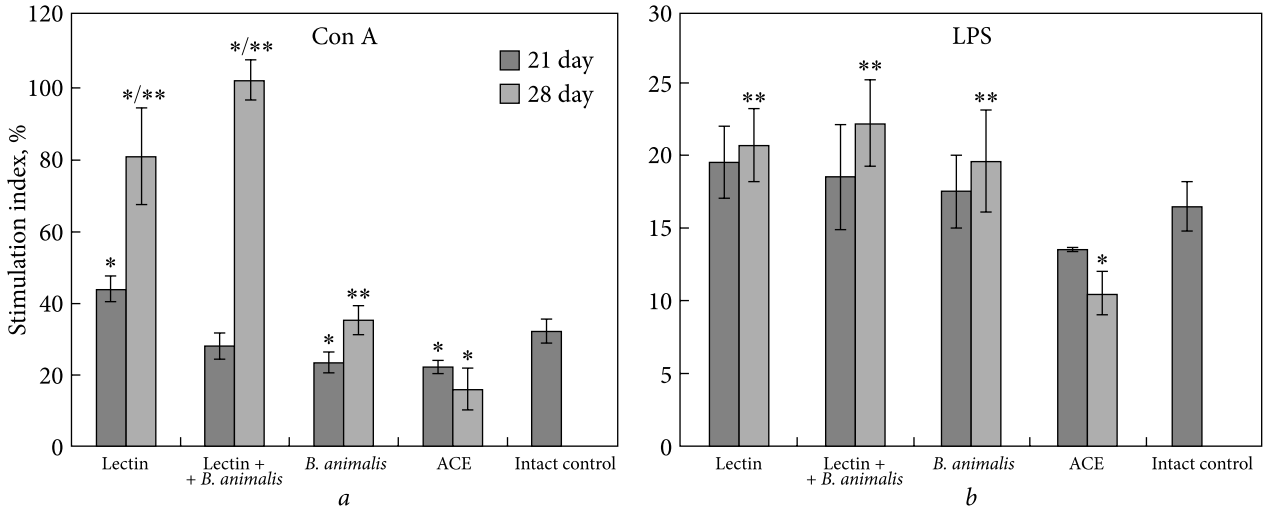

Fig. 4. Effect of administration of *B. animalis* and lectin of *B. subtilis* IMV B-7724 on the ability of PLN lymphocytes to transform into blast cells under the influence of T-cell (*a*) and B-cell (*b*) mitogens. *$p < 0.05$ compared to the intact control; **$p < 0.05$ compared to the ACE group

weight and cellularity of the immunocompetent organs in animals of all experimental groups reached the level of intact mice and significantly exceeded the indicators of control mice with ACE. Such changes were most pronounced under the condition of using the lectin, both separately and in combination with *B. animalis* (Fig. 1, *b*). The detected changes indicated the absence of immunotoxicity of the applied agents, as well as the active proliferation of lymphocytes and the possible activation of the immune response under the influence of bifidobacteria and lectin.

The results of the assessment of the functional activity of the effectors of innate immune responses (NK) and specific adaptive immune responses (CTL) testify to the activation of the immune response in mice with ACE after the course of administration of *B. animalis* and/or the lectin.

As seen in Fig. 2, tumor growth was accompanied by a gradual suppression of the cytotoxic activity of NK cells in mice of the ACE group: on the 21st day, the STAI was 13.8 ± 4.3%, on the 28th day — 10.9 ± 4.9% against 19.2 ± 3.9% in the intact control. Administration of *B. animalis* and/or the lectin resulted in the preservation of NK cytotoxic activity at the level of intact animals on the 21st day of ACE growth (Fig. 2, *a*). Subsequently, on the 7th day after the end of the treatment course (the 28th day of tumor growth), the cytotoxic activity of NK significantly increased compared to the indicators of both the ACE control group and intact mice ($p < 0.05$) (Fig. 2, *b*). The most pronounced effect was observed under the combined use of *B. animalis* and the lectin: in animals of this group, STAI was 40.8 ± 5.6%, while in the ACE group 10.9 ± 4.9%, and in intact control 19.2 ± 3.9%.

Similar results were obtained in the study of the specific cytotoxic activity of spleen lymphocytes. As shown in Fig. 3, on the 21st day of tumor growth, no significant difference was observed between the indicators of cytotoxic activity of CTL in animals of the experimental and control groups (Fig. 3, *a*). On the 28th day of ACE growth, we observed a significant suppression ($p < 0.05$) of CTL activity in untreated mice with tumors compared to intact controls (STAI was 17.3 ± 3.5 and 36.6 ± 2.3%, respectively). Under the use of *B. animalis* and the lectin, alone or in combination, CTL activity remained at the level of the intact control and was significantly higher than that of animals of the ACE group. As for NK, the greatest activity of CTL ($p < 0.05$ compared to ACE) was observed in the case of the combined use of these agents (Fig. 3, *b*). That is, the investigated microbial products contributed to the preservation of a specific immune response in animals with ACE even at the terminal stage of the tumor development.

The preservation of the functional activity of lymphocytes in the animals of the experimental groups throughout the observation period was evidenced by the results of the evaluation of the ability of PLN lymphocytes to transform into blast cells under the influence of T-cell (Con A) and B-cell (LPS) mitogens. An activation of blast transforma-

tion of T-lymphocytes in response to Con A was observed in groups of animals treated with the lectin both alone and in combination with *B. animalis* (Fig. 4, *a*). Moreover, in the latter case, this effect was maximally expressed: the T-lymphocyte stimulation index in mice of this group exceeded that of the ACE group by 6.4 times and that of the intact control group by 3.2 times (in both cases, $p < 0.05$). The use of both microbial preparations also led to an increase in the response of lymphocytes to LPS (Fig. 4, *b*). The indicators of blast transformation of B-lymphocytes under the influence of LPS in animals of all experimental groups were at the level of the intact control and significantly exceeded those in the untreated ACE group.

The results of evaluating the parameters of the functional activity of lymphocytes of PLN and the spleen in mice with ACE demonstrate the influence of both *B. animalis* and the extracellular lectin of *B. subtilis* IMV B-7724 on the effector cells of natural (NK cells) and adaptive (T-lymphocytes) immunity. Their combined use was found to be the most effective. Analyzing our findings and the literature data, one can assume that the activation of NK cells is largely due to the action of the lectin while bifidobacteria play a significant role in increasing the cytotoxic activity of CTL.

The mechanisms of antitumor activity of lectins are related to both direct cytotoxic effect on tumor cells and the indirect effect due to the modulation of immune reactions. In our previous studies on two experimental tumor models (ACE and Lewis lung carcinoma), we have shown that the use of lectin of *B. subtilis* IMV B-7724 as a means of immunotherapy promotes the activation of all the main effectors of antitumor immunity, namely, macrophages, NK, and CTL. The expression of the changes in the activity of these cells was correlated with such characteristics of the tumor development as the growth rate of the primary tumor, the dynamics of metastasis, and the life expectancy of animals with tumors. However, we have noted that the most pronounced effect of the lectin on the effectors of non-specific immunity (macrophages and NK cells), the effect on CTL activity, that is, on the specific immune response, is much less pronounced [14, 21].

According to the literature, representatives of various strains of *Bifidobacterium* administered orally are able to modify the immune response due to the activation of various types of immune cells such as dendritic, NK, and $CD8^+$ and $CD4^+$ T-lymphocytes. However, the largest number of data evidences the ability of bifidobacteria to enhance the antigen-presenting properties of dendritic cells and, accordingly, to cause the activation of specific immune responses. The use of *Bifidobacterium spp.* includes the following aspects of immunoregulatory mechanisms: in the immune microenvironment of the tumor, bifidobacteria contribute to dendritic cell-dependent differentiation of $CD4^+$ helper T-lymphocytes of type I (Th1); the activation of macrophages leads to the stimulation of IL-12 production and further enhancement of the recruitment of tumor-specific CTL antigens; due to the spectrum of produced Th1 cytokines, the activity of antigen-specific $CD8^+$ T-lymphocytes increases; an immune response is induced against microbial antigens that can cross-react with tumor-associated antigens [23—26]. That is, the activating effect of bifidobacteria is mostly aimed at the reactions of specific immunity.

In conclusion, our results evidence that the use of probiotics and microbial metabolites (in particular, lectins) can effectively prevent the suppression of the functional activity of effectors of antitumor immunity, which occurs against the background of tumor growth. The mechanisms of microbial modulation of antitumor immunity reactions and the determination of the influence of various representatives of the endogenous microflora on the immune system require further research.

Acknowledgment

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ВПЛИВ *BIFIDOBACTERIUM ANIMALIS* ТА ЛЕКТИНУ *B. SUBTILIS* IMB B-7724
НА ПРОТИПУХЛИННУ ІМУННУ ВІДПОВІДЬ МИШЕЙ З АДЕНОКАРЦИНОМОЮ ЕРЛІХА

Мета. Дослідити вплив бактерій роду *Bifidobacterium* та позаклітинного метаболіту *B. subtilis* IMB B-7724 на протипухлинну імунну відповідь мишей з модельним пухлинним процесом. **Матеріали та методи**. Дослідження проведено на мишах лінії Balb/c; в якості експериментальної модельної пухлини використано солідну аденокарциному Ерліха (АКЕ). Починаючи з 2-ї доби після трансплантації пухлинних клітин тваринам дослідних груп, вводили лектин *B. subtilis* IMB B-7724 (п/ш, по 1 мг/кг маси), *Bifidobacterium animalis* (per os, по 7×10^5 КУО/мишу) або їх комбінацію. Імунологічні дослідження проводили на 21— та 28 доби росту пухлини. Визначали параметри функціональної активності природних кілерів (ПКК), цитотоксичних Т-лімфоцитів (ЦТЛ), а також здатність лімфоцитів периферичних лімфатичних вузлів трансформуватись у бластні клітини під впливом Т- та В-клітинних мітогенів. **Результати.** Введення пробіотичних компонентів мишам з АКЕ приводило до активації неспецифічної імунної відповіді, зокрема до суттєвого збільшення цитотоксичної активності ПКК, особливо за умови комбінованого застосування *B. animalis* та лектину *B. subtilis*: індекс цитотоксичності ПКК перевищував показники групи АКЕ в 3,7 рази ($p < 0,05$), інтактного контролю — в 2,1 рази ($p < 0,05$). Аналогічні результати отримано при дослідженні специфічної цитотоксичної активності лімфоцитів селезінки: при комбінованому застосуванні мікробних препаратів активність ЦТЛ у 2,5 рази перевищувала показник групи АКЕ. Результати оцінки здатності лімфоцитів периферичних лімфатичних вузлів трансформуватись у бластні клітини під впливом мітогенів Con A та LPS свідчать про збереження функціональної активності лімфоцитів у тварин дослідних груп протягом всього терміну росту АКЕ. **Висновок**. Результати дослідження продемонстрували наявність впливу *B. animalis* та лектину *B. subtilis* IMB B-7724 на ефектори природного та адаптивного імунітету мишей з АКЕ. Найбільш ефективним виявилось комбіноване застосування цих препаратів.

Ключові слова: аденокарцинома Ерліха, лектин *B. subtilis* IMB B-7724*, Bifidobacterium animalis,* протипухлинна резистентність.