Short Communication

Antigenic drift in the evolution of H1N1 influenza A viruses resulting from deletion of a single amino acid in the haemagglutinin gene

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Two genetically distinct lineages of H1N1 influenza A viruses, circulated worldwide before 1994, were antigenically indistinguishable. In 1994, viruses emerged in China, including A/Beijing/262/95, with profound antigenic differences from the contemporary circulating H1N1 strains. Haemagglutinin sequence comparisons of either a predecessor virus, A/Hubei/52/94, or one representative of the cocirculating A/Bayern/7/95-like clade, A/Shenzhen/227/95, revealed a deletion of K at position 134 (H3 numbering) in the antigenic variants. The K134 deletion conferred a selective advantage to the Chinese deletion lineage, such that it eventually gave rise to currently circulating H1 viruses. Using reverse genetics to generate viruses with either an insertion or deletion of aa 134, we have confirmed that the K134 deletion, rather than a constellation of sublineage specific amino acid changes, was sufficient for the antigenic difference observed in the Chinese deletion lineage, and reinsertion of K134 revealed the requirement of a compatible neuraminidase surface glycoprotein for viral growth.

Approximately 20% of the world’s population is infected by influenza A each year, resulting in significant mortality and morbidity (Stohr, 2002). The high incidence of influenza cases is attributable to the ability of the influenza virus to escape immunity induced by prior infection or vaccination. This escape is potentiated by the accumulation of mutations in the surface glycoproteins haemagglutinin (HA), and to a lesser extent neuraminidase (NA), which confer antigenic change to the virus. This phenomenon, known as antigenic drift, necessitates annual vaccine updates to confer protection against the currently circulating strains.

Mutations in the HA molecule are considered to contribute almost entirely to the antigenic drift observed among influenza A viruses (Wilson & Cox, 1990). Those sites at the distal tip of the H1 HA molecule and on the side of the globular head near the receptor-binding pocket appear to be the main targets of the human immune response (Cox & Brokstad, 1999; Raymond et al., 1986; Sato et al., 2004). Influenza A viruses bind to sialic acids on the surface of target cells via a depression in the distal surface of the globular head of the HA molecule (Weis et al., 1988; Wilson et al., 1981). Several residues within this depression are highly conserved across the HA subtypes, including residues 98, 134, 138, 153 and 183 (H3 numbering) (Nobusawa et al., 1991). Amino acid substitutions within the receptor-binding pocket or the ‘second shell’ residues, including 190, 225 and 158, may alter the specificity toward certain types of galactosidic linkages, namely 2–6Gal or 2–3Gal linkages (Aytay & Schulze, 1991; Matrosovich et al., 2000). Because of its location on the HA three-dimensional structure, mutations in the receptor-binding pocket or second shell residues can alter the antigenicity of a virus in addition to, or instead of, modifying receptor specificity or affinity (Daniels et al., 1984).

The HA1 domains of the HA genes from a subset of influenza A/H1N1 viruses collected worldwide from 1977 to 1999 were sequenced and analysed as described previously (Shaw et al., 2002; Xu et al., 2004). These isolates were selected to reflect the genetic and geographical distribution spectrum of influenza A/H1N1 viruses. Accession numbers for new and previously published influenza virus genes and amino acids alignments used in this study can be found in Supplementary Table S1 and Supplementary Fig. S1, respectively, available in JGV online. The obtained sequences were assembled, aligned and edited using the DNASTAR (Madison, WI) and BIOEDIT version 5.0.6 (North Carolina State University) software. Phylogenetic trees were generated with the use of the MEGA version 3.1 software (Kumar et al., 2004).

Sequence alignments and phylogenetic analysis of influenza H1N1 strains isolated before 1994 revealed two clades, the...
A/Bayern/7/95-like clade and the A/Hebei/52/94-like clade, which although genetically distinguishable were antigenically indistinguishable. In 1995, virus isolates emerged in China that had a deletion of aa 134 (Fig. 1). While a comparable deletion had been previously observed in strains from 1935 (A/Alaska/35) and from an immunocompromised child (Rocha et al., 1991), the Chinese deletion lineage became the precursor of the current human H1 viruses (Nakajima et al., 2000). Based on the phylogenetic tree, three strains were chosen for reverse genetic experiments to examine the genetic basis of the antigenic differences that arose during this period; A/Hebei/52/94 was the Chinese virus precursor to the deletion mutant, A/Beijing/262/95, and A/Shenzhen/227/95 from the A/Bayern/7/95-like lineage, which did not give rise to a deletion and eventually became extinct.

Viral RNA (vRNA) was extracted from A/Hebei/52/94-, A/Beijing/262/95- and A/Shenzhen/227/95-infected chicken allantoic fluid using the QIAamp vRNA kit (Qiagen) and the full-length HA and NA genes were amplified and cloned into the bidirectional vector pBD as described previously (Maines et al., 2006). Lysine was inserted at position 134 in the A/Beijing/262/95 HA gene, or deleted in the A/Hebei/52/94 and A/Shenzhen/227/95 HA genes using the QuikChange Site-Directed Mutagenesis kit (Stratagene), and the resulting wild-type and mutant plasmids were sequenced both to confirm the plasmid identities and the absence of additional changes.

Infectious recombinant influenza A viruses were rescued by cotransfecting 293T cells with six A/Puerto Rico/8/34 (PR8) vRNA and protein expression plasmids (pCI-PR8-PB1, pCI-PR8-PB2, pCI-PR8-PA, pCI-PR8-NP, pCI-PR8-M and pCI-PR8-NS) (Subbarao et al., 2003) and two plasmids bearing the wild-type NA and either a wild-type or modified HA gene using TransIT-LT1 (Mirrus) as described previously (Hoffmann et al., 2000). Nine to 11-day-old embryonated chicken eggs were infected with 0.2 ml from each transfection, and recombinant virus and vRNA was isolated following incubation for 72 h at 35 °C. An HA1 amplicon was generated by RT-PCR and subsequently sequenced to ensure virus identity. Aliquots of recombinant viral stocks in allantoic fluid were maintained at −80 °C.

HA and HA inhibition (HAI) assays were performed using standard techniques as described previously (Kendal et al., 1982), using turkey red blood cells (University of Georgia, Athens, GA). Seronegative and post-infection ferret antisera raised against the reference H1N1 viruses were provided by our colleagues in the Diagnostic and Strain Surveillance Branch of the Influenza Division. Briefly, serologically naïve ferrets were inoculated intranasally with 1 ml diluted virus. Serum was collected via cardiac puncture 14 days post-immunization according to Institutional Animal Care and Use Committee regulations (National Research Council, 2002).

Nearly all the recombinant viruses grew well in eggs, reaching HA titres of 1024. The two exceptions were the A/Hebei/52/94 strain bearing a deletion of K134 (Heb/DK134), which grew to a titre of 64, and the A/Beijing/262/95 strain bearing an insertion of K134 (Bei/insK134), which

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**Fig. 1.** Phylogenetic tree of the A/Bayern/7/95-like and A/Hebei/52/94-like clades of H1N1 viruses circulating pre-1994–1995 that gave rise to the K134 deletion. Isolates in bold with an asterisk (*) represent vaccine strains and strains that are boxed are those used in this study. Genetic groups are indicated by brackets.
could only be rescued when the NA gene of A/Beijing/262/95 was replaced with that of A/Hebei/52/94. These two NA polypeptides differ by only 1 aa; A/Hebei/52/94 bears an alanine at position 13, while A/Beijing/262/95 bears a valine [Supplementary Fig. S1(b) available in JGV Online].

The reassortant wild-type (Bei/WT Heb/NA) and mutant (Bei/insK134 Heb/NA) viruses were able to grow to a titre of 1024. In contrast, both the reassortant viruses bearing either the wild-type or ΔK134 mutant HA of A/Hebei/52/94 and A/Beijing/262/95 NA were able to be rescued although at significantly lower titres (256 and 64, respectively). While the role of residue 13 of the NA glycoprotein in the replication of these recombinant viruses is unclear, the functional interaction between the activities of the HA and NA glycoproteins of influenza is well documented (Baigent & McCauley, 2001; Baum & Paulson, 1991; Kaverin et al., 2000; Lu et al., 2005; Wagner et al., 2000).

The effects of K134 insertion or deletion on the three-dimensional structure of the HA trimer were investigated by producing molecular graphic images based on the PR8 and A/swine/Iowa/30 HA trimers using the Chimera package (Computer Graphics Laboratory, University of California, San Francisco, USA) (Gamblin et al., 2004; Huang et al., 1996; Sanner et al., 1996). The models predicted that residue 134 lies along the edge of the receptor-binding pocket, potentially reducing the size of the pocket (Fig. 2). Addition of K134 did not appear to cause gross changes in the three-dimensional structure of HA, but was predicted to introduce a strong-positive charge on the right side of the receptor-binding pocket as well as a slightly reduced negative charge at the bottom of the pocket (Supplementary Fig. S2 available in JGV Online). The predicted change in charge distribution did not impact HA periodate sensitivity of the recombinant A/Beijing/262/95 viruses, while the deletion of K134 in A/Hebei/52/94 HA did result in a modest increase in sensitivity to periodate of the recombinant viruses (data not shown), suggesting that residue 134 might alter the specificity or the avidity of receptor binding. Additional amino acid differences in the receptor-binding pocket of A/Hebei/52/94 and A/Beijing/262/95 HAs, including residues 190, 225 and 226 (H3 numbering) known to impact receptor specificity, may play a role in magnifying the effect of deleting residue 134 in A/Hebei/52/94 HA (Supplementary Fig. S1 available in JGV Online) (Matrosovich et al., 1997, 2000). Interestingly, Kaverin et al. (2000) reported that virus aggregation, resulting from incompatibilities between the HA and NA glycoprotein activities, could be overcome by mutation in the vicinity of the receptor pocket of HA by increasing the local negative charge. This increased negative charge was presumed to decrease the affinity of HA for sialic acid. The failure to rescue Bei/insK134 with its homologous NA may be due to the increased positive charge introduced around the pocket by K134, which may result in stronger affinity for the sialic acid, and consequently require compensatory changes in NA activity to allow viral entry and release. The role of residue 13 in NA activity is the subject of continuing investigation.

**Fig. 2.** Predicted HA trimer surface structure of H1N1 influenza with an insertion of K134. Space-filling representation of HA1 and HA2 domains in white and light blue, respectively. A single receptor-binding pocket is highlighted in yellow. The aa 133 is highlighted in red and aa 135 is highlighted in green. (a) Structure of wild-type PR8, bearing a deletion of residue 134. (b) Predicted structure of PR8 with insertion of K134, depicted in blue. (c) Structure of wild-type A/swine/Iowa/1930, bearing K134, depicted in blue.
The solvent accessibility of position 134, and therefore to neutralizing antibodies, lent support to the hypothesis that this residue could play a role in the antigenicity differences observed in the deletion lineage. All three wild-type recombinant viruses (Bei/WT, Heb/WT and Shen/WT) had HAI and microneutralization titres comparable to the reference antigens (Table 1 and Supplementary Table S2). Furthermore, the A/Beijing/262/95 reassortant bearing its homologous wild-type HA and the wild-type NA of A/Hebei/52/94 did not have an altered antigenic profile compared to the reference antigen. However, insertion of K at position 134 in the A/Beijing/262/95 HA gene resulted in the loss of titre to the homologous antiserum, and a significant gain of reactivity to both the A/Hebei/52/94 and A/Shenzhen/227/95 antisera. Likewise, deletion of K134 in either the A/Hebei/52/94 or the A/Shenzhen/227/95 (Shen/DK134) genes resulted in a virus with antigenic properties characteristic of A/Beijing/262/95. One additional substitution, at residue 166 of the HA1 domain is shared between the A/Hebei/52/94 and A/Shenzhen/227/95 genetic groups versus the A/Beijing/262/95 genetic group (Supplementary Fig. S1 available in JGV Online). This residue lies immediately C-terminal to a potential N-glycosylation site in antibody combining site D on the opposite surface of the HA monomer with respect to the receptor-binding pocket, and lies several residues away from the adjacent pocket of the assembled trimer (Caton et al., 1982). Amino acid substitution at residue 166 did not alter the antigenic properties compared to the wild-type or K134 mutant viruses (data not shown). However, it has been reported that structural conformation of the consensus N–X–T/S can impact the usage of the site (Bause et al., 1982; Bause, 1983). The substitution of N for K C-terminal to this site in A/Beijing/262/95 may alter the local conformation, resulting in differential usage of the site or altered conformation of the carbohydrate. In H3N2 influenza A, glycosylation near the receptor-binding pocket can alter the dependence on NA activity in viral growth (Baigent & McCauley, 2001). At present, it is unknown whether A/Beijing/262/95 HA is differentially glycosylated compared with that of A/Hebei/52/94 or if glycosylation at this site may also affect NA activity requirements in coordination with the presence or absence of K134.

The results presented suggest that K at position 134 acted as an immunodominant epitope in the immune response to H1N1 viruses during this period of evolution. Loss of this residue would have allowed the Chinese deletion lineage to escape neutralizing antibodies not only to the predecessor lineage, but also to the second H1N1 clade circulating at that time. By escaping herd immunity induced by both lineages, the deletion lineage gained a significant advantage allowing it to replace both circulating clades.

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References


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Table 1. Antigenic characterization of H1N1 recombinant viruses bearing K134 insertion or deletion in the HA glycoprotein

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<thead>
<tr>
<th>Virus strain</th>
<th>Antisera*</th>
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<tbody>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td>A/Hebei/52/94</td>
<td>40</td>
</tr>
<tr>
<td>A/Shenzhen/227/95</td>
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<tr>
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</tr>
<tr>
<td>Bei/insK134 Heb/NA†</td>
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<td>Shen/WT†</td>
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<tr>
<td>Shen/AK134†</td>
<td>2560</td>
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*The titre of a reference antigen against its homologous antiserum is indicated in bold, while the titre defining the antigenic group of the recombinant strains is indicated by underlining.

†Recombinant viruses.


