Immunodominance of Antigenic Site B in the Hemagglutinin of the Current H3N2 Influenza Virus in Humans and Mice

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ABSTRACT The hemagglutinin protein of H3N2 influenza viruses is the major target of neutralizing antibodies induced by infection and vaccination. However, the virus frequently escapes antibody-mediated neutralization due to mutations in the globular head domain. Five topologically distinct antigenic sites in the head domain of H3 hemagglutinin, A to E, have been previously described by mapping the binding sites of monoclonal antibodies, yet little is known about the contribution of each site to the immunogenicity of modern H3 hemagglutinins, as measured by hemagglutination inhibition activity, which is known to correlate with protection. To investigate the hierarchy of antibody immunodominance, five Δ1 recombinant influenza viruses expressing hemagglutinin of the A/Hong Kong/4801/2014 (H3N2) strain with mutations in single antigenic sites were generated. Next, the Δ1 viruses were used to determine the hierarchy of immunodominance by measuring the hemagglutination inhibition reactivity of mouse antisera and plasma from 18 human subjects before and after seasonal influenza vaccination in 2017–2018. In both mice and humans, mutations in antigenic site B caused the most significant decrease in hemagglutination inhibition titers compared to wild-type hemagglutinin. This study revealed that antigenic site B is immunodominant in the H3N2 influenza virus strain included in the current vaccine preparations.

IMPORTANCE Influenza viruses rapidly evade humoral immunity through antigenic drift, making current vaccines poorly effective and antibody-mediated protection short-lived. The majority of neutralizing antibodies target five antigenic sites in the head domain of the hemagglutinin protein that are also the most sequence-variable regions. A better understanding of the contribution of each antigenic site to the overall antibody response to hemagglutinin may help in the design of improved influenza virus vaccines.

KEYWORDS influenza A virus, hemagglutinin, immunodominance, antigenicity

Infections with influenza viruses cause the death of 12,000 to 56,000 people in the United States annually (1–3). Two influenza A virus subtypes, H1N1 and H3N2, as well as influenza B viruses are currently circulating in humans. The protection provided by seasonal vaccination is only modest, especially against H3N2 viruses, with an average vaccine effectiveness of 33% being reported between 2004 and 2015 (4) and an estimated effectiveness of 25% being reported in the 2017–2018 season (5). One of the reasons for the poor effectiveness is the rapid accumulation of mutations in the hemagglutinin (HA) surface glycoprotein, the major target of neutralizing antibodies (6–8). This antigenic drift makes it necessary to reformulate and readminister current vaccines almost yearly (9). The HA protein consists of two structurally distinct domains:

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the globular head, composed of the majority of the HA1 polypeptide, and the stalk domain, made up of portions of HA1 and the entire ectodomain of the HA2 polypeptide (10). From 1968 to 2010, 108 amino acid changes in the major epidemic strains occurred within HA1 at 63 residue positions, whereby 85.5% were clustered into regions called antigenic sites (11). Four antigenic sites, A to D, were identified in 1980 by Webster and Laver by determining the reactivity patterns of monoclonal antibodies (MAbs) using hemagglutination inhibition (HI) assays (12). A fifth site, E, was later described by Skehel and colleagues in 1984 and 1987 by identifying escape mutations from a panel of MAbs (13, 14). MAbs to each of the five antigenic sites have shown HI activity (12, 15).

Early studies on the HA of the A/Memphis/1/1971 H3N2 virus suggested that site A was immunodominant, as the HI reactivity of ferret antiserum was completely ablated by mutations in this site (16). Moreover, human plasma samples collected in 1976 showed decreased binding to mutants with mutations in antigenic site A of the A/Aichi/2/1968 virus (17). Further research suggested that the immunodominance hierarchies may vary over time. Computational analyses provided evidence that site A was immunodominant between 1968 and 1971 and between 1989 and 1995, while site B was dominant from 1972 to 1987 and from 1996 to 2003 (18). Studies on plasma samples collected after seasonal vaccination in the 2006–2007 and 2008–2009 seasons suggested that site B was immunodominant over site A in these years; however, the other antigenic sites were not investigated (11).

To systematically study the hierarchy of antibody immunodominance of all antigenic sites, five Δ 1 influenza viruses expressing the HA of the A/Hong Kong/4801/2014 virus (the H3N2 component of the 2016–2017 and 2017–2018 seasonal vaccines; abbreviated HK2014 in the following) were generated in the A/Puerto Rico/8/34 (PR8) backbone, each with one antigenic site mutated. These viruses served as probes to interrogate the immunodominance, as measured by HI reactivity, in mouse antisera and human plasma samples obtained before and after vaccination in the 2017–2018 season. We demonstrate that site B is immunodominant in both mice and humans.

RESULTS

Rescue and characterization of Δ 1 viruses. A 7:1 reassortant virus expressing the H3 protein of the HK2014 virus (the H3 wild type [H3-wt]) was successfully rescued in the PR8 backbone. Using the same backbone, we next sought to rescue Δ 1 viruses in which single antigenic sites of the HK2014 HA were replaced with the corresponding sequences of the HA of the avian A/Jiangxi-Donghu/346-1/2013 (H10N8) virus (19). Both H10 and H3 are group 2 HAs (Fig. 1A) with highly similar crystal structures (Fig. 1B) but little amino acid sequence similarity in their head domains (Fig. 1C). Various mutant HA proteins were tested for their ability to generate viable Δ 1 viruses, as determined by hemagglutination assays. In the following, the rescued viruses are designated H3- Δ A through H3- Δ E, depending on which antigenic site was mutated. A previous analysis of H3N2 strains from 1968 to 2003 demonstrated that one to three amino acid substitutions within an antigenic site were sufficient to cause major antigenic change, as measured by HI reactivity (20, 61). Similarly, two to three mutations within an antigenic site of the PR8 H1 hemagglutinin were previously shown to be sufficient to ablate binding of antigenic site-specific MAbs (20). Thus, we aimed at rescuing Δ 1 viruses with at least three amino acid substitutions in the respective antigenic site that should thereby be antigenically altered. Additionally, several amino acids that were mutated are at positions of previously described escape mutations from MAbs or polyclonal sera (11, 14–16, 20–22).

The amino acid residues that were successfully exchanged in the HA protein of the five Δ 1 viruses are depicted in Fig. 2A. In the H3- Δ A virus, HA residues 140 and 142 to 145 of site A, which forms an exposed loop, were replaced (Fig. 2B). Site B, located at the tip of the head domain, consists of a loop (residues 155 to 160) and an α -helix (residues 187 to 196). The H3- Δ B virus contains mutations in both regions, at positions 159 and 189, as well as an additional compensatory mutation at position 188 in the

FIG 1 Comparison of the H3 and H10 proteins. (A) Phylogenetic tree of influenza A and B virus HA proteins (57). The scale bar represents a 7% change at the amino acid level. (B) Models of the H3 (left) and the H10 (right) HA monomers. The head domains are shown in dark gray, and the stalk domains are shown in light gray. The H3 model is based on the crystal structure of A/Victoria/361/2011 (H3N2) influenza virus HA (PDB accession no. 4O5N) (58), and the H10 model is based on the crystal structure of A/Jiangxi-Donghu/346-1/2013 (H10N8) influenza virus HA (PDB accession no. 4XQO) (59). The models were visualized with UCSF Chimera software (60). (C) The amino acid sequences of the H3 and H10 HAs are aligned. Only regions including the H3 antigenic sites are shown. Conserved amino acids are marked with asterisks. The indicated amino acid numbers are according to the H3 numbering.

helix. The H3-ΔC virus was rescued with a total of 15 amino acid substitutions in site C, which is located in the interface between the head and stalk domains. A compensatory mutation in the H3-ΔC virus was observed at amino acid position 48. Site D, located at the trimer interface, tolerated three substitutions at positions 207 to 209, which, according to the crystal structure, are exposed on the surface of the HA trimer (Fig. 2A). An additional compensatory mutation was observed in site D at position 212. Mutations in site E, which is located at the center of the head domain, were successfully introduced in three different regions in the primary sequence. This virus, termed H3-ΔE, contained a total of six amino acid substitutions. A virus containing all the mutations of the five Δ1 viruses, termed H3-Δ5, was also successfully rescued. The H3-Δ5 virus, in which all these amino acid changes were combined, did not contain the compensatory mutation in site B at position 188 but contained one at residue 246 (asparagine to histidine), outside the antigenic sites. In addition, a virus with a chimeric HA (9, 23–26) designated cH10/3, with the entire head domain of the HK2014 HA replaced with the H10 head, was successfully rescued. All compensatory mutations appeared after viral rescue and were not templated in the original plasmids.

After growing for 48 h in embryonated chicken eggs, the various plaque-purified reassortant viruses reached titers of between 4.9×10^7 (H3-ΔC) and 3.5×10^9 (cH10/3) PFU/ml (Fig. 2C). Hemagglutination titers ranged between 64 (H3-ΔC and H3-Δ5) and 512 (H3-ΔD) hemagglutination units per 50 μ l (Fig. 2D). Immunofluorescence microscopy experiments of virus-infected Madin-Darby canine kidney (MDCK) cells, using MAb

FIG 2 Recombinant influenza viruses expressing H3 HA with mutated antigenic sites. (A) Model of the H3 HA trimer. Residues that were mutated are highlighted in color code according to the antigenic sites, as indicated. The three monomers are shown in three shades of gray. The three-dimensional model is based on the crystal structure of A/Victoria/361/2011 influenza virus (H3N2) HA (PDB accession no. 4O5N) (58) and was visualized using UCSF Chimera software (60). (B) Amino acid sequences of parts of the antigenic sites of HK2014 HA (top sequences, H3 numbering) are aligned with the corresponding sequences of the H10 HA (bottom sequences). Amino acids that have been exchanged in the mutant HAs of the H3- Δ A through H3- Δ E viruses are highlighted in the color code of panel A. Amino acids 188 (site B), 48 (site C), and 212 (site D) are compensatory mutations. (C and D) Bars represent the mean viral titers, expressed as the number of PFU per milliliter of allantoic fluid (C) or the HA titer per 50 μ l of allantoic fluid (D) after the virus was grown in eggs for 48 h at 37°C. (E) Representative images of MDCK cells infected with the indicated viruses for 16 h obtained by

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9H10, which recognizes a conformational epitope in the stalk of group 2 HAs (27), verified surface expression of the various HA proteins (Fig. 2E). A similar staining pattern was observed with polyclonal mouse antiserum generated against a 6:2 reassortant virus expressing HA and neuraminidase (NA) of HK2014 in the PR8 backbone, designated PR8-H3N2(HK2014) in the following. The results obtained by immunofluorescence microscopy were confirmed by whole-cell enzyme-linked immunosorbent assays (ELISAs) that indicated comparable HA expression levels for the various recombinant viruses (Fig. 2F). Binding of the antiserum to the surface of cells infected with the H3-Δ5 and cH10/3 viruses indicated the presence of stalk-specific antibodies. To assess their presence, we performed ELISAs using plates coated with recombinant HA proteins (28). As expected, the mouse antiserum bound to the recombinant HK2014 H3 protein (Fig. 2G). Weaker binding was observed for the cH14/3 recombinant protein, which consists of the HK2014 stalk domain and the HA head domain of the avian A/mallard/Gurjev/263/1982 (H14N5) virus. In contrast, no binding was observed when the full-length H14 protein was coated. Although stalk-reactive antibodies raised with H3 are expected to partially cross-react with the H14 stalk domain (both are group 2 HAs), their abundance was likely too low to give a detectable signal for full-length H14 protein. Overall, the ELISA measurements confirmed the presence of H3 stalk-reactive antibodies in the murine antiserum.

Hierarchy of immunodominance in immunized mice. A recent study in mice showed various antibody responses to the antigenic sites of the PR8 (H1N1) HA protein, depending on the genetic background of the mice (BALB/c versus C57BL/6 strains) and whether the animals were infected with virus intranasally or immunized intraperitoneally or intramuscularly (29). To investigate if recognition of HK2014 HA epitopes also depended on the mouse strain and the route of virus administration, groups of four to five animals were primed with the live PR8-H3N2(HK2014) virus either intranasally or intraperitoneally and boosted 4 weeks later either intranasally or intraperitoneally (four combinations per mouse strain). Serum drawn 4 weeks after the second immunization was further analyzed (Fig. 3A). To test whether mice developed antibodies to the HK2014 HA protein, pooled sera were subjected to ELISA measurements, whereby the H3-wt virus was coated onto the ELISA plates (Fig. 3B). All immunization regimes elicited detectable levels of IgG in both strains. The strongest ELISA signals were observed for BALB/c mice that were primed intranasally.

To determine the contribution of each antigenic site to the immunogenicity of H3 HA, we performed HI assays with the panel of eight recombinant viruses described above (Fig. 3C). HI titers have been shown to correlate with neutralizing activity (30) and with influenza immunity (31–33). All animals mounted HI titers of 1:80 or higher against H3-wt virus. HI titers against the cH10/3 virus were below the level of detection in all mice, suggesting that antibodies against the HK2014 head domain do not cross-react with the H10 head domain. On average, HI titers against the H3-Δ5 virus were about 8-fold lower than those against H3-wt and below the limit of detection in some animals, indicating that the antigenic sites were successfully antigenically altered. Irrespective of the mouse strain or route of immunization, HI titers against the H3-ΔB virus were consistently lower than those against the H3-wt virus, indicating that site B was immunodominant by HI reactivity. HI titers to the H3-wt virus were variable between individual mice, ranging from 1:80 to 1:2,560. To compensate for these differences in overall titers and only compare the relative contributions of each antigenic site, HI titers against the Δ1 mutant viruses were divided by the HI titers

FIG 2 Legend (Continued)

immunofluorescence microscopy. Surface staining with MAb 9H10 (top) or polyclonal mouse antiserum (bottom) is shown. (F) Results from whole-cell ELISA of MDCK cells infected with the indicated viruses for 16 h. Bars show the mean $\pm$ SD. OD490, optical density at 490 nm. (G) Antibody response in polyclonal mouse antiserum, as measured by ELISA. ELISA plates were coated with recombinant HA proteins. H3 is the full-length HA of the HK2014 virus, cH14/3 is a chimeric HA with an H14 head and the HK2014 stalk, and H14 is the full-length H14 HA protein. Data points show the mean $\pm$ SD for three replicates. Positive control MAbs are 9H10 (for H3) and 2F11, an in-house-produced MAb against H14. n.d., not detectable.

FIG 3 HI titers of mouse antisera. (A) Immunization regime. Mice received two doses of PR8-H3N2(HK2014) (6:2) virus either intranasally (i.n.) at a dose of 10^7 PFU or intraperitoneally (i.p.) at a dose of 4×10^6 PFU at the indicated time points. This virus expresses the surface glycoproteins of the HK2014 H3N2 virus in the PR8 backbone. Serum drawn 4 weeks after the second immunization was used for the ELISAs and HI assays. (B) IgG response measured by ELISA. ELISA plates were coated with whole H3-wt virus. The different routes of immunization and mouse strains are indicated to the right of the graphs. Data points show the means for two replicates. (C) HI titers to the indicated viruses are shown. Symbols representing individual mice are color coded as in panel B, and the bars show the mean value for each group. Statistical significance compared to the H3-wt was inferred by the Newman-Keuls corrected one-way analysis of variance (ANOVA) of the $\log_2$ -transformed HI titers (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Data points represent individual mice in all subpanels except the first subpanel (BALB/c i.n. → i.p.), which shows pooled serum from five mice measured in triplicate. For this reason, statistics for the latter group could not be calculated. (D) This panel shows the same data as in panel C, but for each serum sample the HI titer against the H3-ΔA through H3-ΔE viruses was divided by the respective HI titer obtained for the H3-wt virus. Individual serum samples are shown as light gray dots, many of which overlap. The mean values for all samples are shown as black dots. Statistical significance compared to H3-wt was inferred by performing Dunn-corrected Kruskal-Wallis tests (##, $P \leq 0.01$; ###, $P \leq 0.001$). (E) This panel shows the same data as in panel C but plotted as an antigenic map (20). The viruses (H3-wt and H3-ΔA through H3-ΔE) are shown as black data points, whereby the data point for H3-ΔD is hidden. Sera are color coded as indicated to the right of the map. The spacing between grid lines corresponds to a factor-of-2 difference in HI titers. Numbers indicate overlapping data points; e.g., 2 indicates that the data point represents two serum samples with identical or nearly identical HI profiles.

against the H3-wt virus observed for each mouse (Fig. 3D). The normalized data revealed a significant contribution of site B and, to a lesser extent, sites A and C to the immunodominance hierarchy. Mutating the other two antigenic sites, D and E, had no significant effect on HI reactivity. Plotting the HI data of all mice on one map by using

TABLE 1 Plasma samples analyzed in this study

Subject no.	Sex ^b	Age group (yr)	Vaccine trade name ^a	No. of vaccine components	Produced in:	Manufacturer
1	F	50–59	Flucelvax	4	Cell culture	Seqirus, Inc.
2	M	30–39	Flucelvax	4	Cell culture	Seqirus, Inc.
3	M	>60	Fluzone high dose	3	Eggs	Sanofi Pasteur
4	F	>60	Fluzone high dose	3	Eggs	Sanofi Pasteur
5	F	40–49	Flucelvax	4	Cell culture	Seqirus, Inc.
6	F	50–59	Flucelvax	4	Cell culture	Seqirus, Inc.
7	M	40–49	Flucelvax	4	Cell culture	Seqirus, Inc.
8	M	50–59	Flucelvax	4	Cell culture	Seqirus, Inc.
9	M	30–39	Fluarix quadrivalent	4	Eggs	GSK
10	M	>60	Fluvirin	3	Eggs	Seqirus, Inc.
11	M	50–59	Fluarix quadrivalent	4	Eggs	GSK
12	M	40–49	Fluarix quadrivalent	4	Eggs	GSK
13	F	30–39	Fluzone quadrivalent	4	Eggs	Sanofi Pasteur
14	M	20–29	Fluarix quadrivalent	4	Eggs	GSK
15	F	40–49	Fluarix quadrivalent	4	Eggs	GSK
16	M	30–39	Flublok quadrivalent	4	Insect cells	Protein Sciences Corp.
17	F	30–39	Fluarix quadrivalent	4	Eggs	GSK
18	M	30–39	Fluzone quadrivalent	4	Eggs	Sanofi Pasteur

^aVaccine components are as follows: Fluarix quadrivalent, A/Singapore/GP1908/2015 (H1N1) IVR-180 [an A/Michigan/45/2015 (H1N1) 2009 pandemic (pdm09)-like virus], A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, B/Brisbane/60/2008, and B/Phuket/3073/2013; Flucelvax, A/Singapore/GP1908/2015 IVR-18 [an A/Michigan/45/2015 (H1N1) pdm09-like virus], A/Singapore/GP2050/2015 (wild type) [an A/Hong Kong/4801/2014 (H3N2)-like virus], B/Utah/9/2014 (a B/Phuket/3073/2013-like virus), and B/Hong Kong/259/2010 (a B/Brisbane/60/08-like virus); Fluzone high dose, A/Michigan/45/2015 X-275 (H1N1), A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, and B/Brisbane/60/2008; Fluvirin, A/Singapore/GP1908/2015, IVR-180 [an A/Michigan/45/2015 (H1N1) pdm09-like virus], A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, and B/Brisbane/60/2008; Flublok quadrivalent, A/Michigan/45/2015 (H1N1), A/Hong Kong/4801/2014 (H3N2), B/Brisbane/60/2008, and B/Phuket/3073/2013; and Fluzone quadrivalent, A/Michigan/45/2015 X-275 (H1N1), A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, B/Brisbane/60/2008, B/Phuket/3073/2013, and B/Brisbane/60/2008.

^bF, female; M, male.

antigenic cartography (20) revealed that there were no measurable differences in the immunodominance hierarchies between the two mouse strains or the route of administration (Fig. 3E).

Hierarchy of immunodominance in humans before and after seasonal vaccination. We next sought to investigate hierarchies of immunodominance in plasma samples obtained from 18 healthy human subjects before and 4 to 8 weeks (average, 35 days; range, 27 to 57 days) following vaccination in the 2017–2018 season (Table 1). Eleven of the individuals received tri- or quadrivalent vaccines manufactured in eggs, all of which contained an HK2014-like virus as the H3N2 component. Six additional subjects received Flucelvax, a vaccine propagated in MDCK suspension cells that contained an A/Singapore/GP2050/2015-like H3N2 component (34). One individual received the quadrivalent Flublok vaccine that is produced in insect cells and contains recombinant proteins instead of inactivated viruses (35). In the 2017–2018 season, Flublok contained an HK2014-like HA protein as the H3N2 component.

To determine the relative contributions of the antigenic sites to the antibody repertoire in the 18 individuals, HI assays with the eight recombinant viruses were performed. Vaccination boosted the HI titers against all viruses with statistical significance, except for H3-ΔE ($P = 0.058$) and cH10/3 (no measurable titer) (Fig. 4A). The geometric mean HI titers against the H3-wt virus pre- and postvaccination were 1:59 and 1:97, respectively. Ten of 18 individuals had higher HI titers postvaccination than prevaccination against H3-wt virus, with 6 showing a 2-fold increase and 3 showing a 4-fold increase; 1 individual had an undetectable titer before vaccination and a titer of 1:40 postvaccination. Six subjects had equal titers pre- and postvaccination, and two showed a 2-fold decrease in HI titers postvaccination. None of the plasma samples had a measurable HI titer against the cH10/3 virus, confirming the absence of HI-reactive H10 head-specific antibodies in these subjects. Both pre- and postvaccination HI titers were significantly lower against the H3-ΔB virus than against H3-wt virus.

The HI titers against the H3-wt virus were highly variable. Therefore, to better compare the immunodominance profiles, the HI titers against the Δ1 mutant viruses were divided by those observed for the H3-wt virus for each individual (Fig. 4B). The

FIG 4 HI titers of human plasma samples pre- and postvaccination. (A) HI titers against the indicated viruses are shown. White circles represent individual plasma samples from 18 subjects. The bars show the mean for each group prevaccination (black) and at 4 to 8 weeks postvaccination (blue). (B) This panel shows the same data as in panel A, but for each plasma sample, the HI titer against the H3-ΔA through H3-ΔE viruses was normalized to the HI titer obtained for H3-wt virus. Individual plasma samples ($n = 36$) are shown in light gray and blue dots, many of which overlap. The mean values are shown as black and dark blue dots, as indicated. (C) This panel shows the same data as in panel A but plotted as an antigenic map (20). The viruses (H3-wt and H3-ΔA through H3-ΔE) are shown as black data points. Plasma samples are color coded as indicated to the right of the map. The spacing between grid lines corresponds to a factor-of-2 difference in HI titers. Numbers indicate overlapping data points; e.g., 2 indicates that the data point represents two serum samples with identical or nearly identical HI profiles. In panel A, statistical significance pre- versus postvaccination was determined by paired Student's *t* tests of the log₂-transformed HI titers (+, $P \leq 0.05$; ++, $P \leq 0.01$). Statistical significance compared to H3-wt was inferred by the Newman-Keuls corrected one-way analysis of variance (ANOVA) of the log₂-transformed HI titers (**, $P \leq 0.01$; ***, $P \leq 0.001$), whereby pre- and postvaccination groups were analyzed separately. Statistical significance in panel B was inferred by Dunn-corrected Kruskal-Wallis tests (###, $P \leq 0.001$), with pre- and postvaccination groups being analyzed separately. The normalized HI titers for H3-ΔB in panel B are significantly lower than those for H3-wt both pre- and postvaccination.

normalized data confirmed a significant contribution of site B to the HI-reactive antibody response. Exchanging the other four antigenic sites had no significant effect on the HI reactivity. Furthermore, immunodominance hierarchies were similar in plasma taken pre- and postvaccination. Plotting the HI data for all subjects on one antigenic map confirmed that there were no significant differences in the immunodominance hierarchies before and after vaccination (Fig. 4C). In addition, the age of the subjects did not have a significant impact on the immunodominance profiles.

DISCUSSION

In this study, a panel of $\Delta 1$ influenza viruses expressing HA of the current H3N2 vaccine component, each with mutations in one of the five antigenic sites, was generated. In addition, a $\Delta 5$ virus with mutations in all five sites was rescued. These viruses were used to interrogate the hierarchy of immunodominance in mice and humans, as measured by HI reactivity. ELISA studies with recombinant HA proteins revealed that the majority of serum antibodies in immunized mice, which are usually HI reactive, are directed against the head domain. Stalk-specific antibodies, which are generally HI inactive, are also present, but to a lower extent, which confirms the known immunodominance of the head over the stalk (36). A substantially lower HI reactivity to the H3-Δ5 virus than to the H3-wt virus indicated that the introduced mutations successfully altered the antigenicity of the antigenic sites. Residual HI reactivity to this virus may have been due to antibodies binding to head domain epitopes outside the major antigenic sites or antibodies targeting the receptor binding pocket directly using a long CDR3 region. Such MABs have been isolated from humans and typically exhibit binding that is unaffected by mutations in the major antigenic sites (37–42). In addition, the fact that the H3-Δ5 virus was missing the compensatory mutation at position 188

in site B, which was present in the H3-ΔB virus, may have caused the residual HI reactivity of that antigenic site.

It has to be noted that the HI assay provides discrete titer thresholds (i.e., 10, 20, 40) and is therefore limited in detecting subtle differences in antibody levels. Other measures, such as ELISAs or plaque reduction neutralization (PRNT) assays, may be able to detect smaller differences in antibody levels. However, an advantage of the use of the HI assay to determine the contributions of antigenic sites to the HA head-specific polyclonal antibody repertoire is that HI titers are not influenced by stalk-specific antibodies (Fig. 2G) (43). In contrast, ELISAs and neutralization assays are sensitive to stalk-specific antibodies (43), which could increase background signals when measuring head-specific responses.

The HI assay does not differentiate among immunoglobulin isotypes. While IgG is the major systemically induced isotype in serum, IgA is predominant in mucosal tissues, including the respiratory tract. To our knowledge, the antigenic site-specific epitope recognition patterns of purified IgA have not been studied. However, previous studies found that polyclonal IgA provides better heterosubtypic immunity to influenza virus than IgG (44, 45). A possible explanation for this observation is that IgA preferentially targets the conserved HA stalk domain compared to IgG (46). It remains to be studied whether the broader reactivity of IgA could also be explained by IgA-specific reactivity to epitopes within the HA head. For the IgM isotype, differences in epitope recognition patterns from those of IgG are not expected, as it was shown previously in mice that there was no difference between IgG and IgM in the hierarchy of immunodominance to H1 hemagglutinin, as measured by ELISA (29).

The results of this study in humans are in good agreement with those of a previous study that reported the immunodominance of site B in human subjects vaccinated in the 2006-2007 and 2008-2009 seasons, as measured by ELISA using recombinant HA proteins (11). The latter study, however, investigated the effect of mutations only in antigenic sites A and B on serum reactivity. Here we show that mutations in sites A, C, D, and E of the HK2014 HA did not significantly affect HI reactivity in the human plasma samples tested.

Ten out of 18 individuals showed increased HI reactivity against the H3-wt virus after vaccination, whereas 8 subjects had similar or lower HI reactivity postvaccination. Despite the various responses to vaccination with respect to HI reactivity, antigenic site B was dominant both pre- and postvaccination, suggesting that vaccination did not alter the immunodominance hierarchy of HI-reactive antibodies. There were no apparent differences in the postvaccine HI reactivities between vaccines based on egg- or cell culture-propagated viruses and the vaccine based on recombinant proteins. However, the sample sizes were too small to allow for statistically meaningful analyses. Irrespective of the age of the subjects, antigenic site B was immunodominant, suggesting that the immune history to influenza virus (47) did not majorly affect the immunodominance hierarchy of HI-reactive antibodies in the tested individuals.

In contrast to humans, the HI reactivity of mouse antisera was also significantly decreased by mutations in sites A and C, albeit to a lower extent than through mutations in site B. The immunodominance hierarchy in mice was independent of whether antiserum was raised in the BALB/c or C57BL/6 strain and was also not affected by the route of immunization, intranasal versus intraperitoneal. In contrast, the immunodominance hierarchy to antigenic sites of the PR8 (H1N1) virus, as measured by ELISA, was previously reported to vary depending on the mouse strain and route of antigen delivery (29). In accordance with our findings, however, the latter study found that irrespective of the mouse strain and route of immunization, at 4 weeks postimmunization, most of the antibodies bound to antigenic sites Sa and Sb of the PR8 HA, which correspond to site B in the H3 HA (29). The fact that site B is immunodominant with respect to HI reactivity in both mice and humans suggests that the rules governing the immunodominance of the current H3 protein are largely conserved between the two species.

A better understanding of the immunodominance hierarchy may assist in the efforts

toward the development of vaccines that provide better and longer-lasting protection (36). For instance, vaccines eliciting a more balanced immunity against the head domain may be superior to current vaccines, as thereby susceptibility to drifted strains will be lower (48). Using our panel of mosaic viruses, we observed no change in the immunodominance hierarchy pre- and postvaccination, indicating that the seasonal vaccines were likely boosting preexisting antibody responses to the antigenic sites, which are prone to antigenic drift (11). Knowledge on the immunodominance may help to design antigens that are able to focus antibody responses to more conserved but immunosubdominant epitopes in the H3 head (22, 38, 49, 50) or stalk domain (27, 51, 52), which could limit immune evasion by H3N2 influenza viruses.

MATERIALS AND METHODS

Recombinant HA genes and cloning. To obtain the HA gene segment of the A/Hong Kong/4801/2014 virus, RNA was isolated with a High Pure viral RNA kit (Roche) from the New York Medical College (NYMC) X-263 strain, a 6:2 reassortant virus expressing the HK2014 surface glycoproteins on a PR8 backbone obtained from NIBSC. The viral RNA was used as a template for reverse transcription-PCR (RT-PCR) amplification using a SuperScript III one-step RT-PCR system with Platinum *Taq* High Fidelity DNA polymerase (Thermo Fisher) and primers H3-for (CCGAAGTTGGGGGGAGCAAAGCAGGGGATAA TTC) and H3-rev (GGCCGCCGGGTTATTAGTAGAAACAAGGGTGTTTTAATTAATG). Cycling conditions were 15 min at 60°C; 2 min at 94°C; 15 s at 94°C, 30 s at 60°C, and 2 min at 68°C for 40 cycles; 5 min at 68°C; and then a hold at 4°C. The PCR product was purified from a preparative agarose gel with a NucleoSpin gel and PCR cleanup kit (Macherey-Nagel). The HA gene segment was cloned into an ambisense pDZ vector that was digested with the *SapI* restriction enzyme (New England BioLabs), using an In-Fusion HD cloning kit (Clontech). HA gene segments with mutations in the antigenic sites were designed by aligning the HA gene sequences of HK2014 and A/Jiangxi-Donghu/346-1/2013 (H10N8) (the sequence was obtained from the Global Initiative on Sharing Avian Influenza Data [GISAID; <http://gisaid.org>], accession no. EPI530526) with the Clustal X (version 2.0) program (53) and replacing the respective coding sequences with the H10 virus sequences. The mutant HA gene fragments were either ordered as synthetic genes from Integrated DNA Technologies or generated by overlap extension PCR using the CloneAmp HiFi PCR premix (Clontech) and cycling conditions that were adapted to the amplicon lengths. All HA gene segments included 15-bp overhangs at both ends that allowed for cloning into the *SapI*-digested pDZ vector, as described above. The sequences were confirmed by Sanger sequencing (MacroGen for plasmids and GeneWiz for PCR fragments). Primers were obtained from Integrated DNA Technologies.

Viral rescue. To generate reassortant viruses, human embryonic kidney 293T cells were transfected with 0.7 μ g of the pDZ plasmid expressing HA, 2.8 μ g of a pRS-7 segment plasmid that drives ambisense expression of the seven gene segments of the A/Puerto Rico/8/34 (PR8) virus except HA, described elsewhere (54), and 0.5 μ g of a pCAGGS plasmid expressing the HA protein of PR8 virus to facilitate viral rescue, using TransIT-LT1 transfection reagent (Mirus Bio) according to the manufacturer's recommendations. A 6:2 reassortant virus, designated PR8-H3N2(HK2014), with the HA and neuraminidase (NA) surface glycoproteins of HK2014 and the remaining six gene segments of PR8 was generated analogously. After 48 h, cells were treated for 30 min with 1 μ g per ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Supernatants were collected, clarified by low-speed centrifugation, and injected into 8- to 10-day-old specific-pathogen-free embryonated chicken eggs (Charles River Laboratories) that were incubated at 37°C (23, 24). At 48 h postinjection, the eggs were cooled to 4°C for at least 4 h, and then allantoic fluids were harvested and clarified by low-speed centrifugation. The presence of influenza virus in the allantoic fluid was determined by performing HA assays as described below. Positive virus cultures were plaque purified on confluent MDCK cell layers in the presence of TPCK-treated trypsin and expanded in embryonated chicken eggs. Virus titers were determined by plaque assays on MDCK cells. Plaques were stained using a commercial anti-NP antibody (catalog no. PA5-32242; Invitrogen) or MAb 9H10 (27). The sequences of the HA and NA genes were confirmed by Sanger sequencing, as described in the previous paragraph.

Generation of mouse antisera and whole-virus ELISA. To generate antisera to the surface glycoproteins of HK2014, 6- to 8-week-old female BALB/c or C57BL/6 mice obtained from The Jackson Laboratory were primed with the 6:2 reassortant PR8-H3N2(HK2014) virus described above either intranasally with 10^7 PFU or intraperitoneally with 4×10^6 PFU and boosted 4 weeks later either intranasally or intraperitoneally with the same virus. At 4 weeks after the booster immunization, mice were euthanized and blood was drawn. Sera were separated from red blood cells by centrifugation. Animal experiments were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). The presence of antibodies to the H3 HA protein was determined by whole-virus ELISA as described in the following. Ninety-six-well plates were coated with H3-wt virus (allantoic fluid) diluted 1:100 in coating buffer (100 mM NaHCO₃, pH 9.4) for 16 h at 4°C, washed once with phosphate-buffered saline (PBS), and blocked with 5% (wt/vol) skim milk powder in PBS for 1 h. Then, the plates were washed once with PBS and serial 2-fold dilutions of mouse sera in PBS were added. After incubation for 1 h, the plates were washed three times with PBS and the wells were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare) diluted 1:5,000 in 5% (wt/vol) skim milk powder in PBS for 1 h. The plates were washed three times with PBS and developed with SigmaFast o-phenylenediamine (OPD; Sigma-Aldrich)

for 10 min. The reaction was stopped using 3 M hydrochloric acid, and the absorbance at 490 nm was measured on a Synergy 4 plate reader (BioTek).

Immunofluorescence and whole-cell ELISA. MDCK cell monolayers in 96-well tissue culture plates were infected at a multiplicity of infection (MOI) of 5 with the different viruses and incubated for 16 h at 37°C. The medium was aspirated, and the cells were washed twice with PBS and fixed with a methanol-free 4% paraformaldehyde solution for 15 min. After washing three times with PBS, the wells were blocked for 30 min with a 5% (wt/vol) bovine serum albumin (BSA) solution in PBS. The cells were washed once with PBS and incubated for 2 h with MAb 9H10 (27) at 5 μ g per ml or pooled mouse serum (five mice) diluted 1:400 in 1% (wt/vol) BSA in PBS and then washed three times with PBS. For immunofluorescence studies, the cells were incubated with fluorescence-labeled anti-mouse IgG Alexa Fluor 488 antibody (Life Technologies) diluted 1:2,000 in 1% (wt/vol) BSA in PBS for 1 h and then washed three times with PBS before pictures were taken on a Zeiss LSM 880 Airyscan laser scanning confocal fluorescence microscope at the Microscopy Core of the Icahn School of Medicine at Mount Sinai. For whole-cell ELISAs, cells were incubated with the same primary antibodies used for immunofluorescence. The cells were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (GE Healthcare) diluted 1:5,000 in 1% (wt/vol) BSA in PBS for 1 h and then washed three times with PBS and developed with SigmaFast OPD (Sigma-Aldrich) for 10 min. The reaction was stopped using 3 M hydrochloric acid (HCl), and the absorbance at 490 nm was measured on a Synergy 4 plate reader (BioTek).

ELISA with recombinant HA proteins. Recombinant HA proteins (28) were coated at a concentration of 2 μ g per ml in PBS (50 μ l per well) for 16 h at 4°C. After washing once with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T), the wells were blocked with 5% (wt/vol) skim milk powder in PBS for 1 h. The wells were washed once with PBS-T, and pooled mouse antiserum diluted in PBS (50 μ l per well) was added. After incubation for 1 h, the wells were incubated with HRP-conjugated anti-mouse IgG antibody (GE Healthcare) diluted 1:5,000 in 5% (wt/vol) skim milk powder in PBS for 1 h and then washed three times with PBS-T and developed with SigmaFast OPD (Sigma-Aldrich) for 15 min. The reaction was stopped using 3 M HCl, and the absorbance at 490 nm was measured on a Synergy 4 plate reader (BioTek).

Hemagglutination assays. Serial 2-fold dilutions of influenza virus samples (allantoic fluids) were prepared in 96-well V-well microtiter plates, using PBS as the diluent to a final volume of 50 μ l per well. Then, 50 μ l of a 0.5% suspension of turkey red blood cells (Lampire) in PBS was added to each well. The plates were incubated at 4°C until the red blood cells in the PBS control samples settled to the bottom. The hemagglutination titer (hemagglutination units) was defined as the reciprocal of the highest dilution of virus that caused red blood cell hemagglutination.

RDE treatment of plasma and serum. Human plasma samples were pretreated at 56°C for 30 min. One volume of pretreated human plasma or mouse serum was treated with 3 volumes of *Vibrio cholerae* receptor-destroying enzyme (RDE; Denka Seiken, Chuo-ku, Tokyo, Japan) solution in PBS at 37°C for 16 h according to the manufacturer's recommendations. To the RDE-treated samples was added 3 volumes of a 2.5% sodium citrate solution. After incubation at 56°C for 30 min, 3 volumes of PBS was added to each sample for a final dilution of 1:10.

HI assays. Hemagglutination inhibition (HI) assays were performed as described previously (25, 55). Allantoic fluid samples of each influenza virus strain were diluted with PBS to a final HA titer of 8 hemagglutination units per 50 μ l. Twofold dilutions (25 μ l) of RDE-treated plasma/serum in PBS prepared in 96-well V-well microtiter plates were then combined with 25 μ l of the diluted influenza viruses. The plates were then incubated for 30 min at room temperature to allow HA-specific antibodies in the plasma/serum to bind to the virus. Then, 50 μ l of a 0.5% suspension of turkey red blood cells (Lampire) that was washed once with PBS was added to each well, and the plates were incubated at 4°C until the red blood cells in PBS control samples settled to the bottom. Human plasma samples were tested in duplicate, and pooled mouse sera were tested in triplicate. Because of the limited volume, individual mouse sera were tested once. The HI titer was defined as the reciprocal of the highest dilution of plasma (serum) that inhibited red blood cell hemagglutination.

Human subjects. Eighteen individuals provided informed consent and donated blood before/on the day of seasonal influenza vaccination as well as 4 to 8 weeks later. Plasma samples were stored at -80°C until use. The Institutional Review Board (IRB) of the Icahn School of Medicine at Mount Sinai approved the study.

Statistics. Statistical data were generated using GraphPad Prism (version 5.03) software (GraphPad Software). Statistical significance between groups was determined by transforming HI titers into logarithmic values and performing one-way analysis of variance (ANOVA) with the Newman-Keuls posttest, as described previously (56). Normalized HI titers were compared by Dunn-corrected Kruskal-Wallis tests.

Accession number(s). All constructed HA genes have been deposited in the NCBI nucleotide database and can be accessed under accession numbers [MH712767](#) to [MH712773](#).

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