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Efficiency of Xenogeneic Antitumor Vaccine *In Vivo*

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Abstract

The antitumor efficiency of Xenogeneic Antitumor Vaccine (XAV) which is constructed with chicken embryonic proteins of higher molecular weight and cytotoxic metabolite of *Bacillus subtilis* B-7025 was shown at the experimental models of tumor growth (Lewis lung carcinoma and Ehrlich carcinoma). The use of XAV in animals with tumor process and in animals with surgical tumor removal significantly reduces the metastatic potential of tumors by maintaining the functional activity of lymphocytes and macrophages and by stimulating the transformation of plasminogen to angiostatin. At the later stages of tumor growth the antibody-dependent cytotoxicity of lymphocytes and macrophages increased in animals which were vaccinated.

Keywords: Cancer vaccine; Circulating immune complexes; Cytotoxic activity; Tumor growth inhibition

Introduction

The limited possibilities of the existing treatment of patients with cancer (especially at the late stages of the pathological progress) cause the search and the development of new more effective means of treatment. Higher hopes are put on biotherapy, which main objective is to develop methods for amplifying immune response of the body. The main methods are the usage of Antitumor Vaccines (AVs), co-stimulatory cytokines and molecules to enhance antitumor immune response.

The autologous AVs are widely used in modern medicine, which are constructed on the basis of tumor materials of patients. A common problem of such vaccines is that the Tumor-Associated Antigens (TAAs) have low immunogenic activity [1,2]. We could enhance immunological potency with the help of microbial-derived adjuvants. They are able to attract Toll-Like Receptors (TLRs) on the surface of lymphocytes to enhance the immune response and thus they can stimulate the synthesis of cytokines.

Another approach to overcome immune tolerance and induction of immune response against its own endogenous proteins is the use of foreign counterparts TAA [3,4]. Although most TAAs are conserved proteins may indicate their high degree of homology between proteins of human tumors and different animal species [5]. Therefore inter specific minor structural differences can be successfully used to induce a complete immune response to poorly immunogenic tumor antigens [6-8].

It is a well-known fact that tumors synthesize oncofetal antigens. These antigens are proteins that are expressed at certain stages of embryonic development. The usage of embryonic proteins as "universal" specific immunogens opens new possibilities for constructing AVs which are based on them [6,9].

The studies and projects of RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology gave a chance to develop a new approach to the creation of anti-tumor immunological products, based on the use of extracts of embryonic xenogeneic proteins with microbially-derived adjuvants [10,11]. The constructed Xenogeneic AV (XAV) consists of chicken embryonic proteins of high molecular weight and cytotoxic metabolite of *Bacillus subtilis* B-7025.

Materials and Methods

In vivo animal studies

Research of antitumor efficacy of CEP was carried out *in vivo* in mice C57Bl (males 2.5 months, 20-22 g, vivarium RE Kavetsky IEPOR NAS of Ukraine). Animal management and work with animals are carried out according to international common rules of research with experimental animals. Lewis Lung Carcinoma (LLC) was used as experimental model. LLC was injected intramuscular in

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the thigh hind limb (3×10^5 cells per animal).

Animals study groups:

1. Control group of tumor growth without surgery (without surgery);

2. Group of animals without tumor surgery, using vaccinotherapy (without surgery + XAV);

3. Group of animals with Removed Tumor (RT);

4. Group of animals with removed tumor, using vaccinotherapy (RT + XAV)

Drug treatments

Xenogeneic Antitumor Vaccine (XAV) was constructed and studied at RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Science of Ukraine. The XAV consists of chicken embryonic proteins of high molecular weight, which are available from EDTA extraction (2% in Tris buffer, 1 h) [12], and cytotoxic metabolite of *Bacillus subtilis* B-7025 which has an effect on the CEPs. Whereas CEPs change and become more immunogenic. Xenogeneic Anticancer Vaccine (XAV) composed of chicken embryonic proteins of high molecular weight (0.3 mg/ml of the vaccine) and cytotoxic metabolite of *Bacillus subtilis* B-7025 (0.3 mg/ml of the vaccine) was used for vaccine therapy [14]. Vaccination started on the second day after tumor transplantation (0.3×10^6 tumor cells per animal in the thigh of the animal) and continued for 5, 12 and 19-day of tumor growth (0.3 mg/ml in 0.3 ml per animal per injection). At the operated animals vaccine therapy started on the second day after tumor removal (day 15 and tumor growth) and continued at 17, 24 and 31-day. The slaughter of animals and taking sampling material for immunological studies were made on the 36^{th} day after tumor transplantation, according to the conditions of international convention.

Methods of experimental oncology

The dynamics of growth of the primary tumor was determined by the change of volume of the primary tumor node using the formula: $V=1/6\ \pi d^3$, where d - the diameter of the primary tumor site.

The survival rate of animals in each experimental group was determined by the percentage of alive animals in the group to the total number of animals that were taken at the beginning of the experiment.

The average life expectancy of animals in each group was determined by the life expectancy of each mouse in a group.

Ex vivo metastasis assays

The level of metastases in lungs was determined by counting the number and the square of lung metastases that were squashed between two special lenses, the distance between them was 0.25 mm.

The volume of metastases was determined by the formula: $V= \pi r^2 \times 0.25$, where r: radius of metastases.

Electrophoresis

Electrophoretic assessment of the components of the vaccine or tumor extracts was carried out using SDS electrophoresis in 12% polyacrylamide gel. The machine LKB (the USA) was used as the current source, the voltage was 180W and the electric current intensity was 40 mA. The protein was dispensed in quantity of 0.3 mg/ml 30 mcL per well. Color Burst Electrophoresis Marker (M.W. 8,000-220,000), Sigma (USA), C1992 was used as markers in quantity of 5 mcL per well.

Immunoblotting

Immunoblot tests were carried out according to the standard protocols. Transfer of proteins was carried out passively in sandwich system at the nitrocellulose paper Invitrogen (USA) (0.2 μm pore size), blocking of the non-specific sites was made by using 2% solution of BSA (Sigma-Aldrich, A7906, USA). Anti-mouse IgG (whole molecule) - alkaline phosphatase, developed in goat, affinity isolated antigen specific antibody was used as antibodies for detection. Heat shock proteins were detected using antibodies HSC70/HSP70, mAb (N27F3-4) (AP conjugate) (Enzo, USA). 5-Bromo-4-Chloro-3-Indolyl Phosphate p-toluidin salt (BCIP) (5 mg/4 ml of 0.1 M Triss-HCl (pH=90) (Sigma, USA)) was used as the substrate. Serum of animals diluted five times was used in this test [13].

ELISA

The presence of cross-reacting of sera among chicken embryonic proteins and tumor proteins, and levels of IgG to tumor antigens were determined in ELISA. The antitumor antibodies in serum were evaluated by ELISA with the use of tumor antigens (50 μg per well); the last ones were obtained by a triple freeze/thaw procedure on the tumor cells (S37, LLC, B16 melanoma and Ehrlich cancer). The extracts from tumor cells were obtained by the same method which was used for the embryonic [12] with further centrifugation of the lysate. Serum (diluted 1:1000) and anti-mouse immunoglobulin (Fab Specific) labeled with peroxidase (Sigma-Aldrich, USA, Product number A9917) were used; orthophenyldiamine was used as substrate. ELISA results were recorded using a Tiertek Multiscan immunoenzyme analyzer (Finland) at a wavelength of 492 nm.

MTT assay

For determination the cytotoxic activity of immune cells the ratio of immune cells to target cells was 3:1, incubation time was 18 h, 5% CO_2, 100% of humidity, 37°C. MTT (Sigma, USA, M5655) in concentration of 5 mg/ml was added in quantity of 0.02 ml per well and MTT with cells were incubated for 4 h under similar conditions, after that it was washed twice. 0.12 ml (2 mol/l) of KOH and 0.14 ml of 50% DMSO solution were added to the sediment. Absorbance was measured at λ=540 nm. All samples were in triplicate. The results were calculated using the formula:

$$\frac{A_i + A_t - A_{i+t}}{A_i + A_t} \times 100\%, \textit{where}$$

A_i - absorbance in samples with Lph or Mph

A_t - absorbance in samples with target cells

A_{i+t} - absorbance in research samples

Antitumor effect was estimated by Inhibition Index (II) of the primary tumor, the quantity and the volume of metastases in the lungs. The functional activity of immune cells was evaluated in the MTT-assay according to ability to kill the target cells *in vitro* [15,16]. The research results were shown into cytotoxic index. The Cytotoxic Index (CI) was calculated using the formula: CI = (A-B)/A, where A is the percentage of viable tumor cells in the control group and B is the percentage of viable tumor cells in the experiment.

Influence of Serum (SC) was analyzed by Potentiation Index (PI): PI = [(ADCALph/Mph-CALph/Mph)/CALph/Mph] × 100%. Antitumor cytotoxic of Serum (SC) was also studied at MT-test.

Table 1: Level of responsiveness of serum of mice with tumors at the different components of CEPs in ELYSA test.

Type of tumor	Day	Proteins with Mr≈ >70 kD	Proteins with Mr≈ 30-50 kD	Proteins with Mr≈ 20-30 kD	Proteins with Mr≈ <20 kD
Sarcoma 37	14	**3.17 ± 0.037***	0.78 ± 0.043	0.98 ± 0.091	0.72 ± 0.014
	21	**3.03 ± 0.087***	0.53 ± 0.018	0.54 ± 0.044	0.47 ± 0.011
Ehrlich cancer	14	**3.24 ± 0.031***	0.42 ± 0.017	0.54 ± 0.092	0.44 ± 0.011
	21	**1.95 ± 0.035***	0.38 ± 0.019	0.37 ± 0.02	0.33 ± 0.001
LLC	14	**1.29 ± 0.019***	0.36 ± 0.011	0.36 ± 0.018	0.38 ± 0.019
	21	**1.25 ± 0.021***	0.4 ± 0.006	0.34 ± 0.003	0.37 ± 0.02

Note: * - p<0.05 comparing with other factions, compared with CEPs of ≤ 50 kDa

Table 2: Antitumor efficiency of the vaccine in animals with Lewis lung carcinoma on the 34th day of tumor growth.

Group	Mass of primary tumor, g	Frequency of metastasis, %	Quantity of metastases, n
Without surgery	0.67 ± 0.12	100	34.1 ± 7.9
without surgery + XAV	0.10 ± 0.06*	30.0	1.2 ± 0.9*
RT	-primary tumor was removed	82.0	3.6 ± 0.8
RT + XAV	-primary tumor was removed	12.0	0.12 ± 0.10*

Note: * - p<0.05 comparing with control group of tumor growth

Statistical analysis

Student's t-test was used for evaluation of the validity of differences (at P<0.05).

Numerical calculations and graph plotting were conducted using the application Origin Lab.

Results

Experimental study of using embryonic xenogeneic proteins for constructing antitumor vaccines

High level of identity between tumor antigens and surface proteins which were extracted from tissue of chicken embryo has been proven by Enzyme Immunoassay (ELISA). After immunization of one group of intact animal by tumor antigens, and another group by an extract of chicken embryo's surface proteins, we obtained the polyclonal antibodies with common property to respond to antigenic complexes from tumor cells (antigens of LLC and S37) and to respond to surface proteins of chicken embryo CEPs (Figure 1).

Analyzing the results of ELISA (Figure 2), it was found that in animals which were immunized with proteins of Sarcoma 37 (S37), there was an increase in the level of antibodies to these proteins in the serum by 30%. Analyzing the cross responses at CEPs, the level of antibodies increased by 80%, comparing to the response of serum in intact mice. In animals that were immunized with LLC, the level of antibodies in serum increased by 25% (towards protein LLC) and by 30% (towards CEPs).

Analyzing ELYSA results of some fractions of CEPs (Figure 3), it was found that serum of mice, that was obtained on the 14th and 21st day after tumor transplantation (S37, Ehrlich cancer , LLC), reacted strongly with fraction, which contains proteins with molecular mass more than 70 kD (Table 1).

A more detailed assessment was made using immunoblotting analysis. The test showed that serum of animals with tumors (S37, LLC, B16 melanoma and Ehrlich cancer) contacted with CEPs (45 kD to 120 kD). Different binding range may indicate considerable variability of tumor-associated antigens which are the part of the tumor model and have common antigenic determinants with CEPs. The most high-grade reaction was observed at the proteins with a molecular mass close to 84 and 89 kD (Figure 4).

Table 3: Cytotoxic activity of lymphocytes and macrophages of animals with Lewis lung carcinoma on the 34th day of tumor growth.

Group	CALph (CE, %)	CAMph (CE, %)
Without surgery	24.84 ± 0.64	25.73 ± 1.19
without surgery + XAV	36.03 ± 1.26*	35.76 ± 2.11*
RT	26.25 ± 4.54	29.68 ± 2.28
RT + XAV	29.26 ± 3.80	42.47 ± 1.67*

Note: * - p<0.05 comparing with control group of tumor growth

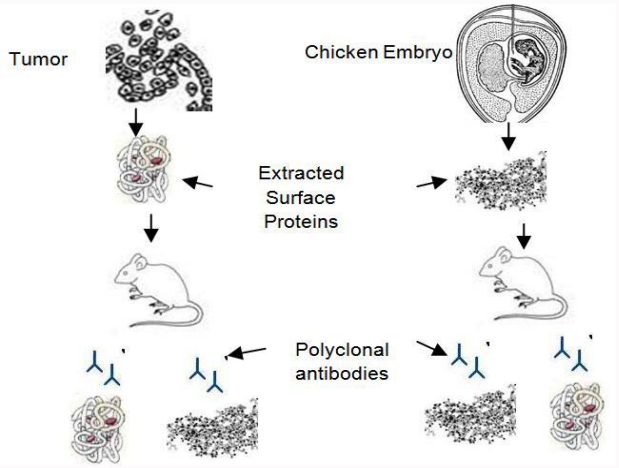

Figure 1: The scheme of studying the homology between the tumor proteins and xenogeneic embryonic proteins.

In addition, CEPs has heat shock proteins with molecular mass 70 kD (HSP-70) (Figure 5). The level of these proteins in fetal tissue is in 1.68 times higher than the level in EDTA extracts of Ehrlich carcinoma tissue. The heat shock proteins, which are included in complex, enable the presentation of antigens to antigen-presenting cells.

Thus, chicken embryonic proteins have partial homology to tumor proteins. In future they can be used as components in the construction of xenogeneic antitumor vaccines. The findings provided the basis for further study of the efficacy of antitumor vaccine, which are made of CEPs, *in vivo* in animals with typical tumor growth.

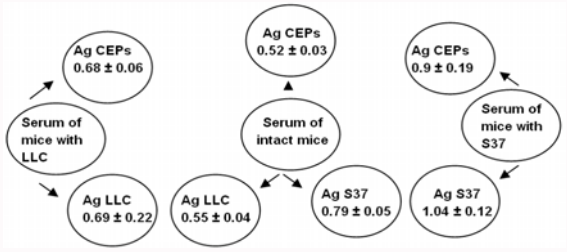

Figure 2: ELISA test of cross-responses between serum of mice which were immunized with tumor proteins and surface proteins of chicken embryo.

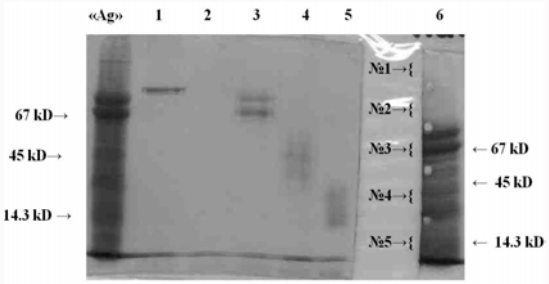

Figure 3: Electropherogram of the components of CEPs (1-5) which were derived from fragments of native phoresis gel of CEPs and which was used in the ELISA test (No. 6 and "Ah" - the protein profile of EDTA- extract of chicken embryo).

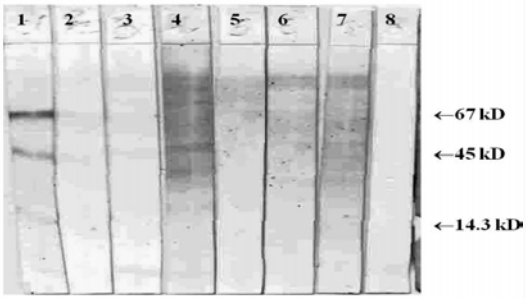

Figure 4: Immunoblot test with CEPs of serum of mice: 1) C57Bl/6 mice, immunized with CEPs; 2) Balb/c mice, immunized with CEPs; 3) intact mice; 4) Balb/c mice with S-37; 5) C57Bl/6 mice with LLC; 6) C57Bl/6 mice with B16 melanoma; 7) Balb/c mice with Ehrlich cancer. 8) Conjugate control.

Studying the efficiency of xenogeneic antitumor vaccine in animals with Lewis lung carcinoma

Animals with LLC, which were administrated XAV, have longer life expectancy, reduced size of the primary tumor and significant delay of development of metastases. Data of anticancer efficiency XAV in the groups of animals with tumor or after surgical removal of tumor are presented in Table 2.

Studying the immunological parameters, it was found that the levels of CALph and CAMph remain at a high level in vaccinated animals at the terminal stages of tumor growth comparing with those levels in unvaccinated animals (Table 3).

Thus, the usage of XAV, in operated and non-operated animals with LLC, significantly reduces the level of metastasis in the lungs by maintaining the functional activity of lymphocytes and macrophages.

Studying the efficiency of xenogeneic antitumor vaccine in animals with lewis ehrlich cancer

The vaccination scheme was the same as in the application of XAV in animals with Lewis lung carcinoma.

According to the results of these experiments, it was found that the

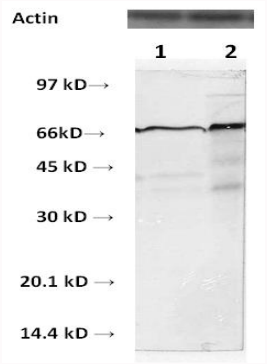

Figure 5: Immunoblot test of HSP-70 in extracts of Ehrlich cancer (1) and chicken embryo tissue (2).

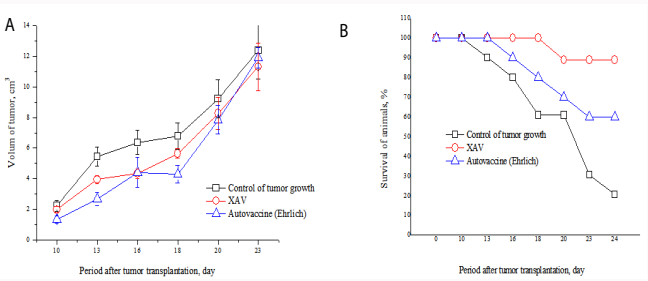

Figure 6: The dynamics of tumor growth (A) and survival of animals with Ehrlich cancer, (B) who were administrated the vaccine, made from homologous tumor or CEP.

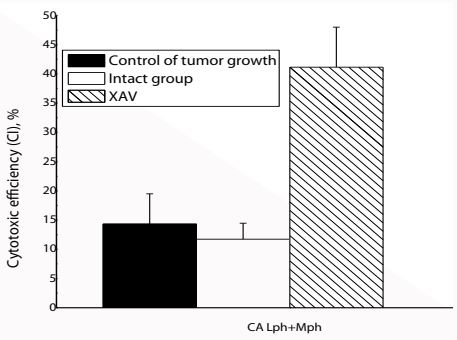

Figure 7: Cooperative cytotoxic activity of lymphocytes and macrophages (24th day of tumor growth).

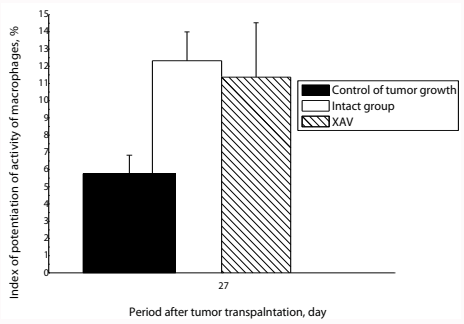

Figure 8: Index of potentiation of activity of macrophages, by adding zymosan in NBT-test.

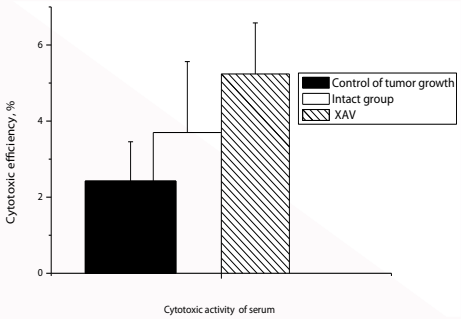

Figure 9: The cytotoxic efficiency of serum of animals with Ehrlich cancer.

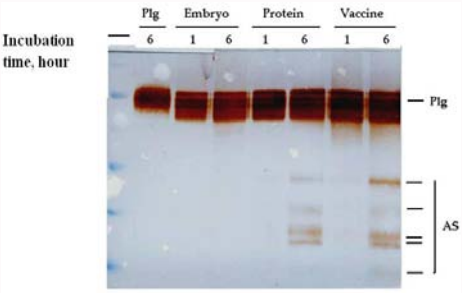

Figure 10: Hydrolysis of plasminogen to angiostatin during the incubation with examining drugs (Plg: Plasminogen; Embryo: chicken embryo proteins; Protein: Cytotoxic metabolite *B. subtilis* B-7025; Vaccine: Xenogeneic antitumor vaccines; AS: Angiostatins with different molecular weight).

vaccine based CEP has more prominent antitumor effect comparing with the vaccine, made from extracts of Ehrlich cancer cells. It was manifested in the retention of the primary tumor and increasing the life expectancy of experimental animals (Figure 6).

Analyzing the immunological data, it was found that the antitumor efficiency of XAV, which were made of CEP and metabolite *B. subtilis* B-7025, related to direct stimulation CALph and CAMph (Figure 7).

At the same time, the index of potentiation (changes in the respiratory activity after adding zymosan) of Mph in animals in the control group was reduced. This fact indicates the exhaustion of reserve capacity of Mph, while the vaccinated animals had the results which were very similar to the intact group (Figure 8).

The cytotoxic activity measures of serum in the control group of animals with tumor growth were reduced. This may indicate the low level of serum humoral factors with a direct cytotoxic effect. In the vaccinated group, this figure increases (Figure 9).

One of the possible mechanisms which provide the antitumor effect of XAV is its ability to stimulate the conversion of plasminogen to angiostatin. That has been shown by immunoblot (Figure 10).

So the results of studying the therapeutic potency of vaccines, which are based on CEP, demonstrate its significant antitumor efficacy. This efficiency manifested by stimulating the functional activity of cellular and humoral factors of immune system.

Discussion

The use of embryonic tissue homogenates in antitumor therapy began in the 30s of the last century. Ficher et al. were achieved of showing antitumor efficacy of extracts derived from fetal liver and thymus [17]. At the same time, it was shown that the administration of embryonic tissue to rats 2 to 3 weeks before Jensen sarcoma transplantation leads to tumor rejection. Nowadays the scientific society continues working on the use of drugs based on embryonic tissue in antitumor therapy and have received quite encouraging results. Animal embryos are used for manufacturing such drugs [18].

This work is a continuation of works that show the use of birds' embryonic proteins, as a material for making antitumor vaccines. It is shown that embryonic proteins with high molecular weight have partial homology with tumor proteins of tumor models; thereof they can be used as a universal antigenic material for vaccines. The cytotoxic proteins of *B. subtilis* B-7025 are used as the adjuvants in this antitumor vaccine. These cytotoxic proteins have been used for a long time for construction of autologous vaccines in Ukraine [19,20].

Newly constructed embryonic vaccine was very effective for the treatment of animals with model tumors (Lewis lung carcinoma and Ehrlich carcinoma), which was manifested in delaying the progression of primary tumor node and metastasis. Antitumor effect of the vaccine was shown by maintaining the functional activation of immune cells (lymphocytes and macrophages) and by the reduction of immunosuppressive factors in the serum of treated animals. In addition, the high level of angiostatin was fixed in the serum of animals, which probably blocks the development and formation of metastases.

Conclusions

1. The proteins that cross-react with sera was obtained from the extract of 7-day chicken embryos. The extracts were derived from animals with tumor process of different genesis (Lewis lung carcinoma, with S-37 and Ehrlich carcinoma) at different periods of tumor growth. In addition, it was shown that proteins with high molecular weight are more reactive comparing with proteins with low molecular weight. As there is the presence of high reactivity of embryonic proteins in the ELISA test with serum which was obtained from animals with tumors of different origins, they can be used as a material in the construction of cancer vaccines.

2. It is stated that the extracts obtained from 7-day chicken embryos have sufficiently high proportion of heat shock proteins with mol. mass 70 kD.

3. The antitumor vaccine, designed on the basis of a separate faction embryo extract (proteins with molecular weight above 50 kD) and cytotoxic metabolite of *B. subtilis* B-7025 makes an inhibitory effect on the development of primary tumor node and the development of metastases at the different models of tumor growth.

4. At the terminal stages of tumor growth, the high level of angiostatin was fixed in serum of animals treated with antitumor vaccine; angiostatin is one of the mechanisms of antitumor protection of the organism.

5. Antitumor effect of xenogenic antitumor vaccine was shown due to maintaining the functional activation of immune cells (lymphocytes and macrophages) and to the reduction of immunosuppressive factors in the serum of treated animals.

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