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POLUDANUM'S INFLUENCE ON REPRODUCTIVE AND RESPIRATORY SYNDROME, TRANSMISSIBLE GASTROENTERITIS AND AFRICAN SWINE FEVER VIRUSES IN PRIMARY AND CONTINUOUS CELL CULTURES

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SUMMARY

Today studies of the influence of interferon synthesis inducer on human and animal body is a topical issue. Interferon synthesis inducers are substances of natural or artificial origin stimulating production of body's own interferon. One of the synthetic inducers of interferon is Poludanum. It is used for stimulation of cell immunity which is capable of preventing infection and disease development and has antiviral and immunomodulatory effect. Poludanum mainly stimulates induction of α-interferon and, to a lesser extent, β- and γ-interferon in cells and tissues which prevent virus reproduction in a cell. The paper presents results of poludanum's influence on reproduction of some porcine viruses in primary and continuous cell cultures by induction of interferon synthesis and other cytokines lowering the level of cell infection. As a result of performed tests it was found out that there is a link between introduction of interferon inducer and change in the PRRS, TGE, ASF virus reproduction. There was detected a high level of Poludanum's interferon inducing activity in pig testicular primary cell cultures and spleen compared to the continuous pig embryo kidney (SPEV) and rhesus monkey kidney (MARC-145) cell lines. Poludanum's interferon inducing activity was moderate for MARC-145 cells and it was not determined for SPEV.

Key words: porcine reproductive and respiratory syndrome; porcine transmissible gastroenteritis, African swine fever, Poludanum, interferon.

INTRODUCTION

Currently many scientists are focused on studies of body non-specific protection factors, in particular on the role of interferons and immune modulators in prevention and treatment of different viral infections [5].

Protection against infections is also ensured by such innate immunity factors as α-, β-, γ-interferons, natural killers (NK), properdin and complement systems, lysozyme, phagocytosis etc., as well as specific factors of humoral (B cells secrete appropriate immunoglobulins) and cell immunity (CD4, CD8, T cells) [8, 9].

Interferons (IFNs) is a class of proteins known as cytokins, immunity mediators, which are active against viruses and involved into antimicrobial and antitumour protection and possess antiproliferative, anti-inflammatory, immunomodulatory and radioprotective properties [2, 8, 9].

IFNs are released by cells in response to an infectious virus introduction into the body. They are multifunctional, because they prevent introduction of infectious agents into sensitive cells; inhibit protein synthesis in a cell and thus slow down the propagation of the virus; they trigger cytotoxic effect and adaptive immunity, ultimately influencing the course and outcome of a disease [8, 9].

Use of exogenous IFNs for successful treatment of acute and chronic diseases or for infection prevention may lead to some side effects, like allergic reactions. That is why a good alternative here is the use of endogenous interferon inducers, i.e. of high molecular and low molecular natural and synthetic compounds, able to somehow stimulate the synthesis of intrinsic IFNs [8, 9].

These immune modulators are effective in IFN induction and as a rule do not cause any allergic reactions, are safe and non-toxic, as they are natural or synthetic nucleic acid products (Poludanum, Ampligen, Polyguacil, Larifan, Ridostin) or natural polyphenols, including fluorenones and acridinones, which are carboxylic acid derivatives [7, 8].

Polyguacil, Ampligen, and Ridostin are natural and synthetic nucleic acid preparations (dsRNAs); Poludanum is a biosynthetic polyribonucleotide complex, comprised of polyriboadenylic and polyribouridylic acids [7].

Inducers belonging to different classes of compounds are different in their dose ranges, stimulating IFN release. Among known inducers, the most effective IFN production is caused by methylglucamine acridonacetate, which induces up to 1 million U/ml in animals and up to 1,280–2,560 U/ml of IFNs in human lymphocyte cell cultures. As little as 4–14 mg/kg of methylglucamine acridonacetate induces α-, β-, γ-, λ-IFNs starting from hour 2 to 72 post inoculation, thus ensuring antiviral and immunomodulatory activity [1, 2, 5, 8].

One of the most available IFN inducers is Poludanum, having antiviral and immunomodulatory effects. It stimulates mostly α-IFN and less β- and γ-IFNs in body cells and tissues, which prevents virus propagation in a cell [6, 11, 16].

In its turn, to determine the effectiveness of IFN inducers *in vitro,* different cell cultures are used, such as primary human lymphocyte cell line and continuous IFN-sensitive BSC-1 cell line. Nevertheless, the selection of primary and continuous animal cell lines to study antiviral activity of different immune modulators is a topical issue for veterinary medicine too.

The aim of this study was to analyze Poludanum antiviral activity *in vitro* using different porcine viruses and appropriate cell lines.

MATERIALS AND METHODS

Reproductive and respiratory syndrome, transmissible gastroenteritis and African swine fever viruses were used in the work.

PRRSV strains of American ("Irkutsky-2007-V" and NVSL are weakly virulent, "BD" is an avirulent vaccine strain) and European genotypes ("KPR" is an avirulent vaccine strain, "Italian 2165" is weakly virulent), TGEV ("Leningradsky" is a weakly virulent, vaccine strain; "Ilyinogorsky" and "Krasnodonsky" are avirulent vaccine strains); ASFV isolate "Lazarevskoye 01/14" isolated from domestic pig spleen (Tula Oblast, Schekinsky Rayon, OOO Lazarevskoye farm and ASFV strain 8-No. 2 "Odintsovo 02/14", isolated from spleen sample from a shot wild boar (Moscow Oblast, Odintsovsky Rayon, Tarakanovskoye forest area).

In order to study IFN inducers effect on freeze-dried Poludanum (OOO "Lans-Farm", Branch Company of OAO "Verofarm", Moscow), 100 Units were dissolved in Eagle's nutrient medium with subconfluent monolayer of cell cultures at the dose of 25–100 Units 24 hours before infection.

Continuous PEK (pig embryonic kidney) and MARC-145 (monkey kidney) cell lines from the Cell Culture Sector under the Innovation Department of the FGBI "ARRIAH" were used.

Primary cell lines of pig testicles and spleen were prepared in the Reference Laboratory for African Swine Fever by trypsinization of tissues using Eagle's medium with 20% bovine fetal serum (PAN-Biotech, Germany) and broad spectrum antibiotics added [2].

Suspension of PEK, MARC-145 and pig testicular cell lines at the seeding concentration of 500, 150–200 and 250 thousand cell/cm^3 correspondingly were used for seeding in 25 cm^2 flasks and 96-well cultural micro plates. Cells were counted using automated cell counter Countess™ (Invitrogen™, Korea). Plates with cell cultures were incubated in a CO_2-incubator, containing 5% CO_2.

Studies with Poludanum were carried out using the following scheme: cultural medium was removed from flasks with monolayer; one group of flasks was filled with 0.5 см^3 of Poludanum solution at the dose of 25–100 U, the same amount of nutrient medium was added to the second group of flasks; and two flasks were used for cell culture control. Poludanum-containing flasks and flasks without it were incubated in a thermostat for an hour at 37 ± 0.5 °C. Then 9 cm^3 of nutrient medium was added to every flask. In 24 hours 1 cm^3 of virus preparation was added to every flask in the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, which had been prepared in advance in penicillin vials. All samples were examined using "Biolam P-1" inverted microscope (OAO "LOMO").

72–144 hours post virus introduction, when a pronounced cytopathic effect (CPE) was observed, Poludanum-containing flasks and flasks without it were placed into a freezer (–70 °C) for 1–2 hours. Then the virus-containing liquid was frozen-thawed and sampled for virus infectivity testing.

Titrating was performed in 96-well cultural microplates with appropriate cell culture. Virus infectious titer was calculated using Karber method (1931) modified by I. P. Ashmarin (1959, 1962) and expressed as lg $TCID_{50}$/cm^3.

RESULTS OF STUDY

Primary analysis of Poludanum interferonogenic effect was carried out using MARC-145 cell culture, infected with PRRSV. As for strain "Irkutsky-2007-V" the virus dilution was 10^{-6}, that is ~3 $TCID_{50}$/cm^3, for the rest of the strains used in the work it was 10^{-5}, that is 10–17 $TCID_{50}$/cm^3. 24–48 hours post virus inoculation slight changes in the monolayer, that is 5–10% of rounded cells, were observed in all flasks, whether containing Poludanum or not. Gradually the monolayer detached; some cells exfoliated to the nutrient medium and in 120 hours 85% of the monolayer was affected, with monolayer in cell culture control remaining intact.

CPE of PRRSV strain "Irkutsky-2007-V" was more distinct in the flasks with Poludanum treated cell culture at the dose of 50 U, compared to Poludanum non-treated cell culture (Table 1).

It was demonstrated that Poludanum inoculated into MARC-145 cell culture decreased the titre of PRRSV strain "Irkutsky-2007-V" by 0.75 lg $TCID_{50}$/cm^3, and by 1.50 lg $TCID_{50}$/cm^3 of NVSL strain. It means that for PRRSV, Poludanum restricted infectivity by 1.25 lg $TCID_{50}$/cm^3 in average. At the same time Poludanum treatment did not significantly influenced PRRSV strain "BD" reproduction.

Thus, the studies performed showed that PRRSV titres in MARC-145 cell culture decreased due to Poludanum treatment, because it induced production of IFNs by these cells.

The effect of IFN induction on reproduction of TGEV adapted to growth in PEK cell culture ("Leningradsky", "Ilyinogorsky" and "Krasnodonsky" strains), was studied using the abovementioned cell culture.

In PEK cell culture, TGEV CPE was manifested as cell rounding and degeneration. Cell degeneration signs involved their shrinking, changes in nuclear envelope and cell death in 24–36 hours post virus inoculation.

The experiments showed that when cells were infected at the dose of 10 $TCID_{50}$/cm^3 (10^{-6} dilution) and exposed to virus for 144 hours, whenther Poludanum at the dose of 50 U was added or was not added at all, no differences in TGEV CPE were observed (Table 2).

Table 1
PRRSV infectivity titres in Poludanum treated and non-treated MARC-145 cell culture ($n = 3$)

Virus strain	Virus genotype	Virus titre (lg ± 0.25 lg $TCID_{50}$/cm^3)	
		treated	non-treated
«Irkutsky-2007-V»	American	5.75	6.50
«BD»		6.25	6.50
NVSL		5.00	6.50
«Italian-2165»	European	4.50	4.75
«KPR»		5.75	6.50

All three TGEV strains under study, when treated with Poludanum, did not demonstrate any changes in reproduction compared to culturing of this virus without Poludanum added.

In order to determine potential Poludanum interferonogenic dose for PEK cells a number of experiments was performed to study changes in viral activity when different amounts of Poludanum were added (Table 3).

It was concluded that Poludanum treatment at the doses of 25 to 100 U does not influence significantly TGEV infectivity. Thus, Poludanum is not interferonogenic in relation to PEK cells and use of this cell line for study of IFN effect on viral reproduction is not feasible.

During the next step, Poludanum interferonogenic effect in primary porcine testicular cell culture using TGEV strain "Leningradsky" was studied.

For this purpose 10^{-1} to 10^{-7} TGEV dilutions were prepared. The last dilutions (from 10^{-5} to 10^{-7}) were used for inoculation of porcine testicular monolayer-containing flasks. During first 24 hours of TGEV reproduction enlarged rounded cells were observed in porcine testicular monolayer. In 48 hours TGEV started destroying monolayer more actively: 35–45% of affected monolayer with Poludanum added; 75–80% of Poludanum non-treated monolayer.

During the period of maximum difference of virus CPE in flasks (48 hours post inoculation), samples were frozen, and subsequently titre of the virus, accumulated in porcine testicular cells in plates with initial cell concentration of 250 and 500 thousand cell/cm^3, was determined (Table 4). Poludanum dose was 50 U.

The results showed 100 times or more difference (from 2.25 to 5.37 lg $TCID_{50}$/cm^3) in titres. This suggests that Poludanum significantly facilitates IFN production in primary porcine testicular cell lines.

Taking into account that it was proved that IFNs significantly influence ASFV propagation depending on an isolate virulence, study of Russian isolates' sensitivity to IFNs was of special interest [15]. For this purpose, two virus variants of different virulence for pigs were selected: "Lazarevskoye 01/14" isolate was 100% lethal, "Odintsovo 02/14" 8-No. 2 strain was at least 80% lethal. Poludanum dose for porcine spleen cells was 50 U.

The study results (Table 5) demonstrated that decrease in virus accumulation level for "Lazarevskoye 01/14" isolate was statistically significant, but not substantial and was not more than 1.00 lg $HAdU_{50}$/cm^3. As for strain 8-No. 2 "Odintsovo 02/14" the difference in titres of Poludanum treated and non-treated samples varied more widely: from 0.50 to 3.00 lg $HAdU_{50}$/cm^3.

Thus, the tests performed revealed that Poludanum is more interferonogenic for primary porcine testicular and spleen cell cultures, which are able to produce more IFNs, than continuous cell cultures. This is expressed by inhibition of virus CPE and decrease in virus accumulation.

DISCUSSION

Based on publications made, it is known that primary culture cells secrete IFN I (including α- and β-IFNs), this protects neighboring cells from infection with different viruses, for example PRRSV, TGEV (Table 6), and inhibit their replication to a larger degree than continuous culture [12, 17, 18]. In their turn, some viruses are able to suppress IFN synthesis in cells using certain non-structural proteins thus simplifying their reproduction in some cell cultures. For example, PRRSV non-structural protein 2 is not only deubiquitinating, but moreover is an IFN antagonist [18].

Table 2
TGEV titration in Poludanum treated and non-treated PEK cell culture ($n = 3$)

Virus strain	Virus titre (lg ± 0.25 lg $TCID_{50}$/cm^3)	
	treated	non-treated
«Leningradsky»	7.00	7.00
«Krasnodonsky»	7.75	7.75
«Ilyinogorsky»	4.75	4.75

Table 3
Comparative data of TGEV Leningradsky strain infectivity titres in PEK cell culture treated with different Poludanum doses and non-treated ($n = 3$)

Virus dilution	Virus titre (lg ± 0.25 lg $TCID_{50}$/cm^3)			
	treated			non-treated
	25 U	50 U	100 U	
10^{-5} (100 doses)	7.75	7.75	7.75	7.50
10^{-6} (10 doses)	7.50	7.50	7.50	7.75

As for ASFV, genes were revealed, which express products inhibiting IFN production in porcine monocytes and macrophages. Moreover, it was proved that in the process of virulent isolate reproduction, a significant decrease in IFN reproduction by infected cells is observed [13].

According to L. Reis et al., if MGF 360 (multigene family) and MGF 505 genes, responsible for IFN inhibitor synthesis, are deleted from virulent ASFV genome, it decreases its lethality for domestic pigs [14].

The experiments performed demonstrated a significant difference in sensitivity to IFN exposure of ASFV "Lazarevskoye 02/14" isolate and 8-No. 2 "Odintsovo" strain. This fact is consistent with the conclusions of A. L. Reis et al., because deletion in MGF 505 4R gene, was detected in 8-No. 2 "Odintsovo 02/14" strain and is absent in "Lazarevskoye 02/14" isolate genome [3].

Thus, the basic parameters of Poludanum interferonogenic activity effect were determined while studying reproduction peculiarities of PRRS, TGE and ASF viruses. Summarized results are given in Table 6.

Thus, taking into account IFN crucial role in protective immunity development *in vivo*, it is important to learn how to determine *in vitro* the ability of different virus strains under study to stimulate and/or inhibit IFN production in cells; to select the most effective IFN inducers, which can

Table 4
TGEV strain "Leningradsky" infectivity titres in Poludanum treated and non-treated porcine testicular cells ($n = 3$)

Virus dose, $TCID_{50}$/cm^3	Virus titre (lg ± 0.25 lg $TCID_{50}$/cm^3)			
	250 th. cell/cm^3		500 th. cell/cm^3	
	treated	non-treated	treated	non-treated
100	5.00	7.25	4.00	7.37
10	4.75	7.25	3.37	7.37
1	2.50	7.25	2.00	7.37

Table 5
Results of ASFV titration in Poludanum treated and non-treated porcine spleen cells ($n = 5$)

Infecting dose, $HAdU_{50}/cm^3$	Virus titre (lg ± 0.25 lg $HAdU_{50}/cm^3$)			
	«Lazarevskoye 01/14»		8-No. 2 «Odintsovo 02/14»	
	treated	non-treated	treated	non-treated
1000	7.00	7.25	6.50	7.00
100	6.75	7.25	5.25	7.00
10	6.50	7.25	4.00	7.00

be used for prevention and treatment of different animal diseases.

CONCLUSION

Based on the work done a moderate interferonogenic activity of Poludanum for MARC-145 continuous cell culture was detected, which was expressed in the decrease in PRRSV titre by 0.25–1.50 lg $TCID_{50}/cm^3$, depending on the strain. These results are consistent with the data elaborated by L. C. Miller et al. [17].

At the same time we did not manage to detect Poludanum effect on TGEV reproduction when using PEK continuous cell line, which may be explained whether by the absence of Poludanum interferonogenic effect on these cells, or by the fact that PEK cells cannot effectively produce IFN and thus cannot be used for the study of Poludanum interferonogenic activity.

As for porcine testicular cells, two or more times decrease in TGEV "Leningradsky" strain titers was observed (from 2.25 to 5.37 lg $TCID_{50}/cm^3$) in Poludanum-treated cell culture compared to the titres of the virus, accumulated in Poludanum non-treated culture.

When analyzing Poludanum interferonogenic activity and its effect on ASFV reproduction in primary porcine spleen cell culture, the decrease in titres of accumulated "Lazarevskoye 01/14" isolate by 0.25–0.75 lg $HAdU_{50}/cm^3$ was noted, and by 0.50–3.00 lg $HAdU_{50}/cm^3$ for 8-No. 2 "Odintsovo 02/14" strain, which is associated with its genome structure peculiarities.

Table 6
Poludanum effect on reproduction of tested viruses

Virus	Strain	Strain characteristics	Cell culture	Poludanum treatment effect
PRRS	"Irkutsky – 2007-V"	Weakly virulent	MARC-145	+
	"BD"	Avirulent	MARC-145	±
	NVSL	Weakly virulent	MARC-145	+
	"Italian-2165"	Weakly virulent	MARC-145	±
	"KPR"	Avirulent	MARC-145	+
TGE	"Leningradsky"	Weakly virulent	PT	+
	"Leningradsky"	Weakly virulent	PEK	–
	"Ilyinogorsky"	Avirulent	PEK	–
	"Krasnodonsky"	Avirulent	PEK	–
ASF	"Lazarevskoye 01/14"	Lethality 100%	PS	±
	8-No. 2 "Odintsovo 02/14"	Lethality ≥ 80%	PS	+

«+» – positive; «±» – inconclusive result;
«–» – negative result, PT – porcine testicles; PS – porcine spleen.

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