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The Antiviral Action of Polyhexamethylene Guanidine Derivatives

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Abstract: Although the PHMG (polyhexamethylene guanidine) and other oligomer guanidines are known as highly efficient biocides against a broad spectrum of microorganisms and eukaryotic cells, the cell protection by PHMG derivatives has been established firstly in this study. The antiviral protection was also exhibited after 15 min pretreatment of different cell cultures with low-concentration of PHMG salts. Monolayers of the continuous bovine tracheal cells culture (TCC) and primary culture of chicken embryo fibroblasts (FCE) were treated with aqueous solutions of PHMG chloride salts or PHMG succinate. The molecules of PHMG polycation adhered to the plasma membrane of the cells tested as they were treated with PHMG for 15-30 min. The viral material was added to the cell cultures after the wash-out carried out twice to rid of unbound PHMG. The viruses of *Equine herpesvirus type 1*, *Rhinotracheitis infectious bovine* and *Equine infectious anemia virus* were used. The protective effect from the cytopathic action of herpes and retroviruses was exhibited after 15 min pretreatment of cell monolayer with PHMG chloride at the TCC concentrations of 10^{-3} - 10^{-2}% and FCE concentrations of 10^{-5} - 10^{-4}%. The unique antiviral properties of PHMG salts represented in our research had never been shown before.

Key words: Polyhexamethylene guanidine, tracheal cell culture, fibroblasts, viruses.

1. Introduction

Polymeric guanidines are commonly used as antiseptics and disinfectants. The correct application of these biocides plays a profound role in the elimination of infection for in- and out-patient treatment and in veterinary [1-3]. The PHMG (polyhexamethylene guanidine), a member of the polymeric guanidine family, has broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, yeasts and viruses.

PHMG is most effective against viruses which shell contains lipids, such as influenza, human immunodeficiency virus, Hepadnaviridae, Retroviridae, Caliciviridae, Orthomyxoviridae, Paramyxoviridae, Reoviridae, Picornaviridae, Adenoviridae, Parvoviridae groups and some others [4].

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PHMG hydrochloride is a highly water soluble polymer. It is odorless, colorless, noncorrosive [5] and much less toxic than common disinfectants [6] to humans and animals within a concentration range of 0.01%-1.0%. PHMG has been widely used for many years as antiseptic and disinfectant in Russia, Ukraine and other countries.

The net negative charge often stabilized by the presence of divalent cations such as Mg^{2+} and Ca^{2+}, it is the important characteristic of the outer envelope of different bacterial cells. Antimicrobial cationic polymers including PHMG interact with phospholipids of outer membrane and the plasma membrane (PM) of cell. PHMG can also interact with weakly charged membranes containing phosphatidylcholine that is typical for eukaryotic cells [7]. The PHMG adsorption is likely to change the surface charge of the cell membrane and the transmembrane potential, membrane permeability to water and small ions,

mobility of phospholipids and regular activity of enzymes.

It is possible to expect that changes of plasma membrane properties, caused by PHMG adsorption are capable of changing cellular sensitivity to viruses. The authors have expected that nontoxic for the cell concentrations of PHMG can protect cells from some viruses [7]. To demonstrate antiviral properties of PHMG, the authors used the primary culture of fibroblasts of the chicken embryo (FCE) and interwoven culture of the tracheal cells of calf (TCC).

2. Materials and Methods

2.1 Chemicals

The salts of PHMG chloride (•HCl) and PHMG succinate (•HOOC-C_2H_4-COOH) were kindly provided by "Termite" (Rivne, Ukraine). The PHMG was the mixture of its oligomers with *n* ranging from 2 to 20 and a mean value of 10. The salts of PHMG were dissolved in water, phosphate buffer (pH 7.2) and Hanks balanced salt solution at the volume ratio of 1:9. The water solutions of PHMG salts were used at the concentrations ranging from 1×10^{-7} to 1×10^{-2} % or 0.001 to 100 mg/L (or $\approx 10^{-9}$-10^{-4} mM).

2.2 Cell Cultures and Viruses

The primary culture of fibroblasts of the chicken embryo (FCE) and interweaved culture of the tracheal cells of calf (TCC); virus-liquids from group of Herpesviridae: *Equine herpesvirus type 1* (vaccine strain CB-69) and *Rhinotracheitis infectious bovine* (strain TK-A), from group of Retroviridae: *Equine infectious anemia virus* (field strain). The average activity of used virus strain was ≈ 100 lg TCD_{50}/cm^3.

2.3 Methods

The cell cultures and laboratory tests were made by standard methods with modifications [8, 9]. Cells were grown in the solution that contained a mixture of 199 medium (45%), a minimum Eagle medium or MEM (45%) and blood serum of cattle (10%). The monolayer was grown after seeding cell suspensions in 96-well plastic plates at 0.1 mL per well. PHMG solution at the concentrations of 0.1, 0.2 and 0.3 mL per well was placed into the plastic plates where the monolayer of cells was formed. The nutrient media was carefully removed from the monolayer before PHMG addition. PHMG was removed after 15 or 30 min preincubation with the cells. The viral material was added into the nutrient medium as monolayer cells were washed with sterile buffer solution and Hanks. The Hanks solution was kept with the cells for 15 or 30 min instead of PHMG in the control experiment No. 1. After pretreatment with PHMG and rinsing with the sterile buffer solution in control experiment No. 2 the Hanks solution was added to the monolayer of cells instead of the viral material. Plates with the monolayer of cells were incubated on air up to 5-7 days in the thermostat at 37 °C. The state of the cells monolayer at the determination of cytopathic viral action and cell destruction was estimated visually by the binocular laboratory microscope (magnification × 70).

3. Results and Discussion

The viruscides concentration of PHMG salt that neutralized the virus was determined upon the addition to viruses-containing suspension. This concentration was 10^{-5}% higher for PHMG chloride [7]. Short 15 min pretreatment of FCE monolayer formed with PHMG chloride solutions at the concentrations of 10^{-5}-10^{-4}% reliably protected the cells against viral infection of rhinopneumonia horses (*Equine herpesvirus type 1*). The results of these experiments are represented in Table 1.

PHMG showed a protective effect after washing the monolayer with a sterile buffer solution and Hanks. It is, therefore, possible to suggest that its molecules are closely connected with the PM of the cells and thus provide an antiviral protection. The virus in the environment surrounding of the cells remained intact. It is likely that in this case the PHMG-induced PM

Table 1 Antiviral effect of PHMG on fibroblasts of the chicken embryo (FCE).

The concentrations of PHMG chloride, %	Condition of the cell monolayer
10^{-4}	Monolayer remained unchanged during the 3 days of observation, the virus did not damage the cells
10^{-5}	Monolayer remained unchanged during the 3 days of observation, the virus did not damage the cells
10^{-6}	Monolayer began to damage within the first 24 h, there were areas of damaged cells, complete destruction of monolayer during the next 24 h, the virus damaged the cells
10^{-7}	During the first 7-12 h monolayer began to damage, there were some areas of destroyed cells, during 12-24 h subsequent destruction of the monolayer (~ 90%), on the next day the monolayer was fully destroyed, the virus damaged the cells
Control No. 1	Monolayer of cells affected by a virus was destroyed within 12-36 h of incubation
Control No. 2	Monolayer was normal, no changes found over 6 days time

$P > 0.95$.

Table 2 The action of virus on the tracheal cells of (TCC) calf after 15 min incubation with PHMG salines.

The concentrations of PHMG, %	Cell destruction by the virus, % damaged part of TCC monolayer					
	Equine infectious anemia virus		*Equine herpesvirus type 1*		*Rhinotracheitis infectious bovine*	
	in 2 days	in 6 days	in 2 days	in 6 days	in 2 days	in 6 days
PHMG succinate						
10^{-2}	0	0	10	50	0	40
10^{-3}	10	50	20	70	20	100
10^{-4}	30	100	30	100	40	100
10^{-5}	70	100	70	100	80	100
PHMG chloride						
10^{-2}	0	0	0	0	0	0
10^{-3}	0	0	0	0	0	0
10^{-4}	20	70	0	10	10	70
10^{-5}	30	100	30	100	20	100
Control	80	100	80	100	90	100

$P > 0.95$.

destruction never occurred. Cell monolayer was infected and destroyed during the first-second days of incubation as in the control No. 1 should this virus-containing solution was transferred to another one not treated with PHMG.

Chicken embryo fibroblasts are not quite adequate for the investigation of the action of herpes virus of horse rhinopneumonia and bovine rhinotracheitis. Pathogen rhinotracheitis has a pronounced tropism to epithelial cells of the mucous shells of the upper respiratory tract and genitals. Therefore, TCC cells were used for the next set of experiments. The results of the antiviral action of different PHMG salts concentrations on TCC monolayer after 15 min preincubation with PHMG salts are shown in Table 2.

PHMG chloride was found to protect the tracheal cells from cytopathic action of all tested viruses within 6 days at the concentrations of 10^{-3}-10^{-2}% after 15 min preincubation time. The concentration of 10^{-4}% showed only partial antiviral protection, while the lower concentrations (10^{-5}% or less) exhibited almost no cells protection against the viruses. The PHMG chloride antiviral action was insufficient on TCC at the concentrations of 10^{-5}-10^{-4}% that quite reliably protected fibroblasts against virus infection of horse rhinopneumonia. This could result from the structural differences of transformed tracheal cells PM, and their greater sensitivity towards the most adequate object, the virus of rhinopneumonia.

The virus protective action of PHMG succinate was lower. It showed full (*Equine infectious anemia virus*) or partial (*Equine herpesvirus type 1* and

Rhinotracheitis infectious bovine virus) protective effect only at the concentration of 10^{-2}%. At the lower concentrations PHMG succinate did not protect cells from damage. The antiviral protection was insignificant even though in some cases it was all else observed. Therefore, in the next set of experiments the time of TCC exposure to PHMG was increased from 15 min to 30 min. PHMG succinate was found to completely protect cells from damage by rhinopneumonia virus (Equine herpesvirus type 1) at a concentration of 10^{-2}%. The cytopathic effect of the virus was not shown within 6 days time interval. The other strains of viruses weren't tested in this study. PHMG succinate at the concentration of 10^{-3}% exhibited only ~50% of antiviral protection. Hence, the time of PHMG adsorption on the PM depends on the anionic composition of PHMG salts.

In conclusion, the obtained data suggest that antiviral protection by PHMG salts depends on their anionic composition (chloride, succinate or others), concentration and the incubation time. PHMG polycations bind membrane lipids and lipoproteins and block the adsorption of the virus. Thus, the antiviral action of PHMG is likely to have no species specificity since similar results have been obtained for different types of viruses.

Perhaps, the potential of the cell surface change due to increased ionic conductivity of the damaged membrane and the positive charge of the PHMG molecule. The screening of PM lipid receptors by PHMG polycation molecules impairs or even prevents the viruses from penetration into the cells. One may also suggest that adaptation stress caused by the adsorption of xenobiotic PHMG on the PM surface increases the overall cell resistance.

The analysis of the degradation of cell monolayer indicates that PHMG concentrations insufficient for the cell protection are still capable of temporary antiviral action that prevents cells from the damage by viruses. A similar short-term or semi-protective effect was shown at the application of specific antibodies or viral inhibitors. Perhaps, there is a gradual chemical degradation of the polymer structure or the phospholipids that lost their properties and functional significance after incubation with PHMG were rejected by the cell and removed from the cell PM.

The mechanism of antiviral action of PHMG is likely to be similar with its antimutagenic action [10]. In this research the authors checked on the possibility to induce the reverse mutation by the application of N-methyl-N1-nitro-N-nitrosoguanidine to some microorganisms like *Salmonella typhimurium*. The efficacy of PHMG protection revealed for this and other relatively strong mutagens was high enough as it even prevented the formation of auxotrophic mutants of pretreated cells. The authors believe that PHMG-induced membrane action that prevents the interaction of the mutagen with the intracellular content including DNA may underlie another mechanism of PHMG antimutagenic action.

All viruses tested contained lipids in their membranes. However, those viruses did not contain fully formed virions, only. Instead, there were viral particles with and without lipoprotein membrane formed. It also matters that the charge of polyanion (and not otherwise) of viral nucleic acid (RNA or DNA) is negated by electrostatic binding with PHMG polycation.

4. Conclusions

The antiviral protection of PHMG salts first revealed in this research may result from the adsorption of PHMG polycation to the plasma membrane of target cell and its strong binding with membrane phospholipids that screen the cell from virus. To display this effect the concentrations of PHMG must be higher than those for non-transformed FCE while processing transformed cells TCC. Short 15 min pretreatment by PHMG chloride at the concentrations of 10^{-5}-10^{-4}% protected the monolayer of fibroblasts against horse rhino pneumonia. PHMG chloride provided an antiviral protection of the

tracheal cells monolayer at the concentrations of 10^{-3}-10^{-2}% after 15 min preincubation whilst less efficient PHMG succinate protected cells at the concentration of 10^{-2}% after 30 min preincubation.

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