

The Cytotoxic Effect of the Wild-Type Newcastle Disease Virus Strain on Tumor Cells *in vitro*

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Abstract—Oncolytic Newcastle disease viruses (NDVs) make up a group of antitumor agents for the experimental treatment of oncological diseases that nonpathogenic for human beings. It is known that realization of the cytotoxic effect of NDV strains on the tumor cells depends on the peculiarities of viral genome structure, strain virulence, and tumor cell culture (National Institutes of Health, 2018). At the same time, the mechanism of NDV-induced death has been poorly studied. The aim of this work was to study the nature of morphological changes in HeLa and Hep-2 (HeLa derivative) human tumor cells after infection with the wild-type mesogenic NDV strain NDV/Altai/pigeon/770/2011. The calculation of living tumor cells using MTT test demonstrated that the cytotoxic activity of NDV/Altai/pigeon/770/2011 strain in 144 h after infection decreases the cell viability till $13.08 \pm 8.29\%$ (HeLa) and $4.74 \pm 3.29\%$ (Hep-2, HeLa derivative). A routine staining detected morphological traits of degradation of infected cells, while morphometric studies detected an increase in the value of the nuclear–cytoplasmic ratio (NCR). The presence of a virus in the tumor cells was visualized by the method of immunocytochemical staining (ICS) using antibodies to the HN glycoprotein of the NDV envelope. The results of immunodetection of tumor necrosis factor α (TNF α) suggest the activation of cell death along the apoptosis pathway against the background of viral infection. The results obtained indicate a pronounced cytotoxic activity of wild-type NDV/Altai/pigeon/770/2011 strain regarding HeLa and Hep-2 (HeLa derivative) adenocarcinoma cells and sensitivity of the studied cell lines to the oncolytic effect of NDV.

Keywords: Newcastle disease virus, cytotoxic effect, tumor cells, apoptosis

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INTRODUCTION

The use of oncolytic viruses is a promising method for treatment of oncological diseases due to the ability of viral particles to have a targeted cytolytic effect on tumor cells without affecting healthy cells. Newcastle disease virus (NDV) is a virotherapeutic agents. NDV is a member of the group of avian paramyxoviruses (*Avulavirus* genus, Paramyxoviridae family) and contains about 150-kb single-stranded negative RNA (Miller et al., 2010). The advantages of this virus for virotherapy consist in a high stability of the genome, absence of interaction with the host cell DNA, and mild side effects in cancer patients (Zhao, Liu, 2012). The processes of virus penetration into the cell and its replication have been described (Puhlmann et al., 2010); however, the mechanisms of cell death against the background of NDV oncolytic activity remain poorly studied. There are conflicting reports about which of the mechanisms is primary. In one study of a

Hitchner B1 NDV lentogenic strain, it was noted that the tumor cells die by virus-mediated activation of necroptosis (Koks et al., 2015). An analysis of A549 cells infected with three different NDV strains (LaSota (lentogenic), Beaudette C (mesogenic), and FMW (velogenic)) demonstrated the activation of both external and internal apoptotic pathways (Bian et al., 2011); at the same time, these data contradict findings that the lentogenic LaSota strain induced the process of mitophagy followed by the inhibition of apoptosis in non-small cell human lung carcinoma A549 cells (Cuadrado-Castano et al., 2015). Such an ambiguous clinical picture when using different NDV strains is spurring new studies in this field.

The aim of the present work is to study the cytotoxic activity of wild-type NDV/Altai/pigeon/770/2011 strain regarding HeLa and Hep-2 (HeLa derivatives) human tumor cell lines.

The NDV/Altai/pigeon/770/2011 strain, which was isolated from a rock dove and belonging to NDV group (pigeon paramyxovirus serotype-1), was selected for subsequent studies as being a strain with a

Abbreviations: NDV—Newcastle disease virus, ICS—immunocytochemical staining, NCR—nuclear–cytoplasmic ratio, TNF α —tumor necrosis factor α .

very pronounced oncolytic potential regarding tumor cell lines in vitro from the collections of NDV isolates (Yurchenko et al., 2018). The human tumor cell lines HeLa and Hep-2 (HeLa derivative), which are frequently used in experimental oncology and relate to carcinoma (the most frequently diagnosed histogenetic type of tumor, which accounts for 90% of all oncological diseases) (Cooper and Hausman, 2013), served as objects of the study of the mechanism of cytotoxic effect of the wild-type NDV strain. In a number of works, the antitumor efficacy of vaccine and attenuated NDV strains regarding human carcinoma was described (Ginting et al., 2017; Yan et al., 2017; Abdullahi et al., 2018), as was the high oncolytic potential of NDV during the infection of HeLa lines was noted (Cheng et al., 2016; Rajmani et al., 2016; Chu et al., 2018).

In the present work, immunocytochemical detection of tumor necrosis factor (TNF- α), which is a major participant in programmed cell death that proceeds by activating the external receptor-mediated apoptosis pathway or RIP1, as well as RIP3-dependent necroptosis, was carried out to analyze the activation of cell death mechanism. In addition, based on the results of studies by Molouki et al. (2010, 2011), in which HeLa cells were infected with the velogenic NDV strain, the portion of cells expressing anti-apoptotic Bcl-2 protein was estimated after NDV infection in order to analyze a possible shift toward antiapoptotic regulation and a Bax/Bcl-2 ratio with a possible NDV-mediated activation of internal pathway of apoptosis.

MATERIALS AND METHODS

Human tumor HeLa cells—HeLa (cervical adenocarcinoma) and Hep-2 (HeLa derivative, laryngeal adenocarcinoma) were used in the work. They were cultivated in DMEM growth medium (Gibco Inc.) with 10% fetal bovine serum (Gibco Inc.) and 60 μ g/mL gentamicin sulfate under standard conditions at a temperature of 37°C and in an atmosphere with 5% CO₂ content.

Cytotoxic activity of wild-type mesogenic NDV/Altai/pigeon/770/2011 strain (Yurchenko et al., 2015) was tested on daily monolayers of tumor cells in 96-well plates. Dilutions of the virus in a concentration of 10 MOI were prepared in a supporting medium (MEM) (Gibco Inc.) containing 1% fetal bovine serum (Gibco Inc.). The cells were incubated with the virus for 1 h 30 min under standard conditions. The control tumor cells without the virus were incubated in the growth medium. The medium was then substituted with the supporting one.

The cell viability after infection with the NDV/Altai/pigeon/770/2011 strain was estimated by a colorimetric method using the MTT test in 24, 72, and 144 h. MTT was diluted in a concentration of

0.5 mg/mL in fetal bovine serum. A 10% working MTT solution was prepared on the supporting medium. The optical density was measured 40 min after the addition of DMSO at wavelengths of 540 and 630 nm (background) on a Lonza Biotek ELX808 Absorbance Microplate Reader microplate photometer (United States). The percentage of living cells was calculated according to the formula: $(E_{540} - E_{630}) / (C_{540} - C_{630}) \times 100\%$, where E are indices obtained for the samples from virus-infected wells, while C are indices obtained for the samples from the control wells.

For light-optical study, preparations of cell lines infected with NDV strains in a concentration of 10 MOI were prepared. In 24, 72, and 144 h after infection with NDV/Altai/pigeon/770/2011 strain, the cells were fixed in 2.5% glutaraldehyde on phosphate-buffered saline (pH 7.4) at room temperature for 1 h. The preparations were stained according to a standard method with hematoxylin and eosin. To quantify the degree of morphological changes in the tumor cells, a ratio of the nucleus area to the cytoplasm area (NCR) was calculated according to the formula $NCR = S_N / S_C$, where S_N is the nucleus area and S_C is the cytoplasm area.

For immunocytochemical staining (ICS), a washing solution of 0.25% Triton X100 on FBS was used. Nonspecific sorption of the cells was blocked in the solution of 0.25% Triton X100 on FBS with 5% goat serum (30 min). The time of exposure of the cells with the antibodies to HN-glycoprotein of NDV envelope (IgG fraction of rabbit antisera obtained from the blood of animals immunized with the NDV/Altai/pigeon/770/2011 strain after purification by affinity chromatography), TNF α (1 : 50, ab6671, Abcam), and Bcl-2 (1 : 50, ab59348, Abcam, United Kingdom) was 90 min. To visualize ICS, an HRP/DAB (ABC) Detection IHC Kit detection system (Abcam, United Kingdom) was used. The visualization was conducted on an AxioImagerA1 microscope with an AxioCam MRc photcamera (Carl Zeiss, Germany) in the Center for Collective Use of Microscopic Analysis of Biological Objects (Siberian Branch, Russian Academy of Sciences) and the Modern Optical Systems Center for Collective Use of the Research Institute of Experimental and Clinical Medicine, Federal Research Center of Fundamental and Translational Medicine.

Measurements of the area of the studied cells were conducted using an ImageJ program. The statistical processing of data on MTT test and NCR detection with a routine staining was conducted using the Statistica 6.0 statistical software package. Student's *t*-criterion was used to estimate a significance of differences between the samples (differences at $p < 0.05$ were considered significant).

RESULTS

To determine the cytotoxic activity of the NDV/Altai/pigeon/770/2011 strain (causing the death of the cell population), we estimated the viability of tumor cells in dynamics after infection with the virus. At a virus concentration of 10 MOI, the survival of HeLa and Hep-2 cells after 24 h remained high and was 95.26 ± 11.85 and $92.83 \pm 9.65\%$, respectively. In 72 h, the portion of living HeLa cells decreased to $77.78 \pm 7.85\%$, and that of Hep-2 cells to $57.84 \pm 9.27\%$. The maximum cytotoxic effect was reached in 144 h, when the portion of living cells was $13.08 \pm 8.29\%$ (for HeLa) and $4.74 \pm 3.29\%$ (for Hep-2) (Fig. 1).

A light-optical study of the control HeLa and Hep-2 cells after routine staining with hematoxylin and eosin demonstrated a low level of differentiation of the studied cell cultures. The cytoplasm has a uniform staining, without inclusions, and the presence of a large, centrally located nucleus is typical (Fig. 2a).

Twenty-four hours after infection with the NDV strain, the nuclei of HeLa cells become less pronounced as a result of a decrease in the intensity of staining and visual clarity of their nucleus borders. One or two nuclei are clearly determined. The cytoplasm becomes heteromorphic, and small inclusions are registered (Fig. 2b). In 72 h, changes become more pronounced. The cells begin to acquire an uncharacteristic form, and violations of monolayer integrity are noted. The cell nuclei shift to the periphery (Fig. 2c).

Similar morphological changes are detected in the Hep-2 cell line infected with the NDV strain, and a decrease in the cytoplasm area is visually registered (Fig. 3).

The mean values of NCR increase over time after infection with the NDV/Altai/pigeon/770/2011 strain. In 72 h, this index increases by 69% for the HeLa cell line and by 126% for the Hep-2 cell line (Fig. 4).

The presence of the virus in the cells of HeLa and Hep-2 human tumor lines was confirmed by means of ICS using antibodies to HN glycoprotein of the NDV/Altai/pigeon/770/2011 envelope. No antibody-labeled accumulations and aggregations were found in the cytoplasm and nuclei of the control (not infected with the virus) tumor cells (Figs. 5a, 5b).

The preparations of virus-infected cells of both human tumor lines demonstrate a positive result of immunostaining (Figs. 5c, 5d), which confirms the ability of the virus to infect tumor cells and to persist in them. It was noted that viral particles are dispersedly distributed inside the cells, with accumulations of them not being found in nuclei.

The TNF α protein, which is one of the markers of apoptosis, was not detected in the control cells of ade-

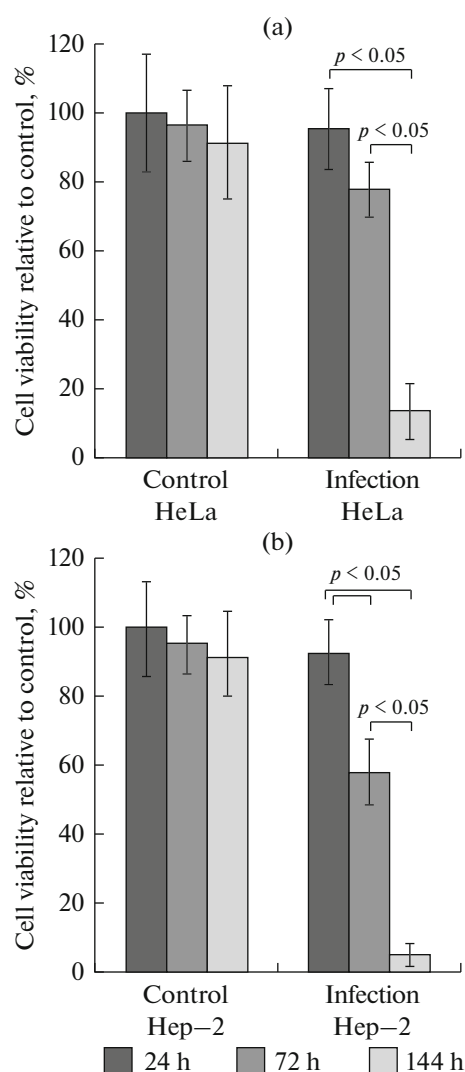


Fig. 1. Reduction in viability of (a) HeLa and (b) Hep-2 cells after infection with NDV/Altai/pigeon/770/2011 NDV strain (concentration of 10 MOI) according to the results of the MTT test. The calculation of the percentage of living cells was performed according to the formula $(E_{540} - E_{630}) / (C_{540} - C_{630}) \times 100\%$, where E are colorimetric indices obtained for virus-infected cells and C are colorimetric indices for the control cells. Data are presented as mean values and standard errors.

nocarcinomas by means of ICS (Figs. 6a, 6b). However, immunopositive accumulations were registered in the cells of both studied lines 24 h after the transduction of NDV (Figs. 6c, 6d).

The amount of cells expressing the Bcl-2 protein was insignificant in both cell lines (HeLa and Hep-2) after infection with the NDV strain (single Bcl-2-positive cells were found) and did not differ from the appropriate control indices (not shown).

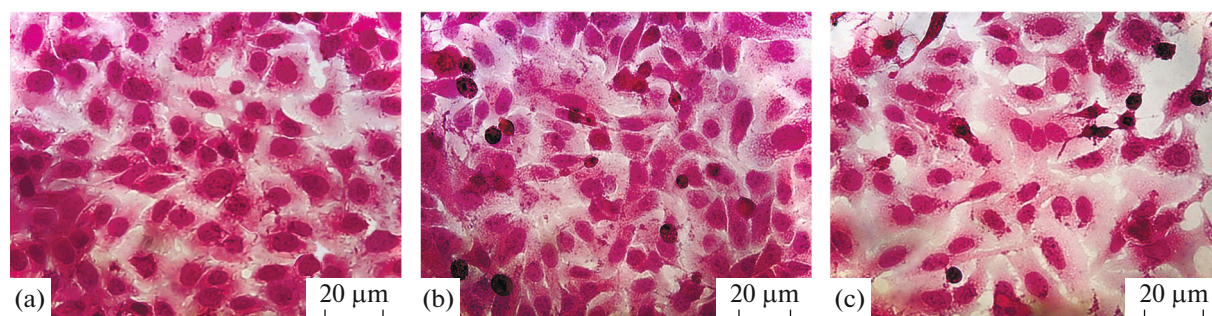


Fig. 2. Change in morphology of HeLa tumor cells after infection with NDV/Altai/pigeon/770/2011 strain. (a) Control HeLa cells; (b) HeLa cells 24 h after infection; (c) HeLa cells 72 h after infection. Ob. 40 \times .

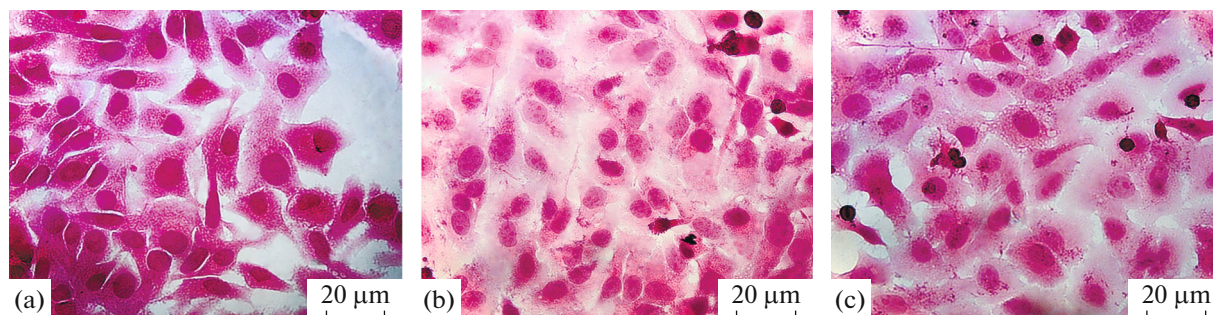


Fig. 3. Change in morphology of Hep-2 tumor cells after infection with NDV/Altai/pigeon/770/2011 strain. (a) Control Hep-2 cells, (b) Hep-2 cells 24 h after infection, and (c) Hep-2 cells 72 h after infection. Ob. 40 \times .

DISCUSSION

Despite the existence of large-scale studies of various oncolytic strains of viruses (including Newcastle disease virus) and the fact that series of preclinical and initial phases of clinical trials of the drugs based on them have been carried out, there is still a problem of

lack of knowledge about the mechanisms of cytotoxic oncolysis, which makes the problem of virus studies quite important. There is no developed idea about the tropism of NDV strains to a particular type of tumor, and possible distant effects of such treatment also remain unstudied.

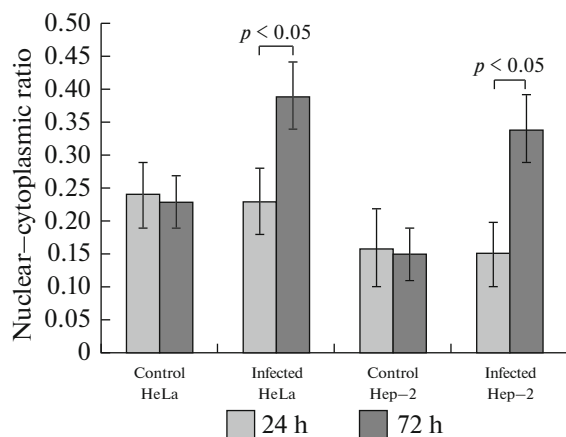


Fig. 4. Dynamics of the nuclear–cytoplasmic ratio after the cell transduction with NDV/Altai/pigeon/770/2011 NDV strain (concentration of 10 MOI). Data are presented as mean values and standard errors.

Based on our results, it is possible to draw the conclusion that cytotoxic effect of NDV on the HeLa and Hep-2 (HeLa derivative) tumor cells is pronounced and leads to an efficient decrease in the viability of both cell lines. The obtained evidence of the oncolytic effect of NDV relative to human tumor cells are confirmed by facts already described in the literature (Mansour et al., 2011; Shestopalova et al., 2012; Yurchenko et al., 2018). The mechanism of antiviral defense associated with the activation of protein kinase R is often disrupted in tumor cells. In this regard, RNA viruses have favorable conditions for reproduction in tumor cells. NDV is able to replicate in the tumor cell 10000 times more efficient than in normal cells (Schirmacher and Fournier, 2009).

The confirmation of the presence of viral particles in infected cells of the studied lines allows it to be concluded that all the observed morphological changes, as well as peculiarities of ICS using antibodies to apoptosis factors (TNF α) distinguishing infected cells from

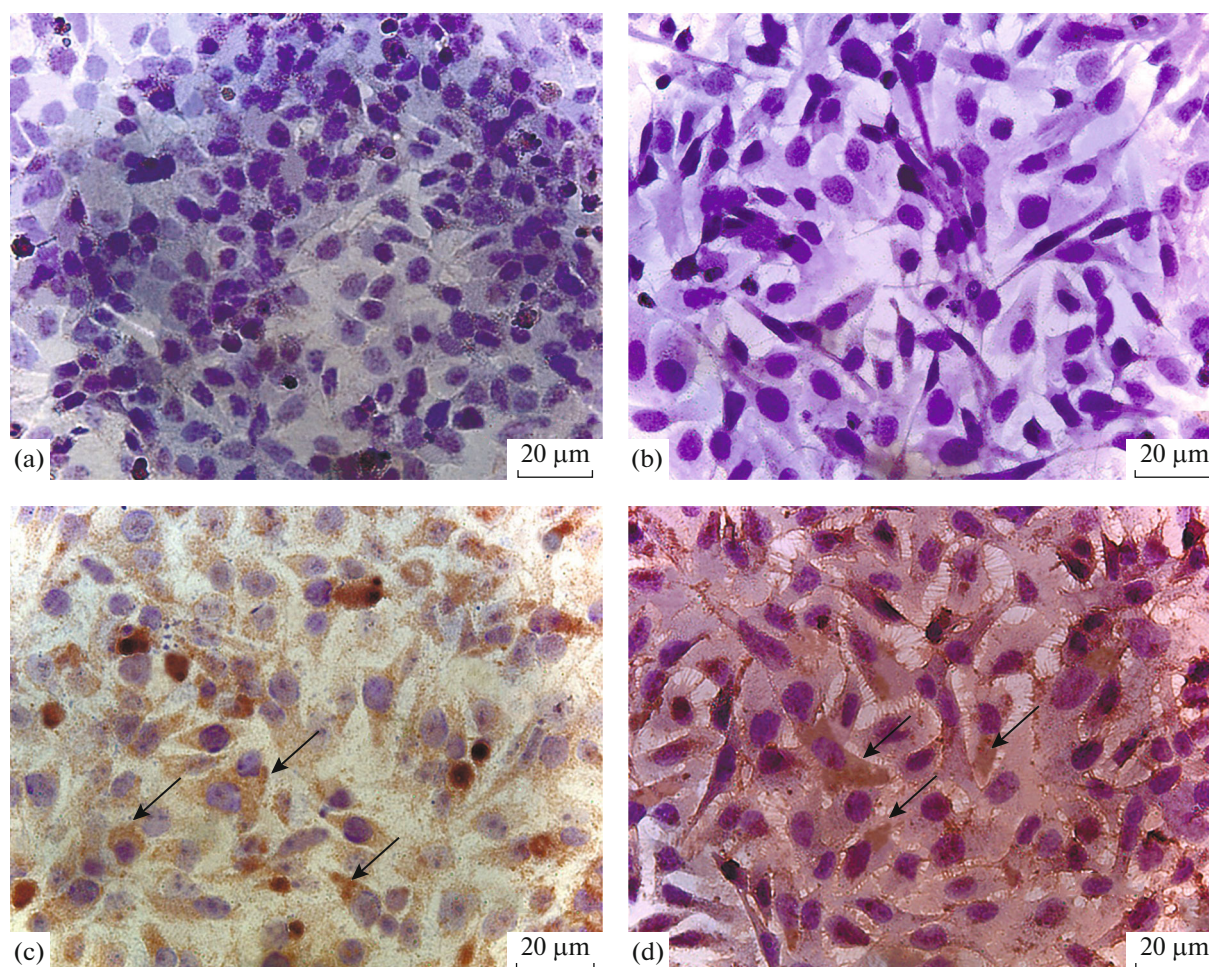


Fig. 5. Immunocytochemical staining of (a, c) HeLa and (b, d) Hep-2 cells using antibodies to NDV HN glycoprotein. (a, b) Control cells; (c, d) cells 24 h after infection with NDV/Altai/pigeon/770/2011 strain. Immunopositive zones in the cytoplasm are marked by arrows. Ob. 40 \times .

the control cells, are associated with the oncolytic effect of NDV.

Based on the results obtained by the method of routine staining with hematoxylin and eosin, as well as the results from the measurement of NCR, it can be concluded that the synthetic apparatus of infected cells is in a state of increased activity. It is obvious that NDV uses the host cell synthetic processes for its replication, as a result of which the latter eventually loses the ability to maintain its basic vital functions and dies (Auer and Bell, 2012).

The results of immunohistochemical reactions with antibodies to TNF α indicate that a single case of infection with the NDV/Altai/pigeon/770/2011 NDV strain can activate or enhance the process of the cell death of HeLa and Hep-2 carcinomas by apoptosis. Thus, the HN viral protein induces apoptosis mediated by a death receptor through the activation of caspase 8 (Rajmani et al., 2015). Obviously, an increase in death is also associated with the effect of viral infection and NDV reproduction on the pro-

cesses of the external pathway of apoptosis activation in carcinoma cells. The presence of NDV or its proteins (HN) enhances the sensitivity of HeLa cells to the effect of TNF α due to the inhibition of transcription of NF- κ B nuclear factor responsible for the control of apoptosis (Rajmani et al., 2016). However, it should be noted that the necroptotic death pathway can be also triggered indirectly through death receptors, including TNFR1(CD120) and TNFR2 tumor necrosis factor receptors and TRAILR1 and TRAILR2 receptors (Vandenabeele et al., 2010). As a consequence, it is difficult to say for sure which pathway of death prevails during the transduction of tumor cells with the NDV/Altai/pigeon/770/2011 strain. This feature requires further study.

Regarding data on immunohistochemical staining using antibodies to Bcl-2, it can be noted that, according to the results of similar works, no significant changes in total levels of anti- and proapoptotic proteins Bcl-2 and Bax are detected during the infection of HeLa cell line before and after the infection with

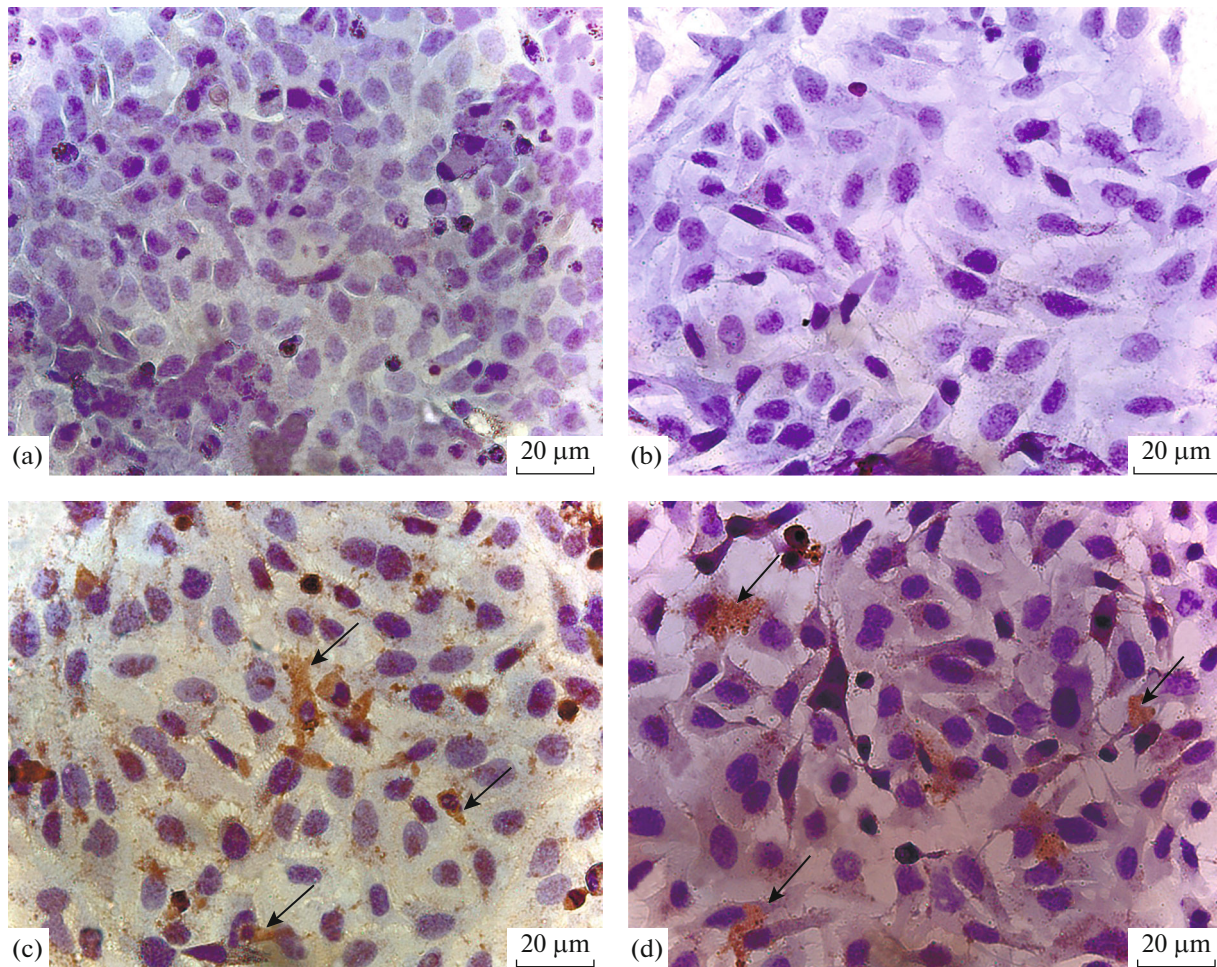


Fig. 6. Immunocytochemical staining of (a, c) control HeLa and (b, d) Hep-2 using antibodies to TNF α . (a, b) Control cells; (c, d) cells 24 h after infection with NDV/Altai/pigeon/770/2011 strain. Immunopositive zones in the cytoplasm are marked by arrows. Ob. 40 \times .

velogenic AF2240 NDV strain, which indicates that the Bax/Bcl-2 ratio at the protein level remained constant. In addition, the introduction of endogenous Bcl-2 did not affect the process of realization of HeLa cell death. Thus, a change in the Bax/Bcl-2 ratio is not involved in NDV-mediated death of HeLa cells (Molouki et al., 2010; 2011). In the present work, no change in the amount of Bcl-2-expressing cells was found during infection with the virus, as a result of which it can be supposed that the NDV/Altai/pigeon/770/2011 strain realizes its cytolytic potential without the activation of internal apoptosis pathway, at least in the early stages of infection.

Thus, the HeLa cervical adenocarcinoma and Hep-2 (HeLa derivative) laryngeal adenocarcinoma cell lines (which have a single histogenetic origin) exhibit a high sensitivity to the oncolytic effect of the NDV/Altai/pigeon/770/2011 strain.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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