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THE ANTICANCER EFFICIENCY OF THE XENOGENEIC VACCINE AND THE INDICATION FOR ITS USE

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***Aim*: To investigate the anticancer efficiency of the xenogeneic vaccine in different tumor models and to assess the possibility whether level of antibodies (Ab) specific for vaccine's proteins can be used as an indication for its use. *Methods*: Mice with Lewis lung carcinoma (LLC), Ehrlich carcinoma (EC) or Sarcoma 37 (S37) were immunized with a xenogeneic anticancer vaccine based on chicken embryo proteins (CEP) and its anticancer activity was examined. The level of specific Ab in the blood serum of non-immunized tumor-bearing mice was studied by ELISA. *Results*: CEP application statically significantly inhibited the growth of LLC (the index of tumor growth inhibition was 42.10–53.13% depending on the day of tumor growth); vaccinated mice with EC showed significant tumor growth inhibition and life prolongation by 34.48%. Among mice with S37, there was noticed no antitumor effect. The number of tumor-bearing non-immunized mice which have had pre-existing CEP-specific Ab did not differ depending on the tumor model. The level of CEP-specific Ab among mice with LLC and EC increased with the growth of the tumor volume, but it decreased among mice bearing S37. Probably, the low level of CEP-specific Ab alongside huge tumor burden shows it is futile to apply the CEP-based vaccine. *Conclusion*: Different tumor strains vary in their susceptibility to CEP-based vaccine. Probably, the low level of CEP-specific Ab when a tumor burden is huge shows it is futile to apply the CEP-based vaccine.**

***Key Words:* xenogeneic anticancer vaccine, chicken embryo proteins anticancer activity, Lewis lung carcinoma, Ehrlich carcinoma, Sarcoma 37, CEP-specific antibodies.**

The construction of xenogeneic anticancer vaccines (AV) is a comparatively new but fairly promising field in cancer biotherapy. The development of AV based on xenogeneic analogues of tumor associated antigens (TAA) was brought about by two facts: 1) tumor antigens are generally products of expression of unmutated patient genes which are tolerated by the body's immune system; 2) the use of homological xenogeneic antigens can overcome immunological tolerance to these proteins [1, 2].

Now a number of researches showed the ability of xenogeneic analogues to overcome immunological tolerance to tumor antigens or proteins connected to carcinogenesis [3–6]. The antitumor efficiency of some xenogeneic vaccines was proved by a number of experimental [2, 3, 5–8] and clinical researches [9–11]. On the other hand, indications for use of xenogeneic AV are not evident enough. In general, it is not a problem in a case of those vaccines which are based on the limited number of antigens: the tumor's expression of these antigens or proteins involved in carcinogenesis is an indication for use of a relevant vaccine. Nevertheless, the identification of tumor antigenic spectrum by every patient requires much time and expense. The problem is more acute for polyvalent vaccines or vaccines based on tissue homogenates and extracts. That is to say, it is urgent to find simple and quick methods to predict the expedience of xenogeneic vaccine use. Xenogeneic vaccines certainly cannot pretend to be "universal" vaccine, therefore there is a still open question how to assess "the applicability spectrum" of every xenogeneic vaccine, i.e. to choose the factors of their expedience for use.

Tumor and embryonic cells are believed to have common features: embryonic cells express antigens which are similar to oncofetal antigens of tumor cells [12, 13]. Moreover, it is known from the literature that the embryo cells of the chicken express proteins, which share homology with the human and mouse tumor antigens [3, 5, 14–16]. The literature tells us about successful application of some proteins or genes of chicken origin as a xenogeneic AV [3, 5, 8]. So, the development of a xenogeneic vaccine based on chicken embryo proteins (CEP) looks promising. In R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of NAS of Ukraine, the work is proceeding with elaboration of the xenogeneic AV based on CEP.

The aim of this particular research project has been to study the anticancer efficiency of the xenogeneic vaccine based on CEP on different models of cancerous growth and assess the possibility to apply the level of CEP-specific antibodies (Ab) as an indication for use of the vaccine.

MATERIALS AND METHODS

The study has been carried out on male C57Bl/6 and Balb/c mice 2–2.5 months old weighting 19–20 g, bred in the vivarium of R.E. Kavetsky IEPOR of NAS of Ukraine. The use and care of experimental animals have been performed in accordance with standard international rules on biologic ethics and was ap-

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Abbreviations used: Ab – antibodies; AV – anticancer vaccine; CEP – chicken embryo proteins; EC – Ehrlich carcinoma; ILSP – index of life span prolongation; ITGI – index of tumor growth inhibition; LLC – Lewis lung carcinoma; MII – metastasis inhibition index; S37 – sarcoma 37; TAA – tumor associated antigens.

proved by Institutional Animal Care and Use Committee [17, 18].

Anticancer efficacy of CEP was examined when vaccination was applied after tumor transplantation — so called therapeutic vaccination. Three different tumor strains were used: Lewis lung carcinoma (LLC), Ehrlich carcinoma (EC) and Sarcoma 37 (S37). To establish tumors, cancer cells suspension was injected *i.m.* in the right hind leg at a dose of $4 \cdot 10^5$ cells/mouse (LLC, EC) or $5 \cdot 10^5$ cells/mouse (S37). Unvaccinated mice with the tumor of relevant strain are referred as a control.

LLC bearing mice were immunized on days 1st, 8th and 15th after tumor injection.

EC bearing mice were immunized on days 2nd, 5th, and 8th after tumor injection.

Vaccination of S37 bearing mice has been performed by three schemata: at 1st, 8th, 15th days (scheme 1, group 1), at 2nd, 5th, and 8th days (sheme 2, group 2) and at 7th, 14th, 28th days after tumor cell transplantation (scheme 3, group 3).

In all the cases, immunizations were performed *s.c.* with 0.3 ml of CEP solution per mouse (protein concentration 0.3 mg/ml).

Mouse sera were collected on days 7th, 14th, 21st and 28th after tumor transplantation. Sera were frozen and stored at −20 °C. By an enzyme-linked immunosorbent assay (ELISA) sera were tested for CEP-specific or TAA specific Ab as described in [19]. Briefly, the CEP or TAA at 0.3 mg/mL were incubated for 24 h at 4 °C on 96-cells microtiter plates. Nonspecific binding was blocked with 3% BSA for 1 h at 37 °C. Sera were added at dilution 1:20 (sera dilution was selected in preliminary tests). Bound Ab were revealed using goat antimouse IgG and IgM peroxidase conjugate (Dako) and *o*-phenyldiamine/H_2O_2 substrates. Plates were read at 492 nm in an MicroELISA (Stat Fax 2100, USA) auto-reader. The negative control consisted of naïve mouse sera in the same dilution. The results are presented as factor F [20]:

$$F = OD_{experiment}/OD_{control}, \quad (1)$$

where $OD_{experiment}$ stand for optical density of cells with serum of tumor-bearing mice, $OD_{control}$ stand for optical density of cells with naïve mice serum. The F value exceed 2 was taken as indication of Ab-positive serum.

CEP was prepared as follows [21]. Briefly, 7 days chicken embryos were rinsed two times in cold NaCl 0.9% solution, homogenized and then extracted with NaCl 0.9% solution, containing 0.1% EDTA, for 60 min at 4 °C by agitation. Following extraction, chicken embrio tissue was removed by centrifugation at 1.500 g for 30 min. The resulting supernatant was collected and frozen at −20 °C. TAA of LLC, EC and S37 were prepared by three consecutive cycles of freezing and melting of cells 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. Concentration of proteins in the extracts was measured by Greenberg and Craddock assay [22]. The same extracts were used in all the experiments, described in the article.

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

$$tumor\ volume = 4/3\pi \cdot width^2 \cdot length \cdot 0{,}5 \quad (2)$$

Index of tumor growth inhibition (ITGI) was calculated according to the formula:

$$ITGI = \frac{V_{control\ mice} - V_{immunized\ mice}}{V_{control\ mice}} 100\%, \quad (3)$$

where $V_{control\ mice}$ and $V_{immunized\ mice}$ stand for mean tumor volume in control unimmunized and immunized mice respectively [23].

Index of life span prolongation (ILSP) was calculated as following:

$$ILSP = \frac{survival\ time_{immunized\ mice} - survival\ time_{control\ mice}}{survival\ time_{control\ mice}} 100\%, \quad (4)$$

where survival $time_{immunized\ mice}$ and survival $time_{control\ mice}$ stand for survival time (days) in immunized and control groups respectively [23].

Metastasis inhibition index (MII) was calculated as following:

$$MII = \frac{A_c \cdot B_c - A_i \cdot B_i}{A_c \cdot B_c} 100\%, \quad (5)$$

A_c and A_i stand for number of mice bearing lung metastases in groups of control and immunized mice respectively. B_c and B_i stand for mean number of lung metastases in groups of control and immunized mice respectively [24].

Pearson correlation coefficient (r) was adjusted for sample size [25].

RESULTS

Study into anticancer activity of the CEP-based vaccine on the model of LLC. According to obtained results, tumors formed on 9–11th day after the LLC cells injection in six out of ten (60%) animals of the control and treatment groups. The tumor volume (Fig. 1) in immunized animals for the whole period of observation was smaller compared to the results of control mice ($p < 0.05$ before day 20 and $p < 0.1$ since 25th until 28th day of tumor growth).

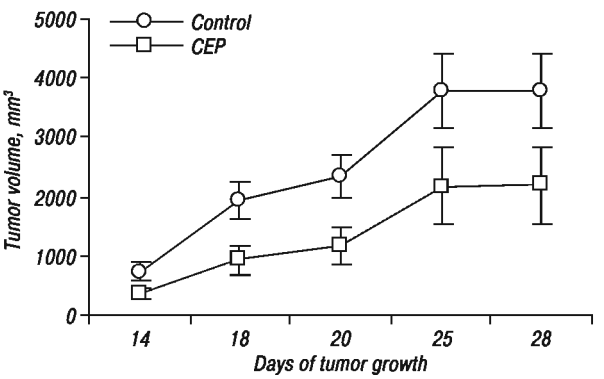

Fig. 1. The tumor volume of vaccinated with CEP-based vaccine and control mice bearing LLC

On the 28th day after the LLC implantation, the animals were euthanized and metastases were assessed

(Table 1). According to the obtained data, the mean volume of metastases in the group CEP was 2.2 times smaller comparing to the control group. The mean number of metastases per mouse in the group tended to decrease among the immunized mice ($0.05 < p < 0.1$ comparing to the control group). MII for the immunized animals was 77.56% and 66.35% — per group in general and per animals bearing metastases respectively.

Table 1. Metastases in vaccinated and unvaccinated mice bearing LLC

Group	Metastases rate, % (sample size/mts*)	Metastases volume, mm³	Metastases number	
			per mouse bearing metastases	per mouse in a group
Control	85.71 ± 12.37 (5/5)	10.11 ± 5.39	10.4 ± 3.4	10.4 ± 3.4
CEP	66.67 ± 19.25 (6/4)	4.61 ± 3.98	4.5 ± 1.97	3.0 ± 1.57*

Notes: * $p < 0.1$ comparing to the control group.

Hence, the AV based on CEP had a significant antitumor and some antimetastatic effect.

Study into anticancer activity of the CEP-based vaccine on the model of EC. An anticancer effect of the CEP-based vaccine was evident in the model of EC. Although tumors formed in every unvaccinated and vaccinated animal on the 5–7th day after the transplantation (Table 2), the immunized mice showed significant inhibition of tumor growth (Fig. 2).

Table 2. The latent period of tumor formation and the survival time for vaccinated with the CEP-based vaccine and control mice bearing EC

Group (sample size)	Latent period of tumor formation (days)	Survival time (days)	Median survival (days)
Control (14)	5.50 ± 0.34	43.77 ± 2.35	44.0
CEP (14)	6.36 ± 0.78	58.86 ± 4.09*	55.0

Notes: *$p < 0.05$ comparing to control group.

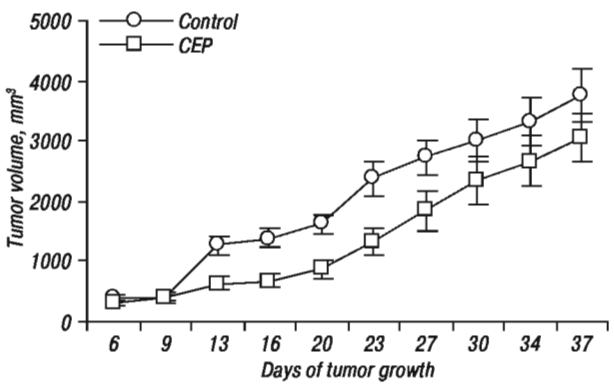

Fig. 2. The tumor volume of vaccinated with CEP-based vaccine and control mice bearing EC

Significant difference ($p < 0.05$) in tumor volume between unvaccinated and vaccinated animals was evident since the 13th day, i.e. after the end of vaccination. In particular, on the 13th and 16th day of tumor growth ITGI in the group of vaccinated mice was 50.57 and 50.74% respectively. Later the difference is gradually getting smaller (46.11 and 43.27% on the 20th and 23rd day of tumor growth respectively), but it stays higher than by 20% until the 30th day, when animals in the control group start dying. As a result of tumor growth inhibition, survival time in the group of vaccinated mice increased ($p < 0.05$): the ILSP in the CEP group reached 34.48%.

To sum up, using CEP on the model EC inhibited tumor growth, and, as a result, significantly lengthened the life span of tumor-bearing animals.

Study into anticancer activity the CEP-based vaccine on the model of S37. The anticancer effect of CEP in case of S37-bearing mice was investigated applying different schemata of vaccination; however, there was no significant difference between groups of vaccinated and unvaccinated animals. So, Table 3 illustrates the data about the latent period of tumor forming and the survival time of immunized animals under different schemata: none of indices between the groups differ significantly.

Table 3. The latent period of tumor formation and the survival time for vaccinated with the CEP-based vaccine and control mice bearing S37

Group (sample size)	Latent period of tumor formation (days)	Survival time (days)	Median survival (days)
Control (15)	6.93 ± 0.29	45.67 ± 3.79	43.0
CEP № 1 (6)	6.67 ± 0.37	42.50 ± 3.81	41.0
CEP № 2 (7)	7.14 ± 0.44	34.57 ± 2.05	33.0
CEP № 3 (15)	7.00 ± 0.20	41.93 ± 2.12	42.0

The tumor volume of unimmunized and immunized mice bearing S37 did not differ significantly, though different schemata of vaccination were applied (Fig. 3). In other words, immunization did not affect significantly any indices of tumor growth; so we considered S37 were resistant to the CEP-based vaccine.

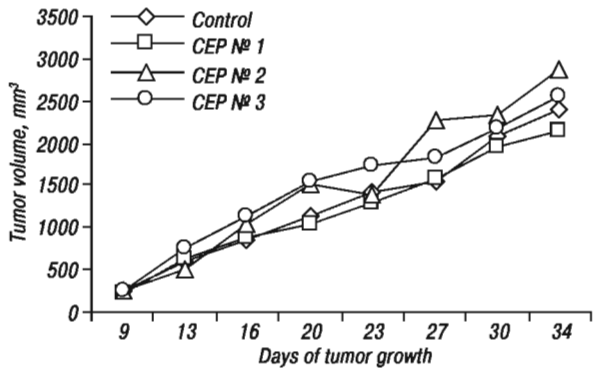

Fig. 3. The tumor volume of vaccinated with CEP-based vaccine and control mice bearing S37

As it appeared, different tumor strains differ in susceptibility to the CEP-based vaccine. The question arises whether there are any criteria according to which it would be possible to predict the efficiency of the CEP-based vaccine.

The evaluation of CEP-specific Ab in blood serum of tumor-bearing mice as an indication for use of the anticancer xenogeneic vaccine based on CEP. The blood serum from unvaccinated mice bearing tumors (LLC, EC and S37) on different days after tumor cells injections (on the 7th, 14th, 21st and 28th day) was taken and checked for its ability to react with its own tumor antigens (TAA) and CEP and the correlation coefficient between the level of CEP-specific or TAA-specific Ab and tumor volume was calculated.

Table 4 compares the portion of animals bearing different tumors whose blood serum was positive for TAA- and CEP-specific Ab. It is obvious that the number of animals in whose blood serum CEP- and TAA-specific Ab were detected was approximately equal. However, the portion of S37-bearing mice whose blood serum was positive for CEP-specific Ab was slightly

lower than that ($0.05 < p < 0.1$) whose serum was positive for their own TAA.

Table 4. The portion of blood serum samples positive for TAA- and CEP-specific Ab depending on tumor strains

Tumor strain	Portion of blood serum samples positive for	
	TAA-specific Ab, % (n)	CEP-specific Ab, % (n)
S37	91.67 ± 5.64 (22 out of 24)	70.83 ± 9.28 (17 out of 24)*
EC	81.82 ± 8.42 (18 out of 22)	77.27 ± 9.17 (17 out of 22)
LLC	83.33 ± 7.61 (20 out of 24)	79.17 ± 8.29 (19 out of 24)

Notes: *$0.05 < p < 0.1$ comparing to a portion of blood serum samples which are positive for TAA-specific Ab

The level of TAA-specific Ab in blood serum of tumor-bearing mice was almost the same and did not depend on the tumor strain or the day after tumor transplantation (Fig. 4, *a*). As a result, there was not found any correlation between the level of TAA-specific Ab and tumor volume (see Table 5, where correlation coefficients are presented).

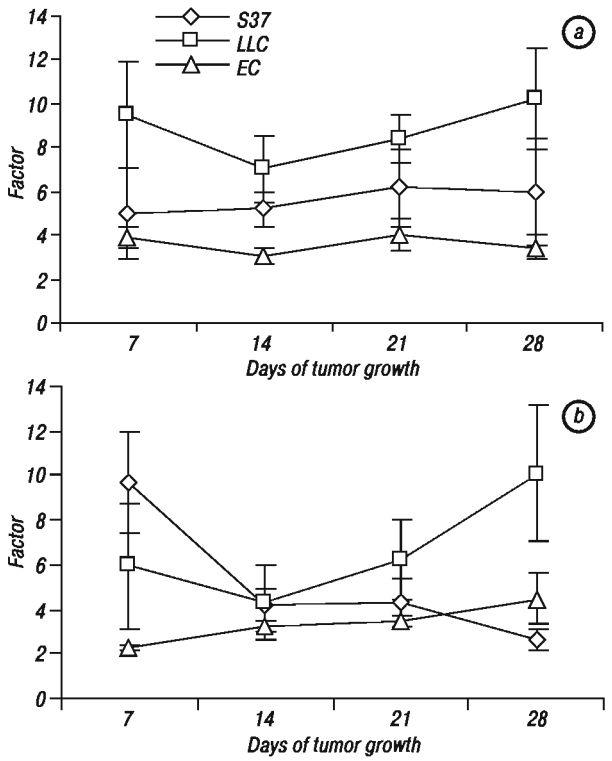

Fig. 4. The level of TAA- (*a*) or CEP-specific (*b*) Ab in blood serum of mice bearing different tumors, depending on the term after the tumor cells injection

Table 5. Pearson correlation coefficient (r) between the tumor volume and the level of TAA- or CEP-specific Ab depending on the tumor strain

Tumor strain	Correlation coefficient between tumor volume and level of	
	TAA-specific Ab	CEP-specific Ab
S37	0.04	−0.48**
EC	−0.09	0.67*
LLC	0.26	0.42**

Notes: *$p < 0.05$; **$p < 0.07$.

The level of CEP-specific Ab varied and differed more significantly (Fig. 4, *b*). For example, mice with S37 showed the highest level of CEP-specific Ab on the 7th day after tumor transplantation, which was decreasing gradually and significantly ($p < 0.05$ comparing to the 7th day) until the 28th day of the experiment. In contrast, EC-bearing mice had the lowest level of CEP-specific Ab on the 7th day, but it statistically significantly grew until the 28th day of tumor growth. In the group of LLC-bearing mice, the level of CEP-specific Ab gradually increased, but there was no statistically significant difference between indices on different days of tumor growth.

So, there was a strong, but multidirectional correlation between the level of CEP-specific Ab and tumor volume (see Table 5): the correlation was positive for mice bearing LLC and EC ($r = 0.42$; $p < 0.07$ and $r = 0.67$; $p < 0.05$ respectively), but it was negative for mice bearing S37 ($r = -0.48$; $p < 0.07$).

So, the detection of CEP-specific Ab in blood serum *per se* cannot be applied as an indication for use of the CEP-based vaccine, as long as the same portion of mice bearing resistant and nonresistant tumor strains were positive for it. However, if the level of CEP-specific Ab is low, but a tumor burden is huge or the level of Ab is decreasing while a tumor is growing, it possibly can indicate the tumor is resistant to the CEP-based vaccine.

DISCUSSION

According to the results, the antitumor efficiency of CEP was evident in the case of two out of three tumor strains used in the study. The high anticancer efficiency of CEP was shown on two models of carcinoma: EC (the tumor came into being as spontaneous breast cancer [26]) and LLC (the tumor came into being as lung cancer [26]); however, sarcoma (S37 started to exist as a breast tumor, but through many transplantations it turned into undifferentiated polymorphous cell sarcoma [26]) turned out to be resistant to the CEP-based vaccine both at different therapeutic schemata of vaccination and at prophylactic — before tumor was transplanted — one (the data are not shown). Since the tumor models, used in the study, differ in histogenesis, we can assume that the anticancer efficiency of the CEP-based vaccine depends on this factor. However, it is impossible to prove or disprove this assumption in the scope of this work, because the number of model tumors (only 3) is not enough for this.

The fact should be pointed up that both "sensitive" to the vaccine tumors (LLC and EC) are undifferentiated or poorly differentiated carcinomata. The low level of tumor differentiation generally is associated with a worse prognosis [27]. It can be assumed that the application of the vaccine based on CEP, probably, can improve the results of treatment in case of un- and poorly differentiated carcinomata.

On the other hand, the obtained results — different anticancer efficiency in the case of different tumor models — once more point to the necessity to find a reliable indication for use of the CEP-based vaccine. Most prognostic and diagnostic markers that are used now in clinical and laboratorial practice are based on detecting in serum specific proteins, so-called oncomarkers or Ab specific to them. Now a growing body of literature points to the importance to detect in serum oncomarkers specific Ab as a more sensitive method [28–30]. The advantage of the latter is due to several factors. Particularly, the levels of oncomarkers sufficient for detection in serum appear at relatively later stages of tumor

growth when it already has clinical signs. Meanwhile, Ab specific to these oncomarkers can be detected at earlier stages of tumor growth, sometimes even before clinical signs of a tumor [31, 32]. Therefore, we decided to check whether the presence of CEP-specific Ab can serve as an indication for use of the vaccine.

It was shown that some portion of mice bearing resistant or nonresistant tumor strains was positive for CEP-specific Ab: 70.73 ± 9.28% of S37 bearing mice (considered as resistant tumor) and 77.27 ± 9.17% and 79.17 ± 8.29% of EC and LLC bearing mice respectively expressed CEP-specific Ab in their blood serum. So, CEP-specific Ab in the blood serum *per se* cannot be applied as an indication for use of the CEP-based xenogeneic vaccine. Changes in CEP-specific Ab levels were more informative. In the case of S37 bearing mice the level of CEP-specific Ab was decreasing while tumor was growing (correlation coefficient was −0.48). Probably, CEP-specific Ab have formed circulating immune complexes, so they could not be detected in ELISA, or the level of CEP-specific Ab decreased during the formation of immune response to the tumor as having lower affinity to TAA of S37. On the contrary, in the case of "sensitive" LLC or EC the level of CEP-specific Ab was constantly increasing. Considering that, in the process of immune response formation the number of Ab with higher affinity is growing (otherwise known as affinity maturation [33]), we may assume that CEP more resemble EC's or LCC's TAA (or contain more proteins which share homology with these tumor antigens), than that of S37. That is why immunization with CEP has no effect on S37. So, the low level of CEP-specific Ab together with a huge tumor burden may indicate that tumor is resistant to the CEP-based AV therapy.

In conclusion, the application of the CEP-based vaccine to animals with LLC and EC had anticancer and antimetastatic effects: statistically significant tumor growth inhibition (both models), statistically significant lengthening of survival time by 34.48% (EC) and the inhibition of metastasizing of LLC — Index of metastases inhibition reached 77.56%. The CEP-based vaccine did not have any anticancer effect in the case of S37. Detection of CEP-specific Ab in a blood serum *per se* cannot be applied as an indication for use of the CEP-based vaccine. However, the low level of CEP specific Ab when tumor burden is huge points to the unfeasibility of vaccine based on CEP.

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