Specific Sites of N-Linked Glycosylation on the Hemagglutinin of H1N1 Subtype Influenza A Virus Determine Sensitivity to Inhibitors of the Innate Immune System and Virulence in Mice

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Specific Sites of N-Linked Glycosylation on the Hemagglutinin of H1N1 Subtype Influenza A Virus Determine Sensitivity to Inhibitors of the Innate Immune System and Virulence in Mice

Michelle D. Tate,* Andrew G. Brooks,* and Patrick C. Reading*,†

Oligosaccharides on the hemagglutinin (HA) of influenza A virus (IAV) attach through N-glycosidic linkages to asparagine (Asn) residues of the conserved glycosylation site motif Asn-X-Ser/Thr, in which X may represent any amino acid except proline (1). Oligosaccharides attached to the stalk region are well conserved between different IAV strains and are important in determining appropriate folding and conformation of HA (2–5). In contrast, the location and number of glycans on the globular head of HA differ markedly between IAV strains (6, 7).

HA serves as the major target for neutralizing Abs, and glycans expressed on the head of HA are likely to shield or modify antigenic sites (8), thereby preventing recognition by Abs elicited to previously circulating strains. Since its re-emergence in 1977, glycosylation sites have been added and lost from the HA of seasonal H1N1 IAV, although the majority of strains have maintained three to five sites (9, 10). Loss or gain of glycans can also affect biological properties of IAV by altering the affinity of HA for host cell receptors (11, 12), modulating proteolytic cleavage of the HA0 precursor (13) or disrupting the balance between receptor binding activity and efficient particle release (14). In addition, glycosylation on HA is important in determining sensitivity of IAV to recognition by lectins of the innate immune system.

Surfactant protein (SP)-D, a calcium-dependent (C-type) lectin of the collectin family, binds to mannosic-rich glycans on influenza virus HA/neuraminidase (NA) glycoproteins to mediate a range of antiviral activities in vitro (15–17). In addition, the macrophage mannose receptor (MMR), a membrane-associated C-type lectin, also binds oligosaccharides on HA/NA to facilitate virus uptake and destruction by macrophages (Mφ) (18, 19). Previous studies have reported a correlation between the degree of glycosylation on the head of HA and: 1) susceptibility to neutralization by surfactant protein D (SP-D) (17, 20, 21); 2) ability to bind MMR and infect murine Mφ (18, 19); and 3) virulence of different IAV in mice (17, 20, 22). The mouse-adapted A/PR/8/34 strain (PR8, H1N1) lacks glycosylation on the head of HA (23), is resistant to SP-D (17, 24), poor in its ability to infect Mφ (18, 19, 25), and highly virulent in mice (22, 26). A mutant of A/Brazil/11/78 (Brazil, H1N1), resistant to the bovine collectin conglutinin, was found to lack N-glycosylation site Asn104 (H1 numbering) from the head of HA (27) and showed a modest reduction in sensitivity to rat SP-D (28). Detailed analysis of the role of that particular glycosylation site in determining sensitivity to innate immune defenses and virulence has not been reported.

Abbreviations used in this article: Asn, asparagine; BAL, bronchoalveolar lavage; Brazil, A/Brazil/11/78; HA, hemagglutinin; IAV, influenza A virus; Mφ, macrophages; MDCK, Madin–Darby canine kidney; MMR, macrophage mannose receptor; NA, neuraminidase; Neu5Ac, N-acetylneuraminic acid; NP, nucleoprotein; PI, propidium iodide; PR8, A/PR/8/34; RG, reverse genetics; SA, sialic acid; SP, single-positive; SP-D, surfactant protein-D; TCID50, 50% tissue culture-infective dose; WT, wild-type.

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We have investigated the role of glycosylation on the head of H1 subtype IAV in modulating sensitivity to components of the murine innate immune system (SP-D and Mβ) in vitro and virulence in mice. Site-directed mutagenesis allowed for sequential removal or addition of potential glycosylation sites to the HA, and reverse genetics (RG) was used to engineer 7:1 reassortant viruses expressing either wild-type (WT) or glycosylation mutant HA on a PR8 backbone. We have removed each of the four potential sites from the head of Brazil HA, as well as generating mutants in which multiple sites were deleted. We have also added individual N-glycosylation sites to PR8 HA and generated a mutant expressing two sites on the head of HA. Data presented in this study demonstrate that HA glycosylation was a critical factor determining sensitivity to SP-D but was not a major factor influencing susceptibility of murine Mβ to infection. RG viruses expressing reduced glycosylation were resistant to neutralization by mouse lung fluids and caused severe disease in mice. Using the HA of PR8 or Brazil, we demonstrate that Asn₁₄₄ was particularly important in determining sensitivity to murine mannose-specific lectins in vitro and disease in mice.

Materials and Methods

Mice and viruses

C57BL/6 (B6) mice were bred and housed in specific pathogen-free conditions at the Department of Microbiology and Immunology, University of Melbourne (Melbourne, VIC, Australia). Male mice 6–8 wk of age were used in all experiments. The influenza virus strains A/PR/8/34 Mount Sinai (PR8, H1N1) and B/1×109 (H3N2) were obtained from the World Health Organization Collaborating Centre for Reference and Research on Influenza, North Melbourne, VIC, Australia. Viruses were grown in 10-d embryonated eggs by standard procedures and titrated by plaque assay under Madin–Darby canine kidney (MDCK) cells as described (29).

Construction of glycosylation mutant viruses using RG

Reassortant IAV used in this study were generated by eight-plasmid RG as previously described (30). Site-directed mutagenesis was performed to sequentially remove N-linked glycosylation sites from the Brazil HA (H1) or to add sites to the globular head of PR8 HA (H1). Removal of N-glycosylation sites was achieved by substituting Asn with Ala to abrogate glycosylation sequence motif Asn-X-Ser/Thr (i.e., Asn₁₀₄ [NGT → AGT], Asn₁₁₄ [NIT → AIT], Asn₁₇₂ [NGS → AGS], and Asn₁₇₇ [NLS → ALS]). To add N-glycosylation sites to PR8 HA, nucleic acid mutations were performed to facilitate amino acid substitutions that created glycosylation motifs (i.e., Asn-X-Ser/Thr) at sites identical to those in the sequence of Brazilian HA (i.e., Asn₁₀₄ [NGT → NGT], Asn₁₁₄ [NTN → NTNTRG], Asn₁₁₇ [NGS → AGS], and Asn₁₁₇ [NLS → ALS]). To add N-glycosylation sites to PR8 HA, nucleic acid mutations were performed to facilitate amino acid substitutions that created glycosylation motifs (i.e., Asn-X-Ser/Thr) at sites identical to those in the sequence of Brazilian HA (i.e., Asn₁₀₄ [NGT → NGT], Asn₁₁₄ [NTN → NTNTRG], Asn₁₁₇ [NGS → AGS], and Asn₁₁₇ [NLS → ALS]). Reassortant IA V used in this study were generated by eight-plasmid RG as previously described (30). Site-directed mutagenesis was performed to sequentially remove N-linked glycosylation sites from the Brazil HA (H1) or to add sites to the globular head of PR8 HA (H1). Removal of N-glycosylation sites was achieved by substituting Asn with Ala to abrogate glycosylation sequence motif Asn-X-Ser/Thr (i.e., Asn₁₀₄ [NGT → AGT], Asn₁₁₄ [NIT → AIT], Asn₁₇₂ [NGS → AGS], and Asn₁₇₇ [NLS → ALS])

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Desialylation and resialylation of erythrocytes

Methods for the enzymatic modification of erythrocyte oligosaccharides have been described previously (31). In brief, suspensions (10%) of human erythrocytes were desialylated with 600 μU/ml sialidase from Clostridium perfringens (Sigma-Aldrich) for 1 h at 37°C, washed twice, and resialylated using 1.5 mM CMP-N-acetylneuraminic acid (Neu5Ac; Sigma-Aldrich) and 4 μl either β-galactosidase-α,2,3-sialyltransferase (Japan Tobacco, Shizuka, Japan) or β-N-galactosyl-β-D-glucosamine-α,2,6-sialyltransferase (Merck) or with buffer alone ( sham-treated) at 37°C for 4 h. Erythrocytes were washed in TBS and used in standard HA titrations.

Virus neutralization assay

The ability of mouse bronchoalveolar lavage (BAL) fluids and recombinant rat SP-D to neutralize virus infectivity was measured by fluorescent-focus reduction in monolayers of MDCK cells cultured in 96-well plates as described (17). Naïve mice were euthanized, and BAL fluids were obtained by flushing the lungs three times with 1 ml TBS through a blunt 23-gauge needle inserted into the trachea. BAL fluids were clarified by centrifugation and supernatants frozen at −20°C. Recombinant rat SP-D was a generous gift from Prof. Erika Crouch (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO). BAL fluids or SP-D diluted in TBS supplemented with 10 mM CaCl₂, incubated with virus for 30 min, and added to MDCK cell monolayers. The number of IAV-infected cells was determined at 7 to 8 h postinfection using immunofluorescence as described above. The total number of fluorescent foci in four representative fields were counted and expressed as a percentage of the number of foci in the corresponding area of duplicate control wells infected with virus alone (i.e., percent of virus control).

Infection of mouse Mβ and epithelial cells

Mouse peritoneal exudate cell Mβ or the murine LA-4 lung epithelial cell line were prepared and infected with virus in eight-well chamber slides as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as described (17). Slides were stained for expression of IA V NP as described above. Titers of infectious virus were determined using standard plaque assay or immunofluorescence as des...
with 10% FCS. Cell numbers and cell viability were determined via trypan blue exclusion.

For flow cytometric analysis, single-cell suspensions prepared from the blood, BAL, and thymi were incubated on ice for 20 min with supernatants from hybridoma 2.4G2 to block Fc receptors, followed by staining with appropriate combinations of FITC, PE, and allophycocyanin conjugated to Gr-1 (RB6-8C5), CD45.2 (104), CD8a (53-6.7), CD4 (GK1.5), B220 (RA3-6B2), TCRβ (H57-597), and NK1.1 (PK136). Addition of 10 µg/ml PI to each sample was used to identify viable cells. Cells were analyzed using an FACSCalibur flow cytometer (BD Biosciences), and a minimum of 50,000 PI− cells were collected. Airway Mδ were identified following cytospin and differential staining of BAL cells.

Pulmonary histopathology
Lungs were perfused, inflated, and fixed in a solution of 4% formaldehyde as previously described (33) before 4-µm sections were prepared and stained with H&E. Airway inflammation of H&E-stained lung sections was evaluated on a subjective scale of 0–5 (0, no inflammation; 1, very mild; 2, mild; 3, moderate; 4, marked; and 5, severe inflammation) by three independent readers, as described (33). Sections were blinded and randomized, and samples corresponding to the least severe and most severe inflammation were assigned scores of 0 and 5, respectively. All samples were then graded for peribronchiolar inflammation (around three to five small airways per section) and alveolitis in multiple random fields per section by three independent readers as described (34). Lung sections were viewed on a Leica DMI3000 B microscope (Leica Microsystems) and photographed at ×100 original magnification unless otherwise stated using a Leica DFC 490 camera running from the Leica Application software (Leica Microsystems).

Cytokine bead array for the detection of inflammatory mediators
The levels of IFN-γ, TNF-α, IL-6, IL-10, IL-12p70, and MCP-1 in BAL supernatants and serum were determined with the use of a BD Cytometric Bead Array kit (BD Biocytex) according to manufacturer’s instructions. Cytokine levels were determined by BD Cytometric Bead Array mouse inflammation kit (BD Biosciences) according to manufacturer’s instructions. The detection limit was 5 pg/ml for all cytokines tested.

Assessment of lung edema and vascular leak
The lung wet-to-dry weight ratio was used as an index of lung fluid accumulation during influenza virus infection. Lungs were surgically dissected, blotted dry, and weighed immediately (wet weight). Note that lungs used in these studies had not been subject to BAL prior to analysis. The lung tissue was then dried in an oven at 60˚C for 72 h and reweighed (dry weight). BAL from hybridoma 2.4G2 to block Fc receptors, followed by staining with appropriate combinations of FITC, PE, and allophycocyanin conjugated to Gr-1 (RB6-8C5), CD45.2 (104), CD8a (53-6.7), CD4 (GK1.5), B220 (RA3-6B2), TCRβ (H57-597), and NK1.1 (PK136). Addition of 10 µg/ml PI to each sample was used to identify viable cells. Cells were analyzed using an FACS Calibur flow cytometer (BD Biosciences), and a minimum of 50,000 PI− cells were collected. Airway Mδ were identified following cytospin and differential staining of BAL cells.

We first compared sensitivity of Brazil and PR8, as well as RG-Brazil-HA, to neutralization by mouse BAL fluid (Fig. 1B). Viruses were incubated with dilutions of mouse BAL (in the presence or absence of 10 mg/ml mannan) and the amount of infectious virus remaining determined by immunofluorescence (18, 22). The potential neutralizing activity of mouse BAL against Brazil was reversed in the presence of mannan (Fig. 1B), indicating that SP-D (or a related mannos-specific lectin) was the major neutralizing activity in lung fluids (22). PR8 was largely resistant to mouse BAL, whereas RG-Brazil-HA was neutralized to equivalent levels to Brazil. Therefore, expression of Brazil HA was associated with enhanced sensitivity to neutralization by mouse BAL.

We next examined the role of HA in determining the ability of IAV to infect murine Mδ (Fig. 1Ci). Consistent with previous reports (18, 22, 25, 26), PR8 was poor in its ability to infect Mδ. In contrast, Brazil and RG-Brazil HA infected ~60% of Mδ and infection was inhibited by mannan, a ligand of MMR. PR8, Brazil, or RG-Brazil HA did not differ in their ability to infect the murine lung epithelial (LA-4) cell line and infection of LA-4 cells was unaffected by mannan (Fig. 1Ci).

Next, mice were infected with 105 PFU of either Brazil, PR8, or RG-Brazil-HA and monitored daily for weight loss and disease. Mice infected with Brazil or RG-Brazil-HA did not lose weight (Fig. 1D) or show visible signs of disease (data not shown) over the 10-d monitoring period, whereas PR8-infected mice rapidly lost weight, and all animals succumbed to disease. Thus, expression of Brazil HA led to increased sensitivity to neutralization by mouse BAL and enhanced susceptibility of Mδ to infection. In mice, expression of Brazil HA was associated with a marked attenuation in virulence.

Generation of RG influenza viruses with differing numbers of N-glycosylation sites on HA
Expression of different H1 HA was critical in determining sensitivity to lectin-mediated innate defenses and virulence in mice (Fig. 1). Next, a strategy was devised to sequentially remove (Brazil) or add (PR8) N-glycosylation sites to the globular head of HA, and RG IAV were generated on a PR8 backbone. RG viruses expressing WT Brazil or PR8 HA were generated as controls. For Brazil HA, we generated single-step mutants lacking Asn177 (−104, −144, −172, and −177), double-step mutants (−104/144 and −172/177), and a mutant lacking all four glycosylation sites (−104/144/172/177). For PR8 HA, we generated single-step (+104, +144, +172 and +177) mutants and a double-step mutant (+104/144). Despite multiple attempts, infectious virus could not be rescued for the +104/144/172/177 mutant, suggesting that addition of glycosylation interferes with function and/or stability of PR8 HA. In addition, particular combinations of glycosylation sites could not be added to PR8 HA, and rescued virus reverted to HA consensus (e.g., double-step +172/177).

Glycosylation on the head of HA modulates virulence of IAV in mice
To examine the effect of the removal (Brazil) or addition (PR8) of N-glycosylation sites to the head of the HA, mice were infected with 105 PFU (Fig. 2A) or 104 PFU (Fig. 2B) RG viruses expressing WT HA or expressing a mutant HA with altered glycosylation. Infection of mice with 105 PFU RG-Brazil-HA (WT) was not associated with weight loss (Fig. 2Ai), and similar results were obtained using single-step glycosylation mutants lacking Asn104 (−104) or Asn172 (−172). Glycosylation mutants lacking Asn144 alone (−144) or in conjunction with Asn104 (−104/144) rapidly lost weight and were euthanized 4 d postinfection. Single-step mutants lacking Asn177 (−177) induced mild, but significant,
Data are representative of two independent experiments. Data are representative of two independent experiments. Inhibition of neutralization was examined by addition of mannan (mn; final concentration of 10 mg/ml) 30 min prior to virus addition (gray bars). Data represent the mean percent infection ($\pm$ 1 SD) from three independent experiments. D, Groups of five mice were infected with 10$^7$ PFU Brazil, PR8, or RG-Brazil-HA. Mice were monitored daily for weight loss, and all mice recovered from infection, whereas mutants lacking both Asn$_{172}$ and Asn$_{177}$ (+172/177) were virulent, and all animals were euthanized. Mice infected with the glycosylation mutant lacking all four sites from the head of HA (-104/144/172/177) succumbed to disease 4 d postinfection.

Mice infected with 10$^3$ PFU RG viruses expressing RG-PR8-HA (WT) rapidly lost weight and succumbed to disease (Fig. 2Ai). Addition of single sites of N-glycosylation at Asn$_{104}$, Asn$_{144}$, Asn$_{172}$, or Asn$_{177}$ (+104, +144, +172, or +177 mutants, respectively) or addition of Asn$_{104}$ and Asn$_{144}$ (+104/144) did not reduce virulence, and all mice were euthanized 4 to 5 d postinfection.

We next inoculated mice with a 100-fold lower dose (i.e., 10$^3$ PFU) of RG viruses to highlight more subtle differences between viruses in ability to induce disease. The -144 Brazil HA mutant induced disease and death in mice following inoculation with 10$^3$ PFU (Fig. 2Ai) but did not induce disease at the lower dose (Fig. 2Bi), whereas -104/144 and -104/144/172/177 retained virulence. Addition of single sites to PR8 HA (i.e., +104, +144, +172 or +177 mutants) did not alter weight loss or mortality, whereas the +104/144 virus was attenuated, and all animals survived infection (Fig. 2Bi). Thus, deletion of glycosylation from Brazil HA was associated with enhanced virulence, whereas addition of glycosylation to PR8 HA attenuated disease.

Receptor specificity of HA glycosylation mutant viruses

We compared RG viruses in a range of assays to investigate factors that influence virulence in mice. Addition or loss of glycosylation sites can alter binding of HA to α(2,3)-Gal– or α(2,6)-Gal–linked sialic acid (SA) (12, 37, 38). We first compared RG viruses for ability to agglutinate erythrocytes from different species, and RG viruses bearing Brazil HA did not differ in ability to agglutinate human, chicken, turkey, guinea pig, or horse erythrocytes (data not shown). Furthermore, when human erythrocytes were desialylated and enzymatically resialylated, RG viruses expressing Brazil HA did not differ in ability to agglutinate erythrocytes bearing α(2,3)-Gal– or α(2,6)-Gal–linked Neu5Ac (Table I). Addition of glycosylation to PR8 HA did not alter binding to human, chicken, turkey, or guinea pig erythrocytes (data not shown). However, compared with WT, the +144 and +104/144 mutants were less efficient at agglutinating erythrocytes bearing α(2,3)-Gal–linked Neu5Ac (Table I), indicating differences in the receptor-binding properties of PR8 HA.

Ability of viruses differing in HA glycosylation to infect murine MΦ and epithelial cells

IAV infection of airway epithelial cells results in productive virus replication (26, 39, 40), whereas influenza virus infection of MΦ is abortive, and infectious virions are not released (18, 26, 41). Therefore, the ability of glycosylation mutants to infect different cell types might be an important factor influencing virulence. Monolayers of murine MΦ or LA-4 epithelial cells were infected with increasing doses of RG viruses, and immunofluorescence was used to determine the percentage of infected cells. Compared to virus bearing appropriate WT HA, glycosylation mutants bearing the HA of Brazil (Fig. 3A, upper panel) or PR8 (Fig. 3A, lower panel) did not differ in ability to infect epithelial cells. In the mouse model, MΦ play a critical role in limiting disease severity (26, 42), and the MMR has been implicated as a receptor for IAV

FIGURE 1. The HA of Brazil modulates sensitivity to innate immune defenses and virulence in mice. A, Schematic of HA monomers of PR8 and Brazil, which carry zero and four potential sites of N-linked glycosylation, respectively, on the head of HA. Red and blue indicate the HA1 and HA2 domains, respectively, and green circles indicate the location of glycosylation motif Asn-X-Ser/Thr. Images are derived from the crystal structure of the HA of A/Aichi/2/68. The amino acid numbering of specific N-glycosylation sites on the head of HA is indicated (H1 numbering). Images were generated using the MacPyMol software and the solved HA structure (ID 3EYM; National Center for Biotechnology Information database). B, Dilutions of mouse BAL in TBS containing 10 mM CaCl$_2$ were incubated with Brazil, PR8, or RG virus bearing the HA of Brazil (RG-Brazil-HA) for 30 min at 37°C and the amount of infectious virus remaining determined by fluorescent focus assay and are expressed as a percentage of a virus only control. Inhibition of neutralization was examined by addition of mannan (mm; final concentration of 10 mg/ml) 30 min prior to virus addition. Data are representative of two independent experiments. C, Monolayers of murine MΦ (i) or LA-4 cells (ii) were incubated with 10$^6$ PFU Brazil, PR8, or RG-Brazil-HA (black bars). After 1 h, monolayers were washed, incubated 7 h, fixed, and stained for expression of viral NP. Inhibition of infection was examined by addition of mannan (mm; final concentration of 10 mg/ml) to cell monolayers 30 min prior to virus addition (gray bars). Data represent the mean percent infection ($\pm$ 1 SD) from three independent experiments. D, Groups of five mice were infected with 10$^7$ PFU Brazil, PR8, or RG-Brazil-HA. Mice were monitored daily for weight loss, and animals that lost $\geq$25% of their original body weight were euthanized. Data represent the mean percent weight change ± 1 SD. Data are representative of two independent experiments.
infection of murine MΦ (18, 19). Despite this, we did not observe major differences between viruses bearing WT or glycosylation mutant HA in their ability to infect MΦ (Fig. 3B). It should be noted that the +104/144 PR8 HA mutant was, however, associated with a modest increase in ability to infect MΦ compared with its corresponding WT virus. RG virus bearing the WT Brazil HA and the Brazil HA −104/144/172/177 mutant were equally efficient in their ability to infect murine MΦ, and infection of MΦ by either virus was reduced to <15% in the presence of 10 mg/ml mannan (data not shown).

Sensitivity of glycosylation mutants to neutralization by mouse BAL or rat SP-D

Pulmonary surfactant contains proteins of the innate immune system and acts as a barrier to influenza virus infection of the airways. SP-D, a mannos-specific lectin of the collectin family, represents the major neutralizing activity in mouse BAL against glycosylated influenza viruses (Fig. 1) (22). Therefore, we determined if addition or removal of glycosylation sites on HA impacted on sensitivity to mouse BAL or to recombinant rat SP-D. RG virus bearing WT Brazil HA was sensitive to neutralization by mouse BAL (Fig. 4A, left panel) or rat SP-D (Fig. 4B, left panel). Removal of Asn104 or Asn177 did not alter sensitivity to neutralization by BAL or SP-D and viruses did not induce disease (Fig. 2A). Glycosylation at Asn144 and Asn177 was, however, important in determining sensitivity to both mouse BAL and SP-D, and single-step mutants lacking each of these sites showed enhanced virulence in mice (Fig. 2A, left panel). RG −104/144 and −104/144/172/177 Brazil HA mutants were largely resistant to neutralization by either BAL or SP-D. Note that the neutralizing activity of mouse BAL (Fig. 4A, right panel) and rat SP-D (Fig. 4B, right panel) for RG virus bearing WT Brazil HA was reversed in the presence of mannan, whereas an equivalent amount of BAL/SP-D did not neutralize −104/144/172/172 Brazil HA. The neutralizing activity of BAL/SP-D for −104, −144, −172, or −177 Brazil HA mutants was also reversed in the presence of mannan (data not shown).

Addition of a single glycosylation site at Asn144 of PR8 HA (+144) increased sensitivity to neutralization by mouse BAL (Fig. 4C, left panel) and SP-D (Fig. 4D, right panel), but this virus did not differ from WT in virulence (Fig. 2). Sequential addition of glycosylation sites (+104/144) led to a further increase in sensitivity to both mouse BAL and SP-D, as well as attenuation of virulence. Neutralization of the +104/144 mutant by mouse BAL (Fig. 4C, right panel) and SP-D (Fig. 4D, right panel) was reversed in the presence of mannan. Overall, these results demonstrate an inverse correlation between sensitivity to mouse BAL and disease severity in mice. For Brazil and PR8, glycosylation at Asn144 and, to a lesser extent Asn177, are important determinants of sensitivity to mouse BAL and disease severity.

Table I. Receptor specificity of RG viruses

<table>
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<th>HA Expressed by RG Virus</th>
<th>HA Glycosylation Mutant</th>
<th>Native</th>
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<th>α(2,6)-SA</th>
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<td></td>
<td>+104/44</td>
<td>64</td>
<td>&lt;1</td>
<td>4</td>
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HA assays were performed using standard procedures, and data are expressed as HA units.

*Viruses bearing WT or glycosylation mutant HA were compared for their ability to agglutinate desialylated (Asialo) and enzymatically resialylated erythrocytes. Human erythrocytes were desialylated with bacterial sialidase from *C. perfringens* for 1 h at 37˚C and enzymatically resialylated with Neu5Ac in the presence of either α(2,3)-ST or α(2,6)-ST. Native human erythrocytes were included for comparison.
Repliection of RG glycosylation mutant viruses in vitro and in vivo

Next, we investigated parameters associated with disease severity following infection of mice with RG virus expressing: 1) WT Brazil HA (“RG-Brazil WT”) or RG-Brazil HA -104/144/172/177 (“RG-Brazil -4”); or 2) WT PR8 HA (“RG-PR8 WT”) or RG-PR8 HA +104/144 (“RG-PR8 +2”). Analyses were performed at day 6 postinfection, the time at which mice infected with RG-Brazil -4 and RG-PR8 WT viruses succumbed to disease.

First, mice were infected with 10⁵ PFU each virus, and titers of infectious virus in the upper (nasal tissues) and lower (lung) respiratory tract were determined (Fig. 5A). Infectious virus was not detected in lungs (Fig. 5Ai) and was present at low titers in nasal tissues (Fig. 5Aii) of mice inoculated with RG-Brazil WT, whereas high titers were detected in the airways of RG-Brazil -4-infected mice (∼2600-fold and ∼20-fold higher titers in lungs and nasal tissues, respectively). Viral titers in lungs and nasal tissues of mice infected with RG-PR8 +2 were lower than from mice infected with RG-PR8 WT (∼30-fold decrease in both lungs and nasal tissues).

Given the marked differences in replication of RG viruses in vitro, we compared their ability to replicate in mouse LA-4 cells to determine if there were intrinsic differences between viruses in their ability to replicate in airway epithelial cells. In these experiments, LA-4 cells were infected with 10⁶ PFU RG-Brazil WT, RG-Brazil -4, RG-PR8 WT, or RG-PR8 +2 viruses, and supernatants removed at 2 and 24 h postinfection were assayed for infectious virus by TCID₅₀. Titers of virus were similar at 2 and 4 h for RG-Brazil WT versus RG-Brazil -4 and for RG-PR8 WT versus RG-PR8 +2 (data not shown). These data indicate that differences in the ability of virus to replicate in the airways of mice are likely due to differences in susceptibility to host defenses rather than defects in their ability to infect and replicate in target cells.

Pulmonary histopathology following infection of mice with RG glycosylation mutants

We examined lung sections from mice infected with 10⁵ PFU RG-Brazil WT, RG-Brazil -4, RG-PR8 WT, or RG-PR8 +2 viruses at day 6 postinfection. Lung sections from naive animals were included for comparison. Lung sections were blinded and scored for peribronchial inflammation and alveolitis by three independent readers (Fig. 5B). Both peribronchial inflammation and alveolitis were more severe in the lungs of mice infected with RG-Brazil -4 compared with animals infected with RG-Brazil WT, and lung scores from RG-Brazil -4-infected mice were equivalent to those from mice infected with RG-PR8 WT (Fig. 5C). Pulmonary pathology was not significantly different between RG-PR8 WT and RG-PR8 +2 in two independent experiments.

Cellularity and production of inflammatory mediators in the airways of mice infected with RG-Brazil -4 and RG-PR8 +2

The total number of cells in BAL was determined 6 d postinfection with 10⁵ PFU RG-Brazil WT, RG-Brazil -4, RG-PR8 WT, or RG-PR8 +2 viruses. Low cell numbers were recovered from the airways of mice infected with RG-Brazil WT (Fig. 6A). Infection of mice with an equivalent dose of RG-Brazil -4 led to recruitment of large numbers of leukocytes, and cell numbers were ∼3.5-fold higher than from mice infected with RG-Brazil WT. Infection with RG-PR8 WT or with RG-Brazil -4 resulted in similar BAL cell numbers. BAL cell numbers from mice infected with RG-PR8 +2 were reduced ∼2-fold compared with mice infected with RG-PR8 WT.

To examine the cellular infiltrate in detail, BAL cells were analyzed by flow cytometry at day 6 postinfection and numbers of neutrophils, NK cells, T cells, CD8⁺ T cells, and B cells determined (Fig. 6B). Numbers of airway Mφ were determined by cytoospin and differential staining of BAL cells. Neutrophils were the predominant cell type following infection with RG-Brazil -4, RG-PR8 WT, or RG-PR8 +2, whereas few neutrophils were recovered from mice infected with RG-Brazil WT. Infection with RG-PR8 WT or with RG-Brazil -4 resulted in similar BAL cell numbers. BAL cell numbers from mice infected with RG-PR8 +2 were reduced ∼2-fold compared with mice infected with RG-PR8 WT.

Compared to mice infected with RG-Brazil WT, pulmonary inflammation in RG-Brazil -4-infected animals was associated with enhanced BAL IL-6 (Fig. 6C; p < 0.05, one-way ANOVA), but not MCP-1, TNF-α, or IFN-γ. Levels of IFN-γ showed a tendency to be enhanced from RG-Brazil -4-infected mice; however, this was not significant two independent experiments. Infection of mice with RG-PR8 WT resulted in BAL levels of TNF-α, IFN-γ, MCP-1, and IL-6 that were significantly higher than those from mice infected with RG-PR8 +2 (p < 0.05, one-way ANOVA).

Vascular leak, pulmonary edema, and systemic responses following infection of mice with RG viruses

Severe influenza infections are associated with vascular leak and pulmonary edema (26, 33) as well as systemic manifestations,
including leukopenia and thymic atrophy (43–46). Compared to mice infected with RG-Brazil WT, high levels of total BAL protein were recovered from RG-Brazil 2-infected mice (Fig. 7A) and enhanced wet/dry lung ratios observed (Fig. 7B). RG-PR8 +2-infected mice showed reduced vascular leak and lung edema compared with mice infected with RG-PR8 WT. We also recorded reductions in total blood leukocyte numbers from mice infected with RG-Brazil 2 or RG-PR8 WT compared with naive animals (Fig. 7C) and this was associated with reduced B cells, CD8+ T cells, and CD4+ T cells. Infection with RG-PR8 +2 led to increased neutrophil and B cell numbers, whereas CD8+ T cells and CD4+ T cells were unaffected. No differences were noted in cell numbers between mice infected with RG-Brazil WT and naive animals. Finally, the cellularity of thymi from mice infected with RG-Brazil 2 or RG-PR8 WT viruses were reduced compared with mice infected with RG-Brazil WT and RG-PR8 +2 viruses, respectively (data not shown). In each case, flow cytometric analysis indicated a specific reduction in double-positive thymocytes and more single-positive (SP) CD4+CD8+ thymocytes (Fig. 7D), whereas numbers of CD4+CD8+ SP and CD4+CD8+ double-negative thymocytes did not differ.

Discussion

Analysis of H1 sequences (1918–2010) indicate that glycosylation on the receptor binding domain of HA is absent from 1918 and 2009 pandemic virus strains but is present on nearly all seasonal IAV (47), consistent with a role for glycosylation in mediating evasion of Ab-mediated neutralization in the human population. Accumulation of glycans in the vicinity of antigenic sites on HA may provide an evolutionary advantage in an immune population by shielding antigenic sites (7, 48, 49); however, these beneficial effects are balanced by the enhanced recognition of glycosylated IAV by collectins of the innate immune system (17, 20, 50). Glycosylated IAV are highly sensitive to neutralization by collectins and show marked attenuation in mice (17, 20, 51). In this study, we have used a mouse model of IAV infection to systematically define the role that particular glycosylation sites on seasonal H1 IAV play in modulating sensitivity to innate immune defenses and virulence.

Hemagglutination inhibition studies using different H1N1 IAV led Hartshorn et al. (50) to propose that Asn144 and Asn172 were important determinants of sensitivity to human SP-D. Using RG viruses, we confirm that Asn144 is an important determinant of sensitivity to mouse BAL, a rich source of SP-D (22), as well as virulence in mice. Deletion of Asn144 from Brazil HA reduced...
sensitivity to mouse BAL and increased virulence in mice. Moreover, simultaneous addition or deletion of Asn104 and Asn144 from PR8 or Brazil HA, respectively, led to marked changes in sensitivity to mouse BAL and virulence when compared with loss of either site alone. These findings suggest that simultaneous binding of SP-D to multiple glycans on IAV HA is likely to increase the overall affinity of binding and therefore antiviral activity. Single-site deletion of Asn177, but not Asn172, reduced sensitivity to mouse BAL and virulence in mice, a finding that may reflect differences in ligand specificity between human and mouse SP-D (52). Of interest, different collectins also display a distinct specificity for oligosaccharides expressed on IAV. A mutant of Brazil (H1N1) selected for resistance to conglutinin, a bovine collectin, lacked Asn104 from the head of HA (27) but remained somewhat sensitive to SP-D (28). In our studies, RG-Brazil −104 was resistant to conglutinin (data not shown); however, its sensitivity to mouse BAL and purified SP-D was similar to that of virus expressing WT HA. Thus, Asn104 represents a critical ligand for conglutinin on H1 IAV, but does not represent the major glycan species recognized by rodent SP-D. A number of studies have reported an association between loss of potential glycosylation sites from HA and adaptation of human IAV to growth in mice. Smee et al. (53) reported elimination of glycosylation sites Asn71 and Asn104 following adaptation of A/New Caledonia/20/99 (H1N1) to growth in mouse lung. Furthermore, sequential passage of A/USSR/90/77 (H1N1) in mouse lung led to loss of Asn104 and Asn144, such that only Asn172 and Asn177 remained on the head of HA, and the resultant virus replicated to high titers in mouse lung (54, 55). However, Shilov et al. (54) reported that loss of Asn144 alone was associated with resistance to inhibitors in mouse serum, and this mutant was as pathogenic for mice as the mutant lacking both Asn104 and Asn144. During adaptation of human IAV to growth in mice, studies have shown that mutations in additional genes, including PB2, PB1-F2, PA, M, and NS1 (56-60), can enhance virus replication and contribute to virulence. Moreover, mutations in HA that affect receptor binding

**FIGURE 5.** Increased glycosylation of H1 correlates with reduced virus growth and inflammation in the respiratory tract of mice. Mice were infected with 10^3 PFU RG viruses expressing WT HA of Brazil (RG-Brazil WT) (i) or Brazil HA lacking glycosylation sites Asn104, Asn144, Asn172, and Asn177 (RG-Brazil −4) (ii) or the WT HA of PR8 (RG-PR8 WT) (iii) or the PR8 HA with glycosylation sites added at Asn104 and Asn144 (RG-PR8 +2) (iv). Mice were killed and analyzed at day 6 postinfection. A, Titers of infectious virus in lung and nasal tissue homogenates. Bars represent the mean viral titer from a group of five mice ± 1 SD. The detection limit of the assay (0.9) is indicated by the dotted line. *Virus titer different to those from mice infected with RG virus bearing corresponding WT HA (p < 0.01, one-way ANOVA). B, Representative images of inflammation in lung sections following H&E staining. Images are shown at original magnification ×100 (left panels) and ×400 (right panels). C, Histopathological scores for lung sections from naive (N) animals and from mice infected with RG viruses. Lung sections were randomized and scored blinded for alveolitis (i) and peribronchiolar inflammation (ii) on a scale of 0-5. Data shown represent scores from individual mice (as indicated by circles) and median values (as indicated by bar) obtained from one out of three readers. Samples were compared for statistical significance using the Kruskal-Wallis test. For each reader, significant differences were observed in immunopathology scores from mice infected with RG-Brazil WT compared with those infected with RG-Brazil −4 mutant (p < 0.01, alveolitis and peribronchiolar inflammation). Data are pooled from two independent experiments for analysis (n = 5 per experiment).
or cleavage have also been implicated in adaptation of human IA V to mice (53, 61, 62). A strength of our RG studies lies in the use of genetically defined viruses to determine whether specific changes in HA impact on virulence, removing complications associated with introduction of mutations in additional genes during adaptation to growth in mice. Using this approach, we have defined the importance of particular glycosylation sites on H1 HA in modulating sensitivity to mouse BAL (as well as purified SP-D) and virulence.

IA V strain PR8 is a mouse-adapted strain that induces an interstitial pneumonia similar to that seen in human cases of viral pneumonia (63, 64). PR8 was adapted to mice by 300 sequential passages in mouse lung (65) and is likely to have acquired mutations associated with increased virus replication in murine tissues and/or evasion of innate host defenses. Using RG and eight plasmids expressing each of the genes of PR8, we demonstrate that addition of two potential glycosylation sites (Asn104/Asn144) to PR8 HA was sufficient to mitigate other mutations introduced into the PR8 genome as a result of adaptation to mouse lung. RG-PR8 +2 was sufficiently attenuated such that it was no longer lethal in mice despite replicating to similar levels as RG-PR8 WT in vitro.

FIGURE 7. Vascular leak, pulmonary edema, lymphopenia, and thymic atrophy are associated with severe disease. Groups of five mice infected with 10^4 PFU RG-Brazil WT, RG-Brazil −4, RG-PR8 WT, or RG-PR8 +2 were killed and analyzed 6 d postinfection. Naive animals (N) were included for comparison. Total protein levels in cell-free BAL (A) and lung wet-to-dry ratios (B) were determined. Bars represent the mean ± 1 SD. C, Cell types in blood were determined by flow cytometry. Numbers of total leukocytes (CD45+), neutrophils (Gr-1^high), NK cells (NK1.1^+CD3e^-), CD820^+ cells, and CD8^+ cells (CD8^+) are shown. Airway macrophages were identified following cytospin and differential staining of BAL cells. Bars represent the mean cell number ± 1 SD. C, Levels of IL-6, MCP-1, IFN-γ, and TNF-α in cell-free BAL were determined by cytokine bead array. Bars represent the mean concentration (pg/ml) and individual mice are shown as circles. Pooled data from two independent experiments are shown and were used for statistical analysis (n = 5 mice per experiment). The detection limit of each mediator (5 pg/ml) is indicated as a dotted line. A–C, *Significantly different compared with samples from mice infected with RG virus bearing the corresponding WT HA (p < 0.05, one-way ANOVA).

LAV strain PR8 is a mouse-adapted strain that induces an interstitial pneumonia similar to that seen in human cases of viral pneumonia (63, 64). PR8 was adapted to mice by >300 sequential passages in mouse lung (65) and is likely to have acquired mutations associated with increased virus replication in murine tissues and/or evasion of innate host defenses. Using RG and eight plasmids expressing each of the genes of PR8, we demonstrate that addition of two potential glycosylation sites (Asn104/Asn144) to PR8 HA was sufficient to mitigate other mutations introduced into the PR8 genome as a result of adaptation to mouse lung. RG-PR8 +2 was sufficiently attenuated such that it was no longer lethal in mice despite replicating to similar levels as RG-PR8 WT in vitro.

RG-Brazil −4 and RG-PR8 WT both induced severe disease and death in mice, although viral titers in the lungs of RG-Brazil −4-infected mice were ~100-fold lower than in mice infected
with RG-PR8 WT. Although resistance to neutralizing proteins in BAL is likely to be a major factor contributing to virulence of H1 IAV, other intrinsic features of HA contribute to enhanced PR8 replication in the airways. Receptor specificity differences between Brazil [specificity for \( \alpha(2,3) \)-Gal and \( \alpha(2,6) \)-Gal-linked SA] and PR8 [specificity for \( \alpha(2,3) \)-Gal-linked SA] are likely to be important, as \( \alpha(2,3) \)-Gal-SA is the predominant linkage expressed in mouse lung (66, 67). Moreover, studies have demonstrated that adaptation of human influenza viruses to growth in mouse lung is associated with a switch in receptor specificity to favor binding to mouse respiratory cells (61, 62).

Results presented in this study implicate potential glycosylation sites at Asn144 and Asn177 on H1 as important determinants in modulating sensitivity to SP-D and virulence in mice. These findings suggest that Asn144 and Asn177 carry glycans readily bound by SP-D (e.g., high mannose-type glycans), whereas other sites on the head of HA (Asn104 and Asn172) carry glycans that are not readily accessible and/or bear terminal glycans bound less efficiently by SP-D (such as complex glycans terminating in galactose). We could not definitively confirm a change in molecular mass of HA for any of the single-step mutants relative to WT HA using Western blot, and both HA (nonreducing) and HA1 (reducing) appeared as diffuse bands, likely due to microheterogeneity of glycans on the HA of egg-grown virus (data not shown).

Although our data suggest that the mutations introduced alter glycosylation, it is possible that changing the underlying sequence of the protein alone (i.e., in the absence of a glycan attachment) might influence sensitivity to mouse BAL and virulence. However, neutralization by mouse BAL and purified rodent SP-D were reversed in the presence of mannan, demonstrating that mannose-specific lectins are the major neutralizing activity in mouse BAL. Biochemical analyses of the HA of A/USSR/90/77, an H1N1 virus with an identical pattern of HA glycosylation to that of Brazil (68), demonstrated a mixture of complex, high-mannose, and hybrid-type glycans (69), although the nature of glycans expressed at each glycosylation site was not determined. Further experiments are required to confirm the presence of glycan as well as the nature of specific oligosaccharides expressed at Asn104, Asn144, Asn172, and Asn177 of the Brazil and PR8 HA.

It is well established that glycosylation of HA can inhibit recognition by Abs (48, 70). In recent studies, addition of Asn142 and Asn177 to pandemic H1N1 HA trimers was associated with reduced sensitivity to neutralizing Abs raised to WT pandemic HA (47), suggesting that glycan shielding may be an important mechanism by which pandemic viruses entering the human population evolve into seasonal influenza virus strains. However, analysis of pandemic H1N1 HA sequences submitted to the National Centre for Biotechnology Information indicate the addition of potential glycosylation sites at Asn179 (e.g., A/Quito/WR1589N/2009 and A/Tallinn/INS3374/2010), accession numbers ADM14775 and ADM31858, respectively) and Asn136 (e.g., A/Netherlands/1493b/2009 and A/Athens/INS257/2009, accession numbers ADJ40554 and ADK33831, respectively) in some H1N1 pandemic strains, and these are distinct to sites recorded on the globular head of seasonal H1N1 IAV. It will be of major interest to define the role of novel glycans added to the pandemic H1N1 HA in regard to masking antigenic epitopes on HA as well as modulating sensitivity to collectins of the innate immune system.

Influenza virus infection of murine Mφ involves binding of HA to cell-surface SA as well as interactions between MMR and glycans expressed on HA/NA glycoproteins (18, 19). We have previously demonstrated that receptor specificity of HA is important in determining ability to infect murine Mφ as these cells express high levels of \( \alpha(2,3) \)-Gal-linked SA (71). Moreover, receptor-specific Mφ expressing \( \alpha(2,3) \)-Gal–linked SA were permissive to MMR-mediated infection by PR8, a virus lacking glycans on the head of HA but with a receptor preference for \( \alpha(2,3) \)-Gal-linked SA. In this study, RG-Brazil WT and RG-Brazil \( \sim 4 \)-infected native Mφ to similar levels (Fig. 3B), and infection was inhibited in the presence of mannan (data not shown). Both viruses show a preference for \( \alpha(2,6) \)-Gal–linked SA (Table I), the predominant SA linkage expressed by Mφ surface. The enhanced virulence of the RG-Brazil \( \sim 4 \) was not associated with reduced ability to infect Mφ, but rather with reduced sensitivity to soluble mannone-specific lectins present in murine lung fluids. Together, our data highlight the importance of specific sites of N-linked glycosylation on the H1 HA in determining sensitivity to SP-D and therefore virulence in mice.

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Disclosures
The authors have no financial conflicts of interest.

References