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Antigenic Drift of the Influenza A(H1N1)pdm09 Virus Neuraminidase Results in Reduced Effectiveness of A/California/7/2009 (H1N1pdm09)-Specific Antibodies

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ABSTRACT The effectiveness of influenza vaccines against circulating A(H1N1)pdm09 viruses was modest for several seasons despite the absence of antigenic drift of hemagglutinin (HA), the primary vaccine component. Since antibodies against HA and neuraminidase (NA) contribute independently to protection against disease, antigenic changes in NA may allow A(H1N1)pdm09 viruses to escape from vaccine-induced immunity. In this study, analysis of the specificities of human NA-specific monoclonal antibodies identified antigenic sites that have changed over time. The impact of these differences on in vitro inhibition of enzyme activity was not evident for polyclonal antisera until viruses emerged in 2013 without a predicted glycosylation site at amino acid 386 in NA. Phylogenetic and antigenic cartography demonstrated significant antigenic changes that in most cases aligned with genetic differences. Typical of NA drift, the antigenic difference is observed in one direction, with antibodies against conserved antigenic domains in A/California/7/2009 (CA/09) continuing to inhibit NA of recent A(H1N1)pdm09 viruses reasonably well. However, ferret CA/09-specific antiserum that inhibited the NA of A/Michigan/45/2015 (MI/15) very well in vitro, protected mice against lethal MI/15 infection poorly. These data show that antisera against the homologous antigen is most effective and suggest the antigenic properties of NA should not be overlooked when selecting viruses for vaccine production.

IMPORTANCE The effectiveness of seasonal influenza vaccines against circulating A(H1N1)pdm09 viruses has been modest in recent years, despite the absence of antigenic drift of HA, the primary vaccine component. Human monoclonal antibodies identified antigenic sites in NA that changed early after the new pandemic virus emerged. The reactivity of ferret antisera demonstrated antigenic drift of A(H1N1)pdm09 NA from 2013 onward. Passive transfer of serum raised against A/California/7/2009 was less effective than ferret serum against the homologous virus in protecting mice against a virus with the NA of more recent virus, A/Michigan/45/2015. Given the long-standing observation that NA-inhibiting antibodies are associated with resistance against disease in humans, these data demonstrate the importance of evaluating NA drift and suggest that vaccine effectiveness might be improved by selecting viruses for vaccine production that have NAs antigenically similar to those of circulating influenza viruses.
Antigenic drift, influenza, monoclonal antibodies, neuraminidase, vaccines

From 2009 to 2016, A/California/7/2009 (CA/09), a very early influenza A(H1N1) pdm09 virus isolate, was the recommended H1N1 component for vaccine production. It was used to manufacture monovalent A(H1N1)pdm09 vaccines administered in October 2009 and to manufacture trivalent or quadrivalent seasonal influenza vaccines from 2010 to 2016. The effectiveness of these vaccines in children was modest; the rate of effectiveness was approximately 60% at best in 2009 to 2010 (1) and 2010 to 2011 (2) but was much lower for live attenuated vaccines in 2013 to 2014 and 2015 to 2016 (3). The poor effectiveness of influenza vaccines is most often attributed to antigenic drift of hemagglutinin (HA), since vaccine-induced antibodies (Abs) are not able to inhibit virus binding to cellular receptors and entry into cells. While there is evidence in a ferret model that antigenic changes can be selected under conditions of immune pressure (4), antigenic drift of HA was not observed with ferret antisera (5, 6), and it was the loss of human serum reactivity (7) which prompted the recommendation to update the A(H1N1)pdm09 virus component of influenza vaccines.

Neuraminidase (NA)-inhibiting antibody titers are a known correlate of immunity against influenza (8, 9), but antigenic drift of this antigen is not widely examined and the impact of an antigenic change on vaccine effectiveness has not been investigated. To examine whether the NA of A(H1N1)pdm09 viruses has undergone antigenic drift since its first introduction in 2009, we examined genetic changes related to the specificity of human monoclonal antibodies (MAbs), evaluated the reactivity of post-vaccination human sera, and performed classical antigenic analysis using ferret antisera. Our data show that there has been substantial antigenic change that is aligned with NA genetic evolution.

In a past study of NA drift, it was noted that differences in antigenicity measured by the classical “two-way” analysis with ferret antisera are often observed to occur in one direction only (10) such that antiserum against one of a pair of viruses reacts similarly against NAs of both viruses, while the other antiserum reacts poorly with the nonhomologous virus. This raises the possibility that antigenic drift may not always result in a loss of immunity. Since one-way drift was observed in the current reported analysis, an in vivo study was performed to assess whether in vitro NA inhibition (NI) antibody titers accurately predicted the ability of the polyclonal antiserum to protect mice against a lethal influenza virus challenge. In spite of being used at similar titers, NA-specific antibodies in polyclonal antiserum raised against CA/09 were not as effective as those raised in response to A/Michigan/45/15 (MI/15), a more recent A(H1N1)pdm09 virus which contains an NA that has experienced antigenic drift.

**RESULTS**

**Genetic changes in NA.** There have been significant changes in the amino acid sequence of NA since 2009. A phylogenetic tree representing the viruses tested in this study is shown in Fig. 1, highlighting the amino acid changes introduced since 2009. The prevalences of sequence changes in viruses isolated each year since 2009 are shown in Fig. 2, with the data supporting this figure provided in Table S1 in the supplemental material. Sequence differences between the NAs used to examine antigenic structure in vitro are shown in the alignment provided in Table S2.

N248D was a change that was introduced into the NA of circulating viruses very early in the pandemic (11) and was fixed in the population by October 2009. Consequently, >80% of NA sequences isolated in 2009 had this change, which has been retained in the NA of all subsequent isolates (Fig. 2). Other early changes in NA sequence occurred at N44S, V241I, and N369K; these were present in a few 2009 isolates, increased in prevalence over the ensuing years, and were present in most viruses isolated in 2015 (Fig. 2; see also Table S1). The N44S mutation introduced a predicted glycosylation site in the stalk of NA which is likely to have stabilized the
tetramer (12) and increased enzyme activity (13). A change in NA surface residue N2005 (this position was never glycosylated) was first observed in 2011 viruses, increased in frequency in subsequent years, and is now present in most A(H1N1)pdm09 isolates. In 2012, a few viruses were isolated that had mutations at residue 451 of NA (A/Bangladesh/2012 is an example); however, this change was not maintained in the virus population or in associated HA genetic group 6A. Instead, HA genetic group 6B emerged and has prevailed. In 2013, clade 6B viruses predominated; however, the NA genetic sequences were heterogeneous at that time. The NAs of some 2013 viruses, including A/Bolivia/559/2013, which was a component of the 2016 live attenuated vaccine, contained a N397K change in charge that did not increase in prevalence in subsequent years, while a K432E change, together with conservative changes at I34V and I321V, increased in prevalence. The prevalence increase occurred along with the
introduction of N386K, which resulted in the loss of a predicted glycosylation site. Amino acid changes V264I and N270K (this is not a predicted glycosylation site), which were also observed in a few isolates in 2013, increased in prevalence and are present in the NAs of current A(H1N1)pdm09 viruses. HA clade 6B.1 viruses have been the predominant circulating A(H1N1)pdm09 virus strains to date, with additional changes in sequence introduced since 2015 (Fig. 1). A recent change in NA residue N449D that was observed in a few isolates in 2015 and 2016 increased to over 50% of the NA sequences reported in 2017 and was present in the sequences of almost all NAs of 2018 A(H1N1)pdm09 viruses reported up to 1 March 2018 (Fig. 2; see also Tables S1 and S2).

Antigenic domains of A(H1N1)pdm09 NA. The specificities of NA-reactive human monoclonal antibodies were examined to pinpoint antigenic domains. Eight NA-specific antibodies cloned from plasmablasts isolated from peripheral blood of four A(H1N1)pdm09-infected patients reacted with NA expressed on pCAGGS-NA-transfected 293 cells, allowing the specificity of antibodies to be determined by introduction of specific mutations (14). The NA inhibition patterns (Table 1) seen with a range of A(H1N1)pdm09 and previously seasonal A(H1N1) and H5N1 viruses demonstrated the breadth of reactivity and confirmed the location of each epitope. The full name of each MAb is shown in Table 1 along with a letter code that was assigned retrospectively to simplify reporting; two MAbs with similar specificities from the same donor were assigned codes D1 and D2. A description of some of these MAbs was published previously (15); therefore, only limited additional information is provided in this report.

MAb A (EM-3C02) was the most effective inhibitor of CA/09 NA activity (Table 1), with the lowest 50% inhibitory concentration (IC50) against CA/09 (28 ng/ml). It was unique in that it was specific for the NA of CA/09 and did not inhibit the enzyme activity of previous seasonal A(H1N1) viruses such as A/Brisbane/59/2007 (BR/07). Binding to cells expressing mutant NAs demonstrated that NA amino acid 369 was critical for MAb binding (Fig. S1); this amino acid is on the top surface of NA (Fig. 3). Amino acid change N369D was an early introduction into the viral population; approximately 50% of A(H1N1)pdm viruses isolated in the Northern Hemisphere during the winter of 2010 to 2011 contained this change. MAb A consequently does not inhibit the NA of A/Brisbane/70/2011 (BR/11) and subsequent A(H1N1)pdm09 viruses, including MI/15 (Table 1).
**TABLE 1** Functional properties of human NA-specific MAb

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<thead>
<tr>
<th>FDA no.</th>
<th>Clone no.</th>
<th>CA/09</th>
<th>BR/11</th>
<th>BA/12</th>
<th>HK/13</th>
<th>BO/13</th>
<th>MS/13</th>
<th>CR/13</th>
<th>IA/14</th>
<th>IN/16</th>
<th>MT/16</th>
<th>QU/17</th>
<th>HI/17</th>
<th>BR/07 (H1N1)</th>
<th>VN/04 (H5N1)</th>
<th>HK/14 (H3N2)</th>
<th>Critical amino acid</th>
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<td>NT</td>
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</tbody>
</table>

*The indicated MAb also inhibits cleavage of MU-NANA.*
The remaining 7 human MAbs tested were broadly reactive, inhibiting the NAs of previously circulating influenza A(H1N1) viruses as well as a number of other A(H1N1)pdm09 viruses. Except for D1 and D2, which had similar inhibition properties, the reactivity of each MAb was unique. Binding to NA mutants in cell-based enzyme-linked immunosorbent assays (ELISAs) (Fig. S1) and inhibition profiles (Table 1) revealed that amino acid N248 is critical for MAb C binding, G249 for MAb D binding, and D451 for MAb E and N273 for MAb F reactivities. The locations of epitopes critical for binding MAbs B and G have not been identified. The reactivity pattern of MAb B was similar to the patterns determined for MAbs D1 and D2, but MAb B had lower binding avidity (i.e., a larger amount of MAb is required to inhibit NA) and regained the ability to inhibit the NA of A/Quebec/RV1925/2017 (QU/17), suggesting that the specificities of MAb B and D1/D2 are different. MAb G bound all NAs of N1 subtype tested but inhibited NA activity poorly. MAb G did not lose binding to any of the mutants tested (Fig. S1B), and therefore its fine specificity is not known.

MAb F bound the NA of CA/09 (H1N1)pdm09 and BR/07 (H1N1) in ELISAs but inhibited the activity of previous seasonal virus BR/07 only, and not CA/09, in enzyme-linked lectin assays (ELLAs) (Table 1; see also Fig. S1). This suggests the plasmablasts from which this MAb was derived had been activated as the result of a recall response, an idea that is supported by data demonstrating that residue 273, a conserved amino acid in the NA of seasonal and A(H1N1)pdm09 viruses, was essential for binding of MAb F and by data from studies which demonstrated that residue 273 is the target of many broadly reactive mouse MAbs isolated following immunization with BR/07 (14). Although inhibition of CA/09 activity was not observed in ELLAs, the plaque size was reduced when MAb F was included in the agar overlay of cells infected with CA/09, demonstrating a potential functional benefit of antibodies with this specificity in reducing virus spread (Fig. S2). Interestingly, MAbs which bound amino acids conserved in NAs of previous seasonal and pandemic viruses had evidence of somatic hypermutation (Table S3), providing additional evidence of reactivation of memory B cells. This is similar to the HA-specific recall responses observed for these plasma cell donors (16).

The locations of amino acids critical for binding of MAbs A, C, D1, D2, and F are shown in Fig. 3. Residues highlighted in red are conserved, while those highlighted in purple have changed since the original 2009 isolates. Additional amino acids on the surface of NA that differ between CA/09 and MI/15, the virus most recently recommended for production of the A(H1N1)pdm component of seasonal influenza vaccines, are identified on the same dimer.
Antigenic analysis of NA with ferret antisera. While antigenic mapping with MAbs locates antigenic epitopes, this information does not identify amino acid mutations that result in loss of polyclonal antiserum binding. To examine antigenic drift of NA, inhibition titers of ferret antisera raised against individual viruses are measured against the homologous parent antigen as well as heterologous antigens. In performing drift analysis of NA, it is essential to perform two-way analysis since many NA-specific antibodies bind to conserved epitopes (10, 14). This two-way analysis is performed using H6N1 reassortant viruses as the source of each antigen to avoid potential inhibition by HA-specific antibodies. The antigenic relatedness of the NAs from two viruses can then be assessed by comparing NI antibody titers (data from multiple assays provided in Table S4), calculating percent relatedness (method of Archetti and Horsfall [17]) (Table 2) or by performing antigenic cartography (10) to generate an antigenic map (Fig. 4).

As previously observed (10), the antigenic differences between NAs of A(H1N1)pdm09 viruses occurred in one direction only. For example, most ferret antisera against CA/09 inhibited the NA of viruses isolated from 2011 to 2017 very well and only some anti-CA/09 sera had 4-fold-lower reactivity with the NAs from some more recent viruses such as MI/15 and A/Indiana/21/2016 (IN/16; Table S4). As a result, the antigenic relatedness of CA/09 and some more recent virus NAs, expressed as the ratio of anti-CA/09 titers against the heterologous/homologous antigens, was 50% or greater (Table 3). In contrast, sera against more-recent viruses did not inhibit earlier NAs well. For example, ferret anti-MI/15 has a homologous NI titer of 10,240 but a titer of 320 against CA/09 (Table S4; assay performed 11 July 2017), a 32-fold difference in titer, indicating there has been significant antigenic drift (i.e., a 4-fold or greater difference

### Table 2 Antigenic relatedness of H1N1pdm neuraminidases

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Genetic group</th>
<th>CA/09</th>
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<th>WA/12</th>
<th>PA/13</th>
<th>BO/13</th>
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aData from assays conducted on 11 July 2017 were used in calculations; values less than or equal to 25% relatedness are in bold font.

### Table 3 Percent reactivity of homologous antiserum with heterologous antigens (one-way)

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<td>IA/14</td>
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<td>100</td>
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<tr>
<td>MI/15</td>
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<td>IA/15</td>
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</table>

aData from assays conducted on 11 July 2017 were used in calculations; values less than or equal to 25% reactivity are in bold font.
in titers). Therefore, calculating the antigenic relatedness between CA/09 and MI/15, taking both anti-CA/09 and anti-MI/15 titers into account, showed that the NAs of these viruses are only 18% related (Table 2).

Cartographic analyses of genetic differences (amino acid sequence differences) (Fig. 4A) and antigenic differences (Fig. 4B and C) provide a way to interpret differences in sequence and data from multiple assays. Antisera were tested against multiple antigens, and therefore many NI measurements were used to determine the cartographic position of each antigen as well as each antiserum. The fundamental calculations needed to perform cartography resulted in distances between sera and viruses on in titers). Therefore, calculating the antigenic relatedness between CA/09 and MI/15, taking both anti-CA/09 and anti-MI/15 titers into account, showed that the NAs of these viruses are only 18% related (Table 2).
the antigenic map that are inversely related to the NI antibody titers. The one-way drift described above and demonstrated in Table 3 is therefore also evident in cartography since the position of each serum reflects how well it reacts with each antigen. Antisera with similar titers against homologous and heterologous antigens are positioned between the corresponding two antigens, whereas antisera that react poorly with heterologous antigens are positioned far from that antigen but close to the homologous antigen. For example, anti-CA/09 is positioned between CA/09 and MI/15 antigens, while anti-MI/15 is close to its homologous antigen (Fig. 4C). An additional advantage of antigenic cartography is that the analysis is performed with data from multiple assays, building a map to represent overall antigenic differences.

Examining the relatedness of antigens by method of Archetti and Horsfall (Table 2), showed that the differences in the antigenicity of the NA began with viruses isolated in 2013; however, the 2013 antigens themselves differed from one another as indicated by the reactivity of antisera against the NAs of A/Pennsylvania/7/2013 (PA/13), A/Bolivia/S59/2013 (BO/13), A/New Hampshire/4/2013 (NH/13), and A/Hong Kong/5008/2013 (HK/13). This analysis clearly shows that PA/13, a virus in HA genetic group 6C, has an NA that is antigenically distinct from those of BO/13, NH/13, and HK/13, all group 6B viruses. The same analysis indicated the NA of BO/13 and the NA of NH/13 are similar (50% relatedness) but are antigenically distinct (relatedness is 25% or less) from the NAs of earlier viruses. Of the viruses tested, the only amino acids accessible to antibodies on the top surface of the NAs that differed between BO/13 or NH/13 and A/Costa Rica/5802/13 (CR/13), a 6C virus with NA that was poorly inhibited by the anti-6B sera, are residue 397 (K in 6B but N in the 6C virus) and residue 432 (E in 6B but K in the 6C virus). These are within neighboring loops at the top of NA, close to the enzyme’s active center (Fig. 3).

The ferret antisera against NH/13 and BO/13 had lower titers against the NAs of HK/13 and later viruses than against the homologous NAs (and vice versa; Table S4). The only difference in sequence that is retained in the more recent viruses and is present in HK/13 but not in BO/13 is N386D (Table S2), which results in a change in charge, as well as in the loss of a potential glycosylation site. The data shown in Fig. 3 revealed that this amino acid is located on the side of the NA monomer, and therefore it is anticipated that removing a carbohydrate group that potentially masks this surface would expose antigenic domains previously not accessible to antibodies.

Interestingly, although the sequences of NAs from A(H1N1)pdm09 HA genetic groups 6B.1 and 6B.2 show that, like the HA, the NAs are genetically distinct (Fig. 4A), reactivity with ferret antisera indicated that they are antigenically similar (Fig. 4C). To examine whether NAs of viruses isolated in 2016 and 2017 are antigenically similar to the NA of the virus recommended for the 2017–2018 vaccine production, a number of H6N1 constructs were prepared and tested for reactivity with ferret antiserum generated against MI/15. The NI antibody titers against MI/15 and viruses isolated in 2017 were similar, suggesting that the amino acid differences (Table S2) did not impact the antigenic structure.

Analysis of human antisera. NI antibody titers were measured for sera collected from adults vaccinated with the 2016–2017 formulation of inactivated seasonal influenza vaccine. The sera were obtained under an approved protocol at a workplace where influenza vaccination is offered on an annual basis. The vaccine administered contained a CA/09-like A(H1N1)pdm09 component. Of 32 vaccinees, 9 exhibited a 4-fold or greater response to the NA of CA/09. All 9 vaccinees who responded to the CA/09 NA component reacted well against recent A(H1N1)pdm09 NAs (Table 4), reminiscent of the reactivity of ferret antiserum generated by infection with CA/09 and suggestive of the presence of antibodies that bind conserved antigenic domains (14, 15, 18).

NI antibody titers for sera collected from a small cohort of patients infected with A(H1N1)pdm09 in 2009 were also measured at the time of study enrollment and approximately 4 weeks later (19). The sera that exhibited a ≥4-fold increase in titer
against the homologous CA/09 NA antigen were also tested for inhibition of NA from more-recent viruses. In contrast to the adult vaccinees, two of four infected patients had a significantly lower (4-fold or greater) titer against more-recent viruses, including MI/15 (Table 5), showing that the CA/09-like viruses induced antibodies that discriminate between the NAs of the infecting strain and more-recent circulating viruses.

Protection mediated by NA-specific antibodies. Given that ferret anti-CA/09 inhibits the NA of recent viruses well in vitro, one might expect that NA-specific antibodies in this serum would be as effective in vivo as antibodies raised against a more recent virus. Previous experiments have demonstrated that while broadly reactive MAbs are beneficial, significantly greater amounts are needed to protect against lethal virus challenge (20, 21). This raised the concern that the antibodies in serum elicited by CA/09 which bind conserved epitopes of MI/15 NA may be poorly effective in vivo.

To compare the effectiveness of NA-specific antibodies induced by CA/09 and MI/15, we transferred ferret anti-CA/09 and anti-MI/15 to mice and then challenged them with a lethal dose (LD) of a reassortant H6N1 virus expressing the NA of MI/15. This H6N1MI/15 reassortant was used to avoid neutralization of infection by HA-specific antibodies present in the transferred ferret antiserum. Any differences in protection observed in DBA/2 mice treated with ferret sera raised against CA/09 and MI/15 were therefore likely due to NA-specific antibodies. Mice that received no serum or that received normal ferret antiserum served as controls. Two different amounts of sera were transferred in separate experiments. Ferret anti-CA/09 had the same NA inhibition antibody titers against the CA/09 and MI/15 antigens (1,280), while the titers of ferret anti-MI/15 tested against CA/09 and MI/15 antigens were 320 and 5,120, respectively.

A 1:2 dilution of ferret antiserum was transferred to naive mice in experiment 1 to evaluate the greatest ability for the sera to protect. Separate groups of mice treated with a 1:2 dilution of each serum were challenged with an H6 reassortant expressing the NA of CA/09 and MI/15 were therefore likely due to NA-specific antibodies. Mice that received no serum or that received normal ferret antiserum served as controls. Two different amounts of sera were transferred in separate experiments. Ferret anti-CA/09 had the same NA inhibition antibody titers against the CA/09 and MI/15 antigens (1,280), while the titers of ferret anti-MI/15 tested against CA/09 and MI/15 antigens were 320 and 5,120, respectively.

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Virus titers, levels of weight loss, and survival rates of groups of mice that received anti-CA/09 and anti-MI/15 sera 18 h prior to challenge with a 10× 50% lethal dose (LD50) of H6N1MI/15 were compared (Fig. 5). Mouse survival was monitored over 14 days, and virus titers were measured in lungs collected on day 6 postinfection. The H6N1CA/09 virus was more pathogenic than the H6N1MI/15 virus, resulting in rapid weight loss and
death of all control mice challenged with the H6N1CA/09 preparation by day 5 compared to the mice challenged with H6N1MI/15, which succumbed on day 7 or 8 (Fig. 5B, D). Nevertheless, anti-CA/09 protected 50% of the H6N1CA/09-challenged mice whereas none of the mice treated with anti-MI/15 survived. The survival curves show that anti-CA/09 provided some protection against heterologous H6N1MI/15 infection when the NI antibody titer was high that but the most effective protection was associated with transfer of anti-MI/15 serum (Fig. 5B). Virus levels were not measurable in the lungs of mice treated with a 1:2 dilution of ferret anti-MI/15, but virus was recovered from the mice treated with anti-CA/09 (Table 6). When the dose of antiserum was normalized to transfer an amount of anti-CA/09 and anti-MI/15 that inhibited the NA of MI/15 to the same extent, the challenge virus was recovered on day 6 postinfection in all groups of mice, as expected for this permissive infection model; however, the group treated with the homologous antiserum (anti-MI/15) had significantly lower lung virus titers than the anti-CA/09-treated group (Table 6). Furthermore, all H6N1MI/15-challenged mice treated with a 1:20 dilution of ferret anti-MI/15 survived, whereas only one of eight mice treated with a 1:5 dilution of anti-CA/09 survived (Fig. 5F).

**DISCUSSION**

CA/09 is an early influenza A(H1N1)pdm09 virus that was recommended for production of the monovalent pandemic vaccine in 2009 and for production of the A(H1N1)pdm09 component of trivalent and quadrivalent seasonal influenza vaccines from 2010 to 2016. The data provided in this report indicate that during this period, the
NA experienced changes in amino acid sequence which impacted antigenicity and reduced the effectiveness of NA-specific antibodies raised against CA/09. Loss of binding to NA by some human MAbs suggests that the observed changes in amino acid sequence (N248D, N369K, and N449D) are the result of immune pressure. Other changes in sequence that have accumulated on the NA surface may also impact antibody binding. Despite the amino acid sequence changes, polyclonal antisera raised against CA/09 inhibited the NA of circulating A(H1N1)pdm09 viruses well, most likely due to the presence of antibodies which bind conserved antigenic domains. These may include amino acids 249, 273, and 451, which were identified as critical for binding of some human MAbs.

Due to the broad reactivity of polyclonal CA/09-specific antiserum, identification of NA drift required testing of the reactivity of sera raised against more-recent A(H1N1)pdm09 viruses. Drift was indeed evident, with ferret antisera raised against recent viruses such as MI/15 showing poor inhibition of the NA of earlier viruses, including CA/09. The biggest change in antigenic relatedness was observed in 2013. During that season, a number of antigenically distinct NAs cocirculated—all with K432E, and some with N397K (for example, BO/13 and NH/13)—while others had N386K (HK/13), the latter resulting in loss of a predicted glycosylation site. All 3 changes are present in the NAs of current A(H1N1)pdm09 viruses.

Considering that the NI antibody titers of antisera raised against CA/09 measured in vivo were approximately equivalent against the immunogen and against the NA of circulating strains, it was anticipated that these antibodies would offer good protection against infection. However, our studies comparing the efficacies of treatment with different NA-specific MAbs demonstrated greater protection against lethal CA/09 infection by strain-specific antibodies than by an antibody that bound to an epitope which is conserved across the subtype (21). Since ELLA, the test used to measure NA inhibition titers, cannot discriminate between antibodies that bind to strain-specific and antibodies that bind to cross-reactive epitopes, there was a possibility that the polyclonal ferret antisera against CA/09 and MI/15 were qualitatively different and might differ in effectiveness. The mouse challenge studies demonstrated that this was indeed the case, providing additional evidence of antigenic drift.

Determining whether there is a need to update influenza vaccines with viruses that have NAs antigenically matched to circulating viruses is not straightforward. Analysis of drift cannot rely on measuring NA inhibition titers of ferret sera against the vaccine strain alone because titers may be confounded by broadly reactive antibodies that are poorly effective in vivo. Future development of an assay that can discriminate between strain-specific and broadly reactive antibodies would facilitate NA drift analysis. Until then, it will be critical to conduct two-way antigenic analyses, with ferret antisera raised against both the vaccine and circulating viruses tested for reactivity against homologous and heterologous antigens.

However, even this may not always provide a clear understanding of antigenic drift from which to make vaccine recommendations for seasonal influenza. Since antisera are collected from ferrets that have experienced a single virus infection, the reactivity is

<table>
<thead>
<tr>
<th>Group</th>
<th>Ferret antiserum</th>
<th>% survival</th>
<th>Avg virus titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1 (1:2 dilution of ferret antiserum)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>0</td>
<td>4.36 ± 0.17</td>
</tr>
<tr>
<td>B</td>
<td>Anti-CA/09</td>
<td>60</td>
<td>2.13 ± 2.01</td>
</tr>
<tr>
<td>C</td>
<td>Anti-MI/15</td>
<td>100</td>
<td>&lt;LOD$^a$</td>
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</table>

| Expt 2 (anti-CA/09 at 1:5; anti-MI/15 at 1:20) | | |
| A     | Naive ferret serum | 0          | 3.95 ± 0.54             |
| B     | Anti-CA/09        | 12.5       | 4.46 ± 0.10             |
| C     | Anti-MI/15        | 100        | 2.95 ± 0.47             |

$^a$LOD, limit of detection.
quite distinct from that of human sera which contain antibodies with specificities shaped by prior infections and vaccinations (22). The nature of the recall response to
NA is likely similar to the observed back-boost in HA antibody reactivity, as numerous monoclonal antibodies that bind conserved epitopes of N1 (this publication) and N2
(15) have been isolated from plasmablasts of patients. Interestingly, MAb A, the single human monoclonal antibody in our study that is strain-specific, was isolated from a
patient likely to have experienced a primary influenza virus infection (16). Asn at 369 of
NA was identified as critical for binding of MAb A; however, it was replaced by Asp very
early after the emergence of A(H1N1)pdm09 viruses in humans. This change was not
sufficient to alter the reactivity of either ferret or human polyclonal antisera.

Ferret antisera do not always identify antigenic changes in HA that are relevant to the
human response. For example, a mutation in the HA of A(H1N1)pdm09 viruses that
reduced binding of antibodies in a substantial proportion of individuals born between 1965
and 1979, and that likely contributed to reduced vaccine effectiveness in this age group,
was not revealed by serum from ferrets previously infected with CA/09 alone (7). One way
to overcome this gap in information using ferret antisera is to include age-stratified human
sera for antigenic analysis of vaccine and circulating influenza viruses.

The consequences of antigenic drift of NA with respect to influenza vaccine effec-
tiveness have not previously been investigated in an animal model and are difficult to
assess in clinical studies because antibodies to HA dominate the response and are a
critical element of immunity. In addition, it is often argued that influenza vaccines
contain inconsistent or very small amounts of NA, resulting in small increases in
NA-specific antibody titers that may not contribute to immunity. However, multiple
studies have shown increases in NA inhibition antibody titers following vaccination
with either inactive or live attenuated vaccines (23–25), and clinical challenge studies
demonstrate a correlation between NA inhibition titers and reduced symptoms of
disease (26). These results demonstrate the importance of considering NA drift when
selecting viruses for vaccine production and suggest the antigenic changes in NA may
have contributed to past vaccine failures. While the poor effectiveness of live attenu-
ated vaccines in the influenza seasons of 2013 to 2014 and 2015 to 2016 was attributed
to poor replication of the A(H1N1)pdm09 component (27), past clinical challenge
studies demonstrated that NI antibody titers correlate with protection against disease
following vaccination with live attenuated viruses (8), raising the possibility that
antigenic drift of NA was an additional contributing factor to this vaccine’s failures.

The mechanisms that contribute to protection by NA-binding antibodies include
inhibition of enzyme activity and reduction in virus load through complement (C)-
mixed or FcR-mediated lysis of infected cells. Our studies focused on direct inhibi-
tion of enzyme activity by using DBA/2 mice that have impaired innate immune
mechanisms, including NK cell (28) and complement (29, 30). Further studies are
needed to establish the contribution of C-mediated or FcR-mediated mechanisms to
clearance by NA-binding antibodies.

The availability of tools to perform immunogenicity studies (31, 32) and the ability
to create suitable reassortant viruses at will by reverse genetics (33) have enabled many
recent clinical studies to measure responses to NA. Further studies of NA antigenic drift
and of the in vivo consequences of antigenic differences between vaccine and chal-
lenge viruses are warranted to establish the clinical value of NA in current and future
influenza vaccines.

Conclusion. Phylogenetic analyses and the specificities of human MAbs suggest
that significant changes in the antigenic structure of NA of A(H1N1)pdm09 viruses have
occurred since these viruses first emerged in 2009. This study used classical analysis of
ferret antisera in NA inhibition assays to demonstrate that antigenic drift has indeed
occurred. Our study brought to light a shortcoming of NI antibody titers measured in
vitro: because the in vitro assay does not differentiate between strain-specific and
broadly reactive antibodies, NI antibody titers may not accurately predict in vivo
effectiveness. Our results demonstrate that the best protection against lethal virus
challenge is obtained with antiserum generated against a virus containing the homologous NA. Given the extensive evidence that NA-inhibiting antibodies contribute to resistance against disease (8), milder epidemics on a population basis (34, 35), and shorter duration and severity of illness (26), it is therefore best to recommend viruses with both HA and NA antigenically matched to those of circulating viruses for vaccine production.

**MATERIALS AND METHODS**

**Viruses.** A(H1N1)pdm09 viruses were kindly supplied by the U.S. Centers for Disease Control and Prevention, Atlanta, GA, or by the WHO Collaborating Center (Victorian Infectious Diseases Reference Laboratory [VIDRL]) in Melbourne, Australia. H6N1 reassortant viruses were generated by transfecting 293 and Madin-Darby canine kidney (MDCK) cell cocultures with eight plasmids, each representative of an individual influenza gene segment, as previously described (33). The NA genes were cloned from the targeted A(H1N1) or A(H1N1)pdm09 virus or created by site-directed mutagenesis of a related plasmid. Viruses in cell supernatants were then amplified in embryonated chicken eggs. The HA and NA genes were sequenced by CBER’s core facility to confirm that the sequences were the same as those reported to the Global Initiative on Sharing All Influenza Data (GISAID).

**Human MAbs and antisera.** Human MAbs were generated by cloning various immunoglobulin genes from plasmablasts and expressed in 293 cells as previously described (36). Secreted MAbs were purified from the supernatant using protein A beads and screened for reactivity with influenza virus in enzyme-linked immunosorbent spot (ELISpot) assays (16). MAbs that did not have HA inhibition (HI) activity and that did not bind to recombinant HA were tested for NA specificity by ELISA with NA of CA/09 transiently expressed on 293 cells, as previously described (14).

Human sera were collected from patients enrolled in protocol no. 07-l-0229 (Influenza in Non-immunocompromised and Immunocompromised Hosts) at the NIH Clinical Center. Participants were enrolled during a period of active confirmed influenza virus infection, and sera were collected at the time of diagnosis and again at 1 and 2 months after diagnosis. This study was approved by the National Institute for Allergy and Infectious Diseases (NIAID) Institutional Review Board (NIAID, NIH, Bethesda, MD) and was conducted in accordance with the provisions of the Declaration of Helsinki and good clinical practice guidelines. A separate study approved by the Institutional Review Board, National Institute of Biological Standards and Control (London, United Kingdom), collected serum samples from healthy adult volunteers immediately before and 3 weeks after administration of standard seasonal trivalent influenza vaccine (split virion, inactivated, containing the following antigens: A/California/7/2009 NYMC X-179A (H1N1)pdm09, A/Hong Kong/4801/2014 X-263B (H3N2), and wild-type B/Brisbane/60/2008). Blood was allowed to clot at room temperature for 6 h, and sera were collected after centrifugation. The serum was heat inactivated at 56°C for 30 min and then stored at −20°C.

**Antigenic analysis of NA.** Influenza seronegative ferrets were infected with wild-type A(H1N1)pdm09 viruses following protocols approved by institutional animal care and use committees. Blood was collected 3 weeks later, and the sera were stored in aliquots at −30°C. The serum was subjected to heat treatment (56°C for 45 min), and the HI antibody titers were measured against H6N1 viruses in ELLAs performed following a previously published method (32). Each H6N1 virus expressed the NA of either a reference or circulating virus. A 50% endpoint titer (the inverse of the lowest dilution that resulted in at least 50% inhibition of enzyme activity) was recorded from the average determined with duplicate serum dilution series. The antigenic relatedness was calculated using data collected for viruses in a single assay performed on 11 July 2017 (see Table S4 in the supplemental material). Nonlinear regression analysis was performed using GraphPad Prism to determine 50% inhibition concentrations (IC50) for MAbs.

**Phylogenetic analysis.** Sequences of all NAs were obtained from GISAID. Initial phylogenetic trees were constructed using RaxML (version 8.2.3), followed by further optimization of the tree topology and branch lengths using Genetic Algorithm for Rapid Likelihood Inference (GARLI), version 2.0 (37), for 10,000 generations using GTR+I+G4 as the evolutionary model. Over 31,000 A(H1N1)pdm09 NA sequences were downloaded from the GISAID database (1 March 2018). Each sequence was analyzed for the presence of particular sequence motifs that contained specific amino acids at positions 44, 200, 241, 248, 264, 270, 321, 369, 386, 432, and 449. The amino acids at these sites were (respectively) NNVNVN (consensus sequence for NA of CA/09-like NA sequences), NNVDVNINKN (change in consensus at N248D), NIDVNVNINKN (in addition to N248D, changes at V241I and N369K), NIDVNIKNN (in addition to the preceding change, N44S, resulting in the introduction of a predicted glycosylation site at residue 42 in the NA stalk), SISIDVKKNKNKNN (in addition to the preceding change, I321V and K432E), SSIDVNVKKEN (in addition to the preceding change, N386K, resulting in loss of potential glycosylation site), SSIDVNSKKKN (in addition to the preceding change, V264I and N270K), and SSIDVNSKKED (in addition to the preceding change, N449D). Viruses that did not contain any of these sequence strings were grouped and reported as “other” in Fig. 2.

**Cartography.** Genetic and antigenic analyses were performed as described (10). Data from all ELLA NI assays (provided in Table S4) were used in the analysis. Antigenic cartography methods for analysis of HI data for human influenza A (H3N2) viruses were followed as described previously (38). Modified multidimensional scaling methods were used to arrange the antigen and antiserum points in an antigenic map to best satisfy the target distances specified by the NI data. Because antisera were tested against multiple antigens and because antigens were tested against multiple antisera, many measurements were used to determine the positions of the antigen and antiserum points in each
antigenic map. In the ultimate antigenic maps, the distances between sera and viruses are inversely related to the Ni antibody titers. The method used for making the genetic map was the same as that used for making the antigenic maps, except that the target distances represented the number of amino acid substitutions between the members of each set of virus NA sequences (38).

Mouse challenge studies. Groups of 8-week-old female DBA/2 mice (The Jackson Laboratory, Bar Harbor, ME) were treated intraperitoneally (i.p.) with ferret antiserum raised against CA/09 (H1N1pdm09) at either a 1:2 (experiment 1) or a 1:5 (experiment 2) dilution or with ferret antiserum raised against MI/15 (H1N1pdm09) at either a 1:2 (experiment 1) or a 1:20 (experiment 2) dilution. The mice were challenged intranasally 18 h later with 10 LD50 of H6N1 reassortant virus containing the NA of MI/15. The reassortant virus with HA of a different subtype was engineered as previously described (33) and used to overcome inhibition by HA-specific antibodies. No serum or an equal volume of normal ferret serum was transferred i.p. to control mice. Lungs were collected for viral titration (n = 3 per treatment group) on days 4 and 6 postinfection. Survival rates and body weights of the remaining mice (n = 8 per treatment group) were monitored for 14 days. Mice were euthanized if ≥25% of initial weight was lost. All mouse experiments were conducted in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee (CBER/FDA).

Data availability. Antibodies and reassortant viruses may be obtained through a Material Transfer Agreement (MTA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00307-19.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.5 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.05 MB.
TABLE S4, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

All NA sequences were retrieved from GISAID. We are indebted to the National Influenza Centers and WHO Collaborating Centers for providing these sequences to the scientific community. We thank the Division of Veterinary Services (CBER, FDA) for animal care and CBER’s core facility for providing sequencing services. Plasmids carrying internal genes of A/Puerto Rico/8/34 (PR/34) (H1N1) and used for cloning HA and NA were kindly supplied by St. Jude Children’s Research Hospital, Memphis, TN, USA.

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J.G. created reassortant viruses and performed serologic analysis; L.C. analyzed MAbs and performed animal experiments; D.F.B. and D.J.S. performed phylogenetic and prevalence analysis; H.W. developed methods and generated ferret antisera; P.W. produced human MAbs; M.J.M. and J.K.T. performed clinical study to collect postinfection sera; X.X. provided viruses and generated ferret antisera; R.H. performed study to collect postvaccination sera; J.W. and R.A. collected samples for production of human MAbs; R.A.M.F. performed cartographic analysis; M.C.E. conceived the hypothesis, designed experiments, analyzed data, and initiated writing of the manuscript. All of us reviewed the manuscript and contributed to the final version.

We have no conflicts of interest to declare.

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