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Cross-Neutralization of 1918 and 2009 Influenza Viruses: Role of Glycans in Viral Evolution and Vaccine Design

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Abstract

New strains of H1N1 influenza virus have emerged episodically over the last century to cause human pandemics, notably in 1918 and recently in 2009. Pandemic viruses typically evolve into seasonal forms that develop resistance to antibody neutralization, and cross-protection between strains separated by more than 3 years is uncommon. Here, we define the structural basis for cross-neutralization between two temporally distant pandemic influenza viruses—from 1918 and 2009. Vaccination of mice with the 1918 strain protected against subsequent lethal infection by 2009 virus. Both were resistant to antibodies directed against a seasonal influenza, A/New Caledonia/20/1999 (1999 NC), which was insensitive to antisera to the pandemic strains. Pandemic strain–neutralizing antibodies were directed against a subregion of the hemagglutinin (HA) receptor binding domain that is highly conserved between the 1918 and the 2009 viruses. In seasonal strains, this region undergoes amino acid diversification but is shielded from antibody neutralization by two highly conserved glycosylation sites absent in the pandemic strains. Pandemic HA trimers modified by glycosylation at these positions were resistant to neutralizing antibodies to wild-type HA. Yet, antisera generated against the glycosylated HA mutant

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SUPPLEMENTARY MATERIAL

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Fig. S1. Orientations of the RBD-A region relative to the H1N1 HA trimer.
Fig. S2. Analysis of purified wild-type and glycosylation mutant HA proteins by SDS-PAGE and MALDI-MS.
Fig. S3. Neutralization of wild-type and glycosylation mutant pseudotyped lentiviral vectors by mAb C179.
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Table S1. Protective efficacy of 2009 CA HA DNA vaccine against 1918 SC and 2009 CA viruses in mice.
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References

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Accession numbers: The sequences of the HA proteins A/South Carolina/1/1918, A/PR/8/1934, A/New Caledonia/20/1999, and A/California/04/2009 can be found as GenBank AF117241, ABD77675, AY289929, and FJ966082, respectively. The sequences of the NA proteins A/Brevig Mission/1/1918, A/New Caledonia/20/1999, and A/California/04/2009 can be found as GenBank AAF77036, CAD57252, and FJ966084, respectively.

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neutralized it, suggesting that the focus of the immune response can be selectively changed with this modification. Collectively, these findings define critical determinants of H1N1 viral evolution and have implications for vaccine design. Immunization directed to conserved receptor binding domain subregions of pandemic viruses could potentially protect against similar future pandemic viruses, and vaccination with glycosylated 2009 pandemic virus may limit its further spread and transformation into a seasonal influenza.

INTRODUCTION

The pandemic influenza A (H1N1) 2009 has spread widely after its adaptation to humans. Its rapid global dissemination led to its designation as a pandemic strain by the World Health Organization less than 2 months after the virus was first identified (1). The prototypic pandemic H1N1 influenza virus emerged in 1918 and then gave rise to periodic seasonal strains that began to diminish in frequency during the late 1950s (2, 3). A resurgence of H1N1 viruses occurred in 1977, reestablishing the H1N1 seasonal strains that are presently in circulation. In contrast to these human-adapted viruses, the current pandemic influenza A (H1N1) 2009 represents a recent cross-species transmission of a virus that has been previously predominantly confined to swine (4). Here, to better understand how such pandemics evolve, we have examined in mice the structural basis for differences in sensitivity to antibody neutralization among pandemic and seasonal influenza viruses. These findings identify neutralization targets that have increased cross-reactivity among pandemic strains and can inform our understanding of H1N1 virus evolution and vaccine design.

RESULTS

Cross-neutralization and protection between 1918 and 2009 pandemic H1N1 viruses

Mice were immunized with DNA vaccines encoding A/California/04/2009 (2009 CA) or A/South Carolina/1/1918 (1918 SC) as described (5), and the specificity of the resulting immune response was initially assessed with a previously described H1N1-pseudotyped lentiviral reporter assay (6). Antisera from the 1918 SC immune mice unexpectedly neutralized heterologous 2009 CA virus entry with a high titer, almost as high as the homologous strain (Fig. 1A, 1918, left versus middle panel). Antisera from 2009 CA immune mice likewise neutralized both viruses with a high titer, in contrast to nonimmune sera or to antisera to a seasonal influenza virus, A/New Caledonia/20/1999 (1999 NC) (Fig. 1A, 2009 versus control and 1999 NC, left and middle panels). In contrast, antisera to the seasonal 1999 NC virus showed strong neutralization toward homologous virus but failed to neutralize either the 2009 CA or the 1918 SC virus (Fig. 1A, 1999 NC, right versus left and middle panels). These results were unanticipated, given the longer chronologic separation of the 2009 CA outbreak from 1918 than from 1999.

Similar cross-reactivity was observed in the hemagglutination inhibition (HI) assay, in which neutralizing antibodies inhibit virus-induced aggregation of red blood cells (RBCs). Antisera directed to 1918 SC showed the highest HI titer to an identical, matched virus and recognized 2009 CA but not seasonal 1999 NC virus (Fig. 1B, 1918). Similarly, antisera raised to 2009 CA reacted with 2009 CA and, to a lesser extent, 1918 SC but not 1999 NC

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virus (Fig. 1B, 2009), whereas 1999 NC immune sera exhibited HI reactivity only to the homologous virus (Fig. 1B, NC). Competition studies revealed that purified recombinant 1918 SC or 2009 CA trimeric hemagglutinin (HA) blocked neutralization of 1918 SC or 2009 CA (Fig. 1C, left and middle panels). Although these trimeric HA proteins were able to inhibit neutralization by antisera to both pandemic viruses, they failed to inhibit the seasonal 1999 NC virus (Fig. 1C, right panel). In contrast, the 1999 NC trimeric HA inhibited homologous virus but did not block neutralization by either the 1918 SC or the 2009 CA antisera (Fig. 1C). Recent studies have described a highly conserved region in the stem of the viral HA protein, a structure that might potentially be involved in cross-neutralization of diverse viruses (7–9); however, there is >94% identity between the seasonal and pandemic viruses in this region. Because no inhibition was observed with HA from the 1999 NC strain, and this virus is sensitive to neutralization by antibodies directed to this stem structure (7, 10), these data suggested that the conserved stem region of the spike was not likely to be the target of neutralization. Instead, these cross-inhibition studies implicated an alternative domain of the viral spike, within the head region, as the site of neutralization.

To determine whether this in vitro immune cross-reactivity also mediated cross-protection in vivo, we immunized mice with inactivated virus vaccines derived from pandemic [Table 1; A/South Carolina/1/1918 (1918 SC) and A/Mexico/4108/2009 (2009 Mex)] or seasonal influenza viruses [Table 1; A/Panama/2007/1999 (H3N2), A/New Caledonia/20/1999, and A/Brisbane/59/2007] and challenged with a highly lethal mouse-adapted 2009 CA pandemic virus. The prechallenge antibody responses to homologous virus were measured in individual serum samples collected before the lethal virus challenge. Each vaccine elicited HI titers of ≥40 to the homologous virus, with geometric mean titers of 57 to 80 (Table 1). Animals immunized with the 1918 SC or 2009 Mex vaccines were completely protected from lethality and showed a more than five-log reduction in viral titers, in contrast to the results with seasonal influenza vaccines or nonimmune controls (Table 1). Immunization with the 1918 SC pandemic strain vaccine therefore conferred protection against the 2009 CA virus, documenting its ability to cross-protect in vivo. Immunization with the 2009 CA vaccine also protected mice from lethal 1918 SC challenge (table S1). Similar to the inactivated virus vaccine, a DNA vaccine encoding 2009 CA HA also protected mice from 2009 CA or 1918 SC viral challenge (table S1). DNA HA vaccines encode proteins with glycosylations terminated by sialic acid because they are generated in the absence of neuraminidase (NA). This would not affect the result, however, as we have shown previously that sialation of HA does not significantly alter its immunogenicity (11).

Evolution of glycosylation and RBD sequence diversity in human H1N1 HAs

To determine the molecular basis of cross-neutralization further, we examined the amino acid diversity and conservation of glycosylation sites among diverse HAs. The amino acid identity between the 1918 SC and the 2009 CAHAs within the globular head is ~79.8% (amino acids 64–286, 1918 SC numbering). This amount of amino acid divergence was similar to the divergence among seasonal influenza viruses and would be expected to confer resistance to antibody neutralization. The globular head of HA comprises two domains: the RBD, an ~148–amino acid domain that includes the sialic acid–binding site, and the

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vestigial esterase domain, a smaller 75-residue region just below the RBD. The top part of the RBD adjacent to the 2,6-sialic acid recognition sites includes a large subregion (referred to throughout the text as RBD-A; amino acids 131 to 143, 170 to 182, 205 to 215, and 257 to 262, 1918 SC numbering), with >6000 Å^2 of solvent-exposed surface area per trimer (or 2000 Å^2 per monomer) that is 95% conserved between these two pandemic strains (fig. S1). By comparison, an average epitope surface area buried by an antibody is ~800 Å^2 (12). Furthermore, neither pandemic strain is glycosylated on the top of the HA head (Fig. 2, A and B, left panels). In marked contrast, the seasonal strains have two highly conserved glycosylation sites (142 and 177, 1918 SC numbering) on the head of the spike (Fig. 2, A and B, right panels) and only 58 to 67% sequence identity with the pandemic 1918 SC or 2009 CA HA in the RBD-A region. Both of these glycosylations occur within the sialic acid–binding or Sa site (13), an immunodominant antigenic region of H1 HA in the center of the RBD-A region noted above. Modeling of these two head glycosylation sites reveals extensive additional chemical structure conferred by carbohydrate modification, which would be expected to shield the receptor binding domain from antibody neutralization (Fig. 2B, right versus left panel). We analyzed these glycosylation sites on H1 HAs further by examining sequences from the National Center for Biotechnology Information Influenza Virus Resource (14), which revealed the acquisition of two glycosylation sites on the top of the RBD by the early 1940s (Table 2 and Fig. 2C). From 1977 to 2008, the presence of at least one of the two glycosylation sites is observed in 97.8% of seasonal strains, and both glycosylation sites are seen in 87.8%(table S2). The few exceptions that lack these RBD glycosylation sites include the swine-related flu strains detected in 1976 as well as viruses that caused limited outbreaks detected in 1967, 1988, and 1991 (table S2). Before the acquisition of this glycosylation, the RBD-A region experienced rapid genetic drift, reaching sequence identities of only ~66% with the 1918 SC HA by the early 1940s (Table 2 and Fig. 2C). However, after the addition of glycosylations to this region, yearly antigenic drift in this region slowed considerably, presumably because glycosylation shielded this region from the antigenic pressure of antibodies. These findings indicate that glycosylation on the top of the RBD is absent from pandemic viruses but present on nearly all seasonal influenza viruses, and suggest that these glycosylation sites of the RBD play a role in evading the human immune response.

The role of glycosylation sites in evading antibody neutralization

To confirm the role of these glycans in protecting against viral neutralization, we created site-directed mutants that introduced these glycosylation sites into the HA proteins of 1918 SC and 2009 CA influenza viruses. These HAs were coexpressed in 293F cells cotransfected with the relevant NA to generate the physiologic trimer spike, as described (11). Addition of the N-linked glycans to 1918 SC and 2009 CA HA proteins was confirmed by Endo H digestion and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (fig. S2A) and by matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) (fig. S2B). Comparable expression of wild-type and mutant HAs with the two glycosylation sites (2G) was confirmed by flow cytometry and the fact that we saw similar neutralization sensitivity with a stem-directed neutralizing antibody, C179 (fig. S3). Absorption with cells expressing wild-type 1918 SC or 2009 CA HA removed the neutralizing antibody activity against both pandemic viruses (Fig. 3A, 1918 and 2009). In contrast, when the RBD-A glycosylation

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sites were introduced into the HA trimer, or when seasonal 1999 NC was used, they failed to absorb these neutralizing antibodies [Fig. 3A, 1918 (2G), 2009 (2G), and NC], indicating a marked decrease in relative binding affinity for the glycosylated HA. The seasonal 1999 NC HA effectively absorbed homologous neutralizing antibodies (Fig. 3A, right panel).

Both the wild-type and the mutant 1918 SC and 2009 CA HAs were able to mediate viral entry into cells comparably with a pseudotyped lentiviral reporter (Fig. 3, B and C, left panels), showing that glycosylation did not compromise trimer function. The neutralization sensitivity of the glycosylated mutant HAs was assessed by incubation of the mutant reporters with antisera to 1918 SC or 2009 CA. Incubation of the glycosylated mutant reporters with antisera to 1918 SC or 2009 CA greatly increased the concentration of antibody needed to inhibit entry by 50% [that is, a lower IC_{50} (median inhibitory concentration) titer]. Therefore, the 1918 SC (2G) and 2009CA(2G)mutants were markedly resistant to neutralization relative to their wild-type, nonglycosylated counterparts [Fig. 3, B and C, middle and right panels, 1918 versus 1918 (2G) and 2009 versus 2009 (2G); see also fig. S4]. The glycan at amino acid 142 was largely responsible for neutralization resistance [table S3; 1918 SC (1G-142)]. In contrast, 1999 NC with glycosylation at either position 142 or 177 was sensitive to homologous neutralization but resistant to neutralization by 1918 or 2009 antisera [table S3; 1999NC(1G-142) and (1G-177)]. Because the RBD-A region has only 58 to 67% sequence identity between seasonal and pandemic strains, 1918 SC or 2009 CA antisera would likely not neutralize seasonal strains, even without glycosylation. Neither 1918 SC nor 2009 CA antiserum neutralized A/PR/8/1934 (1934 PR8), a strain with one glycosylation site on the head region of HA (144, 1918 SC numbering) (table S3). Deglycosylation of the 1934 PR8 at that position did not confer complete sensitivity to 1918- or 2009-neutralizing antibodies [table S3, 1934 PR8 (N144Q)], confirming a role for amino acid variability in immune evasion.

To explore the efficacy of glycan-modified and wild-type HA as vaccine immunogens, we immunized mice with plasmid DNAs encoding 1918 SC or 1918 SC (2G). Antisera from mice immunized with wild-type 1918 SC neutralized homologous virus but poorly inhibited the 2G derivative. In contrast, 1918 SC (2G) immune sera neutralized both wild-type 1918 SC and mutant 1918 SC (2G) viruses (Fig. 3D). These data demonstrate that immunization with the RBD-A–glycosylated HA confers improved protection against a glycosylated variant that might evolve into a seasonal form.

DISCUSSION

Here, we have evaluated the cross-neutralization of pandemic and seasonal H1N1 influenza viruses. Despite more than 90 years of separation between these viruses that both caused human pandemics, the 1918 SC and 2009 CA viruses raised immune responses in mice that demonstrated cross-neutralization, whereas they were both resistant to antisera directed to a relatively recent seasonal influenza virus of the same subtype. To understand the molecular basis for cross-neutralization, we examined the specificity of antibody recognition by protein competition studies, as well as by site-directed mutagenesis and protein structural modeling. The RBD-A region was defined as the target of neutralization, and we demonstrated that glycosylation sites in this region are important in allowing evasion of

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antibody neutralization in seasonal strains. Specifically, introduction of glycosylation sites into these strains eliminated their ability to bind neutralizing antibodies, suggesting that glycan shielding from antibodies is a mechanism by which seasonal influenza viruses evolve after the emergence of a viral pandemic strain. Similar cross-neutralization of these viruses has been reported recently by others and mapped to a similar region of the RBD (15, 16); however, the role of glycans in modulating immune recognition and its influence on viral evolution were not defined in those studies.

The findings of the present study have implications for the evolution of pandemics and the development of herd immunity that may prevent viral spread. As a pandemic H1N1 strain evolves into a seasonal form in humans, it acquires glycosylation sites on the RBD (Fig. 2C) that can effectively mask antigenic regions from recognition by antibodies. A related process likely occurred with H2N2, H3N2, and H5N1 viruses, where glycosylation of HA has been shown to inhibit recognition by antibodies (17–20) and is associated with their antigenic drift (21). Here, we have defined a role of specific glycans in the evolution of H1N1 strains and described the structural basis for antibody resistance. In addition to the changes in glycosylation, we also find that amino acid changes in the RBD-A region contribute to neutralization resistance and viral evolution in humans, and in some cases, the glycosylation is also required for the generation of functional viral spike. For example, the 1999 NC HA lacking both 142 and 177 glycans did not give rise to a functional spike that mediated entry in the context of a pseudotyped lentiviral vector, suggesting that antigenic drift in the RBD-A region has resulted in an HA that now requires these glycans for entry into cells.

Neutralizing antibodies to the pandemic viruses of 1918 and 2009 are not elicited by either vaccines or infections from seasonal influenza viruses. Therefore, a segment of the population that had not been exposed to either pandemic virus would fail to develop protective herd immunity to the 2009 virus and remain susceptible to infection by this otherwise sensitive virus. The presence of H1N1 strains without glycosylation on the top of the RBD and relatively high sequence conservation with the 1918 SC HA in the RBD-A region in the earlier decades of the 20th century is a likely explanation for the relative degree of protection seen in the elderly against the present 2009 pandemic. This is especially true for those born around 1910, who were presumably exposed to the 1918 virus and have antibodies that neutralize A (H1N1) 2009 (22, 23).

The data reported here define the molecular basis of the recognition of chronologically distant pandemic influenza viruses by antibodies raised to other pandemic viruses and have significant implications for vaccine preparedness, both in preventing future pandemics and for the evolution of the current swine-related pandemic influenza virus. The sensitivity of 1918 SC and 2009 CA to RBD-A neutralization suggests that childhood immunization with such prototypic viruses might protect against future pandemic outbreaks with viruses of this type. Knowledge of the structures and mechanisms of cross-neutralization therefore facilitates rational vaccine design and may help to contain such outbreaks. The current A (H1N1) 2009 pandemic virus will likely develop resistance to the present vaccine through the evolution of escape mutants. The data collected in this study suggest that an effective mode of escape would be conferred by acquisition of glycosylation sites in the RBD-A region. In addition, based on analysis of H1N1 sequence variation in RBD-A and elsewhere

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over time, changes in primary amino acid sequence may further promote immune evasion. Surveillance for their presence will facilitate the identification of next-wave viruses. This information can be used preemptively to develop vaccine strains that could prevent the emergence of RBD-A–glycosylated viruses, thus constraining its evolution into a seasonal influenza and limiting its further spread.

Note added in proof: Recently, four new pandemic isolates of the 2009 H1N1 virus have been added to the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/select.cgi?go=1) that show glycosylation in the RBD-A region of HA defined in this report. These strains include A/Beijing/SE2649/2009, A/Russia/178/2009, A/Russia/180/2009, and A/Salekhard/01/2009, all of which contain amino acid mutations predicted to add a glycosylation site at position 179 (1918 numbering).

MATERIALS AND METHODS

Immunogen and plasmid construction

Plasmids encoding different versions of HA proteins (A/South Carolina/1/1918, GenBank AF117241; A/PR/8/1934, GenBank ABD77675; A/New Caledonia/20/1999, GenBank AY289929; and A/California/04/2009, GenBank FJ966082) and NA proteins (A/Brevig Mission/1/1918, GenBank AAF77036; A/New Caledonia/20/1999, GenBank CAD57252; and A/California/04/2009, GenBank FJ966084) were synthesized using human-preferred codons as described (5) by GeneArt. The glycosylation site mutations were introduced using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies).

Vaccination and infectious challenge studies

Female BALB/c mice (6 to 8 weeks old; Jackson Laboratories) were immunized intramuscularly (i.m.) with 15 μg of plasmid DNA in 100 μl of phosphate-buffered saline (PBS) (pH 7.4) at weeks 0, 3, and 6 unless indicated otherwise. Blood was collected 14 days after each immunization, and serum was isolated. Animal experiments were conducted in full compliance with all relevant federal regulations and National Institutes of Health guidelines.

For inactivated virus vaccines, the virus was concentrated from allantoic fluid and purified on a linear sucrose gradient. Purified whole virus was inactivated by treating purified virus at a concentration of 1 mg/ml of 0.025% formalin at 4°C for >3 days. This treatment resulted in complete loss of infectivity of the virus. Groups of mice were anesthetized with Avertin (Sigma-Aldrich) and injected i.m. with 10 μg of vaccine. Mice received two inoculations at an interval of 3 weeks and were challenged 6 weeks after initial vaccination. Sera collected from mice before virus challenge were treated with receptor-destroying enzyme (Denka Seiken) and tested for reactivity to homologous viruses by the HI assay using 0.5% turkey RBCs, as described (24).

For challenge, anesthetized mice received 50 μl of infectious virus [10^6 plaque-forming units (PFUs)] diluted in PBS and inoculated intranasally. The lethal strain of the 2009 H1N1 challenge virus was generated by eight passages through mouse lungs. Four days later, four mice from each group were killed and lungs were collected and homogenized in 1 ml of cold

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PBS. Solid debris was pelleted by centrifugation, and tissues were titrated for virus infectivity in a standard plaque assay (25, 26). The eight remaining mice in each group were checked daily for disease signs and death for 21 days after challenge.

Protein expression and purification

Plasmids expressing a secreted HA were transfected into the human embryonic kidney cell line 293F with 293fectin (Invitrogen) according to the manufacturer's instructions. 293F cells were cultured in Freestyle 293 Expression Medium (Invitrogen) with or without the presence of swainsonine (10 mg/liter) and kifunensine (2.5 mg/liter), and supernatant was collected 72 to 96 hours after transfection and cleared by centrifugation and filtration. HA proteins were purified as previously described (11). Endo H (New England Biolabs) digestion of HA proteins was performed according to the manufacturer's instructions. MALDI-MS was performed as described (20).

Production of pseudotyped lentiviral vectors

The recombinant lentiviral vectors expressing a luciferase reporter gene were produced as described (6). For the production of H1N1 pseudo-viruses, a human type 2 transmembrane serine protease TMPRSS2 gene was included in transfection for the proteolytic activation of HA (27).

Neutralization and cell absorption assays

HA NA-pseudotyped lentiviral vectors encoding luciferase were first titrated by serial dilution. Similar amounts of virus (p24 ≈ 6.25 ng/ml) were then incubated with indicated amounts of mouse antisera for 20 min at room temperature and added to 293A cells (10,000 cells per well in a 96-well plate) (50 μl/well, in triplicate). For 1918 SC–pseudotyped and 1999 NC–pseudotyped vectors, plates were washed and replaced with fresh media 2 hours later, and luciferase activity was measured after 24 hours. For 2009 CA–pseudotyped vectors, 293A cells were incubated with virus overnight and luciferase activity was measured after 72 hours. Monoclonal antibody (mAb) C179 was used to standardize the input virus used for the neutralization assay (fig. S3B). For cell absorption, mouse antisera diluted in 250 μl of culture medium were incubated for 30 min with 8×10^6 293F cells transfected with wild-type or mutant HA and relevant NA for 30 min. Preabsorbed antisera were then collected and used for the neutralization assay.

HI assay

Seed stocks of the A/New Caledonia/20/1999 and A/California/04/2009 viruses were obtained from the Centers for Disease Control and Prevention. Stock virus was expanded in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 hours and stored at −80°C. Sera were treated with receptor-destroying enzyme by diluting one part of serum with three parts of enzyme and incubated overnight in a 37°C water bath. The enzyme was inactivated by 30-min incubation at 56°C followed by addition of six parts of PBS for a final dilution of 1:10. HI assays were performed in V-bottom 96-well plates using four hemagglutinating units of 2009 CA or 1999 NC virus and 0.5% turkey RBCs. Hemagglutination of chicken RBC by 1918 SC–pseudotyped virus was performed as

previously described (6). Inhibition of 1918 SC HA was performed by adding indicated mouse immune sera to the pseudovirus.

Cell surface staining of HA

293 cells were cotransfected with wild-type or mutant HA with relevant NA using the Profection Mammalian Transfection System (Promega). Twenty-four hours after transfection, cells were removed using PBS containing 2 mM EDTA. Cells were then washed twice with cold PBS and transferred to a 96-well plate (0.5×10^6 cells per well). Cells were incubated with C179 mAb (7) (fig. S3A, blue line, 5 μg/ml) or purified naïve mouse immunoglobulin G (IgG) control (fig. S3A, red line, 5 μg/ml) for 30 min on ice, washed, and incubated with Alexa Fluor 488 goat antibody to mouse IgG (Invitrogen) (1:2000) for 30 min on ice. Cells were washed twice with cold PBS and fixed with 0.5% paraformaldehyde. Samples were analyzed using an LSR II cell analyzer (BD Biosciences) and FlowJo software (Tree Star).

Computational analysis

Exposed surface area was calculated for the 1918 HA structure [Protein Data Bank (PDB) entry 1RUZ] using AREAIMOL in the CCP4 suite (28).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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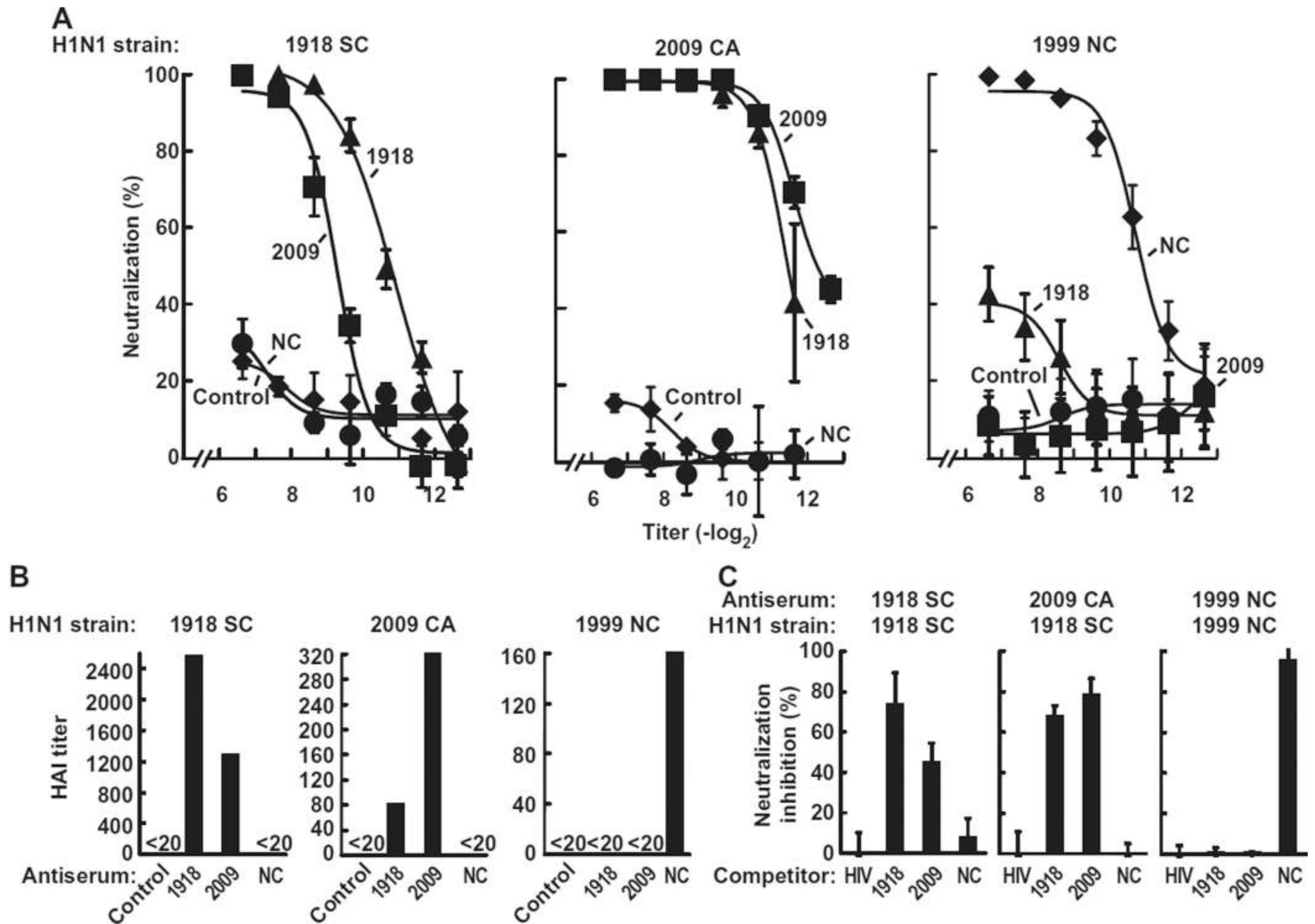

Fig. 1.

Cross-neutralization, HI reactivity, and specificity of antisera to 1918 SC and 2009 CA in contrast to a seasonal strain, 1999 NC. (**A**) Neutralization activity of antisera from mice immunized with the indicated HA plasmid expression vectors or no insert (control) plasmid was measured by luciferase assay with 1918 SC (left panel), 2009 CA (middle panel), or 1999 NC (right panel) HA-pseudotyped lentiviral vectors. (**B**) HI by antisera from mice immunized with control or the indicated HA expression vector was performed with 1918 SC HA-pseudotyped virus and 2009 CA and 1999 NC viruses. (**C**) Antisera from mice immunized with 1918 SC, 2009 CA, or 1999 NC HA plasmid were preabsorbed with HIV (control), 1918 SC, 2009 CA, or 1999 NC HA trimers, and the neutralization activities of the preabsorbed antisera were measured with 1918 SC, 2009 CA, and 1999 NC HA-pseudotyped lentiviral vectors. Percent reduction in neutralization was recorded at 1:800 serum dilution.

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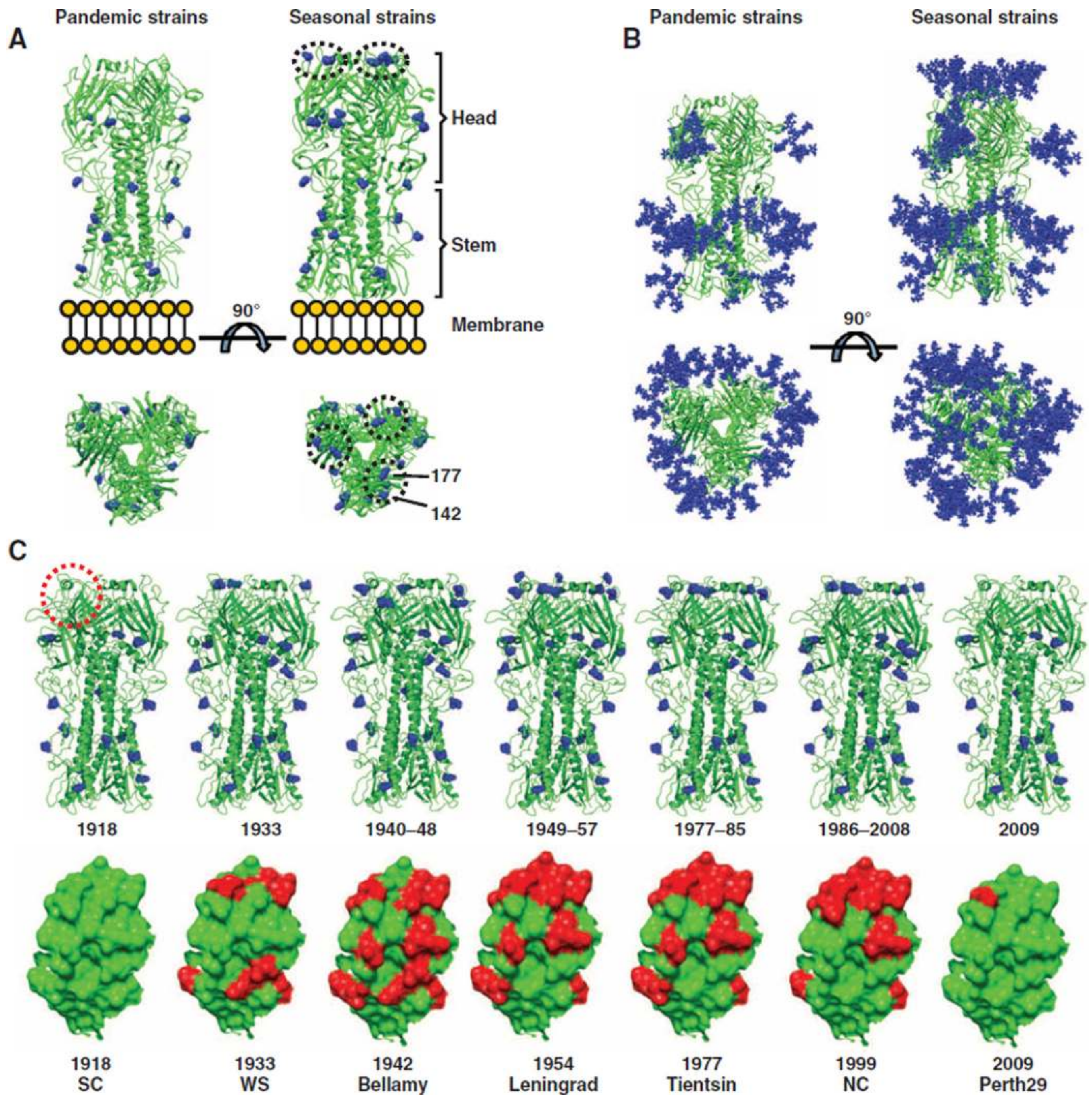

Fig. 2.

Glycosylation patterns and RBD-A sequence conservation of human H1N1 HAs. (**A**) Ribbon diagrams (side and top views) of HA depicting N-linked glycosylation on the pandemic 1918 SC and 2009 CA strains (left panels) and the seasonal 1999 NC H1N1 strain (right panels). The asparagine side chains of glycosylation sites were rendered as blue Corey-Pauling-Koltun (CPK) models. The glycosylation sites 142 and 177 (1918 numbering) on the top of the RBD are circled by a dotted line. (**B**) Same as in (A), except that glycosylations were modeled as complex glycosylations (with the terminal sialic acid

residues removed) with the GlyProt Server (29) and rendered as blue stick models. (**C**) The top panel illustrates the placement of these glycosylation sites on ribbon diagrams of 1918 SC HA using blue CPK models for glycosylated asparagine side chains. A red dotted circle on the leftmost ribbon diagram indicates the placement of the RBD-A region. The bottom panel depicts sequence identity between the 1918 pandemic strain and representative strains in the RBD-A region mapped onto a surface representation. Conserved residues are colored green and altered residues are colored red. The orientation is the same as in fig. S1 (middle two panels). PDB entry 1RUZ (1918 SC) was used for displaying the H1N1 pandemic strain HAs, and the seasonal H1N1 HAs were displayed using the structure of the A/PR/8/34 HA (PDB entry 1RU7). All structural panels were generated with the molecular graphics program UCSF Chimera (30).

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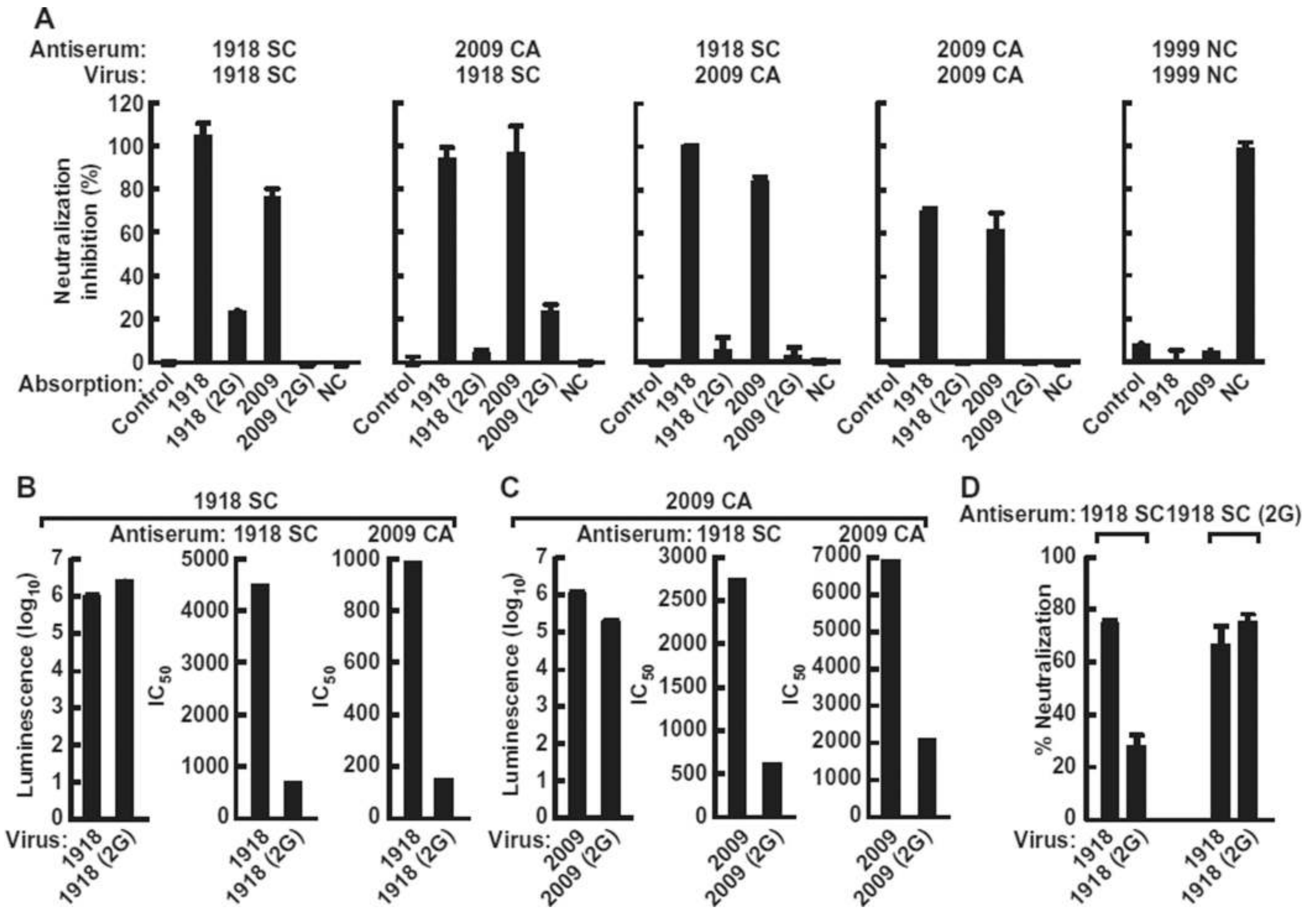

Fig. 3.

Addition of two glycosylation sites to 1918 SC or 2009 CA confers resistance to neutralization. (**A**) Inhibition of neutralizing antibodies to 1918 SC and 2009 CA measured on 1918 SC–pseudotyped and 2009 CA–pseudotyped lentiviral vectors or 1999 NC on 1999 NC reporter after absorption of sera with cells expressing the indicated HA or without absorption (Control). Percent reduction in neutralization was recorded at 1:400 serum dilution. (**B** and **C**) Comparable activity of 1918 SC and 2009 CA, and glycosylation mutants [1918 (2G) and 2009 (2G)] for viral entry using pseudotyped lentiviral vectors (left panels) and relative resistance of the 2G mutant pseudotyped versus wild-type reporters to neutralization by wild-type 1918 SC antisera (middle panel) or 2009 CA antisera (right panel) derived from DNA-vaccinated mice. (**D**) Neutralization of wild-type and glycosylation mutants of 1918 SC viruses by antisera from mice immunized twice with wild-type or glycosylation mutant 1918 SC HA DNA vaccines. Percent reduction in neutralization was measured at a 1:200 serum dilution. The sera raised to the 1918 2G were unable to neutralize the 1999 NC virus.

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Table 1

Protective efficacy of H1N1 vaccines against pandemic influenza A (H1N1) 2009 virus in mice.

Vaccine group*	HI antibody titer to homologous virus†	Weight loss (%)‡	Mean virus titer in lung ($\log_{10}$ PFU/ml)§	No. protected/ total no.//
PBS	<10	18.6 ± 0.98	6.8 ± 0.28	0/8
A/Panama/2007/1999 (H3N2)	76	18.8 ± 0.00	6.1 ± 0.20	0/8
A/New Caledonia/20/1999	80	17.9 ± 4.40	5.5 ± 0.14	1/8
A/Brisbane/59/2007	57	13 ± 7.48	5.6 ± 0.3	2/8
A/South Carolina/1/1918	59	3.7 ± 3.23	≤0.9	8/8¶
A/Mexico/4108/2009	50	1.7 ± 3.38	≤0.9	8/8¶

*Groups of BALB/c mice (*n* = 12) were injected i.m. with 10 μg of formalin-inactivated vaccine. A second dose was administered 3 weeks after the first and challenged 3 weeks later with 10^6 PFU (17,000 LD_{50}) of mouse-adapted A/California/04/2009 virus.

†Sera collected 3 weeks after the second vaccination were tested by HI against homologous virus shown. HI assays used 0.5% turkey RBCs. Antibody titers are expressed as the geometric mean titer of 12 mice per group.

‡The percentage mean maximum weight loss is shown.

§Whole lungs (*n* = 4) were collected on day 4 after challenge, and virus titers were determined on standard plaque assay.

//Protection against death measured for 21 days after challenge.

¶The statistical significance between the 1918 SC–immunized and the 2009 Mex–immunized groups and the PBS control group is *P* = 0.0002 by Fisher's exact test.

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Table 2

Evolution of H1N1 glycosylation and sequence variation. Summary of the presence of glycosylation sites on H1N1 strains as well as RBD-A sequence conservation during various time frames from 1918 to the present. Except for the last column, the numbers indicate residues (1918 SC numbering) predicted to have glycosylations in at least 50% of the sequences for that particular time period. The last column of the table shows the average amino acid sequence identity for the RBD-A region between 1918 strain and H1N1 strains within indicated time frames.

Time period	Stem		Side of head				Top of head					Side of head	Stem			RBD-A conservation (%)
1918	28	40				104							304	498	557	100
1933	28			73			142[*]				179[*,†]	286	304	498	557	80.5
1934–1939	28	40						144[*,†]				286	304	498	557	73.9
1940–1948	28	40				104		144[*]			179[*]	286	304	498	557	66.1
1949–1957	28	40			90	104		144[*]	172[*]	177[*]		286	304	498	557	63.2
1977–1985	28	40				104		144[*]		177[*]		286	304	498	557	65
1986–2008	28	40	71			104	142[*]			177[*]		286	304	498	557	67.1
2009–present	28	40				104							304	498	557	97.2

[*] Glycosylations on the top of the head.

[†] Glycosylations predicted in <50% of HA sequences.