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H5N1 receptor specificity as a factor in pandemic risk

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Abstract

The high pathogenicity of H5N1 viruses in sporadic infections of humans has raised concerns for its potential to acquire the ability to transmit between humans and emerge as a highly pathogenic pandemic virus. Because avian and human influenza viruses differ in their specificity for recognition of their host cell receptors, receptor specificity represents one barrier for efficient transmission of avian viruses in human hosts. Over the last century, each influenza virus pandemic has coincided with the emergence of virus with an immunologically distinct hemagglutinin exhibiting a ‘human-type’ receptor specificity, distinct from that of viruses with the same hemagglutinin circulating in zoonotic species. Recent studies suggest that it is possible for H5N1 to acquire human type receptor specificity, but this has not occurred in nature. This review covers what is known about the molecular basis for the switch between avian and human-type receptor specificity for influenza viruses that have successfully adapted to man, the potential for H5N1 to evolve to human-type receptor specificity and its relevance to pandemic risk.

Keywords

Hemagglutinin; receptor specificity; H5N1; sialic acid; pandemic risk

Introduction

Influenza A virus is an ever-present threat to human health, causing annual epidemics of seasonal flu, and occasional pandemics that coincide with the emergence of a new virus strain into the human population (Bouvier and Palese, 2008). The potential for new zoonotic viruses to adapt to humans has heightened concerns that a highly pathogenic virus, will acquire the ability to transmit in humans, resulting in a pandemic of the likes of the 1918 Spanish flu, which caused an estimated 50,000,000 deaths worldwide. H5N1 viruses are exemplary for their pathogenic potential because they cause repeated epidemics in domestic poultry worldwide, and have infected over 600 people since 1997 (de Jong et al., 1997; Kandun et al., 2006), with 60% mortality reported amongst confirmed cases (Gambotto et al., 2008). Fortunately, however, most infections are associated with close contact of humans with infected poultry, and the virus has not acquired the ability to transmit between humans (Ungchusak et al., 2005; Wang et al., 2008). Because of the concern that adaptation to man will result in a major pandemic, there is an interest in identifying the mutations that would be required for transmission of zoonotic viruses that occasionally infect humans. Such knowledge would help monitor H5NI in zoonotic populations and occasional human infections for the emergence of a virus with pandemic potential (Krauss and Webster, 2012; Peiris et al., 2012).

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Influenza is a membrane-enveloped virus with two surface glycoproteins that are responsible for interaction of the virus with cell surface receptors, and are also the main targets of the immune response. The hemagglutinin (HA) is essential for binding to sialic acid containing receptors and fusion with the target membrane (Skehel and Wiley, 2000), while the neuraminidase (NA) specifically cleaves sialic acids from the receptor, an activity required for release of the virus from the host cell (Skehel and Wiley, 2000). Although there are a total of 17 HA and 10 NA serologically distinct influenza virus subtypes known (Tong et al., 2012), only three H1N1, H2N2 and H3N2 have circulated in humans (Kuiken et al., 2006). Most influenza strains, including H5N1, are found in wild waterfowl species, which serve as a natural reservoir that can occasionally cross species barriers to infect, other species including, domestic poultry, horses, pigs, seals and man (Webster et al., 1992).

Influenza virus receptor specificity is currently considered to be a significant barrier for efficient transmission of influenza virus in humans, and has been established as a risk factor for emergent pandemic viruses (CDC, 2012; Imai and Kawaoka, 2012; WHO, 2010). Human influenza viruses of each pandemic, 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) were found to exhibit preference for sialic acid receptors with the NeuAc 2–6Gal linkage (2–6Sia or human type), while avian and equine virus strains with the same hemagglutinin exhibit preference for receptors with the NeuAc 2–3Gal linkage (2–3Sia or avian type) (Connor et al., 1994; Gambaryan et al., 2005b; Gambaryan et al., 1997; Glaser et al., 2005; Rogers and D'Souza, 1989; Rogers and Paulson, 1983). These sialic acid linkages are commonly found on glycans of cell surface glycoproteins on cells of all mammalian and avian species, but their expression varies in different cell types within a single species, and in the same cell type from different species. For example, the epithelial cells of the upper respiratory tract in humans infected by airborne human viruses contain predominantly 2–6Sia (Baum and Paulson, 1990; Couceiro et al., 1993; Nicholls et al., 2007; Shinya et al., 2006; van Riel et al., 2007), while the intestinal epithelial cells of waterfowl species infected by waterborne avian viruses contain primarily 2–3Sia (Gambaryan et al., 2002; Ito et al., 1998).

Compelling evidence that receptor specificity is relevant to the virulence and transmission of influenza virus has emerged from the analysis of the efficiency of influenza virus receptor variants in the infection of human primary epithelial cells (Matrosovich et al., 2007; Matrosovich et al., 2004; Thompson et al., 2006), the propagation in chicken embryos (Ito et al., 1997b; Rogers et al., 1985), and in infection and transmission in avian and mammalian animal models (Belser et al., 2008; Chen et al., 2012; Herfst et al., 2012; Imai et al., 2012; Leigh et al., 1995; Naeve et al., 1984; Tumpey et al., 2007). Based on the weight of this evidence, it is now presumed that an avian virus must adapt to human type 2–6Sia receptor specificity to become a pandemic virus that efficiently transmits between humans (Imai and Kawaoka, 2012).

Over the past decade there have been numerous reports investigating the receptor specificity of H5N1 influenza virus and mutations that cause a shift towards human type receptor specificity. Recently, several groups have documented H5N1 receptor variants with human type receptor specificity that support airborne transmission in ferrets, a mammalian species susceptible to human viruses, documenting that a change in specificity sufficient to support aerosol transmission can potentially occur in nature (Chen et al., 2012; Herfst et al., 2012; Imai et al., 2012). Less clear, however, is the degree to which solutions to achieve the change in receptor specificity are unique, and how to recognize them as H5N1 evolves in zoonotic species. This review summarizes what is known about the receptor specificity of H5N1 influenza virus and the potential for emergence of a H5N1 pandemic virus with human type receptor specificity. In particular we cover: 1) Methods for analysis of receptor specificity, 2) Structural basis of receptor specificity of human H1, H2 and H3 virus HAs; 3)

Mutations of H5 HA that promote binding to human type receptors; 4) Relevance of receptor specificity for transmission in mammals; and 5) Potential for monitoring receptor specificity for assessment of pandemic risk.

Assessing influenza receptor specificity

In the 40's and early 50's Influenza viruses were recognized to differ in their receptor binding properties based on differential agglutination of animal erythrocytes, graded sensitivity to glycoprotein inhibitors of hemagglutination, and sensitivity to destruction of receptors by influenza or bacterial 'receptor destroying enzymes' (Burnet and Bull, 1943; Choppin and Tamm, 1959; Hirst, 1941; Stone, 1949; Stone, 1951). Later, the 'receptor-destroying enzyme' was identified as neuraminidase, simultaneously identifying sialic acid as the key receptor determinant of the virus (Gottschalk, 1957; Klenk and Lempfrid, 1957). Decades later, influenza viruses were found to exhibit differential specificity for sialoglycans with terminal NeuAc 2–3Gal and NeuAc 2–6Gal linkages, providing an underlying molecular basis for receptor binding differences (Carroll et al., 1981; Gambaryan et al., 1997; Paulson et al., 1979; Rogers and D'Souza, 1989; Rogers and Paulson, 1983). With the realization that receptor specificity was correlated with species of origin and was relevant to virulence in host species, several methods emerged to assess receptor specificity and are now used to explore its biological significance.

Hemagglutination of receptor modified red blood cells (RBCs)

One of the earliest methods used for assessing influenza virus specificity was based on a hemagglutination assay using human RBCs modified to contain either the NeuAc 2–6Gal or NeuAc 2–3Gal linkages on cell surface glycoproteins. Although native RBCs normally contain a mixture of sialoside linkages, they can be engineered to contain a single structure by first stripping off sialic acids with sialidase, and then replacing them with a specific sialyltransferase that forms either the NeuAc 2–6Gal 1–4GlcNAc (ST6Gal I) or NeuAc 2–3Gal 1–3GalNAc (ST3Gal III) sequence on cell surface glycoproteins (Paulson and Rogers, 1987; Paulson et al., 1979). The modified cells can then be used in a standard hemagglutination assay to assess the receptor specificity influenza viruses. This assay was successfully used to demonstrate differences between the specificity of H1, H2 and H3 isolates from humans vs avian and equine viruses isolates bearing the same HA subtype (Connor et al., 1994; Rogers and D'Souza, 1989; Rogers et al., 1983a). The clear advantage of the assay is that the final readout is hemagglutination, a standard assay used in most influenza labs. The major disadvantage is the need for enzymatic manipulation of the RBCs and the difficulty in obtaining purified the specific sialyltransferases to modify the cells since stable commercial sources have not emerged. Nonetheless, variations of this assay using RBCs from other species (e.g. chicken, turkey) are still in use (Chutinimitkul et al., 2010; Glaser et al., 2005; Herfst et al., 2012; Nobusawa et al., 2000).

Animal RBCs have been investigated for their ability to distinguish the specificities of human and avian influenza viruses based on the assumption that different animal RBCs will display sialic acid receptors that support preferential binding viruses with specificity for 2–6 or 2–3Sia, respectively. However, although the spectrum of sialosides present on RBCs are varied, the degree of variation is too great to be a reliable means to discriminate the classic specificities of these viruses (Aich et al., 2011; Ito et al., 1997a; Kumari et al., 2007).

ELISA-type plate assays

Another accepted method for assessing receptor specificity evaluates the binding of influenza viruses to synthetic multivalent sialosides in ELISA-type plate assays. The two basic approaches are illustrated in Figure 1, which differ in the coating of the ELISA plate

with either virus or the polymeric sialoside (Gambaryan and Matrosovich, 1992; Totani et al., 2003).

Gambarayan and Matrosovich developed a robust assay involving coating of the plastic wells with virus, followed by overlaying the virus with a polymer containing a synthetic sialoside coupled to polyacrylamide labeled with biotin (Sia-PAA). After washing the plate, the bound sialoside is then detected with streptavidin-horseradish peroxidase (SA-HRP) or a suitable fluorescent tag (Gambaryan et al., 2006; Gambaryan and Matrosovich, 1992; Ilyushina et al., 2004a; Ilyushina et al., 2004b). By varying the amount of sialoside, a saturation curve is obtained, which allows calculation of an apparent K_d value using a Scatchard plot, providing relative measure of affinity. By comparing the binding of 2–3Sia-PAA and 2–6Sia-PAA, this assay is a highly reliable and sensitive method to assess the specificity of influenza virus towards human type (2–6Sia) or avian type (2–3Sia) receptors. Other aspects of receptor specificity can be explored with a panel of Sia-PAA's displaying synthetic sialoside structures that feature other aspects of sialoside structure (Gambaryan et al., 2006; Gambaryan et al., 2005a; Gambaryan et al., 2005b; Gambaryan et al., 2008; Gambaryan et al., 2004).

Another version of the plate assay involves binding of the sialoside ligand to the well, followed by adsorbing virus and detecting bound virus with anti-virus antibody, as illustrated in Figure 1. In one variation the 2–6Sia and 2–3Sia are coupled to a polyglutamate (Sia-PG) polymer, and the polymer is in turn coupled to plastic coated wells (Auewarakul et al., 2007; Shinya et al., 2005; Totani et al., 2003; Watanabe et al., 2011; Yamada et al., 2006). Another variation is to use monovalent sialosides attached via a linker to biotin, which allows adsorption of the glycans to streptavidin coated plates (Chandrasekaran et al., 2008; Pappas et al., 2010; Xu et al., 2012b). In both cases, virus is overlaid and bound virus is detected with labeled antibodies. Differences in receptor specificity are readily detected by varying the amount of sialoside used to coat the wells. A sialoglycan of complementary specificity will promote increased binding of the virus as the density on the plate is increased, while a sialoglycan that is poorly recognized will not bind virus even at high density. While the binding of the sialoside to the plate creates a surface analogous to the cell membrane, all versions of the ELISA-type assay (Figure 1) involve binding of a multivalent virus to a multivalent sialoside, and have yielded qualitatively similar results.

A limitation relative to the hemagglutination assays is that they involve detection of bound virus, so that suitable antisera must be available, or alternatively, the virus must be labeled. Another disadvantage of the plate assay is that the glycan structures coupled to polymers are typically small fragments of the glycans found on glycoproteins or glycolipids found in cells. Moreover, the most widely used pair of glycans are 3'- and 6'- sialyl-N-acetyl-lactosamine (NeuAc 2–3Gal 1–4GlcNAc -R and NeuAc 2–6Gal 1–4GlcNAc -, respectively), sequences that typically terminate N-linked glycans of glycoproteins, but can also terminate some O-linked glycans and glycolipids. In contrast, the sialyltransferases used to modify cells in the RBC assay produce the same 2–6Sia, namely NeuAc 2–6Gal 1–4GlcNAc - elaborated by ST6Gal I, but produce a different 2–3Sia, namely NeuAc 2–3Gal 1–3GalNAc- found almost exclusively on O-linked glycans of glycoproteins. These distinctions are often lost when results are reduced to a binary 2–3 or 2–6 specificity, but may be biologically important.

Yet, a major advantage of the ELISA-type assays is that they use a plate format that is a familiar assay in most virology, immunology and biochemistry laboratories. Moreover, while the polyglutamate based polymers used by some laboratories are not commercially available, the polyacrylamide (PAA) versions are commercially available (Lectinity Corp.,

GlycoTech Corp.) or can be obtained by request from the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>). As will be discussed further below, another significant advantage of the ELISA-type assays is that they appear to have high sensitivity for detecting small but biologically relevant changes in receptor specificity.

Glycan microarray binding assays

Glycan microarrays have emerged as a way to evaluate the specificity of influenza viruses and recombinant HA towards a wide variety of sialic acid containing glycans in a single experiment (Blixt et al., 2004; Childs et al., 2009; Liao et al., 2010; Nycholat et al., 2012; Rillahan and Paulson, 2011; Song et al., 2011; Stevens et al., 2006b). Glycan arrays assays are analogous to the ELISA-type plate assays in which the sialosides are coupled/adsorbed to a surface (Figure 1), and virus or recombinant HA is subsequently applied, and detected after washing to assess which glycans support binding. In the short time that they have been available they have been increasingly used to investigate the details of influenza virus receptor specificity and relevance to natural glycans expressed on host cells (Amonsens et al., 2007; Bateman et al., 2010; Belser et al., 2008; Belser et al., 2011; Childs et al., 2009; de Vries et al., 2011; Gulati et al., 2009; Kumari et al., 2007; Liao et al., 2010; Liu et al., 2010; Lugovtsev et al., 2009; Nycholat et al., 2012; Song et al., 2011; Stevens et al., 2006a; Stevens et al., 2006b; Stevens et al., 2010; Wang et al., 2009; Wang et al., 2012; Xu et al., 2010; Xu et al., 2012b; Yang et al., 2010; Yang et al., 2007; Yen et al., 2011).

A distinguishing feature of this assay is the fact that a large number of glycans can be simultaneously evaluated. Examples of some of the glycans elaborated on custom glycans microarrays used to assess influenza virus receptor specificity of H5N1 are illustrated in Figure 2. Although a large library of glycans could in principle be evaluated in the plate assay, with a few exceptions (Gambaryan et al., 2006; Gambaryan et al., 2003; Gambaryan et al., 2004), most studies employing ELISA type assays evaluate only a few representative sialosides that are linear fragments of the larger glycans found on glycoproteins and glycolipids. In particular, it is common to assess specificity using 2–3 and 2–6 linked sialyl-N-acetyl-lactosamine (NeuAc 2–3/6Gal 1–4GlcNAc) and/or related glycans extended with an additional N-acetyl-lactosamine unit (Chandrasekaran et al., 2008; Chen et al., 2012; Pearce et al., 2012; Srinivasan et al., 2008; Yamada et al., 2006). By contrast glycan microarray libraries now carry a diverse group of both linear and branched glycans representing complete natural N-linked and O-linked glycans with terminal sialic acids that are potential receptors for influenza virus (Chen et al., 2012; Liao et al., 2010; Liu et al., 2010; Nycholat et al., 2012; Yen et al., 2011). The most widely used glycan microarray for assessment of influenza virus receptor specificity is that of the Consortium for Functional Glycomics (CFG; <http://www.functionalglycomics.org>), which currently maintains a glycan library with over 100 sialic acid containing glycans (Blixt et al., 2004; Rillahan and Paulson, 2011). The CFG also maintains a database of all glycan microarray experiments that are accessible from its website.

An advantage of the glycan microarray is the simplicity in obtaining a wealth of information about the interactions of influenza virus hemagglutinins towards a variety of naturally occurring sialosides. However, it is not yet clear how this additional information is relevant to influenza virus biology. At a top level, it is clear that the classic ‘2–6’ and ‘2–3’ specificity of human and avian influenza isolates is recapitulated in glycan microarray experiments. In addition, however, isolates of the same serotype from a single species reveal different fine specificities towards glycans that contain the terminal NeuAc 2–6Gal or NeuAc 2–3Gal sequences (Belser et al., 2008; Kumari et al., 2007; Liu et al., 2010; Nycholat et al., 2012; Stevens et al., 2008; Stevens et al., 2006a; Stevens et al., 2010). Yet, the biological consequences of this additional level of specificity have not been systematically explored. Investigation into the significance of such differences in specificity

has been hampered by a notable lack of information on the types of glycans present on host epithelial cells that are targets of infection by influenza. Indeed, only fragmentary information is currently available for epithelial cells of natural and laboratory hosts (Bateman et al., 2010; Chandrasekaran et al., 2008; Sriwilaijaroen et al., 2009).

Structural basis of receptor specificity for H1, H2 and H3 HAs

Investigations into the ability of H5N1 to adapt to human type receptor specificity have been facilitated by accumulated knowledge on the basis for receptor specificity switch in the H1, H2 and H3 viruses that co-circulated in man and zoonotic species. As illustrated in Fig. 3A for the H1 HA, the receptor binding site (RBS) of the influenza virus HA is a shallow pocket at the tip of the hemagglutinin framed by an α -helix at the top (190 helix) and two loops at the side (220 loop) and bottom (130 loop) (Skehel and Wiley, 2000). This pocket was recognized as a potential RBS with the first crystal structure of the H3 hemagglutinin because of its location and the juxtaposition of highly conserved amino acid residues (Wilson et al., 1981). Soon thereafter, sequence analysis of a receptor variant of a human virus showed that the switch from 2–6 to 2–3 specificity resulted from a single amino acid change at residue 226 (H3 numbering is used throughout), providing strong support for this pocket as the receptor binding site (Rogers et al., 1983a). It was confirmed to be the RBS when co-crystals with a sialic acid containing receptor fragments were solved for the H3 HA (Weis et al., 1988). The structure revealed that the sialic acid interacts with four conserved residues, Y98, W153 and H183 and Y195 that form the base of the RBS, and are conserved in almost all HA serotypes to date. Other non-conserved residues are also important in sialic acid binding (Daniels et al., 1987; Eisen et al., 1997; Martin et al., 1998; Weis et al., 1988), including several that have been implicated in receptor specificity.

Insights into the structural basis for receptor specificity were revealed through selection of receptor variants based on differences in the receptor binding properties of the viruses (Rogers et al., 1985; Rogers et al., 1983a; Rogers et al., 1983b). It was found early on that the human type receptor variants of H2 and H3 viruses were also sensitive to glycoprotein inhibitors in horse serum, as first demonstrated by Choppin and Tamm in the early 60s (Choppin and Tamm, 1959; Rogers and Paulson, 1983; Rogers et al., 1983a; Rogers et al., 1983b). This property allowed selection of 2–3 receptor variants of human viruses by passage in horse serum (Rogers et al., 1983a). Conversely, 2–6 specific receptor variants of an H3 avian virus could be selected by adsorption to cells containing NeuAc 2–6Gal receptors (Rogers et al., 1985). In both cases, the switch in receptor specificity was mediated by a single amino acid at residue 226, with Leu conferring 2–6 specificity and Gln conferring 2–3 specificity. Surprisingly however, while the 2–6 specific avian virus variants were stable when grown in MDCK cells, they reverted back to wild type specificity within a single passage in eggs, suggesting that the single amino acid human type receptor variant was subject to selection of the wild type 2–3 specificity in this laboratory host (Rogers et al., 1985).

Subsequent sequence analysis of H2 and H3 isolates revealed that Leu at 226 was conserved from 1957–1982 in human isolates, while Gln at 226 was conserved from 1963–1987 among avian and equine isolates, providing strong evidence of natural selection (Connor et al., 1994; Matrosovich et al., 1993). Moreover, the change at residue 226 co-varied in natural isolates with a second change at residue 228, which was serine in human isolates and glycine in avian isolates (Connor et al., 1994). Thus, it was concluded that these two amino acids were responsible for the change and maintenance of receptor specificity of H2 and H3 isolates in their respective hosts (see Figure 3B–3C). It should be noted, however, that recent human H3N2 have been difficult to recover by growth in laboratory hosts, and while they largely retain binding to 2–6Sia, they exhibit quite varied specificity using glycans

microarrays (Kumari et al., 2007; Stevens et al., 2010). Moreover, the conserved Leu 226 has been replaced by conservative substitutions with Ile/Val (Medeiros et al., 2001; Nobusawa et al., 2000; Stevens et al., 2010). These changes suggest that the H3N2 strain continues to evolve receptor specificity, perhaps in response to immune selection (Daniels et al., 1987; Daniels et al., 1984; Underwood et al., 1987), yet maintain the ability to bind to human type receptors.

Human and avian H1 influenza isolates exhibit the same receptor preferences as their H2 and H3 counterparts (Childs et al., 2009; de Vries et al., 2011; Matrosovich et al., 2000; Matrosovich et al., 1993; Rogers and D'Souza, 1989). However, it was recognized early that the amino acids responsible must be different, since both human and avian H1 isolates retained Gln and Gly at residues 226 and 228, respectively (Matrosovich et al., 2000; Nobusawa et al., 1991; Rogers and D'Souza, 1989). It is now clear that the residues responsible for the switch between avian and human type receptor specificity in H1 isolates are residues 190 and 225, with Glu190 and Gly225 required for binding to avian type 2–3 receptors, and Asp190 and Asp225 supporting binding to human type 2–6Sia receptors (Fig. 3 B) (Gamblin et al., 2004; Glaser et al., 2005; Stevens et al., 2006a; Xu et al., 2012a). It is striking that the receptor specificity based on species of origin is conserved, and that different solutions have emerged to accommodate this preference in the different HA frameworks.

The molecular basis for the changes in receptor specificity of human and avian H1, H2 and H3 hemagglutinins has been investigated by co-crystallization of hemagglutinins with receptor fragments containing sialic acid (Eisen et al., 1997; Gamblin et al., 2004; Liu et al., 2009; Russell et al., 2004; Sauter et al., 1992; Weis et al., 1988; Xu et al., 2010). Direct binding studies of 3'- and 6'-sialylactose to influenza H3 hemagglutinin have shown that the intrinsic binding affinity of monovalent sialosides for the hemagglutinin is very weak (e.g. 1–3 mM), and that the difference in the affinity of 2–3 and 2–6 linked sialic acids is only 1.5–2 fold (Sauter et al., 1992). On the one hand, this fact underscores the critical role of multivalency in influenza interaction with host receptors, resulting in the amplification of small differences in affinity to provide biological effects that are highly significant. On the other hand, it poses a challenge to identify molecular features that are responsible for these small but significant differences. Initial results evaluating the structures of the H3 hemagglutinin with 3'- and 6'-sialyllactose and two pentasaccharides (LSTa and LSTc) with structures analogous to structures 2 and 7 in Fig. 2, showed that sialic acid interacted with the conserved residues nearly identically for all structures, and that the 2–6Sia were more ordered, particularly for the longer glycans (e.g. LSTc) (Eisen et al., 1997; Weis et al., 1988). Systematic comparison of the crystal structures of H2 hemagglutinins from 2 human pandemic isolates (1957) and 3 avian progenitor isolates, both with and without the 2–3 (LSTa) and 2–6 (LSTc) pentasaccharides revealed that Leu226 in the human H2 HAs creates a hydrophobic environment that stabilizes binding of the human type receptor (LSTc) and destabilizes the binding of the avian type receptor (LSTa) (Liu et al., 2009). This work also revealed that while the H2 HA is more homologous to the H1 HA based on primary sequence, the structure of the H2 HA is more similar to the H3 HA, explaining why the H2 and H3 HA share a common solution for preferential binding of the 2–6 human type receptors (Leu226 and Ser228).

Structures of the human H1 HAs have similarly provided a rationale for the preferred binding of 2–6 human type receptors. Several structures of H1 HAs have been reported in complex with the pentasaccharides representing human and avian receptors (LSTc and LSTa, respectively), including a representative seasonal strain from the 1918 pandemic (Human 1934), one from the recent 2009 pandemic (A/Cal/04/09), and a structure from a recent avian strain (A/WDK/JX/12416/2005) (Gamblin et al., 2004; Lin et al., 2009; Xu et al.,

2012a). In the structures from the human strains, the 2–6 receptor (LSTc) is well ordered, revealing hydrogen bonds between the two amino acids responsible for the switch in receptor specificity. The Asp 225 forms a hydrogen bond with the 2 OH of the Gal-2 and the Asp 190 forms a hydrogen bond with the nitrogen of the acetamido group of GlcNAc-3. Notably, the 1.65 Å Cal/04 structure captures electron density for all 5 residues, with the last three residues projecting out of the pocket nearly perpendicular to the plane of the bound sialic acid as shown in Fig. 3E (Xu et al., 2012a). This results in the glycans projecting from the pocket in the middle of the 190 helix, in contrast to the same receptor structure in the H2 HA structure (Fig. 3C), which is folded more over the sialic acid and projecting out of the pocket towards the left of the 190 helix. The avian type receptor (LSTa) is less ordered in all three structures, with electron density showing just 2–3 residues. In the avian H1 structure Gln226 makes favorable hydrogen bonds with Gal-2 of LSTa, which are absent in the Cal/04 structure (Lin et al., 2009; Xu et al., 2012a).

In summary, while all three subtypes that have adapted to transmission in man have acquired human type 2–6 specificity, the change from avian to human type specificity for H2 and H3 isolates is accomplished with Q226L and G228S as a ‘switch’, while H1 isolates employ E190D and G225D as a switch. However, the differences in intrinsic affinity of the 2–3 and 2–6 linked receptor glycans are small, and co-crystal structures of both types of glycans have been obtained for H1, H2 and H3 hemagglutinins. The structures reveal that the receptor glycans have a similar topology in each case, with the 2–3 linked glycans projecting out of the pocket, and the 2–6 glycans projecting up towards the 190 helix (Chandrasekaran et al., 2008; Eisen et al., 1997; Lin et al., 2009; Liu et al., 2009; Weis et al., 1988; Xu et al., 2012a; Xu et al., 2010). However, the amino acids involved in the receptor switch provide for favorable interactions that stabilize the preferred receptor type, and this difference is amplified by through the multivalent interaction of the virus with cell surface receptors, resulting in efficient propagation and selection in the respective host species.

Receptor site mutations that affect a switch of H5N1 to human type receptor specificity

Based on the high mortality of human infections of H5N1, and evidence that receptor specificity is considered a barrier for efficient transmission in the human population, numerous groups have investigated the potential for H5N1 to acquire human type receptor specificity, (summarized in Table 1) (Auewarakul et al., 2007; Ayora-Talavera et al., 2009; Chen et al., 2012; Chutinimitkul et al., 2010; Gambaryan et al., 2006; Herfst et al., 2012; Ilyushina et al., 2008; Imai et al., 2012; Maines et al., 2011; Shinya et al., 2005; Stevens et al., 2008; Stevens et al., 2006c; Watanabe et al., 2011; Yamada et al., 2006; Yang et al., 2007). The accumulated evidence suggests that 3–4 mutations may be required for H5N1 to completely switch to a receptor specificity equivalent to that exhibited by H1, H2 and H3 isolates. This fact may partially explain why H5N1 has not yet acquired human type receptor specificity in nature. However, it is now clear that mutations which increase the propensity to bind to human type receptors occur naturally in H5N1 isolates from birds and humans infected with H5N1 (Auewarakul et al., 2007; Gambaryan et al., 2006; Watanabe et al., 2011; Yamada et al., 2006). Moreover, several reports have documented mutagenesis-assisted evolution of H5N1 to human type receptor specificity sufficient to support transmission in a ferret model of human infection (Chen et al., 2012; Herfst et al., 2012; Imai et al., 2012). Here we review the evidence for natural selection of mutations that increase human type receptor specificity, and efforts using directed evolution to understand what it will take for H5N1 to acquire human type receptor specificity sufficient to support transmission in man.

Natural variants of H5N1 with increased binding to human type receptors

Initial analysis of H5N1 and H5N3 isolates from avian species using a series of sialoglycan polymers revealed that H5 had the typical 'avian type' specificity for 2–3Sia with no significant binding to a single 2–6Sia used in the panel (Gambaryan et al., 2004). This study also found that some avian isolates exhibited remarkably higher affinity (10–100 fold) for sialosides with sulfate on the GlcNAc residue (see Fig. 2, structure 4), a structure that was suggested could occur in human lung tissues based on the expression of enzymes known to be involved in its biosynthesis (Gambaryan et al., 2004). While it remains to be seen if this structure is present on the surface of human epithelial cells, this possibility is particularly relevant to the observation that cells deeper in the lungs express 2–3Sia and the presence of sulfated structures could contribute to H5N1 tropism to the lower lung (Nicholls et al., 2007; Shinya et al., 2006; van Riel et al., 2007).

Sequence analysis of H5N1 viruses isolated from human infections revealed mutations near the receptor-binding pocket, stimulating investigations into their potential to increase binding to 2–6Sia, and their relevance to adaptation to human type receptors. Several mutations including N186K, Q196R, Q196H, S227N and several double mutants L133V/A138V and 133/I155T were demonstrated to exhibit increased virus binding to 2–6Sia without significantly decreasing binding to 2–3Sia in ELISA type plate assays (Auewarakul et al., 2007; Gambaryan et al., 2006; Shinya et al., 2005; Watanabe et al., 2011; Yamada et al., 2006). One of these mutations, N186K is of interest because it was previously identified to contribute to the receptor-binding properties for several HA subtypes (Das et al., 2011; Eisen et al., 1997; Gubareva et al., 1994; Ha et al., 2002; Hardy et al., 1995). These striking results raised questions into of the origin of the mutations and their relevance to infection of humans. Did they emerge during the human infection, providing an advantage for replication in the host? Alternatively, could they have been preexisting mutations that aided infection of the human host, or were they simply preexisting mutations that had no consequence to replication in the human host?

Several pieces of evidence suggest that such naturally occurring mutations provide a selective advantage in the human host. Analysis of the ability of H5N1 to bind to epithelial cells of the human airway, wild type virus adsorbs to epithelial cells in histological sections of the lower airway, which express 2–3Sia, but fails to bind epithelial cells of the human upper airway, which express 2–6Sia (Chutinimitkul et al., 2010; Shinya et al., 2006; van Riel et al., 2006). In contrast, a virus with the N186K mutation was also found to bind to epithelial cells of the upper airway, consistent with a selective advantage of this mutation in a human host (Chutinimitkul et al., 2010). In an elegant study, Shinya et al. passed a human isolate with S227N back into ducks, and found that it reverted back to wild type (Shinya et al., 2010). The fact that the mutation is not stable in a natural host of H5N1 is consistent with idea that this mutation may have been selected for during replication in humans.

Evidence for preexisting mutations in occurring in avian H5N1 that increase binding to 2–6Sia emerged from analysis of isolates following a large number of human H5N1 cases in Egypt (Abdel-Moneim et al., 2011; Kayali et al., 2011; Watanabe et al., 2011). Extensive sequence analysis of isolates from birds and infected humans identified several mutations, a single mutant Q196H and double mutant 133/I155T, that had a clear increase binding to 2–6Sia in ELISA type plate assays (Watanabe et al., 2011). The mutations were documented to have occurred in birds prior to the outbreaks in humans, and occurred in human isolates with increased frequency relative to concurrent avian isolates, suggesting that they provided a selective advantage for initiation and/or propagation in humans.

Taken together, the data suggest that naturally occurring mutations have occurred in H5N1 that increase specificity for 2–6Sia and appear to provide selective advantage for

replication in humans. The degree to which these mutations occur randomly or by selection in avian or human hosts is not entirely clear. However, it is already well documented for other influenza virus serotypes that human hosts select exert selective pressures on human viruses for acquisition of 2–6Sia specificity (Imai and Kawaoka, 2012). Moreover, it has also been documented that some terrestrial avian species, such as quail, pheasant, and chicken, express human type 2–6Sia on airway and/or intestinal epithelial cells, and that some avian influenza viruses (e.g. H9N2) with 2–6Sia specificity persist in these populations (Costa et al., 2012; Guo et al., 2007; Kimble et al., 2010; Matrosovich et al., 2001; Sorrell et al., 2009; Wan and Perez, 2006; Wan et al., 2008; Yamada et al., 2012). At present it is not clear if the specificity of the 2–6Sia specificity of these viruses resulted from selection in avian hosts (Yamada et al., 2012), but the potential for terrestrial bird species to play a role in selection of human type receptor specificity in future human pandemic viruses is clear. Despite these concerns, it is notable that all of the naturally occurring H5N1 mutants exhibiting increased 2–6Sia specificity still retain strong binding to avian type 2–3Sia receptors, and the viruses have not acquired the capacity to transmit between humans.

Combining natural mutations with receptor specificity mutations from other HA serotypes

Several groups have investigated the possibility that H5N1 might achieve human type receptor specificity through mutations known to affect receptor specificity in other hemagglutinin serotypes. The basic strategy used is to introduce mutations into the H5 gene, and evaluate the receptor specificity of the corresponding recombinant hemagglutinin, or virus produced by reverse genetics (Chutinimitkul et al., 2010; Stevens et al., 2008; Stevens et al., 2006a). Mutations that cause a specificity change in human H1 isolates (E190D and G225D) were found to produce an H5 hemagglutinin with no detectable binding activity. However, mutations responsible for human type specificity in H2 and H3 hemagglutinins (Q226L and G228S) resulted in modest increased binding to 2–6Sia, coupled with a significant reduction in binding to 2–3Sia (Stevens et al., 2008; Stevens et al., 2006c). Combining the Q226L and G228S mutations with natural H5 mutations (N186K or S227N) resulted in significantly enhanced binding of H5N1 viruses to human type receptors, as evidenced by increased binding to epithelial cells of the human airway (Chutinimitkul et al., 2010).

Based on the appearance of a T160A mutation in recent clades of H5N1 isolates that disrupted the NxT/S sequon of an N-linked carbohydrate at N158 at the edge of the receptor-binding pocket (see Fig. 4A), Stevens et al. reasoned that the loss of the carbohydrate might also influence receptor specificity (Stevens et al., 2008). Although the T160A mutation by itself had little effect on receptor specificity, further introduction of the Q226L and G228S mutation resulted in increased binding to 2–6Sia and greater reduction in binding to 2–3Sia. The same three mutations introduced into other backgrounds (VN/1204 and Indo/05) resulted in a similar shift in specificity towards 2–6Sia. An even more dramatic shift was observed introducing the three mutations into H5N1 viruses by reverse genetics, documenting that the increased valency of the virus amplifies the shift in specificity.

Directed evolution of H5N1 receptor specificity

So what will it take for H5N1 to acquire human type receptor specificity? Given that some selection is already occurring in nature, is it possible for H5N1 to fully adapt to ‘human type’ receptor specificity, removing one barrier for transmission in the human population? Is there one solution involving a combination of mutations, or are there many solutions to achieve full adaptation? Such questions are highly relevant for pandemic preparedness, since the knowledge would arm the community to watch for mutations of H5N1 that would signal the potential for transmission in man. To address this potential, several groups have

employed *in vitro* and/or *in vivo* selection methods to identify a combination of mutations that achieve human type receptor specificity, and have succeeded to identify combinations that change specificity and support transmission in a ferret model (Fig. 4) (Chen et al., 2012; Herfst et al., 2012; Imai et al., 2012).

To select mutations that increased 2–6Sia binding, Donis and coworkers employed cycles of adsorption of H5N1 virus to 2–6Sia coated beads followed by elution and growth in MDCK cells. Sequencing of 139 plaque purified clones revealed amino acid mutations at 8 distinct sites, 4 of which were in or near the receptor-binding pocket. Tested in a plate-binding assay, 5 of the 8 showed increased binding to 2–6Sia, including all 4 near the receptor-binding pocket. Two of the mutations had been previously reported in natural isolates (Q196R and S227N) and shown to increase specificity for 2–6Sia (Table 1). Two others had not been reported, E190G and D187G, although other mutations at or near these mutations have been shown to affect receptor binding specificity (Table 1). Remarkably, while the ELISA based plate assay revealed clear increased binding to 2–6Sia, only S227N exhibited increase in 2–6Sia binding detected in the glycan microarray assay (Fig. 5) (Chen et al., 2012). However, combining two mutations (e.g. S227N/D187G and S227N/Q196R) resulted in increased binding of 2–6Sia in the glycan microarray assay without incremental change in the plate assay (Chen et al., 2012). The positive effects of the D187G and especially Q196R single mutations were particularly apparent when combined with the Q226L/G228S mutation on the Egret/06 background missing the carbohydrate at N158. In the glycan microarray assay, the result was nearly complete suppression of binding to 2–3Sia while further enhancing binding to 2–6Sia (Fig. 5C).

To assess the impact of the change in specificity on transmission, the triple mutant Q196R/Q226L/G228S introduced into a Egret/06 background without a glycan at N158 was tested for contact and aerosol transmission in a ferret model (Fig. 4B). While the wild type virus showed no transmission, the mutant virus transmitted efficiently by direct contact, but not by aerosol. To address the possibility of a mismatch in specificity between the neuraminidase and mutant hemagglutinin, viruses were engineered to contain the N1 or N2 from seasonal human viruses (H1N1 and H3N2, respectively, with the remaining genes from Egret/06. Both viruses also efficiently transmitted by direct contact, and the one containing the human virus N2 neuraminidase also showed partial transmission by aerosol droplets (Chen et al., 2012).

Kawaoka and colleagues started with a similar *in vitro* selection strategy using a laboratory influenza strain (PR8) engineered to contain a H5 hemagglutinin containing random mutations in the receptor binding domain (aa 120–259). This was adsorbed to turkey RBCs treated with a sialidase specific for 2–3Sia to generate RBCs enriched in 2–6Sia. After selection, 370 viruses were propagated and screened for binding 2–6Sia turkey RBCs. Of 9 viruses identified to exhibit binding to 2–6Sia modified cells, each contained 1–4 mutations in the hemagglutinin, and several exhibited increased binding to 2–6Sia in plate binding assays employing 2–6Sia- and 2–3Sia-PAA polymers. In particular, one mutant with four mutations, E119G/V152I/N224K/Q226L, showed strong binding to 2–6Sia and little or no binding to 2–3Sia. This specificity was also seen with a virus engineered to contain just two mutations, N224K/Q226L. To assess the impact on transmission in ferrets, these mutations were engineered into a ‘reassortant’ H1N1 Cal/04 pandemic strain, with the H1 hemagglutinin replaced by the H5 hemagglutinin from VN/1204 with and without the mutations conferring 2–6Sia. The mutations failed to support contact transmission in ferrets, suggesting that the change in receptor specificity alone was not sufficient.

However, it was noticed that viral titers remained high in the nasal turbinates of several ferrets beyond 6 days, resulting from an additional mutation, N158D, which results in the

loss of the carbohydrate at this site. Virus with this additional mutation transmitted by direct contact. Sequence analysis of virus in the contact ferrets revealed yet another mutation in the H5 hemagglutinin, T318I, shown subsequently to stabilize the hemagglutinin trimer, compensating for reduced stabilization introduced by the mutations near the receptor binding pocket. Virus with all four mutations (N158D/N224K/Q226L/T318I) transmitted efficiently by direct contact and aerosol (Fig. 4C). In this example, a series of four mutations were identified using a combination of *in vitro* selection for 2–6Sia binders, and *in vivo* selection for additional mutations that increased efficiency of transmission in a ferret model (Imai and Kawaoka, 2012).

Fouchier and coworkers chose yet another strategy involving passage in ferrets after making three mutations in the wild type Indo/05 strain by reverse genetics (Herfst et al., 2012). Two mutations were introduced in the hemagglutinin (Q226L/G228S) to bias the specificity towards 2–6Sia, and one mutation (E627K) was introduced in the polymerase PB2, which is known to increase replication at lower temperatures in the mammalian airway. This virus was passaged in ferrets 10 times (P1–10) to allow mutations that would provide efficient replication. Then, the P10 virus was tested for airborne transmission with analysis of the sequences. Sequencing of the P10 virus and virus from each ferret that were infected by airborne transmission revealed 9 additional amino acid changes, but only two were consistently found in all isolates, and both were in the hemagglutinin, T160A and H107Y. Interestingly, the T160A mutation also disrupts the sequon for N-linked glycosylation at N158, resulting in no glycan at this position (Fig. 4D). The H107Y mutation is at the trimer interface, and is believed to stabilize the hemagglutinin trimer, although this has not been directly demonstrated.

Unlike the other studies employing directed evolution, this latter study reports transmission of an H5N1 virus with no human virus gene segments. Remarkably, successful transmission of an H5 virus in ferrets appears to be the result of just 5 mutations, three of which were introduced by reverse genetics, and two of which were selected for by passage in ferrets. Although a systematic study of the other mutations found in the ferret isolates has not yet been done and may ultimately be found contribute to transmission efficiency, 4 of the five mutations were in the hemagglutinin, and at least 3 were associated with a change in receptor specificity.

In summary, the three studies demonstrate different solutions for achieving ‘human type’ receptor specificity in the H5 hemagglutinin that supports transmission in ferrets (Fig. 4). It is striking that the solutions have several elements in common (Yen and Peiris, 2012). First, all have the Q226L mutation found in H2 and H3 human isolates. In two cases, this mutation was introduced by reverse genetics (Chen et al., 2012; Herfst et al., 2012), but in one case it was selected through adsorption of mutagenized virus to 2–6Sia containing red blood cells (Imai et al., 2012). They each have one or two additional mutations that enhance 2–6Sia binding. Finally, in all three cases the N-linked glycan at residue 158 is missing, either as a result of intentionally using a strain (Egret/06) that was already missing the glycan, or by natural selection during propagation in ferrets that resulted in a mutation that disrupted the NxT/S sequon required for glycan addition. It is also important to note that mutations to adapt the HA to human type receptor specificity were not alone sufficient for transmission in ferrets, since in two cases an additional mutation in the stalk region or trimer interface was required (Herfst et al., 2012; Imai et al., 2012), and in the third case a N2 neuraminidase from a human seasonal strain was required (Chen et al., 2012). It will be of considerable interest to understand the mechanistic details of how these factors complement each other for creating a transmissible virus.

Relevance of H5N1 receptor specificity to the pandemic potential of H5N1 virus

Can H5N1 naturally acquire human type receptor specificity? If so, will this be sufficient for transmission of H5N1 from human to human? Naturally occurring mutations in H5N1 isolates from birds and humans with increased human type receptor specificity, and mutants derived through *in vitro* and *in vivo* evolution with a complete shift in receptor specificity suggest that it is entirely feasible for H5N1 to naturally acquire human type receptor specificity. The answer to the second question is more complex, since naturally occurring mutants in humans have to date had only modest changes in receptor specificity, and no transmission has yet been documented. However, to the extent that the ferret model is an adequate model of transmission in humans, it seems clear that 2–6Sia receptor specificity is a critical factor for transmission, and that minimal other changes may be required for the H5N1 virus to spread by airborne transmission from animal to animal (Chen et al., 2012; Herfst et al., 2012; Imai et al., 2012).

It is significant that combinations of 3–4 mutations were required for H5N1 to acquire sufficient specificity for 2–6Sia to support transmission in ferrets (Fig. 5), while only 1–2 mutations are required for a change to human receptor specificity for the H1, H2 and H3 hemagglutinins in human pandemic viruses (Belser et al., 2011; Childs et al., 2009; Connor et al., 1994; de Vries et al., 2011; Liu et al., 2009; Liu et al., 2010; Rogers et al., 1985; Rogers et al., 1983a; Tumpey et al., 2007; Xu et al., 2012a; Xu et al., 2010). Because additional mutations are needed, the probability that an appropriate combination of mutations will occur naturally is dramatically reduced, and may be a major factor in why it has not occurred to date (Ayora-Talavera et al., 2009; Russell et al., 2012). However, as alluded to the above, several of the mutations identified that contribute to 2–6Sia specificity have already been detected in avian H5N1 isolates. Notably, the critical mutation affecting the glycosylation site at position 158 (either via N158D, S160A, or both) is present in all but one of over 100 H5 sequences from human infections of clade 2.2 viruses. Another mutation, Q196R, used by Chen et al. (Fig. 4) has also been found in human H5N1 isolates (Chutinimitkul et al., 2010; Yamada et al., 2006), and in a related mutant in a bird isolate in Egypt, which has been associated with increased infections in humans. Other natural mutants (e.g. S227N, 133del / I155T) have also been reported to contribute to 2–6Sia (Watanabe et al., 2011; Yamada et al., 2006). Thus, infection of humans with zoonotic viruses with preexisting natural mutations will increase the probability that additional mutations will occur by natural selection during the course of infection.

As H5N1 evolves in zoonotic species, monitoring mutations that could contribute to acquisition of human type receptor specificity would be of significant value in assessing the potential for pandemic risk. Indeed, analysis of the specificity of natural isolates has already contributed to our understanding of what is needed for the H5 hemagglutinin to bind 2–6Sia receptors and human airway epithelial cells (Auewarakul et al., 2007; Chutinimitkul et al., 2010; Watanabe et al., 2011; Yamada et al., 2006). Of the assays currently used for monitoring receptor specificity, the plate assay and glycan microarray assay provide consistent and complimentary information. Various versions of the plate assay appear well suited for detecting weak 2–6Sia binding properties that result in increased binding to human non-ciliated airway epithelial cells (Ayora-Talavera et al., 2009; Gambaryan et al., 2006; Gambaryan et al., 2008; Gambaryan et al., 2004; Watanabe et al., 2011; Yamada et al., 2006). It is now clear, however, that detection of binding to 2–6Sia in this assay is not alone a sufficient predictor of transmission in mammals. More predictive of transmission is strong binding to 2–6Sia and reduced or minimal binding to 2–3Sia. The glycan microarray assay has a higher threshold for detecting 2–6Sia, showing little change in receptor specificity with single mutations that show clear binding to 2–6Sia in the plate

assays (Fig. 5) (Chen et al., 2012; Watanabe et al., 2011; Yamada et al., 2006). However, the glycan microarray provides additional information about the specificity towards a wide variety of glycans bearing 2–3Sia and 2–6Sia in a single experiment. Thus, it is notable that several reports using glycan microarrays have documented that the simultaneous acquisition of robust binding to glycans with 2–6Sia and reduced binding to the majority of glycans with 2–3Sia correlates with transmission in the ferret model (Belser et al., 2008; Chen et al., 2012; Wan and Perez, 2006; Wan et al., 2008). It is generally assumed that the specificity for 2–6Sia is required for efficient adsorption of the virus to cell surface receptors on human airway epithelial cells. Reduced binding to 2–3Sia has been suggested to prevent the influenza from adsorbing to mucins in the airway (Couceiro et al., 1993), but there has been little done to date to test this hypothesis or its relevance to ferret transmission. This type of information will also become increasingly valuable as more is learned about the types of glycans present on cells lining the airway epithelium and in airway secretions (Bateman et al., 2010; Chandrasekaran et al., 2008).

On a practical level, plate binding assays are well suited for routine monitoring of receptor specificity because of their sensitivity for detecting increased binding to 2–6Sia and because the ELISA type format can be performed in most laboratories. The glycan microarray assay can provide more detailed information for mutations that require more detailed characterization. It should also be noted that bioinformatics approaches for *in silico* assessment of receptor specificity is emerging (Chandrasekaran et al., 2008; Newhouse et al., 2009; Xu et al., 2009), and may ultimately be of utility for large-scale analysis of sequence information once the *in silico* assessment of receptor specificity is documented to be a reliable predictor of receptor specificity assessed with laboratory assays.

Conclusion

Recent reports have documented that the H5 hemagglutinin can acquire the ability recognize human-type receptors. However, the number of mutations required for a receptor switch is greater (3–4) than that required for receptor switch in the H1, H2 and H3 hemagglutinins (1–2) of influenza viruses that have successfully adapted to humans. This may explain why the switch in receptor specificity has not occurred in nature, even during replication in human hosts. Based on analysis of natural isolates and laboratory efforts to evolve receptor specificity, there are a growing number of mutations documented to increase the tendency to bind human receptors. Thus, it is likely that other solutions for achieving human-type receptor specificity in H5 will be discovered. A deeper understanding of how multiple mutations coordinate to confer human-type specificity, while retaining hemagglutinin stability, would greatly facilitate efforts to monitor the pandemic risk of H5N1 as it evolves in nature.

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Figure 1.

Influenza virus hemagglutinin receptor specificity assays in plate format. (A). Virus or recombinant hemagglutinin (HA) is adsorbed to the plate. Biotinylated polymers displaying multivalent 2–3Sia or 2–6Sia are then added, and after washing, bound sialoside is detected with labeled streptavidin (Gambaryan and Matrosovich, 1992). (B). Sialoside is bound to the plate, followed by detection of bound virus (Chandrasekaran et al., 2008; Totani et al., 2003; Yamada et al., 2006).

Figure 2.

Examples of common sialic acid containing glycans found in glycoproteins and glycolipids on cells of mammalian and avian hosts of influenza. Illustrated are linear fragments that occur as variations at the tips of glycoprotein or glycolipid glycans, **1–8** (L), intact O-linked Core 1–4 glycans linked to threonine or serine, **9–21** (O1–O4), and N-linked glycans linked to asparagine, **22–27** (N). In most cases, sialic acids (purple diamonds) can be linked in either $\alpha 2\text{--}3$ or $\alpha 2\text{--}6$ linkage to the next sugar (e.g. galactose).

Figure 3.

Structural basis for human-type receptor specificity in H1, H2 and H3 hemagglutinins. (A) Structure of the hemagglutinin receptor-binding domain (RBD) from Cal/04/09 H1N1 (PDBID: 3UBE). Structural features that frame the RBD including the 130 and 200 loops and the 190 helix are labeled in red. Side chains are shown in green for amino acids involved in binding sialic acid (Y95, W153, H183 and Y195) or are known to influence receptor binding in human influenza viruses (D190, D225, Q226 and G228.). (B) Tabular representation for amino acids involved conferring human type receptor specificity in red, and corresponding sequences in avian isolates. (C) Structure H2 hemagglutinin (A/Sin/1/52 H2N2, PDBID: 2WR) co-crystallized with the 2–6 sialoside, LSTc. Side chains of amino acids that confer specificity for 2–6 sialosides at positions S226 and L228 are shown in green. (D) Structure representation of H2 A/Sin/1/52 H2N2 co-crystallized with the 2–3 sialoside LSTb (PDBID: 2WRB), showing only the terminal NeuAc-Gal sequence, and the ‘avianized’ mutations S226G and L228Q. (E) Structure H1 derived from Cal/04/09 H1N1 co crystallized with LSTc (PDBID: 3UBE). Side chains of amino acids that confer specificity for 2–6 sialosides at positions D190 and D225 are shown in green. (F) Structure of H1 derived from Cal/04/09 H1N1 co crystallized with the 2–3 sialoside LSTb (PDBID: 3UBQ), showing only the terminal NeuAc-Gal sequence, and the ‘avianized’ mutations D190E and D225G. Structural representations are made using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC <http://www.pymol.org>. with ‘avianized’ mutations modeled in Swiss Model (Arnold et al., 2006).

Figure 4.

H5 structures of WT and transmissible H5 viruses. (A) Structure representation of H5 derived from H5N1 with complex N-glycans attached at its N-glycosylation sites (PDBID: 2IBX). The N-linked glycan at position 158 is shown in yellow, and was modeled by Glyprot (Bohne-Lang and von der Lieth, 2005). Side chains of amino acids at position 158, 160, 196, 224, 226 and 228 known to influence receptor specificity and transmissibility are highlighted in green. (B) Transmissible H5 HA from Chen et al. (Chen et al., 2012) with mutations at position 196, 226 and 228 shown in green. The H5 used in this study was from Egret/EG/06, which does not contain a NxT/S sequon at position 158. (C) Transmissible H5 HA from Imai et al. (Imai et al., 2012) with side chains of mutations at position 158, 224

and 226 shown in green. (D) Transmissible H5 HA from Herfst et al. (Herfst et al., 2012) with side chains of mutations at positions 160, 226 and 228 shown in green. In panels A-D a glycan is modeled in appropriate to the specificity of that hemagglutinin for illustration only. Panel has superimposed the NeuAc 2–3Gal structure from LSTb co-crystallized with H1N1 Cal/04/09 (PDBID: 3UBQ). Panels B-D have the superimposed the 2–6 sialoside from LSTc co-crystallized with H2 A/Sin/1/52 H2N2 (PDBID: 2WR7). Structural representations are made using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC <http://www.pymol.org>.

Figure 5.

Comparison of ELISA-type and glycan array assays for assessing receptor specificity of H5 mutations. Shown is the influence of E190G and Q196R mutations on receptor specificity of H5 hemagglutinin assessed in two different assays. (A) Receptor specificity of mutations made on the A/Vietnam/1203/04 background the ELISA-type assay. (B) Receptor specificity of mutations made on the A/Vietnam/1203/04 (right) and clade 2.2 A/Egret/Egypt/06 (right) strains using a glycan array with a variety of 2–3Sia and 2–6Sia. Data are excerpted and reproduced with permission with full details and list of glycans in the glycan array available in the original article (Chen et al., 2012).

Summary of receptor specificity analysis of H5 HA mutations

Table 1

H5 Mutants ^b	Strain ^d								Receptor Assay			Specificity ^c		Ref ^e
	HK/486	VN/1203/04	VN/1194/04	Indo/NL/05	1-KAN-1/05	Indo/05	Egret/EG/06	Duck/EG/07	Modified RBCs	ELISA	Glycan Array	2-3	2-6 ^d	
WT	X	X	X		X	X	X	X	X	X	X	+++	+	1-14
A39T		X								X	X	+++	-	2
E79K			X							X		+++	-	3
S124N								X		X		+++	-	4
S127P			X							X		+++	-	3
I33del								X		X		+++	-	4
L133V					X					X		+++	-	3
S133V						X			X			+++	-	5
S137A					X	X			X			+++	+++	5,6
A138V					X				X	X		+++	-	7
G144R			X	X						X		+++	++	3
I154T								X		X		+++	-	4
K157Q		X								X		+++	-	2
N158S						X			X			+++	+	5
N186K			X	X		X			X	X		++	++	3,5,6
D187G		X								X	X	+++	++	2
E190D		X			X				X		X	++	-	6,8
E190G		X								X	X	+++	++	2
T192I					X	X			X			++	++	5,6
N193K						X				X		+++	++	3
Q196H								X		X		+++	+++	4
Q196R		X	X		X	X			X	X	X	+++	++	3,5,6
N197K				X		X			X	X		+++	++	3,5
V214I								X		X		+++	-	4
S221P		X									X	+++	-	8
K222E						X			X			+	-	5
G225D		X			X	X			X	X	X	+++	+	5,6,8
Q226L		X	X		X	X			X	X	X	++	++	6,8,9

H5 Mutants ^b	Strain ^d								Receptor Assay			Specificity ^c		Ref ^e
	HK/486	VN/1203/ 04	VN/1194/ 04	Indo/N1/ 05	1-KAN-1/05	Indo/05	Egret/EG/06	Duck/EG/07	Modified RBCs	ELISA	Glycan Array	2-3	2-6 ^d	
S227N	X	X	X			X			X	X	X	+++	++	2, 3, 5, 8, 9, 10, 11
G228S		X	X			X			X	X	X	+++	++	8, 9
S239P								X		X		+++	++	4
E255K		X								X		+++	++	2
H295Q		X								X		+++	-	2
T318I		X								X		+++	-	12
R171K (HA2)			X							X		+++	-	3
E79K / R171K (HA2)								X		X		+++	+++	4
H107Y / T160A						X			X			+++	-	13
E119G / Q226L		X								X		++	-	12
S124N / I33del								X		X		+++	-	4
S124N / V214I								X		X		+++	++	4
S127P / N197K			X							X		+++	++	3
S127P / R171K (HA2)			X							X		+++	++	3
H130Q / K157E		X								X		+++	-	12
I33del / I155T								X		X		+++	+++	4
L133V / A138V					X				X	X		+++	++	7
S133V / A138V						X			X			+	-	5
S137A / T192I					X	X			X			+++	+++	5, 6
G144R / N186K			X		X	X			X	X		+++	++	3, 5
V152I / Q226L		X							X			-	++	12
W153R / T160I		X							X			++	-	12
N186K / M230I		X							X			++	++	12
D187G / S227N		X							X	X		+++	+++	2
E190D / K193S					X				X			+++	-	6
E190D / G225D		X			X				X	X		-	-	6, 8
K193S / G225D					X				X			+++	-	6
K193S / Q226L					X				X			+++	-	6
Q196R / S227N		X	X							X	X	+++	+++	2, 3
Q196H / S239P								X		X		+++	+++	4
N197K / R171K (HA2)			X							X		+++	+++	3

H5 Mutants ^b	Strain ^d								Receptor Assay			Specificity ^c		Ref ^e
	HK/486	VN/1203/ 04	VN/1194/ 04	Indo/N1/ 05	1-KAN-1/05	Indo/05	Egret/EG/06	Duck/EG/07	Modified RBCs	ELISA	Glycan Array	2-6 ^d		
												2-3	2-6 ^d	
I202T / R220S		X								X		+++	-	12
R216E/ S221P		X									X	+++	++	8
N224K / Q226L		X								X		-	++	12
Q226L / G228S	X	X			X	X	X		X	X	X	++	++	2, 5, 6, 8, 10, 12, 13, 14
Q226L / E231G		X								X		++	++	12
S227N / G228A		X								X		++	++	12
E79K / S127P/ N197K			X							X		+++	+++	3
E79K / S127P/ R171K (HA2)			X							X		+++	++	3
E79K / N197K / R171K (HA2)			X							X		+++	++	3
E119G / V152I / N224K		X								X		+++	-	12
E119G / V152I / Q226L		X								X		++	++	12
E119G / N224K / Q226L		X								X		-	++	12
S127P / N197K / R171K (HA2)			X							X		+++	++	3
I33del / I155T / V214I								X		X		+++	+++	4
S140N / I33del / I155T								X		X		+++	+++	4
V152I / N224K / Q226L		X								X		-	++	12
N158D / N224K / Q226L		X								X		-	++	12
T160A / Q226L / G228S		X				X					X	++	++	10
N169I / H184L / I217M		X								X		+++	-	12
N186K / Q226L / G228S						X			X			-	+++	5
D187G / Q226L / G228S							X				X	++	++	2
E190D / K193S / G225D					X						X	+++	-	6
E190G / Q226L / G228S							X				X	++	+++	2
K193R / Q226L / G228S		X							X		X	+++	+++	10, 14
K193S / Q226L / G228S					X							-	-	6
Q196R / Q226L / G228S						X						-	++	5
Q196R / Q226L / G228S							X				X	-	+++	2
Q226L / S227N / G228S						X						-	+	5
E79K / S127P/ N197K/ R171K (HA2)			X							X		+++	+++	3
E119G / V152I / N224K / Q226L		X								X		-	+++	12
S124N / I33del / I155T / V214I								X		X		+++	+++	4

H5 Mutants ^b	Strain ^d								Receptor Assay			Specificity ^c		Ref ^e
	HK/486	VN/1203/ 04	VN/1194/ 04	Indo/N1/ 05	1-KAN-1/05	Indo/05	Egret/EG/06	Duck/EG/07	Modified RBCs	ELISA	Glycan Array	2-3	2-6 ^d	
H107Y / T160A / Q226L / G228S						X			X			++	+++	13
N158D / N224K / Q226L / T318I		X								X		-	++	12
N186K / Q226L / S227N / G228S						X			X			-	+++	5
A189K / K193N / Q226L / G228S		X							X			+++	-	6
E190D / K193S / Q226L / G228S	X	X							X		X	-	-	6, 14
Q196R / Q226L / S227N / G228S						X			X			-	+	5
N186K / Q196R / Q226L / S227N / G228S						X			X			+	+++	5
E187G / E190D / K193S / Q226L / G228S	X								X		X	++	++	14

^aIrrespectively of viral background: A/Hong Kong/486/97; A/Vietnam/1203/04; A/Vietnam/1194/04; A/Chicken/Indonesia/N1/05; A/Thailand/1-KAN-1/04; A/Indonesia/5/05; A/Egret/Egypt/1162/06; A/Duck/Egypt/D1Br12/07

^bBased on H3 numbering, numbering for the HA2 domain are given separately and are indicated as such.

^cReceptor specificity: +++ Strong; ++ Moderate; + Weak/Variable; - Not detected

^dSpecificity for 2–6 linkages may be assay and/or strain dependent

^eReferences: 1 (Wickramasinghe et al., 2011); 2 (Chen et al., 2012); 3 (Yamada et al., 2006); 4 (Watanabe et al., 2011); 5 (Chutinimitkul et al., 2010); 6 (Yang et al., 2007); 7 (Auewarakul et al., 2007); 8 (Stevens et al., 2006); 9 (Ayora-Talavera et al., 2009); 10 (Stevens et al., 2008); 11 (Gambaryan et al., 2006); 12 (Imai et al., 2012); 13 (Herfst et al., 2012); 14 (Maines et al., 2011)