

ANTICANCER EFFECTIVENESS OF VACCINATION BASED ON XENOGENEIC EMBRYO PROTEINS APPLIED IN DIFFERENT SCHEDULES

*T.V. Symchych**, *N.I. Fedosova*, *O.M. Karaman*, *L.M. Yevstratieva*, *H.S. Lisovenko*, *I.M. Voyeykova*, *H.P. Potebnia*
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine

The aim: To evaluate anticancer activity of vaccination with chicken embryo proteins (CEP) applied in different schedules. **Materials and Methods:** C57Bl mice were vaccinated with CEP before (prophylactic schedule) or after (different therapeutic schedules with or without preliminary tumor removal) the Lewis lung carcinoma cells transplantation. The latent period of tumor development, tumor volume and metastasis rate were evaluated. **Results:** Potent antimetastatic effect of CEP-based vaccination was seen in case of therapeutic regimen after primary tumor removal. The metastasis inhibition index (MII) reached 96.9 and 97.8% on 18th and 34th day after tumor removal, respectively. When CEP vaccination was performed in the settings of therapeutic regimen without primary tumor removal the anticancer effect was evident only if vaccinations started as early as 24 h after the cancer cells injections. The highest MII achieved in such condition was 77.6%, tumor volume in the group of vaccinated animals was by 53.1–42.1% lower than in the control tumor-bearing mice. CEP vaccination before tumor challenge (prophylactic immunization) led to a statistically significant prolongation of the latent period of tumor development, a reduction of tumor volume (35.8–48.8% compared to control unvaccinated mice) and a marked inhibition of metastasis (MII was 71.1%). **Conclusion:** Vaccination based on CEP exhibited both prophylactic and therapeutic anticancer effects. The last one is more pronounced when the vaccination starts shortly after the primary tumor resection. **Key Words:** chicken embryo proteins, anticancer activity, Lewis lung carcinoma.

The development of xenogeneic anticancer vaccines (XAV) started at the end of the 1990s, when it was shown that the use of xenogeneic analogues of tumor associated antigens enables the body to overcome immunological toleration for its own proteins [1]. Now, it is proven that a number of tumor associated antigens and protein have their counterparts of animal or avian origin which can serve as antigens in XAV. These proteins or genes are exploited in the construction of XAVs, some of which have been shown to have anticancer effect [2, 3]. Some XAV successfully passed I–II phases of clinical trials. Their safety and ability to induce immune response without autoimmune complications have been proven [4–8]. Among others, genes and proteins of chicken origin which share homology with human counterparts are exploited in the XAV construction [9–14].

At the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of the National Academy of Science of Ukraine XAV based on chicken embryo proteins (CEP) is under development. It is known that anticancer vaccines based on one or several antigens can lead to an immune editing of the tumor so that it loses antigens targeted by the vaccine. Moreover, polyantigenic vaccines potentially can elicit an immune response to a wider range of cancer antigens including unidentified ones [15]. That is why the vaccine which is being constructed is designed to be polyantigenic and is based on proteins extracted from the chicken embryo. In preliminary experiments,

it was shown that blood serum of mice bearing different tumor strains has antibodies which react with CEP [16]. When injected to intact mice CEP caused no side effect or allergy reactions [17]. The aim of the current work is to evaluate the anticancer activity of CEP-based vaccine administered by different vaccination schedules.

MATERIALS AND METHODS

The study has been carried out on male C57Bl/6 mice 2–2.5 months old weighing 19–20 g, bred in the IEPOR. The use and care of the experimental animals have been performed in accordance with the standard international rules of biologic ethics and was approved by Institutional Animal Care and Use Committee [18, 19]. The anticancer and antimetastatic efficacy of CEP was examined when vaccination was applied prior to tumor cells injection (prophylactic schedule), after tumor transplantation (therapeutic vaccination) or after tumor removal (post surgery vaccination). Lewis lung carcinoma (LLC) was used as the model of tumor growth.

CEP was prepared as follows [20]: 7 days chicken embryos were rinsed two times briefly 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 the 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. Tumor associated antigens of LLC (LLC-Ag) were prepared by three consecutive cycles of freezing and melting of cell suspension. Following the last melting, cell debris was removed by centrifugation at 1.500 g for 30 min. The resulting supernatants were collected and frozen at –20 °C. The concentration of proteins in the extracts was measured by Greenberg and Craddock assay [21]. The same extracts were used in all the experiments described in the article.

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*Correspondence: E-mail: symchychtv@gmail.com

Abbreviations used: CEP – chicken embryo proteins; ITGI – index of tumor growth inhibition; LLC – Lewis lung carcinoma; LLC-Ag – Lewis lung carcinoma associated antigens; MII – metastasis inhibition index; XAV – xenogeneic anticancer vaccine.

Irrespective of vaccination schedule, CEP or LLC-Ag immunizations were performed s.c. with 0.3 ml solution per mouse (protein concentration 0.3 mg/ml).

According to the prophylactic experiment, mice were immunized with CEP or LLC-Ag (three weekly injections); LLC cells were transplanted 30 days after the last immunization.

Therapeutic vaccination has been performed by three different schemes: on the 1st, 8th, 15th days (group #1); on the 7th, 14th, 28th days after the tumor cell transplantation (group #2); in the third group, vaccination started when the tumor nodule had become clearly palpable and was followed with two additional injections on the 3rd and 10th days after the first vaccination (that corresponds to the 12th, 15th and 22nd days after the tumor transplantation).

Post surgery vaccination started on the 1st, 8th, 15th days after the tumor removal, which corresponds to the 18th, 24th and 31st days after tumor transplantation.

In the prophylactic and treatment vaccination experiments cancer cells suspension was injected *i.m.* into the right hind leg at a dose of 4×10^5 cells/mouse. Unvaccinated mice with the tumor were used as the control.

In the post surgery vaccination experiment, LLC cells were injected per foot at a dose of 2.5×10^5 cells/mouse. The tumor removal was performed on the 17th day after the tumor transplantation. Mice which have undergone surgical tumor removal but received no vaccination are referred as the control.

Tumor dimensions were measured with calipers, and tumor volumes were calculated according to the formula:

$$\text{Tumor volume} = 2/3 \pi \cdot \text{width}^2 \cdot \text{length}.$$

The Index of Tumor Growth Inhibition (ITGI) was calculated according to the formula:

$$\text{ITGI} = 100\% \cdot (V_{\text{control mice}} - V_{\text{immunized mice}}) / V_{\text{control mice}},$$

where $V_{\text{control mice}}$ and $V_{\text{immunized mice}}$ stand for the mean tumor volume in control and immunized mice respectively.

To assess metastasis burden mice were sacrificed and in each animal lungs were removed; surface lung metastases were counted and measured. The metastases volume was calculated as following:

$$V = 4\pi r^3/3,$$

where r — stands for the metastases radius. The percentage of mice bearing metastases is referred as metastases rate. The mean number of metastases was calculated per all the mice in group and per mice bearing metastases.

Metastasis Inhibition Index (MII) was calculated as following:

$$\text{MII} = 100\% \cdot (A_c \cdot B_c - A_i \cdot B_i) / (A_c \cdot B_c),$$

where A_c and A_i stand for the number of mice bearing lung metastases in groups of control and immunized mice respectively. B_c and B_i stand for the mean number of lung metastases in groups of control and immunized mice respectively [22].

The results were analyzed for statistical significance by paired *t*-test using StatSoft STATISTICA 7.0. Values $p < 0.05$ were considered as statistically significant [23, 24]. The data in figures and tables are presented as $M \pm SD$.

RESULTS

The anticancer activity of CEP applied before tumor transplantation (prophylactic immunization). CEP or LLC-Ag were injected three times with one-week intervals. Then 30 days after the last immunization, LLC was transplanted into both unvaccinated animals (the control) and mice vaccinated with CEP or LLC-Ag. LLC tumor appeared in 81.0% (17 out of 21) of the control mice (Table 1). In the treatment groups, 77.8% (7 out of 9) and 81.8% (9 out of 11) of mice immunized with LLC-Ag or CEP, respectively, developed LLC tumors. The difference between all the groups was not significant. The latent period of tumor development was shorter ($p < 0.05$) in the group of the control mice (7.8 days) compared to the mice pre-vaccinated with LLC-Ag (10.0 days) or CEP (10.9 days).

Table 1. The latent period of tumor development and tumor transplantation efficacy in the vaccinated and control LLC-bearing mice

Group	Tumor transplantation efficacy, %	Latent period of tumor development, days
Control	81.0 ± 13.1	7.8 ± 0.4
LLC-Ag	77.8 ± 13.9	10.0 ± 0.7*
CEP	81.8 ± 11.6	10.9 ± 0.6*

Note: * $p < 0.05$ compared to the control group.

The tumor growth kinetics is shown in Fig. 1. During the experiment, the smallest tumor volume was observed in the group of mice immunized with CEP ($p < 0.05$ compared to the control group). In the group of CEP-immunized mice, the ITGI reached 35.8–48.8% depending on time after the tumor transplantation. The tumor volume of mice immunized with LLC-Ag did not differ significantly compared to both control and CEP-immunized mice. In the group of LLC-Ag-immunized mice, the maximal ITGI (28.4%) was observed on the 14th day after the tumor transplantation (Table 2).

On day 28 after LLC transplantation, all the mice of the control and treatment groups were euthanized so the metastases rate to be evaluated. The results are shown in Table 3.

In the mice vaccinated with CEP, 73.4% reduction of the mean metastasis volume was registered, in particular, 51.5 and 72.1% decrease of the metastases number per mouse or per mouse in the group correspondingly. So, in this group MII reached 59.5% per metastases-bearing mouse and 71.1% per group. Contrary to CEP, LLC-Ag vaccination was not effective against metastases development.

Table 2. Tumor volume and ITGI in control and vaccinated before tumor transplantation mice bearing LLC

Group	Parameter	Days after LLC transplantation					
		10	14	17	21	24	28
Control	Tumor volume, mm ³	165.0 ± 22.5	493.7 ± 62.2	981.0 ± 120.1	1523.7 ± 85.1	2087.8 ± 114.1	2492.4 ± 268.2
	ITGI, %	0.9	27.4	12.7	23.1	25.7	24.1
LLC-Ag	Tumor volume, mm ³	163.5 ± 35.8	353.6 ± 65.0	856.6 ± 125.0	1172.2 ± 190.3	1550.9 ± 291.2	1891.5 ± 332.7
	ITGI, %	48.8	38.9	47.7	41.4	36.7	35.8
CEP	Tumor volume, mm ³	84.6 ± 8.3	301.5 ± 54.6	512.8 ± 73.0	892.3 ± 132.0	1321.1 ± 215.3	1600.8 ± 246.7
	ITGI, %	48.8	38.9	47.7	41.4	36.7	35.8

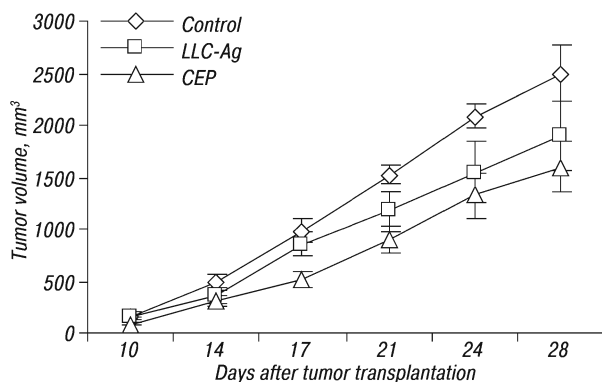


Fig. 1. The growth kinetics of LLC in control and pre-vaccinated animals

Table 3. Metastasis burden in control and vaccinated before tumor transplantation mice bearing LLC

Group	Metastases rate, %	Volume of metastases, mm ³	Number of metastases	
			per mouse bearing metastases	per group
Control	70.0 ± 14.5	38.9 ± 13.9	20.6 ± 6.5	14.4 ± 5.5
LLC-Ag	88.9 ± 9.9	30.4 ± 10.3	22.9 ± 7.1	22.9 ± 7.1
CEP	50.0 ± 20.4	10.4 ± 3.0	11.7 ± 3.5	5.8 ± 3.2

The anticancer activity of the CEP-based vaccination applied after the tumor transplantation (therapeutic vaccination). Therapeutic vaccination with CEP has been performed according to three different schedules of vaccination (described in details in the Materials and Methods section). Any of immunization schedules appeared to be superior in transplantation efficacy and latent period of LLC development, as far as 85.9–89.6% of the vaccinated and control mice developed tumors on the 9–11th day after the LLC cells transplantation.

When it comes to the tumor volume, the most evident effect on tumor growth was observed in the group of mice vaccinated according to the schedule #1 (Fig. 2). Compared to the control group, the difference was significant ($p < 0.05$) till the 20th day after the tumor challenge. The ITGI reached 53.13% on 14th day after the LLC transplantation and was decreasing slowly till the 28th day of the experiment. Although the ITGI (42.1%) observed at this time point of the follow-up period (the 28th day after the LLC transplantation) was the lowest for the group #1, it remained to be the highest among the other groups. The tumor volume of the mice vaccinated according to the two other schedules did not differ significantly compared with the control group.

On day 28 after the tumor transplantation, all the mice were euthanized to assess the metastasis loading (Table. 4). The results of the group #1 were out-standing. In this group, the lowest mean metastases number per group was recorded ($0.05 < p < 0.1$ compared to the control group). The mean metastases

volume was by 54.4% lower than that in the control group. So, the MII in group #1 reached 77.6% (per group) or 66.3% (per mice bearing metastases) and was the highest among all the treatment and control groups. In other treatment groups, the results did not differ significantly from that of the control group.

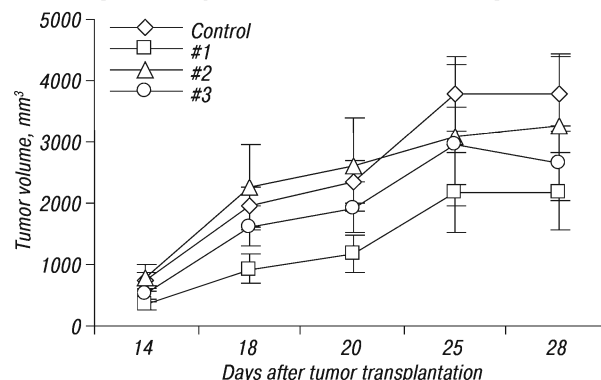


Fig. 2. The tumor growth kinetics in animals vaccinated with CEP and control mice

It is worth mentioning that only in the group #1 all the mice were still alive till the end of the experiment (28 days after tumor transplantation). The worst survival rate was in the group #3 (here the vaccination started after the tumor nodule could be palpable), in which 50% of immunized mice died before the experiment termination.

Table 4. Indexes of metastasis rate in animals vaccinated by different therapeutic schedules and in control LLC-bearing mice

Group	Metastases rate, %	Volume of metastases, mm ³	Number of metastases	
			per mouse bearing metastases	per group
Control	85.7 ± 12.4	10.1 ± 5.4	10.4 ± 3.4	10.4 ± 3.4
#1	66.7 ± 19.2	4.6 ± 3.9	4.5 ± 2.0	3.0 ± 1.6*
#2	80.0 ± 19.4	31.3 ± 15.7	17.7 ± 8.4	13.2 ± 7.6
#3	66.7 ± 27.2	13.2 ± 6.2	23.0 ± 8.5	15.3 ± 10.3

Note: * $0.05 < p < 0.1$ compared to the control group.

The anticancer activity of CEP applied after the tumor resection (post surgical therapeutic vaccination). As far as the most prominent anticancer results were observed in the group of mice immunized on the 1st, 7th and 14th days after tumor transplantation (group #1), the same schedule was chosen to be applied in the study of post surgical therapeutic vaccination. Mice were transplanted with LLC cells (per foot); on the 17th day after transplantation the tumor nodule was removed. All the mice were divided into two groups. The mice in the CEP group underwent vaccinations with CEP on the 1st, 7th and 14th days after tumor resection. On the 35th and 50th days after the tumor transplantation (the 18th and 34th days after the tumor removal, respectively) the mice of both (control and treatment) groups were euthanized to assess the metastases burden (Table 5).

Table 5. Metastasis indexes in mice vaccinated with CEP after surgical removal of LLC

Group	The day 18 after tumor removal				The day 34 after tumor removal			
	Metastases rate, %	Volume of metastases, mm ³	Number of metastases		Metastases rate, %	Volume of metastases, mm ³	Number of metastases	
			per mouse bearing metastases	per group			per mouse bearing metastases	per group
Control	72.7 ± 13.4	69.3 ± 25.8	16.5 ± 3.6	12.0 ± 3.5	66.7 ± 13.6	133.1 ± 98.5	4.2 ± 2.0 ²	2.4 ± 1.1 ²
CEP	27.3 ± 13.4 ¹	1.2 ± 0.9 ¹	3.7 ± 1.1 ¹	1.0 ± 0.6 ¹	15.4 ± 10.0 ¹	0.8 ± 0.4	1.5 ± 0.7	0.2 ± 0.2

Note: ¹ $p < 0.05$ compared to the control; ² $p < 0.05$ compared to the 18th day after tumor removal.

In this experimental setting, CEP showed evident and long-lasting antimetastatic effect. Independently on observation time, CEP immunization led to reduction of the metastases rate, metastases number and volume. For example, on the 18th day after the tumor removal, only 27.3% of the immunized mice had metastases, while in the control group this index reached 72.7% (the difference was statistically significant). The metastases volume in the group of vaccinated mice was by 98.3% lower ($p < 0.05$) when compared to the control mice. The mean number of metastases per metastases-bearing mouse or per group in total was statistically significantly lower in the group of CEP vaccinated mice. So, MII reached 91.7% per mouse and 96.9% per group.

On the day 34th after the tumor removal, mice in the control group showed disease progression. For example, the metastases volume increased by 1.92 times, compared with the 18th day after the tumor removal. The mean number of metastases slightly decreased possibly due to the merging of small metastases. The metastasis rate did not change significantly (66.7 ± 13.6 and $72.7 \pm 13.4\%$ of control mice had metastases on the 34th and 18th days after the tumor removal respectively).

On the other hand, mice immunized with CEP showed stabilization of metastatic process. In particular, the metastases volume was $0.8 \pm 0.4 \text{ mm}^3$ (to compare, it was $1.2 \pm 0.9 \text{ mm}^3$ on the 18th day after the tumor removal); the metastases number per mouse bearing it was 1.5 ± 0.7 (3.7 ± 1.1 on the day 18th of examination). In control mice, metastasis rate in the group of vaccinated mice did not differ significantly from the previous point of observation.

So, we can assume that on the 18th day after the tumor removal almost all the mice (of both control and treatment groups) which were prone to develop metastases developed them, as long as the metastasis rate did not differ significantly on the 18th and 34th days of observation. But the metastasis rate was statistically lower in the CEP vaccinated group during all the experiment (i.e. on the 18th and 34th days after tumor removal) compared to the control. What is important, the vaccinated mice showed inhibition of metastases growth, whereas in the control group the mean metastases volume increased by almost 2 times. As a result, the mean metastases volume in the group of immunized mice was by 94.4% lower than that in the control group. In the CEP group, the MII calculated per group was equivalent to 97.8%. So, the antimetastatic effect of CEP-based vaccination was observed for a prolonged period of time even after the termination of the vaccination.

DISCUSSION

So, as it was shown in the model of LLC, vaccination with CEP appears to have anticancer and antimetastatic effects. In the previous experiments it has been shown that there were CEP-specific antibodies in the blood serum of mice bearing different tumor strains (LLC, sarcoma 37, Ehrlich carcinoma, melanoma B-16) [16]. The presence of CEP-specific antibodies

in the blood serum of unimmunized tumor-bearing mice may be explained by at least two reasons: poly-specific antibody circulation [25, 26] and the presence of some homologous proteins in CEP. It is known that some proteins of chicken origin share homology with mammals proteins, including that of human and mice [9, 10, 12, 27–29]. The anticancer effect of CEP seems to be based on the last assumption. Especially, it looks possible when we consider the antitumor and antimetastatic effects of CEP applied before the tumor challenge — in so called prophylactic settings. According to the prophylactic schedule which was applied in the experiment, the tumor cell injection was performed on the 30th day after the last immunization. Till the 30th day after the CEP injection, the immune response induced by the immunization was expected to terminate [30], but immune memory cells had been already established [31]. The immune memory is capable of mounting a rapid response to subsequent antigen stimulation [32]. In the experiment, LLC cell suspension in the dose sufficient to induce tumors was used instead of the antigen re-challenge. Since a statistically significant prolongation of the latent period of tumor development in the groups of immunized mice was observed, it points to the generation of the rapid immune response to the cancer cells injection. That is, the mice immunized with CEP or LLC-Ag in the prophylactic mode mounted a rapid immune response to cancer cells as if it was an antigen re-challenge.

Subsequently, the observed results indicate with high probability that CEP contains some proteins which share homology with LLC antigens and immunization with CEP leads to immune memory formation. Moreover, in terms of its antimetastatic activity, vaccination with CEP was much more effective than application of LLC-Ag. This finding can be considered as an additional demonstration that xenogeneic homologous proteins are useful for breaking immune tolerance towards autologous cancer antigens.

In case of therapeutic immunization, the anticancer effect of CEP was evident only when applied at the very early stage of tumor formation (24 h after tumor cells injection, group #1), when tumor burden is minimal. When vaccination was postponed to only 7 days (group #2) the anticancer effect could hardly be observed. Furthermore, vaccination with CEP has no anticancer effect when applied to mice with the already established tumor (group #3). So, it can be concluded that without prior tumor removal the application of anticancer vaccine based on CEP will have a minimal anticancer effect in clinical settings. On the other hand, it confirms a generally acknowledged statement that benefit of an anticancer vaccine is most evident when it is applied in earlier and less aggressive disease settings, that is in settings of minimal residual disease [33, 34].

Owing to this, the third experiment — application of CEP after the surgical resection of the tumor — was conducted. In this case, CEP application had a pronounced and long-lasting antimetastatic effect. The number of metastases bearing mice and the mean

metastases volume were significantly reduced in the group of treated mice. These effects were evident till the 34th day after the tumor removal — the day of the experiment termination. It should be mentioned that the mean metastases volume in the CEP group was 60 and 168.5 times smaller than that of the control group on the 18th and 34th days after the tumor resection respectively. MII was very high and reached 96.9 and 97.8% per group in total on the 18th and 34th day respectively. It can be assumed that this vaccine when applied after the surgical removal of a tumor may dramatically improve therapeutic efficacy of cancer treatment, as long as metastatic spread of a tumor is the main death cause of cancer patients [35].

It has been shown that some genes or proteins of chicken origin, when used as a xenogeneic vaccine, can elicit anticancer effect or tumor specific immune response. For example, xenogeneic vaccines based on chicken HSP70 [11], MMP-2 [10, 14], Tie-2 [9] or FGFR [12, 13] were effective in case of LLC [10, 14], fibrosarcoma Meth A [13, 10], hepatoma H22 [9, 10], melanoma B-16 [9], CT26 colon adenocarcinoma [14], canine cancer [11]. Anticancer effects of CEP are comparable with these of the vaccines mentioned above. Whether CEP contains some of abovementioned proteins or its anticancer effect is based on other antigens it remains to be elucidated.

CONCLUSION

Vaccination based on CEP exhibited both prophylactic and therapeutic anticancer effects. The last one is more pronounced when the vaccination starts shortly after the primary tumor resection. In this case, the MII reaches 91.7%. So, CEP are suitable to be used in xenogeneic cancer vaccine construction.

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