

Conformation and Linkage Studies of Specific Oligosaccharides Related to H1N1, H5N1, and Human Flu for Developing the Second Tamiflu

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Abstract

The interaction between viral HA (hemagglutinin) and oligosaccharide of the host plays an important role in the infection and transmission of avian and human flu viruses. Until now, this interaction has been classified by sialyl(α 2-3) or sialyl(α 2-6) linkage specificity of oligosaccharide moieties for avian or human virus, respectively. In the case of H5N1 and newly mutated flu viruses, classification based on the linkage type does not correlate with human infection and human-to-human transmission of these viruses. It is newly suggested that flu infection and transmission to humans require high affinity binding to the extended conformation with long length sialyl(α 2-6)galactose containing oligosaccharides. On the other hand, the avian flu virus requires folded conformation with sialyl(α 2-3) or short length sialyl(α 2-6) containing trisaccharides. This suggests a potential future direction for the development of new species-specific antiviral drugs to prevent and treat pandemic flu.

Key Words: Conformation, Linkage type, Oligosaccharide, H1N1, H5N1, Tamiflu

INTRODUCTION

Yearly occurring epidemics are normally due to human-adapted viruses, such as H1N1, H3N2, or influenza B strains; while pandemics occurred in 1918 (Spanish flu), 1957 (Asian flu), 1968 (Hong Kong flu) and 2009 (Reassorted H1N1 flu) due to antigenic shift or accumulated antigenic drift. Each threatened flu pandemic was responsible for about 20~50 million deaths in the world (Belshe, 2005; Tumpey *et al.*, 2005; Hayden and Pavia, 2006; Perrone and Tumpey 2007; Collins *et al.*, 2008; Pappas *et al.*, 2010). In the spring of 2013, the H7N9 viruses evolved by reassortment of genes from viruses found in Korean wild birds, Beijing bramblings, and Zhejiang duck (Cohen, 2013; Hvistendahl *et al.*, 2013). Recently, concerns have also been raised over the possibility of new influenza pandemics from H5N1, H7N7, H7N9, and H9N2 avian virus subtypes and their reassorted or mutated viruses (Ison, 2011; Samson *et al.*, 2013). Recently, numerous avian H9N2 viruses in South East and Middle East Asia and H7N2 viruses in North America have acquired human-type receptor binding specificity through antigenic drift (Belser *et al.*, 2008; Imai and Kawaoka, 2012). The newly raised and mutated viruses

have become resistant to the actions of commercially available antiviral drugs, such as adamantanes (amantadine and rimantadine) and oseltamivir (Tamiflu). The naturally occurring Tamiflu-resistant H1N1 influenza viruses had already spread globally in humans by 2009 (Gubareva *et al.*, 2000; Laver, 2006; Srinivasan *et al.*, 2008). Influenza viruses H1N1pdm09 and highly pathogenic H5N1 strains were reported to be oseltamivir resistant due to point mutation, such as H274Y (N2 numbering system) or H275Y (the N1 numbering system) (Baz *et al.*, 2009; Memoli *et al.*, 2010; Lee and Yen, 2012; Jimenez-Alberto *et al.*, 2013; Mair *et al.*, 2014). Since 2006, the M2 ion channel blocker, adamantanes, has been associated with widespread resistance among H1N1 (2009), seasonal H1N1, and H3N2 viruses (Stoner *et al.*, 2010; Puzelli *et al.*, 2011). Therefore, it is urgently necessary to develop new classes of antiviral drugs to prevent and treat threatening transmission and infections by Tamiflu- and/or amantadine-resistant viruses.

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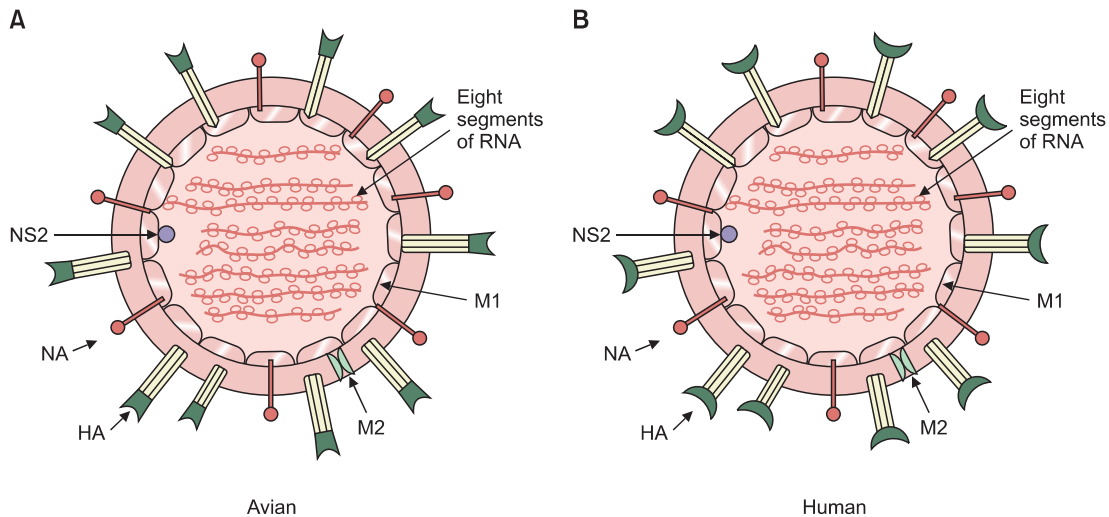


Fig. 1. Schematic representation of influenza A virion (A) Avian influenza A virus (B) Human influenza A virus. HA (hemagglutinin) trimers, NA (neuraminidase) tetramers, and M1 (matrix protein) are spike-like proteins on the surface of the influenza A virus. M2 protein is the target of the antiviral adamantanes. NS2 is one of interior proteins. The interior of the virion contains eight RNA segments.

ANTIGENIC SHIFT AND DRIFT CAUSING MUTATIONS OF INFLUENZA VIRUSES

Influenza viruses are RNA viruses that belong to the *Orthomyxoviridae* family. They include influenza A, B, and C viruses. Among these viruses, influenza A virus has various hosts, including migratory water birds, domestic birds, swine, horses, fowl, dogs, cats, and also humans (Stevens *et al.*, 2006a; Taylo and Drickame, 2011; Imai and Kawaoka, 2012; Rumschlag-Booms and Rong, 2013). Due to the diversity of hosts, influenza A viruses can reassort, mutate, and transmit among a wide range of hosts and also contribute to annual epidemics, as well as to global pandemics. The influenza A virus carries eight single stranded RNA segments, as shown in Fig. 1 (Gubareva *et al.*, 2000; Taylo and Drickame, 2011).

Due to the segmented nature of the viral RNA, the gene segments of one virus can reassort with those of another virus during replication in the infected host cell and form new pathogenic flu viruses. This reassortment event is referred to as antigenic shift (Layne *et al.*, 2009; Yoo, 2011; Rumschlag-Booms and Rong, 2013). The newly formed virus can be especially dangerous if a human-adapted virus, such as H1N1 or H3N2, acquires genes from a highly pathogenic strain, such as H5N1 (Fig. 2). Antigenic drift is another mutation factor of influenza viruses because they have characteristics as flu viral RNA polymerase. Unlike the DNA polymerase, RNA polymerase does not have a proofreading function for when a genomic error occurs. Therefore, influenza virus can accumulate mutations within its genome during replication (Rumschlag-Booms and Rong, 2013). If a highly pathogenic avian virus acquires the ability to enter and replicate in humans through numerous subtle mutations, the virus also becomes highly pathogenic to humans.

The major four influenza pandemics that occurred in 1918, 1957, 1968, and 2009 are thought to have mainly arisen via antigenic shift and drift. The pandemic of 1957, caused by the "Asian Influenza" H2N2 virus, was mutated by the reassortment of an avian virus with a human virus (Tumpey *et*

al., 2005; Collins *et al.*, 2008; Rumschlag-Booms and Rong, 2013). A similar case was seen with the pandemic of 1968, referred to as the "Hong Kong influenza." The HA gene of this virus occurred through the reassortment of human-adapted H3 strain genes and one of eight RNA segments of the avian virus (Neumann and Kawaoka, 2006; Neumann *et al.*, 2009). The 2009 pandemic H1N1 was caused by a reassorted swine strain. Swine acted as an intermediate host for the genetic mixing of avian, swine, and human influenza viruses. Several intermediate species-such as pig, duck, quail, and chicken-may play an important role in the emergence of novel influenza viruses capable of causing a human pandemic (Stevens *et al.*, 2004; Layne *et al.*, 2009; Xu *et al.*, 2012).

LINKAGE SPECIFICITY OF AVIAN- AND HUMAN-INFLUENZA VIRUSES

Many viruses, including influenza viruses, recognize specific oligosaccharides containing sialic acid to attach to the host. The viral surface has two major surface spike-like glycoproteins: HA (hemagglutinin) and NA (neuraminidase), as shown in Fig. 1. HA exists four or five times more abundantly than NA on the virion surface. HA uses a membrane fusion activity, as well as a sialic-acid-binding activity, to initiate the process of entering the host cell; while NA can release the newly formed virus particles from an infected cell (Xu *et al.*, 2012).

Based on structural studies of the highly conserved catalytic site of influenza NA (Colman *et al.*, 1983; Varghese *et al.*, 1983), several NA inhibitors were developed as competitive inhibitors. There are currently two kinds of neuraminidase inhibitors approved worldwide (oseltamivir and zanamivir), and two other NA inhibitors (laninamivir and peramivir) are approved in Japan and Korea but are under investigation elsewhere (Ison, 2011; Lee and Yen, 2012; Clercq, 2013). Because oseltamivir has a large hydrophobic side chain, the binding of oseltamivir to influenza NA requires a conformational change in the side chain of E276 (Collins *et al.*, 2008). In contrast, the molecu-

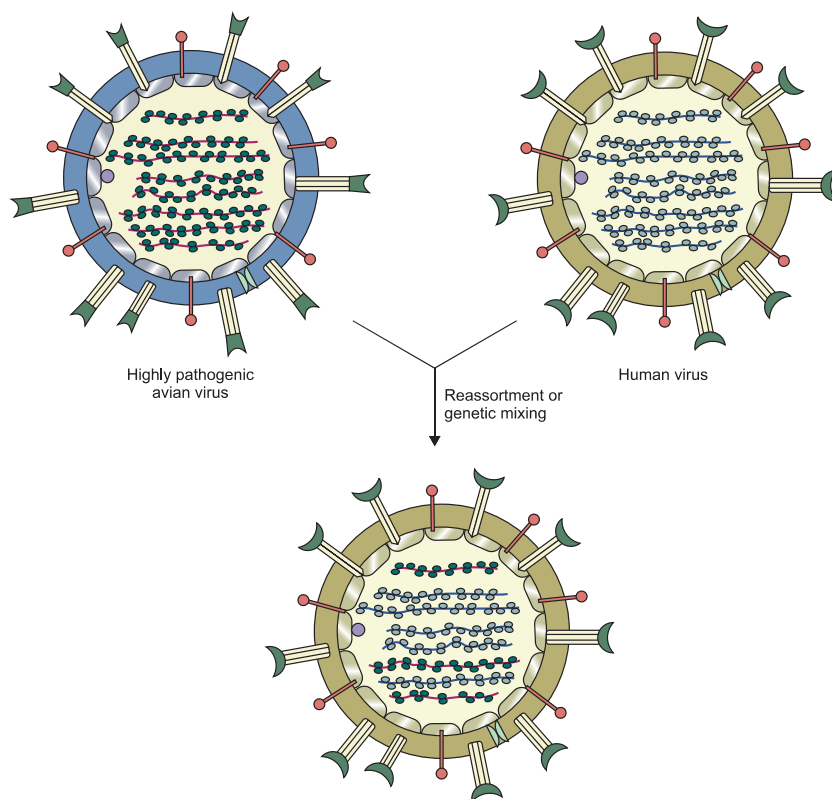


Fig. 2. Schematic representation of antigenic shift or reassortment. Due to the segmented nature of the viral RNA, the gene segments of one virus can reassort with those of another virus during replication in the infected host cell and form new pathogenic flu viruses. The newly formed virus can be especially dangerous if a human adapted virus, such as H1N1 or H3N2, acquires genes from a highly pathogenic strain, such as H5N1.

lar structure of zanamivir includes a guanidine group instead of the hydrophobic group. The guanidine group interacts with the conserved E119 residue without undergoing a side chain conformational change (Zurcher *et al.*, 2006). Peramivir is a cyclopentane derivative with a guanidinyll group similar to that of zanamivir and a hydrophobic group similar to that of oseltamivir (Babu *et al.*, 2000; Bantia *et al.*, 2001). Laninamivir is the active product of the esterified octanoate CS-8958 and has binding properties similar to zanamivir (Vavricka *et al.*, 2011). Due to differences in the modes of action between oseltamivir (Tamiflu) and zanamivir (Relenza), oseltamivir-resistant mutations do not confer resistance to zanamivir or laninamivir (Hayden, 2009). Recently, because of increasing levels of resistance to both oseltamivir (Tamiflu) and zanamivir (Relenza), the development of alternative anti-influenza drugs based on HA or other components of the virus are urgently needed.

The viral glycoprotein HA binds to sialic-acid-containing oligosaccharide moieties to initiate endocytosis. There are various kinds of sialic-acid-containing oligosaccharides in nature, such as sialic acids containing (α 2-3) or (α 2-6) linked to Gal (galactose) or GalNAc (N-acetyl galactosamine), sialic-acid-containing (α 2-6) linked to GlcNAc (N-acetyl glucosamine), or sialic-acid-(Neu5Ac: N-acetyl neuraminic acid) containing (α 2-8) linked to another sialic acid residue. Among these oligosaccharides, influenza viruses do not bind to Neu5Ac(α 2-8)-linked oligosaccharides (Layne *et al.*, 2009). They can recognize only sialyl(α 2-3)- or sialyl(α 2-6)-linked disaccharide

moieties, such as Neu5Ac(α 2-3)Gal or Neu5Ac(α 2-6)Gal, Neu5Ac(α 2-3)GalNAc or Neu5Ac(α 2-6)GalNAc, and Neu5Ac(α 2-6)GlcNAc-containing oligosaccharides (Paulson *et al.*, 2006; Taylo and Drickame, 2011).

Until now, the general paradigm for the determination of avian- and human-virus infection is that human-flu viruses preferentially bind to sialyl(α 2-6)-linked disaccharides, whereas avian flu viruses bind to sialyl(α 2-3)-linked disaccharide moieties of the host receptor binding sites (Paulson *et al.*, 2006; Viswanathan *et al.*, 2010). In the case of H5N1, H7N9, and other mutated flu viruses, classification based on linkage specificity does not correlate with the tendencies of human infection and intra- and inter-species transmissions of flu viruses.

Even though the highly pathogenic H5N1 viruses show strong sialyl(α 2-6)-binding property with weak sialyl(α 2-3)-binding property, they have shown inefficient human infection and human-to-human transmission (Hatta *et al.*, 2001; Nguyen *et al.*, 2005; Russel *et al.*, 2006; Stevens *et al.*, 2006b; Nicholls *et al.*, 2007; Haselhorst *et al.*, 2008). NY18 H1N1, a mixed sialyl(α 2-3) and sialyl(α 2-6)-binding virus, is not transmitted from avians to humans efficiently. On the contrary, Tx91 H1N1, another mixed sialyl(α 2-3) and (α 2-6)-binding virus, is capable of efficient avian-to-human transmission and human-to-human transmission. These mismatched results will be explained by other characteristics of oligosaccharides at the receptor binding sites of the hosts.

Table 1. Correlation between binding specificities and adaptation to humans according to influenza virus types

Virus Strain	Linkage type	Adaptation	Conformation	Ref
H3N2	Long ($\alpha 2-6$)	Human adapted virus	Extended conformation	Matrosovich <i>et al.</i> , 2013 Xu <i>et al.</i> , 2009
H1N1	Long ($\alpha 2-6$)	Human adapted virus	Extended conformation	Chandrasekaran <i>et al.</i> , 2008 Xu <i>et al.</i> , 2008 Xu <i>et al.</i> , 2009
H5N1	Short ($\alpha 2-6$)	Avian virus	Folded conformation	Chandrasekaran <i>et al.</i> , 2008 Wang and Zheng, 2009 Watanabe <i>et al.</i> , 2012
Tx92	Long ($\alpha 2-6$)	Human adapted virus	Extended conformation	Chandrasekaran <i>et al.</i> , 2008 Xu <i>et al.</i> , 2009 Yen <i>et al.</i> , 2007
Sc18	Long ($\alpha 2-6$)	Human adapted virus	Extended conformation	Chandrasekaran <i>et al.</i> , 2008 Xu <i>et al.</i> , 2009 Yen <i>et al.</i> , 2007
Av18	($\alpha 2-3$)	Avian virus	Folded conformation	Bateman <i>et al.</i> , 2010 Chandrasekaran <i>et al.</i> , 2008 Gambaryan <i>et al.</i> , 2005 Xu <i>et al.</i> , 2009 Yen <i>et al.</i> , 2007
NY18	Short ($\alpha 2-6$)	No transmitted to human	Extended conformation	Chandrasekaran <i>et al.</i> , 2008 Gambaryan <i>et al.</i> , 2005 Yen <i>et al.</i> , 2007

CONFORMATIONAL SPECIFICITY OF AVIAN- AND HUMAN-FLU VIRUSES

Claas *et al.* (1998) suggested early on that the size and shape of glycan receptors, rather than the specific linkage type, are important determinants for human adaptation of influenza A viruses by using HA-glycan conformational analysis and glycan-binding assays. Until now, several researchers have found that human-adapted viruses prefer to bind with longer oligosaccharide chains (Kumari *et al.*, 2007; Stevens, *et al.*, 2008; Shriver *et al.*, 2009; Viswanathan *et al.*, 2010; Chen *et al.*, 2012; Yamada *et al.*, 2012). Crystal structure analysis has revealed that human H3 HA has a wider receptor-binding pocket than does avian H5 HA. This property may allow frequent opportunities for H3 HA to make contact with the long $\alpha 2-6$ oligosaccharide receptors (Ha *et al.*, 2001).

Chandrasekaran *et al.* found that H5N1 viruses can bind with short length sialyl($\alpha 2-6$)-linked trisaccharides such as sialyl($\alpha 2-6$)Gal($\beta 1-3$)Glc or sialyl($\alpha 2-6$)Gal($\beta 1-3$)GlcNAc, as well as sialyl($\alpha 2-3$)-linked trisaccharides-but cannot bind with long length sialyl($\alpha 2-6$)oligosaccharides greater than tetrasaccharides, such as Neu5Ac($\alpha 2-6$)(Gal($\beta 1-3/4$)GlcNAc)_n, where n is greater than 2 (Chandrasekaran *et al.*, 2008). NY18 H1N1 also binds with short length sialyl($\alpha 2-6$)-linked trisaccharides but does not have HA-binding specificity to long length sialyl($\alpha 2-6$)oligosaccharides. On the contrary, Tx91 H1N1 shows HA binding to long sialyl($\alpha 2-6$) oligosaccharides (Table 1) (Gambaryan *et al.*, 2005; Xu *et al.*, 2008; Sorrell *et al.*, 2011; Matrosovich *et al.*, 2013).

HA-glycan cocrystal studies indicated that the conformation of the Neu5Ac($\alpha 2-3$)Gal disaccharide occupies a small region of space in the receptor binding site of the host. This is defined

as a folded or cone-like glycan conformation (Shriver *et al.*, 2009; Sorrell *et al.*, 2011). Compared to the Neu5Ac($\alpha 2-3$)Gal linkage (ϕ and ψ rotational bond), the presence of the C6-C5 bond (ω -bond) in the Neu5Ac($\alpha 2-6$)Gal-containing oligosaccharide provides additional conformational flexibility to the Neu5Ac($\alpha 2-6$)Gal linkage (Fig. 3). This flexibility enables the Neu5Ac($\alpha 2-6$)Gal moiety to rotate in a wider region of space and to adapt to an extended or umbrella-like conformation (Ha *et al.*, 2001; Ha *et al.*, 2003; Shriver *et al.*, 2009). This conformation corresponds to the degrees of rotational freedom according to different linkage types of permethylated Gal($\beta 1-3$)GlcNAc- and Gal($\beta 1-6$)GlcNAc-containing trisaccharides (Yoo, 2001; Yoo and Yoon, 2008).

Human-to-human transmission may require not only a specific linkage position but also the conformational flexibility of sialic-acid-containing oligosaccharides as the receptor binding requirement of viral HA. HAs of the human-adapted influenza virus specifically prefer extended conformation with long sialyl-($\alpha 2-6$)-linkage-containing oligosaccharides, such as Neu5Ac($\alpha 2-6$)(Gal($\beta 1-3/4$)GlcNAc)_n, where n is greater than 2 (Chandrasekaran *et al.*, 2008; Shriver *et al.*, 2009). Raman and Sasisekharan (2007) studied conformational aspects of HA-glycan interaction beyond the sialic acid linkages. Also, Xu *et al.* (2009) reported that avian H5 prefers the tightly folded umbrella-like conformation of long $\alpha 2-6$ -linked tetrasaccharides based on the glycan microarray and molecular dynamics studies.

Lectin binding studies in the human respiratory tract demonstrated that the lungs and lower respiratory tract had mainly sialyl($\alpha 2-3$)-linked receptors (Stevens *et al.*, 2006b; Rumschlag-Booms and Rong, 2013; Walther *et al.*, 2013). The Tandem MS/MS study also indicated that the human lungs and

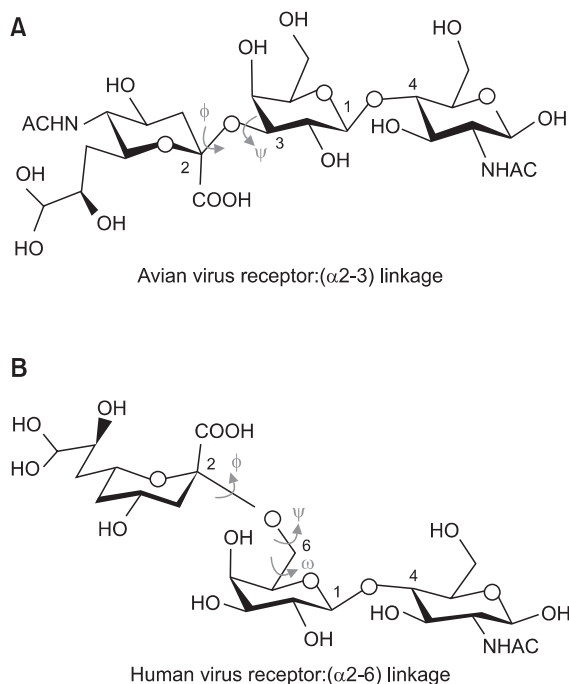


Fig. 3. The constitution of trisaccharides consisting in the receptors of avian influenza viruses and human influenza viruses. (A) Avian virus receptor: Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)GlcNAc and the torsion angle, ϕ and ψ . (B) Human virus receptor: Neu5Ac($\alpha 2-6$)Gal($\beta 1-3$)GlcNAc and the torsion angle, ϕ , ψ and ω .

bronchus contain mixtures of $(\alpha 2-3)$ - and $(\alpha 2-6)$ -linked sialic acid and that the bronchus contains more $(\alpha 2-3)$ -linked sialic acid than does the upper respiratory tract (Fig. 4). As in adults, the lungs and bronchus of children have both $(\alpha 2-3)$ - and $(\alpha 2-6)$ -linked sialic acid but have a greater abundance of sialyl($\alpha 2-3$)-linked oligosaccharides than do adult lungs (Walther *et al.*, 2013). If a human is constantly exposed to an environment of highly concentrated avian viruses, such as a poultry breeding farm or a live poultry market, the human can be directly infected with avian viruses, such as H5N1.

If the newly suggested classification based on conformation types as well as the sugar length of sialyl($\alpha 2-6$)-containing oligosaccharides at the receptor binding sites is adapted, it is possible to detect the evolution of the human-adapted flu virus rapidly and efficiently using tandem MS/MS mass spectrometry and glycan arrays with long length sialyl($\alpha 2-6$) oligosaccharides (Stevens *et al.*, 2004; Xu *et al.*, 2008; Wang and Zheng, 2009; Viswanathan *et al.*, 2010). On the other hand, a sufficient understanding of the avian HA mutations leading to the preference of the extended conformation with long length sialyl($\alpha 2-6$)-containing oligosaccharides will facilitate the development of novel antiviral drugs.

THE THIRD MONOSACCHARIDE TYPE SPECIFICITY OF AVIAN- AND MAMMALIAN-FLU VIRUSES

Influenza viruses adapted to avian animals-such as ducks, gulls, and chickens-can recognize specific types of the third monosaccharides next to the Neu5Ac($\alpha 2-3$)Gal disaccharide of the receptors. Duck viruses preferentially bind to receptors

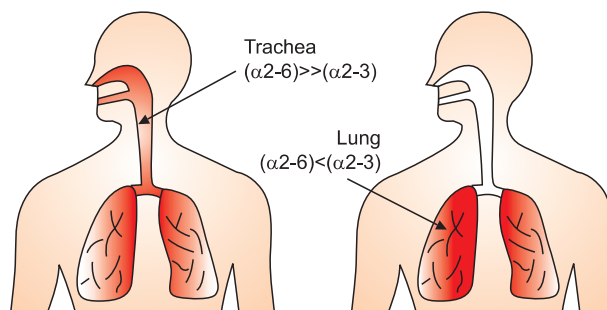


Fig. 4. Distribution of sialyl $(\alpha 2-3)$ and/or $(\alpha 2-6)$ linkages containing oligosaccharides in human respiratory tracts. The human lungs and lower respiratory tract have mainly sialyl $(\alpha 2-3)$ -linked receptors for avian viruses, such as Av 18 and H5N1. The human bronchus and upper respiratory contain mixtures of $(\alpha 2-3)$ - and $(\alpha 2-6)$ -linked sialic acid, which are receptors for human-adapted viruses, such as H3N2, H1N1, Tx92, Sc18, and NY18. The bronchus contains more $(\alpha 2-3)$ -linked sialic acid than does the upper respiratory tract.

with the oligosaccharide sequences Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)GlcNAc and Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)GalNAc. Gull viruses show high binding to the fucosylated sialoligosaccharides Neu5Ac($\alpha 2-3$)Gal($\beta 1-4$)(fuc($\alpha 1-3$))GlcNAc and Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)(fuc($\alpha 1-4$))GlcNAc. On the other hand, chicken viruses preferentially bind to Neu5Ac($\alpha 2-3$)Gal($\beta 1-4$)GlcNAc and sulfated trisaccharides and tetrasaccharides, such as Neu5Ac($\alpha 2-3$)Gal($\beta 1-4$)6-sulfatedGlcNAc and Neu5Ac($\alpha 2-3$)Gal($\beta 1-4$)(fuc($\alpha 1-3$))6-sulfatedGlcNAc, respectively (Russel *et al.*, 2006; Rumschlag-Booms and Rong, 2013). The oligosaccharide Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)6-sulfatedGlcNAc is known to be the ligand of L-selectin (Ha *et al.*, 2003; Yoo and Yoon, 2008). Mammalian viruses can also recognize and bind with Neu5Ac($\alpha 2-3$)Gal($\beta 1-3$)6-sulfatedGlcNAc (Gambaryan *et al.*, 2005). Thus, mammalian viruses bind to the same oligosaccharide as do chicken and gull viruses. Also, fucosylated or sulfated sialic-acid-containing trisaccharides are present in the target tissues of both mammals and chickens (Russel *et al.*, 2006; Rumschlag-Booms and Rong, 2013). Thus, the currently accepted paradigm of classifying viruses as only sialyl($\alpha 2-3$) or $(\alpha 2-6)$ linkage types is not sufficient to explain the infection and transmission of various strains of avian and mammalian influenza viruses. This paradigm must be revised to avoid inappropriate conclusions and applications for developing new species-specific antiviral drugs.

CONCLUDING REMARKS

HA of the influenza virus recognizes the specific linkage type, length, and conformation of oligosaccharides as the receptor binding sites for attachment to the host. HA-oligosaccharide binding has various kinds of inter- and intra-sugar interactions. Sialyl($\alpha 2-3$)-linked oligosaccharides, ionic bonds, inter-, intra-molecular H-bond, and dipole-dipole interactions are related between HA and sialylated oligosaccharides. In addition, longer sialyl($\alpha 2-6$) oligosaccharides with a length greater tetrasaccharide would have the extended conformation through stabilizing Van der Waals interactions between GlcNAc and an acetyl group of Neu5Ac. Also, the extended conformation is involved in interactions between HA amino

acids and the 4th and 5th monosaccharides of the long length oligosaccharides. It is necessary to study the receptor specificities of influenza viruses of various hosts in more detail to keep an eye on the evolution of new pandemic viruses that threaten human health and also to develop the 2nd Tamiflu as their remedy. Also, development of new antiviral drugs based on other HA characteristics and combinations of commercially available antiviral drugs are needed in the near future.

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