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A gel-capture assay for characterizing the sialyl-glycan selectivity of influenza viruses

T. VELKOV[1*], P.E. THOMPSON[1], O. EL-KABBANI[1], F. LINDH[2], J. STAMBAS[3], S. ROCKMAN[4*]

[1]Department of Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville 3052 Victoria Australia; [2]IsoSep AB, Dalkärrsvägen 11, S-146 36 Tullinge, Sweden; [3]School of Medicine, Deakin University, 3217 Victoria Australia; [4]CSL Limited Poplar Road, Parkville 3052 Victoria Australia

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Summary. – Sialic acids (SA) usually linked to galactose (Gal) in an α2,6- or α2,3-configuration are considered the main cell receptors for influenza viruses, in particular for their hemagglutinins (HA). The typing of influenza virus HA receptor selectivity is relevant for understanding the transmissibility of avian and swine viruses to the human population. In this study we developed a simple and inexpensive gel-capture assay (GCA) of the influenza virus HA receptor-binding selectivity. Its principle is the binding of soluble influenza virus to pentasaccharide analogs, representatives of receptors of human and avian influenza viruses, immobilized on a gel resin. The human and avian analogs consisted of a sialyllactose-*N*-tetraose c (LSTc) [Neu5Ac(α2,6)Gal(β1-3) GlcNAc(β1-3)Gal(β1-4)Glc] and a sialyllactose-*N*-tetraose a (LSTa) [Neu5Ac(α2,3)Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc], respectively. Following equilibration, the unbound virus is washed away and the bound one is assayed via HA by densitometry as a function of the analog concentration. Using GCA, the receptor selectivity of three influenza viruses of different HA subtype was investigated. The results showed that the egg-adapted A/California/07/2009 (H1N1) virus exhibited an avian α2,3-linked LSTa selectivity, however, it retained the ability to bind to the α2,6-linked LSTc human receptor analog. Influenza B virus B/Florida/4/2006 showed α2,6-linked LSTc selectivity and a poor α2,3-linked LSTa avidity. The H3N2 virus A/Wisconsin/15/2009 displayed almost comparable avidity for both receptor analogs with a marginally greater α2,3-linked LSTa avidity. The described assay protocol provides a simple and rapid method for the characterization of influenza virus HA receptor binding selectivity.

Keywords: influenza virus; hemagglutinin; receptor; sialyllactose-*N*-tetraose; gel-capture assay

Introduction

The surface HA of influenza viruses initiates virus internalization by binding to the SA attached to membrane glycoproteins, glycophospholipids, and proteoglycans (Skehel and Wiley, 2000; Suzuki, 2005). The host receptor distribution and sialyl-glycan linkage (α 2,6 or α 2,3) specificity of the viral HA largely determines a host range of the virus (Skehel and Wiley, 2000; Suzuki, 2005). HA of avian influenza viruses usually display a preference for α2-3-linked sialyl-glycans, whereas HA of human influenza viruses preferentially bind α2-6-linked sialyl-glycans (Skehel and Wiley, 2000; Gamblin *et al*., 2004; Suzuki, 2005; Stevens *et al*., 2006b). Human influenza B and swine influenza A viruses have been reported to bind both α2,3 and α2,6-linked sialyl-glycans (Ito *et al*., 1998; Gamblin *et al*., 2004; Wang *et al*., 2007; Childs *et al*., 2009; Maines *et al*., 2009).

The determinants of receptor selectivity of many influenza A virus HAs have been extensively characterized by crystallographic analysis of receptor-HA complexes (Skehel and

*Corresponding authors. E-mail: Tony.Velkov@monash.edu.au, Steve.Rockman@csl.com.au ; fax: +61-3-9903 9582.

Abbreviations: 3′SL = 3′-sialyl(*N*-acetyllactose); 6′SLN = 6′-sialyl(*N*-acetyllactosamine); BPL = β-propiolactone; GCA = gel-capture assay; HA = hemagglutinin; LSTa = sialyllactose-*N*-tetraose a; LSTc = sialyllactose-*N*-tetraose c; SA = sialic acids

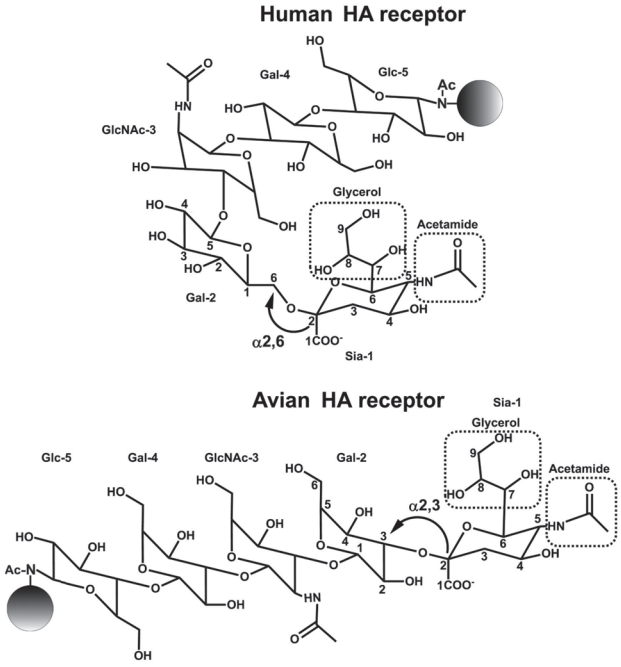

Fig. 1

Structure of analogs of human (LSTc) and avian (LSTa) receptors

The resin-NAc coupling point is drawn schematically.

Wiley, 2000; Ha *et al.*, 2001; Gamblin *et al.*, 2004; Stevens *et al.*, 2004, 2006b; Liu *et al.*, 2009). However, there are far fewer studies of the receptor selectivity determinants of influenza B virus HA (Suzuki *et al.*, 1992; Matrosovich *et al.*, 1993; Gambaryan *et al.*, 1997, 1999; Wang *et al.*, 2007). The importance of developing simple method for typing HA receptor-binding selectivity was heightened by the recent outbreak of swine-origin influenza strains in humans such as the pandemic influenza A 2009 H1N1 virus (Childs *et al.*, 2009; Itoh *et al.*, 2009; Maines *et al.*, 2009). Currently available receptor-characterizing assays include nuclear magnetic resonance, glycan array screening, surface plasmon resonance, hemagglutination or HA inhibition tests, thin-layer chromatography, and ELISA (Rogers and Paulson, 1983; Sauter *et al.*, 1989; Gambaryan and Matrosovich, 1992; Suzuki *et al.*, 1992; Takemoto *et al.*, 1996; Alvarez and Blixt, 2006; Stevens *et al.*, 2006a; Szretter *et al.*, 2006; Hidari *et al.*, 2007; Chandrasekaran *et al.*, 2008; Mandenius *et al.*, 2008; Srinivasan *et al.*, 2008).

In this study, we attempted to develop a simple and inexpensive assay for the selectivity of influenza virus HA binding to its receptors. The GCA utilizes pentasaccharide analogs of the human (LSTc) and avian (LSTa) sialyl-glycan receptors immobilized on a gel resin (Fig. 1). This assay is based on the determination of bound viral HA and the receptor-binding preference can be discerned qualitatively or quantitatively. GCA was used for the characterization of binding selectivity of three influenza viruses, A/California/07/2009 (H1N1), A/Wisconsin/15/2009 (H3N2), and B/Florida/4/2006.

Materials and Methods

Viruses. Reassortant-derived influenza viruses contain HA and neuraminidase genes of the A/California/07/2009 egg-adapted mutant (H1N1) or A/Wisconsin/15/2009 (H3N2) viruses in the genetic background of A/Puerto Rico/8/34. The influenza B virus (B/Florida/4/2006) is an egg-passaged strain derived from a circulating virus. Viruses were propagated by growth for 48 hrs in the allantoic cavities of 10-day-old embryonated chicken eggs at 37°C and infectious allantoic fluids were harvested and pooled. All viruses were purified by rate zonal centrifugation and inactivated by β-propiolactone (BPL) (0.05% v/v) treatment at 4°C. BPL was deactivated by hydrolysis at 37°C overnight. BPL inactivation has previously been shown not to affect HA receptor binding (Goldstein and Tauraso, 1970; Childs *et al.*, 2009). Viruses were quantified by the hemagglutination assay (Szretter *et al.*, 2006).

Preparation of LSTa and LSTc gels. LSTa and LSTc fractogels were synthesized by IsoSep AB (Tullinge) as described by (Blomberg *et al.*, 1993). Fractogel TSK HW65 (F) (Toyopearl) was obtained from Merck. Derivatization to an amino-form was performed by the treat-

ment with epichlorohydrine and subsequent opening of the epoxide with ammonia (Sasaki *et al.*, 1985). The gel in the amino-form (1 g wet weight) was mixed with the reducing oligosaccharide LSTa or LSTc (7–14 μmol) and 3 ml methanol and allowed to react for 10 hrs at 60°C, what resulted in the corresponding glycosylamine. Approximately 1 ml of the mixture in acetic anhydride was kept for 12 hrs at 20°C to give the corresponding glycosylamide.

Gel-capture assay. Inactivated viruses in PBS were standardized to 150 μg/ml HA protein and 100 μl aliquots were incubated with 2–300 μl (400 μmol/l) of either LSTa or LSTc gel slurry (approximately 2.0 μmol/g substitution, determined by weight of oligosaccharide after substitution) at 4°C for 1 hr. The total reaction volumes were 400 μl in PBS buffer. The sialidase inhibitor oseltamivir (10 μmol/l) was included in all incubations. The gel slurry was sedimented by centrifugation at 6000 x g for 1 min and washed four times with 300 μl PBS. Captured virus was released with 100 μl SDS-PAGE sample buffer and approximately 10 μl were resolved on 4–20% gradient polyacrylamide gel. The gels were silver or Coomasie blue stained to visualize proteins, dried, and scanned at 1200 dpi. HA protein bands were quantified densitometrically using LabImage 1D gel analysis software V3.4 (www.kapelan-bioimaging.com). The apparent affinity constant (K_d) of the HA-LST complex was determined by a non-linear regression fitting of the plot of HA band density as a function of LST concentration to standard binding hyperbola generated using GrapPad Prism V5.0 software.

ELISA using the biotinylated sialyl-glycans 3′-sialyl(*N*-acetyllactose) (3′SL; [Neu5Ac(α2,3)Gal(β1-4)Glc]) and 6′-sialyl(*N*-acetyllactosamine) (6′SLN; [Neu5Ac(α2,6)Gal(β1-4)GlcNAc]) was performed essentially as previously described with minor modifications (Gambaryan and Matrosovich, 1992; Chandrasekaran *et al.*, 2008; Srinivasan *et al.*, 2008). In brief, Nunc immobilizer 96 well-plates were coated with influenza virus (0.1mg/ml HA) and blocked with 1% BSA. Various dilutions of biotinylated sialyl-glycans at the desired concentrations in PBS were added into the wells (50 μl/well) and the plates were incubated at 4°C for 1 hr. To inhibit viral sialidase activity, oseltamivir (1.0 μmol/l) was included in all wells. The wells were washed four times with PBS and bound biotinylated sialyl-glycans were quantified with 25 μl/well of HRP-streptavidin (1:2000). Plates were incubated at 4°C for 1 hr. After washing with PBS, the peroxidase activity was assayed with *O*-phenylenediamine solution and the reaction was stopped with 50 μl of 1mol/l HCl. Absorbance was taken at 490 nm. The apparent affinity constant (K_d) for virus binding to 3′SL and 6′SLN was determined from the non-linear regression fit of the plot of biotinylated sialyl-glycan concentration versus the A_{490} to a standard binding hyperbola generated using GrapPad Prism V5.0 software.

Results and Discussion

The receptor specificity of three influenza viruses with different HA subtypes was determined with a novel GCA that measured direct binding to sialyl-glycans with either a sialic acid-α2,3-galactose or sialic acid-α2,6-galactose linkage, representative of avian and human receptors, respectively. In order to validate the assay, identical samples were analyzed by ELISA and the results were quantitatively compared (Gambaryan and Matrosovich, 1992; Matrosovich *et al.*, 1993; Srinivasan *et al.*, 2008).

In the first stage of GCA procedure, virus particles were allowed to bind to the immobilized sialyl-glycan on the gel slurry. The 1 hr incubation of virus with the sialyl-glycan gel slurry at 4°C was sufficient for the binding reaction to reach the equilibrium. During the second step, unbound viruses were removed by successive washing steps. The bound virus was then released from the gel by denaturant treatment with SDS-PAGE sample buffer and resolved on the gradient polyacrylamide gel to maximize resolution. Control incubations were performed with non-derivatized and de-sialylated gel slurry, where the pentasaccharide had been removed by enzymatic deglycosylation. There was no background virus binding detected with the non-derivatized and de-sialylated gel slurry incubations (data not shown). To characterize the interaction quantitatively, densitometric analysis of HA bands were performed and apparent binding affinity constants (K_d) were determined from the plots of HA band intensity versus the sialyl-glycan concentration (Fig. 2, Table 1). Under solution-state equilibrium conditions, the K_d would be equal to the sialyl-glycan concentration at which the available HA binding sites are at half saturation, therefore, a lower K_d value corresponds to a higher binding affinity. However, it should be noted that due to the solid-phase of the sialyl-glycan receptors in GCA and ELISA, the extensive washing steps and multivalent nature of the HA-receptor interaction, the K_d values from these assays did not represent true equilibrium affinity constants. Nevertheless, the K_d values provided a measure of the apparent affinity of the virus HA for the sialyl-glycans.

Generally, the GCA method gave lower sialyl-glycan K_d values compared to those measured by the ELISA method (Table 1). This could be attributed to the greater sensitivity provided by the HRP-colorimetric substrate detection method used in ELISA. In the original ELISA method, the

Table 1. Binding affinities of influenza viruses to sialyl-glycans as determined by GCA and ELISA

Virus	K_d (μmol/l)			
	GCA		ELISA	
	LSTa	LSTc	3′SL	6′SLN
A/California/07/2009 (H1N1)*	12 ± 2.1	24 ± 3.0	0.4 ± 0.07	1.6 ± 0.3
A/Wisconsin/15/2009 (H3N2)	10 ± 2.7	9.4 ± 3.8	0.5 ± 0.1	0.3 ± 0.1
B/Florida/4/2006*	39 ± 9.2	12 ± 4.1	1.8 ± 0.3	1.1 ± 0.1

*Egg-adapted virus.

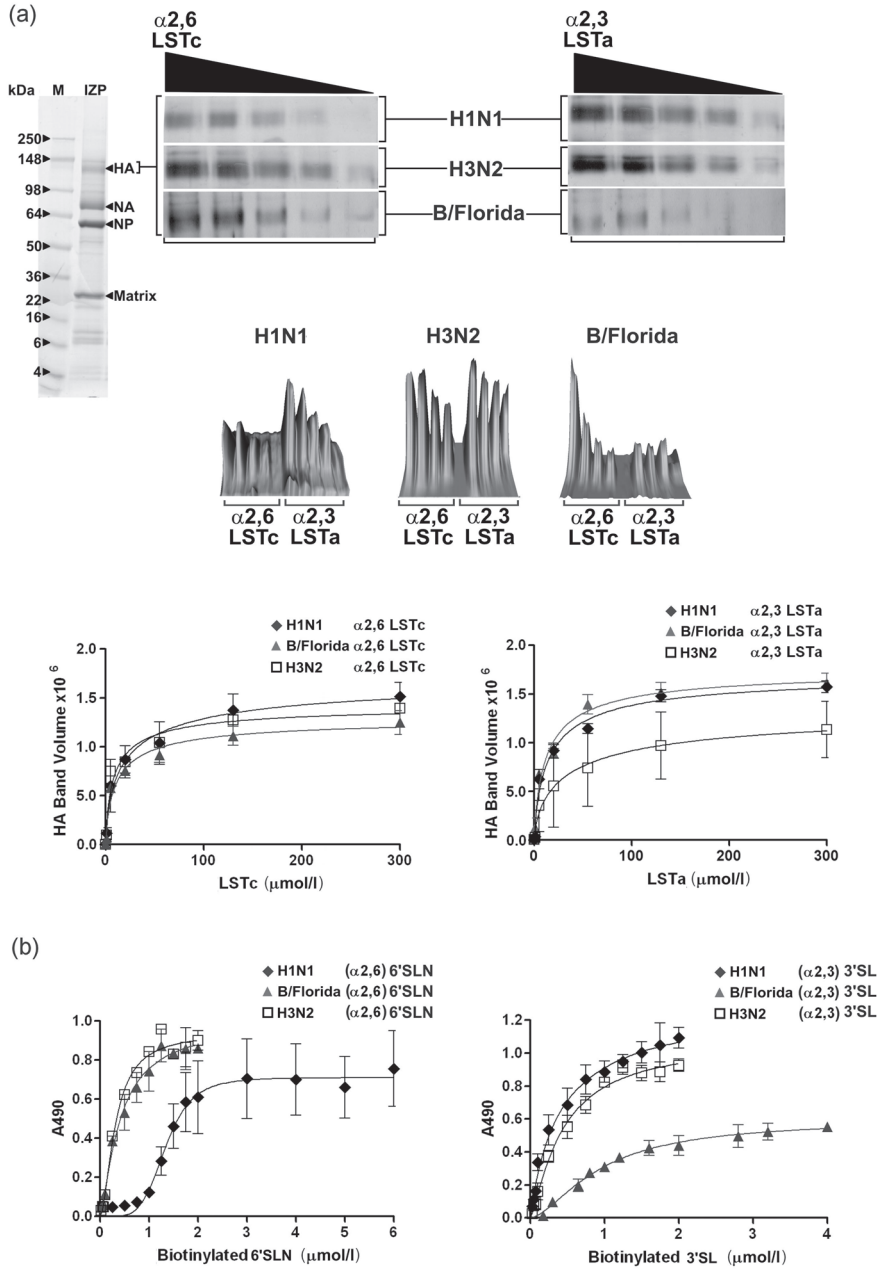

Fig. 2

GCA of influenza virus-HA receptor binding selectivity

(a) SDS-PAGE profile of influenza virus (upper left panel). Molecular weight markers (M) are shown in the left lane. IZP – inactivated virus zonal particle; NA – neuraminidase; NP – nucleoprotein. HA bands of influenza viruses bound to increasing concentrations of LSTc and LSTa, respectively (upper panels) and their densitometry (middle and lower panels). (b) Binding of influenza viruses to increasing concentrations of 6'SLN and 3′ SL, respectively, in ELISA.

virus was immobilized onto microtitre plate wells and the binding of monovalent sialyl-glycans or polyacrylic acid based sialylglycopolymers was subsequently evaluated in a competitive manner, whereby the inhibition of glycan binding to the immobilized virus by HRP-labeled fetuin was measured (Gambaryan and Matrosovich, 1992; Matrosovich *et al.*, 1993, 1997, 2000; Gambaryan *et al.*, 1997, 1999). One shortcoming of this method is that the immobilization of the virus onto the micro-well surface may shield potential binding sites from the sialyl-glycan receptor, what may affect

their binding affinity for the viral HA. However, this may also account for the differences in binding affinity values that we observed in comparison with our procedure. Moreover, ELISA depends on the secondary enzyme-developed colorimetric reaction and further variability is introduced highlighting the indirect measure of binding affinity. Variations of the assay involving HRP-conjugated secondary antibody colorimetric development and detection have been reported and employ biotinylated sialyl-glycans immobilized onto a streptavidin plate array (Chandrasekaran *et al.*, 2008; Srinivasan *et al.*, 2008). The 96-well plate format of the ELISA method means that it is a relatively high throughput procedure. One major limitation of the GCA is the laborious nature of standard laboratory SDS-PAGE. Thus, without expensive semi-automated SDS-PAGE equipment the GCA is a low capacity assay.

The results from both GCA and ELISA showed that the H1N1 A/California/07/2009 egg-adapted virus displays a strong α2,3-sialyl-glycan binding preference with an approximately 2-fold higher binding affinity (LSTa>LSTc) measured by GCA and 4-fold higher binding affinity (3'SL>6'SLN) measured by ELISA (Fig. 2, Table 1). The receptor binding specificity of representative wild-type pandemic H1N1 2009 viruses have also been characterized using glycan microarray analysis with BPL inactivated viruses (Childs *et al.*, 2009) and using soluble recombinant HA (Maines *et al.*, 2009). The findings of both studies indicated the HA of the wild-type H1N1 virus preferentially binds α2,6-sialyl-glycans (human-like). However, the glycan array data also showed a broader specificity with the ability to bind a range α2,3-sialyl-glycans (avian-like), albeit with the reduced avidity (Childs *et al.*, 2009). These observations differ from the strong α2,3-sialyl-glycan preference of the H1N1 A/California/07/2009 egg-adapted virus detected by both GCA and ELISA methods. This discrepancy may result from the wild-type H1N1 virus employed in the glycan array study compared to the egg-adapted isolate we employed. The GCA results for the H3N2 virus isolate, A/Wisconsin/15/2009 indicated a marginally greater α2,6 to α2,3 binding preference (LSTc≥LSTa) (Fig. 2, Table 1). This result was in line with the K_d values measured by ELISA, which showed a marginally greater 6'SLN≥3'SL binding affinity (Table 1).

Influenza B virus almost exclusively infects humans and contributes significantly to the seasonal influenza morbidity and mortality (Wright and Webster, 2001). Unlike the influenza A viruses, the HAs of influenza B viruses have no designated subtypes as they display a greater level of sequence conservation with a mutation rate about 5 times lower (Bootman and Robertson, 1988). However, it remains unclear whether the HAs preferentially bind to α2,6-sialyl-glycans (human-like) or equally well to α2,3-sialyl-glycans (avian-like) (Suzuki *et al.*, 1992; Gambaryan *et al.*, 1997, 1999). This is further complicated by the low sequence homology of influenza B HAs with those of influenza A subtypes (Bootman and Robertson, 1988). The crystal structure of influenza B/HongKong/8/73 HA in complex with LSTa and LSTc revealed that the receptor-binding pocket is capable of making optimal contacts with both receptor analogs (Wang *et al.*, 2007). In the case of the influenza B virus B/Florida/4/06, GCA indicated a strong α2,6-sialyl-glycan preference with an approx. 3-fold greater LSTc>LSTa binding affinity (Fig. 2, Table 1). In comparison ELISA only indicated a marginally greater α2,6- to α2,3-sialyl-glycan binding affinity (6'SLN≥3'SL) (Table 1). Interestingly, ELISA did not discriminate between α2,6- and α2,3-sialyl-glycan binding affinity for the influenza B virus to the same degree as GCA. The poor correlation between GCA and ELISA for the influenza B virus may be attributed to the shorter length of the α2,3-sialyl-glycan 3'-SL (trisaccharide) used in ELISA compared to the pentasaccharide LSTa used in GCA. This would be consistent with the crystallographic structure of influenza B/HongKong/8/73 HA in complex with LSTa showing that receptor interactions with the sugars distal from the SA terminus are most likely responsible for the regulation of binding affinity for the avian receptor (Wang *et al.*, 2007).

In summary, the described GCA protocol provides a simple method for the characterization of influenza virus HA sialyl-glycan binding selectivity. Based on the densitometric analysis of HA bands, it seemed that the GCA is quantitative allowing determination of apparent binding affinities. In this report, the receptor selectivity of only three influenza viruses with different HA subtypes were investigated. Future studies using a broader selection of influenza viruses could help to complete validation of the versatility and robustness of the GCA method. This procedure also offers a simple and convenient method for the affinity purification of virus from a crude material such as allantoic fluid. Furthermore, this assay could prove to be of considerable utility for a drug discovery program aimed at the development of small-molecule attachment inhibitors.

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