UDC 619:578.835.2:57.082.26

DOI 10.29326/2304-196X-2018-4-27-39-43

STUDY OF DNA TRANSFORMATION DYNAMICS IN BHK-21/2-17 CELL CULTURE USING FLOW CYTOMETRY DURING FMD VIRUS REPRODUCTION

M. N. Guseva[1], M. A. Shevchenko[2], D. V. Mikhalishin[3], V. V. Mikhalishin[4], B. I. Manin[5]

[1] Senior Researcher, Candidate of Sciences (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: guseva_mn@arriah.ru*

[2] Leading Veterinarian, FGBI "ARRIAH", Vladimir, Russia, *e-mail: shevchenko_ma@arriah.ru*

[3] Head of Laboratory, Candidate of Sciences (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: mihalishin_dv@arriah.ru*

[4] Chief Expert, Doctor of Sciences (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: mihalishin@arriah.ru*

[5] Leading Researcher, Candidate of Sciences (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: manin_bl@arriah.ru*

SUMMARY

The research tasks covered the study of BHK-21/2-17 cell DNA transformation dynamics during FMDV reproduction process. It was noted that the destruction of major cell population coincided with the increase in apoptotic cell number and detritus amount. Three hours post cell culture infection increase in apoptosis and detritus was observed, G1-phase decreased by 17–21% and polynuclear cells grew by 2.3 times. In seven hours, the drastic rise in cell death was noted. It was established that at all stages of FMDV culture in BHK-21/2-17 suspension cell line, diploid cells G1(2n) were predominant, being basic cells for the virus reproduction. Cells in synthetic (S) and G2- and M-phases were less susceptible to virus. Using flow cytometry technique made it possible to quantify cell cycle phases during reproduction in FMDV cells. We also succeeded in comparing between these phases, virus livability and virus reproduction dynamics. The study of FMDV cytopathic effect in BHK-21/2-17 cells demonstrated that one of the optimization trends in culture vaccine production include proliferation inhibitory factor use at a certain cell cycle phase.

Key words: flow cytometry, cell cycle phases, BHK-21/2-17 cell suspension, foot-and-mouth disease virus.

INTRODUCTION

Foot and mouth disease is an acute highly contagious disease that affects cloven-hoofed animals. The replication of this aphthovirus is cellulicidal, and is followed by the death of infected cells. The virus reproduction cycle consists of the following main stages: virion adsorption on cell surface, cell penetration, deproteinization, synthesis of viral components, formation and release of mature virions from the cell [7].

Replication of viral RNA occurs in the cytoplasm of cells in 70S replicative polymerase complexes found on smooth membranes of cytoplasmic vacuoles, which are formed during infection. These replicative complexes consist of viral RNA, polymerase, and four types of cellular protein [5, 7].

Infection with picornaviruses causes suppression of the synthesis of cellular macromolecules: protein, RNA and DNA. Replication of cellular DNA is inhibited due to disruption of the synthesis of structural and functional proteins (enzymes) caused by reproduction of FMD virus on the membranes of the endoplasmic reticulum. The inhibition of synthesis of cellular proteins by picornaviruses is one of the most controversial questions [5, 7].

FMD virus, like most picornaviruses, causes characteristic cytopathic changes in cells. Membrane vesicles, the number of which gradually increases, appear in the endoplasmic reticulum of infected cells. The leakage of intracellular components, accompanied by cell shrinkage, occurs due to disruption of the permeability of the outer plasma membrane in the late stages of viral reproduction [7].

Replication cycle of the virus consists of two steps: the eclipse period, which is observed in the period between the infection of sensitive cells and appearance of the first virions (up to 2.5 hours); then the number of virions increases, and the cell is destructed in 16–20 hours [5].

Flow cytometry is a modern method that provides fast, high-quality and multimetric analysis of cells and is widely used in such fields of medicine as immunology, pharmacology, cytology, oncology, hematology, genetics, infectious diseases [1, 4]. The use of flow cytometry allows to determine DNA and RNA content in a cell, the total amount

of proteins and the amount of specific proteins recognized by monoclonal antibodies, to study cell metabolism (for example, to measure intracellular pH), to study the transport of calcium ions and the enzyme reaction kinetics [6].

Each cell has its life cycle: from the moment of its formation by division of mother cell to its own division or death. This period is called a cell cycle. The cell cycle consists of two periods: 1) the period of cell growth, called the interphase, and 2) the period of cell division called M phase (from the Greek word μίτος – thread). Each of the abovementioned periods have several phases. Usually, the interphase takes at least 90% of the time of the entire cell cycle. Most of the cell components are synthesized throughout the interphase, therefore it is difficult to identify separate stages in it.

The interphase comprises a G_1, S and G_2 phase. The interphase period, when DNA replicates in the nucleus, was called the S phase (from the word "synthesis").

It should be noted that the interphase comprises not only DNA replication (mainly during the S phase), but also the biosynthesis of structural and functional proteins of the cell.

The period between the M phase and the beginning of S phase is called G_1 phase (from the word "gap"), and the period between the end of S phase and the subsequent M phase is called G_2 phase [3].

The use of flow cytometers allowed to obtain important data on reproduction cycle phases of the cells. The results obtained in this study allowed to estimate the duration and dominance of certain cell cycle phases (G1, S, G2 + M) at different stages of cultivation.

The research tasks covered the study of BHK-21/2-17 cell DNA transformation dynamics during FMDV reproduction process.

MATERIALS AND METHODS

The following materials and equipment were used in the study:

– BHK–21/2–17 cells, USSR Patent No. 240289, 1986;
– Asia-1 Shamir/Israel 3/89 FMD virus strain;
– metal cultivation devices with a capacity of 1,800 dm^3;
– Accuri C6 flow cytometer;
– "BD Cyclents" kit for a cytometer;
– the kit for identification of cell DNA (C6 Flow cells Cytometer Fluid Kit).

Samples were taken every hour during the FMDV reproduction. The concentration of BHK-21/2-17 cells in suspension was determined using a Goryaev chamber for blood count (model 851) corresponding to TU 64-1-816-84. An equal volume of 0.2% Trypan Blue was added to 1 cm^3 of cell suspension, mixed thoroughly and filled into the chamber. The number of cells in 1 cm^3 of suspension was determined using the formula [4]:

$$X = \frac{A \times B \times 4000}{3600} \times 1000,$$

where X – number of cells in 1 cm^3;
A – total number of cells in a chamber;
B – suspension dilution.

Counting was performed at 10x magnification.

RESULTS AND DISCUSSION

The virus reproduction dynamics was determined by the following indicators:

– the correlation between the percentage of cell damage and the time of virus reproduction;
– changes in apoptosis and debris;
– change in stages (duration) of the cell cycle (G_1, S, G_2 + M).

During the cultivation, the pH of the suspension and the percentage of BHK-21/2-17 cell damage were monitored every hour.

An attempt was made to study the changes that occurred in different cell cycle phases during the FMDV cultivation in BHK-21/2-17 suspension cell line. Under standard optimal conditions, intact populations are distributed as follows (by cell cycle phases): G_1 – 15–20%, G_2 + M – 8–10%, S – 65–75%; the number of polyploid cells amounts to 4%, and the number of detritus cells amounts to 1–4% [2].

The table shows the standard dynamics of cytolysis (destruction) of the cells of the studied cell line during FMDV reproduction. Under optimal conditions (pH ≥ 7.25), the main population of BHK-21/2-17 cells was destroyed within 9–24 hours, depending on the virus strain (up to 91% of the entire population).

Table
Number of destroyed BHK-21/2-17 cells in the process of FMDV cultivation

Live cell concentration (mln/ml)	Time (hours)	pH	destroyed cells (%)
4.0	0	7.34	0
4.0	1	7.33	0
4.0	2	7.29	0
4.0	3	7.32	0
3.84	4	7.28	4
3.04	6	7.25	26
2.20	7	7.40	45
1.32	8	7.50	67
0.72	9	7.51	82
0.36	10	7.56	91

When comparing the number of cells that were in apoptosis-debris and in the G_1 phase, some synchronicity between these phases was established: by 3 hours of virus cultivation, the G_1 phase decreased by 15–17%, apoptosis increased to the same extent, then the peak of G_1 decrease (6 hours) and the peak of apoptosis increase was observed at the same time. After 9 hours of cultivation, a snowballing apoptosis increase and cell death was observed (Fig. 1). Such synchronicity in the dynamics of the above stages is probably associated with the increased sensitivity of diploid cells to FMD virus and the rapid degradation of cell membranes to debris.

During visual determination of the extent of cell damage in the Goryaev chamber, it was established that by the 6th hour of cultivation, the FMDV reproduction became very intensive (see the Table). The dynamics of the population destruction coincided with increase in apoptosis cell number and dead cells stained with trypan blue.

It is known that FMDV RNA replication begins approximately 60–90 minutes after infection, depending on the type of cell culture. In BHK-21/2-17 cells, the virus concentration reaches its maximum in 160–170 minutes, and ma-

ture virus particles are detected approximately 30 minutes after the start of RNA synthesis and the formation of virus-specific polypeptides [7].

In these experiments, a jump in apoptosis and debris was observed in 3 hours, which probably coincided with the peak of the synthesis of virus-specific RNA, the formation of provirions and the partial formation of mature virus particles. After 7–9 hours, the process of total cell destruction was observed, with the release into the suspension of the bulk of mature virions.

The diploid phase of the cell cycle (G_1) also prevailed in the cell cycle of the intact population of BHK-21/2-17 cells, during which the synthesis of mRNA, structural proteins, and other cell components began. This phase accounted for 30–75% of the cells, depending on the culture conditions [2]. During the G_1 phase, the cells began to grow in size, mRNA and enzymes necessary for future DNA replication became active.

The number of cells in the G_1 phase in a virus-infected culture was maintained at 60–75% during the time of virus reproduction. The virus-infected cells served as a source of energy and material for the synthesis of viral components and for virion assembly. This was described by A. P. Ponomaryov et al. [5].

The change over from the diploid G_1 phase to the synthetic (S-phase) phase is one of the control points of the cell cycle. Depending on the amount of nutrients and energy, as well as on the external factors of cultivation, the cell "decides" whether to enter the cell cycle or enter the nonproliferating G_0 phase, which changes to apoptosis. The main event of the S-phase is DNA replication [3].

The fact that the percentage of cells in the S-phase (during the virus reproduction) decreased linearly within 6 hours (Fig. 2), indicates that the cells in this phase also participated in the virus reproduction. After 6 h of virus replication, the number of cells in S-phase increased linearly until the end of reproduction, which correlated with two small peaks of G_1 and one peak of G_2 (Fig. 1, 2). Thus, by the end of cultivation the tetraploid G_2-phase and diploid G_1-phase were probably replenished by activation of S-phase and subsequently became quickly destroyed by the virus.

The G_2 phase - the last of three consecutive phases of the interphase stage of the cell cycle – together with the mitotic phase, formed the tetraploid population. In intact BHK-21/2-17 cells, the mitosis preparation phase and mitosis itself accounted for 2–18% of the entire population [2], and in FMDV-infected culture – from 3.68 to 8.0% (Fig. 2). This was probably associated with the inhibition of general biosynthesis and functions of the "host cell" as a result of FMDV-components biosynthesis.

The graph of changes in the number of polyploid cells demonstrates additional mechanism of FMDV effect on cells: in the first 3 hours, their number increased; they did not completely disappear until the end of cultivation. This phenomenon was indicative of the partial resistance of polyploid cells to the virus (Fig. 3).

DNA histograms of BHK-21/2-17 cells during the reproduction of FMDV after infection, and after 3 and 6 hours of cultivation are very informative (Fig. 4). They clarify the graphs presented in Figures 1–3.

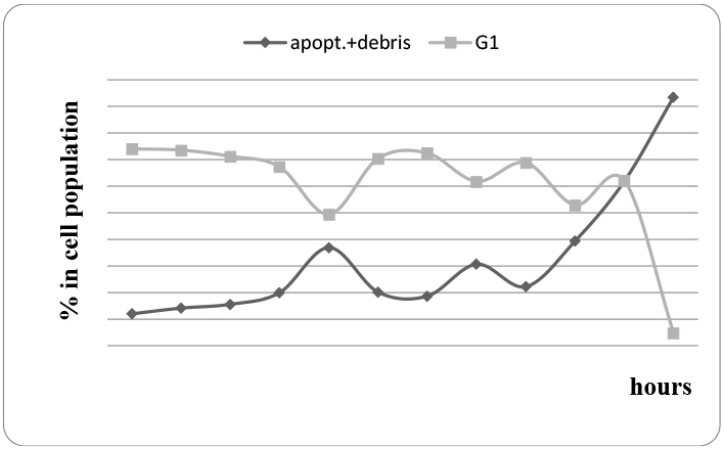

Fig. 1. The change in the percentage of cells that were in apoptosis-debris and G_1 phase, in BHK-21/2-17 culture during the FMDV reproduction

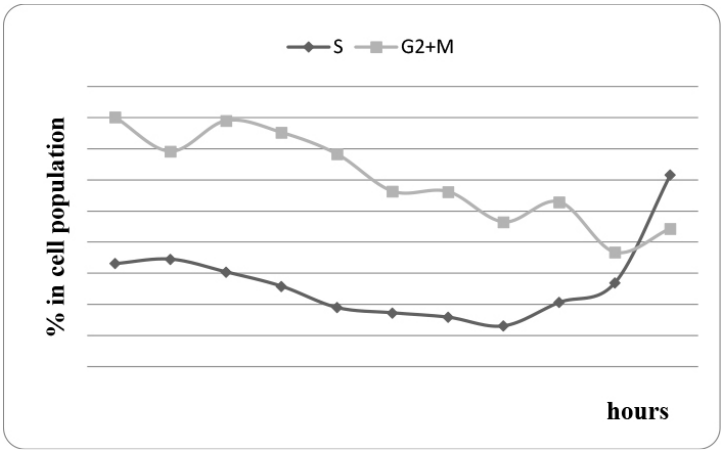

Fig. 2. The change in the percentage of cells that were in S and G_2 + M phases in BHK-21/2-17 culture during the FMDV reproduction

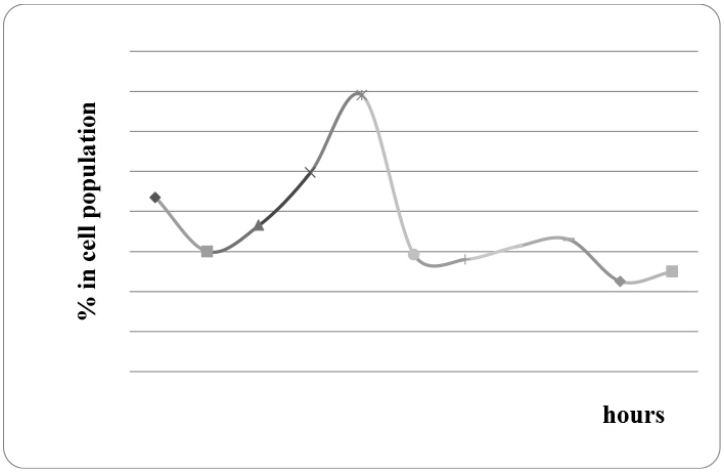

Fig. 3. Changes in the number of polyploid cells in BHK-21/2-17 culture during the FMDV reproduction

CONCLUSION

Thus, when studying the BHK-21/2-17 cell DNA transformation dynamics during FMDV reproduction, an increase of the number of cells in apoptosis and debris was observed 3 and 6 hours post cell culture infection. This correlated with the number of dead cells detected by trypan blue stain. Then the destruction of the cells grew exponentially; by 10 hours of virus cultivation, the percentage of cell damage was 90–91%.

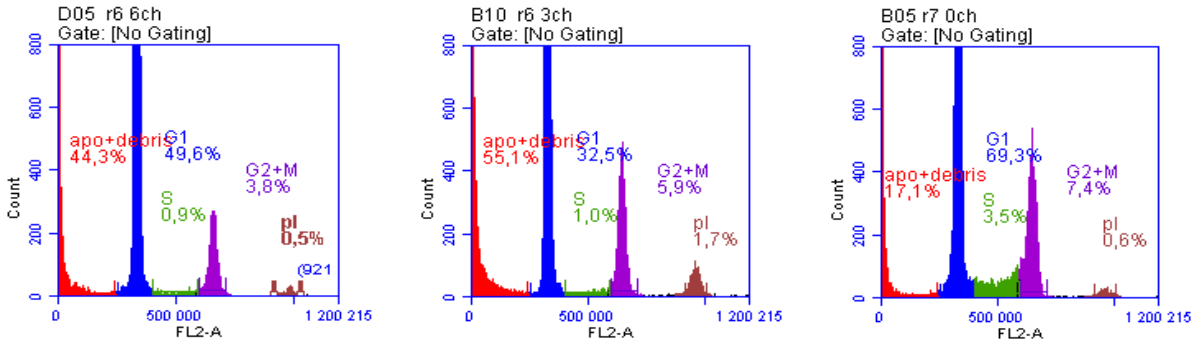

Fig. 4. DNA histogram of BHK-21/2-17 cells during the reproduction of FMDV after infection (A), after 3 hours (B) and 6 hours (C) of cultivation

It was demonstrated that the cells in the G_1 phase, which were completely destructed by the end of cultivation, were the main targets for the FMDV virus.

During the G_2 + M phase, no snowballing cell destruction was observed, intact cells were subsequently involved in the G_1 phase.

Polyploid cells, which were formed during the virus cultivation, were partially resistant to FMDV and probably had the least sensitivity to it. The cell number increase during the cytopathic effect demonstrated the development of cell fusion when affected by the virus.

The study of FMDV cytopathic effect in BHK-21/2-17 cells demonstrated that one of the optimization trends in culture vaccine production include proliferation inhibitory factor use at a certain cell cycle phase.

REFERENCES

1. Voitkova V. V. Apoptosis study using flow cytofluorometry (review) [Izuchenie apoptoza metodom protochnoj citofluometrii (obzor literatury)]. *Acta Biomedica Scientifica*. 2010; 6 (1): 220–225 (in Russian).

2. Study of BHK-21/2-17 cell line cycle using flow cytometry technique [Issledovanie kletochnogo cikla linii VNK-21/2-17 metodom protochnoj citometrii]. M. A. Shevchenko, M. N. Guseva, D. V. Mikhlaishin [et al.]. *Veterinary Science Today*. 2017; 4: 58–62 (in Russian).

3. Cells [Kletki]. Ed by B. Lewin [et al.]. M.: BINOM, Laboratoria znanii, 2011 (in Russian).

4. Basic stages of flow cytometry technique development and use in the FGI RRCRST [Osnovnye ehtapy razrabotki i primeneniya metoda protochnoj citometrii v FGURNCRHT]. A. S. Yagunov, A. V. Kartashev, S. V. Tokalov, L. N. Kiseleva. *Problems in Oncology* [Voprosi onkologii]. 2008; 54 (4): 494–497 (in Russian).

5. Ponomarev A. P., Uzyumov V. L., Gruzdev K. N. Foot-and-mouth virus: structure, biological, physical and chemical properties [Virus yashchura: struktura, biologicheskie i fiziko-himicheskie svojstva] Vladimir: Foliant, 2006 (in Russian).

6. Efficacy of flow cytometry in BHK-21 cell restoration mechanism study during culture and cryopreservation processes [Effektivnost' metoda protochnoj citometrii v izuchenii mekhanizmov reparacii kletok VNK-21 v processe kul'tivirovaniya i kriokonservirovaniya]. B. L. Manin, V. T. Nochevny, S. V. Haidukov, V. N. Laskavy. *Veterinarna medicina: mizhvid. tem. nauk. zb.* Kharkiv, 2011; 95: 66–70 (in Russian).

7. Foot and mouth disease: monography [Yashchur: monografiya]. A. N. Burdov, A. I. Dudnikov, P. V. Malyarets [et al.]; ed. A. N. Burdov. M.: Agropromizdat, 1990 (in Russian).

Submitted on 05.06.18
Approved for publication on 29.11.18