N-Linked Glycosylation in the Hemagglutinin of Influenza A Viruses

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Since the 1918 influenza A virus (IAV) pandemic, H1N1 viruses have circulated in human populations. The hemagglutinin (HA) of IAV determines viral antigenicity and often undergoes N-linked glycosylation (NLG) at several sites. Interestingly, structural analysis of the 1918 and 2009 H1N1 pandemic viruses revealed antigenic similarities attributable to the conserved epitopes and the NLG statuses of their HA proteins. NLG of the globular head of HA is known to modulate the antigenicity, fusion activity, virulence, receptor-binding specificity, and immune evasion of IAV. In addition, the HA of IAV often retains additional mutations. These supplemental mutations compensate for the attenuation of viral properties resulting from the introduced NLG. In human H1N1 viruses, the number and location of NLG sites has been regulated in accordance with the antigenic variability of the NLG-targeted antibody-binding site. The relationship between the NLG and the antigenic variance in HA appears to be stably controlled in the viral context.

Key Words: Glycosylation, hemagglutinin, influenza virus, pandemic

INTRODUCTION

Ever since they adapted from waterfowl, various subtypes of the influenza A virus (IAV) have circulated in avian and mammalian hosts, including humans.[1,2] There is a high level of antigenic variability, as shown by the recent discovery of the 17th hemagglutinin (HA) subtype in a virus from bats.[3] Although most of the IAV-related disease burden in humans has been caused by the H1 and H3 subtypes, novel variants causing disease in humans can emerge at any time due to antigenic drift or shift.[4] To reduce the number of infections due to this respiratory agent, the World Health Organization operates an interdisciplinary surveillance and countermeasures program in cooperation with more than 100 national influenza centers in six different regions (Africa, the Americas, Southeast Asia, the Eastern Mediterranean, Europe, and the Western Pacific), six collaborating centers, and four essential regulatory laboratories. This program largely focuses on the annual recommendation of candidate viruses for use in the inactivated trivalent influenza vaccine and also develops risk management protocols, such as 'the pandemic influenza preparedness framework' for emerging novel variants.[5,6] A mismatch between vaccine vi-

ruses and circulating strains can cause problems attributable to suboptimal vaccine efficacy and can result in greater disease severity, even among the vaccinated population.[7,8] Thus, predicting the evolutionary pathway of HA is like a wheel of fortune.[9] One of the most promising solutions to overcome this mismatch might be a universal vaccine with high cross-reactivity. Universal vaccines can overcome the difficulty in determining the appropriate vaccine virus and can promote the development of protective immunity against various IAVs.[10,11] The conserved regions of HA are prime targets for the identification of universal epitope sequences.[12-15] Even though vaccines targeting the neuraminidase (NA) or M2 proteins of IAVs have also been evaluated for broad reactivity,[16,17] optimal antibody responses comparable to the response to natural infection were only obtained in the trials targeting HA.[18]

Most human H1N1 (hH1N1) viruses harbor multiple N-linked glycosylation (NLG) sites in the head of HA; the 1918 and 2009 pandemic viruses are notable exceptions (Fig. 1).[19,20] In fact, the NLG of HA has been demonstrated to regulate the virulence of IAV by modulating the biological activity of HA.[21-24] In addition, NLG can allow IAVs to evade antibody recognition.[25,26] There have been reports that the NLG status of the receptor-binding domain mediated protective antibody responses to both the 1918 and 2009 pandemic H1N1 viruses[27] and affected the immunogenic hierarchy in mouse antisera.[19] In this review, we discuss how the NLG of the globular head of HA affects the biological function of IAVs and how hH1N1 viruses utilize NLG for immune evasion.

THE HA PROTEIN AND ANTIGENIC VARIATION DUE TO NLG

HA, one of the major surface glycoproteins of IAV, com-

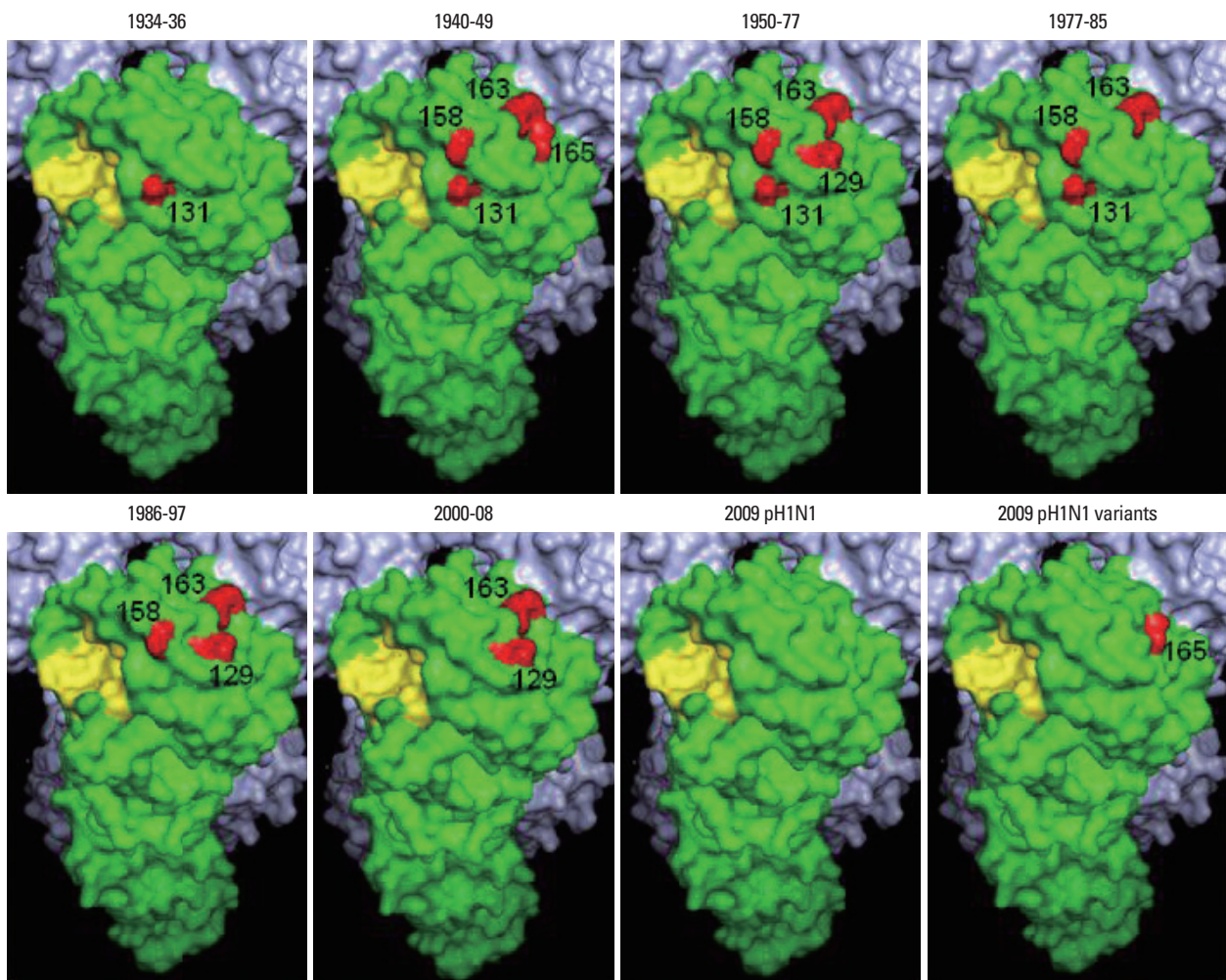

Fig. 1. NLG of the globular head of H1 HA during each time period. Using the HA crystal structure of the A/California/04/2009 virus (PDB ID: 3LZG),[27] the possible N-linked glycans on the head of H1 HA from Table 1 were evaluated. Potential glycosites (red) near the RBS (yellow) are indicated by the H3 numbering. NLG, N-linked glycosylation; HA, hemagglutinin; RBS, receptor-binding site.

mences the infection by binding to sialic acid (SA) moieties present on cell membranes. Once the virus is internalized by endocytosis, the protease-mediated cleavage of the HA protein into the HA1 and HA2 subunits exposes a hydrophobic fusion peptide located in the N-terminus of the HA2 subunit. The exposure of this peptide initiates a fusion process between the viral and cellular endosomal membranes. Then, the eight-segmented genome of IAV encapsidated by viral nucleocapsid protein is released into the cytoplasm for replication and transcription in the nucleus. This replication process is finally terminated by another surface glycoprotein, NA, which facilitates the release progeny virions that then infect neighboring cells.[28,29]

In addition to acting as an infection initiator, HA also functions as a principle immunogen for IAV.[30] When the IAV infection begins, the host's protective machinery immediately reacts by activating components of the first line of defense, including macrophages, dendritic cells, and natural killer cells. The next defensive reactions are mediated by humoral and adaptive immune responses. The humoral immune response induces HA-specific antibodies targeting the globular head or stem regions of this protein. Both types of HA-specific antibodies can neutralize IAV and block the binding of IAV to cellular receptors or the fusion process. Due to the conservation of the stem sequences among divergent IAVs, stem-specific neutralizing antibodies might exhibit cross-reactivity against not only homologous but also heterologous subtypes.[12,31] However, this conserved stem region

seems to be less immunogenic than the globular head; the globular head-specific antibodies are very effective in preventing IAV from binding to host cells.[32]

In response to the immune pressure mediated by vaccination in humans, the HA of IAV often accumulates mutations that result in antigenic drift. Thus, viral evolution can be driven by antigenic variation.[7,8] For some unknown reasons, the HA antigenic sites of hH1N1 viruses sometimes exhibit fewer amino acid changes.[33] The number of potential NLG sites is also controlled in hH1N1 viruses. Five potential NLG sites in the head of each HA monomer are typically selected in hH1N1 viruses (Fig. 2). Only up to three out of the five glycosites are utilized (Table 1).[19,20,33] Fitness costs might explain why IAVs never accept the total possible number of N-linked glycans on the head of HA.[34,35] Interestingly, there might be a specific relationship between antigenic variation and the number and position of N-linked glycans on the globular head of HA in hH1N1 viruses. It is likely that the lower the number of N-linked glycans, the higher the total antigenic variability of the SA antigenic site in the HAs of hH1N1 viruses (Fig. 3).

THE NLG OF THE HA OF IAV

The NLG of glycoproteins takes place in both prokaryotic and eukaryotic cells.[36] Through the attachment of glycans at asparagine residues, NLG promotes protein folding by en-
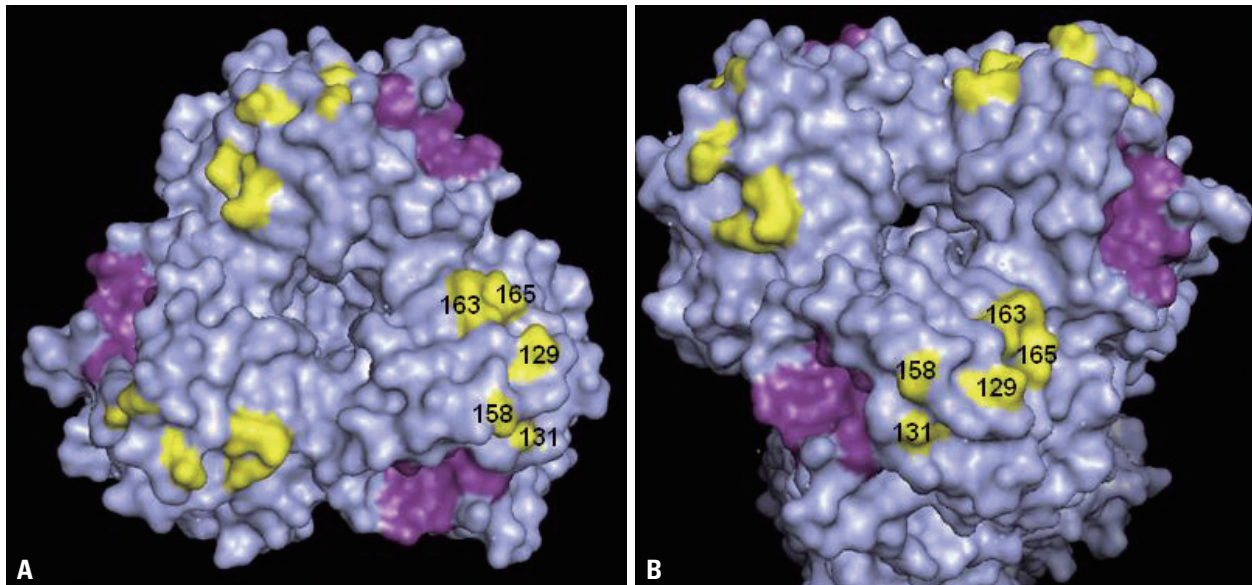

Fig. 2. The prediction of potential glycosites on the head of H1 HA. The 3-D crystal structure of the HA trimer of the A/California/04/2009 virus (PDB ID: 3LZG)[27] was modified by the PyMol program to locate potential glycosites (yellow) at the membrane-distal region with top (A) and tilt (B) views. Among the five glycosites, four of them were identified in the SA antigenic site, and glycosite 131 (H3 numbering) was located at the vicinity of the receptor-binding site (RBS in magenta). HA, hemagglutinin; SA, sialic acid.

Table 1. Amino Acid Residues Harboring N-Linked Glycans on the Head of H1 HAs since the Early 1930s

Time period	Residues harboring NLG on the head of H1 HAs					Max. # of N-linked glycans
1934-36		131				1
1940-49		131	158	163	165	3
1950-57	129*	131	158	163		3
1977-85		131	158†	163		3
1986-97	129		158‡	163		3
2000-08	129			163		2
Since 2009					165§	1

NLG, N-linked glycosylation; HA, hemagglutinin; NCBI, National Center for Biotechnology Information.
This table was modified from Table 2 in the work of Wei, et al.[19] published in 2010. The exceptional NLGs of residues 129 during 1950-57, 158 during 1977-85 and 1986-97, and 165 since 2009 were obtained from HA sequences available from the NCBI.
*GenBank accession ID: ADT78859.
†GenBank accession ID: ABF21277.
‡GenBank accession ID: AEC47164.
§GenBank accession ID: AEO01518.

hancing solubility and mediating interactions between nascent proteins and cellular proteins, such as chaperones.[37] Three key processes in NLG have been identified: 1) the lipid-mediated assembly of monosaccharides into glycans, which is performed by various enzymes in the ER-Golgi network; 2) the acceptance of a glycan by the consensus sequence, N-X-S/T (Asn-X-Ser/Thr; X for any amino acid except proline), of the protein; and 3) the oligosaccharyl-transferase-catalyzed attachment of the glycan to the side chain of the asparagine residue.[36] The surface glycoproteins of influenza, HIV, and other important human pathogens adopt the NLG of the host cell. This process not only diversifies viral glycoproteins through the addition of various glycans but also conveniently allows the evasion of the host immune arsenal by sending 'self signals' to the innate immune machinery and by reducing the induction of antibodies against the viruses.[25]
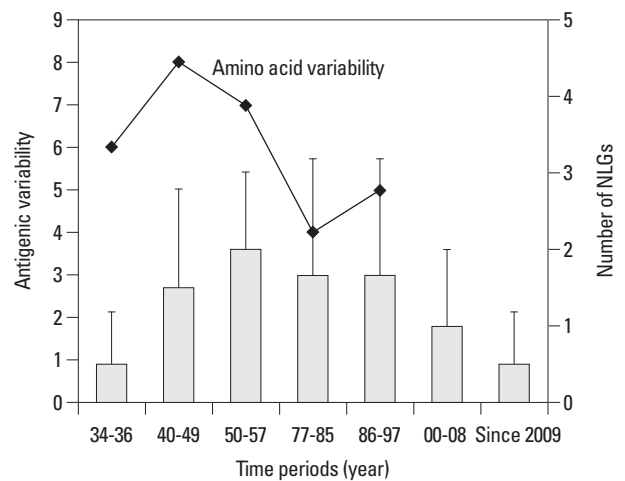

Fig. 3. The antigenic variability and the number of N-linked glycans on the head (Sa antigenic site) of H1 HA. The variance in the amino acids (indicated at the left) in the Sa antigenic site of H1 HA was modified from the work of Sun, et al.[33] published in 2012. For comparison, the numbers of N-linked glycans (indicated at the right) in the membrane-distal region of H1 HA were collected from the works of Wei, et al.[19] and Sun, et al.[20] and were supplemented with the HA sequences of the hH1N1 viruses available from the National Center for Biotechnology Information of the United States. NLG, N-linked glycosylation; HA, hemagglutinin.

THE NLG-REGULATED HA RECEPTOR-BINDING AVIDITY AND VIRULENCE OF IAV

The NLG of the globular head of HA regulates the multivalent receptor-binding avidity of IAV to SA cellular receptors.[38-40] In the hemadsorption assay, the presence of oligosaccharide side chains in the vicinity of the receptor-binding site (RBS) was shown to induce negative effects that interrupt the efficient HA and SA (HA-SA) interaction. When these N-linked glycans were removed, HA receptor-binding avidity was recovered, and erythrocytes were not released.[38] It should be determined whether the strong receptor-bind-

ing avidity of HA benefits viral replication in cells given that the HA-NA balance plays an important role in the viral life cycle. Ironically, the enhanced receptor binding of the NLG-deleted virus to the SA receptor attenuated viral growth in Madin-Darby canine kidney (MDCK) cells.[39] Strong adherence to cells via the interaction between NLG-deficient HA and SA seemed to be too strong for IAV to be released. However, this negative regulation of receptor binding by the attached N-linked glycans might be compensated for by the relative NA activity. In a mutant study with an H7 virus, the N2 NA, which exhibits stronger enzymatic activity than the N1 NA, efficiently supported the growth of NLG-delet-

ed viruses in MDCK cells, whereas mutant viruses with the weak N1 NA exhibited retarded growth.[39] In addition, there seems to be a hierarchy among the glycosites around the RBS. Glycosite 149 (H3 numbering) was shown to more strongly regulate viral growth than glycosite 123 did, regardless of the relative NA activities.[39] In a different study, the introduction of additional N-linked glycans around the RBS decreased both the receptor-binding and the fusogenic activities of HA and resulted in the retarded replication capacity of H2N2 viruses.[40]

The virulence of IAV might also be regulated by NLG of the HA globular head region. In previous studies, an increased number of N-linked glycans attached to the head of HA attenuated the virulence of IAV in mice.[41] In contrast, the loss of NLG increased the virulence of IAV.[42-45] The augmented virulence during the adaptation of IAV in mice was also related to the removal of NLG sites in HA.[46,47] However, innate immune responses were investigated primarily to elucidate the relationship between the virulence of IAV and NLG given that N-linked glycans had been previously shown to be targets of innate immunity.[48] It has also been reported that enhanced resistance to surfactant protein-D[41,45] and decreased susceptibility to collectins[43] and bronchoalveolar lavage fluid[44,45] are associated with the loss of NLG and with elevated virulence in mice. According to all of these results, NLG of the membrane-distal region of HA has critical roles in the regulation of the biological activities of HA and the virus itself. In addition, the impact of NA activity and innate immune responses on NLG modification should also be considered to understand the effects of the presence or absence of NLG on the virulence of IAVs.

THE NLG-MEDIATED ALTERATION OF HA RECEPTOR-BINDING SPECIFICITY

Human IAV preferentially binds to α-2,6-sialic acid (6' SA) moieties, whereas avian IAV favors α-2,3-sialic acid (3' SA) in the cell membrane.[49,50] It was previously revealed that the receptor binding specificity was primarily attributable to several amino acid residues, including 190, 226, and 228, in HA.[50-53] The E190D mutation was demonstrated to be sufficient for a switch from avian to human receptor specificity (3' SA→6' SA) in the H1 subtype, and both the Q226L and G228S mutations were needed for the switching of the H2 and H3 subtypes. Hence, IAV requires adaptation to replicate effectively in new hosts, and these adaptations are usu-

ally accompanied by mutations around the RBS of HA. However, host adaptive mutations seem to emerge in a host cell-dependent manner. In an earlier study, a growth advantage was observed in Madin-Darby bovine kidney cells but not in chicken embryo fibroblast cells after the introduction of one mutation that removed the NLG site at residue 131 from the tip of the parental HA.[54,55] In another study with the A/USSR/90/77 (H1N1) virus, host adaptation was associated with the alteration of receptor-binding specificity.[23] The loss of the NLG site at residue 131 increased the binding affinity of the virus to 6' SA, but there appeared to be no effect on the specificity for 3' SA. When mapped in the HA crystal structure of A/Puerto Rico/8/34, glycosite 131 is located right next to the RBS.[35,56] Due to the bulky side chains of oligosaccharides, NLG at this position might interfere heavily with the viral life cycle by blocking efficient access of HA to the SA receptor. As a result, this virus would require a breakthrough, such as an additional mutation, to survive, and the incorporation of such additional mutations might be a way for novel IAV variants to emerge. The HA of avian IAV prefers the 3' SA receptor for cell binding. In a study of an H5N1 avian virus (A/Vietnam/1203/2004, VN1203), the T160A mutation in HA removed a potential NLG sequon at residue 158, but this mutation had no effect on the receptor preference of the virus. However, when compensated with the additional Q226L mutation, which is known to be one of the requirements for avian IAV to adapt to the 6' SA of human receptors in H2 and H3 subtypes,[50] the T160A/Q226L HA double mutants of the VN1203 virus exhibited altered receptor-binding specificity from 3' SA to 6' SA.[57] The G158Q mutation was also demonstrated to control, in part, the receptor-binding preference of the parental A/WSN/33 strain and its N129D mutant viruses.[22] These observations indicate that NLG itself might have a minor effect on the receptor-binding specificity of IAV. However, the steric interference caused by the attached N-linked glycans might induce viral compensations, which eventually result in altered receptor-binding specificity.

The relationship between changes in NLG and the transmissibility of IAV should be also considered. Given that the NLG of the globular head of HA can change the HA receptor-binding specificity, changes in NLG may modulate the transmissibility of IAV. In a guinea pig model, an H5N1 virus that could bind to both 3' SA and 6' SA lost its affinity for 6' SA as a result of the A160T mutation, resulting in the complete loss of transmissibility of the parental virus.[58] Thus, in addition to the alteration of receptor specificity, changes

in the NLG of the globular head of HA are able to affect viral transmission. Hence, changes in NLG should be monitored by continuous surveillance to prevent the transmission of emerging IAV variants.

THE NLG-MEDIATED CIRCUMVENTION OF THE ANTIBODY RECOGNITION OF IAV

The HA of hH1N1 viruses harbor multiple potential NLG sites in the head and stem regions.[20] Unlike the stem region glycosites, each of the five glycosites (residues 129, 131, 158, 163, and 165) in the head of HA was selectively utilized for N-linked glycan attachment in hH1N1 viruses.[19] By accepting the various changes in NLG, the hH1N1 viruses were able to shield the globular head region of HA and escape antibody recognition.[19,59] The immune evasion mechanism has been studied using mutants that can escape monoclonal antibodies (MAbs).[26,47] In a study with MAb 2D1, which was isolated from a survivor of the 1918 pandemic, the antibody recognition sites were usually matched with the Sa antigenic site at the tip of H1 HA. Considering the amino acid differences with respect to seasonal H1N1 viruses, NLG of the center of the Sa antigenic site might result in evasion from recognition by 2D1 for the seasonal H1N1 viruses.[27] Another study with a 2009 pandemic-specific MAb, EM4C04, also suggested that NLG-mediated immune evasion could occur in response to the introduction of the K123N and G134S mutations.[47] However, these NLG-mediated escape mutants might harbor compensatory mutations to maintain viral competence. It is possible that numerous quasispecies variants could emerge among the survivors of the antibody intervention. Among these variants, only a limited number of clones might maintain sufficient competence for survival. Mutations outside of the defined antigenic sites also yielded the same results. In a study with MAb 5J8, H1N1 HA retained mutations between the RBS and the Ca2 antigenic site.[14] Although these mutations did not induce NLG-mediated evasion, the H1N1 viruses that survived might be supplemented with compensatory benefits. If these benefits have a high fitness cost, the viruses might choose an alternative, cost-effective path. For instance, the D199H mutation in the 1918 HA could not result in escape unless it was coupled with another mutation at residue 133a (147 in H1 numbering; K133aQ mutation), a compensatory change to maximize the effects of the D199H mutation.[14] These results demonstrate that hH1N1 viruses accept mutations in response to antibody pressure, resulting in survival of the fittest virus.

PERSPECTIVES

IAV continuously evolves in various hosts. To achieve optimal competence against natural or transferred host immune responses, IAV adapts itself by modulating its viral properties. NLG of the globular head of HA is one of the tools used to evade immune pressure. By acquiring or losing NLG, IAV can circumvent direct or indirect interventions. In addition, the evolutionary signature of hH1N1 viruses might be embedded in the NLG attachment signal. As observed in the history of hH1N1 viruses, the HAs of hH1N1 viruses have been never equipped with hyperglycosylation. Hence, there might be a balance between the number and location of NLG sites and the antigenic variance in the evolutionary pathway. NLG is certainly a viral countermeasure and should be understood in the comprehensive context of the virus itself and the immune interventions against it.

ACKNOWLEDGEMENTS

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