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ANTITUMOR AND ANTIMETASTATIC ACTIVITIES OF VACCINE PREPARED FROM CISPLATIN-RESISTANT LEWIS LUNG CARCINOMA

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Aim: **To study antitumor and antimetastatic activities of antitumor vaccine (ATV) prepared from cisplatin (CP) sensitive and resistant strains of Lewis lung carcinoma (LLC).** ***Methods:*** **The inhibition of tumor growth, and the mean survival time of the tumor-bearing animals, the number and the volume of metastases were measured as the indices of ATV efficacy. The activity of cytotoxic T-lymphocytes and natural killer cells, peritoneal macrophages (Mph), the level of tumor necrosis factor and the total proteolytic activity of blood plasma (PA) were assessed.** ***Results:*** **ATV from CP resistant LLC prepared using cytolectin (CL) of *B. subtilis B-7025* significantly inhibited growth of CP resistant tumors (by 52%) and increased mean survival time (MST) of animals (by 44.6%). The index of metastasis inhibition for ATV prepared from CP sensitive or resistant LLC was 154.5% and 227.0%, respectively. In all vaccine-treated animals, Mph activity was shown to be significantly increased. In spite of high antitumor and antimetastatic effects of ATV prepared from CP resistant LLC, PA in plasma of animals inoculated with CP resistant LLC was increased significantly upon vaccine administration.**

Key Words: **cancer vaccine, antitumor and antimetastatic activities, drug resistance, Lewis lung carcinoma.**

Natural or acquired drug resistance of tumor cells is one of the reasons of failure of existing methods of cancer treatment. Drug resistance could be mediated, in particular, by the altered expression of cytoplasmic membrane proteins, which bind chemotherapeutic drugs [1, 2].

Within the population of the same tumor, cancer cells are heterogenous in various aspects including those inherent for drug resistance. Even in tumors susceptible to specific cancer treatment, the strong selection in the setting of the chemotherapeutic drugs is a driving factor of the development of the resistant clones and the acquisition of the resistance by the tumor as a whole. The altered expression of such oncogenes as *c-fos, c-mys, H-ras, Her/neu, c-jun, c-abl* as well as *p53* tumor suppressor gene is a decisive factor in formation of resistance to cisplatin (CP) [3, 4]. The study of cell lines derived from stomach cancer resistant to CP, 5-fluorouracil or doxorubicin also identified the altered expression of more than 250 genes [5]. The changes in activities of several proteolytic enzymes in resistant variants of different tumors cells lines was demonstrated both *in vitro* [6, 7], and *in vivo* [8, 9]. Serine proteinases involved in apoptosis are among the known factors associated with mechanisms of drug resistance formation [6, 8, 10]. Another important structure mediating drug resistance in cancer cells is P-glycoprotein [11–13].

Recently, the data have been accumulated suggesting various mechanisms of susceptibility of cancer cells to the chemotherapeutic drugs as well as cytotoxic factors of antitumor immunity [14, 15]. Several reports suggest the possibility of designing anticancer vaccines based on the active components isolated from the cells with drug resistance phenotype [16]. In experimental resistant tumors (intestinal cancer CT-26 and Lewis lung carcinoma) hyperexpressing MDR1, the positive effect of vaccine based on DNA encoding for MDR1 transfected to the attenuated strain of *Salmonella typhimurium* turns has been demonstrated [13]. New prospects are opened with the advent of dendritic cell technology for vaccine production [17, 18].

It is known that drug resistant cancer cells may be still susceptible to the effects of the activated autologic lymphocytes [15, 17, 19, 20]. However, the data on ATV efficacy against the tumors with drug resistance phenotype are still insufficient. Therefore, it is of interest to compare the effects of ATV prepared from the components of drug-susceptible and resistant cancer cells.

Earlier, the development of ATV from CP resistant Lewis lung carcinoma (LLC) cells using the cytotoxic lectine (CL) of *B. subtilis B-7025* have been reported [21]. The aim of the present study was to compare antitumor and antimetastatic activities of ATV prepared from the initial and chemoresistant LLC variants in therapeutic setting of vaccine administration. The parameters characterizing activity of antitumor macrophages were studied in parallel with the analysis of the effects on the proteolitic activity in blood plasma of experimental animals.

MATERIALS AND METHODS

Adult male C57Bl mice (aged 2.5 months) bred in animal facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology were used for the experiments. All experiments were performed according to the regulations of Ethic Committee of the Institute. Cisplatin resistant (CR) LLC strain was originated from the initial LLC strain by the consecutive series of the transplantations in the setting of CP injections [9].

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Abbreviations used: ATV – antitumor vaccine; CL – cytotoxic lectin; CP – cisplatin; CR – cisplatin resistant; CS – cisplatin sensitive; LLC – Lewis lung carcinoma; Mph – peritoneal macrophages; MST – mean survival time; PA – proteolytic activity.

ATV from cisplatin sensitive (CS) LLC and CR LCC cells were prepared using cytotoxic lectin (CL) of *B. subtilis B-7025* according to our method [22]. Protein concentration in ATV was 4.0 mg/ml. The efficacy of ATV was assessed in the therapeutic setting of experiment (Table 1). The effects of ATV were evaluated by tumor incidence, life-span of tumor-bearing animals, the size of the primary tumor, the number and the volume of the metastatic foci.

Table 1. Experimental design

Test	Group No.	n	Tumor		Vaccination		
			LLC variant	Route of inoculation and dose of cells	Origin of vaccine	Dose	Day
I	1	20	CS	4.5×10^5 in femoral muscle	CP sensitive LLC	0.3 ml, subcutaneously	1, 4, 7, 11, 14
	2	20			CP resistant LLC		
	3	20			–	–	–
	4	20	CR	4.5×10^5 in femoral muscle	CP sensitive LLC	0.3 ml, subcutaneously	1, 4, 7, 11, 14
	5	20			CP resistant LLC		
	6	20			–	–	–
II	1	10	CR	5.0×10^5 in femoral muscle	CP sensitive LLC	0.3 ml, subcutaneously	1, 4, 7, 11, 14
	2	10			CP resistant LLC		
	3	10			–	–	–

Notes: CS – cisplatin sensitive; CR – cisplatin resistant.

The antitumor activity of cytotoxic T lymphocytes (CTL) and natural killer cells (NK cells) was estimated by radiometric method [23].

The functional (metabolic) activity of peritoneal macrophages (Mph) was assayed by NBT-test [24]. The content of tumor necrosis factor (TNF) in culture supernatant and blood serum was estimated using TNF-sensitive L-929 cells [25]. The total blood plasma proteolytic activity (PA) was evaluated by digestion of protamine sulfate according to the K.M. Veremeenko spectrophotometric method [26].

RESULTS AND DISCUSSION

Mean survival time (MST) of tumor-bearing mice was shown to increase upon immunization with ATV prepared from either CP sensitive or CP resistant variant of LLC (Table 2). MST of animals with CP sensitive LLC was increased significantly upon administration of homologous ATV (prepared from CP sensitive LLC) (53.85 ± 4.03 *vs* 37.5 ± 1.07 days in untreated animals, $p < 0.05$), while the effect of ATV prepared from CP resistant LLC was not significant. MST of animals with CP resistant LLC was increased significantly upon administration of ATV prepared either from CP resistant or sensitive variant of LLC (see Table 2).

Table 2. MST of mice with CS or CR LLC treated with ATV prepared from CS or CR LLC

Tumor	Treatment	n	MST, days M ± m	t	ILS,%
CS LLC	Vaccine from CS LLC	8	53.85 ± 4.03	2.80*	+43.6
	Vaccine from CR LLC	9	45.15 ± 4.4	1.63	+20.4
	–	7	37.50 ± 1.07		
CR LLC	Vaccine from CS LLC	9	53.71 ± 4.24	3.12*	+39.1
	Vaccine from CR LLC	7	55.86 ± 5.09	2.95*	+44.6
	–		38.62 ± 1.39		

Notes: CL of *B. subtilis B-7025* was used for vaccine preparation. CS – cisplatin sensitive; CR – cisplatin resistant; *$p < 0.05$ in comparison with corresponding values in untreated animals.

ATV prepared from CP sensitive LLC decreased the final incidence rate of transplanted CS LLC by 30.7% and CR LLC only by 15.4%. In mice with transplanted CR LLC, ATV prepared from CR LLC decreased the final incidence rate of CR tumors by 42.9% and CS LLC by 21.4%. These data suggest that ATV prepared from homologous LCC strains (sensitive or resistant) are more effective.

When the growth inhibition was assessed, ATV prepared from CP sensitive LLC inhibited the growth of CP sensitive and CP resistant tumors by 24.14% and 34.62%, respectively. ATV prepared from CP resistant LLC inhibited the growth of CP sensitive and CP resistant tumors by 16.32% and 51.64%, respectively. Therefore, ATV prepared from CP resistant LLC inhibited the growth of the homologous LLC ($p < 0.05$) and did not affect significantly the growth of CP sensitive LLC strain ($p > 0.05$).

The functional activity of the effectors mediating antitumor response was assayed on the 34th day since LLC transplantation, i. e. on the terminal stage of tumor growth. According to our previous data as well the data of other researchers [27, 28], at that time the immunity of the animals is in the state of exhaustion due to inhibition of antitumor immunity partially because of immune suppression.

As shown in Table 3, the functional characteristics of immune response of vaccinated animals to some extend depended on the type of ATV used. Nevertheless, in all cases, Mph activity was increased significantly. On 34th day of tumor growth in mice with initial LLC strain tumors after treatment with ATV based on CP sensitive and CP resistant LLC Mph activities were respectively 3.2 and 4.7 times more ($p < 0.05$) than in untreated animals. In mice with CP resistant LLC, the Mph activities upon treatment with ATV prepared from CP resistant LLC were increased 2.1-folds as compared with that in untreated animals ($p < 0.05$). In this group only the trend of increasing of NK cell and CTL activity was shown ($0.05 < p < 0.1$).

Table 3. Activity of cell immunity effectors tumor-bearing hosts treated with ATV prepared from CS or CR LLC (CL of *B. subtilis B-7025* was used for vaccine preparation)

Tumor	Treatment	Cytotoxicity index,%		
		NK cells	Mph	CTL
CS LLC	–	10.0 ± 8.1	15.4 ± 5.98	19.3 ± 5.4
	Vaccine from CS LLC	13.8 ± 4.8	49.4 ± 11.8*	18.2 ± 2.9
	Vaccine from CR LLC	28.6 ± 1.6*	71.9 ± 5.9*	13.5 ± 3.5
CR LLC	–	18.2 ± 8.1	22.6 ± 6.5	20.3 ± 5.0
	Vaccine from CS LLC	17.4 ± 12.9	43.6 ± 8.2*	11.7 ± 5.9
	Vaccine from CR LLC	25.0 ± 7.9	47.1 ± 9.4*	30.6 ± 5.9

Notes: The cytotoxicity was assessed on Day 34 post inoculation. CS – cisplatin sensitive; CR – cisplatin resistant; *$p < 0.05$ in comparison with corresponding values in untreated animals.

We attempted to assess whether such Mph activation is associated with increasing TNF content in the supernatants (Sn) of Mph cultures. In Mph cultures of intact mice, TNF titer was 2.0 ± 0.2 $\log_2$ and cytotoxicity index (CI) assayed at 1:2 dilution was 34.6 ± 5.8%. On 34th day of tumor growth in untreated animals, these

indices were 1.3 ± 0.2 log_2 and 21.0 ± 1.8% for CP sensitive LLC and 1.5 ± 0.2 log2 and 24.3 ± 1.8% for CP resistant LLC. In ATN-treated animals, the corresponding indices were significantly higher than in intact animals and tumor-bearing untreated animals (Table 4).

Table 4. Patterns of nonspecific immunity upon treatment with vaccines prepared from CS or CR LLC

Group	NBT-test of Mph, in absorbance unit	TNF activity titer log_2	CI, %
Vaccine from CR LLC	0.361 ± 0.004[1, 2]	4.5 ± 0.1[1, 2, 3]	102.7 ± 15.0[1, 2, 3]
Vaccine from CS LLC	0.383 ± 0.009[1]	2.3 ± 0.2	48.9 ± 6.5
Untreted mice inoculated with LLC	0.397 ± 0.002[1]	2.6 ± 0.3	56.1 ± 10.5
Intact control	0.476 ± 0.003	2.8 ± 0.2	62.8 ± 10.6

[1]$p < 0.05$ in comparison with intact control; [2]$p < 0.05$ in comparison with untreated inoculated mice; [3]$p < 0.05$ in comparison with other vaccine treatment.

The results of the assay of PA in plasma of the experimental animals are presented on Figure as percentage relative to control values in untreated animals inoculated with LLC taken as 100%. In plasma of mice inoculated with CP sensitive LLC and treated with homologous vaccine, the trend of decreasing PA was observed ($0.05 < p < 0.1$). On the contrary, PA in plasma of animals inoculated with CP resistant LLC was increased significantly upon administration of either vaccine (from CP resistant or sensitive LLC) ($p < 0.05$). It is suggested that the response of proteolysis system to immunotherapy depended on the susceptibility of tumor to CP rather than the type of ATV.

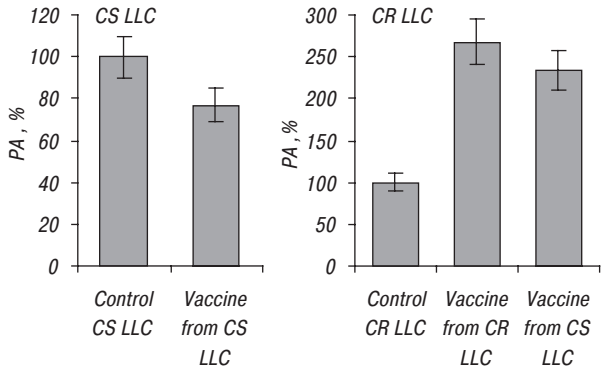

Figure. PA in blood plasma of vaccinated mice with initial and CP resistant LLC strain

CP is known to reduce PA in blood of cancer patients [29]. The same decrease was observed in present study in the animals inoculated with CP sensitive LLC treated with vaccine from CP sensitive LLC. On the contrary, PA in plasma of animals inoculated with CP resistant LLC was increased upon vaccine administration. Such effect may be associated with the activation of the effector cells mediating antitumor immunity, Mph in particular, which are one of the sources providing the increased proteinase level in the blood [30]. This fact is in line with the findings demonstrating the activation of non-specific immunity in the vaccine-treated animals inoculated with CP resistant LLC at the advanced stages of tumor growth.

There are contradictory data reported by various researchers as to the trend of proteolytic enzyme activity in metastasizing tumors of different histogenesis: both increase [31] and decrease [32] of such activity are reported. The views on the effects of proteolytic activity on the survival of cancer cells are also quite different [33, 34].

As was shown in our earlier work [8], the formation of drug resistance phenotype in cancer cells is accompanied by the shift on the balance between the proteolytic enzymes and their inhibitors towards proteinase activation. PA augmentation associated with the formation of drug resistance is associated with the increasing metastatic capability of cancer cells.

Therefore, we have analyzed antimetastatic activity of the vaccine prepared from CP sensitive or resistant LLC in the animals inoculated with CP resistant LLC. The antimetastatic efficacy was assessed by the index of metastasis inhibition. On the 34th day since inoculation, in treated animals from all groups the size of the primary tumor decreased significantly. The ATV prepared from CP resistant LLC was more effective (by 34.4%) than ATV prepared from CP sensitive LLC.

The administration of ATV prepared from either CP resistant or sensitive LLC decreased significantly the rate of metastasizing as well as the volume of lung metastases (Table 5). Index of metastasis inhibition (calculated by the relative decrease of the number of metastasis as compared to untreated animals) for ATV prepared from CP sensitive or resistant LLC was 154.5% and 227.0%, respectively. The antimetastatic effect was most evident when the volume of metastases was measured (decrease by 87.83% and 83.4% for ATV prepared from CP sensitive or resistant LLC).

Table 5. Quantitative characteristics of metastasizing upon inoculation of CR LLC in animals treated with ATV prepared from CS or CR LLC

Group	Mean lung weight, mg	Mean number of metastases	Mean volume of metastases, mm^3
Vaccine from CR LLC	210.9 ± 11.9*	6.0 ± 2.6*	36.17 ± 21.3*
Vaccine from CS LLC	236.0 ± 22.9	7.71 ± 2.2*	49.48 ± 20.9*
Control	374.2 ± 73.9	19.60 ± 3.1	297.18 ± 79.2

Notes: CS – cisplatin sensitive; CR – cisplatin resistant; *$p < 0.05$ in comparison with corresponding values in untreated animals.

On the 34th day of tumor growth, Mph activity in ATV-treated mice measured in NCT-test was the same as in untreated mice inoculated with LLC. Nevertheless, TNF content in serum of mice treated with ATV prepared from CP resistant LLC was 1.7–1.9 times higher comparing with all other groups ($p < 0.05$).

The obtained data demonstrated that ATV from CP resistant LLC prepared using cytotoxic lectin (CL) of *B. subtilis B-7025* significantly inhibited the growth of tumors and the development of metastases (by 52% and 87.83% respectively) with accompanying prolongation of MST by 44.6%. Nevertheless, in spite of antitumor and antimetastatic effects, the administration of such vaccine increased significantly PA in plasma of tumor-bearing animals. This phenomenon needs further investigations.

The elaborated procedure of administrating ATV prepared from CP resistant LLC has been shown to activate the effector components of cell-mediated immunity in the animals inoculated with CP resistant LLC. This is in line with the findings presented N.M. Berezhnaya *et al.* [14, 19, 20] who demonstrated the increased efficacy of several immunotherapeutic modalities such as vaccine administration or LAC therapy

in the animals with resistant tumors (B-16 melanoma, MC-rhabdomyosarcoma) as compared to the initial strains sensitive to chemotherapy. Moreover, the explants of human cancers resistant to chemotherapy (soft tissue sarcoma, cancer of cervix uteri, ovarian cancer) are susceptible to the lysis by the activated autologic lymphocytes [15].

While the interpretation of our findings is difficult because of the lack of sufficient information on the phenomenon of increased susceptibility of chemoresistant tumors to the factors of immune response, the contribution of cell surface molecules is evident. Underlying mechanisms of chemoresistance formation are variable. Among them there are epigenetic modifications of *mdr-1* locus and methylation of *mdr-1* promoter; activation of enzymes of gluthation system; changes of genes and proteins that control apoptosis of cancer cells; increased expression of genes coding for transmembrane transport proteins (P-glycoprotein) etc. [12, 35, 36].

The data on the correlation between the acquisition of drug resistance and increased susceptibility to the effectors of cell-mediated immunity are currently limited. In particular, Azuma *et al.* [12] proved the presence of cytotoxic T cells specific to MDR-1 in experimental murine leukemia, but significant correlation between P-gp expression and increased sensitivity of resistant cancer cells to the factors of immune defense has not been yet revealed.

To sum up, the data obtained indicate the importance of further studies on the mechanisms of the effects of ATV prepared from chemoresistant tumors with the aid of cytotoxic lectin of *B. subtilis B-7025.* Such studies may be advantageous for the improvement of the treatment of resistant tumors in clinical setting.

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