Why glycosylation matters in building a better flu vaccine

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Abstract

Low vaccine efficacy against seasonal influenza A virus (IAV) stems from the ability of the virus to evade existing immunity while maintaining fitness. While most potent neutralizing antibodies bind antigenic sites on the globular head domain of the IAV envelope glycoprotein hemagglutinin (HA), the error-prone IAV polymerase enables rapid evolution of key antigenic sites, resulting in immune escape. Significantly, the appearance of new N-glycosylation consensus sequences (sequons, NXT/NXS, rarely NXC) on the HA globular domain occurs among the more prevalent mutations as an IAV strain undergoes antigenic drift. The appearance of new glycosylation shields underlying amino acid residues from antibody contact, tunes receptor specificity, and balances receptor avidity with virion escape, all of which help maintain viral propagation through seasonal mutations. The World Health Organization selects seasonal vaccine strains based on information from surveillance, laboratory, and clinical observations. While the genetic sequences are known, mature glycosylated structures of circulating strains are not defined. In this review, we summarize mass spectrometric methods for quantifying site-specific glycosylation in IAV strains, and compare the evolution of IAV glycosylation to that of human immunodeficiency virus. We argue that the determination of site-specific glycosylation of IAV glycoproteins would enable development of vaccines that take advantage of glycosylation-dependent mechanisms whereby virus glycoproteins are processed by antigen presenting cells.
Introduction

Viruses replicate by causing infected cells to produce virions that infect other cells. As has been observed by Fodor et al., virion composition determines virus stability, transmissibility, tropism, and immunogenicity (1). In the case of IAV, viral hijacking of the host cell machinery results in error-prone replication. Some host cell proteins are taken up to construct the new virions, which are pleiomorphic in structure. Viral protein mutations caused by error-prone replication result in antigenic drift, which allows the virus to evade neutralization by immune system molecules. Hence, generating new vaccines annually is a critical effort in public health.

There are 18 known IAV HA, divided into two groups, and 11 known neuraminidase (NA) subtypes (2). Among the possible HA and NA combinations, only H1N1, H2N2 and H3N2 have caused pandemics. Today H1N1 and H3N2 circulate seasonally in humans. As shown in Figure 1, IAV is a negative-sense, single-stranded RNA virus with 8 gene segments that code for at least 17 proteins (3). The trimeric HA glycoprotein binds sialylated glycans on the surface of host cells and facilitates subsequent endosomal membrane fusion. The NA glycoprotein cleaves sialic acids, allowing newly formed virions to escape the cell surface. The host adaptive immune system responds to IAV infection by generating antibodies that neutralize HA binding to host receptors.

The need for a better flu vaccine

Vaccines are injected intramuscularly or subcutaneously and must travel to the lymph nodes in order to elicit an antibody response. By contrast, a natural IAV infection occurs in human airways. The acute host infection immune reaction cascade includes cytokine release, influx of white blood cells, and cellular activation, resulting in clinical symptoms (4). This initial innate immune response limits the initial viral load and activates the adaptive immune system. The
IAV infection induces systemic and local antibody responses (known as humoral immunity) and cytotoxic T cell responses (known as cellular immunity). In lymph nodes, naive B cells, through their surface antibodies, bind cognate antigens, become activated, and transition from IgM to IgG production. They then differentiate into memory B cells as they increase their immunoglobin specificity. Immunoglobins of the IgA class transported across the upper airway mucosal epithelium neutralize and clear infection. The lower airway is protected primarily by IgG.

The World Health Organization recommends the compositions of influenza vaccines based on surveillance, laboratory and clinical observations (5). Despite this considerable effort, the effectiveness of the seasonal influenza vaccine remains unacceptably low, ranging from 10-60% (6). Thus far, neither the widespread accessibility of deep gene sequencing for efficient characterization of circulating strains, nor the availability of crystal coordinates for HA has led to improvement of vaccine efficacy. To address concern about future pandemics, the National Institute of Allergy and Infectious Diseases (NIAID) has released a strategic plan to address this problem (7).

Licensed vaccine classes include inactivated virus, live attenuated virus, and recombinant HAs (3). The majority of licensed inactivated or live attenuated vaccines are expressed in embryonated chicken eggs. Egg-based vaccine production has been practiced for 70 years; however, the many drawbacks of this system have spurred the exploration of new expression systems. It has been recognized for many years that IAV adapts to the cell in which it is propagated (8). Thus, IAV passaged in embryonated chicken eggs undergoes selection of strains adapted to growth in chicken cells. HA from such egg-adapted IAV display amino acid mutations near the receptor-binding site (9-12) and typically stimulate lower antibody titers. Mammalian tissue culture-grown IAV have shown superior vaccine protection in animal models compared with corresponding egg adapted IAV (13), and there now exists one inactivated
vaccine expressed in mammalian cells, Flucelvax, approved by the FDA. Investigators have also used insect and plant cells for expression of HA (13-15). FluBlok is a licensed recombinant vaccine containing HA grown in insect cells from baculovirus vectors. Unlike egg-based expression, these alternative expression systems do not depend on a large supply of pathogen-free eggs for vaccine production, making them economical choices as well as ways to avoid the lowered immunogenicity that arises from egg adaptation.

The number of HA sequons increases as IAV circulates seasonally in humans.

Glycosylation in the HA stalk region, at or near residues 15, 26, 289, 483, and 542, occurs in all HA forms (see (16) and references therein). These glycans may interact with glycan binding chaperones in the endoplasmic reticulum and appear to play roles in HA trimer assembly (17,18). In contrast to seasonal strains, pandemic IAV, newly introduced to the human population, evades host antibody and innate immune defenses, and penetrates into the deep lung to infect bronchiolar, alveolar epithelial cells, and alveolar macrophages (19). To circulate in humans, these IAV must evade antibody recognition (20,21). Thus, amino acid residues of the HA globular domain mutate rapidly under evolutionary pressure to avoid antibody recognition (22). Newly emerging pandemic IAV typically begin with a low degree of glycosylation of the HA globular domains, but the number of N-glycosylation consensus sites increases as the strains circulate seasonally. The amino acids shielded by N-glycosylation appear not to mutate at a high rate, relative to those that are exposed to antibody binding (20,23).

The number of sequons on the HA globular head domain increases with the amount of time the IAV sub-type circulates seasonally in humans (21,24-26). Genetic studies show evolutionary changes in the number of N-glycosylation sequons in the IAV protein sequences (16,24). The
number of N-glycosylation sequons for human-circulating H3N2 and H1N1 has increased over time and the pattern has changed (27). All of these findings suggest that amino acid mutations and increased glycosylation affect how HA interacts with immunity. Indeed, Bajic et al. showed that engineered hyperglycosylated HA restricted the resulting antibody repertoire to a subdominant epitope and that such antibodies protected against viral challenge (28). However, little is known about the types and structures of HA glycans and how these influence antibody responses.

**Glycosylation impacts IAV antigenicity, immunogenicity, and immunodominance**

As pointed out by Yewdell et al., among several respiratory RNA viruses with similar mutation rates and antigenic escape frequencies, only IAV undergoes genetic drift (29). While the understanding of the reasons for IAV drift remains incomplete, the fact that immune responses in humans are focused on particular antigenic sites on the HA protein means that single point mutations have large impacts on viral escape.

Antigenicity refers to the capacity of a chemical structure (antigen) to bind antibodies or T cell receptors. Immunogenicity, by contrast, refers to the capacity of the antigen to induce an adaptive immune response. The immune system responds to complex antigens in a hierarchical manner, a concept known as immunodominance (30). Thus, immunodominant antigens may suppress immune responses to subdominant antigens. Broadly protective antibody responses appear to target subdominant conserved epitopes that have low variability due to the need to maintain viral function and display restricted gene usage (28).

Immune responses to viral antigens are governed by factors including antigen structure (concentration, conformation and location), B cell immunodominance, and T cell immunodominance. Aspects of T cell immunodominance have been reviewed (31). Briefly,
because CD8+ T cells help clear viral infections, researchers have tried to develop vaccines that exploit these responses. CD8+ T cells recognize viral peptides processed by cellular proteasomes and presented by the major histocompatibility complex (MHC) class I molecules. Most T cell responses are generated against immunodominant viral peptides, which make up only a small fraction of the thousands of processed viral peptides. T Cell populations that recognize glycopeptides presented by MHC-I and MHC-II have been identified (32,33), indicating the significance of glycosylation on acquired immune responses (34).

Immunodominance reflects many factors, including antigen presentation and T cell activation (35). Immunodominant antigens are recognized by large T cell populations, relative to those of subdominant antigens. This hierarchy is a reproducible pattern among individuals. Immunodominance largely results from the fact that only a small percentage of peptides bind MHC molecules with affinity sufficient for stable presentation to activate CD8+ T cells, a pattern that shows high evolutionary conservation (36). At present, the apparent inability of memory CD8+ T cells to protect against IAV has driven renewed focus on vaccines that elicit an antibody response (29).

Among IAV proteins, the order of antibody immunodominance is approximately HA > NA >>> nucleoprotein (36). It is therefore of interest that the number of HA (and NA) sequons increases during seasonal circulation in humans. In order to evaluate the influence of IAV glycosylation on binding of strain antibodies, researchers currently rely on genetic sequences to predict sequons. It is known that the number of sequons on the HA globular head domain increases with the amount of time the IAV sub-type circulates seasonally in humans (21,24-26). Genetic information has been used to model HA glycosylation by inserting the generic N-glycosylation chitobiose core structure onto HA crystal structure coordinates (26,37-39). However, information about the size and composition of glycans at individual sites and their impact on antigenic integrity is available for only a few strains.
The current understanding of the adaptive immune system derives from peptide antigens (40). Synthetic glycopeptides containing oligosaccharides generated reduced CD4+ T cell responses to IAV strains with glycosylation at the corresponding position (41). Avci et al. have described a mechanism whereby B cells take up glycoconjugates through a carbohydrate–recognizing B cell receptor and process the antigens in the endosome. The peptide portion of the resulting glycan-peptide is presented to carbohydrate-recognizing T cells, the stimulation of which results in a carbohydrate-specific adaptive immune response (40). The number of examples of natural glycopeptides inducing T cell responses is only a few (42). Nonetheless, the influence of site-specific glycosylation structure on recognition of IAV antigens by antigen presenting cells for subsequent T cell stimulation have not been defined clearly.

Comparing how glycosylation is used to evade the human immune system, in IAV and human immunodeficiency virus 1 (HIV-1)

While IAV and HIV-1 differ in their replication mechanisms, both exhibit sufficient antigenic variation in their surface proteins over time in the human population to evade the protection conferred by standard vaccine strategies. In both cases, the ability of the virus to evolve reduces the efficacy of vaccines. Glycosylation of the HIV-1 envelope protein trimer, consisting of gp120 and gp41, corresponds to about half its mass (43). The underlying protein is highly mutative and evolves constantly to evade host antibodies. The high density of N-glycosylation limits the accessibility of glycan biosynthetic processing enzymes, resulting in a shield of primarily high mannose N-glycans that facilitate viral escape by interfering with proteolytic processing of envelope peptides for presentation by the major histocompatibility complex (44,45). While broadly neutralizing antibodies to envelope protein have been identified that either tolerate the dense glycan shield or bind to epitopes that contain glycans, it has not been possible to formulate a vaccine that elicits such responses (46). By contrast, glycosylation of
IAV HA appears to interfere with receptor binding and/or membrane fusion if too many sequons are occupied on the head domain (47-49).

The simplest mechanism whereby IAV escapes neutralizing antibodies of the adaptive immune system occurs through mutation(s) that diminish antibody binding affinity (50). In some cases, mutations can cause allosteric effects that decrease the antibody access to the epitope (51). Mutations that increase the avidity of HA for the host sialic acid receptor may cause IAV to bind host cells more avidly than competing antibodies (52,53). Amino acid mutations may create new N-glycosylation sites (sequons) in the HA globular domain. These mutated sequences are synthesized, extruded into the ER lumen of infected cells, and modified by N-glycans, which can, as shown in Figure 2, subsequently block antibody binding and therefore impact antigenicity (20,38). Depending on their accessibilities, immature high mannose N-glycans may be trimmed by mannosidases and subsequently extended by galactosyltransferases to form complex-type N-glycans.

Most IAV antigenicity studies do not account for changes in HA site-specific glycosylation because adding glycosylation lowers receptor binding avidity, thereby complicating interpretation of hemagglutination inhibition (HI) assays used in most serological analyses (38). The micro-neutralization (MN) assay is an alternative method that can overcome non-antigenic effects caused changes in receptor binding affinity (54-56).

IAV viral fitness, the ability of the virus to propagate, is maintained by balancing receptor binding and membrane fusion with the release of new virions from the cell surface. A mutation that significantly increases receptor avidity may have a negative impact on fitness if virions cannot escape the cell surface. Thus, mutations that strengthen NA activity may help balance such increases in HA receptor binding avidity (57). Head group glycans can increase viral fitness by shielding HA residues from antibody binding and tuning receptor specificity (58). In addition, the subsequent appearance of new glycosylations sites can balance the increased receptor binding
avidity of an amino acid mutation as a mechanism for maintaining fitness (58). Increased glycosylation may also compromise viral fitness by enabling the binding of HA by lectins of the innate immune system (59-62), or by negatively impacting assembly of stable HA trimers in the ER (16,24,38,49,63-65). Lectins, including surfactant protein D (SP-D) and mannose binding lectin (MBL), neutralize IAV by binding to glycosylated HA. While these interactions depend on the glycan structures present at each glycosite, they cannot be predicted from HA sequence information alone.

Glycosylation and antigenic cartography of influenza viruses

Antigenic cartography (66) is used to assess the antigenic distance among HA molecules from different IAV strains (67-69). Antigenic distances are calculated from hemagglutination inhibition and microneutralization assays (69). Wan et al. developed a 3D antigenic cartography construction and visualization resource to study strain candidates for vaccines (68). Given its roles in shielding underlying protein sequences from antibody binding, glycosylation is likely to impact antigenic cartography of a given IAV strain. Expanded knowledge of site-specific glycosylation in different IAV strains, including the range of glycoforms present at each site, would enable the correlation between antigenic distance calculation and HA glycosylation. This would be a boon to efforts in predicting the pandemic potential of zoonotic viruses. It would also facilitate vaccine planning by improving the ability to predict whether a given seasonally circulating virus will likely escape vaccines.

Towards a broadly neutralizing IAV vaccine

The major HA antigenic sites in the head domain show high rates of mutation, including the addition of new sequons (70). At the same time, the evolution of receptor binding sites and the
stem domain is much more limited to conserve their functions (71-73). Antibody escape mutants occur in five major head domain antigenic clusters (50). Neutralizing antibodies appear to target regions proximal to the receptor binding site and mutations responsible for antigenic drift tend to occur within these proximal regions (74-78). This may be related to the accumulation of $N$-glycosylation on the head group that shield underlying antigenic sites (79). Such glycosylation will disrupt binding of sialic acid residues if it occurs too close to the receptor binding site, thus leaving an opening for neutralizing antibodies to bind.

In principle, broadly neutralizing antibodies can target the conserved sites of the receptor binding and stalk regions, respectively, which are present across different IAV strains (79). However, most human antibodies against HA (and NA) bind hypervariable residues, not those conserved among IAV strains. Efforts to generate broadly neutralizing antibodies have focused on the receptor binding site and the highly conserved stalk region (80), for which escape mutants would disrupt key viral functions and therefore have a high fitness cost. For this to work, however, it is necessary to direct the immune system away from the immunodominant variable residues towards subdominant residues that are conserved among strains.

Although most neutralizing antibodies target residues proximal to the receptor binding site, some appear to mimic the sialic acid receptor itself and bind to conserved residues, thus offering the potential for a broadly neutralizing response (78). Some researchers have pointed to the lack of accessibility of the stem region for the lack of broadly neutralizing antibodies from vaccines (81), while others have noted that the stem region of HA in virions should be accessible to antibody binding based on structural studies (82). Improved understanding of the dynamics of IAV immunodominance in human populations will be necessary in order to design vaccine strategies that succeed in generating antibodies against conserved epitopes that confer broadly neutralizing projection against IAV (83).
Structural analysis of HAs from pandemic and seasonal IAV indicates that while the HA fold is conserved, the surface properties and glycosylation patterns differ significantly among subtypes (80). A large-scale in vitro mutational analysis of the H1 and H3 HA receptor binding site identified many replication-competent mutations not yet observed in nature, indicating that the receptor binding site can accommodate much more sequence diversity than previously believed (84). These researchers noted that many deleterious single mutations were viable when present in combination with other substitutions, demonstrating epistatic effects in evolution of the HA receptor binding site. Natural mutations to the receptor binding site become part of a network of epistatic modifications that prevent reversion of individual substitutions (70). The recent decline in effectiveness of IAV vaccines has been attributed in part to HA substitutions that arise during virus growth in chicken eggs that reduce binding and neutralization by a receptor binding site broadly neutralizing antibody by orders of magnitude (85). This work highlighted the fact that much about the receptor binding site of HA remains unknown, despite decades of effort.

For H3N2, the mode of receptor binding has shifted as the virus has circulated since 1968. Thus, mutations that increased H3N2 sialic acid binding in early years after 1968 in H3N2 on are inhibitory more recently due to other substitutions in the receptor binding site (70). This suggests that many residues proximal to the receptor binding site coordinate the receptor binding behavior of HA. Further, after 2003, H3N2 preference moved to binding extended, branched N-glycans, indicating the ability of the virus to evolve to bind a subset of airway glycans as a way of maintaining fitness.

Proteomics of IAV
Mass spectrometric analysis of viral glycoproteins has been summarized in a recent review (86). Downard et al. developed an approach for using accurate mass measurement of proteolytic peptides of IAV proteins, referred to as proteotyping, to identify HA and NA from circulating IAV types and subtypes. The accurate mass values constitute signatures for conserved regions of IAV proteins that enable virus typing (87,88). The investigators used this approach to differentiate seasonal strains from pandemic H1N1 (89-91) and study the evolution of H5N1 strains (92) and NA subtypes (93). They developed computer algorithms to identify virus reassortants from whole virus digests (94). FluShuffle considers combination of viral protein identities that match the mass spectral data using Gibbs sampling. FluResort uses those identities to calculate the weighted distance of each across two or more phylogenetic trees through viral protein sequence alignment. As an extension to this approach, the FluClass algorithm performs phylogenetic classification using MS data starting from DNA- or protein-based phylogenetic trees (95). The MassTree algorithm identifies and displays protein mutations and calculates mutational frequencies across phylogenetic trees for studies of IAV evolution (96-98).

**IAV glycoproteomics**

As reviewed (99), mass spectrometry has been used in proteomics studies of IAV proteins and in mass profiling of tryptic peptides and glycopeptides. An early pioneering study characterized N-glycosylation on three IAV strains (100). An LC/MS method has been used to characterize glycoforms at specific sites using alternating high and low collision energy values (a data independent analysis (DIA) experiment known as MS) combined with multiple reaction monitoring assays as a means of comparing recombinant HA samples as vaccine candidates (14,101). These investigators used this approach to analyze site-specific glycosylation in a series of engineered H3N2 HA variants with added sequons that mirror those that appeared
during seasonal circulation since 1968 (49,102). They also characterized HA glycosylation in a series of engineered H5N7 as part of an effort to define glycosylation structure-function relationships in this avian IAV strain (103). In additional work, they also examined glycosylation in a set of reference HA antigens used in influenza vaccine potency testing (104). We have used site-specific glycosylation information to model interactions between HA and surfactant protein-D (60,102,105-107).

**MS workflows for assigning glycopeptides.**

Confident assignment of glycopeptides requires building a search space consisting of the glycosylation variants of a measured proteome. For IAV, the hypervariable proteome (108), combined with the presence of host proteins in the viral architecture (1), require special consideration when applying proteomics methods. In addition, other post-translational modifications, including phosphorylation, have been observed on IAV proteins (109). Because the proteomics search space must include both IAV and host proteins, the rate of false positive identifications becomes unacceptably high if random mutations are allowed. In light of this, we recommend defining a range of allowable mutations in a custom database that contains sequences for a given IAV strain plus host proteome.

**Mass spectrometry methods for assigning site-specific glycosylation.** For detailed glycoproteomics reviews, see (110-116). As shown in Figure 3, glycopeptide glycoforms elute from a reversed phase chromatography column over a narrow retention time window. Identification of site-specific glycosylation requires tailored analytical and bioinformatics methods. Proteomics workflows identify and quantify proteins based on prediction of peptide tandem mass spectra from genomic databases. While small PTMs have single predictable mass shifts, glycosylation at a given site is heterogeneous, pushing confident site-specific assignment...
of glycosylation beyond the scope of conventional proteomics workflows. In order to assign site-specific glycosylation, one must generate an appropriate list of theoretical glycan and peptide compositions (known as the search space) and use this to assign the most probable glycopeptide composition (117). The number of possible glycoforms at each glycosylation site multiplies the size of the search space and the difficulty in making confident assignments. Assumptions made about the purity or complexity of the sample can greatly affect the quality and confidence of the results. Using a too small search space by assuming incorrectly that a glycoprotein sample is pure may lead to unacceptably high numbers of contaminant glycopeptides incorrectly assigned to the target glycoprotein. Overestimating the size of the search space by including too many glycoproteins and glycoforms leads to decreasing ability to assign glycopeptides with acceptable confidence.

The ability to distinguish HA glycosylation among different IAV strains or expression platforms with statistical significance depends on the quality of the mass spectral data. Typical top-N data-dependent acquisition (DDA) mass spectrometry methods, based on selection of the most abundant precursor ions, give rise to missing values that negatively impact the ability to quantify glycopeptides. In addition, glycosylation variants of a given peptide elute over a narrow retention time window using LC-MS. For this reason, DDA samples only the most abundant glycoforms. The under-sampling and reproducibility problems resulting from the stochastic nature of top-N DDA result in the inability to quantify a given precursor ion in a significant subset of a biological sample cohort. These missing values, the occurrence of which increases as the glycopeptide abundance decreases, necessitate the imputation of glycopeptide abundances, a step that reduces the resulting statistical rigor. The use of targeted quantification (118) solves the missing value problem but limits the number of precursor ions for which tandem mass spectra are available. This suggests the use of the MS\(^1\) dimension of a DDA or targeted quantification
experiment to quantify glycopeptides. For this it would be important to develop metrics for assigning confidence of glycopeptides for which tandem mass spectra were not collected.

Data-independent analysis (DIA) eliminates the need to isolate precursor ions provided that peptides are chromatographically well resolved. In sequential window acquisition of all theoretical fragment ion spectra (SWATH)-MS DIA (119), for example, fragment ion spectra for all precursors are acquired within the specified m/z range and retention time window. However, there are limitations to DIA for the analysis of glycopeptides. First, one cannot construct a rigorous spectral library because of the lack of a comprehensive collection of synthetic glycopeptides. In addition, the glycopeptide oxonium ions and residue losses from the precursor ion do not provide peptide sequence information because these losses occur in all glycopeptides, irrespective of their glycan compositions. In order to be effective, DIA methods must take into account the narrow retention time window over which glycopeptide glycoforms elute in typical reversed phase gradients. Further, if a large precursor window is fragmented, there may be problems determining which precursor peak produced the glycopeptide fragments. Despite these limitations, investigators have used low collision energy settings to produce Y-type ions for identification and quantification of IgG glycopeptides in a complex matrix of human plasma (120-122). A SWATH DIA method was used to quantify high mannose N-glycopeptides from yeast using manually created glycopeptide libraries (123,124). Researchers developed a DIA strategy to quantify 25 N-glycopeptides from plasma using a search space of 161 glycoforms for a study of liver cirrhosis (121). Others have used DIA to produce comprehensive glycosylation maps of human serum IgM using extracted ion chromatograms of shared peptide-specific fragment ions to filter related glycoforms for a given glycosite (125). This approach allowed identification of glycopeptides with unexpected modifications. A targeted DIA method identified N-glycopeptides without predefined glycan compositions from 59 N-glycosylation sites.
from 41 glycoproteins, including 21 IgG glycopeptide glycoforms, from HILIC-enriched human blood plasma tryptic digest (126). Due to the statistical limitations of DDA, therefore, a targeted DIA approach may be necessary to produce a comprehensive catalog of all HA glycoforms with sufficient confidence.

Conclusions

Because of the low efficacy of existing seasonal IAV vaccines, we need a paradigm change in IAV vaccine design. IAV strains are continually mutating under immune pressure, necessitating selection and manufacture of new vaccines every year. To mitigate the disease burden caused by IAV, we need to develop a broadly neutralizing vaccine capable of protecting against multiple strains. IAV researchers currently use genetics and structural biology studies in determining IAV antigenicity and immunogenicity, but as of today, glycosylation state of HA is largely ignored.

In the HIV vaccine field, researchers have moved toward mimicking the glycan shield in order to generate broadly neutralizing antibodies analogous to those that develop naturally in a subset of infected individuals. For IAV, glycosylation does not go to the extreme of forming a sterically restricted shield. There is a strong argument for the need to understand the influence of IAV glycosylation on transport to the lymph nodes and innate immune lectin mediated recognition and processing by dendritic cells and B cells towards generating an effective antibody response.

The technology to correlate glycopopulations of vaccine constructs with breadth of antibody response already exists. Exploitation of the knowledge of the most appropriate glycopopulations of HA, as well as the viral expression systems that can produce them, will mark a major step toward developing more effective IAV vaccines.
Acknowledgements. The authors are supported by NIH grant U01CA221234 and the Graduate Medical Sciences program at Boston University School of Medicine.
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Flu Glycosylation

Figures

Figure 1. Influenza A viral cycle (127). Virion binding to airway cell receptors can be neutralized by antibody binding to the hemagglutinin (HA) head group or by innate immune system collectin binding to high mannose N-glycans on the HA head group. If neutralization does not occur, virion HA molecules bind airway cell sialic acid receptors and the virion is endocytosed. Low endosome pH causes virion membrane fusion and uncoating of ribonucleoprotein. Viral proteins are transported into the nucleus by host cell machinery. The negative sense RNA viral genome is converted to positive sense RNA that serves as a template for viral RNA production. Viral mRNA is exported from the nucleus and translated by cytoplasmic ribosomes. Viral proteins are imported to the nucleus where viral ribonucleoprotein complex assembly occurs. Viral ribonucleoproteins are transported to the plasma membrane. The HA and NA proteins pass through the secretory pathway where they are glycosylated and assembled into trimers.
Viral progeny are assembled at the plasma membrane. Enveloped virions bud from the cell surface with the aid of NA cleavage of cell surface sialic acid residues.

Figure 2. Mechanisms whereby hemagglutinin glycosylation influences IAV fitness (58).
Automated precursor ion selection based on abundance

Precursor ions selected from targeted list

All precursor ions subjected to tandem MS

Advantages

No need to prepare targeted list
Reproducible precursor ion selection
Reproducible scans

Disadvantages

Precursor ion selection varies run-to-run
Time consuming to prepare targeted list; lack of flexibility
Glycopeptide spectral libraries required; selectivity limited
Figure 3. Comparison of acquisition methods for tandem MS of glycopeptides. Extracted ion chromatograms for glycopeptide IADTNITPQGLPSSLTELHLDGNK glycoforms are shown, illustrating that a large number of glycoforms elute over a narrow retention time range using reversed phase chromatography LC-MS as described (128). Automated precursor ion selection using data dependent acquisition, targeted precursor ion selection using parallel reaction monitoring (PRM) and data independent acquisition are compared.