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Towards development of plasmacytoma cells-based expression systems utilizing alphavirus vectors: An NS0-VEE model

Viktoriya V. Keyer[a], Alexandr B. Shevtsov[a], Mikhail M. Zaripov[b], Aliya Zh. Baltabekova[a], Erlan M. Ramanculov[a], Alexandr V. Shustov[a,*]

[a] National Center for Biotechnology, Korgalzhin hwy 13/5, 010000, Astana, Kazakhstan
[b] Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Puschino, 142290, Russia

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ABSTRACT

Plasmacytoma (myeloma) cells have a large protein expression capacity, although their industrial use is confined to stable expression systems. Vectors derived from genomes of viruses from the genus Alphavirus allow obtaining of high yields of target proteins but their use is limited to transient expression. Little information has been published to date on attempts to combine the myeloma cells as hosts with alphaviruses as expression vectors. A plasmid construct which allows rescue of a model alphavirus *Venezuelan equine encephalitis virus* (VEE) upon transfection of a cell culture was created. Mutations in the capsid and nsP2 genes allow for less cytopathogenic propagation of the virus. A cDNA-copy of the genome was placed in a plasmid under the control of the CMV promoter for virus rescue following DNA transfection. Parameters for the virus rescue by electroporating of the infectious clone in murine myeloma cells (NS0) were optimized. The highest FFU counts (1.2×10^5 FFU per 10 ug DNA) were produced with 2 pulses (voltage 250 V, capacitance 960 u F) and the best electroporation buffer was selected from eight buffers. Self-sustained VEE infection was established in NS0 cultures with high titers (8×10^8 FFU/ml) of the virus, despite a fraction of infected cells dying during 5-days observation. Further development of the NS0-VEE expression system may require addressing of apoptosis induced by VEE.

1. Introduction

Plasmacytoma-derived cell lines including murine origin lines NS0, Sp2/0, etc. are often used for development of hybridoma cell lines and large-volume production of monoclonal antibodies (mAbs) and recombinant biopharmaceutical proteins (Griffin et al., 2007). The plasmacytoma (commonly referred to as myeloma) cells are characterized by excellent protein yields (~3 g/L) (Fan et al., 2009). Despite protein glycosylation profiles in murine and human cells are different, and proteins expressed in murine cells can be immunogenic in humans, the NS0 line persists as an industrial workhorse to obtain proteins for human treatment (Morrow, Jr., 2007).

Viral vectors based on genomes of alphaviruses belong to a class of the most productive vectors for heterologous expression in mammalian cells. Alphaviruses are members of the genus *Alphavirus*, family *Togaviridae* and the most studied species in the genus are *Sindbis virus* (SIN), *Semliki Forest virus* (SFV) and *Venezuelan equine encephalitis virus* (VEE). Alphaviruses possess 11–12 kb-long RNA(+) genomes (positive sense means that genomic RNA (gRNA) is translated on ribosomes), do not use reverse transcription in their life cycles and replicate in the cytoplasm. After infection the gRNA is translated to produce non-structural proteins (nsP1-4) which assemble to form the replicase complex. The replicase complex uses the gRNA as a template to synthesize a negative sense RNA strand. In turn, the negative sense RNA strand is a template for replication of gRNA and high-level transcription of an accessory RNA species called subgenomic mRNA (sgRNA).

Studies on replication and translation of the alphaviruses resulted in creation of vectors for use as replicative backbones in live vaccines and to produce recombinant proteins using eukaryotic expression (Lundstrom, 2017). Several features make alphaviruses attractive

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Abbreviations: CFU, colony-forming unit; CMV, cytomegalovirus; EEEV, Eastern equine encephalitis virus; FFU, focus-forming unit; GOI, gene of interest; gRNA, genomic RNA; HDV, hepatitis D virus; HGH, human growth hormone; ICA, infectious centers assay; iPSC, induced pluripotent stem cells; ORF, open reading frame; PEI, polyethyleneimine; RBZ, ribozyme; RT, room temperature; SFV, Semliki Forest virus; sgRNA, subgenomic RNA; SIN, Sindbis virus; SP, subgenomic promoter; UTR, untranslated region; VEE, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus

[*] Corresponding author.

E-mail addresses: keer@biocenter.kz (V.V. Keyer), shevtsov@biocenter.kz (A.B. Shevtsov), mizaripov1969@gmail.com (M.M. Zaripov), Baltabekova@biocenter.kz (A.Z. Baltabekova), ramanculov@biocenter.kz (E.M. Ramanculov), shustov@biocenter.kz (A.V. Shustov).

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vectors: 1. they infect multiple vertebrate and invertebrate species and diverse cell types implying a wide range of suitable expression hosts; 2. for model alphaviruses such as VEE it is characteristic to show vigorous replication with production of large amounts of heterologous proteins and achieving of high titers; 3. alphaviruses have a natural process of synthesizing sgRNA which is not essential for intracellular replication, and is an appealing target for genetic engineering to generate transgene-expressing constructs.

Alphavirus vectors allow obtaining extremely high yields of target proteins (Quetglas et al., 2012), although depending on the vector, the production can be time-limited (transient) because of death of the producing culture as a result of a direct cytopathic effect of the virus replication (Fros and Pijlman, 2016) or induction of apoptosis (Baer et al., 2016; Li and Stollar, 2004). Expression using autonomously replicating fragments of alphavirus genomes (replicons) far outperforms traditional expression methods: cloning genes of interest (GOI) into eukaryotic plasmids or developing stably transformed cell lines having the GOI integrated into the genome (Aranda et al., 2014).

To further increase productivity of transient expression platforms, a combination of highly producing hosts such as myeloma cells and alphavirus vectors are forthcoming. Surprisingly, at present there are not many articles on the topic as only one paper actually gives expression levels using SFV vector with NS0 cells: see Fig. 2 in the work (Blasey et al., 2000), and no information is available on ability of alphaviruses to infect plasma cells during natural infection. Few studies investigate growth properties of alphaviruses in lymphocytes and the published data show inability of VEE to infect unstimulated lymphocytes (Levitt et al., 1979). Different alphavirus species show either tropism (preferential infection) or inability to infect specific cell types of hematopoietic cells. VEE is capable of infecting macrophages and has a lymphoid stage in its pathogenesis (when the infection occurs in organs of the lymphatic system) whereas closely related *Eastern equine encephalitis virus* (EEEV) is unable to replicate in lymphoid tissue (Taylor et al., 2015).

This paper demonstrates the creation of a DNA/RNA-layered system utilizing the VEE genome capable of less cytopathogenic replication and presents optimized conditions for virus rescue in the myeloma (plasmacytoma) NS0 cells.

2. Materials and methods

2.1. Cell cultures

Baby hamster kidney (BHK-21) cells were obtained from the National center for biotechnology (Astana). Adherent BHK-21 cells were grown in DMEM with high glucose (Lonza BE12-604 F/U1) supplemented with 10% fetal calf serum (FBS, Gibco 16000-044), 1% penicillin/streptomycin, 2 mM L-glutamine at 37 °C in atmosphere of 5% CO_2.

Murine myeloma cell line NS0 is from a collection of the Institute of Theoretical and Experimental Biophysics (Puschino, Russia). NS0 cells were grown in RMPI-1640 medium (Sigma R8758) supplemented with 10% FBS, antibiotics and L-glutamine. In culture vessels (T75 flasks, Sigma SIAL0641) NS0 cells grow as a predominantly adherent culture and form monolayers of loosely-attached cells.

2.2. DNA/RNA-layered system

Standard methods of genetic engineering were used to manipulate DNA. The VEE genome is from TC-83 strain (Genbank: L01443), apart from mutations which have been introduced to diminish cytopathogenicity. The mutations were introduced in the genes for nonstructural protein nsP2 and capsid protein (C protein) as described in (Atasheva et al., 2015; Petrakova et al., 2005). The mutation in the nsP2 (Gln739-> Leu) was described in (Petrakova et al., 2005). This mutation allows for less cytopathogenic replication of VEE replicons (fragments of VEE RNA without structural genes), although this mutation alone is not sufficient to provide the non-cytopathenicic phenotype to the replicating full-length genome. Clustered mutations in the gene encoding capsid (C) protein were described which prevent cytopathogenicity during replication of the full-length VEE genome in BHK-21 cells (Atasheva et al., 2015). In the pCMV-VEE-GFP construct, the sequence of the C gene is modified to incorporate the same amino acid changes which were described in (Atasheva et al., 2015).

The genome is placed downstream of the human cytomegalovirus (CMV) immediate-early promoter. The 3′-terminal sequence of the CMV promoter including the preferred transcriptional start is described in (Isomura et al., 2008). The transcription start site (G + 1) is positioned to precede the first nucleotide of the VEE 5′UTR.

To ensure production of viral genomes with proper 3′-ends, a ribozyme and an eukaryotic transcriptional termination signal were engineered downstream of the cloned genome. The antigenomic ribozyme of hepatitis D virus (HDV RBZ) was placed after the oligo-A stretch, which terminates the VEE genome. Following the RBZ, a polyadenylation signal of the human growth hormone (HGH) gene is positioned.

To simplify titering, GFP gene was engineered into the construct. Sequences of the VEE subgenomic promoter (SP) and 5′UTR of the sgRNA were added to the 5′-end of GFP gene and cloned upstream of the open reading frame (ORF) for structural proteins. In the final construct, the first (by order, 5′- > 3′) subgenomic promoter (SP1) drives synthesis of sgRNA for GFP; the second promoter (SP2) is for sgRNA which is translated to produce the structural proteins C-p62-6k-E1.

A sequence of the resulting DNA/RNA-layered system was deposited to Genbank: MH891622, its schematic representation is given in Fig. 1.

Plasmid pCMV-VEE-GFP was prepared using an alkaline lysis method, purified by banding in a cesium chloride gradient and used in transfection experiments.

2.3. Optimization of electroporation conditions

Two protocols describing conditions for electroporation were used as the starting conditions in optimization experiments. One method which in the following sections is referred to as the “high-voltage” protocol is from (Liljestrom et al., 1991). The other methods, referred to as “low-voltage” protocols, are those recommended by Bio-Rad, e.g. for NS0 (Bio-Rad Laboratories Inc, 2019a) and for BHK-21 (Bio-Rad Laboratories Inc, 2019b). For the “high-voltage” protocol electric parameters were: exponential-decay pulse, field strength 7500 V/cm, infinite resistance, capacitance 25 u F. The “low-voltage” protocols, for NS0: exponential wave, voltage 250 V, capacitance 960 u F; for BHK-21: square wave, voltage 140 V, pulse duration 25 msec. Cell cultures BHK-21 (a control for generation of live virus) and NS0 cell cultures were electroporated in amounts of 1×10^7 cells and 1 ug of the plasmid per electroporation, with either the “high-voltage” protocol or the “low-voltage” protocol. During the optimization process, electroporation buffers, incubation temperatures, the number of pulses and DNA concentrations were varied.

For electroporation experiments cell cultures were grown to ~90% confluence. Cells were harvested by trypsinization and collected by centrifugation at 1000 rpm for 5 min at 0 °C. In the majority of experiments the following procedures were done at 0 °C, using an ice-cold electroporation buffer. The cell pellet was resuspended in 7 ml of the electroporation buffer and the cells were collected again. One more wash with the same electroporation buffer was performed as described. The final cell pellet was re-suspended in the electroporation buffer and the cell suspension was diluted to obtain 1×10^7 cells in a 400 ul aliquot. An aliquot (400 ul) of cells was mixed with the plasmid (DNA amounts varying in the range 0.1–10 u g). The mixture was transferred to a chilled electroporation cuvette (2-mm gap, Sigma Z706086) and predetermined number of pulses was done using Bio-Rad Gene Pulser II.

Following the electric pulse(s), the cells were left to recover for various times (10–30 min) at either room temperature (RT, in majority

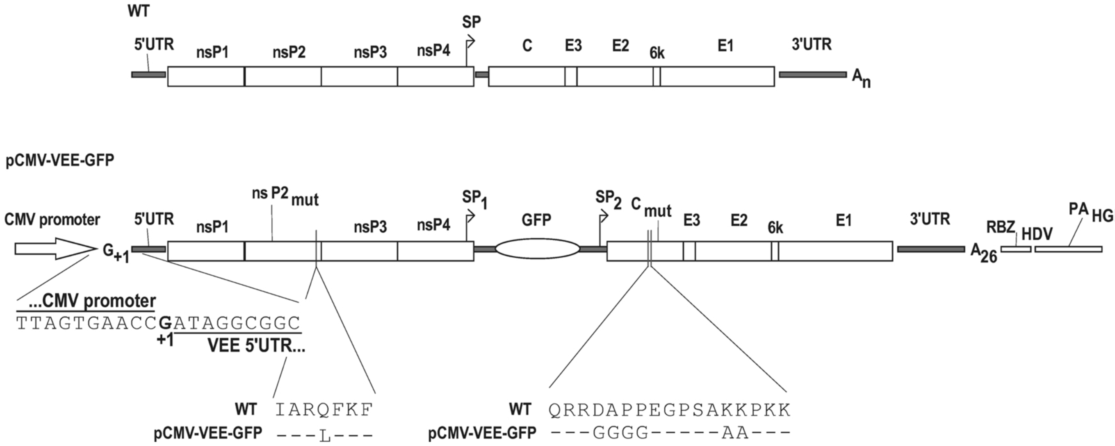

Fig. 1. Upper chart (WT), schematic representation of the VEE genome. Functional genes are shown as open rectangles. Untranslated regions are depicted as solid lines. Arrow sign designates the subgenomic promoter (SP). The genome has a polyA tail (An) at the 3′-terminus. Lower chart (pCMV-VEE-GFP), a part of a plasmid (DNA/RNA-layered system) which is designed to launch VEE replication after DNA transfection. The CMV promoter's transcriptional start site (G + 1) is positioned before the first nucleotide of the VEE genome. The cloned genome terminates at the 3′-end with a stretch of 26 adenine residues (A_{26}). The ribozyme (RBZ_{HDV}) and polyA signal (PA_{HGH}) are placed after the 3′-end of the genome. The cloned genome has cytopathogenicity-reducing mutations in the nsP2 ($nsP2_{mut}$) and capsid (C_{mut}) genes. Alignments are also shown to depict fragments of amino acid sequences of nsP2 (wild type (WT) and $nsP2_{mut}$) and capsid (WT and C_{mut}) proteins. The cloned genome has two subgenomic promoters (SP1, SP2): RNA trancribed from SP1 is translated to produce GFP (depicted as the ellipse figure); RNA trancribed from SP2 is translated to produce structural proteins.

of experiments) or on ice. After this step (recovering) the transfected cells were mixed with 1.6 mL of the complete medium and the resulting mixture (in the following sections referred to as undiluted electroporated cells) was used in an infectious centers assay to measure efficiency of the virus rescue as described in Section 2.4.

2.4. Infectious centers assay, limiting dilution (Reed-Muench) modification

Before starting the electroporation experiments, 96-well plates were seeded with BHK-21 cells (3.75×10^4 cells per well) and the cells were allowed to attach (~4 h).

After electroporation of a cell line, the undiluted electroporated cells were used to produce serial dilutions (from 1:10 to $1{:}10^7$). The dilutions were prepared using the complete medium (based on DMEM for BHK-21, RMPI-1640 for NS0) as a diluent. Medium was removed from 96-well plates with growing BHK-21 monolayers. Aliquots (0.1 ml) of the undiluted electroporated cells or the serial dilutions were distributed into the wells. One long row (12 wells) was used to plate the undiluted electroporated cells and the other rows were used for the dilutions. All plates were incubated in 5% CO_2 at 37 °C and monitored for at least 3 days for GFP fluorescence.

The wells with GFP-positive cells were counted. Focus-forming units (FFU) were calculated using the Reed-Muench method and equation (Ramakrishnan, 2016). Efficiency of the virus rescue is expressed as the FFU counts per 1 ug of the electroporated plasmid.

2.5. Virus production and titering

The NS0 cell culture was electroporated with 10 ug of the DNA/RNA-layered system using conditions of the low-voltage protocol and two pulses. All undiluted electroporated cells were seeded into a P100 culture dish. Medium on the transfected cells was completely replaced every day with the fresh complete medium. The spent medium (virus sample) was collected every day to determine virus titers. 6-well plates with growing BHK-21 monolayers were prepared by seeding of 5×10^6 BHK-21 cells per well and allowing the cells to attach. For titration, serial dilutions ($1{:}10^4 - 1{:}10^9$) were prepared by diluting of the virus samples in PBS with 1% FBS. Medium was removed from wells of the 6-well plates and aliquots (0.2 ml) of the dilutions were distributed into the wells. The plates were incubated for 1 h in 5% CO_2 at 37 °C with occasional shaking. Infectious inocula were removed from the wells and the monolayers were covered with molten agar-containing growth medium (DMEM with 3.3% FBS, antibiotics and 0.5% agar). Agar medium was allowed to solidify and the plates were incubated for 3 days and observed for GFP fluorescence. Foci of GFP-producing cells were counted and the numbers were recalculated into virus titers.

2.6. Statistical analysis

All experiments were performed in triplicate. Mean values were calculated and ranges between the highest and lowest values were recorded. Statistical significance in pairwise comparisons was assessed by unpaired *t*-test using GraphPad Prism 6.0 (GraphPad). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Construction of a DNA/RNA-layered system

Plasmid pCMV-VEE-GFP (deposited to Genbank: MH891622) was produced by cloning of the VEE genome into an eukaryotic expression vector downstream of the CMV promoter. To produce the full-length VEE genome, a part of the genome encoding structural proteins was synthesized de novo and assembled with the previously described GFP-expressing VEE replicon (Kim et al., 2017). Molecular design of the plasmid is presented in Fig. 1.

3.2. Rescue of VEE from the DNA/RNA-layered system: optimal electroporation buffer

To select the optimal electroporation buffer for rescue of the virus, the buffers were purchased or self-made (Table 1). The tested buffers include PBS (Amresco E404) and a set of the previously published buffers (3H, 2S, 3 P, 1S, 1 M, 1SM, 2 M) (Chicaybam et al., 2013). The latter were selected for use in this work because these buffers were optimized for electroporation of T-lymphocytes; it was expected that the same buffers may be suitable for electroporation of plasmacytoma cells which are related to B-lymphocytes (Chicaybam et al., 2013). Efficiency of the virus rescue was measured by counting focus-forming units (FFU) using an infectious centers assay (ICA). One infectious center is a cell in which production of the virus begins as a result of successful transfection with the DNA/RNA-layered system and the virus

Table 1
Electroporation buffers.

Buffer	Composition
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate pH 7.4
3H	5 mM KCl, 15 mM $MgCl_2$, 90 mM NaCl, 10 mM glucose, 0.4 mM $Ca(NO_3)_2$, 20 mM HEPES and 75 mM tris-hydrochloride pH 7.2
2S	5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 50 mM sodium succinate, 150 mM sodium phosphate pH 7.2
3P	5 mM KCl, 15 mM $MgCl_2$, 90 mM NaCl, 10 mM glucose, 0.4 mM $Ca(NO_3)_2$, 40 mM sodium phosphate pH 7.2
1S	5 mM KCl, 15 mM $MgCl_2$, 50 mM sodium succinate, 120 mM sodium phosphate pH 7.2
1M	5 mM KCl, 15 mM $MgCl_2$, 50 mM mannitol, 120 mM sodium phosphate pH 7.2
1SM	5 mM KCl, 15 mM $MgCl_2$, 25 mM sodium succinate, 25 mM mannitol, 120 mM sodium phosphate pH 7.2
2M	5 mM KCl, 15 mM $MgCl_2$, 50 mM mannitol, 15 mM HEPES and 150 mM sodium phosphate pH 7.2

propagates to infect other cells in a monolayer. In this study the ICA has been combined with the end-point dilution approach, i.e. a sample of electroporated cells was used to produce serial dilutions and aliquots of the dilutions were added to cultures in cell culture plates. After development of the GFP fluorescence the GFP-positive wells in the plates were counted. The Reed-Muench method was used to calculate FFU counts which are numbers of the infectious centers in samples of electroporated cells. The virus rescue efficiency is expressed as the FFU count per 1 ug of the electroporated DNA.

The FFU counts are presented in Fig. 2. The high-voltage protocol was consistently more efficient (produced higher FFU counts) than the low-voltage protocol for electroporation of BHK-21 cells if results for one buffer are compared. Surprisingly, the maximum efficiency (1.17×10^4 FFU/ug, high-voltage protocol) was observed when commercial PBS (pH 7.4) was used as the electroporation buffer (Fig. 2A). Nearly equal counts (1.10×10^4 FFU/ug) were obtained with buffer 3H ($p = 0.7$) whereas other buffers were less efficient. Replication of the virus rescued from the pCMV-VEE-GFP construct was not cytopathogenic to BHK-21 cells (data not shown).

Time constants (*tau*) for exponential-decay pulses were recorded from the electroporator during the experiments but no conclusive dependence was found between the time constants and FFU counts (data not shown).

It was unexpected to obtain equal efficiency of the virus rescue (in BHK-21) using the electroporation buffers PBS and 3H, because these buffers significantly differ in the chemical composition and have different pH. It is possible that in this experiment the rescue efficiency reached a certain limit ($\sim 1 \times 10^4$ FFU/ug for the high-voltage

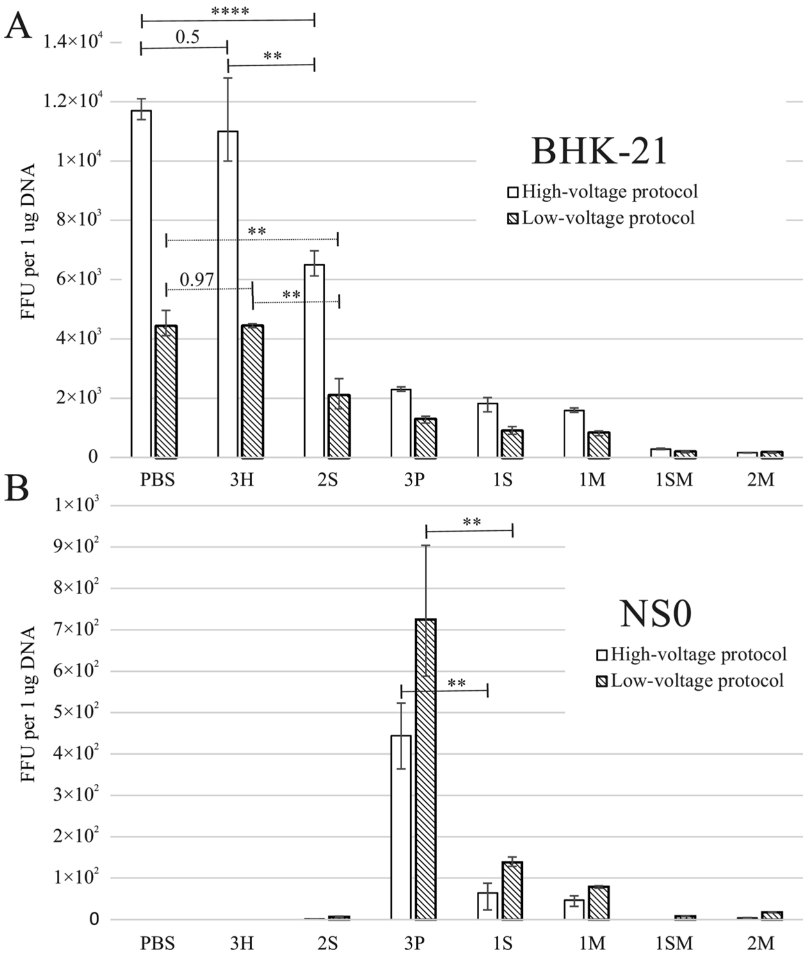

Fig. 2. Efficiency of the virus rescue (FFU counts) after electroporation of the DNA/RNA-layered system in cell cultures. Electroporation buffers are listed in Table 1. White bars represent results for the "high-voltage" protocol, cross-hatched bars are results for the "low-voltage" protocol. The bars show means and the whiskers show ranges (max-min) from three experiments. Panel A, electroporation of BHK-21. Panel B, electroporation of NS0 cells. Statistical significance (p value) for pairwise differences is shown as a number (if $p > 0.05$) or denoted by stars: * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

protocol), and further increase is not dependent on the number of cells, which acquire DNA during the electroporation but on other factors such as the ability of DNA to traffic through the cytoplasm and enter the nucleus, giving rise to infectious RNA, etc. Also for BHK-21, the efficiency of buffer 3 P was 50 times lower than for buffer 3H, despite these buffers having similar chemical composition (the difference is that buffer 3H is HEPES/Tris-buffered, and 3 P is phosphate-buffered).

There are published observations showed that the presence of divalent cations (magnesium or calcium) in the electroporation buffer is necessary for successful electroporation (Wu and Yuan, 2011), although the results show that for electroporation of BHK-21, PBS without divalent cations works better than various buffers containing divalent cations.

In agreement with a widely shared opinion that myeloma cells (NS0 in particular) are difficult to transfect (Khan, 2013; Shi and Yang, 2007), electroporation of the plasmid into NS0 culture led to efficiency of rescue 2 orders of magnitude lower than that for BHK-21 (Fig. 2B). The highest FFU counts (7.25×10^2 FFU/ug) were obtained with buffer 3 P and the low-voltage protocol. In sharp contrast to the results with BHK-21, electroporation of NS0 in buffers 3H and PBS resulted in zero efficiency. The results underscore importance of empiric selection of an electroporation buffer to a particular cell line when electroporation is used as a method of delivery. The low-voltage protocol showed better results than the high-voltage protocol for electroporation of NS0.

3.3. Optimal incubation temperatures and times

There are reports where cultured mammalian cells were electroporated at RT and no benefits were found from lowering incubation temperatures during the electroporation procedure (Anderson et al., 1991; Chicaybam et al., 2016). Here, to optimize the virus rescue, experiments were done with different incubation temperatures before subjecting cells to electroporation and after the pulse. NS0 cells were electroporated in the optimal buffer (3 P) using one pulse (V = 250 V, R = infinity, C = 960 u F); in this experiment several temperature regimens were tried: 1. all electroporation steps at 0 °C; 2. all steps at RT; 3. incubation of cells at 0 °C before the pulse, and at RT after the pulse. The highest virus rescue was observed when cells were maintained at 0 °C before the pulse and the incubation after the pulse (resting) was at RT (Fig. 3A). This optimal temperature regimen was used in all subsequent electroporations.

Incubation times in the protocol were optimized. Duration of the resting step (at RT) after the electric pulse was varied from 10 to 30 min (Fig. 3B). The optimal incubation time after a pulse was 10 min. Also, an effect of pre-incubation of a mixture of NS0 cells with DNA was studied: the mixture was incubated for 10 min on ice before the pulse and upon electropotarion cells were incubated for 10 min at RT. The results of the latter experiment show that the preincubation only reduced the FFU counts.

3.4. Effects of the number of pulses and amount of DNA

The number of electric pulses appeared to largely influence the efficiency of virus rescue (Fig. 4A). With the conditions of the low-voltage protocol, two pulses result in 30X increase in FFU counts compared to one pulse (2.13×10^4 vs. 7.25×10^2), although with three or four pulses efficiency of rescue decreased, possibly because more intense electroporation led to dying of a larger fraction of the electroporated cells.

Too low amount of plasmid during electroporation will understandably decrease the FFU counts, although transfecting cells with excessive amounts of DNA may also be detrimental because cytotoxic effects were attributed to cytoplasmic DNA (Moghimi et al., 2011). Amount of the DNA/RNA-layered system was varied from 0.1 to 10 ug per electroporation and the electroporation was done by the low-voltage protocol with two pulses. Increase in the dosage led to enhancement in the FFU counts (Fig. 4B).

3.5. Virus titers after electroporation of NS0 cells with the DNA/RNA-layered system

Ability of the NS0 cell culture to support propagation of VEE was measured by constructing of a virus growth curve after electroporation. The NS0 cells were electroporated with 10 ug of the plasmid using the optimized conditions (buffer 3 P, low-voltage protocol, two pulses). The transfected cultures grown in P100 dishes continued to grow as monolayers with a small fraction of freely floating cells, although a fraction of the cells changed appearance during the experiment while remaining attached. Unlike the naive NS0 cells which look uniformly spherical with the translucent cytoplasm, in the transfected cultures deformed cells with the dark cytoplasm (supposedly dead cells) and post-cells debris began to accumulate from the first day after the electroporation. In the transfected culture, GFP fluorescence reached maximum levels at 36 h after the transfection and did not change further during several-days observation. Total amounts of cells in the electroporated cultures continued to increase indicating that cells continued proliferation despite the virus infection. The virus growth curve is presented in Fig. 5. The titers were high (8×10^8 FFU/ml at day 2; 6.67×10^8 FFU/ml at day 5) and in fact exceeded titers obtained by electroporation of BHK-21 cells with the same construct (data not shown). The results show that the VEE TC-83 strain with cytopathogenicity-reducing mutations overcomes innate antiviral immunity in NS0 cells. Not all cells in the transfected cultures had GFP-fluorescence during the 5-day observation time. This is probably because at every time point after the transfection the cell populations contain a fraction of dying cells incapable of producing GFP. Accumulation of the dead cells was particularly noticeable at the end of the experiment (Fig. 6).

4. Discussion

Two techniques are commonly used to rescue live viruses from infectious (molecular) clones. The first technique (naked RNA) relies on cloning of a cDNA-copy of a virus genome under transcriptional control of a small bacteriophage-derived promoter which is suitable for *in vitro* transcription. Viral RNA is produced *in vitro* with use of a phage-derived RNA polymerase and this RNA is transfected into cell cultures or may be injected into animals (Beissert et al., 2017; Romani et al., 2017). The second technique has several names, including infectious DNA (iDNA) (Yamshchikov et al., 2017), or DNA/RNA-layered system (Lundstrom, 2014), or DNA-launched virus (Zou et al., 2018). All these names refer to the technique when a cDNA-copy is placed in a plasmid downstream of an eukaryotic promoter, e.g. cytomegalovirus (CMV) immediate-early promoter. Upon entry of a DNA/RNA-layered system into a cell, the plasmid DNA is being transported to the nucleus where the strong RNA polymerase II promoter (CMV) drives production of primary RNA transcripts which resemble the viral genomes. The latter are supposed to be transported to the cytoplasm where they initiate autonomous replication.

Development of DNA/RNA-layered systems using alphavirus backbones has been a fruitful technology to generate RNA vectors, in particular in relation to self-amplifying vaccines but also to produce recombinant proteins (Ratnik et al., 2013; Viru et al., 2011). An area in which these vectors currently outperform competing technologies is utilization of alphavirus vectors for epigenetic reprogramming, in particular to produce medical-grade (autologous and syngenic) induced pluripotent stem cells (iPSC) from adult differentiated donor cells (Yoshioka and Dowdy, 2017; Yoshioka et al., 2013).

DNA is directly amenable to genetic engineering and the DNA/RNA-layered systems approach simplifies experimentation. Also DNA/RNA-layered systems allow circumventing some limitations such as a limit on the maximum length of RNA which can be practically produced *in vitro*; modern *in vitro* transcription kits allow synthesizing of ~15 kb-long

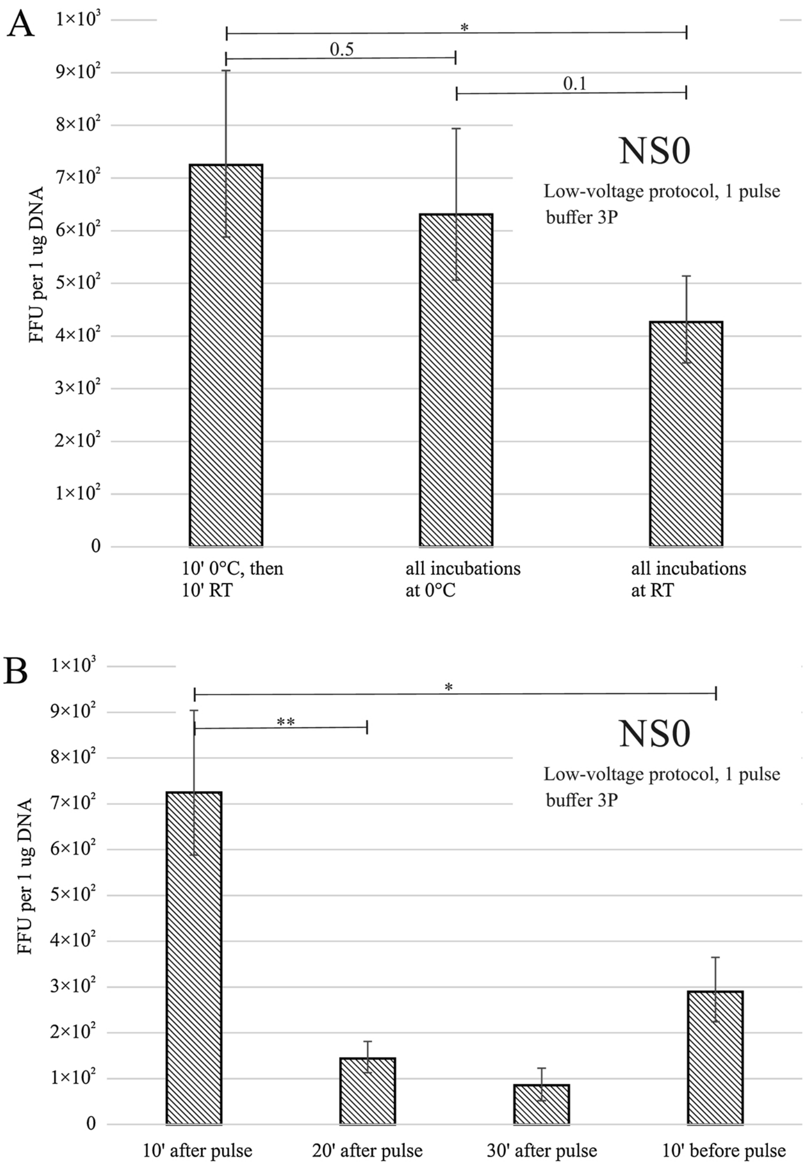

Fig. 3. Panel A. Effects of incubation temperatures before an electric pulse and after the pulse on FFU counts. Three regimens were tested: all steps of the electroporation protocol were done at 0 °C; or at 0 °C before a pulse and at room temperature (RT) after the pulse; or all steps at RT. Panel B. Electroporated cells were incubated for varying times after the electric pulse (10, 20, 30 min). In one experiment, a mixture of NS0 cells with DNA was incubated for 10 min before a pulse and 10 min after the pulse. Designations are as in Fig. 2.

RNA molecules (Yoshioka et al., 2013). The alphavirus replicative machinery is capable of replicating significantly longer genomes (Nanda et al., 2009). This means that the naked RNA approach cannot fully explore possible scope of applications of the alphavirus vectors, whereas DNA/RNA-layered systems can hold inserts of practically any size and limited only by a synthetic potential of the alphavirus replicase. Another limitation is a packaging capacity of alphavirus virions. The natural alphavirus genomes have a length of ~11 kb and it was reported that the packaging capacity of the virions is ~13–14 kb, so packaging-compatible inserts should not exceed 3 kb. Very long genomes will not be packaged into alphavirus virions, although the DNA/RNA-layered systems approach still allow using an infection as a way to deliver overly long RNAs into cells, for which purpose a DNA/RNA-layered system may be incorporated into a suitable DNA-virus, such as *Vaccinia virus*. Mutations in the SIN capsid protein were described which change morphology of SIN capsids (the mutations increase a triangulation number to T > 4 and a diameter up to 205 nm; the natural diameter is 65 nm) (Nanda et al., 2009). The SIN virions with the mutated capsid protein can package genomes up to 32 kb (Nanda et al., 2009). Although no similar mutations have been described for VEE, the VEE-based DNA/RNA-layered system provides a viable technique to handle overly long RNA-vectors.

Transfection efficiency is a factor determining success of experimentation with DNA/RNA-layered systems. Different myeloma cell lines require empiric optimization of transfection conditions (Steinbrunn et al., 2014) and NS0 are considered to be “notoriously difficult” to transfect (Khan, 2013; Shi and Yang, 2007). Electroporation is widely used as it is considered a “universal” transfection technique, allowing optimization to difficult-to-transfect cells (Luft and Ketteler, 2015; Steinbrunn et al., 2014). Optimal electroporation conditions for particular cell types vary significantly, although reasons for this dependence have not been fully explained, and optimization of electroporation protocols remains a trial-and-error process. Among known factors affecting the efficiency are cell size and shape (large-volume cells being easier to electroporate, and elongated cells show less survival after pulses compared to spherical cells) (Luft and Ketteler, 2015). Less understood are differences related to the biological characteristics of cells such as membrane fluidity and cell cytoskeleton

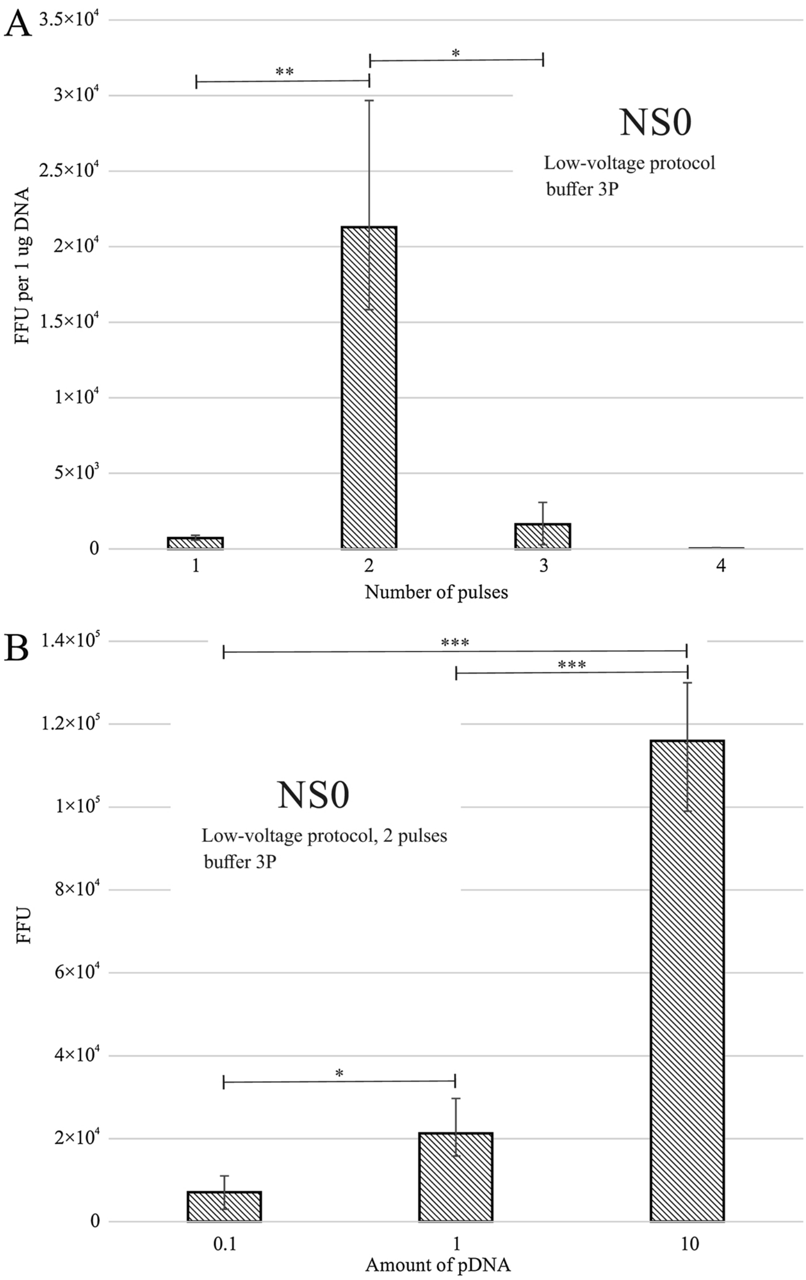

Fig. 4. Panel A. Effects of a number of electric pulses on rescue of the virus: two pulses result in the highest FFU counts. Panel B. Different amounts of the DNA/RNA-layered system were electroporated into NS0 culture using the optimimal conditions. The largest dosage (10 ug) leads to the highest FFU counts. Designations are as in Fig. 2.

which integrity affects membrane resealing and survival (Xiao et al., 2011).

Observations have been published on that adsorption of extracellular DNA on the outer surface of the plasma membrane is important during electroporation and precedes penetration of DNA through the membrane (Wu and Yuan, 2011). It was experimentally demonstrated that presence of divalent cations (Mg^{2+}, Ca^{2+}) in an electroporation buffer is needed for formation of the DNA-membrane complex (Wu and Yuan, 2011). Results of electroporation of the myeloma cell line NS0 (in this paper) are in favor of the above hypothesis because the most efficient rescue of the virus (maximum FFU number) was achieved with buffer 3 P which contains 15 mM Mg^{2+} and 0.4 mM Ca^{2+}, and zero efficiency was found for PBS without the divalent cations. The buffers listed in Table 1 were compared during electroporation of the cell lines Nalm-6 (B cell precursors), P815 (granulocyte lineage, mast cells) and Jurkat (T cell lymphoma) (Chicaybam et al., 2016, 2013), and buffer 3 P also gave the best results, in full accordance with the results in this study.

In contrast, with BHK-21 cells, electroporation in PBS produced the highest efficiency, whereas buffer 3 P was only forth in the efficiency ranking. Buffer 3H (similarly to 3 P contains 15 mM Mg^{2+} and 0.4 mM Ca^{2+}, the pH-controlling components are different) shown the efficiency equal ($p = 0.7$) to that obtained with PBS. This observation again underscores importance of empiric selection of the optimal electroporation buffer for a particlular cell line.

In the ICA, FFU counts in the myeloma cells (NS0) transfected with the DNA/RNA-layered system slightly exceeded 100,000 when 10 μg of the plasmid were electroporated into 100 million cells, which corresponds to rescue of the virus in 1% of the electroporated cells. Non-viral transfection of cells with hematology origin generally results in low

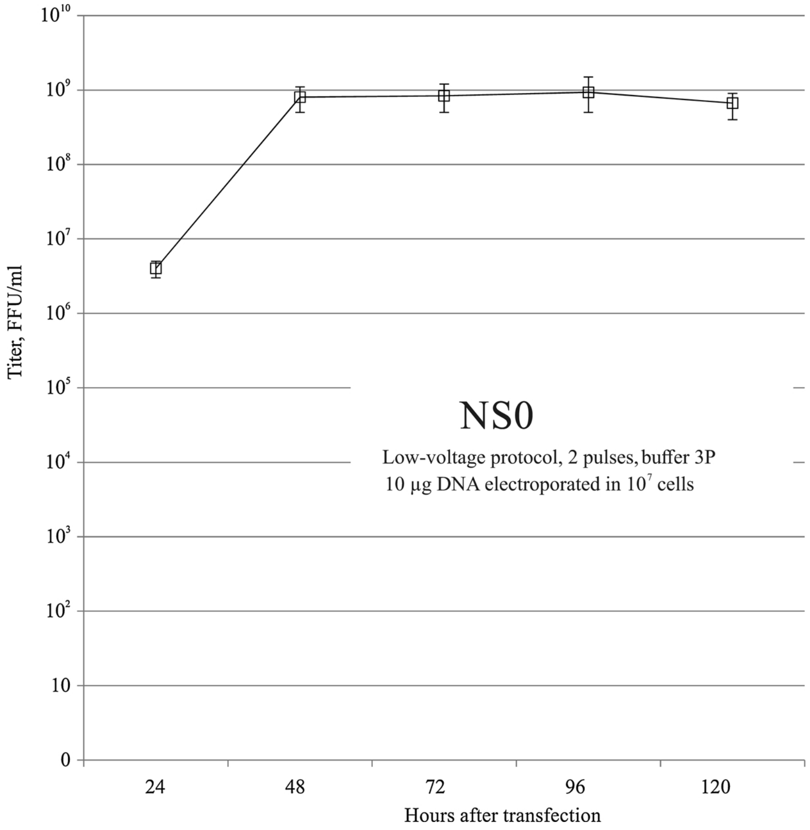

Fig. 5. Titers of the virus in samples of medium collected from NS0 culture transfected with the DNA/RNA-layered system. Each day after the electroporation medium was collected and replaced with the fresh medium. Each data point represents a mean from three experiments and whiskers show a range (max-min).

yields, and electroporation is considered to be a more efficient method for delivery in these cell types than chemical transfection (e.g. liposomes, PEI) (Moghimi et al., 2011). One competing technology with reported high efficiency of delivering into immune cells is lentiviral transduction, although its application is more complex and poses safety concerns. Published papers show low efficiency of electroporation of myeloma cells, e.g. only 0.03–25 CFU per 10^6 electroporated cells (after testing of six lines of B- or T-lymphomas) (Potter, 1988) or 10–1000 CFU per 10^6 electroporated cells (for Sp2/0, P3-X63-Ag8.653) (Wright and Shin, 1991), although the cited sources used different protocols.

Virus rescue after plasmid transfection is a result of a multistep process, in which delivery of the plasmid into the cytoplasm is only the beginning step. Other steps include intracellular trafficking, entry into the nucleus, generation of primary transcripts (complicated by a possibility of aberrant splicing), export of the (unspliced) transcripts into the cytoplasm, translation of viral proteins and initiation of the virus replication. Every step may influence a selection of the optimal method for virus rescue from DNA/RNA-layered systems. It is possible that experimental conditions favorable for virus rescue are not the best for expression of reporter proteins using conventional eukaryotic expression plasmids.

With regard to the steps of transcription and nuclear export, there is a possibility that a fraction of primary transcripts (essentially, RNA genomes) are directed to a spliceosome and undergo RNA splicing. A presence of numerous cryptic splice sites has been predicted in the VEE genome using publicly available tools such as the alternative splice site predictor (http://wangcomputing.com/assp/) (data not shown). Aberrant splicing may take its toll on virus rescue efficiency. A pertinent case and its solution are illustrated in the work (Haseloff et al., 1997): aberrant splicing hampered expression of a reporter until a

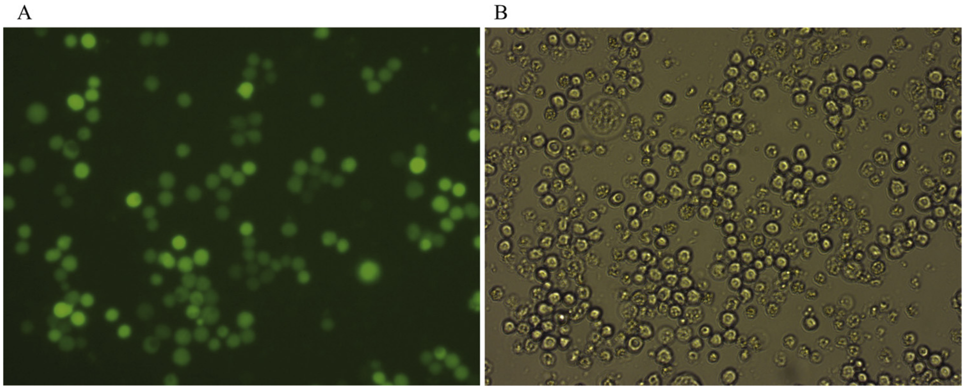

Fig. 6. Photographs of NS0 culture transfected with the DNA/RNA-layered system (the culture used to build the growth curve at Fig. 5). White light and fluorescence images were taken 5 days after the electroporation. Panel A. GFP fluorescence, objective 20×. Panel B. same field, white light.

reporter gene was modified to remove a cryptic splice site.

Constrains on DNA/RNA-layered systems posed by intracellular environment are supposedly different from what is known from utilization of simple expression vectors. It has been found that only three copies of the eukaryotic plasmid expression vector in a cell are required to obtain expression of a reporter (Ludtke et al., 2002). By electroporating of a multicistronic plasmid which incorporates a SIN genome and a different reporter gene under control of its own promoter (in a cistron different from the virus genome), it was demonstrated that the amount of the exported gRNA must be above a certain threshold for the virus to initiate replication in the cytoplasm, and this threshold is higher than the reporter mRNA amount to detect translation (Boorsma et al., 2003).

Applicability of vectors derived from the New World alphaviruses depends on their ability to overcome various intracellular protective mechanisms (innate immunity). EEEV and *Western equine encephalitis virus* (WEEV) are incapable of self-supported infection in lymphocyte cultures because of marginal titers (Levitt et al., 1979); it was found that the lymphocyte-specific micro-RNAs (miRNA) interfere with the replication (Trobaugh and Klimstra, 2017). On the contrary VEE naturally infects lymphoid organs (Steele and Twenhafel, 2010) and is capable of replication in lymphocytes and monocytes to the titers allowing a sustained culture infection (Liprandi et al., 1986). All alphavirus species are sensitive (albeit to various degrees) to type I interferon-dependent inhibition of replication, which is the innate immunity mechanism important to control all RNA-viruses. Cytoplasmic RIG-I-like receptors detect a variety of RNA viruses leading to production of type I interferons and activation of numerous interferon-stimulated genes. Autocrine regulation activates virus-suppressing processes in infected cells. Paracrine action of interferons on uninfected cells in a culture lessens sensitivity to the incoming virus (activation of the antiviral state) (Jensen and Thomsen, 2012). To date, there are no published studies that provide data on whether NS0 cells have normally functioning or defective pathways for induction and response to interferons. One study with a different industrial murine myeloma cell line showed that these cells are susceptible to the alpha-interferon (Plesnicar et al., 2009). The TC-83 virus with attenuating mutations in the nsP2 and capsid proteins is capable of a persistent infection of NS0 cells and reaches high titers indicating that the virus overcomes or suppresses antiviral effects of endogenous interferons in the NS0 cells, or these cells have a defect in interferon signaling. Long-term survival of infected NS0 cultures means that virus replication does not cause transcriptional and translational shutoff. Overcoming endogenous interferon action and absence of transcriptional and translational shutoff make this virus a suitable vector for NS0 cells-based expression systems.

VEE efficiently replicates in NS0, albeit a different phenomenon may limit effectiveness of the system. This is an appearance of dead cells in the infected culture and increased accumulation of dead cells during growth of the infected culture. Replication of alphaviruses was frequently observed to induce programmed cell death (apoptosis) in infected cells (Baer et al., 2016; Li and Stollar, 2004). Various industrial murine myeloma cell lines are reported to be sensitive to induction of apoptosis which develops in response to various stress factors (Krampe and Al-Rubeai, 2010; Lasunskaia et al., 2003). NS0 cell line is described as exceedingly sensitive to apoptosis (Sauerwald and Betenbaugh, 2002). Considering VEE infection as a stress factor, it is possible that the accumulation of dead cells in the infected cultures (this study) is because of apoptosis. Apoptosis is commonly encountered during culturing of NS0-based industrial producers, and strategies were developed to reduce the apoptosis (O'Connor et al., 2006; Tey and Al-Rubeai, 2005).

5. Conclusion

In this study, to be in pace with recognized importance of DNA/RNA-layered systems for DNA vaccines and virotherapy (Lundstrom, 2018), a combination of a VEE-based vector and cultured plasma cells was tried as a step of developing of an expression platform. For the first time it was shown that murine plasmacytoma cell line NS0 is able to support full cycle of VEE replication. A number of variables were tested to optimize an electroporation protocol for the virus rescue in these cells.

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Declaration of Competing Interest

Authors declare that there are no competing interests.

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