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Human Lung Carcinoma (A-549) Continuing Cell Line and Human Endothelial (ECV-304) Continuing Cell Line Responses to the Influenza Virus at Different Multiplicities of Infection

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Abstract—The course of infection upon virus entry into the cell depends not only on the biological characteristics of the cells and of the virus itself, but also on the intensity of the cell infection by the virus, i.e., on the multiplicity of infection. The purpose of our work was to perform a comparative study of the responses of two human cell lines, the lung carcinoma cell line A-549 and the endothelium cell line ECV-304, to the infection with the influenza virus A at different multiplicities of infection. At the first passage, both cell lines responded by enhancement of proliferation and apoptosis induction only to the low doses of influenza virus (ID 1–10). In A-49 cells, the stimulatory effect of the low virus doses was observed 1–2 days earlier than in ECV-304 cells. Enhanced proliferation was observed in both cell lines from the second to the fourth passages, when cells were infected with higher virus doses (ID 100 and 1000). In addition, the response of the A-549 cells to low doses of the H3N2 strain of the influenza virus A depended on the virus propagation conditions—namely, no enhancement of cell proliferation was observed in response to the infection with the virus propagating in chicken embryonated eggs, in contrast to infection with the virus that propagated in cell culture. Immunocytochemistry of A-549 cells has demonstrated that, on the third day after infection, there could be observed a change (in the dose-dependent manner) in the intracellular localization of p53 and cyclin A, proteins involved in the cell cycle progression. At the low virus dose, cyclin A was predominantly detected in the nuclei (63%), while at the high virus dose it was p53 (54%), which was predominantly detected in this cellular compartment, this observation confirming that stimulation of cell proliferation in the case of very low multiplicity of infection and cell division arrest takes place in the case of high multiplicity of influenza virus infection. The study of the influenza virus A reproduction in A-549 and ECV-304 cells using a whole number of virology techniques showed low sensitivity of these cells to the influenza virus, which manifested in the gradual decrease in the viral RNA expression and the impairment of mature viral particles assembly during several passages. Therefore, the decrease in the multiplicity of infection is associated in the A-549 and ECV-304 cells with impairment of production of mature virus particles or certain virus protein synthesis, which is accompanied by cell proliferation enhancement and apoptosis induction. As a result of the comparative study of the two cell lines (A-549 and ECV-304) upon infection with different doses of influenza virus A, we have revealed common principles and specific features indicating the effects of the biological properties of the viruses and cells, as well as of the multiplicity of infection on the course of virus infection.

Keywords: A-549 and ECV-304 cell lines, influenza virus A, cell proliferation, apoptosis, infectious dose, viral RNA expression

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INTRODUCTION

Since 1977, in the human population, there has been observed simultaneous circulation of the two influenza virus A subtypes, A(H1N1) and A(H3N2). In 2009, a new pandemic influenza virus A(H1N1)pdm09 of swine origin, a unique natural reassortant the genome of which encompasses the fragments of the genomes of the swine, avian, and human influenza viruses, forced out the seasonal influenza virus of the A(H1N1) subtype, and, thus, since 2010, cocirculation of the A(H1N1)pdm09 and A(H3N2) viruses has been observed. There also occur

[1] *Abbreviations used:* PC—percent of control; AI—apoptosis index; ID—infectious dose; mAbs—monoclonal antibodies; $TCID_{50}$—50% tissue culture infectious dose, the virus titer able to produce cytopathic effects in 50% of cells in a monolayer; CPE—cytopathic effect; TNF—tumor necrosis factor; FS—fetal serum; HA—hemagglutinin; NA—neuraminidase.

sporadic outbreaks of avian flu caused by the viruses belonging to a number of subtypes (H5N1, H7N1, H7N7, H7N9, etc.), which, fortunately, has not been able to cross the species barrier and, thus, is not capable of being efficiently transferred from human beings to human beings (Kiselev, 2011). For this reason, it remains of importance to study the tropism and transmissibility of the influenza virus, which, in turn, is spurring the search for new cell-based models for the study of virus–cell interactions (van der Brand et al., 2010).

Apart from the biological properties of the cells and viruses, the multiplicity of virus infection, that is the amount of the virus which infects the cell, has a profound effect on the course that the infectious process in the cell will follow. Earlier, we have demonstrated that infection with low doses of influenza virus A leads to the enhancement of proliferation followed by the induction of apoptosis in the following cell lines of human origin: 14 continuous suspension cell lines obtained from patients with hemoblastoses (Smirnova et al., 2009, 2015), glyoblastoma cell line (T-98), and spontaneously transformed endothelial cells (ECV-304) (Smirnova et al., 2011a), as well as primary endometrial cell culture (Durnova et al., 2012).

The aim of our work was a comparative study of the responses of the two human cell lines (A-549 lung carcinoma cell line and ECV-304 endothelial cell line) to the infection with influenza virus A at different multiplicity of infection. The choice of these two cell lines for our study was based on the following important considerations. The principal target for the influenza virus are epithelial cells of the upper and lower respiratory tract. For this reason, the most widely used model system for the study of the specific features of cellular metabolism in the course of the influenza virus infection on molecular level has come to be the human lung carcinoma cell line A-549 represented by the type II pneumocytes (Vester et al., 2009).

Another cellular target that is important for influenza virus pathogenesis, especially for the highly pathogenic avian influenza virus, is endothelial cells (Ocana-Macchi et al., 2009). We used the continuous ECV-304 human endothelial cell line, which was obtained in 1990 (Takahashi et al., 1990) from the spontaneously transformed human umbilical vein endothelial cells. Nevertheless, there being a report suggesting that ECV-304 cells have been derived from human urinal bladder carcinoma T-24 cells (Brown et al., 2000), numerous morphological, immunochemical, biochemical, and cytogenetic studies have clearly demonstrated that ECV-304 cells may be used as an endothelial cell model for the purposes of certain medical and biological studies (Suda et al., 2001; Heath-Engel and Lingwood, 2003; Fujimoto et al., 2006; Yartseva and Fedortseva, 2009). This cell line has been utilized in some virology studies to investigated such viruses as herpes simplex viruses type 1 and type 2 (Chirathaworn et al., 2004; Zhang et al., 2014), rubella virus (Mo et al., 2007), dengue virus type 2 (Liew and Chow, 2004), and Japanese encephalitis virus (Shwetank et al., 2013). However, we have only come across a single work that used ECV-304 cells for studying the role of cellular proteases in the influenza virus pathogenesis (Kido et al., 2012).

In the current work, we analyzed the changes in cell proliferation and the induction of apoptosis in A-549 and ECV-304 cells during several successive passages upon infection with different doses of influenza virus A, as well as virus reproduction, namely, the release of the infectious virus and its hemagglutinin (HA) from the cells and the expression of mRNA for the viral proteins M1, HA, and neuraminidase (NA). In addition, with the aid of immunocytochemistry, we have studied the intracellular localization of proteins that regulate the cell cycle, p53 and cyclin A, at different multiplicities of virus infection. As a result of these studies, we have revealed both general changes in the states of A-549 and ECV-304 cells upon influenza virus infection and the differences between these two cell lines.

MATERIALS AND METHODS

Cell cultures. Continuing human cell lines A-549 (lung carcinoma) and ECV-304 (spontaneously transformed endothelial cells), as well as Madin-Darby canine kidney cell line (MDCK), were obtained from the Cell Culture Collection of the Influenza Research Institute, Ministry of Health Care of the Russian Federation, St. Petersburg. Cells were grown in alpha-MEM medium containing 2% bovine fetal serum without antibiotics. Cells were passaged every sixth or seventh day in dilutions from 1 : 3 to 1 : 5 using a solution containing versene and chymopsin to detach cells. For the experimental purposes, 3-day cultures with the cell content of 5×10^4 cells/mL were used.

Influenza virus A subtypes H3N2 (A/Brisbane/10/07, A/Pert/16/11), H1N1pdm09 (A/St. Petersburg/05/09, A/St. Petersburg/06/14) and H2N2 (A/Singapore/01/57) were propagated in chicken embryos. Influenza virus subtype A(H3N2) A/Astrakhan/06/12 was propagated in MDCK cells.

The virus titers were 5–6 log $TCID_{50}$ (virus titer which causes a cytopathic effect in 50% MDCK cells in a monolayer). The final tenfold dilution of the titrated virus was taken as one infectious dose (ID) per milliliter. Multiplicity of infection (the ratio of ID to infected cell number) corresponded to 0.00002–0.0002 at low virus doses (1–10 ID), and to 0.02 at high virus doses (1000 ID).

Infection of a 1-day cell monolayer grown in 24-well plates was performed by introducing 0.1 mL of virus-containing liquid with known virus titer. Virus was left in contact with cells for 45 min at 37°C in serum-free alpha-MEM medium containing 1 μg/mL trypsin and antibiotics (penicillin/streptomycin). Cells were then

washed once with the same medium to remove the virus, and then a growth medium containing 1% of fetal serum was added. In the course of the experiment, cells were grown during four passages in 50-mL cultural flasks (Nunc, Denmark). The 1-day cell monolayer was infected with the virus using the approach described above, with the inoculate volume being 0.5 mL. On days 5 and 6 after virus infection, cells were passaged with a 1 : 2 dilution. Half of the cell suspension was left to grow in the flask, and the remaining portion of cell suspension was used for inoculation into the wells of 24-well plates for further assessment of proliferation and apoptosis induction and for freezing in tubes at −20°C and subsequent studying of the viral RNA expression by real-time PCR. To study apoptosis induction, coverslips were put in the wells. Cell cultures that were incubated with intact virus-free liquids were used as a control. Two wells were analyzed at each time point, and all experiments were carried out in three replications.

Cell proliferation was assessed by counting trypan-blue-negative cells in Fuchs Rosenthal chamber. For this purpose, cells were detached from the surface using a solution containing versene and chymopsin and cell suspension samples were taken for cell number counting. Proliferation activity was assessed from the ratio of the number of infected cells at a certain point in time to the number of control (intact) cells at the same time point. The obtained ratio was expressed as the proportion of control, the control being taken as 100%.

Apoptosis induction in the cells was assessed by nuclear chromatin fragmentation. Samples mounted onto the coverslips were fixed with methanol and acetic acid mixture in the ratio of 3 : 1 and stained with Hoechst-33258 fluorescent dye, and the proportion of apoptotic cells was determined using a luminescent microscope with 90× magnification and oil immersion and examining not less than 300–500 cells each time. The results were expressed as an apoptosis index (AI) calculated according to the formula $AI = (b/c) \times 100\%$, where b is the number of apoptotic cells and c is the total number of the cells examined. Cell staining with Hoechst-33258 served also for controlling cell contamination with bacteria, L-form bacteria, and micoplasma.

To detect the virus and to study its state in the tested cell cultures, several technical approaches were utilized. (1) The presence of HAs of the virus in the culturing medium was determined by hemagglutination reaction with chicken erythrocytes or human 0 (I) blood group erythrocytes. (2) To detect intracellular virus forms, cells were disrupted by successive freezing and thawing and the obtained lysates were inoculated into permissive MDCK cell culture. Virus presence in the lysates was assessed by the cytopathic effects (CPE) in MDCK cells. (3) Expression of mRNA for virus M1, HA, and NA proteins was detected using real-time PCR and commercially available kits manufactured by the Central Research Epidemiological Institute, Russia, according to the recommendations in the user's manual. An AmpliPrime RIBO-prep kit (Russia) was used for RNA (DNA) extraction from the samples under study, and Reverta-L kit (Russia) was used for cDNA synthesis on RNA matrix in the course of the reverse transcription reaction. The following reagents kits for real-time PCR were used: AmpliSense Influenza virus A/B-FL (Russia) to detect the RNA for M1 protein, AmpliSense Influenza virus A-type-FL (Russia) to identify H1N1 and H3N2 influenza virus A subtypes (allows detection of fragments of HA genes (H1 and H3) and NA genes (N1 and N2) of epidemic virus strains), and AmpliSense Influenza virus A/H1-swine-FL (Russia) to detect HA of the pandemic H1N1pdm influenza virus A. Amplifications were performed in a Rotor-Gene 6000 thermal cycler (Corbett Research, Australia). The expression level was calculated as $50 - C(t)$, where $C(t)$ is the threshold cycle value for the sample under analysis.

Immunocytochemistry was performed using cells grown on coverslips. On the third day after infection with the virus, cells were fixed with a methanol and acetone mixture. Mouse monoclonal antibodies against the proteins under study (p53 and cyclin A) and other reagents necessary for visualization of their expression manufactured by Novocastra (United Kingdom) were used. The results of the study were expressed as the percentage of stained cells calculated by the formula $n = (b/c) \times 100\%$, where n is the percentage of stained cells (%), b is the number of positive cells, and c is the total number of cells examined. In each experiment, no less than 300–500 cells were examined using a light microscope with 40× magnification.

The obtained results were statistically processed with the aid of the MS Office Excel 2007 and Statistica 6.0 software packages. To compare two groups of nonparametric samplings, the Mann–Whitney U test was used. The difference between experiment and control was considered significant at $P < 0.05$.

RESULTS

Comparative analysis of proliferation of the A-549 and ECV-304 cells led to an interesting observation. Both cell lines responded to the infection with influenza virus A subtypes H1N1pdm09 and H2N2 that propagated in chicken embryos with stimulation of proliferation. However, the A-549 cell, in contrast to ECV-304 cells, showed no response to the infection with the influenza virus A subtype H3N2 recovered from allantoic fluid and viral stimulation of cell proliferation in the case of infection with this virus strain took place only when the virus propagated in the cell culture (table).

Sensitivity of cell lines to stimulatory action of influenza virus A (recovered from allantoic fluid or from cell culture) at a low infectious dose (1 ID)

Virus	Portion of live cells, % of control[a]	
	cell lines	
	ECV-304	A-549
Subtype H3N2		
Brisbane/10/07 (allantoic)	182.6 ± 10.3[b]	95.8 ± 4.7
Pert/16/11 (allantoic)	149.4 ± 7.8[b]	96.2 ± 3.4
Astrakhan/06/12 (cell culture)	203.4 ± 14.2[b]	188.4 ± 11.5[b]
Subtype H1N1pdm		
St. Petersburg/05/09 (allantoic)	152.7 ± 9.3[b]	176.3 ± 10.6[b]
St. Petersburg/06/14 (allantoic)	155.9 ± 8.4[b]	165.5 ± 9.2[b]
Subtype H2N2		
Singapore/01/57 (allantoic)	124.9 ± 4.6[b]	127.7 ± 5.1[b]

One infectious dose (ID) in 1 mL corresponded to the final tenfold dilution of the titrated virus; [a] cells grown without virus (control); [b] statistically significant difference from the control, $P < 0.05$ (according to Mann–Whitney U test).

For this reason, all further studies were performed using only virus preparations recovered from allantoic fluid, namely, pandemic influenza virus A H1N1pdm subtype to infect A-549 and ECV-304 cells and epidemic influenza virus A H3N2 subtype to infect only ECV-304 cells. The general response of A-549 and ECV-304 cells was enhancement of proliferation at the first passage when the lowest dose of influenza virus A (1 ID) was used. The difference between the two cell lines consisted in an earlier manifestation of stimulatory effect in A-549 cells: slightly increased proliferation upon the infection with 1 ID of influenza virus as early as on the first day after the infection, a peak of proliferation on the third day, and a decrease in proliferating activity up to the fifth day (Fig. 1). In ECV-304 cells, an increase in proliferating activity started to be observed at a later time—namely, on the third day after infection—and proliferation considerably increased up to the fifth day (Fig. 1). At subsequent passages, stimulation of proliferation become observed also in the cells infected with higher virus doses (100 ID for A-549 cells and 100 and 1000 ID for ECV-304 cells) from the second passage on. A-549 cells infected with a high dose (1000 ID) of influenza virus A/St. Petersburg/05/09 (H1N1pdm09) revealed cytodestructive changes and appeared to be dead by the fourth passage.

Comparative analysis of apoptosis induction in A-549 and ECV-304 cells upon infection with different doses of the influenza viruses A/St. Petersburg/05/09 (H1N1pdm09) and A/Brisbane/10/07 (H3N2). A-549 cells, which are known for their high sensitivity to the apoptosis-inducing action of influenza virus A, showed a high level of apoptosis (29%) as early as on the first day after infection upon infection with the highest virus dose (1000 ID) (Fig. 2). During subsequent days of culture growth, apoptosis began to be observed in cells infected with lower virus doses (100 and 1 ID). The maximum apoptosis level (up to 40–50%) in A-549 cells was registered at the second passage on the fifth day of culture growth (100 and 1 ID). In ECV-304 cells, the apoptotic effect developed more slowly, with the apoptosis level being low on the first and second day after infection and increasing by the days 3–5 after infection (up to 25%) (Fig. 2). At the second passage, a spike in the apoptosis level (from 25 to 50%) was observed in ECV-304 cells on the fifth day, as was the case with the A-549 cells, at all doses of virus used. At the third passage, the apoptosis level decreased in both cell cultures at all doses of influenza virus and then slightly increased again at the fourth passage.

Intracellular distribution of p53 and cyclin A proteins that are associated with the cell progression through the phases of the cell cycle. This series of experiments were performed on A-549 cells infected with different doses of the influenza virus A/St. Petersburg/05/09 (H1N1pdm). When A-549 cells were infected with a high dose of influenza virus (100 ID), p53 was localized in the nucleus in 54% of cells, indicating cell division arrest, while, in the cells infected with the low virus dose (1 ID), p53 was localized predominantly in the cytoplasm (46% of cells) (Fig. 3). At the same time, the proportion of cells in which cyclin A was localized in the nucleus, indicating active cell division, was 63% at the low virus dose and decreased up to 1% at the high influenza virus dose (Fig. 3). Therefore, these observations give support to previous findings indicating that, upon infection with a low dose of influenza virus, the majority of cells enter into the state of active proliferation (their number being statistically higher than in the control cells on the third day in the active growth state).

Influenza virus detection in ECV-304 and A-549 cells using different techniques (hemagglutination reaction, CPE detection in permissive MDCK cells, and PCR). Studying the reproduction of both influenza virus subtypes in ECV-304 and A-549 cells upon infection with doses from 1000 to 1 ID (virus dilution from 10^{-2} to 10^{-6}) revealed low permissivity for these cells to the influenza virus A (virus titers in this cells did not exceed 2–3 $\log TCID_{50}$ as compared to 5–7 $\log TCID_{50}$ in permissive MDCK cells).

The study of influenza virus release from the infected ECV-304 and A-549 cells into the culture liq-

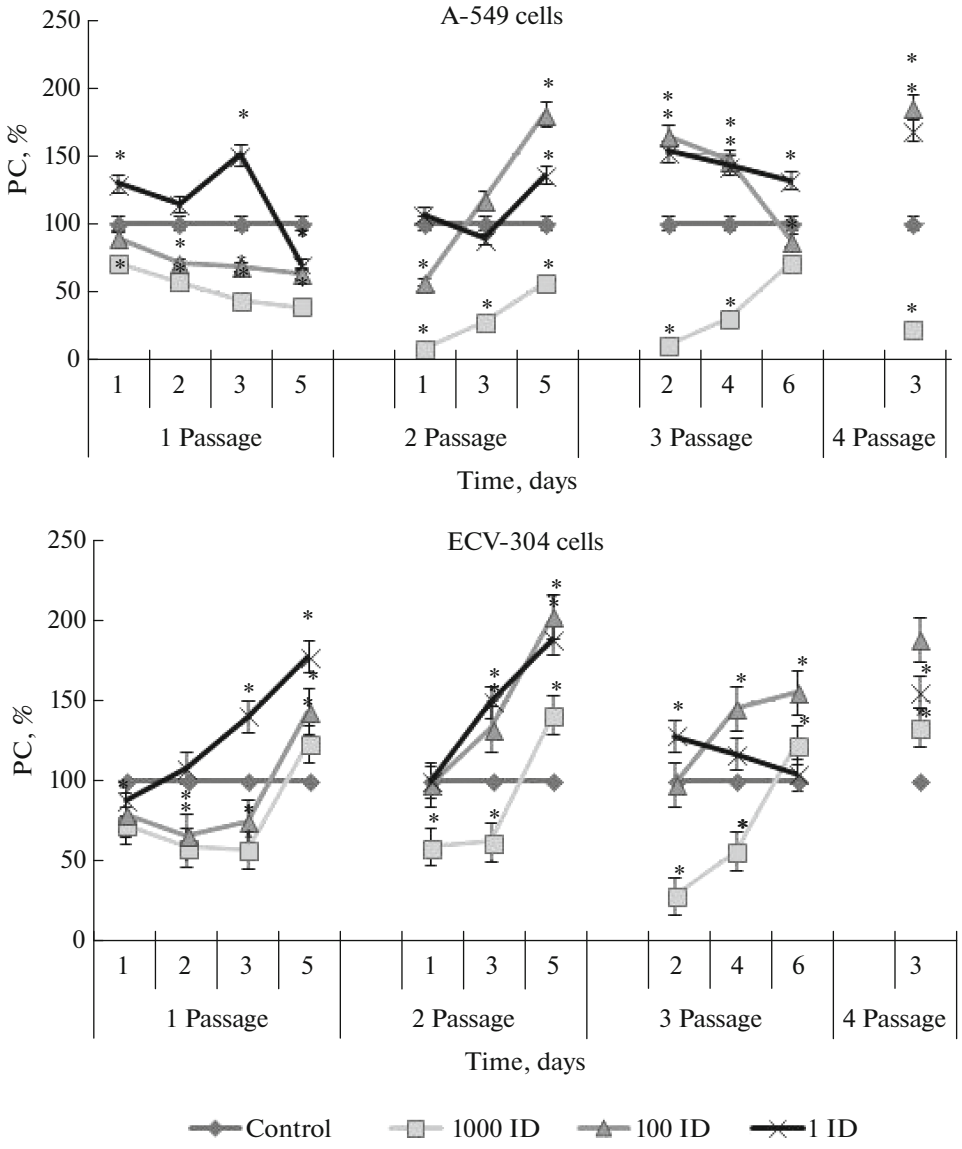

Fig. 1. Proliferation dynamics for A-549 and ECV-304 cells infected with different doses of the influenza virus A/St. Petersburg/05/09 (H1N1pdm) during four passages. PC—percentage of control, ID—infectious dose, control—cell grown without virus; asterisk indicates the difference from control at $P < 0.05$.

uid using hemagglutination reaction revealed extremely low titers of viral hemagglutinin (no higher than 1 : 2), it being detected ever only in cultural liquid samples obtained from the cells infected with the high virus dose (1000 ID). At lower virus doses (1–10 ID), we obtained negative results. Testing cultural liquid for the presence of the infectious virus (by CPE caused in permissive MDCK cells) also gave a negative result. Infectious virus in the titer of 1 $\log TCID_{50}$ was detected only when MDCK cells were inoculated with the lysates of cells (of both cell lines) infected with a high dose of virus (1000 ID). The lysates were obtained as a result of disruption of cells by sequential freezing and thawing. The obtained results indicate impaired influenza virus A reproduction and infectious virus release into the extracellular space in ECV-304 and A-549 cells infected with low virus doses.

Real-time PCR with primers to viral RNAs encoding M1, HA, and NA proteins showed a gradual decrease in the expression of the corresponding genes in the course of three passages depending upon the virus dose. Figure 4 presents the expression of the RNA encoding viral HA in the A-549 and ECV-304 cells infected with different doses of the influenza virus A/St. Petersburg/05/09 (H1N1pdm09). Upon infection with the low virus dose (1 ID), HA RNA was observed only at the first passage (day 4–5), while, in the cells infected with higher virus doses (100 and 1000 ID), viral PNA was observed during no more than three passages (Fig. 4).

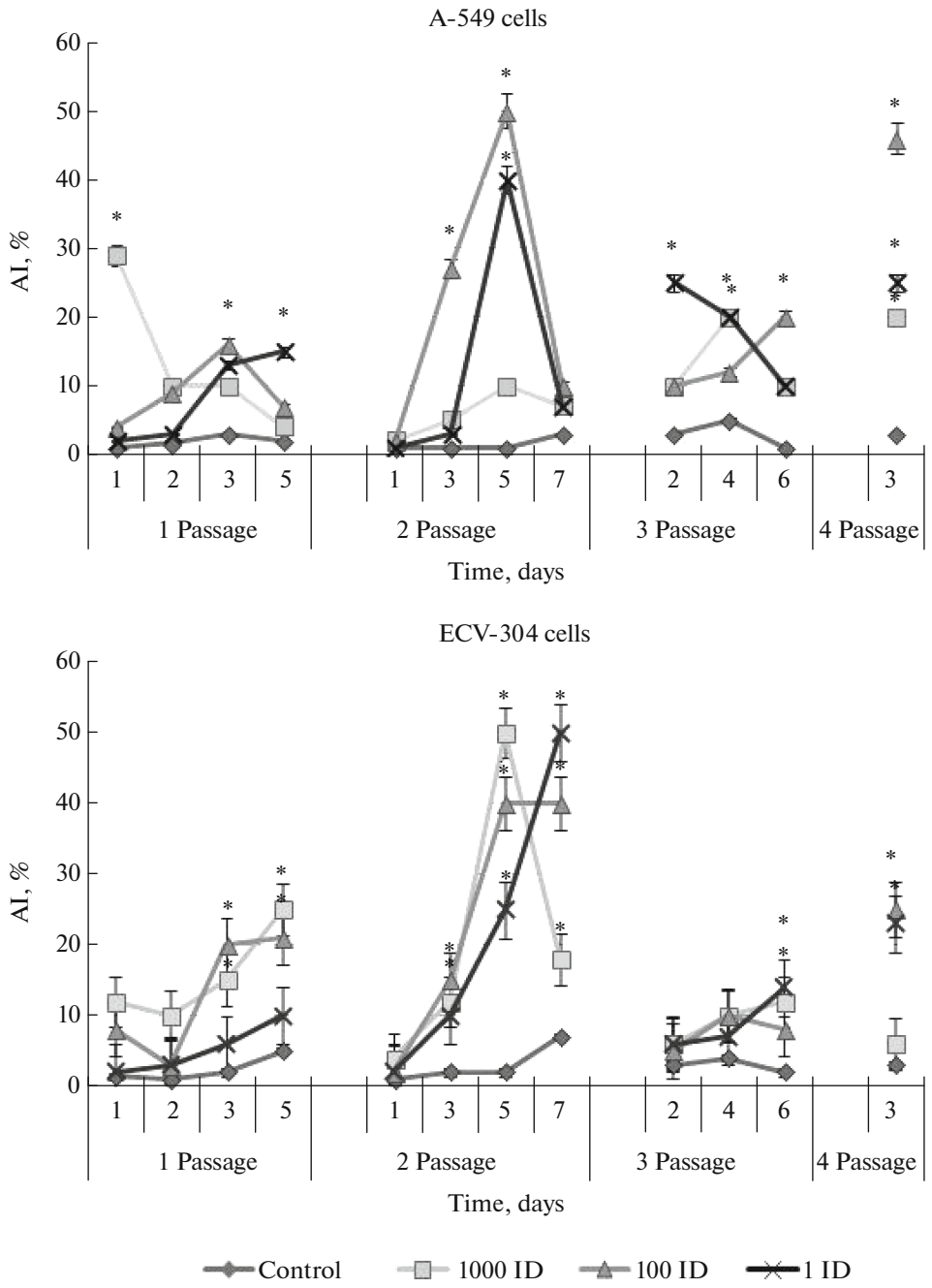

Fig. 2. Dynamics of apoptotic death of A-549 and ECV-304 cells infected with different doses of influenza virus A/St. Petersburg/05/09 (H1N1pdm) during four passages. AI—apoptosis index, ID—infectious dose, control—cell grown without virus; asterisk indicates the difference from control at $P < 0.05$.

DISCUSSION

A comparative study of the cell lines of different origin (A-549 epithelial cell line and ECV-304 endothelial cell line) revealed some differences in the responses of these cells to the infection with the influenza virus A. A-549 cells were unable to increase proliferation upon infection with the influenza virus A subtype H3N2 propagated in chicken embryos. This may be a result of the changes in the level of glycosylation of the influenza virus surface antigens (HA and NA) that occur under different virus propagation conditions, in chicken embryonated eggs or human and animal cells (Mochalova et al., 2003). HA and NA are the main determinants of the influenza virus, directing the interaction with the cells, and changes in their glycosylation levels affect virus absorption and its

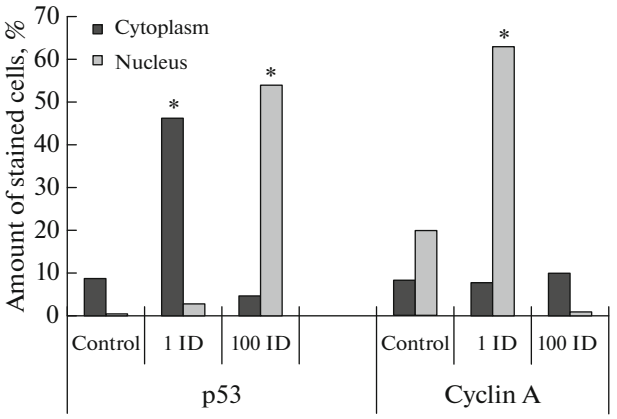

Fig. 3. p53 and cyclin A distribution between the nucleus and the cytoplasm in A-549 cells on the third day after infection depending upon the dose of the influenza virus A/St. Petersburg/05/09 (H1N1pdm). Control—cell grown without virus; asterisk indicates the difference from control at $P < 0.05$.

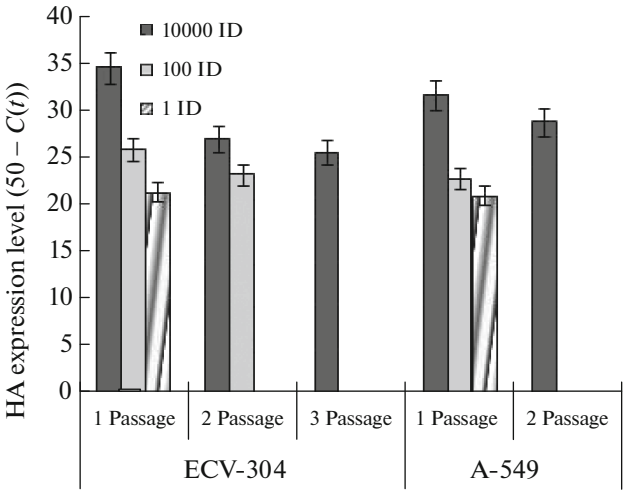

Fig. 4. Expression of mRNA for viral hemagglutinin (HA) in ECV-304 and A-549 cells. $C(t)$—threshold cycle value for the sample under study.

entrance into the host cell; antigenic and immunogenic properties of the virus; and, under in vivo conditions, virus tropism (Klenk et al., 2002). Mutational changes of the influenza virus A surface antigens associated with its absorption properties are studied using lectins, inhibitors, and erythrocytes of various origins (Lin et al., 2012). The effect observed in this work may be used to develop another model system to be used both for studying changes in the glycosylation of the influenza virus A surface antigens and for characterizing different virus subtypes.

It may also seem that the observed specific feature of the A-549 cells is a consequence of the slowed (compared with the MDCK cells) post-translational processing of viral glycoproteins in the endoplasmic reticulum and subsequent HA and NA transporting to the cell membrane (Ueda et al., 2008). As a matter of interest, when the influenza virus of the H3N2 subtype was recovered from allantoic fluid, cytodestructive changes in A-549 cells, viral RNA expression, apoptosis induction, and viral nucleoprotein synthesis were not in any may affected (Smirnova et al., 2011b).

Differences between the A-549 and ECV-304 cells were observed in the case of proliferation stimulation and apoptosis induction, both effects being detected in the A-549 cells 1–2 days earlier than in the ECV-304 cells. We have already observed earlier the differences between these two cell lines, which consisted in extremely low levels of tumor necrosis factor expression (TNF) in the A-549 cells in contrast to the very high levels of its expression in the ECV-304 cell line (Smirnova et al., 2012). This specific property of the A-549 cell has also been noted by other authors in regard to TNF and INF-α, expression of which significantly increased when the cells were primed with the corresponding cytokine (Veckman et al., 2006).

The study of influenza virus A reproduction in A-549 and ECV-304 cells using a number of virological methods revealed that this cell lines possess low sensitivity to the virus under study, which was evidenced by a decrease in the production of mature virus particles and a gradual decrease in the expression of the RNAs encoding viral protein in the course of several successive passages. Normal reproduction of the influenza viruses of the A subgroup that circulate in human populations can be generally achieved in highly differentiated polarized cells, such as the cells of the upper respiratory tract, while the A-549 cells are insufficiently polarized and poorly differentiated type II pneumocytes of lower areas of the lung (Sun and Whittaker, 2007). Human endothelial cells are also not permissive for human influenza viruses, in contrast to the avian influenza viruses (Short et al., 2014), but they may be essential for influenza virus pathogenesis, being the most important source of cytokines in the lung (Teijaro et al., 2011).

It is well-known that influenza virus is able to cause cell cycle arrest in G_0/G_1, when the conditions are the most favorable for virus reproduction (He at al., 2010). In the current work, we have demonstrated that the multiplicity of the influenza virus infection has effects on intracellular localization (distribution between the nucleus and the cytoplasm) of p53 and cyclin A, which regulate cell cycle progression. We have obtained similar data previously when studying influenza virus A infection in human endometrial cells (Durnova et al., 2013).

To summarize, comparison of the A-549 and the ECV-304 cells' responses to influenza virus A infection revealed both common features and characteristic differences. The obtained results indicate that not only the biological characteristics of the viruses and the

cells, but also the multiplicity of virus infection, can affect the infectious process development.

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