

EXAMINATION OF ANTIGENIC STRUCTURE AND SOME BIOLOGICAL ACTIVITIES OF HEMAGGLUTININ-NEURAMINIDASE (HN) AND FUSION (F) GLYCOPROTEIN ANTIGENS OF PARAINFLUENZA 3 VIRUS, *IN VITRO*

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The objective of our study was examination of the antigenic structure, fusional and hemolytic activities of the surface glycoprotein HN and F antigens of purified parainfluenza (PI 3) viruses activated with 0.025 g/dl trypsin-versen (molecular weights of 112 kD, 81-82 kD and 30-31 kD), in vitro. The samples of activated PI3 virions with total protein concentrations of 0.55 and 0.27mg/ml and hemagglutinating titre of 256 and 128 HAU/0.1 ml, induced bovine turbinata (BT) cell fusion and hemolysis of guinea-pig erythrocytes. After treatment of the aforementioned samples with specific hyperimmune sera against PI 3 virus in which the hemagglutination-inhibition (HI) titre was 1:64, cell fusion was not registered. The results show that there are possibilities to use fusional and hemolytic tests for the fast detection of immunologically important glycoprotein antigens of PI 3 viruses and their identification with specific hyperimmune sera.

Key words: PI3 virus, glycoprotein antigens, SDS-PAGE, BT cells, fusional test, hemolysis

INTRODUCTION

The most important investigations of the complex antigenic structure and fusional, hemagglutinating and hemolytic activities of outer glycoprotein antigens of parainfluenza viruses type 1 (Sendai) and type 3 (PI 3) were made by Scheid and Choppin (1974), Wechsler *et al.* (1985), Sondra and Schlezinger (1987), Panigrahi *et al.* (1987) and Ray (1987). Some important studies, related to proteolytic activation of fusional glycoproteins of the Parainfluenza viruses, were described in the doctoral theses of Milic, (1993, Belgrade), Schulze, (1995, Marburg) and in the papers of Tanabayashi and Compans (1996) and Milic *et al.* (1997).

The glycoprotein antigens of the PI 3 virus exist in the form of spikes in the outer envelope, which contain two immunologically important transmembranous glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F). They play an important role in the process of initiating an infection as they make possible the adsorption of viruses to the surface of cell membranes (Klenk *et al.*, 1987). These antigens of the PI 3 virus participate in inducing the synthesis of virusneu-

tralizing antibodies in the organisms of immunized animals and humans (Morein *et al.*, 1983; Ray *et al.*, 1988; Milic, 1993 and Milic *et al.*, 1994 and 1997). The methods of purification, concentration and biochemical characterization of HN and F antigens of PI 3 virus, the confirmation of the presence of these glycoproteins using the fusion test, as well as the possibility of their use in the preparation of specific subunit vaccines were described by Ray *et al.* (1985), Ray (1987), Milic *et al.* (1991), Milic (1993) and Milic *et al.* (1998).

Recovery of the biological activities of F and HN antigens of the outer envelope of PI 3 viruses *in vitro*, would make fast detection and identification of Parainfluenza viruses possible. We decided to examine the fusional, hemagglutinating and hemolytic activities of outer envelope glycoprotein antigens of purified PI 3 virions and the possibility of their identification by inhibition of cell fusion with specific immune sera against PI 3 virus, *in vitro*.

MATERIAL AND METHODS

Virus and Tissue Cultures

The bovine PI 3 virus, strain SD 2, with a titre of LD₅₀ 10^{-3.5} (log 10^{-3.5} TCID₅₀/0.1 ml) was used. It was propagated in MDBK cell lines (Madin Darby bovine kidney cells) in the presence of Minimal Essential Eagles medium (MEM) with 2 g/dl of fetal calf sera for 96 hours at 36°C. The fusional test and inhibition of cell fusion with specific hyperimmune sera against PI 3 virus were performed on BT cell lines. Hemagglutinating and hemolytic activities of HN and F antigens of purified PI 3 virions were examined in micro plates with 0.5% suspension of guinea pig erythrocytes.

Procedures with inoculated cell lines

After an incubation period of 96 h the cell lines were frozen at -25°C and then thawed and centrifuged at 3400 rpm for 25 min at 4°C. The supernatant fluid was separated from the cuvettes which contained suspensions of PI 3 viruses with a hemagglutinating titre of 64 HAU/0.1 ml.

Concentration and purification of PI 3 viruses

Harvested PI 3 viruses were concentrated with PEG-6000 (polyethylene glycol-Serva) and purified through 10-40 g/dl (w/v) potassium-sodium tartrate linear gradients in 0.2 mol/l PBS (pH 7.2). Samples of concentrated viruses resuspended in 1.2 ml of MEM with 2g/dl (w/v) fetal calf sera were centrifuged in the abovementioned gradients at 25000 rpm at 4°C for 2 h. Samples of PI 3 viruses from visible bands of the gradients were then collected and recentrifuged through 10 ml of 0.2 mol/l PBS at 40000 rpm. Purified virions were pelleted at the bottom of cuvettes after 1.5 h and resuspended in aliquots of 1.5ml of 0.2 mol/l PBS (pH 7.2).

Determination of total protein concentrations

Total protein concentration in the samples of purified PI 3 virions was determined by the method according to Lowry *et al.* (1951).

Biochemical identification of the glycoprotein HN and F antigens of purified PI 3 virions

This was done by SDS-PAGE in a discontinuous buffer system, according to Laemmli (1970), with selective staining of viral glycoproteins with Schiff's reagent (PAS staining) according to Gordon (1983).

The vertical SDS polyacrylamide electrophoresis method in glass tubes, 10cm long and 0.5cm diameter, was used to analyse the antigenic structure and purity of the samples of PI 3 virions. The samples of purified PI 3 virions (150 μ l) were placed on to stacking gel of 4g/dl polyacrylamide. Immediately before electrophoresis, samples were treated with solutions of 2g/dl SDS in 0.0625 mol/l Tris/HCl buffer (pH 6.8) in the presence of 5 mg/dl of 2-beta mercaptoethanol and heated for 1 min at 85°C.

Electrophoresis was performed for 3.5 h at 160V with a current of 8mA per tube. Glycoprotein antigens in the electrophoresed samples were detected by selective staining with Schiff's reagent.

Reference samples which contained proteins of known molecular weight from 205 kD to 29 kD (St1-Sigma) and from 66 kD to 17.8 kD (St2-Sigma) were used as coelectrophoresis markers.

Examination of fusional activities of purified PI 3 virions in vitro (fusional test on BT cells).

The fusional activities of the glycoprotein antigens of purified PI 3 virus were examined on BT cells (bovine turbinate cell fusion). Two groups of seven samples (500 μ l) of purified PI 3 virions with hemagglutinating titres of 256 HAU/0.1ml and 128 HAU/0.1ml with total protein concentrations of 0.55 mg/ml and 0.27mg/ml were individually activated with 5 μ l of 0.025g/dl trypsin-versen suspension 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, fourteen samples of purified virion suspensions (500 μ l) were treated with 75 μ l of MEM containing 10g/dl fetal calf sera in order to inactivate trypsin-versen. These samples of activated virions were individually inoculated in seven flasks with BT cell lines and incubated for 1.5 h at 35 °C. Seven flasks with BT cells, inoculated with non-activated purified PI 3 virions were incubated in the same way. After that, the inoculated cell lines were observed under polarized light on a Karl Zeissler microscope, at a magnification of 4x8x3.

Inhibition of cell fusion

Each sample of 500 μ l of purified PI 3 virions, with a hemagglutinating titre of 256 HAU/0.1ml, activated with 5 μ l of 0.025 g/dl trypsin-versen in 0.2 mol/l PBS, was treated individually with an equal volume of hyperimmune sera against PI 3 virus with an HI titre of 1:64. These mixtures of activated virions and sera were incubated for 30 min at 35 °C and individually inoculated into seven flasks with BT cells (bovine turbinate cells). Seven samples of the same volume of activated PI 3 virions without hyperimmune sera were individually inoculated into two flasks with BT cell lines and served as the control. All inoculated BT cell lines were incubated for 1.5 h at 35 °C.

Determination of hemolytic activities of activated PI 3 virions

Seven samples of 25 μ l of purified PI 3 virions activated with 0.025g/dl trypsin-versein in 0.2mol/l PBS were diluted in PBS from 1:4 to 1:512 in microplate wells. Aliquots of 50 μ l PBS containing 0.5% erythrocyte suspension were added to each well. The samples were incubated for 45 min at 37 °C, followed by gentle mixing of the microplates.

The same number of samples of nonactivated purified PI 3 virions were individually treated with 50 μ l of 0.5% erythrocyte suspension and incubated as above.

Hemagglutinating activities of purified PI 3

Hemagglutinating activities of purified PI3 were examined by the method of direct hemagglutination in microplates according to Clarke, (1958) and Mihajlovic, (1984).

RESULTS

The samples of purified virions examined by SDS-PAGE contained seven protein fractions with molecular weights of 112kD to 29kD, among which were glycoprotein antigens with molecular weights of 112kD, 81-82kD and 30-31kD. (Figure 1, 1A, 1B, 1C, 1D).

The glycoprotein HN and F antigens of 112kD, 81-82kD and 30-31kD stained pink with Schiff's reagent.

The samples of purified PI 3 virions with total protein concentration of 0.55mg/ml and hemagglutinating titre of 256 HAU/0.1ml, activated with trypsin-versein, induced cell fusion in inoculated BT cells (+ +). This resulted in the formation of agglomerations of BT cells in groups with multinuclear forms and syncytium (Figure 2).

The samples of purified PI 3 viruses with total protein concentration of 0.27mg/ml and hemagglutinating titre of 128 HAU/0.1ml, activated with trypsin-versein, induced extensive (+) cell fusion in inoculated BT cell lines followed by formation of smaller groups of merged cells (Figure 3).

In all control BT cells inoculated with non-activated PI 3 virions no cell fusion was detected (Figure 4).

On the basis of the results obtained from the fusional tests we noticed that the samples of activated purified PI 3 virions with a hemagglutinating titre of 256 HAU/0.1ml induced greater cell fusion (+ +) than the equivalent samples with a hemagglutinating titre of 128 HAU/0.1ml and the lower total protein concentration (+) – (Table 1).

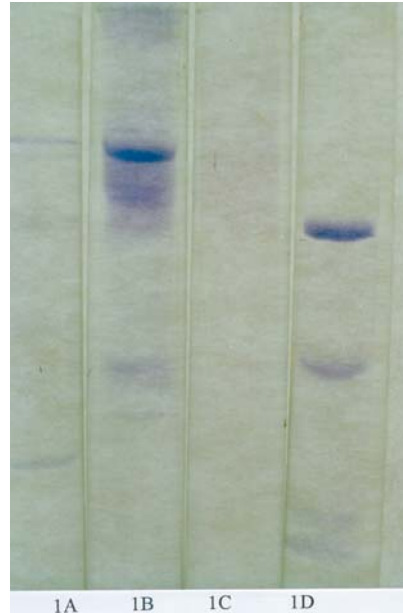


Figure 1. The samples of electrophoresed PI 3 virions purified according to Laemmli and Gordon: 1A- Reference proteins from 205 kD to 29 kD (St1); 1B- Protein fractions of purified virion samples stained with Coomassie Brilliant Blue; 1C- Glycoprotein fractions of virion samples stained with Schiff's reagent (Gordon); 1D- Reference proteins from 66 kD to 17.8 kD (St2)

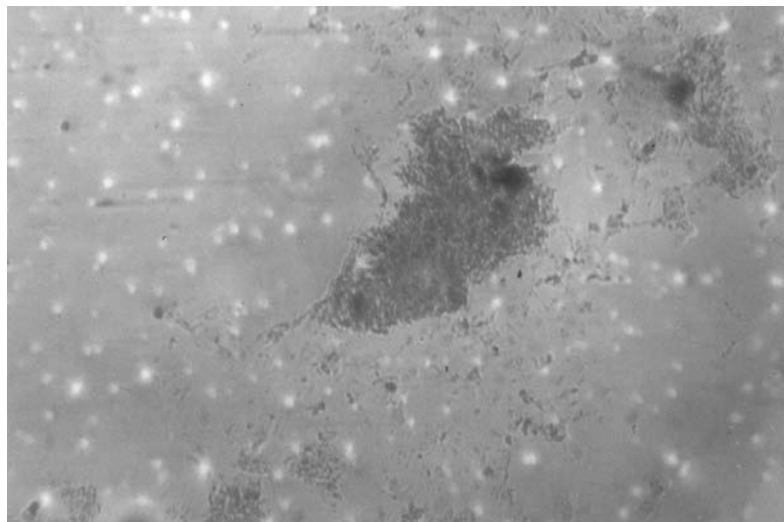


Figure 2. The fusion of BT cells inoculated with the sample of activated PI 3 virions with a hemagglutinating titre of 256 HAU/0.1 ml

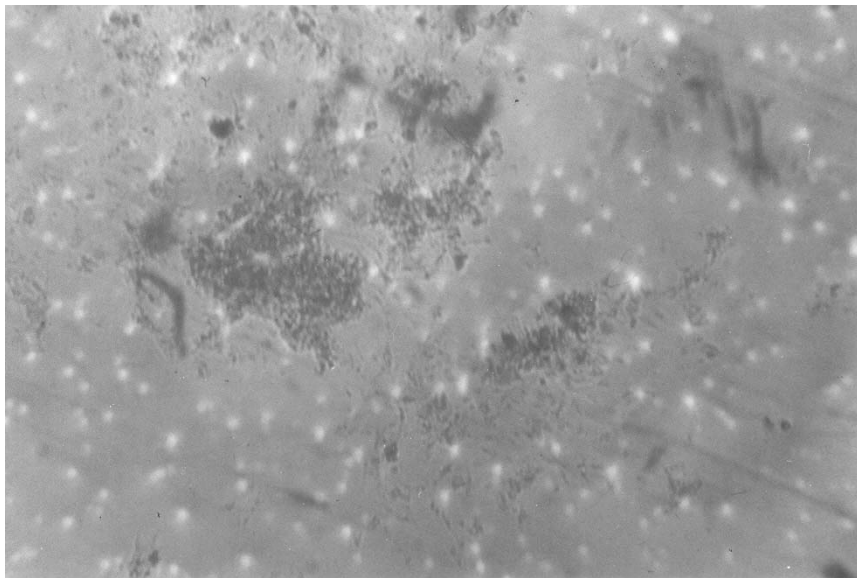


Figure 3. The fusion of BT cells inoculated with the sample of activated PI 3 virions with a hemagglutinating titre of 128 HAU/0.1ml

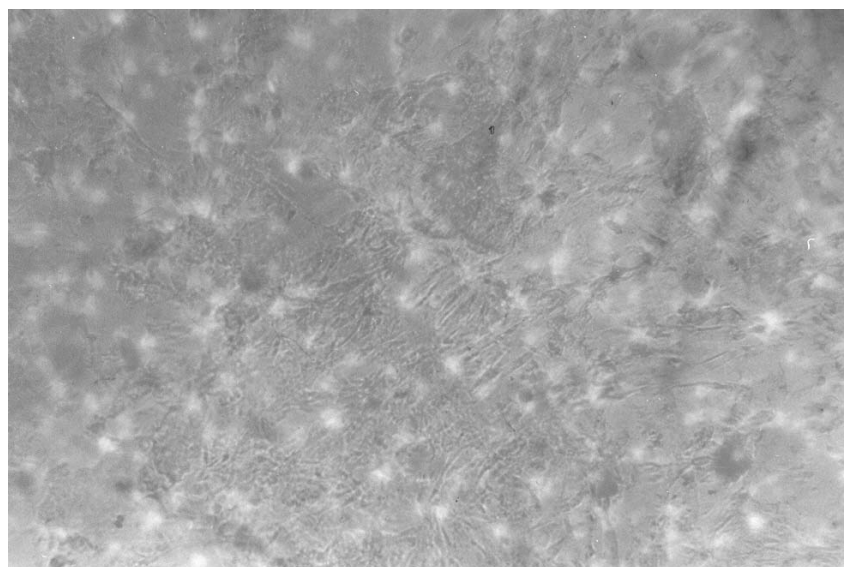


Figure 4. Control BT cells inoculated with non-activated purified PI3 virions without cell fusion

Table 1. Hemagglutinating and fusional activities of glycoprotein antigens of purified PI 3 virions

The groups of samples of purified PI 3 virions	Hemagglutinating titre (HAU/0.1ml)	Total protein concentration (mg/ml)	BT cell fusion
1st group of activated PI 3 virions	256	0.55	++
2nd group of activated PI 3 virions	128	0.27	+
1st control group of non-activated PI 3 virions	256	0.55	-
2nd control group of non-activated PI 3 virions	128	0.27	-

In control BT cells inoculated with the samples of non-activated purified PI 3 virions, cell fusion was not observed. The cell fusions detected in the BT cells inoculated with activated PI 3 virions with purified envelope glycoprotein antigens confirmed the presence of fusional antigens in the samples and their fusional activities.

After treatment with hyperimmune sera against PI 3 virus, the samples of activated purified virions inoculated into BT cell lines did not express the cell fusion. (Figure 5).

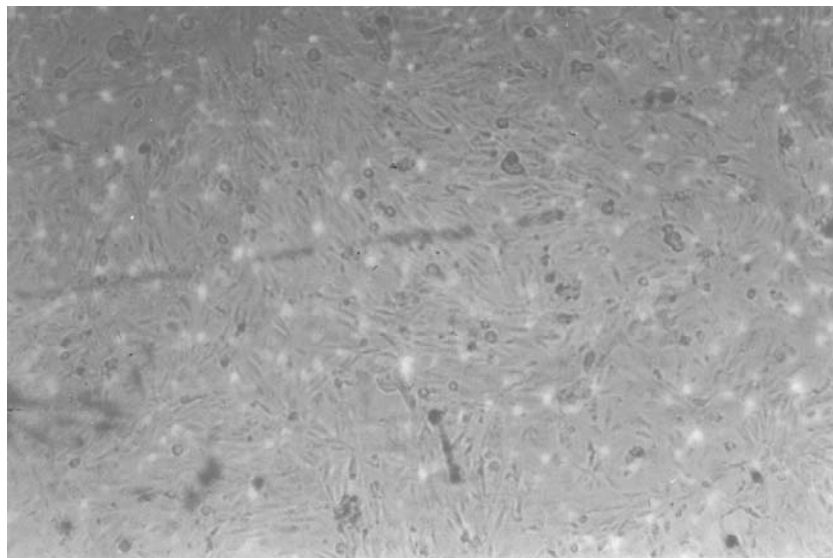


Figure 5. Absence of cell fusion in BT cell line inoculated with the sample of activated PI 3 virions neutralized with specific hyperimmune sera

In all control BT cell lines inoculated with the samples of activated purified PI 3 without specific hyperimmune sera, cell fusion was detected (Table 2).

Table 2. Inhibition of cell fusion with hyperimmune sera against PI3 virus

The samples of purified PI 3 virions activated with trypsin – versen	Hemagglutinating titre (HAU/0.1ml)	BT cell fusion
Purified virions treated with hyperimmune sera against PI 3	256	–
Purified virions without hyperimmune sera-control	256	++

Hemolytic activities of purified PI 3 virions were detected in the lower dilutions of the activated antigens. The samples of purified PI 3 virions activated with trypsin-versen induced the hemolysis of guinea pig erythrocytes at the antigen dilutions of 1:8 and 1:16. The first group of seven samples with a total protein concentration of 0.55mg/ml and hemagglutinating titre 256 HAU/0.1ml induced hemolysis at the dilution of 1:16, whilst the second group of seven samples with total protein concentration of 0.27mg/ml and hemagglutinating titre of 128 HAU/0.1ml expressed hemolytic activity at the dilution of 1:8. In the two control groups of seven samples each, hemolysis was not observed (Table 3).

Table 3. Hemagglutinating and hemolytic activities of the glycoprotein antigens of purified PI 3 virions

The groups of samples of purified PI 3 virions	Hemagglutinating titre (HAU/0.1ml)	Total protein concentration (mg/ml)	Hemolytic activity per 0.1ml
1st group of activated PI 3 virions	256	0.55	1:16
2nd group of activated PI 3 virions	128	0.27	1:8
1st control group of non-activated PI 3 virions	256	0.55	–
2nd control group of non-activated PI 3 virions	128	0.27	–

DISCUSSION

The correlation between the biological activities of MDBK cell and egg-grown Sendai virions and their glycoprotein antigens (Scheid and Choppin, 1974) showed that the precursor of the smallest virion glycoprotein is biologically inactive, and that its proteolytic cleavage *in ovo* to yield the small F glycoprotein was responsible for the activation of hemolyzing and cell-fusing activities and infectiv-

ity to MDBK cells. Sendai virions, which had been grown in MDBK cells, were treated with trypsin *in vitro* which resulted in cleavage of the precursor to the smaller F glycoprotein followed by the fusion of BHK21-F cells inoculated with activated parainfluenza virions. Such treatment of the Sendai virions with trypsin *in vitro*, induced the expression of their hemolyzing activity.

Kruse *et al.* (1981) examined the fusion of fibroblasts with erythrocyte ghosts induced by the HN and F glycoprotein of Sendai virus. The results showed that the whole Sendai virions and their isolated HN and F glycoproteins can be used as the fusogen. The fibroblast cells were exposed to arginase-loaded erythrocytes and individually inoculated with the aforementioned fusogens. The samples of HN and F solution were three times more efficient than the samples of whole virus in stimulating fusion per μg of HN and F proteins added.

Later examinations of antigenic structure and some biological activities of glycoprotein antigens of the PI 3 virus *in vitro* (Milić, 1993; Milić *et al.*, 1997), confirmed the significant role of HN and F glycoproteins isolated from the outer viral envelope, with molecular weights (Mr) of 78, 40 and 35kD and activated with trypsin-venen solution, induced intensive BT cell fusion.

Hu *et al.* (1992), Tanabayashi and Compans, (1996) and Yao *et al.* (1997) confirmed the presence of a functional interaction between the aforementioned viral glycoproteins in inducing cell fusion and hemolysis.

The biochemical characterization of the surface glycoprotein antigens of purified PI 3 virions with molecular weights of 112kD, 81-82kD and 30-31kD (HN and F antigens) and their activation with trypsin-venen in our examinations represent a prerequisite for the application of the cell fusion test, hemolytic test and the test of inhibition of cell fusion with hyperimmune sera against PI 3 virus for the fast detection and identification of fusional and hemagglutinin-neuraminidase PI 3 antigens. On the basis of these results, it can be concluded that the described methods of detection and identification of fusional antigens of PI 3 virus, can be used in virological diagnostics. Moreover, samples of activated glycoprotein antigens of purified PI 3 virions with low total protein concentrations (from 0.27 to 0.55mg/ml) expressed significant fusional and hemolytic activities, *in vitro*.

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ISPITIVANJE ANTIGENSKE STRUKTURE I NEKIH BIOLOŠKIH AKTIVNOSTI HN I F ANTIGENA VIRUSA PARAINFLUENCE 3, *IN VITRO*

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SADRŽAJ

Cilj naših istraživanja je bilo ispitivanje antigenske strukture, fuzionih i hemolitičkih aktivnosti površinskih glikoproteinskih HN i F antigena prečišćenih virusa PI3 aktivisanih sa 0.025g/dl tripsin-versenom (molekulske težine od 112kDa, 81-82 kDa i 30-31 kDa), *in vitro*. Uzorci aktivisanih virusa PI 3 sa ukupnim koncentracijama proteina od 0.55 i 0.27 mg/ml i hemaglutinacionog titra od 256 i 128 HJ/0.1ml, indukovali su fuziju BT ćelija i hemolizu eritrocita zamorca. Posle tretiranja napred navedenih uzoraka specifičnim hiperimunim serumom protiv virusa PI 3 čiji je HI titar bio 1:64, nije registrovana ćelijska fuzija. Rezultati pokazuju da postoji mogućnost korišćenja fuzionog i hemolitičkog testa za brzu detekciju imunološki značajnih glikoproteinskih antigena virusa PI 3 i njihovu identifikaciju sa specifičnim hiperimunim serumom.