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Current Opinion in Virology

# Influenza virus–glycan interactions

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It has been known for many years that influenza viruses bind by their hemagglutinin surface glycoprotein to sialic acid (N-acetylneuraminic acid) on the surface of the host cell, and that avian viruses most commonly bind to sialic acid linked α2-3 to galactose while most human viruses bind to sialic acid in the α2-6 configuration. Over the past few years there has been a large increase in data on this binding due to technological advances in glycan binding assays, reverse genetic systems for influenza and in X-ray crystallography. The results show some surprising changes in binding specificity that do not appear to affect the ability of the virus to infect host cells.

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**Current Opinion in Virology** 2014, **7**:128–133

This review comes from a themed issue on **Virus–glycan interactions and pathogenesis**

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For a complete overview see the Issue and the Editorial

Available online 24th July 2014

http://dx.doi.org/10.1016/j.coviro.2014.06.004



## Introduction

In the spring of 2013 there was an outbreak of a new human influenza virus, serotyped as H7N9, from scattered regions of China. The outbreak did not spread outside China, and disappeared within weeks as live bird markets were closed. However, new cases towards the end of 2013 and into 2014 show that the virus is still present. Like the previous outbreaks of avian influenza H5N1, H7N7 and H9N2 in humans, the H7N9 virus has not been shown to transmit from one human to another. There is ongoing concern that a few key mutations might confer the ability to spread in the human population. Much of the research activity to understand the factors that might lead to a human pandemic is directed to study of receptor binding and recent advances in technology have facilitated these efforts. This review summarizes the information available on receptor binding by influenza viruses using glycan arrays, structural investigation to understand the molecular basis of receptor specificity, together with studies on binding and cleavage of sialylated substrates by the neuraminidase, and compares the H7N9 data with recent information on seasonal human viruses, H3N2, H1N1 and influenza B.

## Historical background

Influenza viruses belong to the Orthomyxoviridae family. These are enveloped viruses with a genome consisting of 8 segments of single-stranded, negative sense RNA, containing coding information for at least 11 functional proteins. Influenza type A and B viruses have two major surface glycoprotein antigens embedded in the viral membrane and forming the outer spikes of the virus particle. They are the hemagglutinin (HA, or H) that binds to sialic acid receptors and fuses the viral and cell membranes to release the viral nucleocapsids, and neuraminidase (NA, or N) which cleaves sialic acid. Neutralizing antibodies target these surface glycoproteins. Type A influenza viruses are divided into subtypes based on lack of cross-reactivity of the surface antigens, currently H1 to H16 and N1 to N9. Two groups of viral genome sequences from bats recently discovered have been tentatively designated H17N10 and H18N11. Live bat virus has not been recovered or propagated as yet, but it has been shown that H17 and H18 do not bind sialic acids and N10 and N11 do not cleave sialic acid and the receptors have not yet been identified [1].

The HA is the attachment protein, binding to sialylated glycans on the host cell surface, while the NA removes the sialic acid from glycans, thus acting as a receptor-destroying enzyme. The specificity of these two activities has become a subject of intense study. In the 1980s James Paulson and his colleagues showed that human influenza A viruses bind to sialylated glycans with an α2-6 linkage to galactose while avian influenza viruses bound to α2-3 linked sialic acid. They grew a human H3 isolate in the presence of horse serum that contains a potent inhibitor of human virus hemagglutination, α2 macroglobulin, and found a change in binding from Siaα2,6Gal to the Siaα2,3Gal linkage. This binding change was accompanied by a mutation in HA of Leu226Gln [2]. Furthermore, by applying selection using cycles of binding of an avian virus to red blood cells treated to display only Siaα2-6 glycans and amplification of bound virus, they isolated mutant viruses that bound only Siaα2-6 glycans. These viruses had the reciprocal single amino acid substitution in the HA of Gln226Leu while passaged in MDCK cells but rapidly reverted to 226Q when grown in eggs [3]. Studies on host specificity of influenza viruses, and in particular on what changes are needed to allow an avian virus to infect and transmit among humans have focused on this finding ever since [4•,5•]. A molecular explanation

for the dramatic difference in binding Siaα2-3 versus α2-6 was found in crystal structures of avian and human HAs bound to sialylated pentasaccharides LSTa or LSTc. The sialic acids are bound identically but the rest of the glycan in Siaα2-3 follows an extended conformation while the glycan in Siaα2-6 bends back on itself, as seen in a recent study of HA of H7N9 viruses in complex with LSTa and LSTc [6].

If NA is truly a receptor-destroying enzyme its specificity should match that of the HA. Trends have been noted in NA specificity that have been interpreted as NA mutations to match HA to humans, but the changes are minor decreases in the ratio of 2-3/2-6 activity and in no case has the influenza NA been found to prefer Siaα2-6 over Siaα2-3 (reviewed in [7]).

## Glycan array analysis

The development of glycan arrays in the early 2000s has revolutionized the study of influenza virus binding specificity. Until then only a few sialylated glycans were available for binding studies, such as sialyllactose, the milk pentasaccharides LSTa and LSTc, and gangliosides [8]. The establishment and funding of the Consortium for Functional Glycomics (CFG) led to development of new chemo-enzymatic methods for glycan synthesis that allowed the printing of several hundred individual glycans on a glass slide [9,10]. The current version of the CFG Glycan Array has 610 glycans, of which 166 are sialylated. Other array platforms have been developed including use of neo-glycolipids [11] and glycans attached to bovine serum albumin [12]. Raw data from the CFG arrays are publicly available, posted on the CFG web site http://www.functionalglycomics.org/glycomics/publicdata/. It is instructive to examine the raw data in addition to the processed and interpreted accounts that are published in journals, where a large amount of information is necessarily lost.

Several studies have been made of the 2009 pandemic H1N1 viruses using various glycan array platforms. At first sight the results are conflicting but the conflicts appear to be from analysis of only a single concentration of virus or recombinant HA along with differing interpretation of the significance of minor binding signals [13]. The conclusion is that pdmH1N1 viruses show preferential binding to α2-6 sialylated glycans with lesser but maybe significant binding to α2-3 sialylated glycans.

We undertook glycan array screening of a comprehensive collection of human H3N2 viruses isolated from 1968 to 2012 and found surprising variation in binding specificities. The study was done using viruses passaged only in mammalian cell lines to avoid changes due to adaptation for growth in embryonated chicken eggs as for vaccine production. H3N2 viruses first appeared in humans in 1968 and isolates from the early years preferentially bind short, branched α2-6 sialylated glycans. In later years the preference changed to long, linear α2-6 sialylated polylactosamine structures (Figure 1). However, there are exceptions to this pattern (Figure 2), including viruses that bind α2-3 sialic acids, preferentially or exclusively, and viruses that bind only a very restricted set of glycans [14••]. While we recognize that the glycan array does not contain all the sialylated structures of a mammalian cell, and the single spot of each glycan cannot be a true reflection of the cell surface, there is no doubt that the binding site changes with antigenic changes to give differences in avidity and specificity without noticeable decrease in viral fitness or transmissivity. All of the viruses we studied spread around the world.

The idea that receptor specificity determines host tropism has been vigorously explored. Most studies aiming to alter host specificity have been done with H5N1 viruses. This subtype, previously only seen in birds, was first recognized as a new human pathogen in 1997 and became more prevalent in the early 2000s, but did not transmit from one human to another except in very rare instances. There was considerable concern that, as in the early Paulson experiments, a single mutation might confer on this virus the ability to transmit between humans and so start a new pandemic to which the human population had no immunity. This turned out not to be the case, and multiple mutations were required for an H5N1 virus to transmit between ferrets [4•,5•]. Very stringent biosecurity was applied to these experiments and there is no knowledge of whether such combinations of mutants, not seen in nature, would enable human-to-human transmission. These mutant viruses have increased affinity for α2-6 linked sialic acids but although a relationship between the sialic acid linkage and infectivity or transmission is inferred, we do not yet know if it is direct.

The same concerns arose over the H7N9 viruses. To the end of February 2014 WHO has reported a total of 375 laboratory-confirmed cases of human infection with avian influenza A(H7N9) virus, including 115 deaths. The vast majority of cases have occurred in China or the Hong Kong SAR, with two in Taipei and one in Malaysia in a traveler from China. Most cases appear to be due to direct transmission from infected poultry. There may have been rare, limited human-to-human infection but to date there have been no sustained human transmissions (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/).

The H7N9 virus HAs bind α2-3 linked sialic acids but some of the 2013 H7 HAs have Leu at 226 and it was thought that this might confer the ability to bind α2-6 receptors. Recombinant HA binds only Siaα2-3 glycans, and only a small subset of them. These are generally short, typical N-linked or O-linked glycans, and some are sulfated [6]. Two human H7N9 viruses with 226 Q or L