The receptor preference of influenza viruses

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Objectives   The cell surface receptor used by an influenza virus to infect that cell is an *N*-acetyl neuraminic acid (NANA) residue terminally linked by an alpha2,3 or alpha2,6 bond to a carbohydrate moiety of a glycoprotein or glycolipid. Our aim was to determine a quick and technically simple method to determine cell receptor usage by whole influenza A virus particles.

Methods   We employed surface plasmon resonance to detect the binding of viruses to fetuin, a naturally occurring glycoprotein that has both alpha2,3- and alpha2,6-linked NANA, and free 3′-sialyllactose or 6′-sialyllactose to compete virus binding. All virus stocks were produced in embryonated chicken's eggs.

Results   The influenza viruses tested bound preferentially to NANAalpha2,3Gal or to NANAalpha2,6Gal, or showed no preference. Two PR8 viruses had different binding preferences. Binding preferences of viruses correlated well with their known biological properties.

Conclusions   Our data suggest that it is not easy to predict receptor usage by influenza viruses. However, direct experimental determination as described here can inform experiments concerned with viral pathogenesis, biology and structure. In principle, the methodology can be used for any virus that binds to a terminal NANA residue.

Keywords   Cell receptor, influenza virus, *N*-acetyl neuraminic acid, surface plasmon resonance.

Introduction

Influenza A viruses initiate infection by binding to cell surface *N*-acetyl neuraminic acid (NANA) that is terminally linked to a carbohydrate moiety of a glycoprotein or glycolipid. NANA residues can be linked either by alpha2,3 or alpha2,6 bonds. Influenza viruses discriminate between these according to structure of the receptor pocket situated on the haemagglutinin major virion surface protein. An early view was that avian influenza viruses bound to NANAalpha2,3Gal, which is the predominant receptor in the respiratory and alimentary tract of birds, and that mammalian influenza viruses bound to NANAalpha2,6Gal, the main receptor of the mammalian respiratory tract. However, recent data suggest that the situation is more complex, in that (i) viruses have a spectrum of receptor binding activity and that (ii) most mammals have both types of receptor, although they may not be located in the same region of the respiratory tract, or alternatively they may be located on different cells within one region. There are extensive studies in humans.[1–13] One mammalian species where there is some uncertainty is the laboratory mouse in which most authors identify only NANAalpha2,3Gal in the respiratory tract,[1,9,14,15] while others report NANAalpha2,6Gal on lung stroma and lung vessels.[16,17] Most work is restricted to C57BL/6 mice, and it would be useful to know the receptor distribution in other inbred strains that are commonly used as models for influenza. The receptor preference of an influenza virus is a key determinant of its host range, the nature of the infection that ensues (e.g. upper respiratory tract or lower respiratory tract), and hence its overall virulence. To understand infection, it is necessary to be aware of the receptor preference of the infecting virus and the receptors present on the potential target tissue. Receptor preference can be determined by virus haemagglutination of natural or derivatized red blood cells that have on their surface predominantly NANAalpha2,3Gal or NANAalpha2,6Gal,[18,19] or by inhibition of haemagglutination by horse serum that is rich in proteins bearing NANAalpha2,3Gal.[19] Alternatively binding of virus to immobilized sugars can be detected by a variety of standard procedures.[20–24] Sugar-HA protein or sugar-whole virus complexes have been detected directly by proton NMR spectroscopy,[25,26] and apparent association constants for sugars binding to immobilized virus have been determined.[27] Binding of purified HA protein to a naturally glycosylated protein was detected by surface plasmon resonance (SPR) or ELISA,

with receptor identification by specific sialidase digestion or competition with sialylglycopolymers.[28,29] Here, we describe a quick and technically simple method to determine receptor usage that employs specific sugars to compete the binding of whole virus to a naturally occurring glycoprotein and SPR to detect such binding.

Methods

Viruses

In this study, we used H1N1, H2N3 and H3N2 subtype viruses (Table 1). All were grown in allantoic cavity of embryonated chicken's eggs, purified by differential centrifugation, and stored in liquid nitrogen at 2×10^5 HAU/ml (approximately 600 μg protein/ml). Although the allantoic cavity contains mainly NANAalpha2,3Gal receptors,[30] this does not necessarily predicate receptor usage as only some NANAalpha2,6Gal-using viruses evolve to preferentially use NANAalpha2,3Gal receptors e.g.[31,32]

Surface plasmon resonance

We used SPR (Biacore2000, Uppsala, Sweden) to detect virus binding to fetuin, a naturally occurring, heavily glycosylated protein obtained from foetal calf serum (Sigma-Aldrich, Gillingham, UK), which has a triantennary oligosaccharides containing NANAalpha2,3Gal and NANA-alpha2,6Gal residues in 2:1 proportions.[33,34] It is generally agreed that fetuin does not contain other sialic acid moieties. The fetuin was covalently immobilized to the dextran surface of the CM5 sensor chip by amine-coupling according to the manufacturer's instructions, and then binding of virus to fetuin was determined by injecting various concentrations of virus in HEPES-buffered saline (0·01 M HEPES, pH 7·4, 0·15 M NaCl, 3 mM EDTA, 0·005% surfactant P20) (Biacore) over the fetuin-immobilized flow cell for 7 minutes at a flow rate of 5 μl/minute at 25°C. The response units (RU) from the reference channel, where no fetuin was immobilized were subtracted, resulting in the RU due to the specific binding between fetuin and virus.

Sequencing of A/PR/8/34 haemagglutinin genes

Virion RNA was extracted and reverse transcribed using random hexamer and reverse transcriptase (Fermentas, York, UK) according to the manufacturer's instructions. This was followed by standard *Taq* DNA polymerase PCR (Fermentas) using the primers (Invitrogen, Paisley, UK):

  ha1:5′-TAGCCCCACTACAATTGGGGAAATGT
  ha2:5′-CAGTGCGAAAGCATACATTGGTGCTAT

to specifically amplify the receptor binding region (amino acid 65-251, H3 numbering). After gel purification, the PCR products were sequenced using the above primers and the ABI PRISM 3130xl Genetic Analyser from the University's Molecular Biology Service. Derived sequences were compared with A/PR8 Cambridge (CAA24272) and A/PR8 Mount Sinai (AAM75158) from GenBank.

Results and discussion

Determination of receptor preference

An example of the dose–response nature resulting from the application of various concentrations of whole virus particles (A/Puerto Rico/8/34; H1N1) to fetuin is shown in Figure 1. Receptor preference was determined by preincubating purified virus (usually 200 μg/ml) with various concentrations of free 3′-sialyllactose (NANAalpha2,3Gal beta1,4Glc) or 6′-sialyllactose (NANAalpha2,6Gal beta1,4Glc) (Carbosynth, Berkshire, UK), and then determining the amount of virus binding to fetuin in the presence and absence of the sugar (Figure 2). As the virion neuraminidase could potentially cleave off NANA,[28] the system was monitored by observing the RU reading from three consecutive injections over the flow cell surface. Of the viruses tested, A/PR8, A/Victoria and A/New Caledonia had a stable RU value, whereas A/mallard failed to give a consistent signal. However, the signal was stabilized when the neuraminidase-inhibitor Zanamivir (400 nM; GlaxoSmithKline, Stevenage, UK) was preincubated with A/mallard before being injected. A/WSN(mouse), A/Udorn and A/Sydney were also assayed in the presence of Zanamivir. Neither Zanamivir nor sugar alone bind to the fetuin-derivative sensor chip suggesting any RU changes on the fetuin surface were due to the virus binding (data not shown).

Figure 3 and the summary in Table 1 show binding data for seven viruses. The 50% point inhibition of virus binding was determined by extrapolating to the sugar concentration at which this occurred. A/mallard was readily inhibited by 3′-sialyllactose and hence bound predominantly to NANAalpha2,3Gal receptors (Figure 1A); 50% inhibition of binding to NANAalpha2,6Gal required a 7·6-fold higher concentration of 6′-sialyllactose. Other viruses with a pronounced preference for NANAalpha2,3Gal receptors were the human strains A/PR8(Reading) (Figure 3D) and A/WSN(mouse) (Figure 3E). In contrast A/Victoria preferred binding to NANAalpha2,6Gal receptors (Figure 3B), with 3′-sialyllactose failing to achieve significant inhibition of binding. Finally A/Udorn (Figure 3F) and A/PR8(Warwick) (Figure 3C) bound to both types of receptor, although they both had a preference for NANAalpha2,6Gal. Inhibition of the binding of A/Sydney (Figure 3G) was low despite varying the virus concentration. The number of sugar moieties is crucial for the binding for some viruses, and it may be that A/Sydney interacts poorly with trisaccharides in general and with sialyllactose in particular.[35] Nevertheless, the data suggest that A/Sydney preferentially binds to NANAalpha2,6Gal.

Table 1. Summary of the receptor binding preferences of some influenza A viruses*

Subtype	Virus	Original host	Source	Passage history	50% inhibition by NANAalpha2,6Gal beta1,4Glc (μM) approximately (a)	50% inhibition by NANAalpha2,3Gal beta1,4Glc (μM) approximately (b)	Ratio (a)/(b)	Receptor preference
H1N1	A/Puerto Rico/8/1/34 (Reading)**	Human	Reading University	Unknown ECE*** passages + transfection of plasmids into 293T cells + 1 MDCK cells + 2 ECE	>500	~500	n.a.	Mainly 2,3
H1N1	A/Puerto Rico/8/1/34 (Warwick)	Human	Warwick University	Unknown ECE passages	190	300	0·6	Both
H1N1	A/WSN(mouse) variant of A/WS/33	Human	JCSMR, ANU, Canberra, Australia	Unknown ECE and mouse passages	>500	130	>3·8	Mainly 2,3
H1N1	A/New Caledonia/20/99	Human	WHO Melbourne, NIBSC Potters Bar, and Retroscreen Virology Ltd	8 ECE passages	190	380	0·5	More 2,6 than 2,3
H2N3	A/mallard/England/7277/06	Duck	VLA, Weybridge	1 ECE passage from a duck intestine isolate	380	50	7·6	Mainly 2,3
H3N2	A/Victoria/3/75†	Human	Reading University	Unknown ECE passages + transfection of plasmids into 293T cells + 1 MDCK cells + 2 ECE	130	>500	<0·3	Mainly 2,6
H3N2	A/Sydney/5/97	Human	Retroscreen Virology Ltd	2 chicken kidney passages + 5 ECE	>500	>500	n.a.	Mainly 2,6
H3N2	A/Udorn/307/72	Human	Dr R. A Lamb	Unknown ECE passages	200	200	1	Both

n.a., not applicable.
*Most data are taken from Figure 3.
**A molecularly cloned virus carrying the 244 DI RNA[37].
***ECE, passages in the allantoic cavity of embryonated chicken eggs.
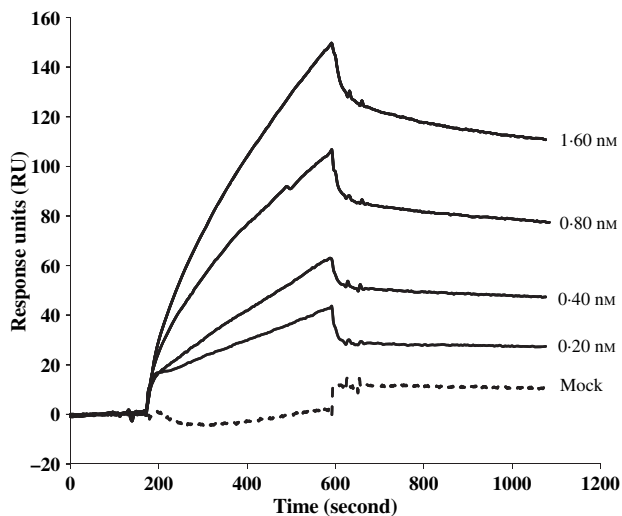†A molecularly cloned virus.

Figure 1. An overlay of sensorgrams of the binding of A/PR/8/34 (Warwick) to a fetuin-derivatized sensorchip after injection of virus (35 μl in HBS buffer) at the indicated concentrations. Data were corrected with reference to virus binding to a control channel with no fetuin. Purified allantoic fluid from mock-infected eggs (mock) failed to bind to fetuin indicating that the binding between virus and fetuin was specific.
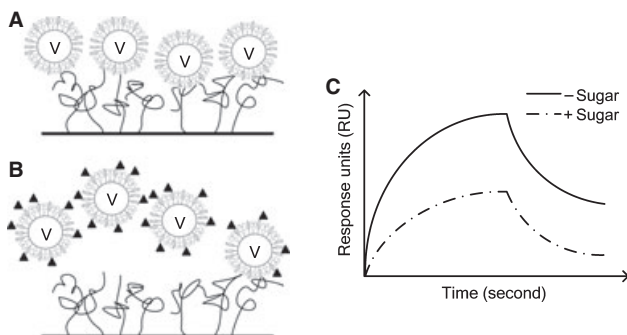

Figure 2. Scheme for determining the receptor preference of influenza viruses (V). Binding of virions to immobilized fetuin (A) is detected in real time by surface plasmon resonance (SPR) (C). This binding can be competed by preincubating virus with various concentrations of free 3'-sialyllactose (NANAalpha2,3Gal beta1,4Glc) or 6'-sialyllactose (NANAalpha2,6Gal beta1,4Glc) (both represented by ▲; B and C).

Figure 3. Receptor binding preference of influenza A viruses as determined by inhibition of binding to fetuin through preincubation of virus with various concentrations of free 3'-sialyllactose (NANAalpha2,3Gal beta1,4Glc) (♦) or 6'-sialyllactose (NANAalpha2,6Gal beta1,4Glc) (■). The error bars are based on at least two measurements; where not shown the error bar lies within the datum point.

The overall conclusion is that while all the viruses tested can bind to both types of receptor under the conditions applied, some have a strong preference for one receptor over the other. We have not addressed the nature of this variation, which could result from there being a heterogeneous population of virions with a preference for NANAalpha2,3Gal or NANAalpha2,6Gal or a homogeneou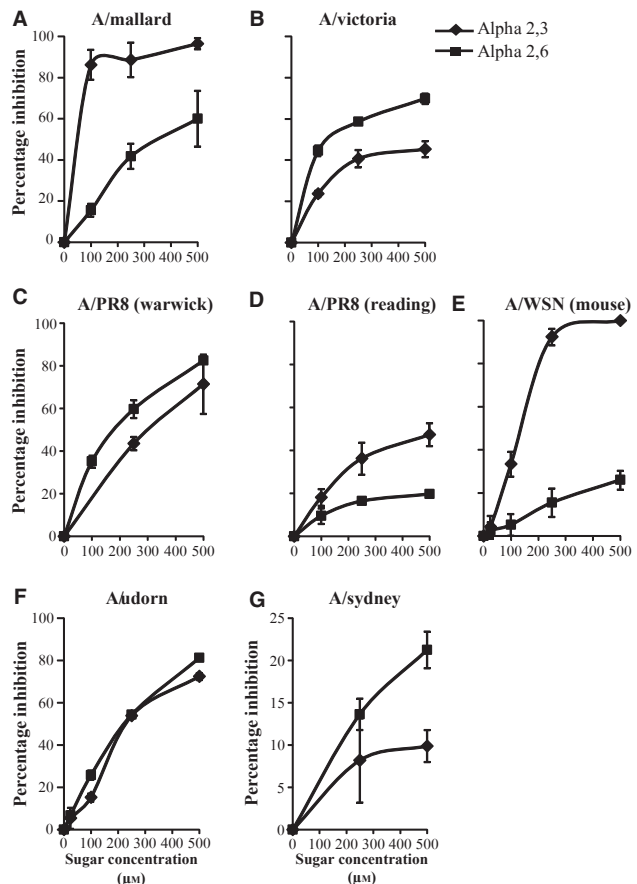s population of virions that recognize NANAalpha2,3Gal and NANAalpha2,6Gal with different efficiencies. However, A/PR8(Reading) and A/Victoria were molecularly cloned viruses which after the transfection/cocultivation step had undergone just two egg passages before testing, and had little chance to develop variants.

Affinity data for the binding of A/New Caledonia and A/PR8(Warwick) to fetuin are compared in Table 2 with data of others. Binding for whole virus particles of a variety of subtypes and receptor preferences were remarkably similar and all had a high subnanomolar KD. The Ka for HA rosettes was much lower, whereas Kd is similar, which suggests that the high KD for virions resulted from the multivalency afforded by the approximately 500–1000 HA trimers per virion. The 2:1 excess of NANAalpha2,3Gal over NANAalpha2,6Gal in fetuin did not seem to affect virus binding.

Table 2. Affinity of binding to NANA receptors as measured in different assay systems

Virus/HA	Subtype	Receptor preference	Reference	Ka (1/Msec)	Kd (1/sec)	KD (nM)	χ^2
A/New Caledonia/20/99	H1N1	More 2,6 than 2,3	This report	2.52×10^6	2.38×10^{-4}	9.45×10^{-1}	6.29
A/PR8/1/34(Warwick)	H1N1	Both	This report	1.02×10^6	4.12×10^{-4}	4.03×10^{-1}	1.5
A/Aichi/2/68	H3N2	Both	46	1.61×10^6	3.15×10^{-4}	1.96×10^{-1}	
A/duck/HK/313/78	H5N3	More 2,3 than 2,6	46	1.85×10^6	0.75×10^{-5}	4.05×10^{-1}	
X31 (HA rosettes)	H3N2	More 2,6 than 2,3	28	2.00×10^3	2.00×10^{-4}	1.00×10^2	

All data (except HA rosettes) were evaluated on a 1:1 binding model (1 virus particle:1 molecule of fetuin or sialylglycolipid); the chi-squared test matches the experimental data with the theoretical model, with a value of <10 being highly significant.

Correlation of receptor binding preference of viruses with their biological properties

A/mallard preferentially binds to NANAalpha2,3Gal receptors which is consistent with its recent isolation from the gut of its avian (duck) host where only NANAalpha2,3Gal receptors are found,[4,36] but despite this and passage solely in chicken's eggs, NANAalpha2,6Gal was able to inhibit its binding to fetuin indicating that it had the ability to recognize NANAalpha2,6Gal receptors.

A/Victoria had a clear preference for NANAalpha2,6Gal receptors. Nonetheless, like all the viruses in this study, A/Victoria was grown in the allantoic cavity of chicken's eggs in which NANAalpha2,3Gal receptors predominate.[30] In other studies[37] A/Victoria was used to deliver a cloned defective interfering RNA intranasally to mice which have predominately NANAalpha2,3Gal receptors (references above). These mice were protected from respiratory disease and weight loss caused by a pathogenic mouse-adapted A/WSN(mouse) that strongly preferred NANAalpha2,3Gal receptors (Figure 3E). As both DI and infectious virus have to infect the same cell for interference to take place,[38] it therefore seems likely that A/Victoria can recognize NANAalpha2,3Gal cell receptors in the murine respiratory tract.

A/New Caledonia and A/Sydney cause clinical influenza in ferrets, an animal that has a high proportion of NANA-alpha2,6Gal receptors in its respiratory tract.[29,39] Their preference in our assay for NANAalpha2,6Gal receptors is consistent with this property. However, like A/mallard they grow well in the NANAalpha2,3Gal receptor-rich milieu of the allantoic cavity of the embryonated chicken's egg.

A/PR8 viruses examined here have demonstrably different receptor preferences. A/PR8(Reading) strongly preferred NANAalpha2,3Gal receptors while A/PR8(Warwick) bound to both NANAalpha2,3Gal and NANAalpha2,6Gal receptors. As specific H1 HA1 residues are known to affect the receptor binding specificity (in particular, residues 138, 190, 193 and 225, (H3 numbering:[40–42]), we sequenced a fragment covering this region (amino acid residues

65–251). Table 3 shows that the sequence of A/PR8(Warwick) is identical to the Mount Sinai variant and that in A/PR8(Reading) 123K changed to E, and 186P changed to S. Both have a conserved change of I194L. The P186S change is common to the majority of human H1 sequences, including the Cambridge variant of A/PR8; 186P corresponds to the avian sequence.[43] Recent human H1N1 viruses contain 186P, but the 2009 pandemic swine-origin A (H1N1) virus contains 186S.[44] Residue 186 forms part of a network which is involved in binding to the third sugar in the receptor (N-acetylglucosamine), and P186S was one of the changes implicated in the lower affinity binding of HA of the 2009 A (H1N1) virus CA/04 to an alpha2,6 oligosaccharide, compared to the HA of the 1918 pandemic virus SC18.[44] The alteration of 123 from the positively charged K (lysine) to a negatively charged E (glutamic acid) is likely to have significant ramifications for the HA1 structure even if 123 is not of one of the known binding residues.

In earlier work A/PR8(Reading) was used to deliver the 244 DI RNA intranasally to mice, and protected them very

Table 3. Sequence changes in the HA1 of variants of A/Puerto Rico/8/34

	A/Puerto Rico/8/34			
	Cambridge (CAA24272)	Mount Sinai (AAM75158)	Reading	Warwick
123*	K	K	E	K
132	T	–	–	–
133	K	N	N	N
142	A	E	E	E
186	S	P	S	P
190	D	E	E	E
194	I	I	L	L

*H3 numbering; the region encoding amino acid residues 65–251 was sequenced.

efficiently from clinical influenza caused by A/WSN(-mouse).[37] The receptor preference of both A/PR8(Reading) and A/WSN(mouse) (Figure 3D,E) are consistent with the predominantly NANAalpha2,3Gal receptors found in the mouse respiratory tract (references above). In the ferret model intranasal DI PR8(Warwick) afforded strong protection against an intranasal challenge with A/Sydney, a ferret-adapted NANAalpha2,6Gal receptor-preferring (Figure 3G) challenge virus,[45] suggesting that both viruses were able to use the NANAalpha2,6Gal receptors that predominate in the ferret respiratory tract.[29,39] However, less effective protection of ferrets against the same challenge virus was observed with by DI PR8(Reading) (N. J. Dimmock, unpublished data), which would be consistent with the mismatch in their receptor preference. Such variation in receptor binding for A/PR8 virus is consistent with the findings of others where PR8 is described as attaching solely to NANAalpha2,3Gal receptors,[8,21] attaching also weakly to NANAalpha2,6Gal receptors,[24] and attaching to both types of receptor.[19] Two variants of PR8 (Cambridge and Mount Sinai) are known which differ in HA sequence, but the foregoing viruses were not so identified; all were grown in embryonated chicken's eggs.

Conclusions

Detection of the binding of whole virions to fetuin by SPR and its inhibition by competition with specific sugars provides a convenient way of determining the receptor preferences of influenza and other viruses that use NANA as their cell receptor. The influenza A viruses studied showed a complete spectrum of receptor preferences which were consistent with their other biological properties.

Conflict of Interest

Nigel J. Dimmock and Anthony C. Marriott hold shares in ViraBiotech Ltd. Bo Meng declares no conflicts of interest.

Acknowledgements

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