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Comparison of the receptor binding properties of contemporary swine isolates and early human pandemic H1N1 isolates (Novel 2009 H1N1)

Konrad C. Bradley [a], Cheryl A. Jones [b], S. Mark Tompkins [b], Ralph A. Tripp [b], Rupert J. Russell [c], Marie R. Gramer [d], Jamie Heimburg-Molinaro [e], David F. Smith [e], Richard D. Cummings [e], David A. Steinhauer [a,*]

[a] Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Rd, Atlanta, GA 30322, USA
[b] Department of Infectious Diseases, University of Georgia, Athens, GA, USA
[c] School of Biology, University of St. Andrews, Fife KY16 9ST, UK
[d] Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN, USA
[e] Department of Biochemistry, and the Glycomics Center, Emory University School of Medicine, O. Wayne Rollins Research Center, 1510 Clifton Road NE, Atlanta, GA 30322, USA

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ABSTRACT

We have utilized glycan microarray technology to determine the receptor binding properties of early isolates from the recent 2009 H1N1 human pandemic (pdmH1N1), and compared them to North American swine influenza isolates from the same year, as well as past seasonal H1N1 human isolates. We showed that the pdmH1N1 strains, as well as the swine influenza isolates examined, bound almost exclusively to glycans with α2,6-linked sialic acid with little binding detected for α2,3-linked species. This is highlighted by pair-wise comparisons between compounds with identical glycan backbones, differing only in the chemistry of their terminal linkages. The overall similarities in receptor binding profiles displayed by pdmH1N1 strains and swine isolates indicate that little or no adaptation appeared to be necessary in the binding component of HA for transmission from pig to human, and subsequent human to human spread.

Introduction

According to the World Health Organization, the viruses responsible for the recent human outbreak of the 2009 H1N1 pandemic subtype (pdmH1N1) were first detected in North America in April of 2009, have since been isolated in more than 200 countries, and have caused greater than 18,000 deaths worldwide. The pandemic H1N1 strains are composed of six segments derived from a triple reassortant swine H1N1 strain (trH1N1), and two segments from the Eurasian "avian-like" swine H1N1 viruses (Garten et al., 2009). Initially, there were concerns that the new human strains were highly pathogenic, due to a high mortality rate of infected patients with complications in a Mexico City hospital (Domínguez-Cherit et al., 2009; Fraser et al., 2009). However, as the virus spread through the United States, the majority of symptomatic infections were found to result in uncomplicated, upper respiratory tract illness with a relatively low mortality rate. The newly emergent H1N1 strains rapidly spread worldwide, and the pdmH1N1 outbreak was declared a phase 6 pandemic by the WHO in June 2010 (Dawood et al., 2009). Despite the fact that pandemic H1N1 viruses (pdmH1N1) are not recognized as highly pathogenic in the classical sense, certain features of their disease profile appear to differ from the seasonal H1N1 viruses that have circulated in humans since 1977. They affected children and younger adults at much higher rates than older adults and the elderly, likely as a result of immunity in the population that had been exposed to H1N1 viruses that circulated prior to 1957 (Hancock et al., 2009). In addition, the disease they caused in children was often more serious than had been observed with the previously circulating seasonal H1N1 strains (Bhat et al., 2005; CDC, 2009; Louie et al., 2009), and had significant effects in terms of years of lost life (Viboud et al., 2010). For these reasons, the characteristics of replication, transmission, and pathogenicity of pdmH1N1 viruses and related human and swine viruses warrant continued and detailed scrutiny.

The triple reassortant virus (trH1N1), which contributed 6 of the 8 gene segments in the pdmH1N1, is made up of segments from avian, human, and swine viruses. The trH1N1 viruses emerged in swine in the late 1990s and were first reported in humans in 2005 (Olsen, 2002; Shinde et al., 2009). These strains periodically infected humans through 2009, typically resulting in mild illness, but did not become established in the human population (Shinde et al., 2009). Currently, it is unclear when or where the reassortant event that combined the six segments from the trH1N1 and the NA and M segments from the

* Corresponding author.
E-mail address: dsteinh@emory.edu (D.A. Steinhauer).

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Eurasian H1N1 swine influenza viruses occurred, but phylogenetic analysis has suggested the pandemic reassortant could have been circulating undetected for several years (Smith et al., 2009). Numerous studies have characterized the pathogenicity and transmissibility of the current pdmH1N1 strains in rodents, ferrets, and swine, as well as the susceptibility of different age groups to the new strain (Belser et al., 2010; Brookes et al., 2010; Ge at al.; Hancock et al., 2009; Itoh et al., 2009; Maines et al., 2009; Steel et al.), and in some experimental settings it seems that pdmH1N1 isolates can replicate more efficiently in the lower respiratory tract compared to seasonal H1N1 strains. Such studies suggest that the receptor binding properties of pdmH1N1 may have unusual features by comparison with other H1N1 viruses that have been characterized previously.

Influenza receptor binding is mediated by the hemagglutinin glycoprotein (HA) and is thought to play a major role in the ability of influenza to infect and propagate in a new species (Horimoto and Kawaoka, 2005; Webster et al., 1992). Influenza binds to cell-surface glycans containing N-acetylneuraminic acid (Neu5Ac) or its derivatives (termed sialic acids) at their termini (Gottschalk, 1959; Klenk et al., 1955). The linkage by which sialic acids are attached to the penultimate sugar of the glycan is thought to play a role in host range specificity of influenza viruses, often based on whether this glycosidic bond is present in an α2,3 or α2,6 conformation (Connor et al., 1994; Ito and Kawaoka, 2000; Rogers and D'Souza, 1989; Skehel and Wiley, 2000). Influenza viruses isolated from avian species commonly replicate in the gut and preferentially bind α2,3-linked sialic acid (α2,3 SA), while influenza in humans is a respiratory disease and tends to prefer α2,6-linked sialic acids (α2,6 SA). Swine contain an abundance of both α2,3 and α2,6 SA in their respiratory tract (Ito and Kawaoka, 2000; Nelli et al., 2010) and therefore are considered to be a potential "mixing vessel" for human, avian, and other swine influenza strains (Scholtissek et al., 1985). In previous studies using enzymatically modified erythrocytes, swine viruses have shown a preference for α2,6 SA binding (Gambaryan et al., 2005; Rogers and D'Souza, 1989; Takemae et al., 2010), and it is generally believed for an avian or swine influenza strains to infect humans and transmit efficiently from person to person, it must first be able to bind α2,6 SA (Horimoto and Kawaoka, 2005; Matrosovich et al., 2000).

Although there have been a selection of studies investigating the binding properties of pdmH1N1 viruses published recently (Childs et al., 2009; Maines et al., 2009; Yang et al., 2010), we have chosen to expand on these reports by conducting a comprehensive study of the binding characteristics of pdmH1N1 viruses using glycan microarrays. For comparative purposes, we include in these studies parallel analyses of H1N1 swine influenza viruses that were isolated from pigs during the same year of the pandemic outbreak, as well as seasonal H1N1 viruses that circulated in humans before 2009. In addition to HA receptor-binding characteristics, it is believed that a functional balance between HA binding and neuraminidase (NA) enzymatic activity may occur during adaptation to a new host possibly by improving transmission efficiency of influenza viruses (Banks et al., 2001; Matrosovich et al., 1999). The NA of pdmH1N1 viruses is derived from avian origins but has been circulating in Eurasian swine for three decades. Therefore, to evaluate the functional balance between HA and NA, we have also examined the NA enzymatic activity of these viruses.

Results

Viruses examined

The details regarding initial transmission of pdmH1N1 to humans have not been established, but the two strains of influenza virus that combined to create the quadruple reassortant pandemic H1N1 virus involved triple reassortant viruses (trH1N1) that had circulated in North American swine since the late 1990s and Eurasian "avian-like" H1N1 viruses, with HA genes being derived from the trH1N1 viruses (Garten et al., 2009). For these studies, we assessed the receptor

Table 1
Receptor binding site sequence alignment of strains examined by glycan array analysis (H3 numbering).

		Residue number																							
		130 Loop						190 Helix				220 Loop													
Subtype	Strain	133	134	135	136	137	138	183	186	190	191	192	193	194	195	217	218	219	220	221	222	223	224	225	226	227	228	229	230
Seasonal H1N1	A/Pennsylvania/08/2008	T	G	V	S	A	S	H	P	D	Q	K	T	L	Y	I	A	K	R	P	K	V	R	D	Q	E	G	R	I
	A/Brisbane/59/2007	T	G	V	S	A	S	H	N	N	Q	K	A	L	Y	I	A	K	R	P	K	V	R	D	Q	E	G	R	I
	A/New Caledonia/20/1999	T	G	V	S	A	S	H	N	N	Q	R	A	L	Y	I	A	K	R	P	K	V	R	D	Q	E	G	R	I
SOIV H1N1	A/California/04/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
	A/Mexico/INDRE4487/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
	A/Texas/15/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
Swine isolates (H1N1)	A/Sw/Minnesota/02749/2009	R	G	V	T	A	A	H	S	D	Q	Q	S	L	Y	I	A	T	R	P	K	V	R	D	Q	A	G	R	M
	A/Sw/Minnesota/02751/2009	K	G	V	T	A	A	H	P	D	Q	Q	T	L	Y	I	A	V	R	P	K	V	R	D	Q	A	G	R	M
	A/Sw/Illinois/02860/2009	T	G	V	S	A	S	H	P	N	Q	R	T	L	Y	I	T	K	R	P	K	V	R	D	Q	E	G	R	I
H3 subtype	H3N1 WT	N	G	G	S	N	A	H	S	E	Q	T	S	L	Y	I	G	S	R	P	W	V	R	G	L	S	S	R	I
	A/Sw/Minnesota/02719 (H3N2)	D	G	S	S	Y	A	H	G	D	Q	T	N	L	Y	I	G	S	R	P	W	V	R	G	V	S	S	I	I

Residues which differ from the reference strain (A/Pennsylvania/08/2008) are labeled in red. H3N1 WT refers to a laboratory virus with the HA from A/Aichi/2/68 on a A/WSN/34 genetic background.

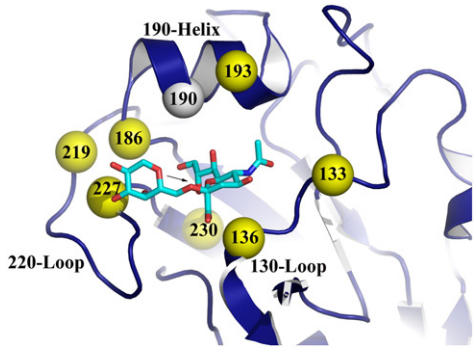


Fig. 1. Schematic diagram of the HA receptor binding site and major structural characteristics (dark blue), and the location of bound Neu5Acα2,6Gal (light blue) in the binding pocket. The arrow indicates the α2,6 glycosidic linkage, and the yellow balls show the locations of residues that differ among strains analyzed in this study and discussed in the text. The white ball indicates residue 190, which is known to affect receptor binding, but does not differ between pdmH1N1 and trH1N1 swine isolates. All numbering is based on H3 the subtype prototype HA from A/Aichi/2/68 virus.

Table 2
Agglutination of erythrocytes by purified influenza strains.

Subtype	Virus	Erythrocytes			
		Chicken	Turkey	Horse	Guinea pig
Seasonal H1N1	A/Brisbane/59/2007	1024	1024	0	2048
	A/New Caledonia/20/1999	512	256	16	2048
	A/Pennsylvania/08/2008	1024	1024	16	2048
SOIV H1N1	A/California/04/09	64	256	0	1024
	A/Texas/15/2009	16	64	0	256
	A/Mexico/INDRE4487/2009	32	128	0	512
Swine isolates (H1N1)	A/Swine Iowa/1976/1931	128	128	0	2048
	A/Swine/Minnesota/02749/2009	64	512	0	2048
	A/Swine/Minnesota/02751/2009	128	256	0	256
	A/Swine/Illinois/02860/2009	16	64	0	64
H3 Subtype	H3N1 WT	128	128	0	256
	A/Swine/Minnesota/02719/2009 (H3N2)	128	512	0	1024

HA assays were performed using 0.5% erythrocytes from each animal, as described in the Materials and Methods.

binding properties of three 2009 human pandemic H1N1 strains, three contemporary strains of 2009 swine isolate trH1N1 viruses, and three seasonal human H1N1 strains that had circulated in previous years. We also examined the classical 1931 swine H1N1 isolate A/swine/Iowa/1976/31, and two H3N2 viruses for comparative purposes.

Influenza isolates are routinely amplified in embryonated chicken eggs; however, it is well established that viruses passaged in eggs have a propensity to select for mutations in the HA receptor binding region that may alter the HA affinity for various sialic acid containing cell surface receptors (Burnet and Bull, 1943; Gambaryan et al., 1999; Katz et al., 1990; Katz and Webster, 1988; Lugovtsev et al., 2009; Robertson et al., 1987, 1985; Stevens et al., 2010). Therefore, we utilized minimal passage in MDCK cells to amplify viruses whenever possible, and the HA and NA genes for stock viruses were sequenced and compared to the sequences obtained from initial isolates as well as published sequences to ensure against laboratory-generated selection of viruses with altered binding characteristics. The A/Brisbane/59/2007 and A/New Caledonia/20/1999 human vaccine strains, as well as the 1931 swine isolate A/Swine/Iowa/1976/1931 were included in this study for comparative purposes, but their passage histories are not well documented, and almost certainly include propagation in eggs at some stage. The viruses and their sequences at HA positions known to be relevant for receptor binding properties are shown in Table 1. Fig. 1 shows the structure of the H1 pdmH1N1 A/California/04/2009 receptor binding site (Xu et al., 2010) and highlights the locations of residues pertinent to this study. Fig. 2 shows the phylogenetic relationships of viruses utilized in this study.

Erythrocyte agglutination properties of pandemic H1N1 and swine isolates

The collection of viruses shown in Table 1 was first assessed for their capacity to agglutinate erythrocytes from different species, which are known to vary in their expression of cell-surface glycans

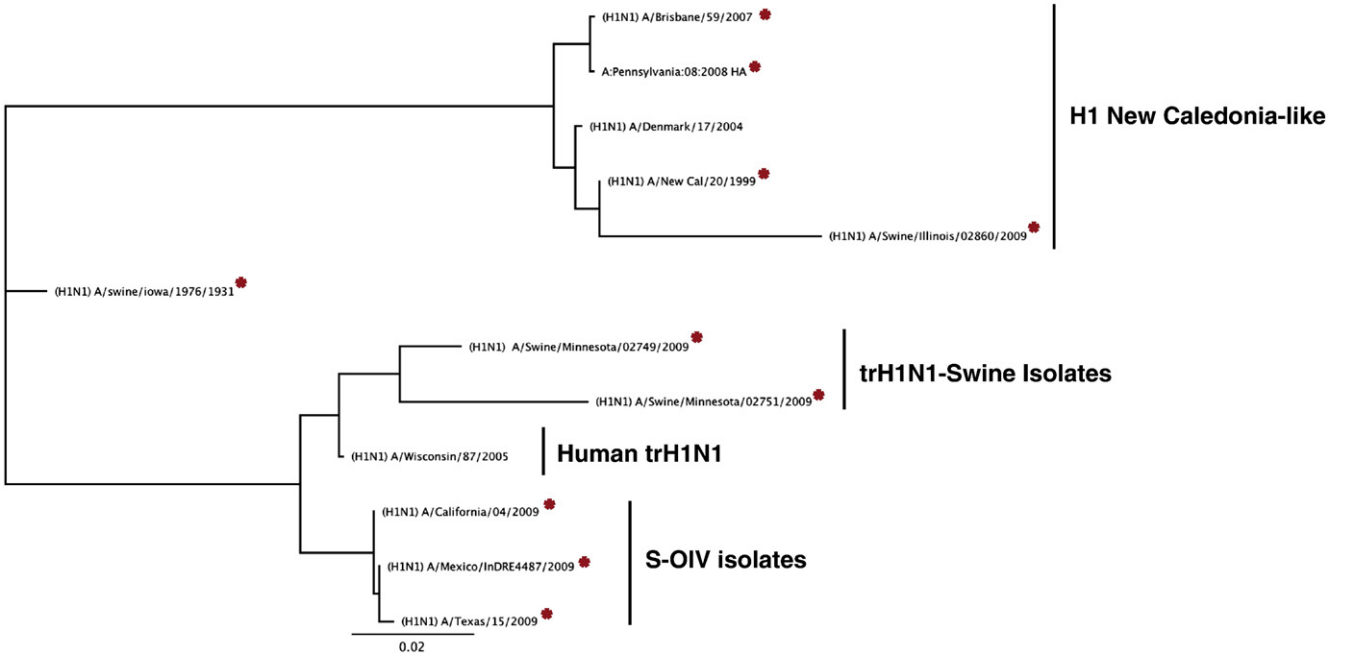


Fig. 2. Basic phylogenetic tree of related influenza isolates, as well as isolates utilized in this study. The tree was assembled using the genetic distance model HKY and the tree building neighbor-joining methodology with no outgroups. Strains marked with a red asterisk indicate that the isolate was used in this study.

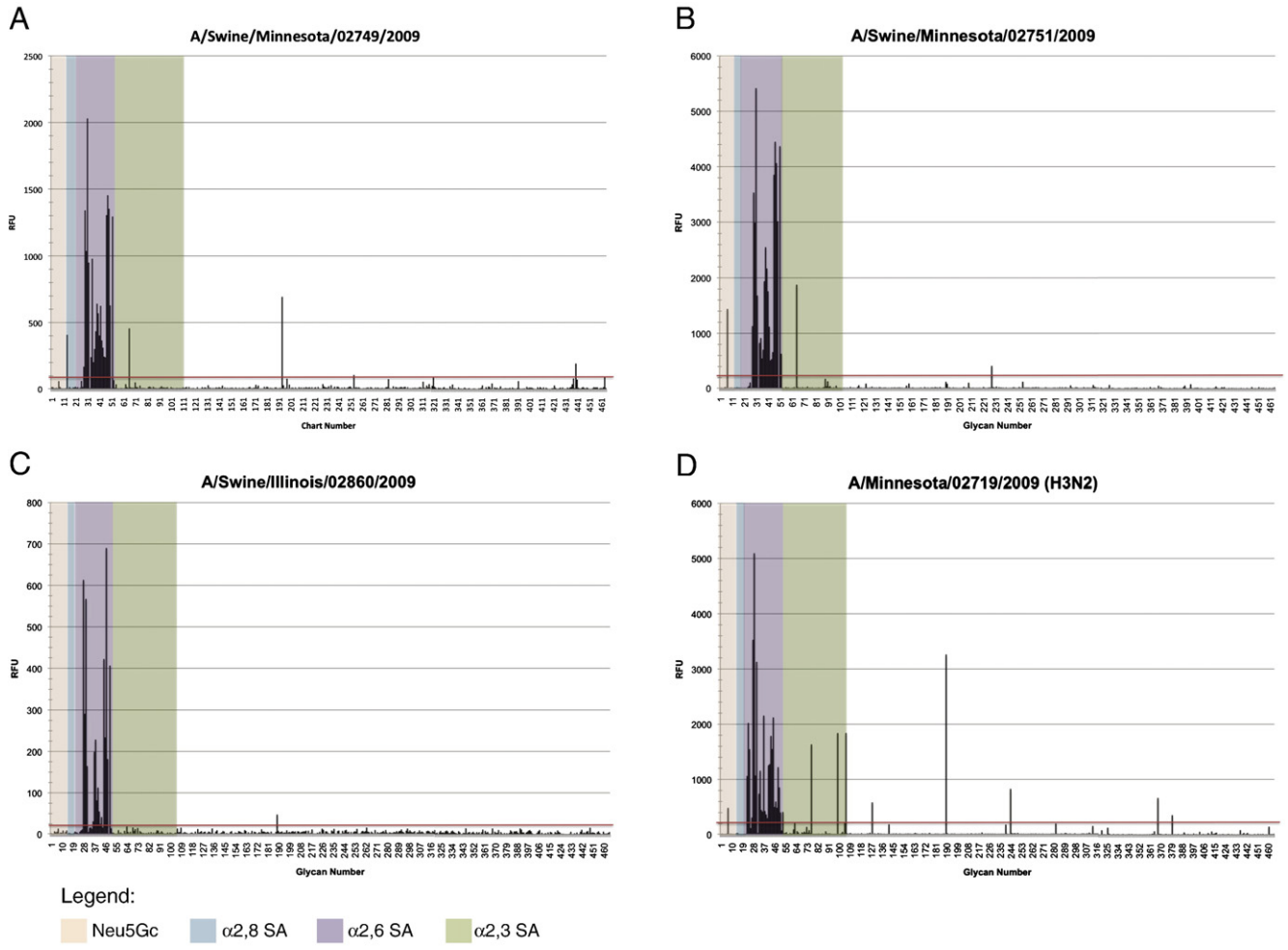


Fig. 3. Glycan microarray studies were performed at the Consortium for Functional Glycomics using CFG microarrays. Alexafluor488 labeled viruses were incubated for 1 hour at 4 °C prior to scanning. Glycans in the graph were sorted based on their glycan structure to group α2,6 and α2,3 SA. Background is indicated by the red line and was determined by multiplying the average of all glycans on the microarray by two. (A) A/Swine/Minnesota/02749/2009 (H1N1), (B) A/Swine/Minnesota/02751/2009 (H1N1), (C) A/Swine/Illinois/02860/2009 (H1N1), (D) A/Swine/Minnesota/02719/2009 (H3N2).

with sialic acids in the α2,3 versus α2,6 composition (Ito et al., 1997). Chicken red blood cells (cRBCs), turkey red blood cells (tRBCs) and guinea pig red blood cells (gpRBCs) are commonly used to characterize influenza isolates and laboratory stocks, as these erythrocytes contain both α2,3- and α2,6-linked glycans, with the α2,6 conformation being predominant on gpRBCs (Medeiros et al., 2001). By contrast, horse red blood cells (hoRBCs) contain a predominance of cell surface glycans with sialic acids in α2,3 configuration (Ito et al., 1997). Nearly all of the viruses in our panel were found to agglutinate guinea pig erythrocytes with high efficiency, while agglutination using turkey and chicken erythrocytes were generally observed to have slightly lower HA titers (Table 2). In contrast, among the viruses in our panel, only the A/New Caledonia/20/1999 vaccine strain and the A/Pennsylvania/08/2008 human seasonal strain demonstrated detectable HA titers using horse erythrocytes, suggesting that these viruses may have the capacity to recognize receptors in α2,3 conformation.

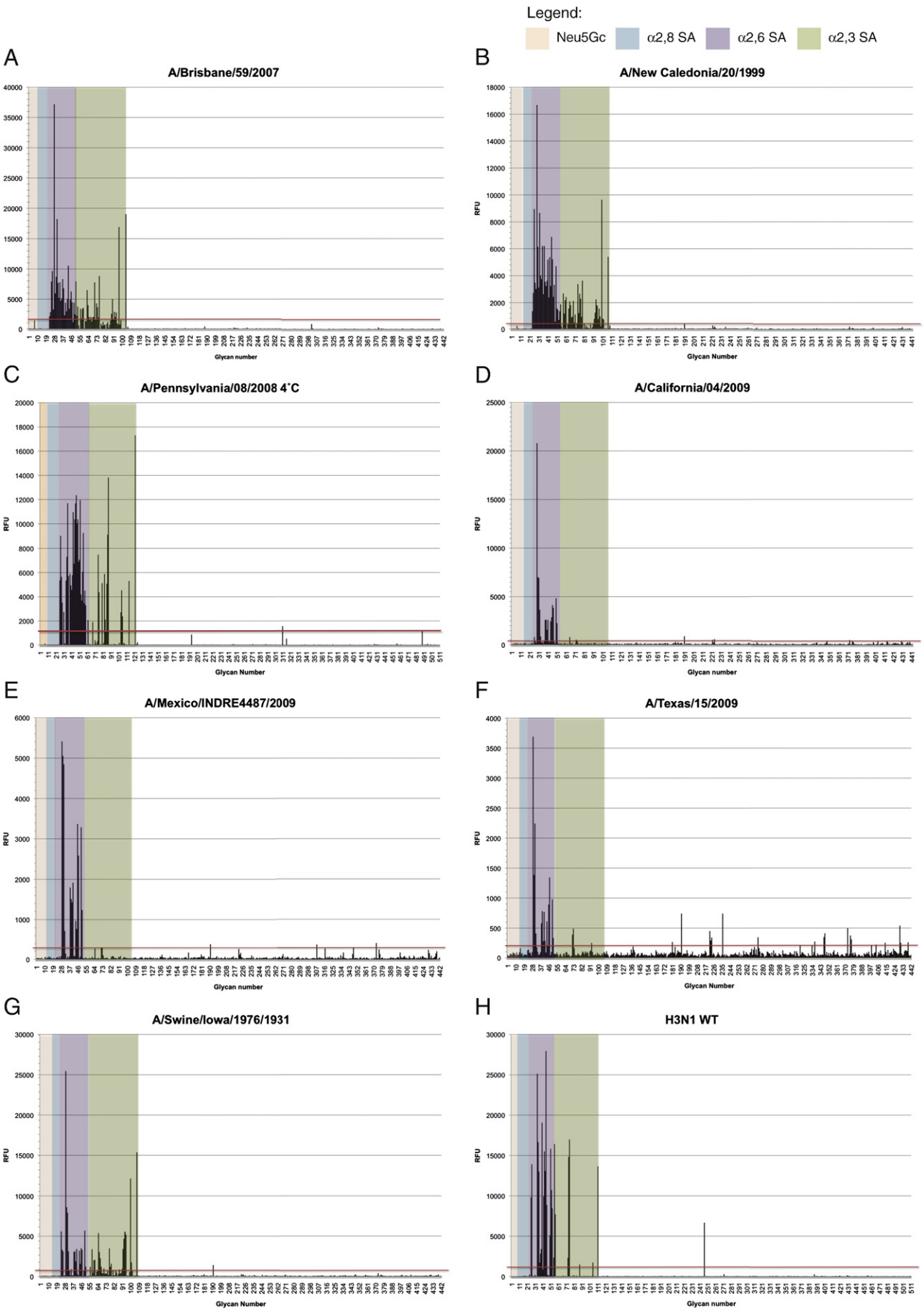
Glycan microarray analyses

For a more direct analysis of the receptor binding capabilities of the viruses shown in Table 1, we utilized glycan microarray analysis (Blixt et al., 2004). Purified viruses were labeled using the Alexafluor 488 amine reactive dye, as described in Materials and methods, and bound to glycan arrays provided by the Protein-Glycan Interaction Core (Core H) of the Consortium of Functional Glycomics. The plots of binding data are presented in histogram form in Figs. 3 and 4. The binding results were sorted based on their glycan structure in order to group the glycans containing terminal sialic acids on the graph. A peak was considered to be significant if the relative fluorescence units (RFUs) were 2× higher than the average RFUs for all the glycans expressed on the microarray. The overall levels of binding were variable among samples if RFUs of maximum peaks are compared; therefore, the graphs shown in Figs. 3 and 4 have variable ranges on the *y*-axis in order to provide optimal visualization of the distribution of glycan species bound for individual samples. The structures of the top five glycans bound by each virus are presented in diagrammatic form in Fig. 5. These structures do not include the spacers necessary for attaching a glycan to the glass slide.

Contemporary H1 swine isolates

To determine the receptor binding patterns of recent swine isolate strains, three 2009 isolates were examined. The first strain, A/swine/

Fig. 4. Glycan microarray experiments were performed as indicated in Fig. 3, and the Materials and methods. (A) A/Brisbane/59/2009 (H1N1), (B) A/New Caledonia/20/1999 (H1N1), (C) A/Pennsylvania/08/2008 (H1N1), (D) A/California/04/2009 (H1N1), (E) A/Mexico/INDRE4487/2009 (H1N1), (F) A/Texas/15/2009 (H1N1), (G) A/Swine/Iowa/1976/1931, (H1N1), (H) H3N1 Aichi HA WT (H3N1).


Legend:
Neu5Gc
α2,8 SA
α2,6 SA
α2,3 SA
A
A/Brisbane/59/2007
B
A/New Caledonia/20/1999
C
A/Pennsylvania/08/2008 4˚C
D
A/California/04/2009
E
A/Mexico/INDRE4487/2009
F
A/Texas/15/2009
G
A/Swine/Iowa/1976/1931
H
H3N1 WT
RFU
Glycan number
Glycan Number

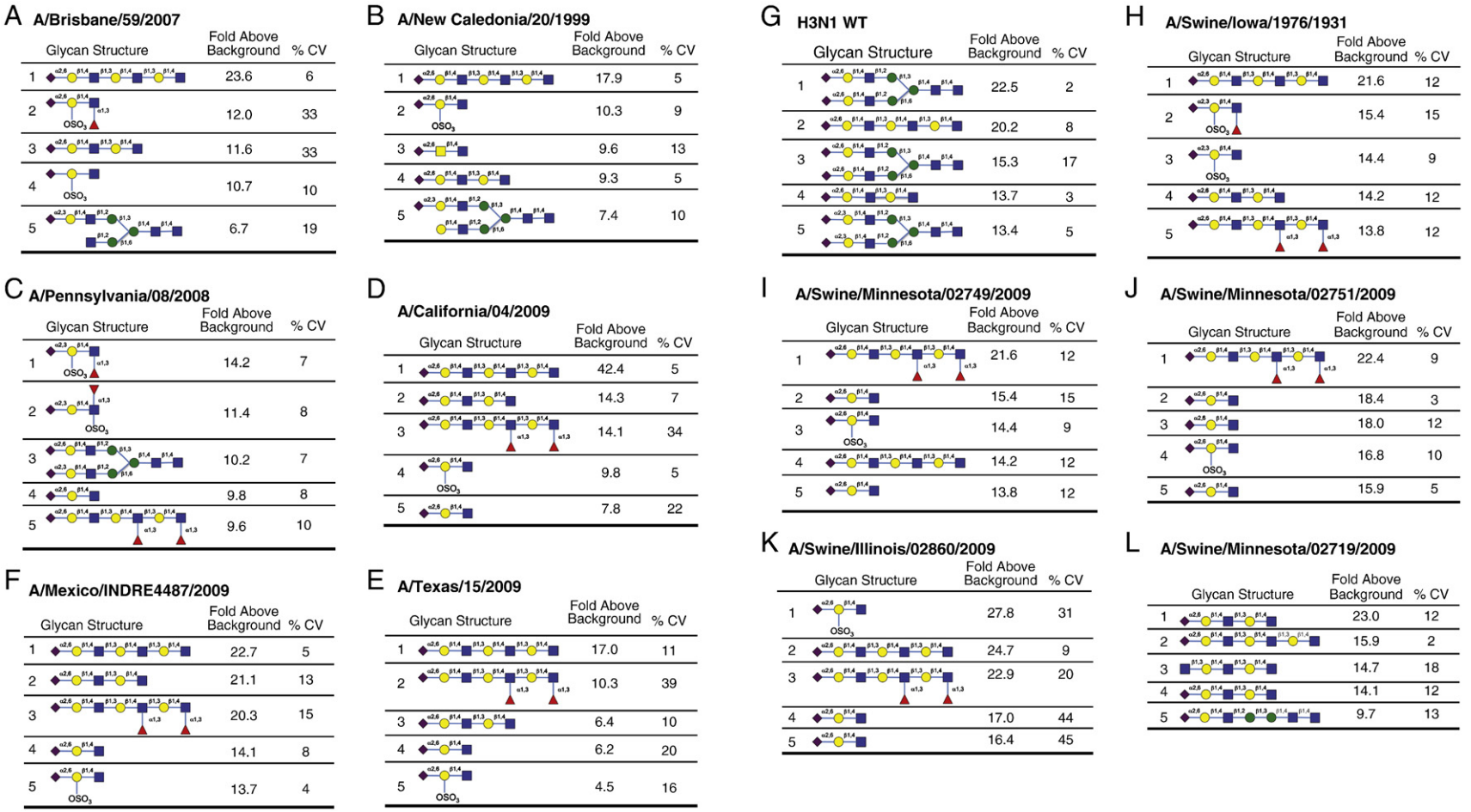


Fig. 5. Top five glycans bound by purified swine isolate viruses on the CFG microarray. The five highest binding glycans with %CVs less than 50% are listed without the spacer used to attach glycans to the consortium slides. The %CV shown is the number provided by glycan array analysis software. Identical structures are indicative of virus binding to glycans linked to the slide with different spacers. Nomenclature is as follows: purple diamond, Neu5Ac; yellow circle, Galactose; blue square, GlcNAc; red triangle, fucose; OSO_3, sulfate group. Linkages are indicated between sugar symbols. (A) A/Brisbane/59/2007, (B) A/New Caledonia/20/1999, (C) A/Pennsylvania/08/2008, (D) A/California/04/2009, (E) A/Mexico/INDRE4487/2009, (F) A/Texas/15/2009, (G) H3N1 WT, (H) A/Swine/Iowa/1976/1931, (I) A/Swine/Minnesota/02749/2009, (J) A/Swine/Minnesota/02751/2009, (K) A/Swine/Illinois/02860/2009, (L) A/Swine/Minnesota/02719/2009.

Minnesota/02749/2009 (Mn/02749), revealed a highly restricted binding phenotype, binding exclusively to α2,6 SA containing glycans, with no binding to glycans containing α2,3 SA that did not also contain a terminal α2,6 SA (Fig. 3A and Fig. 6D, glycans 3–5). A/swine/Minnesota/02751/2009 (Mn/02751) also preferentially bound α2,6 SA-containing glycans (Fig. 3B), with greater binding to branched glycans over single chain glycans (Fig. 6D, glycans 1 and 3), but the addition of a 6′-O-SO_3 did not seem to increase binding over identical glycans lacking a sulfate (Fig. 6F, glycans 1 and 5). The receptor binding domains of Mn/02749 and Mn/02751 are identical, with the exception of three residues at positions 133, 193, and 219. One difference observed for these strains was that Mn/02751 displayed significant binding to a single α2,3-linked N-glycolylneuraminic acid (Neu5Gc) present on the microarray, a Neu5Gcα2,3Galβ1,4(Fucα1,3) GlcNac glycan (Fig. 3B). However, the full breadth of Neu5Gc binding for these two strains is difficult to ascertain due to the limited number of Neu5Gc glycans present on the microarray. We attempted to determine how the number of sugars in the glycan chain altered binding, but effects varied greatly depending on the type and conformation of internal sugars and therefore do not appear to be a major determinant of binding preference. A/swine/Illinois/02860/2009 (Il/02860) virus is divergent from Mn/02749 and Mn/02751, differing at several positions in the receptor binding site (Table 1). Relative to the other swine isolates, it bound poorly to the microarrays, recognizing less than 2/3 of the glycans bound by both Minnesota strains (Fig. 3C). Despite the D190N mutation, previously shown to alter receptor binding, it was also highly restricted to α2,6 SA, with preferential binding to an unbranched poly-*N*-acetyllactosamine glycan with a terminal α2,6 SA. In addition, Il/02860 bound to the same Neu5Gc glycan recognized by Mn/02751. However, any trends relating to length or additional charged sugars are difficult to determine due to the overall poor binding of Il/02860 to the microarray.

Analysis of pdmH1N1 isolates

To compare the binding of trH1N1 swine isolates with early isolates from the pdmH1N1 outbreak, we examined three prototype pdmH1N1 strains that were isolated from patients presenting with a representative spectrum of disease during the early stages of the outbreak. All three pdmH1N1 strains bound primarily α2,6 SA-containing glycans (Fig. 4D–F), with very limited binding to glycans with α2,3 SA. Overall, the strongest binding glycans bound by the 2009 pdmH1N1 isolates were highly restricted to the same structure (Fig. 5D–F).

A/California/04/2009 (Fig. 4D) was isolated on 1 April from a 10-year-old male patient, and was an early isolate of the pandemic. This virus displayed a highly restricted α2,6 SA binding preference. Although this virus showed slight binding to a long biantennary structure with both α2,3 SA and α2,6 SA, the relative binding was reduced relative to an identical glycan containing two terminal α2,6 SA (Fig. 6C glycans 3 and 4). The addition of a sulfate (6′-O-SO_3) or fucose to a glycan did not seem to affect binding of A/California to α2,3 SA- or α2,6 SA-containing glycans, either positively or negatively (Fig. 6E, glycans 1, 2 and 5). The glycans to which A/California bound were long, unbranched, poly-*N*-acetyllactosamine glycans, and there appeared to be a preference for structures containing Galβ1,4GlcNAc as the second and third sugar from the terminus in the glycan chain. A/California preferentially bound biantennary glycans present on the microarrays over identical monoantennary glycan chains (Fig. 6, glycans 1 and 2), but curiously, the biantennary glycan chain did not require a terminal sialic acid on both chains to increase binding (Fig. 6C, glycans 1, 3, and 6). This could indicate that the sialic acid on an unbound branch of the biantennary glycan does not increase avidity, but rather increases steric hindrance with the globular head of HA.

A/Mexico/INDRE4487/2009 (A/Mexico) was isolated from a 29-year-old female with a severe URT infection (Belser et al., 2010). A/Mexico showed a similar overall binding phenotype to A/California with a few notable differences. Again, a high preference for glycans having terminal α2,6 SA, was observed, but in some cases, binding to monoantennary glycans with terminal α2,3 SA was visualized above background (Fig. 4E). In each case where A/Mexico bound a monoantennary glycan having a terminal α2,3 SA, a fucose was branched off of the third sugar, GlcNAc (data not shown). Interestingly, the data indicated a preferential binding of A/Mexico to glycans having a Galβ1,4GlcNac linkage at the fourth and fifth sugar, as opposed to a Galβ1,3GlcNAc at the same location (data not shown).

The A/Texas/15/2009 virus (A/Texas) was isolated from a fatal influenza infection of a 1-year-old pediatric patient (Belser et al., 2010). A/Texas bound similar glycans as A/Mexico, but among the human pdmH1N1 strains examined, it showed the greatest capacity for binding to α2,3 SA glycans (Fig. 4F). Similar to A/Mexico, all bound glycans with a terminal α2,3 SA also contained a branched fucose off of the third sugar, GlcNAc. Further, A/Texas bound a surprisingly large number of non-sialylated glycans present on the microarrays, most of which contained at least one fucose branched off of the third GlcNAc sugar, but the significance of this is unclear. Further studies will be needed with additional types of fucosylated glycans to explore this phenomenon.

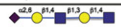
Glycan microarray analysis of seasonal H1N1 vaccine strains

Three viruses were examined to compare the receptor binding patterns of pdmH1N1 strains to those of H1N1 seasonal and vaccine strains from previous years. A/Pennsylvania/08/2008 (A/Penn) is a pre-pandemic seasonal H1N1 human isolate from October of the 2008–2009 influenza season, minimally passaged in MDCK cells. We also examined the receptor binding phenotype of vaccine strains A/Brisbane/59/2007 (A/Brisbane) and A/New Caledonia/20/1999 (A/New Cal), which were both vaccine strains for seasonal H1N1 strains in circulation prior to the pdmH1N1 outbreak, but their passage history is undocumented.

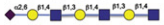
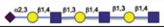
The A/Penn human isolate preferentially bound glycans having α2,6 terminal SA, but displayed some interesting characteristics that included α2,3 SA binding as well. Notable among these were a number of short, sulfated glycans with α2,3 terminal SA (Fig. 5C). In fact, eight out of twelve glycans having α2,3 terminal SA that showed significant binding by A/Penn were either sulfated or fucosylated, while the other four glycans were long biantennary structures with either α2,3/α2,6 terminal SA combination or α2,3/α2,3 SA present at the glycan termini (data not shown). In general, A/Penn bound both branched and unbranched glycans equally, provided they contained at least one terminal α2,6 SA. Of note, the glycan with the second highest level of binding was a sialylactosamine (SLN) glycan containing a sulfate and fucose branched from the same GlcNAc (Fig. 5C, glycan 2). This glycan was recently modeled into the H5 and H7 crystal structures and predicted to create additional contacts with residues K222 and K193 in H5 and H7 influenza subtypes, respectively (Nicholls et al., 2008). All of the pdmH1N1 strains and human isolates utilized in our study contain a K at position 222, but there is significant variation at residue 193 between the pdmH1N1 strains and vaccine strains (Table 1).

The highest peaks associated with A/Brisbane binding represented glycans having long, monoantennary, terminal α2,6 SA linked to poly-*N*-acetyllactosamine glycans. We compared virus binding of glycans having α2,3 or α2,6 SA, which revealed a clear preference for α2,6 SA (Fig. 6A, glycans 1–4). In a comparison of the ability of A/Brisbane to bind biantennary versus monoantennary glycans, the data showed a preference for monoantennary counterparts (Fig. 6C, glycans 1–4). This could indicate that long biantennary glycans create steric hindrance in and around the receptor binding pocket, causing reduced

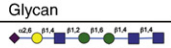
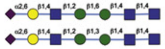
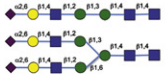
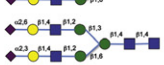
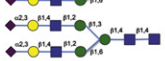
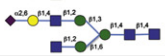
A

	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		3.8	6.6	6.0	14.3	6.4	21.1	13.4
2		2.2	2.6	0.0	0.1	0.1	0.1	0.0
3		23.6	17.9	4.4	42.4	17.0	22.7	20.2
4		2.1	2.3	1.6 (103%)	0.2	0.1	0.1	0.1

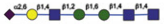
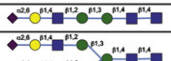
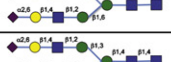
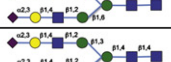
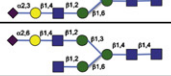
B

	Glycan	Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		9.7	14.2	6.9	6.6	14.1
2		2.3	0.0	0.0	0.0	0.0
3		28.9	10.1	14.6	24.7	15.9
4		2.2	0.3	0.0	0.0	0.0

C

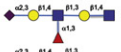
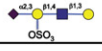
	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		4.9	4.3	4.8	1.7	0.8	0.6	2.7
2		3.2	6.6	9.0	0.8	0.4	0.3	8.0
3		1.5	3.9	8.5	3.2	1.3	6.0	16.5
4		1.9	2.5	10.2	0.7	1.3	0.3	7.1
5		2.5	1.9	3.6	0.2	2.8	0.1	13.7
6		6.7	3.4	5.7	5.0	0.4	51.4	0.1

D

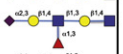
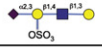
	Glycan	Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		0.8	4.6	3.4	1.2	3.3
2		0.1	2.5	2.9	0.5	9.7
3		2.1	4.3	9.0	3.3	1.3
4		0.3	3.9	4.6	2.2	5.7
5		3.4	0.1	0.1	0.1	0.1
6		3.6	2.6	2.2	1.7	7.0

Legend:

E

	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		0.8	1.7	0.1	0.1	0.1	0.1	0.0
2		2.7	2.8	1.7	0.1	0.2	0.1	0.0
3		2.2	2.6	0.0	0.1	0.1	0.1	0.0
4		4.9	3.6	0.1	0.4	0.7	0.4	0.0
5		10.7	10.3	4.4	0.2	0.2	0.1	0.0

F

		Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		0.7	0.1	0.1	0.2	0.9
2		0.2	0.0	0.0	0.0	0.6
3		2.3	0.0	0.0	0.1	0.0
4		0.3	0.1	0.0	0.2	0.2
5		13.8	0.0	0.0	0.1	8.3

Legend:

Fig. 6. Comparison of glycans bound on CFG microarrays. The fold of background is indicated. In the instance where the %CV is greater than 50%, the %CV is indicated in parenthesis. Compared glycans are all bound to the same spacer that links the glycan to the CFG slide.

binding to the receptor. Generally, the highest binding α2,3 SA receptors contained either a fucose or a sulfate near the glycan terminus, and their absence lead to a reduction in binding (Fig. 6E, glycans 1–5).

Similar to A/Brisbane, A/New Cal showed a preference for long, monoantennary glycans with a terminal α2,6 SA, and generally bound similar glycans as A/Brisbane (Figs. 4B and 5B). The increased binding of A/New Cal to fucosylated glycans was less pronounced than for that observed for A/Brisbane (Fig. 6E, glycans 1–4), but the addition of a sulfate to the sugar near a terminal α2,3 SA clearly leads to increased binding. Sequence analysis of the two strains indicated that the receptor binding site is highly conserved between the two viruses, except for a mutation, K192R, in the A/New Cal strain. Both strains contain the D190N mutation that is commonly associated with egg adaptation, and has been shown to increase binding to glycans with a terminal α2,3 SA.

A/Swine/Iowa/1976/1931 and H3N2 isolate glycan microarrays

We also determined the receptor binding phenotype of A/Swine/Iowa/1976/1931 (Sw/IA/1931), a classical swine H1N1 strain, which is a descendent of the 1918 pandemic strains that entered the North American swine population (Shope, 1936). Sw/IA/1931 bound to a mixture of glycans having α2,3 and α2,6 terminal SA (Fig. 4G) with a clear preference for long poly-*N*-acetyllactosamine glycans having α2,6 terminal SA (Fig. 6B, glycans 1 and 3), but did not bind well to monoantennary glycans containing mannose sugars (Fig. 6D, glycans 1 and 2). Because the passage history of this virus is unknown, these results are most likely representative of binding patterns acquired during multiple passages in animals, eggs and/or tissue culture (Francis, 1937; Horsfall et al., 1941).

Finally, we examined the receptor binding phenotype of a swine H3N2 influenza isolate, A/swine/Minnesota/02719/2009 (swine/02719). Similar to the H1N1 isolates, this strain also preferentially bound glycans having α2,6 terminal SA (Fig. 3D). Interestingly, binding to a branched glycan having terminal α2,6/α2,6 SA was decreased relative to the binding observed for a monoantennary glycan with similar sugar composition having a terminal α2,6 SA; however, a branched glycan having α2,6/α2,3 SA at the termini did not exhibit a similar decrease in binding ability (Fig. 6D, glycans 1–4). Significant binding to monoantennary glycans having α2,3 terminal SA was only visualized when a sulfate was present (Fig. 6F, glycans 1 and 5). This is in contrast to binding of our H3 Aichi laboratory adapted strain, which bound primarily α2,6 linked sialic acid, but was still capable of binding biantennary glycans having α2,3/α2,3 or α2,3/α2,6 SA at their termini (Fig. 4H and Fig. 6C, glycans 4 and 5).

Binding of pdmH1N1 strains to various cell monolayers

The type and distribution of cell-surface glycans vary from species to species, and are known to vary among tissue culture cell lines. As a complementary assay, we compared the relative binding of the Alexafluor 488 labeled pdmH1N1 and swine isolates to two different cell lines commonly used to propagate influenza viruses: MDCK cells, a widely-utilized canine kidney cell line, and A549 cells, a human lung carcinoma cell line. Viruses were adsorbed to cell monolayers, washed, and binding was quantified by fluorimetry. Each strain was normalized to the WT level of binding, and is shown as a percentage of the laboratory strain, H3N1 WT. Fig. 7A shows that the laboratory-adapted H3N1 strain bound extremely well to MDCK cells. In contrast, H1N1 pdmH1N1 isolates and the swine/02749 isolate bound to MDCK

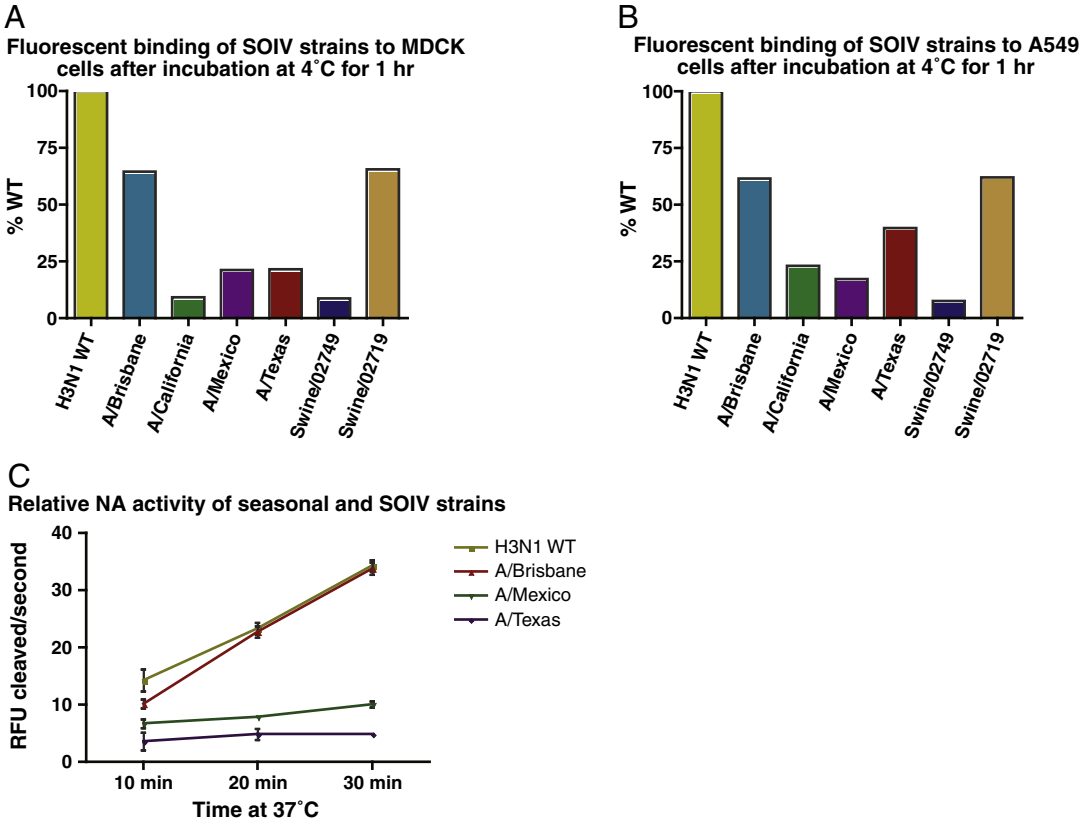


Fig. 7. Fluorescent binding and neuraminidase activity assays of influenza isolates. For the fluorescent binding assay, Alexa488 labeled influenza virus was incubated at an MOI = 3.0 for 1 hour at 4 °C, washed and scanned. Neuraminidase activity assays were performed using 0.01 mg MUNANA and 10^4 PFU of virus. Fluorescence was determined at 10 min, 20 min, and 30 min. Panels—Fluorescent binding results in panel (A) MDCK and (B) A549. Panel C—Neuraminidase assay with influenza isolates.

cells at less than 25% of WT levels (Fig. 7A). Showing intermediate levels of binding were A/Brisbane and swine/02719 strains, which bound 64.6% and 65.5% of WT, respectively. A comparison of relative binding to A549 cells shows a similar pattern as that observed on MDCK cells. The primary differences were that overall binding by all viruses was increased in A549 cells (data not shown) and that increased binding was observed for A/California and A/Texas strains. The fact that A/Brisbane bound both MDCK and A549 cells greater than any of the pdmH1N1 isolates is largely consistent with the increased number of glycans bound on the glycan microarrays.

Relative NA activity of pdmH1N1 strains

As mentioned previously, the HA/NA functional balance has been shown to be critical for viral fitness and propagation. In order to compare the relative neuraminidase activity of pdmH1N1 strains, we utilized the fluorescent chemiluminescent substrate methylumbelliferone N-acetyl-neuraminic acid MUNANA, which fluoresces when cleaved by a neuraminidase. Ten thousand (10^4) PFU of virus was diluted with PBS and MUNANA substrate into cold 96-well plates. Plates were then incubated at 37 °C for 10, 20, or 30 min, at which time the reactions were stopped and assayed by fluorimetry. Interestingly, the A/Brisbane and H3N1 WT had increased levels of NA activity relative to the two pdmH1N1 strains (Fig. 7C). Sequence alignments indicated few mutations between the pdmH1N1 NA proteins, and all catalytic sites remained identical between pdmH1N1 strains and A/Brisbane (Colman et al., 1983). However, a recent structural analysis of a 2009 H1N1 pdmH1N1 isolate revealed that a V149I mutation in the NA of pdmH1N1 isolates alters the 150 loop, and appears to form a smaller catalytic pocket. This alteration could affect the highly conserved catalytic residues D151 and R152 (Li et al., 2010) which would most likely affect NA activity.

Discussion

The 1997 emergence of highly pathogenic strains of avian H5N1 viruses and their sporadic transmission to humans raised serious concerns over the genesis of a new human pandemic, but fortunately, these strains have yet to establish themselves in the human population, or transmit readily from person to person (Miller et al., 2009). By contrast, the current H1N1 pdmH1N1 strains spread rapidly in the human population from the time they were first detected, and do not appear to be the result of multiple introduction events (Garten et al., 2009). Understanding the molecular determinants necessary for the adaptation of influenza viruses to humans is essential to improved pandemic preparedness. In particular, the role of HA-mediated receptor binding should always be examined, as this property is one of the first obstacles to overcome for adaptation to a new species. Here, we examined the receptor binding properties of H1N1 pdmH1N1 strains currently circulating in the human population, and compared them to earlier seasonal H1N1 strains, as well as to trH1N1 swine influenza isolates. Swine are of particular interest to the ecology and emergence of influenza viruses, as that they are capable of propagating viruses of both human and avian origins (Scholtissek et al., 1983), which allows the segmented influenza genome to reassort in co-infected animals, potentially giving rise to novel influenza viruses.

The early pdmH1N1 isolates examined almost exclusively recognized α2,6-linked sialic acids. For the A/California virus, the only example of binding to a glycan having α2,3 SA is with a glycan that also contained a terminal α2,6 SA (Fig. 6C, glycan 4 and data not shown). Binding of glycans having terminal α2,3 SA by both A/Texas and A/Mexico was highly correlated with the presence of fucose, since all monoantennary α2,3 SA glycans bound by A/Mexico and A/Texas were fucosylated. The 2008 H1N1 seasonal strain, A/Penn, showed a preference for glycans having a terminal α2,6 SA as well, but demonstrated a greater ability to recognize glycans with terminal α2,3 SA than the pdmH1N1 strains that we examined. However, similar to A/Texas and A/Mexico, the majority of the glycans containing terminal α2,3 SA that were bound by A/Penn contained either a sulfate, fucose, or both.

The two glycans that were bound at the highest levels by the pdmH1N1 isolates and the A/Penn isolate were a simple SLN and a seven-sugar, monoantennary, sialylated poly-*N*-acetyllactosamine glycan that contained two additional fucose residues at the fifth and seventh positions in the glycan, both having terminal α2,6 SA (Fig. 5C glycan 5, Fig. 5D glycan 3, Fig. 5E glycan 3, and Fig. 5F glycan 2). Both of these glycans were recently shown to bind the majority of both egg-adapted and MDCK-passaged H3N2 isolates (Stevens et al., 2010). The possibility exists that if the pdmH1N1 HA receptor binding affinity is relatively weak, as may be indicated by the relatively low binding to MDCK and A549 cells shown in Fig. 7A and B, the addition of a fucose could lead to greater interaction with residues in the 220 loop, which has been visualized previously in modeling experiments (Nicholls et al., 2008).

Interestingly, the H3 subtype MN/02719 was the only swine isolate to not bind the monoantennary seven-sugar glycan with fucoses linked to the 5th and 7th sugar residues at levels above background (depicted in Fig. 5I, glycan 1). The other H3 virus used in this study, the laboratory strain H3N1 WT, also did not bind the structure, which could indicate a subtype-specific binding preference. Swine isolates generally had the same pattern of binding as pdmH1N1 strains, consistently binding short SLN glycans (i.e., Fig. 5J, glycans 2, 3, and 5). These isolates also preferred glycans with terminal α2,6 SA versus α2,3 SA (Fig. 6B, compare glycans 1 and 2, 3 and 4). The near absence of α2,3 SA binding by the swine isolates utilized in this study is interesting considering the presence of both α2,3 and α2,6 SA in the swine respiratory tract (Van Poucke et al., 2010). This could indicate that a reassortment event occurring in the swine respiratory tract could select for variants that preferentially bind α2,6 SA. However, we note that the small sample size tested on these glycan microarrays may not be representative of all circulating swine influenza isolates in North America or Europe.

Overall, the results confirm the hypothesis that human isolates generally adopt high specificity for α2,6-linked terminal SA, and that this is probably favored over mixed or dual α2,3 and α2,6 SA binding capacity. Previous glycan microarray binding experiments performed with seasonal H3N2 isolates have shown a marked preference for glycans with terminal α2,6 SA (Kumari et al., 2007), and seasonal non-egg-adapted H1N1 isolates bind α2,6 SLN over α2,3 sialyllactose (Gambaryan et al., 1999, 1997). The vaccine strains, A/Brisbane and A/New Cal, bound to glycans having both α2,3 and α2,6 terminal SA, but this is likely due to repeated passage through eggs, rather than an accurate reflection of the binding preferences of the original strain. We obtained similar results for A/Swine/1976/1931 virus, the classical swine influenza isolate, and this also is likely to reflect an extensive laboratory passage history that included replication in eggs. Although it has been known for decades (Burnet and Bull, 1943), these results emphasize the importance of passage history for any viruses to be analyzed for receptor binding properties. Even minimal passage in eggs is likely to select for mutants with altered binding profiles (Burnet and Bull, 1943; Gambaryan et al., 1999; Katz et al., 1990; Katz and Webster, 1988; Lugovtsev et al., 2009; Robertson et al., 1987, 1985; Stevens et al., 2010). Although the use of MDCK cells may not be the ideal substitute for amplification of virus stocks, it would appear to be preferable to eggs for binding studies, at least for human and swine isolates (Stevens et al., 2010).

Data from numerous laboratory studies, and observations of evolving phenotypes of early strains in the 1957 and 1968 pandemics indicate that the HA and NA proteins operate in a reciprocal balance due to their opposing functions (Mitnaul et al., 2000; Wagner et al., 2002, 2000). Viruses with a tightly binding HA coupled with a weak NA protein have difficulty releasing from cell membranes upon viral release, while poorly binding HA and a strongly active NA may prevent efficient binding to glycans present on the cell membrane. In experiments examining NA activity, we found that pdmH1N1 isolates had relatively weak neuraminidase activity, indicating that the HA

and NA functions are fairly well matched, and indicative of the lengthy period of time the pdmH1N1 strains have been predicted to be circulating undetected in nature (Smith et al., 2009). The role of the neuraminidase in viral infection has been linked to cleaving sialic acids present on the cell surface, as well as in releasing mucin bound virus. Human mucins have been shown to contain a abundance of glycans having α2,3 terminal SA and are known to be inhibitory to influenza strains (Burnet, 1948; Couceiro et al., 1993). These data, in combination with the predominance of α2,6 SA found in the upper respiratory tract of humans (Shinya et al., 2006), could indicate a selective advantage for strains that bind strictly to glycans containing terminal α2,6 SA over those with dual specificity. Presumably, virus strains that bind glycans containing either α2,3 or α2,6-linked terminal SA can evade the upper respiratory mucins with a weakly binding HA, but the lack of glycans containing α2,3 SA in the upper respiratory tract could lead to poor transmission capabilities (Maines et al., 2006; Shinya et al., 2006).

The overall binding levels observed for pdmH1N1 strains and contemporary swine H1N1 viruses seemed to be low relative to H3N1 WT and vaccine isolates. This was reflected in our fluorescent binding assays, and we also observed high PFU:HA titer ratios for these viruses (data not shown). Glycosylation in the globular head of HA has been associated with weaker binding (Wagner et al., 2000), but in the pdmH1N1 strains, no additional glycosylation is present that could cause steric hindrance with any potential receptor (Igarashi et al., 2010). Alternatively, the possibility exists that the strains are binding to host glycans that are not present on the microarray. The current glycan microarray consists of approximately 500 glycans, but the overall mammalian glycome is likely to be much larger with a larger set of glycan determinants (Cummings, 2009). In addition, swine, unlike humans, express Neu5Gc on their cell membranes, and there is evidence that swine, but not human influenza strains recognize Neu5Gc (Suzuki et al., 1997). Because the glycan microarrays used in this study contain fewer than ten Neu5Gc glycans, an absence of binding may not necessarily indicate an inability to bind. However, the weak binding of pdmH1N1 isolates observed in our studies may, in fact, be reflective of their inherent biology. Weak HA binding might confer a selective advantage with regard to transmission efficiency, as there are examples with other viruses of weaker binding allowing for greater dissemination, systemic spread, and increased pathogenicity within a host animal (Byrnes and Griffin, 2000; Stehle and Harrison, 1996).

The viruses examined here differ at several positions in and around the glycan receptor binding site (Table 1 and Fig. 1), although it is not currently possible to directly correlate binding properties to the presence of particular residues at specific HA positions. For H1 viruses such as those responsible for the 1918 pandemic, mutations at positions 190 and 225 have been shown to heavily influence a strains ability to bind glycans having α2,3 versus α2,6 terminal SA; however, these residues are unchanged between the A/Penn, pdmH1N1, and trH1N1 isolates (Table 1). Residues in the receptor binding site that are altered include 133, 136, 138, 192, 193, 219, 227, and 230 (H3 numbering). With the exception of 136 and 138, most of these residues do not contact sialic acid directly but may interact with contact residues, or potentially interact with internal sugars of the glycan receptor. In the case of glycans with α2,6 SA, mutations at 219 and 227 are thought to interact with internal sugar residues in the glycan, as are residues 192 and 193, which were recently modeled to interact with a Glc5 sugar in glycans similar to those found in Fig. 6A (Maines et al., 2009). Structural interpretations for binding to glycans with α2,3 SA are more difficult to determine, largely due to the reduced electron density for such receptors beyond the 3rd sugar residue in HA receptor complexes (Gamblin et al., 2004). However, as discussed previously, a sulfate or fucose on the 3rd sugar residue of a receptor could potentially interact with the residues at 222 or 193. Another of these mutations, I230M, has been observed previously by selection in mice during passage of an H3 Y98F mutant with reduced receptor binding activity (Meisner et al., 2008). This residue is considered to be a "second shell" mutation, in that it does not directly interact with sialic acid, but appears to interact with the Y98 residue, potentially altering the structure of the glycan binding pocket. It is interesting that the two strains associated with severe disease, A/Texas and A/Mexico, bound several glycans with terminal α2,3 SA. Although increased α2,3 binding has been associated with higher pathogenicity in the past (Hatta et al., 2001; Liu et al., 2010; Shinya et al., 2005) and has been observed to correlate in one case with changes at position 225 (Chen et al., 2010), differences in patient age, as well as unknown patient history, make concrete conclusions relating to pathogenicity difficult to determine.

Increased surveillance and improved techniques have vastly increased our ability to identify potential pandemic strains in swine and avian species. However, the combinations of conditions necessary for a strain to efficiently transmit to humans from another species are not well understood. Influenza infection and transmission in a new host species are clearly a multifactorial trait, but as the HA and NA are critical to the initial events of infection and transmission, it is important to examine the characteristics of early pandemic isolates and compare them to closely related strains. Such studies may make it possible to identify critical properties of viruses with a greater likelihood for initiating new pandemics.

Materials and methods

Viruses and cells

Influenza virus stocks for glycan microarray analysis were grown in cell culture using standard methods. In brief, Madin-Darby Canine Kidney (MDCK) cells (ATCC, Manassas, VA) were seeded into 75 cm^2 or 175 cm^2 tissue culture flasks and grown in DMEM (Hyclone, Logan, UT), 5% FBS, 2 mM L-glutamine until approximately 90% confluent (~8×10^6 cells total). Cells were rinsed twice with 1× PBS and 5 ml MEM (Hyclone) with TPCK-treated trypsin (10 μg/ml, Worthington Biochemical, Lakewood, NJ) was added to each flask. Original stock virus was added at 1:100 dilution in 5 ml MEM/TPCK trypsin and incubated 2–4 hours at 37 °C, 5% CO_2 before an additional 5 ml of MEM/TPCK trypsin was added. Infection continued for 48–72 hours and was monitored for CPE. Supernatant was harvested and centrifuged at 1000 rpm, 5 min to clarify cell debris. Virus titer was evaluated by a hemagglutination (HA) and plaque assay and/or $TCID_{50}$ on MDCK cells. Viral RNA was purified using a QiaAmp Viral RNA purification kit (Qiagen), and reverse transcribed using SuperScript III 1st strand synthesis (Invitrogen). The HA and NA genes were amplified using HA- or NA-specific primers designed according to published sequences on the Influenza Research Database (www.fludb.org).

The human influenza viruses used in these studies were kindly provided by Drs. Alexander Klimov and Xiyan Xu at the Centers for Disease Control and Prevention (Atlanta, GA): A/California/04/09, A/Mexico/INDRE4487/09, A/Texas/15/09, A/Brisbane/59/2007, A/New Caledonia/20/99, and A/Pennsylvania/08/2008. The recent swine influenza viruses used in this study were isolated by the University of Minnesota Veterinary Diagnostic Laboratory (Saint Paul, MN): A/Swine/MN/02719/2009, A/Swine/MN/02749/2009, A/Swine/MN/0251/2009, A/Swine/IL/02861/2009. The classical swine H1N1 A/Swine/1976/31 was kindly provided by Dr. Richard Webby (St. Jude Children's research Hospital, Memphis, TN).

Virus purification

Harvested virus was purified through a 25% sucrose cushion in NTE buffer, consisting of 100 mM NaCl, 10 mM Tris buffer, and 1 mM EDTA. Viruses were centrifuged for 3 hours at 28,000 rpm, resuspended in NTE buffer, and kept frozen at −80 °C until needed.

PdmH1N1 labeling

Purified influenza strains were labeled with the amine reactive dye Alexafluor488 (Invitrogen). Twenty-five micrograms of amine reactive Alexa 488 was incubated with 100 µl of virus and 1 M NaHCO3 (pH 9.0) for 1 hour. The labeled virus was dialyzed overnight in a 7000 MWCO Slide-A-Lyzer MINI dialysis unit (Thermo Scientific) against PBS + 1 mM EDTA, and binding experiments were carried out by members of the CFG Core H facility. HA titers were monitored before and after labeling to ensure labeling did not alter receptor binding capabilities. Viral titers for glycan microarray studies were as follows: A/Penn—6.2×10^6 PFU/ml; A/California—1.9×10^7 PFU/ml; A/Mexico—4.3×10^6 PFU/ml; A/Texas—1.4×10^7 PFU/ml; A/Brisbane—1.7x10^6 PFU/ml; A/New Cal—3.2×10^7 PFU/ml; Sw/IA/1976—4.7×10^6; MN/02719—2.6×10^6; MN/02749—1.8×10^7 PFU/ml; MN/02751—2.4×10^6 PFU/ml; IL/02860—1.8×10^5 PFU/ml.

Glycan microarrays and analysis

Core H of the Consortium of Functional Glycomics performed glycan microarray binding experiments (www.functionalglycomics.org) using CFG array version 4.0 or 4.1. Briefly, 70 µl of fluorescently labeled virus was incubated on a glycan microarray slide at 4 °C for 1 hour, prior to scanning by a Perkin-Elmer ProScanAray. Significant peaks were determined by averaging the relative fluorescent units (RFU) of all glycans on the microarray, which was then multiplied by two to determine the background RFU level. Finally, any glycans above background with a %CV of greater than 50% were not considered to be significant.

Fluorescent binding assays

Live influenza viruses were labeled in the same manner as strains prepared for glycan microarrays. Fully confluent MDCK, A549 and PT-K75 cell lines were chilled at 4 °C for 1 hour prior to influenza binding. 10^4 PFU of labeled virus was bound and incubated on chilled cell lines at 4 °C for 1 hour, washed 3 times with cold PBS, and scanned using a Biotek Synergy 2 fluorimeter using a bottom optics position and excitation/emission of 485/528.

NA activity assays

Cold 96-well plates were kept on ice while cold PBS, 10^4 PFU of virus, and 0.01 mg MUNANA (Invitrogen) were added to each well. Plates were placed in a 37 °C incubator and reactions stopped at 10, 20, and 30 min using 1 M Glycine (pH 10.7). After the stop solution was added, plates were scanned using a Biotek Synergy 2 fluorimeter using an excitation/emission of 360/460 respectively. Each virus at each time point was performed in triplicate and the fluorescence from a mock well containing only PBS and 0.01 mg MUNANA was subtracted from the mean fluorescence obtained from each condition tested. NA activity was determined by subtracting the mock well from the virus-treated well, and divided by the number of seconds passed since the plates had been place at 37 °C.

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