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REVIEW

H3N2 influenza viruses in humans: Viral mechanisms, evolution, and evaluation

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ABSTRACT

Annual seasonal influenza vaccines are composed of two influenza A strains representing the H1N1 and H3N2 subtypes, and two influenza B strains representing the Victoria and Yamagata lineages. Strains from these Influenza A and Influenza B viruses currently co-circulate in humans. Of these, strains associated with the H3N2 subtype are affiliated with severe influenza seasons. H3N2 influenza viruses pre-dominated during 3 of the last 5 quite severe influenza seasons. During the 2016/2017 flu season, the H3N2 component of the influenza vaccine exhibited a poor protective efficacy (~28–42%) against preventing infection of co-circulating strains. Since their introduction to the human population in 1968, H3N2 Influenza viruses have rapidly evolved both genetically and antigenically in an attempt to escape host immune pressures. As a result, these viruses have added numerous N-linked glycans to the viral hemagglutinin (HA), increased the overall net charge of the HA molecule, changed their preferences in receptor binding, and altered the ability of neuraminidase (NA) to agglutinate red blood cells prior to host entry. Over time, these adaptations have made characterizing these viruses increasingly difficult. This review investigates these recent changes in modern H3N2 influenza viruses and explores the methods that researchers are currently developing in order to study these viruses.

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Introduction

Influenza is a respiratory illness that infects between 5–15% of the global population annually. The World Health Organization (WHO) estimates that these infections result in 250,000 to 500,000 deaths every year.[1] Those suffering from an influenza infection commonly display symptoms such as fever, sore throat, coughing, nasal discharge, headache, and myalgia. More severe cases can also lead to the development of conditions such as bronchitis or pneumonia.[2] Influenza outbreaks have caused widespread illness to humans many times throughout history. In 1968 an avian reassortant virus of the H3N2 subtype was introduced into the human population that caused a global pandemic associated with more than one million deaths worldwide.[3] More recently in 2009 an influenza pandemic caused by a novel strain of H1N1 resulted in millions of infections in more than 214 countries.[4] Since their introduction in 1968, H3N2 influenza viruses have undergone extensive genetic and antigenic evolution leading to numerous seasonal epidemics, exemplified by the WHO recommending 28 vaccine strain changes over that period of time. H3N2 influenza viruses have also altered their receptor binding properties over the last half century, and are beginning to display a reduced affinity for oligosaccharide analogues of cell surface receptors.[5] Recent H3N2 influenza vaccine effectiveness studies in the U.S. and in Europe from the 2016/2017 season in people showed a poor efficacy (~28–42%) for all age groups.[6] The rapid evolution of influenza viruses creates difficulties for experts to recognize and predict current and future epidemiological threats.[7] The WHO meets twice a year to recommend strains that will be included in the seasonal influenza vaccine. This is based primarily from results of hemagglutination inhibition (HAI) assays that compare antibody titers of reference ferret antiserum to currently circulating influenza isolates.[8] The HAI assay detects antibodies that bind to the viral hemagglutinin and prevent the virus-mediated agglutination of erythrocytes.[9] However recent studies have demonstrated that most modern H3N2 strains have gained the ability to agglutinate red blood cells through neuraminidase-sialic acid interactions.[10] Therefore, many researchers have modified existing assays and developed new ways to characterize these modern H3N2 influenza viruses. One of the more common modifications includes the addition of Oseltamivir carboxylate, a neuraminidase inhibitor, into the HAI assay in order to ablate neuraminidase binding prior to host entry.[11] This review seeks to investigate these recent changes in modern H3N2 viruses and explore the methods that researchers are currently developing to analyze this ever-adapting pathogen.

Viral life cycle

There are currently three types of influenza viruses: A, B, and C.[4] Influenza and other members of the family Orthomyxoviridae are enveloped viruses characterized by having eight

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segmented, negative-sense RNA genomes.[12,13] Influenza A viruses (IAV) can infect humans, birds, pigs, horses and other animals while influenza B and C viruses are only found in humans.[1] IAV are subtyped according to their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA).[14,15] Currently there are 18 known subtypes of HA (H1–18) and 11 of NA (N1–11), but only a limited number of these i.e., H1N1, H3N2, and H3N3, currently circulate in humans.[2,16] HA is a trimeric glycoprotein that is typically classified in two groups: group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16 while group 2 includes H3, H4, H7, H10, H14, H15. Recently two more HA subtypes, H17 and H18, have been discovered in bats. NA is a tetrameric glycoprotein that is divided into three groups: group 1 (N1, N4, N5, and N8), group 2 (N2, N3, N6, N7, N9), and group 3, which contain NA from B influenza viruses. N10 and N11 have only recently been discovered and primarily circulate in bats.[17] The IAV surface is composed of a host cell derived lipid membrane that is studded with glycoprotein projections of HA and NA at a ratio around 4:1 respectively.[18] Also present on the outer membrane of IAV are a number of smaller matrix (M2) ion channels, which aid in viral un-coating, and M1 proteins which form a matrix that encloses the viral core.[12] The viral core houses the eight viral RNA segments. Two of the segments encode pre-mRNA that is eventually spliced to produce the nuclear export protein (NEP also known as NS1) as well as M1 and M2. The other six segments consist of m-RNA that are translated into nucleoprotein (NP), polymerase subunit (PA, PB1, or PB2), HA, and NA.[2]

The influenza infection cycle begins when HA on the viral surface binds to sialic-acid (SA) containing glycan receptors on the host cell surface.[10] SAs are commonly found on the surface of many cells located in the respiratory tract such as epithelial cells, dendritic cells, and alveolar macrophages.[2] The efficiency of the binding to SA depends both on the type of SA, and the type of linkage that connects the SA residue with the oligosaccharide of the receptor molecule. It has ben shown that human influenza viruses preferentially bind to SA attached to galactose by an α2,6 linkage, while equine and avian viruses prefer α2,3 linked SA molecules.[15,19,20] After binding to SA the virus is then internalized by the host cell through the process of endocytosis, and the virus is encapsulated in an endosome. The endosome posses a low pH around 5 to 6 which triggers the fusion of the viral membrane with the endosome.[10] For membrane fusion to occur the HA precursor (HA_0) must be cleaved into two polypeptides, HA_1 and HA_2, which are linked by a disulphide bond.[7] This exposes a fusion peptide on HA_2 that mediates the merging of the viral envelope with the endosomal membrane. Meanwhile the M2 ion channel provides a route for hydrogen ions to diffuse into the virus particle which disrupts the internal protein-protein interactions, and allows the viral RNPs to be released from the viral matrix into the host cell cytoplasm.[12]

Once the viral RNPs have been released they are directed into the host nucleus with the help of viral localization signals.[4] In the nucleus, negative sense viral RNA is transcribed into positive sense mRNA using viral polymerase. The polymerase snatches 5′ caps from cellular RNA and 3′ RNA is polyadenylated in order to make viral pre-mRNA.[2] Also synthesized from the viral RNA is complementary RNA that RNA polymerase uses to transcribe more copies of negative–sense genomic viral RNA allowing the virus to efficiently replicate its genomic material.[12] Once the viral mRNA is capped and polyadenylated it is ready to be exported out of the nucleus and translated into proteins by the host ribosomes. The newly assembled proteins are then transported to the apical side of the cell membrane where the virions are assembled.[2] At this point discrete packaging signals on all viral RNA segments ensure that a full genome and the surface proteins HA, NA, and M2 are properly incorporated into the newly formed virus particles.[12] The mature HA protein is composed of three pairs of the HA_1/HA_2 subunits comprising a trimer, while NA is assembled as a tetramer.[21]

Viral release from the host cell is initiated by an accumulation of M1 at the cytoplasmic side of the cell membrane. When budding is complete the HA continues to bind the SA on the host cell until it is cleaved by the sialidase activity of the NA protein.[12] This facilitates the release of progeny virions from the host cell. Additionally sialidase activity of NA is able to remove SA residues from the viral surface helping prevent newly assembled viruses from aggregating near the cell surface and enhancing infectivity.[4] Influenza viruses have a relatively simple replication cycle. However their segmented genome, and their error-prone RNA-dependent RNA polymerases enable them to evade adaptive immune responses in a range of host species. Therefore developing a long lasting vaccine against these viruses can be very challenging.[12]

H3N2 introduction & evolution in humans

H3N2 is responsible for one of the three major influenza pandemics that occurred in the last century. In 1968, a novel strain of H3N2 influenza virus emerged in Hong Kong (A/Hong Kong/1/1968 [HK/68]) that quickly led to a global epidemic that was associated with more than one million deaths world-wide.[3] Prior to this outbreak there was no documentation of H3N2 viruses circulating in humans. Most likely, circulating human H2N2 viruses re-assorted with avian H3N2 influenza viruses that resulted in a novel H3N2 viral strain that possessed the ability to infect and transmit between humans.[13,22] Further examination of this virus provided evidence that the HA and PB1 fragments of avian H3N2, and the NA from the H2N2 pandemic strain of 1957 combined into a new viral strain of H3N2.[3,17] H2N2 and H2N3 viruses continued to co-circulate in humans until 1971 when H2N2 viruses began to wane from human circulation. However since 1968 H3N2 IAVs have circulated seasonally in the human population resulting in numerous epidemics, significant morbidity, and substantial mortality.[13]

Hemagglutinin proteins on the surface of pandemic influenza viruses typically differ from their avian precursors by at least one or two mutations in the receptor binding site (RBS) that alter viral receptor specificity from preferentially binding to α2,3 linked SAs with α2,6 SAs. A combination of five amino acid substitutions in the HA of the Hong Kong 1968 H3N2 isolates were associated with the bird-to-human adaptation and pandemic emergence of these viruses.[22] Reassortment between influenza viruses, mutations, and genomic evolution are some of the ways for the virus to increase diversity in viral protein sequences, escape antibody pressure, and evade the host's immune system.[13] IAVs typically evade the host immune

response by changing the antigenicity of HA and NA, both gradually through antigenic drift, and abruptly through what is known as antigenic shift.[16] Groundbreaking work in the 1980s identified 131 amino acid positions in five antigenic sites (A-E) in the globular head of H3 near the RBS as the main targets for specific antibodies, and suggested that antigenic shift is likely caused by substitutions in these sites.[23]

The virus can also escape immune pressure through the addition of N-linked glycosylation sites on the globular head of its glycoproteins. These sites support the attachment of sugar molecules to the side chain amide nitrogen of Asn (N) found in the seqon Asn-X-Ser/Thr where X may represent any amino acid except Pro.[3] The N-glycosylations of the HA and NA act to mask antigenic epitopes, limit binding to host antibodies, and protect the enzymatic sites of NA.[14] To date there has been no O-linked glycosylation (e.g. attachment of a sugar molecule to the hydroxyl group of Ser or Thr on the polypeptide chain) reported for IAVs. The globular head of HK/68 contained two N-glycosylation sites at residues 81 and 165. Since introduction into the human population, H3N2 viruses have evolved and gained up to 7 additional N-glycosylation sites on the HA globular head, and 5 on the HA stem region.[3,20] The HA of the most recent H3N2 vaccine strain, A/Hong Kong/4801/2014, has 11 N-glycosylation sites, while the NA contains 8 motifs.[14] N-glycans are beneficial to the virus because they can physically interfere with the binding of antibodies to antigenic sites, but they may also be detrimental to viral fitness since these glycans may mask the RBS and reduce the receptor binding activity.[24-26] The receptor binding properties of HA are determined not only by amino acid residues forming the RBS, but also by glycan interactions with HA residues near the binding pocket.[3] This progressive accumulation of N-glycosylation sites allows for the virus to escape antibody-mediated responses, thus playing a role in antigenic drift.[27]

In addition to gaining N-glycosylation sites H3 HAs have also increased their overall electric potential. The net charge of the HA molecule is an important feature that effects viral antigenicity and receptor binding affinity. During viral adsorption a higher positive charge could promote the binding of HA to its negatively charged host cell sialic acid receptors. Since 1968 the net number of positively charged amino acids in the H3 HA molecule has increased on average from +7 to +17.8.[14] These charged amino acid changes raise the avidity of the virus possibly allowing it to bind to the host cell receptor before recognition by antibodies. The change in the positive charge of H3 HA appears to parallel the addition of N-glycosylation sites on the molecule. Therefore, the positive charge of HA may have increased to compensate for the deleterious effects of gaining more N-glycosylations during the evolution of the human H3N2 virus.[24]

Since its introduction to the human population in 1968, H3N2 has been constantly evolving to evade host immune pressures. Escape is primarily achieved through the addition of N-glycosylation sites, antigenic drift, and charged amino acid substitutions near the RBS. Adaptations of H3 IAVs have appeared so quickly that since 1968 the WHO has recommended 28 vaccine strain changes.[5] In the last ten years alone there have been six suggested H3N2 influenza vaccine strain changes. This is primarily due to the emergence of novel clades and sub-clades of H3N2 IAVs. Clades are typically defined by amino acid substitutions that occur as the virus progeny diversify from parental strains. These substitutions can be functionally relevant as they may influence the antigenicity and susceptibility to neutralizing antibodies induced by infection with other lineages of H3N2.[28] The rapid mutation rate of human H3N2 IAVs results in the appearance of antigenically novel viruses every 2 to 5 years.[29] Sequence evolution within these periods can confine antigenic drift of the virus to a group of sequence variants with similar antigenic properties, which is referred to as an antigenic clade.[7] Currently the majority of H3N2 IAVs in circulation belong to clades 3C.2a and 3c.3a.[30] WHO reference laboratories suggest that the strains in the A (H3N2) clades, 3c.2a and 3c.3a were diversified from 3c.2 and 3c.3, respectively and thereafter the 3c.2a clade dominated over 3c.3a.[5,21] This is exemplified by the WHO choosing a representative of clade 3c.2a, A/Hong Kong/4801/2014 (HK/14), to be included in the northern hemisphere vaccine for the 2016/2017 and 2017/2018 seasons.[6]

H3N2 IAVs belonging to clade 3c.2a first emerged during the 2014/2015-influenza season.[21] These viruses differed from the previous vaccine strain, A/Texas/50/2012 ((Tx/12)(Clade 3c.1)), by ~10–12 amino acids. Notably substitutions F159Y and K160T occurred at antigenic site B together with an existing N at site 158 represented the potential gain of a glycosylation site that is likely to mask viral epitopes and reduce antibody access to the antigenic site (Fig. 1) [30, 31]. These mutations in antigenic site B significantly decrease the binding of ferret, sheep, and human antibodies elicited by the Tx/12 vaccine strain. During the same influenza season another A (H3N2) clade, 3c.3a, also co-circulated in humans.[21] Both clade 3c.2a and 3c.3a viruses were determined to be antigenically distinct from Tx/12, and therefore the WHO recommended that the 2015/2016 H3N2 component of the northern hemisphere influenza vaccine be updated with an A/Switzerland/9715293/2013-like (Sz/13)(clade 3c.3a) virus.[31] However antigenic site B of clades 3c.2a and 3c.3a viruses differ due to the glycosylation at site 159 found on 3c.2a viruses, and by early 2015 the 3c.3a viruses began to wane from human circulation (Fig. 1).[21,31] It is possible that the additional glycosylation site conferred a selective evolutionary advantage that that allowed 3c.2a viruses to be more efficient at human-to-human transmission than 3c.3a viruses.[21] As a result the WHO once again recommended a vaccine strain change for the northern hemisphere to include a clade 3c.2a representative, A/Hong Kong/4801/2014 (HK/14), for the 2016/2017 season.[6,30]

Clade 3c.2a viruses can currently be divided into sub-clades 3c.2a1 and 3c.3a2 both of which carry the mutation N121K which separates them from clade 3c.2a (Fig. 1).[28] Viruses belonging to 3c.2a1 carry a signature amino acid substitution N171K located on antigenic site D. These viruses can be further characterized by substitutions D122N and T135K in antigenic site A that each cause the loss of N-linked glycosylation sites.[6] Clade 3c.2a2 viruses belong to a newly emerging group that is not yet recognized as an official clade by the WHO.[6,28] Viruses from this clade are distinguished by the S144K substitution, which is located in an antigenic site flanking the RBS. 3c.2a2 viruses can be further categorized into two clusters. Cluster I possesses substitutions I58V and S219Y, and cluster II contains

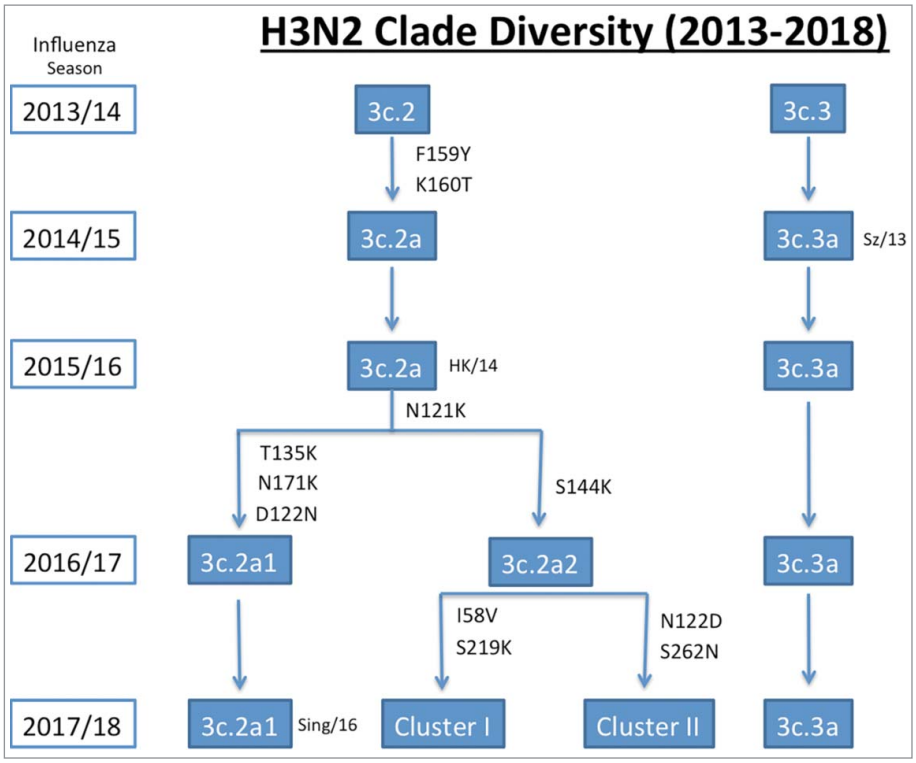

Figure 1. An overview of H3N2 IAV clade diversity from 2013–2018. During the 2013/14 season clades 3c.2 and 3c.3 represented the majority of H3N2 strains in human circulation. In the 2014/15 season, 2 new clades began to emerge, 3c.2a, which is characterized by F159Y and K160T mutations, that lead to a potential gain of a glycosylation site, and 3c.3a, from which the vaccine strain A/Switzerland/9715293/2013 emerged. By the 2015/16 season 3c.3a viruses began to wane from circulation resulting in another vaccine strain change to A/Hong Kong/4801/2014 (HK/14), which belongs to clade 3c.2a. In the 2016/17 season clade 3c.2a began diverging into two new clades, 3c.2a1, possessing the characteristic amino acid changes T135K, N171K, and D122N, which resulted in the loss of a glycosylation site, and 3c.2a2, which possess the mutations N121K and S144K. In the 2017/18 season clade 3c.2a2 began to split into cluster I, which is defined by the I58V and S219K mutations, and cluster II, which contains N122D and S262N mutations, with the N122D mutation representing the potential loss of a glycosylation site. Clades 3c.2a1 of which A/Singapore/INFIMH-16–0019/2016 (Sing/16) belongs, and 3c.3a were also still in circulation during the 2017/18 season.

N122D and S262N substitutions, with N122D resulting in the loss of a potential N-linked glycosylation site (Fig. 1).[28] Influenza surveillance and vaccine efficacy studies from the 2016/2017 influenza season in Greece, London, Canada, and Japan have all linked recent A (H3N2) epidemics to newly emerged clade 3c.2a viruses.[6,21,28,30] The recommendation by the WHO to include the same H3N2 vaccine virus, HK/14, in the 2017/2018 vaccines indicates that the viruses of the newly emerged sub-clades are antigenically similar to HK/14. Nevertheless, there is an increased level of genetic diversification observed among circulating 3c.2a1 viruses, leading the WHO to once again recommend another vaccine strain change for the upcoming 2018/2019 season to a clade 3c.2a1 virus, A/Singapore/INFIMH-16–0019/2016 (Sing/16)(Fig. 1).[6,32] Therefore as new H3N2 strains emerge and spread, the genetic composition of these viruses need to be closely monitored due to the frequency of antigenic drift variants emerging and escaping vaccine induced protection.

Hemagglutination of modern H3N2 viruses

Assays that detect serum antibody concentrations are often used as correlates of protection for influenza virus infections. One of the most common assays for quantifying serum antibody titers is the hemagglutination inhibition assay (HAI). The HAI assay identifies antibodies that can prevent the agglutination of erythrocytes by the HA of an influenza virus.[8] The antibody concentration can then be quantified by taking the reciprocal of the highest serum dilution that completely inhibits the binding of the virus to the red blood cells (RBCs). An HAI titer $\geq$1:40 is associated with a 50% or higher protection against influenza infection compared to an HAI titer <1:10.[9,33,34] However, this correlate of protection has been established from studies performed in healthy adults, and is not appropriate for predicting protection in children.[9,34]

The HAI assay also faces a few technical challenges, such as variations among erythrocytes from different species and inconsistencies between batches of erythrocytes from the same species.[9] The binding efficiency of influenza HA to the host SA is dependent on both the type of SA and the type of linkage that connects the SA with the oligosaccharide of the receptor molecule.[19] RBCs from different mammalian and avian species express surface SAs of various linkages, which make selecting the optimal RBCs to perform HAI assays challenging.[26] Equine and bovine RBCs display mainly α2,3 linked SA molecules, while avian and human RBCs display both α2,3 and α2,6 linked SA molecules. Typically, human influenza viruses agglutinate human, guinea pig, and avian RBCs, but not RBCs from horses or cows. However, H3N2 viruses from 2005 to present no longer agglutinate avian RBCs, but retain the ability to agglutinate guinea pig and human RBCs.[19,35] Guinea pig and human RBCs display nearly threefold more α2,6 than α2,3 linked

SA molecules when compared to avian RBCs indicating that modern H3N2 viruses may preferentially bind to α2,6 linked SA molecules.[19,26] Surface biolayer interferometry (BLI) assays analyzing H3N2 viral binding to polyacrylamide-linked polyvalent receptor analogues of α2,3-sialyl lactosamine and α2,6-sialyl lactosamine show a similar trend of recent H3N2 viruses preferentially binding to α2,6-linked molecules.[15,26,35,36] As a result, many researchers now use guinea pig RBCs as targets in their HAI assays when analyzing recent H3N2 viruses.[36,37]

Viral growth and propagation

The ability to isolate and propagate H3N2 IAVs is essential to perform annual surveillance of circulating viruses as well as to determine the antigenic and antiviral sensitivity of different strains. Traditionally, IAVs are propagated in embryonated chicken eggs, and many manufacturers still use this medium to produce their vaccines today. Immortalized mammalian cell lines have also been used to propagate and isolate IAVs in scientific laboratories since the 1960′s. The process is a relatively simple, sensitive, and cost-effective method.[26,38] Numerous cell lines such as BHK-21, SPJL, LLC-MK2, and Vero cells have been successfully used for viral growth, but Madin-Darby canine kidney (MDCK) cells are the most reliable, sensitive, and easy to handle cell line. Influenza viruses isolated from MDCK cell cultures retain the same HA sequence as the original virus isolated from influenza infected subjects, whereas viruses propagated in embryonated chicken eggs occasionally mutate and display a different HA from the original source.[19,25]

MDCK cells express both α2,3 and α2,6-linked SA receptors on their surface allowing both human and avian influenza viruses to be isolated from MDCK cultures with high hemagglutination (HA) titers.[19,38] However, in recent years most H3N2 IAV isolates have exhibited poor growth in MDCK cultures, and display a preferential binding to α2,6-linked SA molecules.[19,20,25,26] When compared to human respiratory cells the amount of α2,6-linked SA receptors on MDCK cells is relatively low, and thus, MDCK cells may no longer be the ideal medium for viral propagation of modern human H3N2 viruses.

To circumvent this development, researchers have engineered a novel cell line, MDCK SIAT1, to overexpress α2,6-linked SA receptors on their surface.[38,39] Increasing the amount of α2,6-linked SA receptors on the cell surface increases the number of potential interactions between influenza virions and the MDCK SIAT1 cells allowing this modified cell line to be useful for the propagation of recent human H3N2 viruses.[15,38] MDCK SIAT1 cells are produced by stable transfection of MDCK cells with human CMP-N-acetylneuraminate: β-galactoside α2,6-sialtransferase, an enzyme that catalyzes the α2,6-sialylation of galactose moieties on glycoproteins or glycolipids.[38,39] Unlike H3N2 viruses isolated from 1968 to 2001, viruses that have emerged after 2001 have decreased ratios of infection in MDCK cells when compared with MDCK SIAT1 cells.[5,37,38] As a result, MDCK SIAT1 cells are currently the preferred medium for propagation of modern H3N2 viruses because of their ability to maintain the faithful replication and infectivity of the virus, while avoiding the generation of defective viruses.[5] This shift towards propagating recent H3N2 viruses in cell cultures is further evidenced by the inclusion of cell propagated H3N2 viruses in the recommended list of vaccine viruses for the 2017/18 influenza season.[6]

NA binding in modern H3N2 viruses

Traditionally the roles of HA and NA during the influenza life cycle are associated with viral entry and release from the host cell respectively. However, the roles of these proteins in modern H3N2 IAVs may not be so clearly partitioned.[18] The NA glycoproteins of recent H3N2 viruses can facilitate viral entry into target cells during infection.[4,10,11,18] Oseltamivir carboxylate, rather than increasing HA titers by inhibiting viral release from RBCs actually repressed the hemagglutination of both turkey and guinea pig RBCs.[11] In addition to its role in viral release, NA promotes viral access to target cells in airways through mucous degradation, aiding in the removal of decoy receptors on mucins, cilia, and cellular glycocalyx, which would impede viral access to the receptors on host cell surfaces.[4,10,40]

The SA receptor binding activity of NA is associated with amino acid residue 151. A D151G mutation in the active catalytic site of NA causes the protein to bind to SA with increased affinity without cleaving sialic acid moieties.[4,10,11,18,41] The active site of NA consists of 8 functional residues (R118, D151, R152, R224, E276, R292, R371, and Y406) and 11 framework residues (E119, R165, W178, S179, D198, I222, E227, H274, E277, N294, and E425). These residues are highly conserved among all NA subtypes.[4,42] Thus, a mutation in one of the functional residues at site 151 could be responsible for a change in the function of NA. The location of residue 151 at the edge of the NA active site, and its sensitivity to oseltamivir suggest that D151 is a critical residue involved in the catalytic activity of NA.[10,11,20] Residue D151 may function in wild-type NA to cleave the virus from the SA receptor, but once it is replaced by glycine the NA gains the ability to bind to SA receptors on RBCs.[4]

The ability of NA to initiate viral binding to host cells is not unique to N2 subtypes. The G147R mutation in N1 NA also induces receptor-binding activity on the NA in the lab adapted H1N1 strain A/WSN/33. Site 147 is located near the NA active site, and it slightly decreases the sialidase activity of the NA.[4,18] The G147R N1 mutation has been found in a small number of sequences including some human 2009 pandemic H1N1 strains, human seasonal strains, and chicken H5N1 isolates of various lineages.[18] NAs with the D151G or G147R mutations not only bind to SA receptors, but they are also sensitive to neuraminidase inhibitors such as oseltamivir and zanamivir.[4,11,18,41] Due to this observation, many researchers are now recommending that 20nM oseltamivir carboxylate be added to the HAI assay protocols when using guinea pig RBCs to inhibit the NA-dependent agglutination caused by modern H3N2 IAVs.[4,11]

H3N2 neutralization assays

The current issues occurring with the H3 HAI assay have raised interest in the use of alternative assays to antigenically characterize H3 IAVs.[43] In addition to the HAI, the micro-

neutralization (MN) assay is another commonly used technique for determining the immune response induced by influenza vaccines.[9] Ideally in these assays a standard number of infectious units (e.g. 100 50% tissue culture infectious doses ($TCID_{50}$)) is incubated with serial dilutions of serum samples in 96-well plates containing monolayers of MDCK cells. The infection of these cells are commonly assessed by either measuring cytopathic effects (CPE), detecting viral protein synthesis using enzyme-linked immunosorbent assay (ELISA), or by immunostaining virus-infected cells.[43] MN assays can be used to measure both the infectivity of an influenza virus, and the ability of antibodies in serum to neutralize virus replication in MDCK cells.[44] Neutralizing antibodies are generally accepted as primary mediators of immunity against influenza virus infection. The quantity of the antibodies in the MN assay is expressed as the reciprocal of the highest serum dilution that induces at least a 50% reduction in the cytopathic effect of an influenza virus on mammalian cell cultures.[33] As opposed to the HAI assay, to date no correlate of protection has been established for the MN assay.[9] MN assays are commonly used as an alternative to HAI assays because they can overcome non-antigenic effects due to affinity changes in the receptor binding of different influenza viruses, which can complicate the interpretation of HAI results.[44]

Until recently, most MN assays were based on CPE or ELISA assays, but in the 1990s MNs based on focus and plaque reduction that rely on counting visible plaques to quantify infectivity were developed. However, visual counting only captures larger plaques that are resolvable by the human eye, and excludes smaller plaques that are produced by many currently circulating H3N2 viruses.[44] Variation in plaque size and non-uniform morphology can also complicate visual quantification leading to disparity among the examiners, and between experiments leading to incomparable results.[45] In order to alleviate these issues, many researchers are now quantifying viral infection with flatbed scanners and counting software that provides users with a high throughput alternative for enumerating viral plaques.[44,45] Conventional neutralization assays based on the inhibition of CPE in MDCK cells are also laborious and time-consuming. MN assays that utilize micro-titer plates and a downstream ELISA to detect infected cells is faster than conventional assays and can produce results in as little as two days[34].

The widespread use of multi-well plate assays along with advances in flatbed scanning technology now allow researchers to perform quantitative MN assays suitable for the antigenic characterization of large numbers of viruses, as well as a means for measuring the antiviral activities of neutralizing antibodies.[44] MN assays of this type allow users to measure virus infections on the cellular level, regardless of size, leading to a more sensitive assay that possesses the ability to characterize viruses with low infectivity.[44,45] Additionally, the automation of the plaque enumerating process allows users to obtain results quickly, while also making their results more comparable between laboratories. Variations in measurements of the same sample were less than 3%, which correlates to the uncertainty introduced during the image acquisition process.[45] This level of error can be further reduced by averaging images of the same well from multiple scans, but the current level of reproducibility is sufficient to provide data for many virological studies.[44,45] Although no correlate of protection exists for these assays, their results have generally been consistent with those results obtained through HAI. These assays are also capable of examining the effects of a wider range of antibodies than HAI, making them an interesting tool for characterizing antigenic similarities or differences between H3N2 viruses and vaccines.[44]

Conclusion

Since their introduction to humans in 1968, H3N2 IAVs have been rapidly evolving both genetically and antigenically in an attempt to escape host immune pressures. As a result, these viruses have altered themselves in many ways, including the addition of numerous N-linked glycans, increases to the overall net charge of the HA molecule, changes to preferences in receptor binding from α2,3 to α2,6-linked SA receptors, and the ability of NA to agglutinate RBCs prior to host entry. In order to adapt to this constantly changing viral landscape, researchers have been modifying existing assays that are commonly used for the antigenic characterization of H3N2 IAVs. The use of guinea pig red blood cells and the addition of 20nM oseltamivir carboxylate to the HAI assay allows investigators to assess modern H3N2 viruses using traditionally accepted techniques with known correlates of protection. Additionally, advances in MN assays allows for an additional method for assessing H3N2 viruses that can further support the results obtained from the HAI assay, while also providing more information about neutralizing antibodies that interfere with infection. The adaptations acquired by modern H3N2 IAVs create difficulties in recognizing and predicting current and future epidemiological threats. However, adopting changes to the standard methods used to analyze H3N2 viruses would allow researchers to continue to study and better understand this ever-changing subtype of influenza viruses.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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