Exp Oncol 2020
42, 2, 120–125

Experimental ONCOLOGY

# CONJUGATION OF NEW DNA VACCINE WITH POLYETHYLENIMINE INDUCES CELLULAR IMMUNE RESPONSE AND TUMOR REGRESSION IN NEUROBLASTOMA MOUSE MODEL

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***Aim:*** **To estimate immunogenicity and antitumor effect of new DNA vaccine against neuroblastoma using tyrosine hydroxylase as an antigen and linear polyethylenimine (PEI) 20 kDa as a synthetic DNA carrier in syngeneic mouse tumor model.** ***Materials and Methods:*** **DNA vaccine was made by cloning the tyrosine hydroxylase minigene fused to the potato virus X coat protein gene into the expression vector. The A/J mice were vaccinated by three intramuscular injections. For immunogenicity study, immune response was estimated by target cells cytotoxicity assay, interferon-gamma production in enzyme-linked immunospot assay and antigen-specific antibodies in 14 days after the final vaccination. Antitumor effect was assessed by measurement of tumor volume and event-free survival rate in mice with engrafted NB41A3 murine neuroblastoma cells following three intramuscular injections of the vaccine: 7 days before, 5 and 10 days after tumor engraftment. The immune response was also assessed on the 30$^{th}$ day after tumor engraftment.** ***Results:*** **The immunogenicity and antitumor effect of the vaccine in the form of aqueous solution of DNA and DNA-PEI conjugate were compared. Splenocytes cytotoxicity was the highest in the group of DNA-PEI vaccines (37.3 ± 6.9% lysis of target cells) compared with the unconjugated DNA vaccine (26.2 ± 4.0%) and placebo control (21.9 ± 3.7%). The production of interferon-gamma in the enzyme-linked immunospot assay was about ten times higher in the DNA-PEI group than in the other groups. The vaccine slowed or prevented the growth of the tumor. Mice vaccinated with the DNA-PEI vaccine had significantly better survival compared to control group ($p < 0.0003$).** ***Conclusions:*** **DNA vaccine against tyrosine hydroxylase, administered as a DNA-PEI 20 kDa conjugate, slows down the growth of neuroblastoma cells engrafted to mice.**

***Key Words:*** **neuroblastoma, DNA vaccine, tyrosine hydroxylase, polyethylenimine.**

**DOI: 10.32471/exp-oncology.2312-8852.vol-42-no-2.14473**

Neuroblastoma (NB) is a malignant extracranial tumor of sympathetic nervous system. It belongs to the most common pediatric tumors, along with lymphomas, kidney tumors and the central nervous system tumors [1]. NB derives from progenitor cells of the sympathetic nervous tissue and can spread to other parts of the body including bones, skin, and liver [2]. Approximately 60% of patients are in the high-risk group because of the concomitant adverse prognostic factors, dissemination of the disease, older age, *MYCN* amplification, etc. However, despite the use of various therapeutic modalities (autologous bone marrow transplantation, myeloablative chemotherapy), it is not possible to achieve long-term survival. About 55% of patients in the high-risk group experience relapse [3]. Immunotherapy has a good therapeutic potential for cancer treatment [4] and might be considered a promising option in the treatment of NB as well.

DNA vaccine is a plasmid vector encoding a tumor antigen against which an immune response is induced. The vaccine administration elicits predominantly cellular immune response via Th1 response followed by activation of $CD8^+$ cells [5]. An important aspect is the presence of competent immune system that will respond to the vaccine components. Therefore, DNA vaccination is applicable mainly for the concomitant therapy of tumors with low-to-medium malignant potential, a high probability of long-term remission, and the risk of subsequent recurrence, and could considered as an adjuvant treatment method, which allows reducing the likelihood of the disease return or the metastases formation.

DNA-vaccine design is a key step for successful outcome. Genetic construct of vaccine includes a tumor-specific antigen in a mammalian expression vector. A number of additional sequences can improve DNA vaccine, e.g. bacterial or viral immune enhancer gene, leader peptides for protein secretion [6], nuclear targeting peptides [7], sequences for ubiquitin dependent degradation [8], etc. In this study, we used tyrosine hydroxylase (TH) as an NB-associated antigen [9–11]. TH is the enzyme of the sympathetic nervous system that converts tyrosine into dioxyphenylalanine and regulates the rate of biosynthesis of dopamine and norepinephrine. All cases of NB are characterized by high and stable expression of TH, regardless of the stage and *MYCN* amplification status. Catecholamine production and metabolism are the established clinical markers for the diagnosis and monitoring of NB patients [12].

Vaccine DNA delivery methods are very important because the bulk of naked DNA is degraded by nucle-

Submitted: August 01, 2019.

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*Abbreviations used:* CTA – cytotoxic activity of splenocytes; DNA-WS – aqueous solution of pDNA; ELISPOT – enzyme-linked immunospot assay; IC– intact control; IFNγ – interferon-gamma; NB – neuroblastoma; pDNA – plasmid DNA; PC – placebo control; PEI – polyethylenimine; PL – peptide library; PVXCP – potato virus X coat protein; TH – tyrosine hydroxylase; TGC – tumor growth control.

ases and large plasmid DNA (pDNA) barely enters the cell nucleus. In order to increase the DNA vaccine half-life, and increase transfection rate, various chemical carriers are used [13]. One of the best *in vitro* studied transfection reagent is polyethylenimine (PEI), a polycation capable of forming small-sized (100–400 nm) positively charged nanoparticles with DNA with [14]. These nanoparticles deliver effectively pDNA to a destination point that was established in cell cultures and in experiments *in vivo* [15, 16]. An increase in the molecular weight and branched polymer structure increases transfection activity as well as toxicity of DNA-PEI polyplex. Conjugation of pDNA with PEI 8kDa had good results in eliciting antitumor response in syngeneic mouse model [17] but immunogenicity can be further improved. In this study, we investigated secreted form of fusion DNA vaccine (TH — potato virus X coat protein (TH-PVXCP)) conjugated with linear PEI 20 kDa on NB mouse model.

## MATERIALS AND METHODS

***Preparation of DNA vaccine.*** The general design of DNA vaccine was essentially the same as described earlier [17]. Plasmid vector pING was the courtesy of Jedd D. Wolchok, USA. DNA vaccine coding highly homologous fragment of TH (124 amino acids from 278 to 401 residue of native TH) and costimulatory gene of PVXCP was modified. Immunoglobulin heavy chain leader peptide was added to the N-end of protein to provide secretion of the protein out of the cell. To increase cell transfection efficacy pDNA was conjugated with linear PEI 20 kDa. Investigation of DNA condensation by various PEI isoforms showed that the conjugate of DNA and PEI 20 kDa in a mass ratio of 1:1.5 with DNA in a concentration of 100 ng/μl provides the best transforming activity.

DNA-PEI conjugate was prepared by mixing 10 μg pDNA with aqueous solution of 15 μg PEI 20 kDa, pH = 7.0 (Sigma, USA) (total volume 100 μl) and administered as intramuscular injection.

***Cells and mice.*** Murine NB cell line NB41A3 was purchased from Russian Cell Culture Collection (Institute of Cytology Russian Academy of Sciences, Saint-Petersburg, Russia). Cells were grown in F10 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin. Syngeneic A/J mice were purchased from the Harlan Laboratories and were bred in the vivarium of the Institute of Bioorganic Chemistry of the National Academy of Sciences. The animals aged 8–10 weeks (n = 74) were used (a female to male ratio 1.6:1). All animal experiments were done according to the Belarusian Guide for the Care and Use of Laboratory Animals.

***Immunogenicity study.*** The mice (two groups, 10 males + 10 females) received three intramuscular injections (every 5$^{th}$ day) of empty vector pING or aqueous solution of 10 μg of pDNA (DNA-WS) without tumor engraftment. Fourteen days after the last injection, the mice were sacrificed and immune responses were evaluated (splenocytes cytotoxic activity, interferon gamma (IFNγ) production and the titer of antigen-specific antibody).

***Study of antitumor effects.*** The mice were randomized into the following groups: intact control (IC) (n = 7), tumor growth control (TGC) — (n = 5), placebo control (PC) — mice vaccinated with empty vector pING (n = 13), mice vaccinated with DNA-WS (n = 14) and vaccinated with pDNA-PEI conjugate (n = 15). Mice received three intramuscular therapeutic vaccinations: 7 days before, 5 and 10 days after tumor engraftment. Mice of all groups except for IC were injected subcutaneously with $10^6$ NB41A3 cells. Tumor growth was measured by digital microcaliper every five days. The tumor volume [mm$^3$] was calculated as width × length × height. The last measurement was performed on Day 25. All mice were sacrificed on the 30$^{th}$ day after engraftment of NB cells. Cytotoxicity test and enzyme-linked immunospot assay (ELISPOT) for IFNγ on mouse splenocytes were started the same day. Collected mouse sera were aliquoted and stored at –80 °C before testing.

***Cytotoxicity assay.*** At the end of the experiment, splenocytes were harvested and incubated in the presence of irradiated NB41A3 cells in RPMI-1640 supplemented with 10% FBS, 1% non-essential amino acids, 50 μmol/l β-mercaptoethanol and 100 U/ml IL-2 (Biotech, Russia) at a target:effector (T:E) ratio of 1:10 and 1:25 overnight. 10,000 of target cells per well were plated into 96-well plates (Sarstedt, Latvia). Effector cells were added to duplicate wells at varying T:E ratios. Cytotoxic activity of splenocytes (CTA) was determined by using a flow cytometry assay (FC500, Beckman Coulter, USA). Target cells were prestained with carboxyfluorescein succinimidyl ester (STEMCELL, Canada) to separate them from the effectors; propidium iodide was used for vitality staining. Cytotoxicity was calculated according to the formula:

*lysis (%) = [experimental cdc — spontaneous cdc] / [100 — spontaneous cdc] • 100%*,

wherein cdc — count of dead cells.

***ELISA.*** The samples of whole blood collected from the heart in aseptic conditions were centrifuged at 5000 g to separate serum from cellular fraction. The sera were tested in duplicate at 1:100 and 1:200 dilutions in 96-well plate coated with PVXCP. Immobilized anti-PVXCP antibody was detected with goat anti-mouse antibody IgG+IgM labeled with horseradish peroxidase (Thermo Fisher Scientific, USA). Two-fold dilutions of mouse serum collected from animals immunized with PVX virus were used as calibrators. Each dilution contained specified amount of relative units per ml (1000–125).

***ELISPOT.*** IFNγ secretion was determined using the BD ELISPOT mouse IFNγ ELISPOT Set (BD Biosciences, USA). Splenocytes were harvested from individual mice after immunization with DNA vaccine. Effector cells were plated in duplicate at 2.5 • $10^5$/well in 200 μl final volume with medium alone, 20 μg/ml of the PVXCP protein or 10 μg/ml of the TH overlapping peptide library (PL) for 24 h. Phytohemagglutinin

(Sigma, USA) (4 μg/ml) was used as a positive control. The plates were analyzed with an ELISPOT reader (CTL ImmunoSpot® S5 UV Analyzer, C.T.L., USA). The mean number of spots from duplicate wells was calculated for each responder animal and adjusted to represent the mean number of spots per $10^6$ spleen cells.

***Statistics.*** The statistical significance of differential findings between experimental groups was determined by nonparametrical Mann — Whitney *U*-test using GraphPad Prism 6.0 software. The differences were regarded as significant if $p < 0.05$. Survival rates were calculated using Kaplan — Meier method, and differences in survival were assessed using the long-rank test.

## RESULTS

***Vaccine immunogenicity study.*** Immunogenicity of DNA vaccine was tested in mice without tumor engraftment to check the immune capacity of the aqueous solution of vaccine. DNA-WS did not influence T-cell cellular immune response in healthy mice after vaccination. Percent of lysed cells was comparable with control group: 3.4% and 4.9% in control *vs* 2.9% and 5% in DNA-WS, effector:target ratio 1:10 and 1:25, respectively). Median (Min; Max) of IFNγ production after incubation with PVXCP protein did not differ between groups (6 (0; 82) spots/$10^6$ splenocytes in control *vs* 1 (0; 366) in DNA-WS). The same results were obtained after stimulation with TH PL (0 (0; 2) in control *vs* 0 (0; 70) in DNA-WS. It is worth noting that there was an obvious division into responders and non-responders in each group. Fifty percent responded to PVXCP and 15% to TH library. Mean value of IFNγ production among responders increased in vaccinated mice after stimulation with both PVXCP protein (3.9 times higher than in control) and TH PL (35 times higher). Anti-PVXCP antibody response was not detected in vaccinated mice (mean ± SEM was 117 ± 10 RU/ml *vs* 113 ± 23 RU/ml in control group).

***Antitumor effect of vaccine.*** In cytotoxicity test against NB41A3 cells, all groups with engrafted tumor demonstrated levels of target cell killing significantly higher ($p < 0.01$) than IC mice even in PC group, where CTA was 21.8 ± 3.7 compared to 11.69 ± 1.9 in IC (1:25 T:E ratio) (Fig. 1, *a*). Injection with DNA-WS led to double increase of CTA compared to PC at a 1:10 T:E ratio. Mice administered with DNA-PEI conjugate had a tendency to the highest CTA (17.97 ± 5.20% and 37.29 ± 6.90% in 1:10 and 1:25 ratio respectively, $p = 0.07$ *vs* PC).

In ELISPOT test with PVXCP protein and TH PL, IFNγ secretion level was equally low in IC, PC and DNA-WS groups. Mean number of spots per $10^6$ cells was 1.4 and 0.3 respectively (Fig. 1, *b*). Only mice vaccinated using DNA-PEI conjugate exceeded the placebo level. The mean value was 10.4 spots/$10^6$ cells for PVXCP and 8 spots/$10^6$ cells for PL.

Significant antibody response to PVXCP protein was not detected in any vaccinated mice. IC and DNA-PEI groups had the lowest titer of antibody (134 ± 27 and 146 ± 23 RU/ml, respectively). PC and DNA-WS groups had a little elevation in anti-PVXCP antibody response (174 ± 42 and 171 ± 19 RU/ml, respectively).

***Tumor growth and survival of vaccinated mice.*** An examination of mice for primary tumor was started on the 5th day after tumor engraftment. First NB foci were established on the 10th day in each group of mice. The differences in tumor growth in treated and untreated mice became detectable on the 20th day. Mean tumor volume in mice vaccinated with DNA-WS on the 20th day was 308.5 ± 155.0 $mm^3$ that is ~4 times smaller than in TGC and PC group (1276 ± 417 and 1226 ± 467 $mm^3$, respectively). These differences become significant on the 25th day where the mean tumor volume of DNA-WS-treated mice was 894 ± 320 $mm^3$, that was 2.4 times smaller than in PC group (2136 ± 524 $mm^3$) ($p = 0.04$) (Fig. 2, *a*). Vaccination with DNA-PEI conjugate had the best inhibiting effect on NB growth on the 20th and 25th days. A mean tumor volume in this group was 252 ± 194 on the 20th day ($p = 0.002$ *vs* TGC and $p = 0.007$ *vs* PC) and 356 ± 192 $mm^3$ on the 25th day ($p = 0.002$ *vs* PC) (Fig. 2, *b*). Beyond that, several mice in the treated groups did not form detectable tumors by the last time point on the 30th day, namely 23% (n = 3) of mice in PC group, 28.5% (n = 4) in DNA-WS and 53% (n = 8) in DNA-PEI group. Even if mice without tumors would be excluded from the experimental groups, it is evident that DNA-

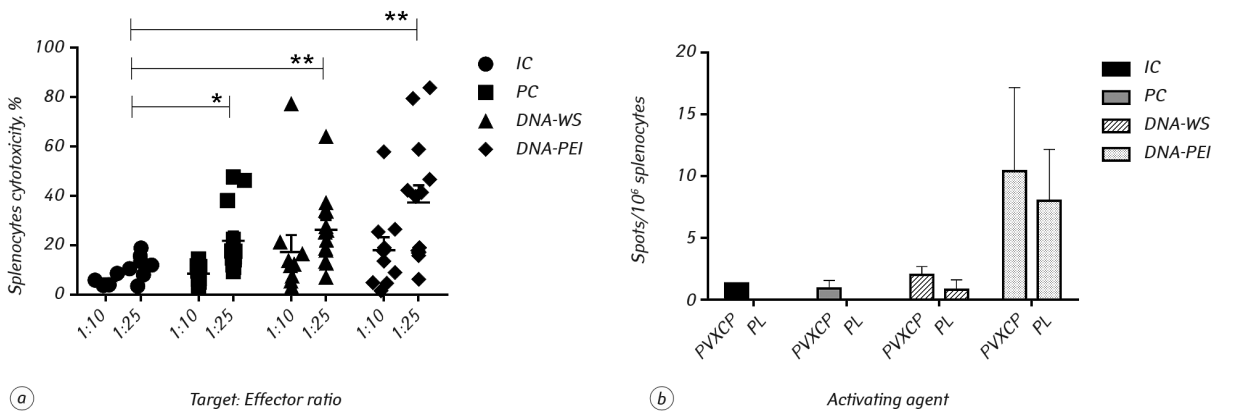


**Fig. 1.** Immune response induced by DNA vaccine in mice with engrafted NB: *a* — Specific cytotoxic activity of splenocytes against murine NB cell line NB41A3 with target: effector ratio of 1:10 and 1:25; *b* — IFNγ production by mouse splenocytes after incubation with PVXCP protein and TH PL. Mean ± SEM. *$p < 0.05$; **$p < 0.01$

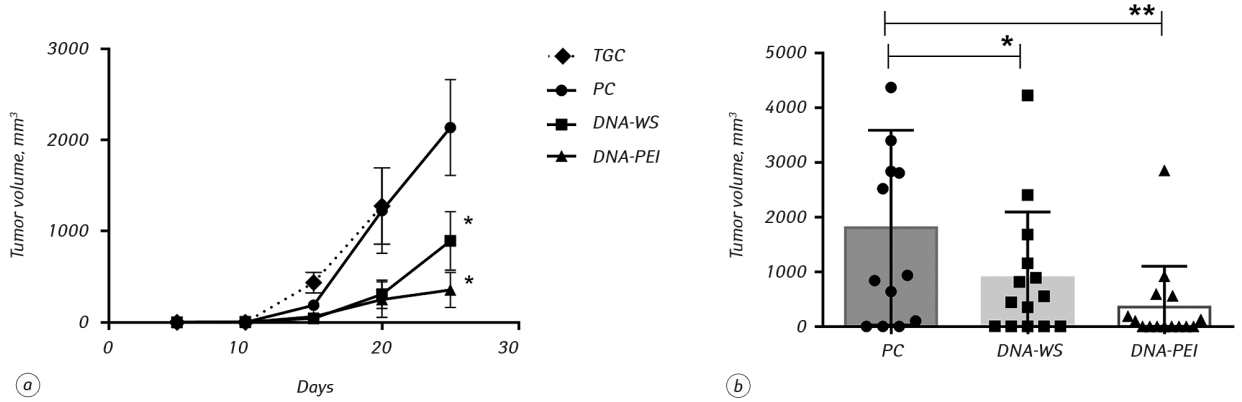


**Fig. 2.** Dynamics of NB growth in vaccinated and non-vaccinated mice: *a* — Tumor growth from day of tumor engraftment until the 25th day of monitoring; *b* — Tumor volume on the 25th day. Mean ± SEM. *$p < 0.05$; **$p < 0.01$. The tumors could not be measured on 25th day in the group of tumor growth control and on 30th day in other groups in the mice that have not yet died at these time points.

PEI vaccination led to the significant reduction of tumor growth comparable to PC (763 ± 365 *vs* 2341 ± 529 mm$^3$, respectively; $p = 0.04$) and DNA-WS group (1252 ± 397 mm$^3$). Moreover, vaccinated mice without tumors had better results in all tests [performed in the study. CTA and IFNγ production in tumor-free mice injected with DNA-WS and DNA-PEI were twice higher. There was also only low negative correlation of immune response with final measured tumor volume in combined groups ($R^2_{CTA} = -0.19$ and $R^2_{IFN\gamma} = 0.26$). There was a significant difference in anti-PVXCP antibody titer in both groups: in DNA-WS group 134.6 ± 53.0 RU/ml (tumor+) *vs* 243.6 ± 57.0 RU/ml (tumor-free) ($p = 0.005$); in DNA-PEI group 64 ± 46 RU/ml (tumor+) *vs* 161 ± 81 RU/ml (tumor-free) ($p = 0.003$) (The data are presented as mean ± SD). A significant negative correlation was found between antibody titer and final tumor volume ($R^2 = -0.41$, $p = 0.02$).

Kaplan — Meier progression-free survival analysis was also used to display the difference in outcome between experimental and control mice groups (Fig. 3). All signs of the increased malignancy (necrosis, tumor volume 1000 mm$^3$, skin ulceration) were considered as complete events. It was shown that mice vaccinated with DNA-WS had significantly better survival compared to placebo group (53.3 ± 12.9% *vs* 11.8 ± 7.8%, $p < 0.002$) and to TGC group ($p = 0.004$). On the final day of monitoring, 28.5% of mice vaccinated with DNA-WS had no detectable tumor *vs* 23.1% in PC. Mice vaccinated with the DNA-PEI vaccine had significantly better survival compared to PC group (76.9 ± 11.6% *vs* 11.8 ± 7.8%, $p < 0.0003$) and to TGC group ($p = 0.006$), but not significant increase in 23.6% compared to DNA-WS vaccine. Only three animals out of 15 vaccinated with the DNA-PEI vaccine achieved the complete event on the 25th day.

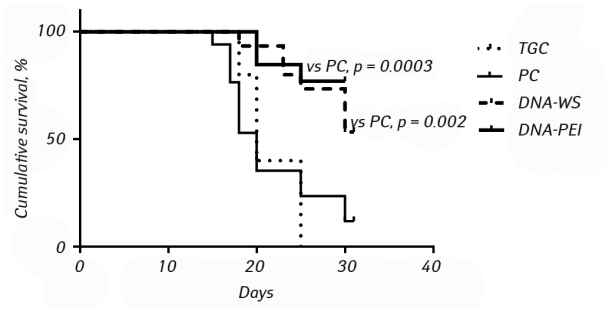


**Fig. 3.** Kaplan — Meier survival analysis

## DISCUSSION

The survival of patients of high-risk group or those with relapsed NB is still negligible despite the use of high-dose chemotherapy and autologous HSCT. New treatment approaches, including active immunotherapy, remain in demand for the treatment of patients with this disease. DNA vaccine is a convenient tool for immunotherapy due to its availability, low cost and high safety. Although DNA vaccines were first delivered in the form of an injection of an aqueous solution of pDNA that triggers an immune response, clinical trials have been quite disappointing due to the low efficiency of DNA immunization. A number of methods have been proposed for improved DNA delivery of vaccines *in vivo*, including electroporation [18, 19] and various chemical carriers [13]. It was previously shown that conjugation of DNA-vaccine coding TH-PVXCP fusion with linear PEI 8 kDa elicits tumor-protective immunity in a syngeneic mouse NB model [17]. PEI is a cationic polymer widely applied as a transfection reagent. PEI is a positively charged polymer capable of forming stable complexes with the DNA (polyplexes). DNA-PEI nanoparticles provide DNA protection from nucleases, cell uptake, "lysosomal escape" and enhance antigen cross-presentation [20]. *In vitro* transfection experiments revealed that PEI isoforms with higher molecular weight and branched structure provide more effective transfection but could be more toxic. After a series of *in vitro* experiments on transfection of HEK293T cell (data not shown), we found that the conjugation of pDNA with PEI 20 kDa in a 1:1 to 1:2 mass ratio had the optimal combination of high efficiency and low toxicity compared to other linear and branched isoforms. Additionally, DNA-vaccine was modified to be secreted outside of the cell what should increase antigen uptake by antigen-presenting cells and T cell priming. We provided the experimental data that conjugation of new TH-PVXCP DNA-vaccine with PEI 20 kDa induces cellular im-

mune response against NB and promotes an increase of event-free survival.

NB41A3 cell line was derived from mouse non-metastatic, rapidly progressing NB. DNA vaccine can be tested in a "prophylactic" (before tumor engraftment) or "therapeutic" (after tumor engraftment) regimen. After a series of preliminary experiments, we chose intermediate option with one vaccine administration before and two after tumor engraftment. First vaccination is supposed to prime immune response and then boost it after the tumor engraftment to enable induction of primary immune response before the formation of a bulky tumor. At the later stages of tumor growth, when the tumor load is high, the vaccine may not be effective.

In this study, we compared the efficiencies of vaccination by intramuscular injection of DNA vaccine in aqueous solution with DNA vaccine prepared as PEI conjugate. Our results demonstrate that DNA conjugation with PEI substantially enhances both immunogenicity and antitumor effect. The immune response to vaccination with DNA-PEI was characterized by more pronounced CTA and the higher level of IFNγ production. 53% of mice vaccinated with DNA-PEI, 28.5% with DNA-WS and 23.1% in PC did not show tumor growth. The fact that the number of animals without detectable tumor was directly proportional to the immunogenicity of the vaccine suggested the direct contribution of vaccination to tumor rejection. All mice vaccinated with DNA-PEI demonstrated significant therapeutic effect of the vaccine at the final point of monitoring on the 30$^{th}$ day. Moreover, cellular immune response was higher than in previous study with PEI 8 kDa [17].

Interestingly, some mice in the placebo group also demonstrated some cytotoxic activity of lymphocytes probably induced by tumor itself or supported by innate immunity due to the adjuvant effect of CpG motifs. We also found weaker IFNγ secretion in vaccinated mice with tumor than in vaccinated IC. The possible explanation might be an immunosuppressive effect of the tumor on lymphocytes.

No significant elevation of specific anti-PVXCP antibodies of IgG and IgM classes was found in all experimental groups despite the modification of the construct with leader peptide and expression of vaccine protein product outside the cells. It could be due to the absence of secretion of a sufficient amount of soluble protein capable of inducing a humoral immune response. In our case, such factors as bacterial pDNA, intramuscular injection, intracellular localization of the native antigen, as well as the use of PEI, led to the formation of a Th1 immune response [16, 21, 22].

In addition, it was found that mice without tumor had better indices of immune response than mice with established NB. It could be explained by the difference in the immune reactivity. Tumor growth might be prevented in the animals with more immune reactivity. The negative correlation between tumor volume and the indices of the immune activity confirms that dynamics of tumor growth depends on activity of immune components. The antigen-dependent response seems to play a key role in tumor rejection. Activation of humoral immunity takes two weeks, and according to our vaccination scheme, antibody should appear in a bloodstream before the tumors became palpable (on Days 10–17).

To summarize, our results show that a DNA vaccine encoding the chimeric TH-PVXCP protein, administered as DNA-PEI 20 kDa conjugate, has anti-NB activity in the experimental model of the aggressive tumor growth. Therefore, prophylactic and antirelapse regimens of DNA vaccination in the setting of surgically resected tumor is worthwhile studying.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding the publication of this paper.

## ACKNOWLEDGMENTS

The study was funded by the Belarus Ministry of Health (Grant № 20171462).

We would like to thank Dr. Aliaksandr A. Yarmishyn (Taipei Veterans General Hospital) for editing this manuscript.

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