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A novel method for detection of Newcastle disease virus with a fluorescent sialidase substrate

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A B S T R A C T

Newcastle disease virus (NDV), belonging to the family *Paramixoviridae*, causes respiratory and neuronal symptoms in almost all birds. NDV has haemagglutinin-neuraminidase (HN) glycoprotein possessing sialidase activity. HN glycoprotein is highly expressed on the surface of NDV-infected cells, resulting in much higher sialidase activity in NDV-infected cells than in non-infected cells. It was reported that mouse and human cancer cells up-regulating sialidase expression were histochemically stained with a fluorescent sialidase substrate, 2-(benzothiazol-2-yl)-4-bromophenyl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (BTP3-Neu5Ac), which deposits water-insoluble fluorescent compound BTP3 on locations of sialidase activity. By using the BTP3-Neu5Ac assay, we showed that NDV-infected cells and HN gene-expressing cells could be simply detected at room temperature after only 5 min. Infection of the cells with the virus resulted in apparent green fluorescence, which disappeared with addition of a sialidase inhibitor. Cells that were stained in the BTP3-Neu5Ac assay were immunostained with an anti-NDV antibody. Moreover, BTP3-Neu5Ac staining was applied to a virus overlay binding assay with NDV particles. NDV-bound protein bands on guinea pig red blood cells were easily and rapidly detected by the BTP3-Neu5Ac assay after Western blotting. BTP3-Neu5Ac offers an easy and rapid protocol for fluorescent staining of NDV and virus-infected cells without antibodies.

1. Introduction

Newcastle disease virus (NDV), belonging to the family *Paramyxoviridae*, contains a single-stranded negative-sense RNA (de Leeuw and Peeters, 1999; Yuan et al., 2012). NDV is highly contagious among almost all birds and mainly causes respiratory and neuronal symptoms, resulting in death. NDV has been studied since its first identification in 1926 in England. Vaccines against NDV have controlled outbreaks in poultry (Alexander et al., 2012; Chong et al., 2010; Dortmans et al., 2011; Kapczynski and King, 2005; Nagy et al., 1991). However, Newcastle disease caused by NDV still occurs throughout the world and is a threat to poultry farms due to enormous economic loss (Alexander et al., 2012; Dortmans et al., 2011).

Detection of viral proteins and virus-infected cells by histochemical immunostaining with anti-virus antibodies has contributed to virological studies so far (Takaguchi et al., 2011; Takahashi et al., 2008, 2012a, 2013b). Once a virus-specific antibody has been prepared, an immunostaining method with the antibody should be selected as the first choice for detecting viruses and virus-infected cells. When the infectious titres of some virus solutions [for example, median tissue culture infectious dose ($TCID_{50}$) or focus-forming units (FFU)] are determined, an immunostaining method could be frequently used (Takaguchi et al., 2011; Takahashi et al., 2008, 2012a, 2013b). However, it takes several months to acquire either monoclonal or polyclonal antibodies (Miyamoto et al., 1997; Suzuki et al., 2001). When antibodies in a laboratory are not reactive

Abbreviations: BSA, bovine serum albumin; BTP, benzothiazolylphenol; BTP3-Neu5Ac, 2-(benzothiazol-2-yl)-4-bromophenyl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid; CPE, cytopathic effect; DANA, 2, 3-dehydro-2-deoxy-*N*-acetylneuraminic acid; DMEM, Dulbecco's modified minimum essential medium; F, fusion; FBS, foetal bovine serum; FFU, focus-forming units; HAU, haemagglutination units; HN, haemagglutinin-neuraminidase; HRP, horseradish peroxidase; LLC-MK_2, Lewis-lung carcinoma monkey kidney; MEM, minimum essential medium; NDV, Newcastle disease virus; Neu5Ac, α-D-*N*-acetylneuraminic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PE, phycoerithrin; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; SFM, serum-free medium; TBS, Tris-buffered saline; $TCID_{50}$, median tissue culture infectious dose; UV, ultraviolet; X-Neu5Ac, 5-bromo-4-chloroindol-3-yl-Neu5Ac.

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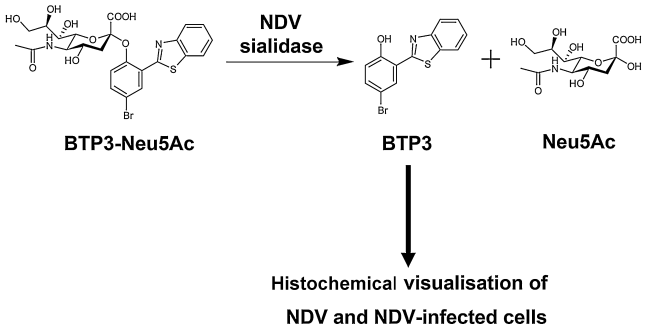

Fig. 1. Visualisation scheme for the detection of NDV sialidase activity by BTP3-Neu5Ac assay.

to some virus strains, FFU are usually determined as the infectious titre by a plaque forming assay, which needs a few days or even a few weeks until distinct plaque formation (Elizondo-Gonzalez et al., 2012; Fukushima et al., 2011; Takahashi et al., 2013a). The viral cytopathic effect (CPE) induces plaque formation, but this assay cannot always be used because some virus strains show no apparent CPE (Fukushima et al., 2011). Therefore, we tried to establish a sensitive, easy, and rapid method for histochemical detection of NDV-infected cells and the viral particles without using antiviral antibodies.

NDV has haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins on the viral surface. These glycoproteins are highly expressed on the surface of NDV-infected cells, where progeny viruses prime budding and release (Pantua et al., 2006; Plattet and Plemper, 2013). HN glycoprotein has sialidase activity, which inhibits self-aggregation among viral particles by removal of terminal sialic acid residues such as α-D-*N*-acetylneuraminic acid (Neu5Ac) from glycoconjugates on the HN and F glycoproteins (Miyagi and Yamaguchi, 2012; Huang et al., 2004; Panda et al., 2004; Plattet and Plemper, 2013; Sanchez-Felipe et al., 2012; Takimoto et al., 2002). Therefore, synthetic sialidase substrates are useful for non-immunological detection of HN glycoprotein. In fact, detection of sialidase activity has been performed by using several reagents including 4-methylumbelliferyl-Neu5Ac, 1,2-dioxetane derivative of Neu5Ac (generally called NA-STAR), and 5-bromo-4-chloroindol-3-yl-Neu5Ac (generally called X-Neu5Ac) (Buxton et al., 2000; Fujii et al., 1993; Minami et al., 2011, 2013; Suzuki et al., 2005; Takahashi et al., 2012b, 2013a). Of these three substrates, X-Neu5Ac can histochemically show cellular localisation of sialidase activity but does not provide high sensitivity. Sensitisers such as Fast Red Violet LB enhance sensitivity of X-Neu5Ac, but unwanted backgrounds are induced in multiple steps of the protocol (Minami et al., 2011; Saito et al., 2002; Suzuki et al., 2005). A benzothiazolylphenol derivative (BTP)-based sialidase substrate (BTP-Neu5Ac) was synthesised in a previous study (Minami et al., 2014). Sialidase reaction releases BTP from BTP-Neu5Ac by hydrolysis of chemical bonding between Neu5Ac and BTP (Fig. 1). BTP is a water-insoluble crystalline fluorescent compound with a large Stoke's shift and deposits on locations of sialidase activity (Kim et al., 2009; Minami et al., 2014; Otsubo et al., 2013). In previous research, one of the BTP-Neu5Ac derivatives, 2-(benzothiazol-2-yl)-4-bromophenyl 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (BTP3-Neu5Ac), fluorescently distinguished Sendai virus- and influenza A virus-infected cells from intact cells (Kurebayashi et al., 2014; Takano et al., 2014). The BTP-Neu5Ac assay is expected to be suitable for fluorescent histochemical staining of NDV-infected cells and viral particles. In this study, Lewis-lung carcinoma monkey kidney (LLC-MK_2) cells were infected with NDV and visualised with BTP3-Neu5Ac at room temperature without cell fixation. Moreover, NDV particles, which were blotted on a polyvinylidene difluoride (PVDF) membrane, were directly detected with the BTP3-Neu5Ac assay. NDV-binding protein bands of guinea pig red blood cell membranes, which were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted on the PVDF membrane, were visualised with the BTP3-Neu5Ac assay.

2. Materials and methods

2.1. Chemicals

BTP3-Neu5Ac was synthesised as described previously (Kurebayashi et al., 2014; Minami et al., 2014; Takano et al., 2014). 2,3-Dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) was purchased from Tokyo Chemical Industry, Tokyo, Japan.

2.2. Cells and viruses

LLC-MK_2 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% foetal bovine serum (FBS). African monkey kidney Cos-7 cells were maintained in Dulbecco's modified MEM (DMEM) supplemented with 10% FBS. NDV (D26 strain and Miyadera strain) was propagated in 11-day-old embryonated chicken eggs and purified by a density sucrose gradient as described previously (Suzuki et al., 1985).

2.3. Antibodies

Rabbit anti-NDV polyclonal antibody was prepared by immunisation of rabbits against NDV (Miyadera strain) as described previously (Suzuki et al., 2001). Horseradish peroxidase (HRP)-conjugated Protein A (Sigma–Aldrich, St. Louis, MO, USA) and phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) were purchased.

2.4. Fluorescent visualisation of NDV-infected cells with BTP3-Neu5Ac

A confluent monolayer of LLC-MK_2 cells on a 96-well plate was infected with the indicated FFU of NDV (D26 strain and Miyadera strain) in 100 μl/well of serum-free medium (SFM), Hybridoma-SFM (Invitrogen, Carlsbad, CA, USA), at 37 °C for 24 h in 5% CO_2. Cells were washed with phosphate-buffered saline (PBS; pH 7.2, 131 mM NaCl, 14 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl) and stained with 10 μM BTP3-Neu5Ac in 50 μl of PBS at room temperature for 5 min. To confirm sialidase dependency of fluorescent staining with BTP3-Neu5Ac, the cells were also stained with 10 μM BTP3-Neu5Ac in 50 μl of PBS at room temperature for 5 min in the presence of 1 mM DANA, a pan-sialidase inhibitor. Then the cells were washed with 100 μl/well of PBS and observed in 100 μl/well of SFM using an IX71 fluorescent microscope (Olympus, Tokyo, Japan) equipped with a fluorescent filter (U-MWU2, DM400, BP336-385, BA420).

2.5. Immunostaining of NDV-infected cells

In order to confirm that BTP3-Neu5Ac specifically stained NDV-infected cells, infected cells were immunostained with a rabbit anti-NDV polyclonal antibody. A confluent monolayer of LLC-MK_2 cells on a 96-well plate was infected with the indicated FFU of NDV (D26 strain) in 100 μl/well of SFM at 37 °C for 24 h in 5% CO_2 and washed with 100 μl/well of PBS. Then the cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 10 min and washed with PBS. The cells were treated with a rabbit anti-NDV polyclonal antibody in PBS,