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THE EFFECTS OF EARLY POSTOPERATIVE IMMUNIZATION WITH XENOGENEIC EMBRYO PROTEINS ON LEWIS LUNG CARCINOMA MODEL

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Aim: **To investigate the effect of chicken embryo proteins (CEP) as a prototype of xenogeneic vaccine on immune reactions in mice immunized after Lewis lung carcinoma (LLC) surgical removal.** ***Materials and Methods*****: C57Bl male mice were immunized on days 1, 8, and 15 after surgical removal of LLC. The immune response was assessed on days 7, 14, 21 and 28 after tumor resection. Cytotoxic activity of natural killer cells (NK) and cytotoxic T-lymphocytes as well as antibody dependent cellular cytotoxicity was estimated in MTT-assay; specific antibodies were detected in ELISA; lymphocyte proliferation was tested in reaction of** ***in vitro*** **blast transformation.** ***Results:*** **None of the immunized mice developed LLC metastases. Immunization with CEP seems to prevent the potential decrease in NK cell cytotoxic activity and spontaneous blast transformation activity of lymphocytes following the surgically induced stress. Further research on improving immunization schedule and elucidating the mechanisms of NK modulation with CEP is needed.**

Key Words: **xenogeneic anticancer vaccine, chicken embryo proteins, Lewis lung carcinoma.**

Anticancer vaccines are believed to improve the outcome of cancer treatment. Until now, a huge variety of anticancer vaccines based on different techniques are elaborated [1, 2]. Among them are xenogeneic anticancer vaccines, which utilize xenogeneic homologous proteins as antigens (Ags) to elicit anticancer immune response [3]. For many reasons xenogeneic vaccines are superior to auto- or allogeneic ones. The main advantage is that xenogeneic homologous proteins in general are more potent immune response inducers than autologous and even allogeneic Ags [4–7]. Homologous immunogens are derived from a number of different species for instance, from the rhesus [4], rat [7], xenopus [8], mouse [9–11], pig [12, 13], quail [14], chicken [5, 6, 15] etc. Proteins of human origin in different rodent [16–18], canine [16, 19–21] or feline [22] tumors have been investigated as well. Up to now, some of xenogenic anticancer vaccines are under clinical testing [9, 23–25]. The Oncept vaccine, based on DNA coding for human tyrosinase, (Merial, Duluth, GA, USA) received a conditional licensure from the United States Department of Agriculture in 2007 for treatment of canine oral malignant melanoma stages II/III [26].

Previously we have shown that immunization with xenogeneic anticancer vaccine based on chicken embryo proteins (CEP) possesses antitumor activity on Lewis lung carcinoma (LLC) and Ehrlich carcinoma models [27, 28]. The effects of CEP application on anticancer immune response in mice bearing Ehrlich carcinoma have been described [28]. The scope of the research was to examine the effects of CEP administration on immune reactions in mice immunized after resection of LLC.

MATERIALS AND METHODS

Animals. The study has been carried out on male C57Bl mice 2–2.5 month old weighting 19–20 g, bred at the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobilogy (IEPOR). The use and care of experimental animals have been performed in accordance with standard international rules on biologic ethics and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [29] and was approved by Institutional Animal Care and Use Committee.

Preparation of CEP. CEP was prepared as described in [30]. Briefly, 7-day chicken embryos were rinsed two times in cold 0.9% NaCl solution, homogenized and then extracted with 0.9% NaCl solution, containing 0.1% EDTA, for 60 min at 4 °C by agitation. Following extraction, chicken embryo tissue was removed by centrifugation at 1,500 g for 30 min. The resulting supernatant was collected and frozen at –20 °C. Protein concentration was determined according to Greenberg and Craddock assay.

Preparation of LLC Ags. Antigens of LLC (LLC-cells-Ag) were prepared by three consecutive freezing-thawing cycles of cell suspension. Following the last thawing, cell debris was removed by centrifugation at 1,500 g for 30 min. The resulting supernatants were collected and frozen at –20 °C. Protein concentration was measured according to Greenberg and Craddock assay.

For Ag of LLC metastatic cells (LLC-mts) to be prepared, metastatic nodules were carefully dissected of lung tissue, homogenized in the Potter homogenizer and thoroughly washed in phosphate buffered

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Abbreviations used: Abs – antibodies; ADCC – antibody dependent cellular cytotoxicity; Ags – antigens; CEP – chicken embryo proteins; CTA – cytotoxic activity; CTAI – cytotoxic activity index; CTL – cytotoxic T-lymphocytes; LLC – Lewis lung carcinoma; LLC-cells-Ag – antigens of LLC; LLC-mts – LLC metastatic cells; NK – natural killer cells; SI – stimulation index.

saline. After the last wash, cells were suspended in a fresh portion of phosphate buffered saline; all the other procedures were performed as for LLC-cells-Ag above.

Scheme of the experiment. C57Bl mice (n = 34) were injected with LLC cells in the hind foot at a dose of $2.5 \cdot 10^5$ cells/mouse. On day 17 after transplantation, the tumor-bearing hind paw was surgically removed by paw amputation on ankle joint under the ether anesthesia.

Mice were randomly divided in two groups, one group was immunized with CEP. The other group received no immunization and was referred to as the surgery control. The data of both (the CEP and the surgery control) groups were compared with intact mice of the same strain, sex and age (referred to as the intact control).

In total, 42 animals were used in the experiment (3 mice per one observation point per group in the immunology tests (12 per group in total), plus 5 mice per group that were checked for metastases only on day 38 after the surgery), plus 8 naïve mice used as the intact control).

Immunizations were performed *s.c.* with 0.3 ml of CEP solution per mouse (protein concentration 0.3 mg/ml) on days 1, 8, and 15 after the surgery. The immune reactions were assessed on days 7, 14, 21 and 28 after tumor resection. The metastatic status was checked on days 21, 28 and 38 after the surgery.

Cytotoxic activity (CTA) assay. CTA of spleen lymphocytes was determined by the MTT assay [31]. K-562 cells were used as target cells for the examination of natural killer cell (NK) CTA, while LLC cells were used as targets for cytotoxic T-lymphocytes (CTL) and antibody dependent cellular cytotoxicity (ADCC).

In brief, target cells ($2 \cdot 10^4$ cells/well) and immune cells ($1 \cdot 10^5$ lymphocytes/well), in RPMI-1640 medium supplemented with 10% fetal bovine serum (all reagents from Sigma, USA) and antibiotics, were placed in a flat-bottom 96-well plate and incubated for 18 h in 100% humidity atmosphere with 5% CO_2 at 37 °C. After that, 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 (1,500 g for 15 min) and washed twice with 0.9% NaCl solution. After all, 0.12 ml of KOH (2 mol/l) and 0.14 ml of DMSO (50% solution) were added into each well. Optical density was measured at $\lambda = 545$ nm *vs* $\lambda = 630$ nm with a MicroELISA reader (StatFax-2100, USA). Each sample was measured in triplicate.

Cytotoxic activity index (CTAI, %) was calculated by the formula:

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

where OD_{lc} — optical density of wells containing only lymphocytes; OD_{tc} — optical density of wells containing only target cells; OD_{lc+tc} — optical density of wells in which tumor cells and lymphocytes where incubated; OD_{blank} — optical density of wells with the culture medium only.

In order to determine ADCC activity, 0.01 ml/well of autologous blood serum was added to target-containing wells and preincubated for 30 min. After that, lymphocytes were added to the wells and all the other steps were the same as described above.

Lymphocyte blast transformation assay. Lymphocytes were obtained from aseptically removed lymph nodes by homogenizing in Potter homogenizer. Aliquots of lymphocytes ($2 \cdot 10^6$ cells/ml) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 µg/ml gentamicin were transferred to flat-bottom plates (200 µl/well) and stimulated by 15 µg protein/well of the LLC-cells-Ag or by 10 µg protein/well of concanavalin A (ConA, Sigma, USA) or left without stimulation (spontaneous reaction). The plates were incubated for 2 days at 37 °C in 5% CO_2 atmosphere. The response was determined by the percentage of transformed cells counted per 100 cells. LLC-cells-Ag was prepared as described above.

Detection of specific antibodies (Abs) in blood serum. The mouse sera were collected on days 7, 14, 21 and 28 after tumor resection and stored at –20 °C. By the enzyme-linked immunosorbent assay (ELISA), the sera were tested for Abs specific to LLC-cells-Ag and LLC-mts as described in [32]. Briefly, 0.1 ml of LLC-cells-Ag and LLC-mts-Ag solutions at 0.3 mg/ml were incubated for 24 h at 4 °C in 96-cell microtiter plates. Nonspecific binding was blocked with 3% BSA for 1 h at 37 °C. The sera were added in 1:20 dilution (the sera dilution was selected in the preliminary tests). Bound Abs were revealed with the goat antimouse IgG peroxidase conjugate (Sigma, USA) and o-phenyldiamine/H_2O_2 substrates. The plates were read at 492 nm in MicroELISA reader (Stat Fax 2100, USA). The naive mouse sera in the same dilution were used as the negative control. The results are presented in conventional units (CU) [33]:

$$CU = OD_{experiment} / OD_{control},$$

where $OD_{experiment}$ stands for optical density of wells with serum of experimental mice; $OD_{control}$ stands for optical density of wells with naïve mice serum. The CU value exceeding 2 was considered as indication of Ab-positive serum.

Detection of cytokines in blood serum. IL-4 and IFN-γ concentration in the blood serum was analyzed with the BD OptEIA (BD Biosciences, USA) kit according to the manufacturer recommendations.

Statistics. The data are presented as mean value ± standard error (M ± m). The statistical analysis was made using Student's *t*-test. The difference was considered as significant when $p < 0.05$; p value higher 0.05 but lower 0.1 ($0.05 < p < 0.1$) was treated as a tendency. The correlation analysis was made using the Pearson correlation coefficient.

RESULTS

No surgery-associated causalities or relapses were recorded. The number and volume of metastases were checked on days 21, 28 and 38 after the surgery.

We did not check for metastases on earlier time points as long as metastases are not expected to be found out. In the immunized group, there were no metastases detected, while in the surgery control group 6 out of 10 mice developed metastases (Table 1).

Starting from day 7 after the surgery, the immune reactions were tested weekly for four consecutive weeks. The most evident immune response on CEP immunization was the increase of spontaneous blast transformation (Fig. 1), which is an unspecific index of *in vivo* lymphocyte activation [34]. The spontaneous blast transformation in the group of immunized mice (Fig. 1, *b*) was significantly higher than in the intact control group ($p < 0.05$ on days 7, 14 and 21). However, the lymphocytes were not exhausted because the blast transformation in response to ConA did not differ from the intact control level. On the contrary, in the surgery control group (Fig. 1, *a*) spontaneous blast transformation did not differ significantly from the intact control level, but reaction on ConA stimulation tended to decline ($0.05 < p < 0.1$ on days 14, 21 and 28 as compared to day 7). Reaction of lymphocytes to stimulation with LLC-cells-Ag was similar in both groups (Fig. 1, *a*, *b*) demonstrating the transient increase ($p < 0.05$ as compared to the intact control) in the number of blast cells on day 21 after the tumor resection.

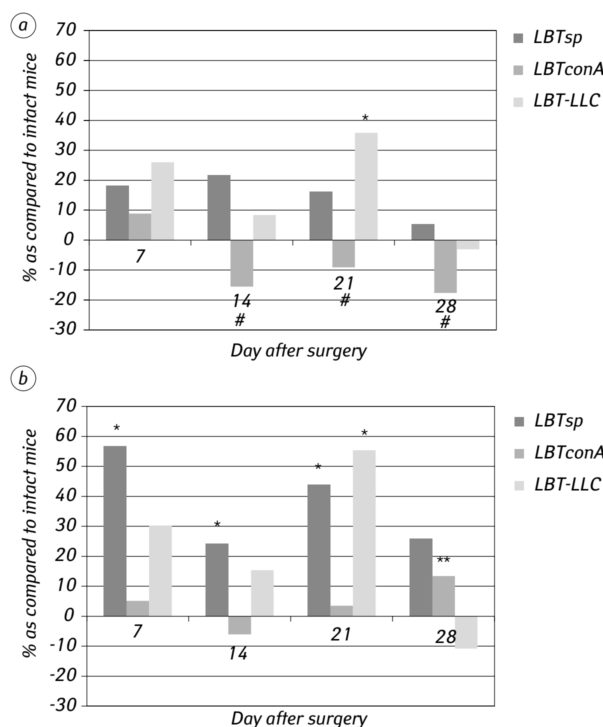

Fig. 1. Lymphocyte blast transformation in *in vitro* reaction of surgery control (*a*) and immunized (*b*) groups of mice after tumor resection. LBTsp — spontaneous blast transformation; LBTconA — blast transformation induced by Con A; LBT-LLC — blast transformation in response to *in vitro* stimulation with LLC-cells-Ag. *$p < 0.05$ compared to the intact control; **$p < 0.05$ compared to the surgery control group; #$0.05 < p < 0.1$ compared to data obtained on day 7 in the group

Surgery is known to impose stress on immune cells, in particular NK cells [35–38]. Indeed, on days 7 and 14 after tumor resection NK CTA (Fig. 2) of the surgery control mice was by 2 and 2.5 times lower compared to the intact control ($0.05 < p < 0.1$ and $p < 0.05$, respectively). On the contrary, in the group of immunized mice, suppression of NK CTA was postponed and observed only on day 14 after tumor removal.

The CTL CTA of the immunized and the surgery control groups did not differ from the intact control level significantly (Fig. 3).

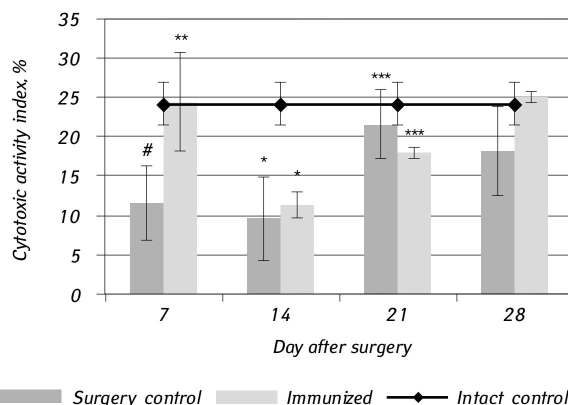

Fig. 2. NK CTA in immunized with CEP after LLC tumor resection or nonimmunized C57Bl mice in comparison to the intact control. *$p < 0.05$ compared to the intact control; **$p < 0.05$ compared to the surgery control group; ***$p < 0.05$ compared to data obtained on day 14 in the group; #$0.05 < p < 0.1$ compared to the intact control

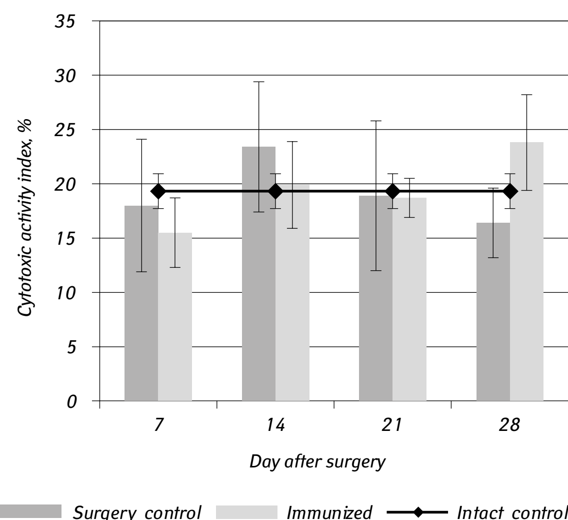

Fig. 3. CTL CTA of control and immunized with CEP after LLC tumor resection C57Bl mice

Table 1. LLC metastasis (mts) in the surgery control group and in the group of mice immunized with CEP after LLC surgical removal

Parameters	Day after surgery					
	21		28		38	
	Surgery control	Immunized	Surgery control	Immunized	Surgery control	Immunized
Total number of mts⁺ mice	2	0	1	0	3	0
Total number of mts	3	–	2	–	7	–
Total volume of mts, mm³	5.23	–	1.05	–	1022.79	–

ADCC of lymphocytes in the immunized group of mice did not differ significantly from the intact control level. While in the surgery control group changes in ADCC of lymphocytes had wavelike character, being slightly increased on day 7 after the operation ($0.05 < p < 0.1$ compared to the intact control), decreased on day 14 ($p < 0.05$ compared to the intact control) and again increased on day 21 ($p < 0.05$ compared to the immunized group) (Fig. 4). The wavelike character of ADCC tightly resembles ones of the Abs level (Table 2) and the percentage of Abs-positive mice (Fig. 5) in the group although did not correlate significantly.

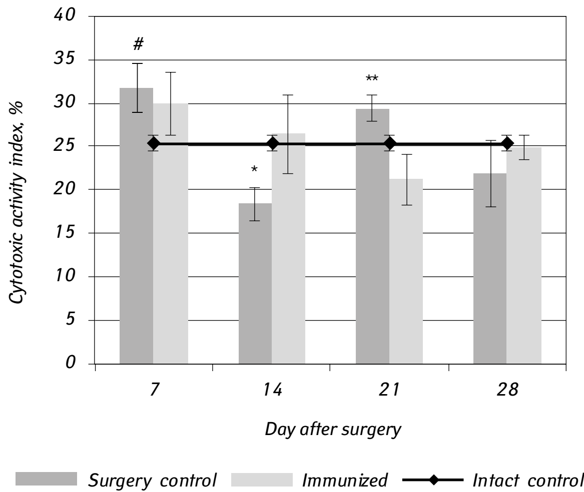

Fig. 4. Lymphocytes of ADCC CTA in immunized with CEP after LLC tumor resection and control C57Bl mice. *$p < 0.05$ compared to the intact control; **$p < 0.05$ compared to the immunized group; #$0.05 < p < 0.1$ compared to the intact control

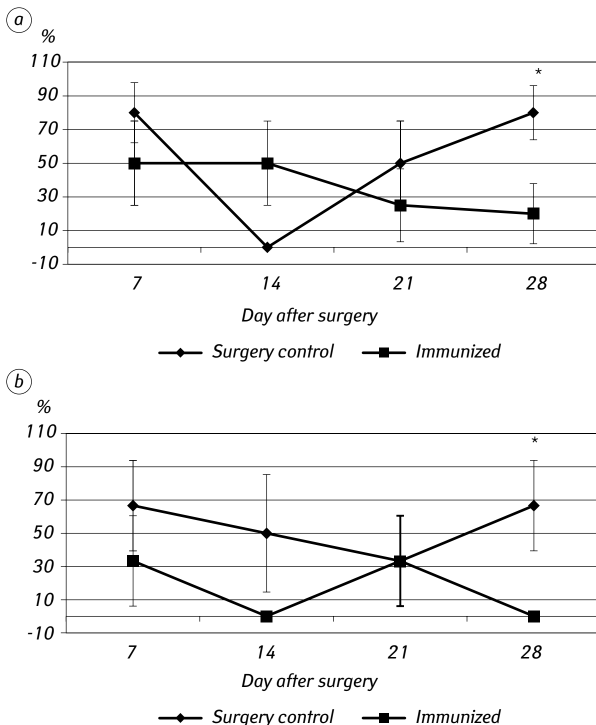

Fig. 5. Percentage of mice expressing antibodies specific for LLC-cells (*a*) and LLC-mts (*b*) Ags. *$p < 0.05$ compared to the immunized group

Table 2. Level of Abs specific for LLC-cells and LLC-mts Ags in the blood serum of control and immunized after tumor resection C57Bl mice

Days after surgery	Anti LLC-cells Abs		Anti LLC-metastatic-cells Abs	
	Surgery control group	Immunized group	Surgery control group	Immunized group
7	10.67 ± 2.1	9.16 ± 6.5	10.03 ± 4.6	13.51 ± 4.8
14	n/d	11.00 ± 8.6	2.05 ± 1.2	n/d
21	10.21 ± 1.6	8.40 ± 3.7	10.39 ± 4.5	2.51 ± 1.1
28	9.19 ± 1.4	14.39 ± 2.8	2.83 ± 0.1	n/d

Note: n/d – Abs were not detected.

In 1979, Fogel *et al.* [39] demonstrated that cells of primary injected LLC and of metastatic nodules of the tumor express different Ags. In our experiment, we checked the dynamics of the expression of Abs specific for Ags of LLC-cells (Fig. 6, *a*) and LLC-mts (Fig. 6, *b*). In the immunized group, the percentage of Abs-positive mice (for both, LLC-cells and LLC-mts specific, Abs) was slowly decreasing from day 7 to day 28. On the contrary, in the surgery control group the percentage of Abs-positive mice started to grow after slight decrease and significantly exceeded the percentage in the immunized group on day 28 after the surgery.

The level of Abs (both anti-LLC-cell and anti-LLC-mts) did not differ significantly between the groups (Table 2). Although in the surgery control group the level of anti-LLC-mts Abs correlated tightly with the volume of metastases (r = 0.93, $p = 0.0009$).

Surgical intervention often leads to a shift in cytokine balance towards Th2 type [36, 40]. In our experiment, in the surgery control group the level of IL-4 was higher than in the intact control group by 1.6, 1.8 and 1.6 times, correspondingly, on days 7, 14 and 28 after tumor resection (Fig. 6). The level of IL-4 in the

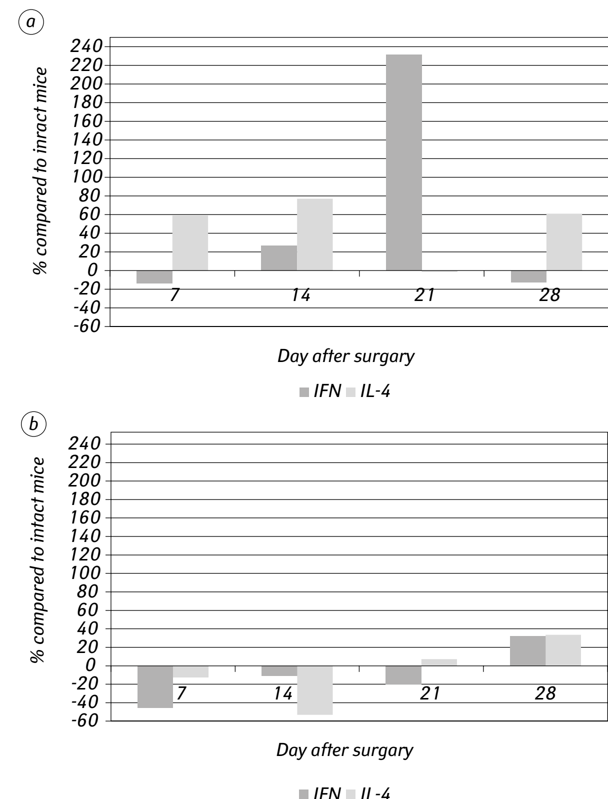

Fig. 6. The level of IL-4 and IFN-γ in the blood serum of surgery control (*a*) and immunized (*b*) mice as compared to intact mice level

immunized group was in the range of the intact control being lower than in the surgery control group ($p < 0.05$ on day 14).

The blood serum level of IFN-γ in the group of immunized mice did not differ from that in the intact control group. On the contrary, in the surgery control group the level of IFN-γ on days 14 and 21 was by 4.0 and 3.3 times higher than in the intact control group ($p > 0.05$ due to high inner group variability). However, it should be mentioned that the increase in IFN level was noted only in the mice which developed LLC metastases (r = 0.95, $p < 0.0005$) that, most probably, indicate ongoing immune response to the metastatic cells.

DISCUSSION

A potent antimetastatic effect of immunizations with CEP was shown in the previous experiments [27] as well as in the current study: throughout the experiment metastases have been found in 6 out of 10 surgery control mice while in the group of immunized mice no metastases were registered (0/11). The aim of the current research was to assess the immune response in immunized mice, which could underlie antimetastatic effect of CEP. We were surprised that only two reactions, i.e. NK CTA and spontaneous blast transformation of lymphocytes, differ between the immunized and nonimmunized mice in the early postoperative period. It is possible, of course, that the major changes up to day 7 after the surgery were not taken into account in our study. On the other hand, maybe the protection of NK cell function in the early postoperative period was sufficiently enough to inhibit the metastatic spread of tumor cells. It is widely accepted that surgery promotes metastasizing [38, 41, 42]. The decrease in the total number of NK cells in a spleen affected NK cells migration and killing activities is among the main causes that facilitate postoperative metastasizing (reviewed in detail in [42, 43]). As reviewed in [38], postoperative NK cell suppression correlates with increased metastasizing in both experimental settings and human trials. On the other hand, perioperative application of immune modulating remedies can result in remarkable antimetastatic effect [37, 38]. For example, the protection against metastatic spread after surgery was reached with the perioperative treatment with influenza vaccine [44], oncolytic parapoxvirus ovis and vaccinia virus [37], CpG-C (along or in combination with propranolol and etodolac) [45], hydrogel loaded with the agonists of Toll-like receptor 7/8 [46], Flt3 kinase ligand along [47] or in combination with a whole tumor cell vaccine [48], IL-2 [49] or with the adoptive therapy with lymphokine-activated killer cells and recombinant interleukin-2 [50]. Moreover, in the studies [44, 45], the authors have proved that the antimetastatic effect of perioperative immunizations is conferred by rescued NK functioning. Abrogation of NK activities diminished the antimetastatic effect of immunizations. In [46], anti-metastatic and anti-recurrence effects depended on NK as well as dendritic cells and T cells functioning.

In our experiment, the postoperative decrease of NK CTA was seen in both, immunized and nonimmunized, groups. However, while in the surgery control group NK CTA suppression was registered on days 7 and 14 after the surgery, in the immunized group NK CTA decreased only on day 14. It is of crucial importance, to our mind, that the functions of NK cells was preserved shortly after the resection of tumor since it could prevent the metastatic spread induced by surgery intervention.

As for the T-cells reactions, they did not differ significantly between the groups, except for the spontaneous blast transformation of lymphocytes. The absence of T-cell CTA and the absence of lymphocytes blast transformation in response to stimulation with LLC-Ag in both groups as well as weak spontaneous blast transformation in the nonimmunized group can be at least partially attributed to the surgery imposed stress too [51]. The absence of lymphocyte blast transformation in response to the stimulation with LLC-Ag on days 7 and 14 and the sharp increase of response on day 21 goes in line with the previously cited research of Ananth *et al.* [51], who have shown that antigen-specific T-cells are functionally impaired following surgery, especially their proliferation in response to specific antigen re-stimulation. The authors reported that the recovery period lasted between day 7 and 28 after surgery. Therefore, it looks like the increase in blast transformation in response to LLC-Ag on day 21 after the surgery appeared after the lymphocytes recovery from the immune suppression. From this point of view, immunization with CEP can be considered as protecting the ability of lymphocytes to form blasts *in vitro* in response to *in vivo* immune stimuli (immunization with CEP and/or compensatory reaction on surgery) while it was not enough to rescue tumor-activated lymphocytes.

Contrary to days 7 and 14 after the surgery, the last two time-points (days 21 and 28 postoperative) evidence little about the results of the immunization but point to the metastatic status of the animals. While in the immunized group, the studied indexes did not differ from the intact mice ones, in the surgery control group the appearance of metastases provoked the activation of some immune reactions. For example, the metastases volume correlated tightly with the IFN-γ level (r = 0.95, $p < 0.0005$) and the level of LLC-mts-specific Abs (r = 0.93, $p = 0.0009$). It looks like that the appropriate schedule of immunization is more important than the magnitude of induced immune activation. The immunization with CEP in early postoperative period although did not elicit a pronounced and long-lasting immune activation, but was able to partially preserve immune cells from surgery imposed suppression that appears to be responsible for protection from metastatic spread of the tumor. That is to say, the early intervention is needed to improve the treatment results.

Finally, it is evident that, although unavoidable, surgery imposes stress on immune cells. On the other hand, the perioperative applications of agents aimed

to positively affect the immune response possess an opportunity to improve outcome of cancer patient treatment. We have shown that the application of CEP in early postoperative period can protect experimental mice from metastases spread by conferring the protection of NK cell CTA and preserving the ability of lymphocytes to proliferate in response to antigenic stimuli. Further research on the improvement of the immunization schedule and the exact mechanisms of NK modulation with CEP is needed.

CONCLUSION

In general, it was shown that immunization with CEP in early postoperative period protected mice from metastatic spread of the LLC tumor. Protection of NK cell cytotoxic function from surgery-imposed stress in early postoperative period may be one of the mechanisms which underlie the antimetastatic effect of CEP.

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