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USE OF XENOGENEIC VACCINE MODIFIED WITH EMBRYONAL NERVOUS TISSUE ANTIGENS IN THE TREATMENT OF B16-MELANOMA-BEARING MICE

I.M. Voeykova, N.I. Fedosova, O.M. Karaman, O.Yu. Yudina, G.V. Didenko, G.S. Lisovenko, L.M. Evstratieva, G.P. Potebnya

R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv, Ukraine

The aim **of the work was experimental study of anticancer efficacy of xenogeneic cancer vaccine (XCV) developed on the basis of rat embryonic nervous tissue and protein-containing metabolite of *Bacillus subtilis B-7015* (70 kDa), in B-16 melanoma-bearing C57Bl/6 mice.** ***Methods:*** **Immunological methods and methods of experimental oncology were used. Effects of XCV on primary and secondary organs of immune system of experimental animals, its anticancer and antimetastatic efficacy were evaluated.** ***Results:*** **It has been shown that XCV did not induced toxic effects on organism, and did not caused inflammatory reactions. The relation between the degree of XCV anticancer efficacy with the regimen of its use and the presence of primary tumor has been analyzed. It has been demonstrated that the developed XCV possesses significant antimetastatic activity if it is used after surgical removal of the primary tumor: in this case lung metastasis inhibition index reached 97.4%.** ***Conclusion:*** **High immunogenecity of new XCV creates perspectives for detailed study of its mechanisms of action.**

Key Words: **oncofetal antigens, xenogeneic cancer vaccine, B-16 melanoma, immunotoxicity, effectors of anticancer defence.**

Despite significant progress in the development of modern methods of anticancer therapy, an efficacy of treatment of cancer patients remains insufficient therefore the development of new approaches in this field is an actual task. Cancer biotherapy including cancer vaccine therapy shows promising results [1–4]. In experimental and clinical studies aimed on the development of immunotherapeutic means, an efficacy of cancer autovaccine use has been demonstrated [5, 6]. Also there were reported the data evidencing on higher efficacy of immunization with xenogeneic analogs of endogenous molecules, because such immunization is capable to overcome immunologic tolerance to tumor antigens and leads to significant suppression of cancer development [7, 8].

A special place in the development of biotherapeutical methods belongs to melanoma — a malignant tumor of neuroectodermal origin which develops from skin melanocytes. Melanoma cells are highly resistant to chemo- and radiotherapy, while local surgical removal of the tumor could not guaranty the development of recurrence and distant metastases [9, 10]. At the same time, it has been shown [9] that tumor antigens induce immune reactions resulting in tumor cell destruction and suppression of cancer development. So, one could conclude that immune therapy may be considered as an important element of systemic melanoma treatment.

An experimental model — B-16 melanoma — was firstly created by Roscoe B. Jackson in 1954 from ear skin region of C57Bl/6 mouse. This experimental tumor is characterized by high immunogenicity and significantly higher growth rate compared to other solid tumors. B-16 melanoma model is appropriate for the study of pathogenetic patterns and metastasis of the disease and for evaluation of new methods of melanoma treatment with CD40L/IFN-γ-matured, IL-12p70-producing DCs [11].

The published results concerning melanoma therapy are controversial and do not allow to chose the optimal ways for prophylaxis and treatment of this pathology. In regard to immune therapy, in clinical trials of xenogeneic vaccination of patients with uveal melanoma for prevention of hematogenous metastases, in 27 patients there has been recorded 49.7 months long recurrence-free period. These data allow consider xenovaccination as an available method for metastasis prophylaxis in uveal melanoma patients [12]. In patients with melanoma cross-immunization with vaccines comprising mouse (xenogenic) or human gp100 plasmid DNA, significantly improved patients survival, including at later stages of the malignant process. The authors have shown that the conjunction of vaccines with gp100 from different species increased vaccination effectiveness, also its biological safety was proved [13].

At present time there have been reported some data on the efficacy of use of embryonic tissues for therapy cancer patients. The use of embryonic tissues for generation of xenogeneic cancer vaccines (XCV) is based on antigenic similarity between tumor and embryonic cells due to expression of a number of antigens which play a key role in overcoming immune tolerance [14]. The means of embryonic origin act at epigenetic level through regulatory systems of an organism what could create grounds for the development of new biotherapeutical approach — construction of effective oncofetal cancer vaccines [14–16].

One of the promising approaches to their design is the use of embryonic nerve tissue. Its choice was

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*Correspondence: E-mail: irina_iepor@mail.ru
Abbreviations used: CIC – circulating immune complexes; MII – metastasis inhibition index; PCM – protein-containing metabolite of *Bacillus subtilis B-7015* with molecular weight of 70 kDa; PFENT – protein fraction of rat embryonic nervous tissue; XCV – xenogeneic cancer vaccine.

justified by the fact that tumor and embryonic cells are similar to each other due to the expression of a number of antigens that can be used to overcome immune tolerance in induction of antitumor response [15–17]. At the same time, embryonic nerve cells possess high adaptive capacity [18]. Therapeutic approaches to the application of embryonic tissues, including nervous, and switch on specific (substitute) and non-specific mechanisms that are based on the modulation process of regeneration, repair, proliferation and differentiation [18–20]. Disclosure of these mechanisms may be crucial for the development of new methods of treatment of diseases, including cancer [9, 21–23].

However, presently the strict criteria for construction of cancer auto- and xenogeneic vaccines are not worked out, and universal indications for vaccine therapy are not formulated as well as the doses and regimens of vaccination alone or in combination with other treatment modalities.

The aim of the work was experimental study of anticancer efficacy of XCV containing protein fraction of rat embryonic nervous tissue (PFENT) and protein-containing metabolite of *Bacillus subtilis B-7015* with molecular weight of 70 kDa (PCM) in mice with B-16 melanoma dependent on schemes and regimens of vaccination.

MATERIALS AND METHODS

The study has been carried out on male C57Bl/6 mice 2.5 months old weighting 18–19 g, bred in the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobilogy of NAS of Ukraine (IEPOR). The use and care of experimental animals have been performed in accordance with standard international rules on biologic ethics and was approved by Institutional Animal Care and Use Committee [24, 25]. In the work healthy laboratory animals were used; prior to experiment animals were carried for 14 days in quarantine conditions. Later on, animals were housed in a facility with constant temperature and received balanced nutrition.

As experimental model, mouse melanoma B-16 cells obtained from National Bank of Human and Animal Tissues of IEPOR, were used. This strain is maintained by passages in C57Bl/6 mice. For transplantation melanoma cell suspension prepared by primary tumor trypsinization according to the method [26], was injected *i.m.* in the right hind leg at a dose of $4 \cdot 10^5$ cells/mouse in a volume of 0.2 ml (for the study of vaccination at the background of tumor growth) or in a foot of right hind leg at a dose of $2.5 \cdot 10^5$ cells/mouse in a volume of 0.04 ml (for the study of vaccination after surgical removal of primary tumor). The course of tumor development was characterized by standard indices: frequency of tumor development, latent period of tumor appearance, number and volume of metastases.

In the study we used the XCV designed in the Department of Construction of Biotherapeutical Means. This vaccine contains protein fraction of nervous tissue from rat embryo of late gestation period (PFENT) and protein-containing metabolite (PCM) of *B. subtilis B-7025* with m.w. 70 kDa [27].

The studied vaccine was standardized by protein content ([C] = 0.3 mg/ml in total). Vaccine or its separate components (PFENT and/or PCM) were injected subcutaneously in the dorsum region in a volume of 0.3 ml/mouse.

Three series of experiments have been performed. In the first series intact mice were triply immunized with vaccine with 3 day intervals for analysis of its toxic, inflammatory and/or immunomodulating effects. At the 7th day after the last vaccination, there have been determined specific indices of weight and cellularity of immunocompetent organs (spleen, thymus, peripheral lymph nodes) by standard method of cell staining using trypan blue. Cytotoxic activity of natural killer cells (NK) has been determined by MTT test (K542 cells were used as targets in a ratio of 1:5 [28]); cytotoxic activity of peritoneal macrophages (PMP) was evaluated using NBT-test [29]. For analysis of peripheral blood indices, blood samples were collected in minitubes with EDTA solution and analyzed in automatic hemoanalyzer PCE-210 (Erma. Inc., Japan). Absolute content of leukocytes, platelets, erythrocytes, absolute and relative content of lymphocytes, monocytes, granulocytes, hemoglobin content have been determined [30]. The level of circulating immune complexes (CIC) in blood serum of mice was determined in precipitation reaction: high molecular weight CIC with the use of 3% polyethylene glycol (PEG), medium molecular weight CIC — 4.5% PEG, low molecular weight CIC — 6% PEG [30, 31].

In the second series, immunization was performed by therapeutic scheme at the background of B-16 melanoma growth. Vaccination has been performed by two schemes: at 1st, 7th, 14th days (scheme 1), and at 7th, 14th, 28th days after tumor cell transplantation (scheme 2). Antitumor effect has been evaluated by the rate of tumor development, duration of latent period, primary tumor growth dynamics, metastatic volumes, and life span of mice.

The third series of the study included animal immunization after surgical removal of primary tumor. Vaccination has been initiated in three days after radical surgery by following schemes: triply with 3-day intervals (scheme 3); triply with 7-day intervals (scheme 4). Indices of B-16 melanoma lung metastasis were evaluated at the 24th day after tumor removal (the 45th day of tumor growth). Vaccination efficacy was evaluated by metastasis inhibition index (MII). In all cases intact animals or unvaccinated animals with transplanted B-16 melanoma served a control. For comparison of parameters obtained in experimental and control groups the modulation indices were calculated [32].

Statistical analysis of the data was performed using Student's *t*-criterion. Values $p < 0.05$ were considered statistically significant [33].

RESULTS AND DISCUSSION

Immunization of intact C57Bl/6 mice with XCV or its separate components did not cause irritation and/or inflammation in the place of preparation injection and had no influence of animal weight (Table 1).

Table 1. Weight and cellularity of immunocompetent organs of vaccinated and not vaccinated C57Bl/6 mice at 7th day after last vaccination

Index	Group of animals (MI, %)			
	Intact animals (n = 10)	PFENT (n = 5)	PCM (n = 5)	Vaccine PFENT + PCM (n = 5)
Animal weight (g)	18.7±0.4	18.7±0.4	19.1±0.9	19.1±0.5
	(17.8÷20.4)	(0.0)	(+2.1)	(+2.1)
Relative organ weight ($\times 10^{-3}$):				
Spleen	6.1±0.9	7.5±0.6	8.8±1.5	7.2±0.8
	(4.0÷8.6)	(+22.9)	(+44.2)	(+18.0)
Thymus	2.1±0.3	1.6±0.2	1.7±0.4	1.8±0.2
	(0.9÷2.6)	(-23.8)	(-19.0)	(-14.2)
Peripheral lymph	3.3±0.2	3.8±0.3	3.2±0.3	2.9±0.3
nodes	(2.2÷3.7)	(+15.1)	(-3.0)	(-12.1)
Cellularity per 1 mg of organ				
Spleen	1.10±0.10	0.97±0.09	1.13±0.09	1.01±0.11
	(1.01÷1.32)	(-11.8)	(+2.7)	(-8.1)
Thymus	2.05±0.15	1.93±0.24	4.30±1.73**	1.96±0.09
	(1.78÷2.41)	(-5.8)	(+109.7)	(-2.9)
Peripheral lymph	1.75±0.15	1.40±0.30	1.61±0.21	1.40±0.12
nodes	(1.50÷2.18)	(-20.0)	(-8.0)	(-20.0)
Viable cells (%)				
Spleen	77.9±1.9	75.8±1.7	75.5±1.4	73.3±0.6**
	(75.0÷83.9)	(-2.6)	(-3.0)	(-5.9)
Thymus	88.5±0.5	88.6±1.4	91.3±2.3	89.1±0.7
	(87.8÷89.7)	(0.0)	(+3.1)	(+0.6)
Peripheral lymph	83.7±3.8	86.9±1.9	87.5±1.7	88.0±1.6
nodes	(76.1÷90.1)	(+3.8)	(+4.5)	(+5.1)

Notes: MI – modulation index of value compared to intact control; PFENT – protein fraction of rat embryonic nervous tissue; PCM – protein-containing metabolite of *Bacillus subtilis B-7015* with molecular weight of 70 kDa. Values are mean ± SE. *Significant at $p < 0.05$, **Significant at $0.1 < p < 0.05$ compared to intact control.

As one may see in Table 1, immunization of experimental animals has no significant influence on weight and cellularity indices of immunocompetent organs (spleen, thymus, peripheral lymph nodes) compared to that in intact control. It evidenced on the absence of toxic effects caused by vaccine and/or its components on central and peripheral organs of immune system of experimental animals. Introduction of the vaccine and its separate protein components also did not affect the studied indices of peripheral blood (Table 2).

We have observed just a decrease of leukocyte counts in response on XCV administration, which occurred possibly due to the decrease of total lymphocyte and monocyte counts; however, such event was transitory and did not affect animal's general state. All mentioned above evidenced on an absence of immunotoxic and inflammatory reactions.

The study of vaccination impact on effector reactions of unspecific immunity demonstrated the absence of significant alterations in intact and vaccinated mice. PMP counts and their activity in the NBT test in intact mice were $4.8 \pm 0.7 \cdot 10^6$ cells and 0.57 ± 0.01 optical units, respectively. The corresponding parameters in all immunized mice were at level of intact control. Administration of separate components (PFENT or PCM) led to suppression of cytotoxic activity of NK (8.9 ± 1.7 and 4.2 ± 1.4 optical units, respectively *vs* intact control: 18.8 ± 2.4 optical units, $p < 0.05$). In the case of vaccination with XCV containing both components such effect was not observed (15.4 ± 3.1 optical units). So, designed vaccine had no negative influence an activity of effector cellular pattern of unspecific immunity — NK and PMP.

Table 2. Peripheral blood parameters of intact and vaccinated C57Bl/6 mice (7th day after last vaccination)

Index	Intact mice (n = 10)	Experimental groups (MI, %)		
		PFENT (n = 5)	PCM (n = 5)	Vaccine PFENT + PCM (n = 5)
Leucocytes	8.95±0.57	7.08±0.86	6.92±1.21	5.00±0.72*
($\times 10^9$/l)	(7.7÷10.1)	(-20.9)	(-22.7)	(-44.1)
Erythrocytes	10.04±0.26	9.07±0.26*	9.41±0.36	9.65±0.35
($\times 10^{12}$/l)	(9.56÷10.56)	(-9.6)	(-6.2)	(-3.8)
Platelets	269.00±34.78	290.25±33.45	311.40±40.89	254.80±21.03
($\times 10^9$/l)	(216.0÷354.0)	(+7.9)	(+15.8)	(-5.3)
Hemoglobin	148.00±2.45	130.75±3.63*	129.00±7.07*	138.40±6.73
(g/l)	(143.0÷152.0)	(-11.7)	(-12.8)	(-6.5)
Leucogram				
Lymphocytes	6.28±0.37	5.28±0.76	4.96±0.97	3.52±0.60*
($\times 10^6$/l)	(5.4÷6.9)	(-15.9)	(-21.0)	(-43.9)
Monocytes	0.98±0.12	0.78±0.14	0.66±0.12	0.44±0.06*
($\times 10^6$/l)	(0.8÷1.2)	(-20.5)	(-32.3)	(-54.9)
Granulocytes	1.70±0.12	1.03±0.12*	1.30±0.28	1.04±0.31
($\times 10^6$/l)	(1.5÷2.0)	(-39.7)	(-23.5)	(-38.8)
Lymphocytes	70.10±0.80	74.20±2.92	71.32±3.16	70.70±5.31
(%)	(68.2÷71.5)	(+5.8)	(+1.7)	(+0.9)
Monocytes (%)	10.83±0.78	11.10±1.89	9.58±0.32	8.98±0.80
	(9.1÷11.9)	(+2.5)	(-11.5)	(-17.0)
Granulocytes	19.08±0.62	14.73±1.40*	19.08±3.40	20.30±5.49
(%)	(17.5÷19.9)	(-22.8)	(0.0)	(+6.4)

Notes: MI – modulation index of value compared to intact control; PFENT – protein fraction of rat embryonic nervous tissue; PCM – protein-containing metabolite of *Bacillus subtilis B-7015* with molecular weight of 70 kDa. Values are mean ± SE. *Significant at $p < 0.05$, **Significant at $0.1 < p < 0.05$ compared to intact control.

Safety of XCV has been evidenced also by CIC levels. As one may see in Figure, immunization did not elevate the content of low, medium and high molecular weight CIC. Their content in blood serum of mice from all experimental groups did not differ significantly from respective indices in intact controls. An absence of elevated level of medium molecular weight CIC after administration of studied vaccine and/or its components additionally evidenced on the absence of inflammatory processes caused by immunization. Level of low molecular weight CIC was also in the margins of intact control, i.e., immunization did not lead to synthesis of incomplete or monovalent antibodies which, being bound to antigen, do not eliminate it but, on the contrary, mask it from attack of immune system [33, 34].

In the second series immunization was performed by therapeutic scheme at the background of B-16 melanoma growth for determination of antitumor and antimetastatic activity of XCV. As it is presented in Table 3, immunization of mice by both schemes (1 and 2) had no effect on latent period of tumor development (visible tumors have developed on 9–12 days after transplantation), and the tumor rates (tumors developed in $80.0 \pm 10.3\%$ (12/15) and $86.7 \pm 8.8\%$ (13/15) of vaccinated animals respectively *vs* $94.1 \pm 5.7\%$ (16/17) in control group). Tumor growth dynamics in vaccinated animals did not differ from that in control group. Also vaccination had no significant influence on the rate, number and volume of metastases (Table 4).

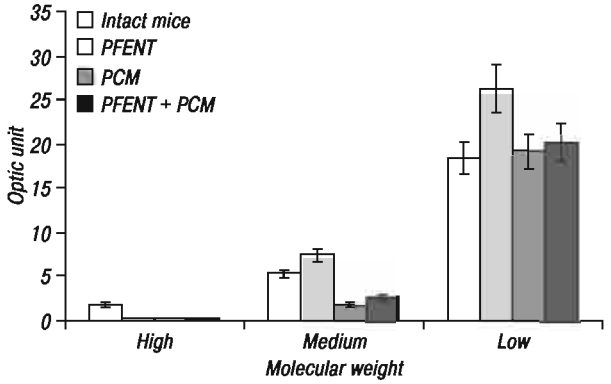

Figure. CIC content in blood serum of mice in response to vaccination (7th day after the last injection)

Table. 3. Growth of melanoma B-16 in unvaccinated and vaccinated C57Bl/6 mice

Group of animals	Efficacy of tumor transplantation (%)	Latent period of tumor formation (days)	Life duration after tumor transplantation (days)
Unvaccinated (n = 17)	94.1±5.7 (16/17)	11.6±1.9	31.2±1.2
Vaccinated by scheme 1 (n=15)	80.0±10.3 (12/15)	8.5±0.4	25.6±0.7*
Vaccinated by scheme 2 (n=15)	86.7±8.8 (13/15)	10.0±1.2	27.7±2.8

Notes: Scheme 1 – vaccination has been performed: at 1st, 7th, 14th days after tumor cell transplantation. Scheme 2 – vaccination has been performed: at 7th, 14th, 28th days after tumor cell transplantation. Values are mean ± SE. *Significant at $p < 0.05$ compared to unvaccinated mice.

Table 4. Metastasis of melanoma B-16 in unvaccinated and vaccinated C57Bl/6 mice

Group of animals	Metastases rate (%)	Metastases number	Volume of metastases (mm^3)
Unvaccinated (n=16)	92.3,0±7.1 (16/16)	6.6±1.1	8.1±5.4
Vaccinated by scheme 1 (n=12)	75.0±12.5 (9/12)	6.9±2.2	8.0±2.9
Vaccinated by scheme 2 (n=13)	91.7±7.7 (13/13)	13.7±5.2	11.3±5.2

Notes: Values are mean ± SE. Insignificant $p > 0.05$ compared to unvaccinated mice.

We suppose that an absence of antitumor and antimetastatic effects of vaccination could be related, firstly, to insufficient activation of effector cells of anticancer defense incapable to eliminate large amounts of B-16 melanoma cells, and secondly, to an action of immunosuppressive factors produced by tumor cells or immune cells of animals [34, 35].

To support this hypothesis, in the third series of our study we have analyzed effects of vaccination after surgical removal of primary tumor for prevention of recurrence and metastases development. Therefore, surgical removal of tumors was performed at 21st day of tumor growth. Evaluation of the results was carried out at 24th day after the surgery (45th day of tumor development).

As it could be seen in Table 5, immunization resulted in significant decrease of metastasis rate (metastases have developed in 41.7 ± 11.2% of control mice and in 16.7 ± 10.8% ($p < 0.05$) and 18.2 ± 11.6% ($0.05 < p < 0.1$) of animals immunized by schemes 3 and 4, respectively). The number and volumes of metastases were significantly lower only in the group of animals immunized by scheme 3: 0.3 ± 0.2 *vs* 4.6 ± 1.1 ($p < 0.05$), and 0.3 ± 0.2 mm^3 *vs* 0.8 ± 0.1 mm^3 ($0.05 < p < 0.1$), respectively, compared to the control.

These results coincide with the results of other authors [9, 36]. In particular, prophylactic use of xenogeneic vaccines developed on the basis of human melanoma cells with the following removal of tumor nodule led to decreased lung metastasis rate in mice with B-16 melanoma. According to author's opinion, such vaccines could be used with high efficacy for prophylaxis of recurrence in melanoma patients after surgical treatment [36].

Table 5. Metastasis in unvaccinated and vaccinated C57Bl/6 mice at 24th day after surgical removal of B-16 melanoma (day 45 after tumor transplantation)

Group of animals	Metastases rate (%)	MII (%)	Metastases number	Volume of metastases (mm^3)
Unvaccinated (n=12)	41.7±11.2 (5/12)		4.6±1.1	0.8±0.1
Vaccinated by scheme 3 (n=12)	16.7±10.8* (2/12)	97.4	0.3±0.2*	0.3±0.2**
Vaccinated by scheme 4 (n=11)	18.2±11.6 (2/11)	60.1	4.2±3.0	10.3±8.3

Notes: Scheme 3 – vaccination has been initiated in 3 days after radical surgery by following schemes: triply with 3-day intervals. Scheme 4 – vaccination has been initiated in 3 days after radical surgery by following schemes: triply with 7-day intervals. MII – index of metastasis inhibition relatively to unvaccinated mice. Values are mean ± SE. *Significant at $p < 0.05$ vs unvaccinated animals; **significant at $0.1 < p < 0.05$ vs unvaccinated animals.

So, triple introduction of XCV or its separate components to intact C57Bl/6 mice had no immunotoxic effect on experimental animals and did not cause inflammatory reactions. In a model of B-16 melanoma it has been shown that its use is accompanied with antitumor and antimetastatic effect only in the case of surgical removal of primary tumor. In mice that underwent surgical removal of tumors and were immunized by scheme 3 we have registered significantly decreased metastasis rate ($p < 0.05$), 15.3-fold decrease of metastases number ($p < 0.05$) and 2.7-fold decrease of metastases volume ($0.05 < p < 0.1$) compared with unvaccinated mice treated only by surgery. For animals from this group, MII achieved 97.4%. It has been shown that antimetastatic efficacy of vaccination was dependent on the regimen of immunization (the most effective one was triple vaccination with 3 day intervals after surgical treatment).

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