Hemagglutinin Receptor Binding Avidity Drives Influenza A Virus Antigenic Drift

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Hemagglutinin Receptor Binding Avidity Drives Influenza A Virus Antigenic Drift

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Rapid antigenic evolution in the influenza A virus hemagglutinin precludes effective vaccination with existing vaccines. To understand this phenomenon, we passaged virus in mice immunized with influenza vaccine. Neutralizing antibodies selected mutants with single–amino acid hemagglutinin substitutions that increased virus binding to cell surface glycan receptors. Passaging these high-avidity binding mutants in naïve mice, but not immune mice, selected for additional hemagglutinin substitutions that decreased cellular receptor binding avidity. Analyzing a panel of monoclonal antibody hemagglutinin escape mutants revealed a positive correlation between receptor binding avidity and escape from polyclonal antibodies. We propose that in response to variation in neutralizing antibody pressure between individuals, influenza A virus evolves by adjusting receptor binding avidity via amino acid substitutions throughout the hemagglutinin globular domain, many of which simultaneously alter antigenicity.

Influenza A virus remains an important human pathogen due largely to its ability to evade antibodies specific for its attachment protein, the hemagglutinin (HA). This "antigenic drift" is due to accumulation of amino acid substitutions in HA epitopes recognized by antibodies that neutralize viral infectivity by blocking interaction of HA with sialic acid residues on host-cell membranes (*1*–*3*). The H1 subtype HA has four antigenic sites recognized by monoclonal antibodies with high neutralizing activity, designated Sa, Sb, Ca, and Cb (*4*). How can HA escape polyclonal antibodies given that the frequency of variants with simultaneous multiple point mutations is exceedingly low (*5*)? A popular model posits sequential selection by different individuals whose antibody responses focus on different individual antigenic sites (*6*, *7*).

To better understand how antigenic drift occurs in human populations, we revisited classical experiments modeling drift in outbred Swiss mice (*8*). We generated three separate infectious stocks of the mouse-adapted strain A/Puerto Rico/8/34 (H1N1) (PR8) in Madin-Darby canine kidney (MDCK) cells using reverse genetics. Each stock was serially passaged in naïve mice or mice immunized with inactivated virus. Mice were infected intranasally with virus prepared from lung homogenates.

After nine passages, HA gene sequencing revealed no detectable mutations in viruses passaged in naïve mice (Fig. 1A). By contrast, each lineage from vaccinated mice contained a predominant population with a different single–amino acid substitution: residue 158 (E to K, lineage I), 246 (E to G, lineage II), or 156 (E to K, lineage III) (E, Glu; G, Gly; K, Lys). Residue 158 is located at the interface of the Sa/Sb antigenic sites, residue 156 is in the Sb site, and residue 246 is located outside the defined epitopes (*4*) (Fig. 1B). E158K, initially detected in lineage I after passage 2, predominated by passage 3 (table S1). In lineage II, E246G abruptly emerged during passage 3. In lineage III, E158K and E156K codominated from passage 2 to 7, with E156K predominating after passage 8. None of the lineages exhibited changes in the neuraminidase (NA) gene.

We measured the mutants' ability to escape antibody responses by hemagglutination inhibition (HAI) and virus neutralization assays using immune serum pooled from 45 PR8-vaccinated mice. Each mutant escaped antibody responses in these ternary (virus, antibody, and cell) assays (Fig. 1, C and D), despite demonstrating only minor (E156K, E158K) or no (E246G) decreases in anti-HA antibody binding (Fig. 1E). More precise antigenic analysis by enzyme-linked immunosorbent assay confirmed that the amino acid substitutions had limited effects on individual monoclonal antibody binding (fig. S1). E156K modified Sb antigenicity, but had no effect on the other sites. E158K altered binding of a subset of Sa- and Sb-specific monoclonal antibodies. Notably, just 1 of 16 monoclonal antibodies tested exhibited (slightly) altered binding to E246G, consistent with the observation that the substitution resides outside defined antigenic sites (*4*).

HA mutations can decrease HAI antibody activities by increasing the viral HA binding avidity for cell surface glycan receptors (*9*–*11*). Relative to wild-type virus, such "adsorptive mutants" exhibit enhanced agglutination of erythrocytes treated with *Vibrio cholerae* neuraminidase receptor destroying enzyme (RDE) to remove terminal sialic acids, the cellular HA ligand. Notably, relative to wild-type virus, each mutant better agglutinated RDE-treated erythrocytes (Fig. 1F). Mutant-virus hemagglutination was also more resistant to competition from horse serum "nonspecific" inhibitors (fig. S2), confirming increased cellular receptor binding avidity. Mutant viruses also exhibited higher binding avidity than wild-type virus to both α2-3 and α2-6 sialylated glycans in a dose-dependent, direct glycan receptor-binding assay (fig. S3).

E156K and E158K mutants were again selected when different PR8 stocks (propagated in either eggs or MDCK cells) were passaged in PR8-immunized BALB/c or C57BL/6 mice, indicating that these are particularly adept escape mutants. Because these substitutions modify antigenicity [unlike E246G and previously described adsorptive mutants (*10*)], this suggests that polyclonal antibody escape favors substitutions that simultaneously increase cellular receptor binding and diminish antigenicity.

These findings prompted us to examine the cellular receptor binding avidities of 40 monoclonal antibody–selected HA-escape mutants (*4*). Each amino acid substitution in the panel exerts similar relatively minor effects on HA antigenicity (*4*). Surprisingly, 23 mutants exhibited altered binding to RDE-treated erythrocytes (Fig. 2A and table S2). There was a strong correlation between cellular receptor binding and polyclonal antibody HAI escape (Fig. 2B). Substitutions modifying receptor binding cover all four antigenic sites (Fig. 2, A and C). Of 18 substitutions that enhance receptor binding, 11 increased HA positive charge (Fig. 2D and table S2). Because virions possess ~900 HA monomers (*12*), increased positive charge may enhance cellular receptor binding by increasing charge attraction with negatively charged cell surfaces (*13*). Retrospective analysis supports a relation between H3 HA charge and receptor binding (*14*).

Upon further passaging of the in vivo–selected virus populations in mice vaccinated with homologous inactivated virus (e.g., E156K virus passaged in E156K-vaccinated mice), we detected minor virus populations with novel amino acid substitutions distant from the sialic acid binding site, often distant from defined neutralizing epitopes (table S3 and fig. S4). Substitutions that enhanced receptor binding (Fig. 3A) enhanced polyclonal HAI antibody escape (Fig. 3B).

Optimizing viral fitness requires balancing host cell receptor binding of input virus with release of progeny virus. Notably, passaging in vivo–selected mutants in naïve mice selected mutants (table S3) with reduced cellular receptor binding avidity (Fig. 3A). Mutations selected by passaging in naïve mice decreased polyclonal HAI antibody escape (Fig. 3B). E158K- and E246G-derived mutants were inhibited at levels similar to those exhibited by wild-type PR8, demonstrating the central role of cellular receptor binding in E158K and E246G single–point mutants escape from polyclonal antibodies (see also Fig. 1E). E156K

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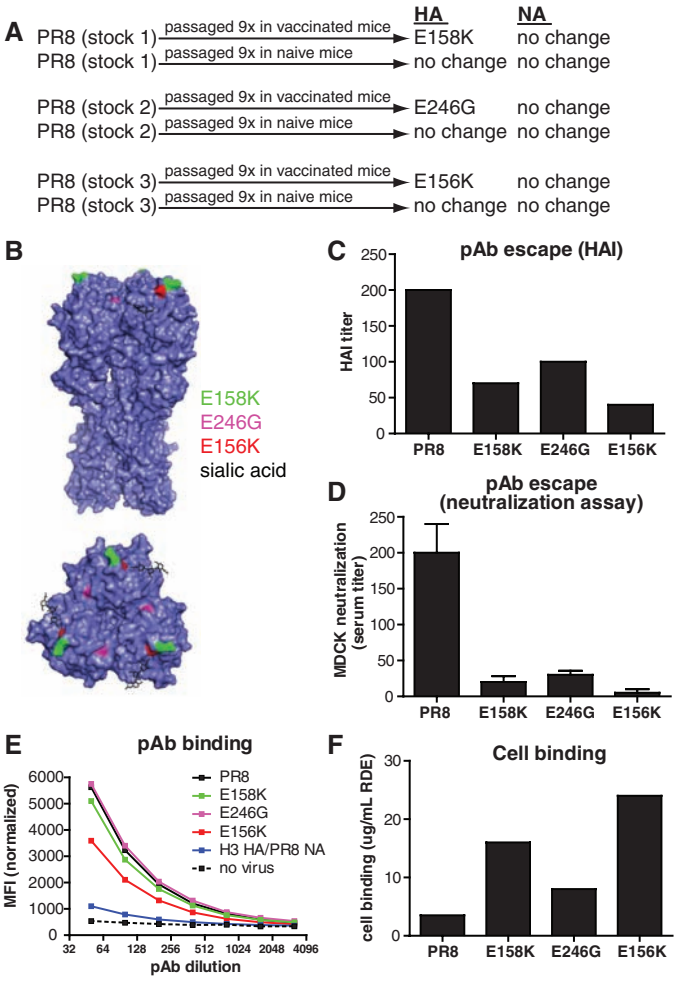


Fig. 1. In vivo influenza A virus passaging selects for mutants with altered binding avidity. (**A**) HA and NA genes were sequenced in lung homogenates from three independent PR8 stocks serially passaged in vaccinated and naïve Swiss mice. [GenBank accession numbers AF389118 (HA) and AF389120 (NA)] (**B**) Location of in vivo–selected HA amino acid substitutions in mutant viruses. (**C**) PR8 and mutant viruses were tested for escape from polyclonal antibodies to PR8 by HAI with turkey erythrocytes or (**D**) by virus neutralization assays with MDCK cells. Data are expressed as inverse dilutions of serum and are representative of three (HAI) or two (virus neutralization) experiments. Data are shown as means ± SEM. (**E**) Polyclonal antibody binding to HA was assessed by flow cytometry after addition of different dilutions of polyclonal antibody to L929 cells infected with the indicated virus followed by the addition of anti-mouse fluorescein isothiocyanate. Shown is mean fluorescence intensity (MFI) after normalizing HA expression based on the binding of a mixture of Ca monoclonal antibodies or a NA-specific monoclonal antibody (for the H3 HA/PR8 NA virus). Polyclonal antibodies bind nearly exclusively to HA, as inferred from their low binding to the H3 HA/PR8 NA–infected cells. Data are representative of three independent experiments. (**F**) Cellular receptor binding avidities were determined by hemagglutination of turkey erythrocytes pretreated with RDE. Data are expressed as the maximal amount of RDE that allowed full agglutination. Data are representative of three independent experiments.

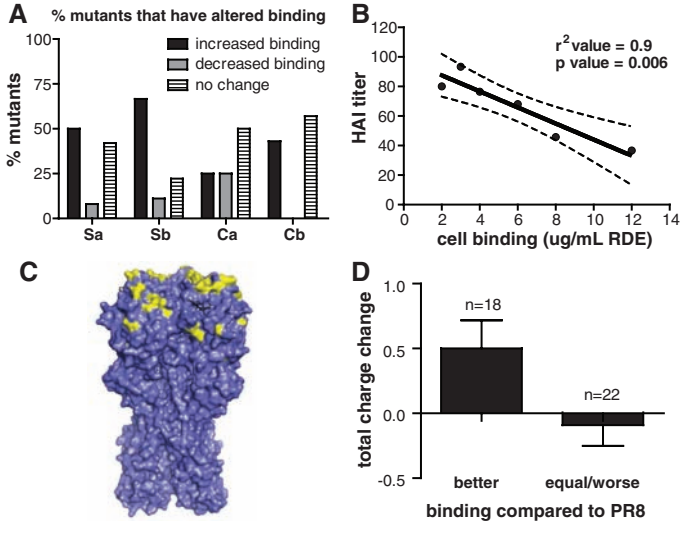
viruses with secondary mutations acquired in naïve mice still escaped wild-type-specific polyclonal antibodies better than wild-type virus, despite a return to wild-type binding avidity, demonstrating that E156K alteration of Sb antigenicity contributes to immune escape.

Despite the absence of antibody selection, some secondary mutations selected in naïve mice modified HA antigenicity (fig. S1). A227T, located near the sialic acid–binding site, reduced binding of the Sa-specific antibody, IC5-2A7. R220G reduced binding of the Sa-specific antibody, H2-6A1. Thus, antigenic drift can be a byproduct of Darwinian selection for mutations that optimize host cell receptor binding during influenza A virus transmission between immune (increased receptor binding) and naïve individuals (decreased receptor binding).

To demonstrate an independent role for cellular receptor binding avidity in polyclonal antibody–mediated evolution, we coinfected mice with wild-type PR8 and AM6, an absorptive mutant with a substitution (P186S) in the receptor binding site that does not modify antigenicity (*10*, *15*) (fig. S5A). AM6 was rapidly selected in vaccinated but not naïve mice (fig. S5B). Next, we coinfected mice with E246G virus (minor antigenic change, high receptor binding) and E246G/A227T (greater antigenic change, low receptor binding). Vaccinated mice selected E246G, whereas naïve mice selected E246G/A227T (fig. S5D), confirming the critical role of receptor binding avidity in antibody-driven viral evolution.

To extend our drift model, we passaged E156K/R220G in mice given a high vaccine dose to generate severe antibody selection pressure. This resulted in the selection of E156K/R220G/I244T (no selection occurred in naïve mice). I244T, located in the Sa/Ca interface (fig. S6A), increased cellular receptor binding (Fig. 3C), and as predicted, increased polyclonal HAI antibody escape to levels exhibited by the E156K progenitor (Fig. 3D). Thus, during these passages between naïve and immune individuals, influenza A virus

Fig. 2. Numerous HA amino acid substitutions simultaneously modulate receptor binding and escape from polyclonal antibodies. (**A**) Cellular receptor binding avidities of 40 viruses with single HA amino acid substitutions were determined with RDE-treated turkey erythrocytes. The percentages of viruses within each antigenic group with altered binding are shown. (**B**) Individual mutants were tested for their abilities to escape polyclonal antibodies to PR8 by HAI. Data are plotted as ability to escape polyclonal antibodies versus cellular receptor binding avidity. Means are represented as dots and the 95% confidence interval is represented by dashed lines. (**C**) Locations of HA amino acid substitutions that promote increased avidity are shown in yellow. (**D**) Net change in the charge of mutant viruses was determined (*n*, number of viruses in each group). Data are shown as the means ± SEM.



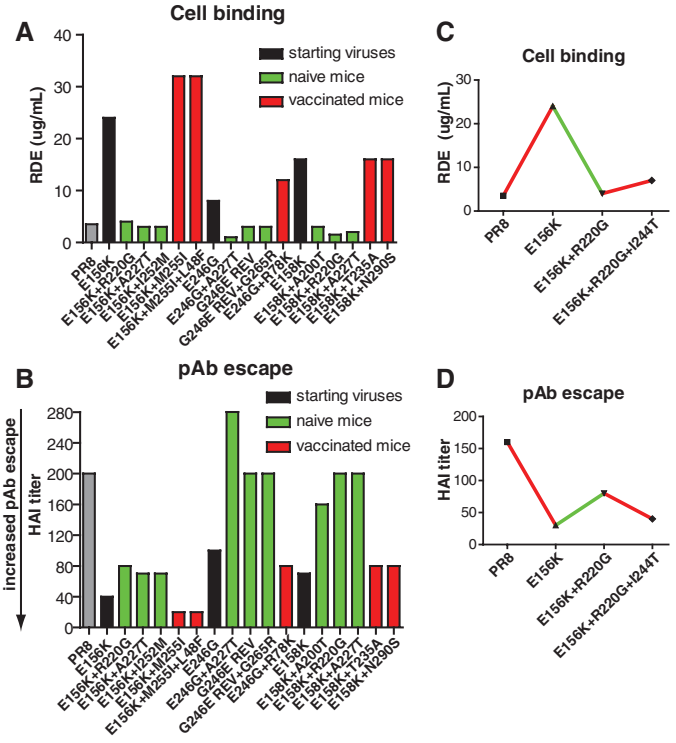


Fig. 3. Removal of polyclonal antibody selection promotes decreased receptor binding of in vivo–selected mutants. (**A**) Mutant viruses were tested for agglutination of RDE-treated turkey erythrocytes. REV denotes virus with amino acid reversion of original mutation. (**B**) Mutant viruses were tested for escape from PR8 polyclonal antibodies by HAI. Receptor binding avidities (**C**) and ability to escape PR8 polyclonal antibodies (**D**) were also determined for the virus that was isolated after further passage in heavily vaccinated mice. Abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; R, Arg; S, Ser; and T, Thr.

exhibited three cellular receptor binding avidity changes that were completely concordant with immune pressure. All of these changes were accompanied by amino acid substitutions that reduced the binding of different monoclonal antibodies (fig. S6B).

On the basis of these findings, we propose a new hypothesis for antigenic drift (fig. S7). Immune individuals select single point mutants with increased receptor binding avidity and, under optimal circumstances (for the virus), diminished antigenicity. Transmission to a nonimmune individual leads to selection of mutants with decreased receptor binding. Because binding avidity–altering substitutions exist throughout the globular domain, they frequently modify antigenicity, even when this is not a selecting factor. Repeating the cycle results in constant modulation of binding avidity along with steady alterations in antigenicity, even in the absence of antibody selection. Our model posits that cellular receptor binding alone exerts selection pressure for a substantial fraction of substitutions in defined antigenic regions and also contributes to the fixation of 30% of mutations in the globular domain outside defined antigenic regions (*16*).

The properties of recent human isolates are consistent with this model. A/Panama/2007/99, the 2003–2004 vaccine strain, did not match the circulating A/Fujian/411/02 strain by HAI assays. This difference was attributed to two amino acid substitutions (*17*). Notably, one substitution is at residue 156, which we identify as playing a critical dual role in Sb antigenicity and receptor binding avidity, while the other is at residue 155, which is immediately adjacent to the sialic acid binding site, and also located in the Sb site. During the antigenic evolution of H3N2 viruses from 1968 to 2003, a limited number of amino acids (in one case, a single N-to-K substitution increasing positive charge) dictate HAI escape from polyclonal sera (*18*).

This model complements recent work emphasizing the importance of HA receptor specificity in influenza A virus species and organ tropism (*19*). Receptor specificity and affinity are intimately related physiochemically, as demonstrated by our observation that each of the first-generation absorptive mutants (E156K, E158K, E246G) exhibits a unique avidity profile against the panel of sialylated oligosaccharides (fig. S3). Because host selection for optimal HA receptor binding avidity generates mutants with altered receptor binding specificity, this is a confounding factor in interpreting the evolutionary selection forces that generate influenza A virus isolates with altered glycan specificities.

In our model, antigenic drift is accelerated by sequential passage of influenza A virus between immune and nonimmune individuals (fig. S7), which in the human population are nearly all children. Therefore, decreasing the naïve population size by increasing pediatric influenza A virus vaccination rates will likely slow antigenic drift and temporally extend the effectiveness of influenza vaccines. Monitoring receptor binding avidity of circulating viral isolates may facilitate the accurate prediction of mutants with epidemic potential.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S7
Tables S1 to S3

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