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N-Linked Glycosylation Attenuates H3N2 Influenza Viruses[▽]

David J. Vigerust,[1] Kimberly B. Ulett,[1] Kelli L. Boyd,[2] Jens Madsen,[3] Samuel Hawgood,[3] and Jonathan A. McCullers[1]*

Department of Infectious Diseases[1] and Animal Resources Center,[2] St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Department of Pediatrics and Cardiovascular Research Institute, University of California San Francisco, San Francisco, California 94043[3]

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Over the last four decades, H3N2 subtype influenza A viruses have gradually acquired additional potential sites for glycosylation within the globular head of the hemagglutinin (HA) protein. Here, we have examined the biological effect of additional glycosylation on the virulence of H3N2 influenza viruses. We created otherwise isogenic reassortant viruses by site-directed mutagenesis that contain additional potential sites for glycosylation and examined the effect on virulence in naïve BALB/c, C57BL/6, and surfactant protein D (SP-D)-deficient mice. The introduction of additional sites was consistent with the sequence of acquisition in the globular head over the past 40 years, beginning with two sites in 1968 to the seven sites found in contemporary influenza viruses circulating in 2000. Decreased morbidity and mortality, as well as lower viral lung titers, were seen in mice as the level of potential glycosylation of the viruses increased. This correlated with decreased evidence of virus-mediated lung damage and increased in vitro inhibition of hemagglutination by SP-D. SP-D-deficient animals displayed an inverse pattern of disease, such that more highly glycosylated viruses elicited disease equivalent to or exceeding that of the wild type. We conclude from these data that increased glycosylation of influenza viruses results in decreased virulence, which is at least partly mediated by SP-D-induced clearance from the lung. The continued exploration of interactions between highly glycosylated viruses and surfactant proteins may lead to an improved understanding of the biology within the lung and strategies for viral control.

Influenza viruses express two envelope proteins that are involved in virulence, neuraminidase (NA) and hemagglutinin (HA). During the virus life cycle these proteins have distinct functions in entry and release of the virus. HA plays a primary role in the binding and uptake of the virus into target cells and is the main target of neutralizing antibodies. Structurally, the HA is a 200-kDa homotrimer with an ectodomain composed of a globular head and a stalk region (49). Both of these regions undergo posttranslational modifications in the Golgi, where glycoconjugates are added to certain sites of N-linked glycosylation. Some of these glycosylation sites, primarily in the stalk region, are indispensable to the proper folding and conformation of the HA molecule (9, 33). During the last 4 decades of circulation in humans, N-linked glycosylation in and around the globular head has gradually increased in H3N2 subtype viruses (36, 48). Carbohydrate attached to glycosylation sites has been previously characterized in tissue culture to be cell type specific and site specific and composed primarily of complex-type and oligomannose oligosaccharide (27, 35). The role of these glycoconjugates in the life cycle of the virus and the evolutionary reasons behind the increasing glycosylation seen since H3N2 viruses began circulating in humans are poorly understood at present.

The presence of carbohydrate on the HA can have either positive or detrimental effects on the virus. For example, glycoconjugates that are positioned close to the cleavage site can interfere with proteolytic activation of the nascent HA0 (22). Alternatively, replication and release of virus may be facilitated by carbohydrate that is located near the receptor binding site through a mechanism of reduced receptor affinity (46, 47). Carbohydrate around the globular head can also potentially shield antigenic sites from immune recognition. This may contribute to antigenic drift of influenza viruses where successive glycosylation events prevent accessibility and recognition by antibodies in an immune population (1). Additional work from Klenk at al. illustrates that carbohydrate is especially important for the interaction of HA and NA, where a balance is needed between receptor binding activity and virus release (23). A virus containing HA with little carbohydrate modification can tightly bind the receptor, requiring greater NA activity to promote particle release. Conversely, an HA with more extensive glycosylation interacts weakly with receptors and requires a less active NA to facilitate release. Overall, the HA depends on a balance of glycosylation to mediate the proper folding of the HA, interaction of virus with receptor, and efficient particle release.

Collectins are a family of collagenous lectin molecules that are calcium-dependent carbohydrate binding proteins previously shown to bind enveloped viruses (6, 26, 32, 41). The function of these proteins is believed to be as a first line of defense against both bacterial and viral pathogens by binding to carbohydrate moieties on the pathogen surface. In support of this concept, children with a deficiency in mannose binding lectin are more prone to a variety of serious infections (21, 40). Multiple studies have shown that lung-resident surfactant proteins A and D (SP-A and SP-D, respectively) neutralize and aid in clearance of influenza A viruses (7, 8, 15, 18, 19). SP-A is more effective at neutralizing influenza viruses that contain low carbohydrate content and does so via sialic acid residues

* Corresponding author. Mailing address: St. Jude Children's Research Hospital, 332 N. Lauderdale St., Memphis, TN 38105-2794. Phone: (901) 495-5164. Fax: (901) 495-3099. E-mail: jon.mccullers@stjude.org.

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present on the carbohydrate recognition domain that compete virus away from cellular sialic acids (24). SP-D directly interacts with carbohydrate on the HA globular head. It preferentially binds to high-mannose oligosaccharides on the HA, most notably the oligosaccharide attached via amino acid 165, which is conserved in all H3N2 viruses isolated to date (8). Data from several labs suggest that the high avidity of SP-D binding to influenza viruses is a key contributor to the virus-neutralizing capacity of the bronchoalveolar fluid (8, 19, 43, 44).

We sought to investigate the potential cost of accumulating additional glycosylation on the globular head of the HA of influenza viruses. We constructed seven viruses expressing mutant HAs containing between 6 and 12 potential sites for N-linked glycosylation. We hypothesize that the level of glycosylation is inversely related to virulence in the naïve host; as glycosylation increases, the severity and sequelae of disease decrease due in part to improved recognition and neutralization by collectins.

TABLE 1. Sequential modifications to the globular head of HA

Amino acid position	HK68 consensus sequence	Sequence modification[b]
Stalk		
8	NSTA	None
22	NGTL	None
38	NATE	None
285	NGSI	None
154 (HA2)	NGTY	None
Globular head[a]		
81	NETW	None
167 (−1)	NVTM	NV**G**M
63 (+1)	DCTL	**N**CTL
126 (+2)	TWTG	**N**WTG
248 (+3)	NSNG	NS**T**G
135 (+4)	NGGS	NG**T**S
144 (+5)	GSGF	**N**SSF

[a] The change in the number of glycosylation sites is given in parentheses.
[b] Changed residues are in boldface.

MATERIALS AND METHODS

Generation of mutant viruses. Plasmids expressing the internal genes from influenza virus A Puerto Rico/8/34 (H1N1, referred to hereafter as PR8) and the HA and/or NA from A/Hong Kong/1/68 (H3N2) (HK68), A/Leningrad/360/86 (H3N2) (Len86), A/Sydney/5/97 (H3N2) (Syd97), and A/Panama/2007/99 (H3N2) (Pan99) were obtained from Robert Webster (St. Jude Children's Research Hospital [SJCRH]) or were cloned from viruses obtained from Webster as described previously (20). The plasmid expressing the HA of HK68 was sequentially modified by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA) to encode additional sites of glycosylation. Oligonucleotide primers were designed using web-based Primer X (www.Bioinformatics.org) from the consensus sequence for HK68 to create the N-X-S/T sequon at positions 63, 126, 248, 135, and 144 using historic sequences found in natural isolates from the past 38 years. Reassortant viruses expressing an H3 HA, an N2 NA (from Syd97), and the internal genes of PR8 were rescued into a coculture of MDCK and 293T cells using the 8-plasmid reverse genetics system, as described previously (20). Viruses were propagated for an additional passage in MDCK cells and then grown in eggs to produce stocks for use in the experiments described here. Glycosylation mutants were generated with the previous virus as a template to generate viruses containing 1, 2, 3, 4, and 5 additional glycosylation sites (Table 1). An additional mutant virus was created by disrupting the glycosylation site at amino acid 165 of the HA. The nucleotide sequences of all plasmids and of the HA and NA of all stock viruses were confirmed by Big Dye Terminator Cycle sequencing (Hartwell Center), and analysis was performed by alignment against published sequences.

Immunoprecipitation and Western blotting. Subconfluent MDCK cells were infected at a multiplicity of infection of 0.1 with reassortant virus. At 48 h postinfection the monolayers were disrupted in phosphate-buffered saline (PBS) containing 0.5% NP-40 and immunoprecipitated with EZview Red Anti-HA Affinity Gel (Sigma Aldrich, St. Louis, MO) targeting amino acid residues 98 to 106 (YPYDVPDYA) of human influenza virus HA. Immunoprecipitates were resolved on a 10 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (Bio-Rad, Hercules, CA). Western blotting was performed with mouse monoclonal anti-HA antibody (Research Diagnostics, Concord, MA) and TrueBlot horseradish peroxidase-conjugated anti-mouse secondary antibody (eBioscience, San Diego, CA) and visualized by enhanced chemiluminescence on radiographic film.

Mice. Female BALB/cByJ and C57BL/6J mice were obtained from Jackson Laboratories and utilized at 6 to 8 weeks of age (Bar Harbor, ME). C57BL/6J mice deficient in SP-D were generated by author S. Hawgood at the University of California at San Francisco (4) and then bred at SJCRH. Mice were housed in groups of 4 to 6 in high-temperature, 31.2-cm by 23.5-cm by 15.2-cm polycarbonate cages with isolator lids. Rooms used for housing mice were maintained on a 12:12-h light:dark cycle at 22 ± 2°C with a humidity of 50% in the biosafety level 2 facility at SJCRH. Prior to inclusion in experiments, mice were allowed at least 7 days to acclimate to the animal facility. Laboratory Autoclavable Rodent Diet (PMI Nutrition International, St. Louis, MO) and autoclaved water were available ad libitum. All experiments were performed in accordance with the guidelines set forth by the Animal Care and Use Committee at SJCRH.

Infection model. The dose infectious for 50% of embryonated chicken eggs (EID_{50}) was determined by interpolation using the method of Reed and Muench and used as the basis to calculate the dose lethal for 50% of mice (MLD_{50}) using serial dilutions of virus delivered to groups of 4 mice. For infection experiments, virus was diluted in sterile PBS and administered at a dose of 1×10^6 EID_{50} intranasally to mice lightly anesthetized with 2.5% inhaled isoflurane (Baxter, Deerfield, IL) in a total volume of 100 μl (50 μl per nostril). Mice were weighed at the onset of infection and each subsequent day for illness and mortality. Mice that were found to be moribund were euthanized and considered to have died that day.

Lung titers. Mice were euthanized by CO_2 asphyxiation. Lungs were aseptically harvested, washed three times in PBS, and placed in 750 μl of sterile PBS. Lungs were mechanically homogenized using an Ultra-Turrax T8 homogenizer (IKA-werke, Staufen, Germany). Lung homogenates were pelleted at 10,000 rpm for 5 min, and the supernatants were used to determine the viral titer for each set of lungs using serial dilutions on MDCK monolayers.

Pathology. Lungs were removed immediately after euthanasia and were insufflated and fixed overnight with 2% neutral buffered paraformaldehyde. After 24 h, the lungs were transferred into 10% neutral buffered formalin for an additional 24 h; the lungs were then embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically for histopathology by an experienced veterinary pathologist (K. L. Boyd) blinded to the composition of the groups. The lung parenchyma and large airways were considered separately and assigned a grade of 0 to 3 based on the histologic character of the lesions. A score of 1 was given to mild findings including minimal infiltrates of lymphocytes and plasma cells around airways and vessels, minimal epithelial hyperplasia, minimal leukocyte infiltration of alveolar spaces, and <10% of the lung affected. A score of 2 was given for moderate findings including moderate infiltrates of lymphocytes and plasma cells around airways and vessels, moderate epithelial hyperplasia with focal necrosis, focally extensive infiltration of the alveolar spaces by leukocytes with some consolidation, focal pleuritis, and >10% but <30% of the lung affected. A score of 3 was given for more severe findings including extensive necrosis of airway epithelium and the interstitium, extensive leukocyte infiltration and consolidation, severe pleuritis, and lobar involvement.

Inhibition of hemagglutination by rhSP-D. Recombinant human SP-D (rhSP-D) was expressed in Chinese hamster ovary cells and purified by maltose affinity chromatography as previously described (16). Inhibition of hemagglutination was done using standard methods. Briefly, virus suspensions titrated to 4 HA units were incubated with serial dilutions of rhSP-D for 30 min at room temperature. A 0.5% suspension of chicken red blood cells was added, and the minimum concentration needed to inhibit hemagglutination was determined in quadruplicate assays. In a second experiment, 1.25 μg of rhSP-D (the minimum amount which would inhibit hemagglutination from all viruses) was incubated with serial dilutions of virus prior to hemagglutination. In parallel, virus was incubated with PBS, and the HA titer of the diluted suspension was determined. The maximal number of HA units which could be prevented from hemagglutinating red blood cells is reported.

Statistical analysis. Comparison of survival between groups of mice was done with a log rank chi-squared test on the Kaplan-Meier survival data. Comparison of viral lung titers, weight loss, and inhibition of hemagglutination between

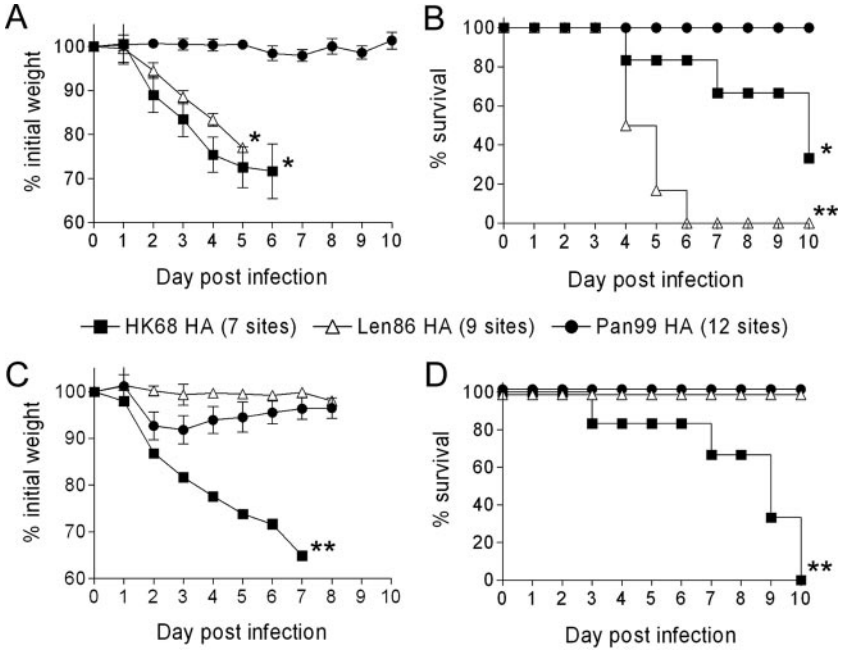

FIG. 1. Viruses with different HAs differ in virulence based on their level of glycosylation. Groups of 6 mice were infected with 1×10^6 EID_{50} of reassortant viruses expressing the HA from HK68 (7 glycosylation sites), Len86 (9 sites), or Pan99 (12 sites) paired with the NA from HK68 (A and B) or Syd97 (C and D) to determine the effect of glycosylation on morbidity (weight loss) (A and C) and survival (B and D). An asterisk denotes a statistically significant difference compared to the group infected with a virus expressing the Pan99 HA; a double asterisk indicates a significant difference compared to both other viruses.

groups was done using analysis of variance. A P value of <0.05 was considered significant for these comparisons. SigmaStat for Windows (version 3.11; SysStat Software, Inc.,) was utilized for all statistical analyses. Due to the small number of SP-D-deficient animals available for use, studies involving those mice were underpowered for statistical analysis.

RESULTS

Viruses differing in glycosylation of the HA differ in virulence. To assess the impact of different amounts of oligosaccharide on virulence, reassortant viruses with different H3 HAs were used to infect naïve mice. Viruses expressed the H3 HA from HK68 (7 potential sites of glycosylation), Len86 (9 potential sites of glycosylation), or Pan99 (12 potential sites of glycosylation) and were paired with both the HK68 NA and the Syd97 NA to control for the contribution of an HA-NA functional match. When matched to the HK68 NA, viruses containing the HK68 (7 sites) or Len86 (9 sites) HAs required nearly 3 logs less virus to kill mice (MLD_{50} of $10^{5.6}$ for both) than did a virus containing the Pan99 HA (12 sites; MLD_{50} of $>10^{7.25}$). These viruses caused significantly more weight loss and mortality when administered to groups of mice at a dose of 1×10^6 EID_{50} (Fig. 1A and B). Only the virus expressing the HK68 HA retained lethality (MLD_{50} of $10^{5.8}$ for that virus, while other viruses had an MLD_{50} of $>10^{7.0}$) when matched to the Syd97 NA (Fig. 1C and D), which has one additional glycosylation site compared to the HK68 NA. These data are consistent with our hypothesis that more heavily glycosylated viruses are less virulent in a naïve host since the viruses with seven potential glycosylation sites (HK68 HA) were clearly more virulent than the viruses with 12 potential sites (Pan99). The difference in the virulence of the virus expressing the Len86 HA when matched to the Syd97 NA may be because of a poor functional match to that NA or may be due to an extra glycosylation site on the Syd97 NA relative to the HK68 NA. We sought to resolve these possibilities by generating viruses differing in glycosylation on an isogenic background.

Generation and characterization of mutant HAs with increasing oligosaccharides on the globular head. To control for differences in HA-NA functional match and glycosylation of the NA, viruses were created by reverse genetics to be isogenic in all respects except the potential level of glycosylation. Mutations in the globular head of HK68 were made to create sites for potential glycosylation (Table 1; Fig. 2A). Western blotting of lysates from MDCK cell culture demonstrates a gradual decrease in electrophoretic mobility as potential glycosylation sites are added stepwise in the HK68 H3 HA (Fig. 2B). The Δ144 (+5) mutant (where Δ indicates a change in glycosylation at this residue and the number in parentheses indicates the change in the number of glycosylation sites) (12 sites total) migrates to the same position as a virus with the Pan99 HA (12 sites). A virus generated to lack the site at position Δ167 (−1) migrates faster than the wild-type HK68 HA, suggesting that this site was occupied by carbohydrate in the wild-type virus. The addition of carbohydrate to some or all of these potential sites did not affect the replication of the viruses. Single and multistep growth curves and peak titers in both eggs and MDCK cells were similar for the seven viruses (data not shown).

Glycosylation decreases the virulence of influenza viruses. Preliminary data suggested that the level of glycosylation could affect the outcome of infections in vivo (Fig. 1). To confirm this finding, groups of six BALB/cByJ or C57BL/6J mice were in-

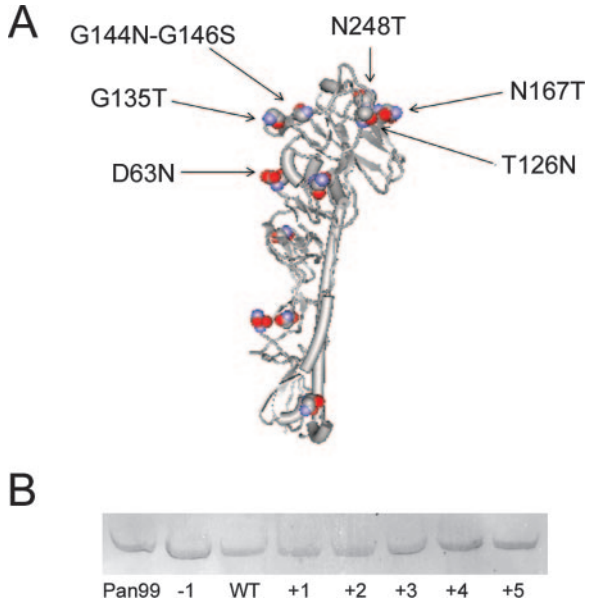

FIG. 2. Sites of glycosylation on the influenza virus HA. Site-directed mutagenesis was used to generate additional sites for N-linked glycosylation. (A) The amino acid identity, position, and location within the globular head of H3N2 HA are indicated where additional sites of potential glycosylation were introduced. (B) To demonstrate that these sites were occupied by carbohydrate, the electrophoretic mobility of purified virus was determined by immunoprecipitation and Western blotting followed by resolution on a 10 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel.

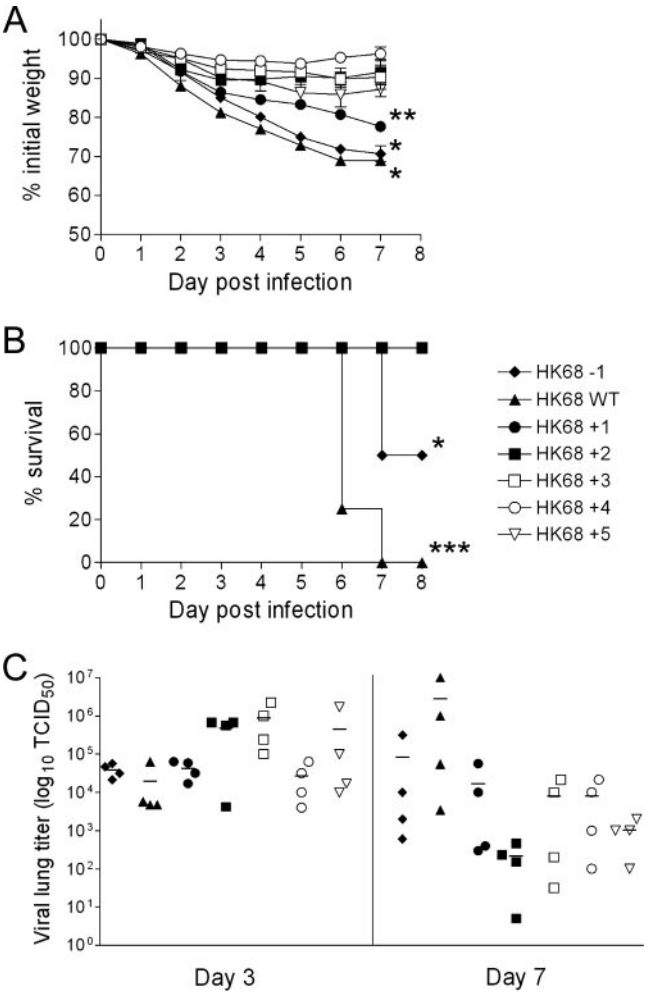

FIG. 3. Increasing glycosylation of the influenza virus HA attenuates the virus. Groups of 6 mice were infected with 1×10^6 EID_{50} of otherwise isogenic influenza viruses engineered to have additional potential sites of glycosylation and followed for weight loss (A) and survival (B). (C) Total viral lung loads were determined for mice infected with viruses for 3 or 7 days. Significant differences are indicated as follows: *, compared to viruses with +1 to +5 additional glycosylation sites; **, compared to the viruses with +2 to +5 additional glycosylation sites; ***, compared to all other groups. $TCID_{50}$, 50% tissue culture infective dose.

fected intranasally with each single-step glycosylation mutant to determine the specific effect of glycosylation without influence from any other factor. A hierarchy of weight loss was seen in BALB/cByJ animals that was dependent on the number of sites of potential glycosylation (Fig. 3A). Animals infected with the least glycosylated viruses (6, 7, or 8 sites) lost significantly more weight, and most mice infected with wild-type or Δ167 (−1) virus succumbed to infection by day 7 (Fig. 3A and B). The relatively increased morbidity of these two viruses in C57BL/6J mice was similar to that of BALB/cByJ mice although no animals succumbed to infection at this dose (data not shown). A possible mechanism for this decreased virulence was clearance of virus, since the mean viral lung load increased between days 3 and 7 for the two lethal viruses but decreased for the other 5 (Fig. 3C).

Severity of lung injury decreases as glycosylation increases. Lungs from infected BALB/cByJ mice were harvested to examine the histological features of disease. Histopathologic changes in the lungs of mice infected with the most virulent virus, the wild-type HK68, included epithelial hyperplasia and hypertrophy of the airways, moderate infiltration of inflammatory cells in a peribronchial distribution with some extension into the parenchyma, and scattered foci of necrosis with edema and fibrin deposition (Fig. 4A, D, and G). In general, the level of pathology in the lung decreased as the level of glycosylation increased. Mild inflammation and hyperplasia were seen in all lungs examined, although both the severity and the extent of the lesions were diminished in lungs from mice infected with more highly glycosylated viruses compared to wild type. With the exception of mild epithelial hypertrophy, the airways of mice infected with viruses containing two to five additional glycosylation sites were normal (Fig. 4E and F and Table 2). The character of the parenchymal disease was much milder in mice infected with viruses containing four or five additional glycosylation sites compared to the other viruses (Fig. 4C and Table 2). Examination of lungs from C57BL/6J mice infected with the same panel of viruses showed a similar pattern of pathology (data not shown).

Inhibition of hemagglutination by SP-D is dependent on level of HA glycosylation. After demonstrating that the virulence of the glycosylation mutants was inversely related to their level of glycosylation in naïve mice, we sought to explore whether clearance by SP-D was responsible for these differences. Groups of 3 to 4 mice deficient in SP-D production were

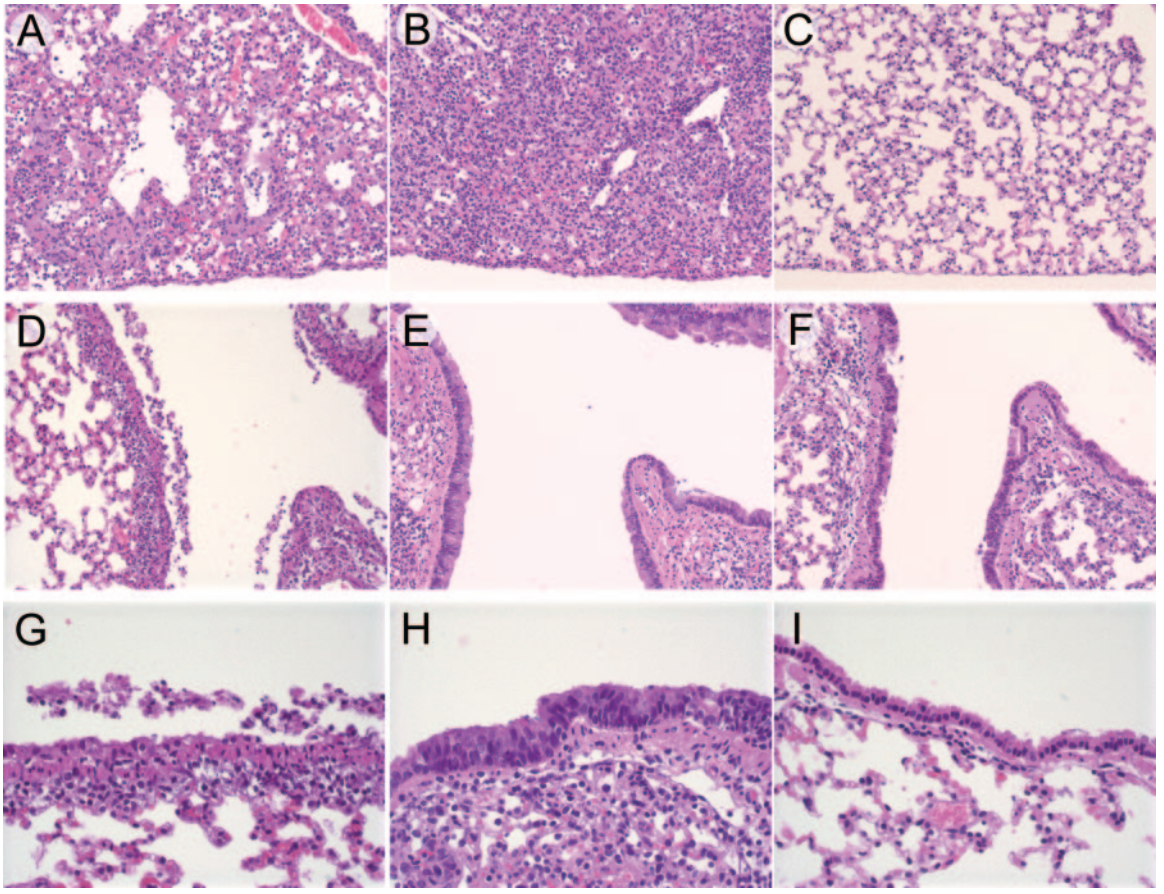

FIG. 4. Increasing glycosylation decreases the histopathologic damage to the lungs. Mice were infected with 1×10^6 EID_{50} of the HK68 WT virus (A, D, and G) the HK68 Δ248 (+3) virus (B, E, and H), or the HK68 Δ144 (+5) virus (C, F, and J). Representative sections of lungs removed 5 days after infection are shown for each virus. Panels A, B, and C show representative areas of the parenchyma (magnification, ×20); panels D, E, and F show large airways (magnification, ×20); and panels G, H, and I show the epithelial border of a bronchus (magnification, ×40).

infected with single-step glycosylation mutants to examine the effect on disease outcome. An inverse pattern of weight loss and mortality was seen compared to wild-type C57BL/6J mice. The viruses expressing a greater level of glycosylation elicited more weight loss than the wild type (Fig. 5A), and only viruses that contained the highest levels of potential oligosaccharide were found to elicit death in SP-D-deficient animals (Fig. 5B). Lung titers from animals deficient in SP-D show an inverse pattern with the Δ135 (+4) and Δ144 (+5) mutants maintaining a 1- to 2-log higher mean titer in the lung compared to wild type and the Δ63 (+1) mutant (Fig. 5C).

TABLE 2. Histopathological scores of lungs from mice infected with glycosylation mutants

Virus	Airway score (mean ± SD)	Parenchymal score (mean ± SD)	Comment(s)
Mock	0.0 ± 0.0	0.0 ± 0.0	No disease
HK68 wild type	2.7 ± 0.5	2.4 ± 0.5	Severe airway necrosis, interstitial pneumonia
HK68 mutant[a]			
Δ63 (+1)	2.0 ± 1.4	1.5 ± 0.6	Moderate airway necrosis
Δ126 (+2)	1.0 ± 0.0	1.8 ± 0.5	Mild edema
Δ248 (+3)	1.0 ± 0.0	2.7 ± 0.6	Moderate interstitial edema, hemorrhage
Δ135 (+4)	0.9 ± 0.4	1.0 ± 0.5	Mild edema
Δ144 (+5)	1.0 ± 1.0	1.0 ± 0.0	

[a] See text for explanation of notation.

In vitro experiments using recombinant human SP-D showed that the purified protein inhibited the hemagglutination of all of the viruses in a dose-dependent manner. In HA inhibition assays, the minimal concentration of rhSP-D required to neutralize 4 HA units of Δ167 (−1), HK68 wild type, Δ63 (+1), and Δ126 (+2) strains was 1.25 μg. A lower amount of rhSP-D, 750 ng, was sufficient for inhibition of the Δ248 (+3), Δ135 (+4), and Δ144 (+5) mutants. The maximum hemagglutination inhibition capacity of 1.25 μg of rhSP-D was 8 HA units of the Δ167 (−1) mutant on the low end and 512 HA units of the Δ135 (+4) mutant on the high end, indicating greater inhibitory capacity for viruses containing greater glycosylation (Fig. 6).

DISCUSSION

Over the past nearly 40 years of circulation, H3N2 viruses have gradually acquired additional oligosaccharide content

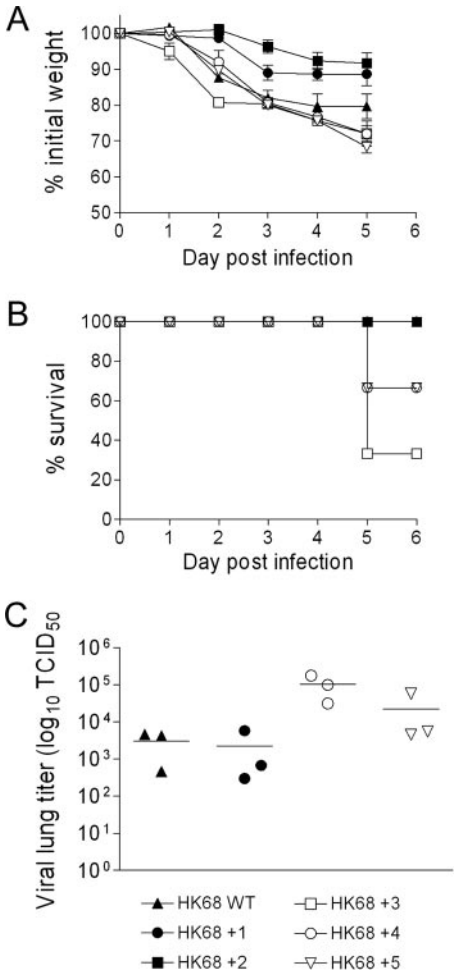

FIG. 5. Mice deficient in SP-D are more susceptible to highly glycosylated viruses. Groups of 3 to 4 mice deficient for SP-D were infected with 1×10^6 EID_{50} of otherwise isogenic influenza viruses engineered to have additional potential sites of glycosylation and followed for weight loss (A) and survival (B). (C) Total viral lung loads were determined for mice infected with viruses for 7 days. $TCID_{50}$, 50% tissue culture infective dose.

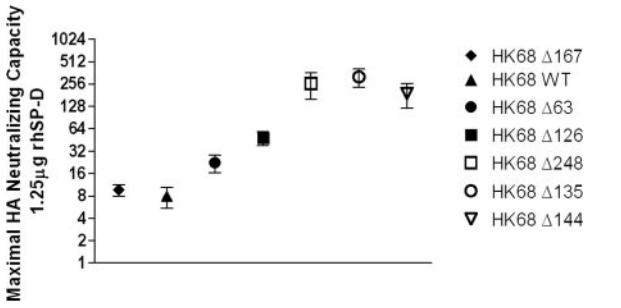

FIG. 6. Hemagglutination inhibition capacity of rhSP-D is inversely related to the glycosylation of viruses. Maximal hemagglutination inhibition of rhSP-D was determined by incubation of virus in the presence of 1.25 μg of rhSP-D.

around the globular head of the protein. These progressive, adaptive changes likely occurred because they provided an evolutionary advantage. However, since the changes have been gradual and are not fixed features of the HA, we reasoned they likely also came at some cost. We constructed a series of mutant viruses differing only in sites of potential glycosylation on the globular head of the HA to test the hypothesis that the level of oligosaccharide content is inversely related to virulence and tested this hypothesis in a mouse model of infection. We demonstrate that the functional outcome of additional N-linked glycosylation on the globular head of H3N2 influenza viruses is to attenuate the severity of infection in naive mice, likely mediated by improved neutralization by SP-D. A breakpoint for virulence in mice was evident at eight total sites of glycosylation on the HA (3 sites on the globular head); for maximal hemagglutination inhibition capacity of recombinant SP-D the breakpoint was nine total sites (4 sites on the globular head). Our conclusions were supported by studies in mice deficient in SP-D, which evidenced an inverse pattern of disease, with viruses possessing the highest potential for glycosylation eliciting significant disease and mortality.

Glycosylation of surface proteins plays a role in the biology of many viruses. including Hendra (5), Hantaan (37), severe acute respiratory syndrome coronavirus (SARS-CoV) (30), West Nile (17), hepatitis C (13), and influenza viruses. The function of surface glycoconjugates in the life cycle of many of these viruses is to aid in entry into target cells. For example, the hepatitis virus E2 and West Nile virus PrM and E proteins rely upon glycosylation to interact with immune molecules such as DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) and the related liver lectin L-SIGN for attachment and entry (10, 11). The glycosylated SARS-CoV S protein and filovirus envelope glycoprotein can interact with the lectin LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin) to enhance viral uptake and infection (14). Human immunodeficiency virus type 1 and influenza virus have also been described as using glycosylated gp120 and HA molecules to interact with and mediate entry via DC-SIGN and mannose receptor molecules on dendritic cells and macrophages (29, 31, 45). In addition, glycosylation of the surface proteins of SARS-CoV, Nipah, Hendra and Hantaan viruses has been described to participate in infectivity, protein folding, tropism, and proteolytic processing (2, 3, 12, 25, 28, 30, 34).

It has become clear that the addition of glycosylation in many viruses is also a mechanism for viral evasion and persistence. Evidence for this view derives from studies where successively adding additional sites for linkage of oligosaccharide by site-directed mutagenesis provided influenza A viruses with the ability to evade the host response without negatively impacting survival and biological activity (1). The additional sugar on the globular head resulted in a decrease in receptor binding and did not affect fusion activity, but, importantly, the viruses were now more resistant to antibody recognition. Skehel et al. showed that the introduction of a site for glycosylation at amino acid position 63 in the X-31 (H3N2) virus resulted in a lack of recognition by monoclonal antibody directed against X-31 (38). Thus, acquisition of carbohydrates on the globular head of the HA of influenza viruses may be an evolutionary adaptation allowing further circulation in an immune population. The trend toward accumulation of sites for potential glycosylation can be seen in both the H3N2 and H1N1 lineages. From its introduction into the human popula-

tion in 1918, the H1N1 viruses have progressed from 4 sites of potential glycosylation, all within the stalk region, to 8 sites for potential glycosylation, 4 of which are now in the globular head. In the H3N2 strains, the pandemic strain at its introduction contained 6 sites within the HA1 subunit, 2 of which were on the globular head. Currently circulating H3N2 viruses now have 13 potential sites for glycosylation, the original 4 sites in the stalk region and 9 sites on the globular head.

In this study we have engineered five additional sites of glycosylation into the globular head of HK68 to recapitulate the acquisition of glycosylation that has occurred in circulating H3N2 strains over the last 38 years. Additionally, we created a reverse mutant by removing the site for glycosylation in antigenic site B [Δ167 (−1)] which has been implicated as a potential site of recognition by the lung collectin SP-D (19). Our data demonstrate that there is generally an attenuation of disease severity in naïve mice as the level of glycosylation increases. However, in our model the Δ167 (−1) virus did not appear to be any more virulent than wild type. We found a breakpoint between 8 and 9 sites of glycosylation (3 to 4 additional sites on the globular head) for virulence and neutralization, suggesting that after this point the effects mediated by SP-D are maximized. From these experiments we cannot distinguish whether this breakpoint derives generally from reaching a plateau in carbohydrate content or is specific to the particular sites we engineered into the virus. Data from SP-D-deficient animals support our proposed mechanism since mice infected with viruses of higher potential glycosylation were not attenuated in these animals as they were in fully competent hosts. In fact, our data suggest that clearance via other mechanisms, such as SP-A, may be more important for the less glycosylated viruses, perhaps because the lack of carbohydrates on the surface improves access of SP-A to its site of binding. The differences between the breakpoint for virulence (8 sites) and inhibition of hemagglutination (9 sites) may be due to the contribution of SP-A. Further study using recombinant SP-A and animals deficient in SP-A or both SP-A and SP-D is warranted to dissect the relative contribution of each.

These findings have important implications for our understanding of influenza biology and host interaction. Pandemic strains from this century have contained few sites for glycosylation on the globular head where the carbohydrates attached there might be accessible to collectins. The HA of the H1N1 strain of 1918 has been shown to play a major role in the virulence of that virus (42) and had glycosylation sites only in the stalk. The H2N2 pandemic strain of 1957 contained only 1 site on the globular head, and the H3N2 strain of 1968 had just 2 sites. Highly pathogenic avian influenza viruses of the H5N1 subtype which have recently crossed over into humans have a total of only six potential sites for glycosylation, excluding 1 site in the cytoplasmic tail which is unlikely to be glycosylated (39). During human infections with H5N1 strains, the lack of neutralization by collectins could potentially contribute to the high virulence.

The presence of glycosylation may have important implications for vaccine design as well. A strong neutralizing antibody response may be dependent on access to the surface of the HA protein, which may be blocked by carbohydrates. Thus, standard vaccines made from recently circulating, highly glycosylated viruses may elicit poor responses; using genetic engineering to remove potential glycosylation sites may alleviate this problem and improve the vaccines (4). However, our data suggest that this approach may affect virulence if applied to live attenuated influenza vaccines. The balance between attenuation and immunogenicity would have to be carefully considered.

In summary, our study demonstrates that the level of potential glycosylation impacts the disease severity and outcome of infection in naïve animals. The likely mechanism explaining this observation is neutralization and clearance of the virus, mediated by the collectin SP-D. This may provide a balance for the benefits, such as evasion of the immune response, garnered as the virus accrues more surface carbohydrates. Further analysis of the impact of both SP-D and SP-A, particularly in more complex systems where preexisting immunity is present, would be of interest. An exploration of the role of collectins in a model such as ferret that better approximates the disease in humans would also be important to better understand the biology of influenza in the lung.

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