

EXAMINATION OF THE ACTIVITY OF HN AND F GLYCOPROTEIN ANTIGENS OF THE OUTER ENVELOPE OF NEWCASTLE DISEASE VIRUS BY USING FUSIONAL, HEMOLYTIC, HEMAGGLUTINATION AND HEMADSORPTION TESTS, *IN VITRO*

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The objective of our study was to examine fusional, hemolytic, hemagglutination and hemadsorption activities of the surface glycoprotein HN and F antigens of Newcastle disease (ND) virus, in vitro. The samples of activated ND virions, induced Vero cell fusion after 6h, 12h, 24h and 48h. After 24h of treatment of the inoculated Vero cells with dilutions of the specific immune sera against ND virus, cell fusion was not registered at dilutions of 1:2 and 1:4. The ND virion samples, activated with 0.025 g/dL trypsin-versen, induced hemolysis of chicken erythrocytes at antigen dilutions of 1:4 and 1:8. The samples of activated ND virions, expressed an intensive hemagglutinating activity of 256 HAU/0,1 mL. After treatment of the abovementioned samples with specific immune sera against ND virus, hemolytic activities were not detected at immune sera dilution of 1:32. The hemadsorption of chicken erythrocytes at the surface of inoculated Vero cells was detected after 6h of inoculation with activated ND viruses. After 24h of treatment of inoculated Vero cells with dilutions of specific immune sera against ND virus, the hemadsorption of chicken erythrocytes was not registered at immune sera dilution of 1:64. These results showed the possibility to use fusion, hemolytic and hemadsorption tests for the detection of immunologically important glycoprotein antigens of ND viruses and their identification with specific immune sera.

Key words: Newcastle disease virus, fusion test, hemagglutination, hemolysis, hemadsorption

INTRODUCTION

The most important investigations of the complex antigenic structure and fusional, hemagglutinating, hemolytic and hemadsorptive activities of the outer glycoprotein HN and F antigens of the Newcastle disease (ND) virus were made by Scheid and Choppin (1974), Polos (1979), Tanabayashi and Compans (1996), Milic *et al.* (2001), Milic *et al.* (2003), Ferreira *et al.* (2004) and Lawrence (2004).

Hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus represents a multifunctional molecule with three distinct activities: receptor binding activity, neuraminidase activity and fusion promotion activity. It recognizes sialic acid – containing receptors on cell surfaces and promotes the fusion activity of the F protein, thereby allowing the virus to penetrate the cell surface. Hemagglutinin-neuraminidase protein acts as a neuraminidase by removing the sialic acid from progeny virus particles to prevent self – agglutination. Thus, the HN protein plays an important role in viral infection.

The fusion (F) protein of Newcastle disease virus is directly responsible for the fusion between the viral envelope and the target membrane. For paramyxoviruses, the fusion mechanism has been proposed to occur at neutral pH. This protein is produced as a single inactive peptide, Fo, once cleaved by cellular protease, becomes the active F1 – F2 form, with two peptides linked by a disulfide bond. Four domains of the F1 polypeptide have been suggested to be involved in the fusion mechanism of NDV: the N – terminal fusion peptide and three heptad repeat (HR) regions of the ectodomain, named HR1, HR2 and HR3. Cleavage of the fusion (F) protein is known to be required for the initiation of an infection and considered to be a major determinant of NDV virulence. At the same time, the fusion protein is directly responsible for the hemolytic activity.

MATERIAL AND METHODS

Virus and Tissue culture

The used ND virus, strain was La Sota, at a titre of $LD_{50} = 10^{-5,45}$ ($\log_{10}^{-5,45}$ TCID₅₀/0.1 mL) and hemagglutination titre of 64HAU/0.1 mL. The fusion test and inhibition of cell fusion, hemadsorption test and hemadsorption inhibition test were performed on Vero cell lines.

Examination of fusional activities of Newcastle disease virus, in vitro (fusion test on Vero cells)

The samples of ND viruses (500 μ L) with hemagglutination titre of 64HAU/0.1 mL were individually activated with 5 μ L of 0.025 g/dL trypsin-versen in 0.2 mol/L PBS (pH 7.2) according to Scheid and Choppin (1974) and Milić (1993). After 10 min of incubation at 37°C, the samples of virus suspension were treated with 75 μ L Eagle MEM containing 10 g/dL fetal sera in order to inactivate trypsin – versen and individually inoculated into the microplate wells with Vero cells. The samples were incubated for time intervals of 6h, 12h, 24h and 48h at 36°C.

Inhibition of cell fusion – neutralization of ND virus with specific immune sera

Identification of the glycoprotein antigens of the outer envelope of ND viruses and determination of the titre of specific antibodies against ND virus in immune sera were performed by the method of virus-serum neutralization according to B. Mihajlovic (1984).

Determination of hemolytic activities of activated ND virus

The samples of 25 µL of activated ND viruses, were diluted in PBS from 1:4 to 1:512 in microplate wells. The aliquotes of 50 µL PBS containing 0.5% chicken erythrocytes suspension were added to each well. Microtitre plates with the samples were incubated for 45min at 37°C, followed by gentle mixing.

Inhibition of hemolytic activities of activated ND viruses

The samples of specific immune sera against ND virus of 25 µL were diluted in PBS from 1:2 to 1:1024 in microplate wells. After that, the 25 µL samples of activated ND viruses (4HAU/0.1 mL) were added in each well. These mixtures of aliquotes of activated virus and specific immune sera were incubated for 30 min at 37°C. The 50 µL PBS aliquotes, containing 0.5% chicken erythrocytes suspension, were added into each well and the samples were incubated for 45 min at 37°C.

Examination of hemagglutination activities of ND virus

Hemagglutination activity of activated ND virus was examined by the method of direct hemagglutination in microplates according to Clarke and Cassals (1958).

Hemagglutination – inhibition test – HI test

Determination of the titre of specific antibodies against ND virus in immune sera and additional identification of the virus were performed by the method of hemagglutination-inhibition test according to Clark and Cassals (1958).

Hemadsorption test

Hemadsorption activities of the glycoprotein antigens of the outer envelope of ND virus were examined by the standard hemadsorption test according to B. Mihajlovic (1984).

Hemadsorption – inhibition test

Identification of the outer glycoprotein antigens of ND virus and determination of the titre of specific antibodies against ND virus in immune sera were performed by the method of hemadsorption – inhibition test according to B. Mihajlovic (1984).

RESULTS

The samples of activated ND virus with hemagglutinating titre of 256 HAU/0.1 mL, induced cell fusion in inoculated Vero cells which was expressed in the form of smaller groups of merged cells and syncytium. After 6h and 12h of incubation of inoculated Vero cells, cell fusion was intensive at antigen dilutions of 1:2 and 1:4 (Figure 1). After 24h of incubating Vero cells inoculated with ND virus, an intensive cell fusion was detected in samples at antigen dilutions

of 1:2, 1:4 and 1:8, low intensive at dilution of 1:16 and least intensive at antigen dilution of 1:32. After 48h of incubation intensive cell fusion was detected at antigen dilutions of 1:2 (Figure 2), 1:4, 1:8, 1:16, 1:32 i 1:64, low intensive at antigen dilution of 1:128 and least intensive at 1:256. In all control Vero cells inoculated with non-activated ND virus with hemagglutination titre of 64 HAU/0.1 mL, cell fusion was not detected (Figure 3).

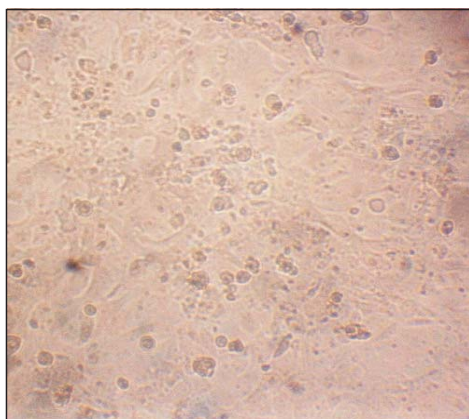


Figure 1. Fusion of Vero cells after 12 h of inoculation with activated ND viruses at the antigen dilution of 1:4

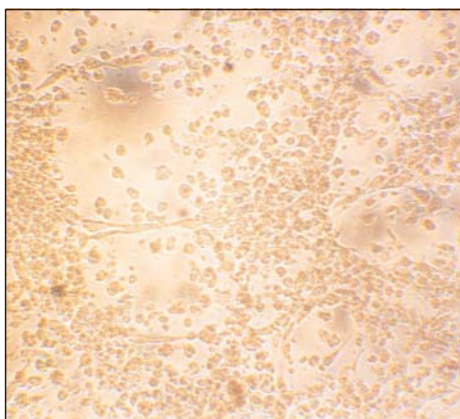


Figure 2. Fusion of Vero cells after 48 h of inoculation with activated ND viruses at the antigen dilution of 1:2

After 48h of inoculation of Vero cells with the samples of activated ND virions and specific immune sera against ND virus at dilutions of 1:2 and 1:4, inhibition of cell fusion was detected. The samples of activated ND virus induced hemolysis of chicken erythrocytes at antigen dilutions of 1:4 and 1:8. This was followed with a significant increase of the hemagglutination activity of activated ND virus which was 256 HAU/0.1 mL. After treatment of the activated ND virus samples with the dilutions of specific immune sera of 1:2, 1:4, 1:8, 1:16 and 1:32, hemolysis of chicken erythrocytes was not registered. In the control group, which contained non-activated ND virions, hemolysis of chicken erythrocytes was not registered, too. After treating the samples with dilutions of specific immune sera against ND virus, agglutination of chicken erythrocytes was not detected at the dilution of 1:64. The hemadsorption of chicken erythrocytes at the surface of inoculated Vero cells was detected in the samples after 6h from inoculation of the cell line with activated ND virus at antigen dilutions of 1:8 and 1:16. After 12h from inoculation of Vero cells with activated ND viruses, the hemadsorption of chicken erythrocytes, on the surface of inoculated cells, was registered at antigen dilutions of 1:2, 1:4, 1:8, 1:16, 1:32 (Figure 4) and 1:64. After 24h of inoculation of Vero cells with activated ND virus, the hemadsorption of chicken erythrocytes on the surface of inoculated cells, was detected at antigen dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. After 24h of treatment of inoculated Vero cells with the

dilutions of specific immune sera against ND virus, the hemadsorption of chicken erythrocytes was not observed at the dilution of immune sera of 1:64.



Figure 3. Vero cells inoculated with non-activated ND viruses – cell fusion was not detected

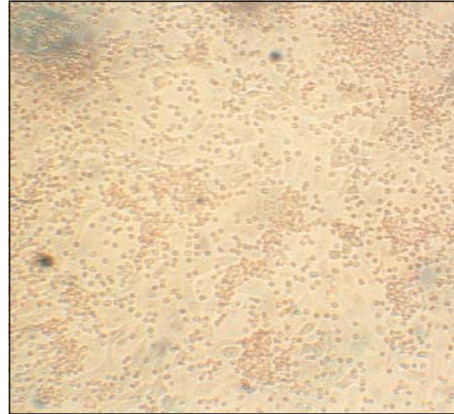


Figure 4. Hemadsorption of chicken erythrocytes after 12 h of inoculation of activated ND virions at the antigen dilution of 1:32

DISCUSSION

Ferreira *et al.* (2004) examined the hemagglutination activities of the HN antigen of the Newcastle disease virus and their results suggest that gangliosides act as primary receptors whilst N-linked glycoproteins represent the second receptors critical for viral entry. Huang *et al.* (2004) confirmed that the hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus plays a crucial role in the process of initiating an infection and that at the same time it determines tropism and virulence of this virus related to host cells.

The objective of our study was to examine fusional, hemolytic, hemagglutination and hemadsorption activities of the surface glycoprotein HN and F antigens of Newcastle disease virus, *in vitro*. Our results showed that the samples of ND viruses, activated with 0.025 g/dL trypsin-versein, induced cell fusion in inoculated Vero cells which was expressed in the form of smaller groups of merged cells. The intensity of cell fusion of inoculated Vero cells got reinforced from low to intensive cell fusion and resulted in the formation of agglomeration of Vero cells into groups with multinuclear cell forms and syncytium. The intensity of cell fusion depended from antigen dilution, as well. After 6h, 12h, 24h and 48h of incubation of inoculated Vero cells, cells fusion was intensive at different antigen dilutions. After 48h of inoculation of Vero cells with the samples of activated ND virions and specific immune sera against ND virus at dilutions of 1:2 and 1:4, inhibition of cell fusion was detected. Milic *et al.* (2001, 2003) examined the antigenic structure, fusional and hemolytic activities of the surface glycoprotein

HN and F antigens of purified PI3 virus activated with 0.025 g/dL trypsin-venesen by using fusional, hemolytic and hemagglutination tests. Their results and the results of our research confirmed that these tests can be used for fast detection of immunologically important glycoprotein antigens of PI3 and ND viruses and their identification with specific immune sera. Scheid and Choppin (1974) established that the fusion (F) protein of the outer envelope of Sendai virus has an important role in the cell fusion process and in hemolysis. Tanabayashi and Compans (1996), Yao *et al.* (1997) confirmed that specific interaction between both paramyxoviral glycoproteins is required to run the cell fusion process and hemolysis. Breker *et al.* (1996) examined hemolytic and fusional activities of the glycoprotein antigens of the outer envelope of PI3 virus. Their results suggested that different strains of PI3 virus which exhibit greater syncytium – inducing activity, *in vitro*, have greater pathogenicity, *in vivo*. Examination of some biological activities of HN and F proteins of the outer envelope of ND virus was done by using the hemadsorption test. The obtained results showed hemadsorption of chicken erythrocytes at the surface of inoculated Vero cells was detected at different antigen dilutions after 6h, 12h and 24h from inoculation of the cell line with the activated ND virus. After 24h of treatment of inoculated Vero cells with the dilutions of specific immune sera against ND virus, the hemadsorption of chicken erythrocytes was not observed at the dilution of immune sera of 1:64. Polos and Gallaher (1979) examined hemadsorption activities of glycoprotein antigens of the outer envelope of ND virus on CHO-15B cell line and their results showed an expression of hemadsorption activities of the glycoprotein antigens of ND virus after 6h and 13h from inoculation.

On the basis of these results, it can be concluded that the described methods, among the other standard methods of virological diagnostics, can be successfully used for the detection and identification of the immunologically important glycoprotein antigens of the outer envelope of Newcastle disease virus.

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ISPITIVANJE AKTIVNOSTI GLIKOPROTEINSKIH HN I F ANTIGENA SPOLJAŠNJEG OMOTAČA VIRUSA NEWCASTLE BOLESTI PRIMENOM TESTOVA FUZIJE, HEMOLIZE, HEMAAGLUTINACIJE I HEMADSORPCIJE, *IN VITRO*

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SADRŽAJ

Cilj ovih istraživanja je bilo ispitivanje fuzionih, hemolitičkih, hemaglutinacionih i hemadsorpcionih aktivnosti glikoproteinskih antigena virusa Newcastle bolesti, *in vitro*. Rezultati testova ćelijske fuzije pokazali su da je virus Newcastle bolesti posle aktivacije sa 0,025 g/dl tripsin-versena indukovao fuziju Vero ćelija posle 6h, 12h, 24h i 48 od inokulacije virusa. Tretiranjem inokulisanih Vero ćelija sa razređenjima imunih seruma protiv virusa Newcastle bolesti, ustanovljena je inhibicija ćelijske fuzije u razređenjima seruma od 1:2 i 1:4. Uzorci virusa Newcastle bolesti, indukovali su posle aktivisanja sa 0,025 g/dl tripsin-versena, hemolizu eritrocita kokoši u razređenjima antigena od 1:4 do 1:8. Isti uzorci su ispoljili i intenzivne hemaglutinacione aktivnosti posle aktivacije tripsin-versenom koje su iznosile 256 HJ/0,1 ml za virus Newcastle bolesti. Posle tretiranja uzoraka aktivisanih virusa Newcastle bolesti sa razređenjima specifičnih imunih seruma, nije ustanovljena njihova hemolitička aktivnost do razređenja seruma od 1:32. Posle 6h, 12h i 24h od inokulacije Vero ćelija aktivisanim uzorcima virusa Newcastle bolesti, ustanovljena je pojava hemadsorpcije eritrocita kokoši u različitim razređenjima antigena virusa. Posle 24h od tretiranja inokulisanih Vero ćelija sa razređenjima specifičnog imunog seruma protiv virusa Newcastle bolesti, nije ustanovljena pojava hemadsorpcije eritrocita kokoši do razređenja imunog seruma od 1:64. Ovi rezultati su ukazali na mogućnost korišćenja fuzionog, hemolitičnog i hemadsorpcionog testa za brzu detekciju imunološki značajnih glikoproteinskih antigena virusa Newcastle bolesti i njihovu identifikaciju primenom specifičnih imunih seruma.