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Hemagglutinin Receptor Binding Avidity Drives Influenza A Virus Antigenic Drift

Scott E. Hensley¹, Suman R. Das¹, Adam L. Bailey¹, Loren M. Schmidt¹, Heather D. Hickman¹, Akila Jayaraman², Karthik Viswanathan², Rahul Raman², Ram Sasisekharan², Jack R. Bennink¹, and Jonathan W. Yewdell^{1,*}

¹Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA

²Harvard-MIT Division of Health Sciences & Technology, Koch Institute for Integrative Cancer Research and Department of Biological Engineering, Massachusetts Institute of Technology, , Cambridge, MA 02139, USA

Abstract

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. 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 MDCK cells using

*To whom correspondence should be addressed; jyewdell@mail.nih.gov.

Supporting Online Material

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Methods

Figs. S1 to S7

Tables S1 to S3

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). 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 following passage 2, predominated by passage 3 (Table S1). In lineage II, E246G abruptly emerged during passage 3. In lineage III, E158K and E156K co-dominated from passage 2–7, with E156K predominating following passage 8. None of the lineages exhibited changes in the neuraminidase 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 & cell) assays (Fig. 1C–D), despite demonstrating only minor (E156K, E158K) or no (E246G) decreases in anti-HA antibody binding (Fig. 1E). More precise antigenic analysis using ELISA 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 one 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 (9,10)(11). Relative to *wt* virus, such “adsorptive mutants” exhibit enhanced agglutination of erythrocytes treated with *V. cholerae* neuraminidase receptor destroying enzyme (RDE) to remove terminal sialic acids, the cellular HA ligand. Strikingly, relative to *wt* virus, each mutant better agglutinated RDE-treated erythrocytes (Fig. 1F). Mutant-virus hemagglutination was also more resistant to competition from horse serum “non-specific” inhibitors (Fig. S2), confirming increased cellular receptor binding avidity. Mutant viruses also exhibited higher binding avidity than *wt* virus to both $\alpha 2-3$ and $\alpha 2-6$ 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. As 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 forty monoclonal antibody selected HA-escape mutants (4). Each amino acid substitution in the panel exerts similar relatively minor effects on HA antigenicity (4). Surprisingly, twenty three mutants exhibited altered binding to RDE treated-erythrocytes (Fig. 2A, 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. 2A,C). Of eighteen substitutions that enhance receptor binding, eleven increased HA positive charge (Fig. 2D) (Table S2). Since 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 relationship between H3 HA charge and receptor binding (14).

Upon further passaging *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, 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. Strikingly, passaging *in vivo* selected mutants in naïve mice selected mutants (Table S3) with reduced cellular receptor binding avidity (Fig. 3A). Mutations selected by naïve mouse passage decreased polyclonal HAI antibody escape (Fig. 3B). E158K- and E246G-derived mutants were inhibited at similar levels as *wt* 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 viruses with secondary mutations acquired in naïve mice still escaped *wt*-specific polyclonal antibodies better than *wt* virus, despite a return to *wt* 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 by-product of Darwinian selection for mutations that optimize host cell receptor binding during influenza 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 co-infected mice with *wt* 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 coinfecting mice with E246G virus (minor antigenic change/high receptor binding) and E246G/A227T (greater antigenic change/low receptor binding). Vaccinated mice selected E246G while naïve mice selected E246G/A227T (Fig. S5D), confirming the critical role of 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 evolution 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 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).

Based on 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 non-immune individual leads to selection of mutants with decreased receptor binding. Since 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, explaining 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). Remarkably, one substitution is at residue 156, which we identify as playing a critical dual role in Sb antigenicity/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 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). Since 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 IAV isolates with altered glycan specificities.

In our model, antigenic drift is accelerated by sequential passage of influenza A virus between immune and non-immune 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 retard antigenic drift and temporally extend the effectiveness of influenza vaccines. Monitoring binding avidity of circulating viral isolates may facilitate the accurate prediction of mutants with epidemic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Air GM, Laver WG, Webster RG. *Contrib Microbiol Immunol* 1987;8:20. [PubMed: 3304832]
2. Dimmock NJ. *J Gen Virol* 1984;65:1015. [PubMed: 6202836]
3. Knossow M, Skehel JJ. *Immunol* 2006;119:1.
4. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. *Cell* 1982;31:417. [PubMed: 6186384]
5. Yewdell JW, Webster RG, Gerhard WU. *Nature* 1979;279:246. [PubMed: 86955]
6. Wilson IA, Cox NJ. *Annu Rev Immunol* 1990;214:737. [PubMed: 2188678]
7. Wang ML, Skehel JJ, Wiley DC. *J Virol* 1986;57:124. [PubMed: 3941436]
8. Gerber P, Loosli CG, Hambre D. *J Exp Med* 1955;101:627. [PubMed: 14367684]
9. Fakekas de St Groth S. *Top Infect Dis* 1977;3:25.
10. Yewdell JW, Caton AJ, Gerhard W. *J Virol* 1986;57:623. [PubMed: 2418215]
11. The term avidity refers to the combined strength of the multivalent interactions between viral HA and cell surface glycan receptors.
12. Harris A, et al. *Proc Nat Acad Sci USA* 2006;103:19123. [PubMed: 17146053]
13. Gambaryan AS, Matrosovich MN, Bender CA, Kilbourne ED. *Virol* 1998;247:223.
14. Underwood PA, Skehel JJ, Wiley DC. *J Virol* 1987;61:206. [PubMed: 3783824]
15. Matrosovich MN, et al. *Virol* 1997;233:224.
16. Nelson MI, et al. *PLoS Path* 2006;2:e125.
17. Jin H, et al. *Virol* 2005;336:113.
18. Smith DJ, et al. *Science* 2004;305:371. [PubMed: 15218094]
19. Nicholls JM, Chan RW, Russell RJ, Air GM, Peiris JS. *Trends Microbiol* 2008;16:149. [PubMed: 18375125]

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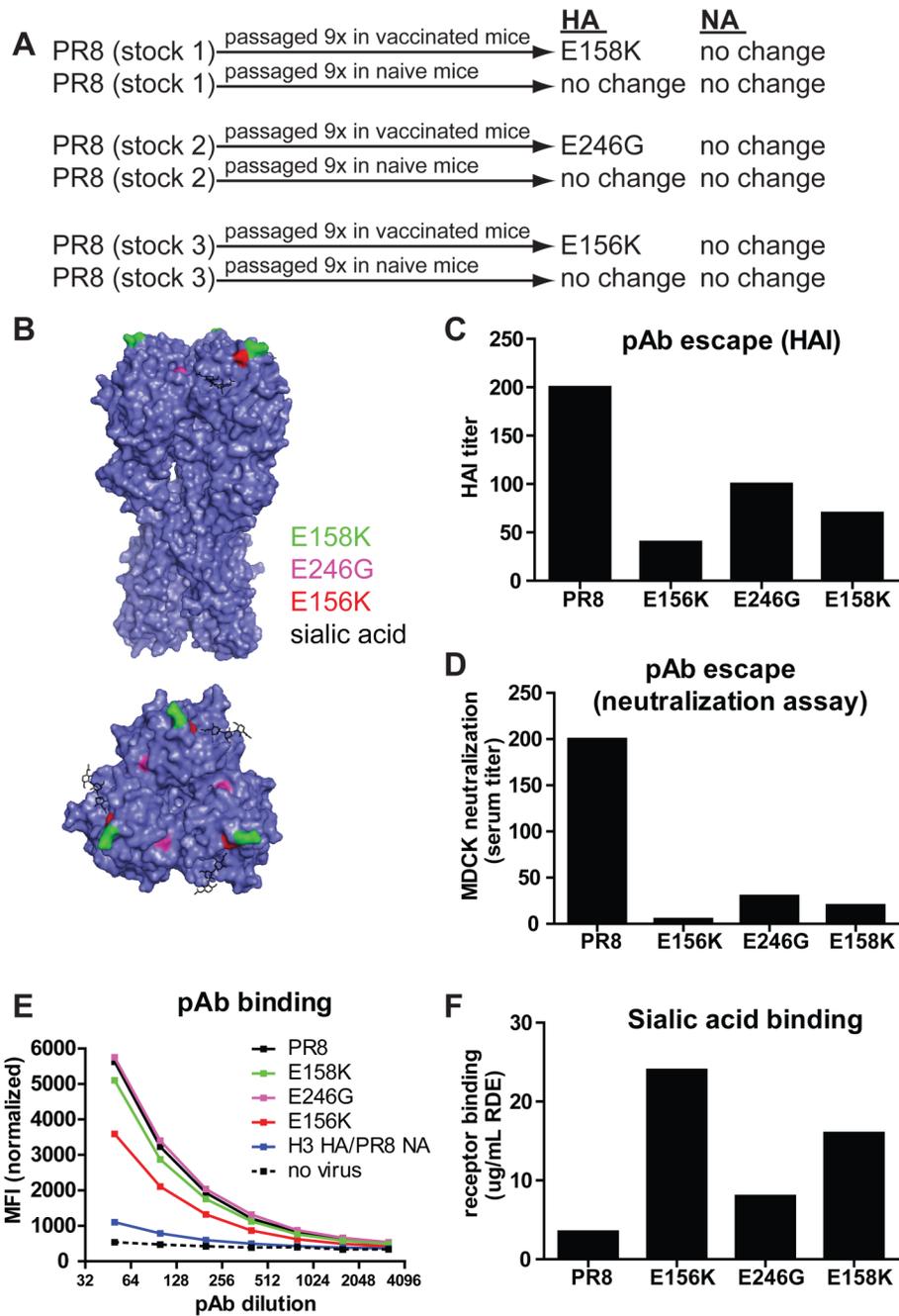


Fig. 1. *In vivo* influenza virus passaging selects for mutants with altered binding avidity
(A) HA and NA genes were sequenced in lung homogenates from 3 independent PR8 stocks serially passaged in vaccinated and naïve Swiss mice. **(B)** Location of *in vivo* selected HA amino acid substitutions in mutant viruses. **(C)** PR8 and mutant viruses were tested for escape from anti-PR8 polyclonal antibodies by HAI using turkey erythrocytes or **(D)** virus neutralization assays using MDCK cells. Data are expressed as inverse dilutions of serum and are representative of three (HAI) or two (virus neutralization) experiments. Means \pm SEM are shown in panel D. **(E)** Polyclonal antibody binding to HA was assessed by flow cytometry after adding different dilutions of polyclonal antibody to L929 cells infected with the indicated virus followed by the addition of anti-mouse FITC. 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 pre-treated with RDE. Data are expressed as the maximal amount of RDE that allowed full agglutination. Data are representative of three independent experiments.

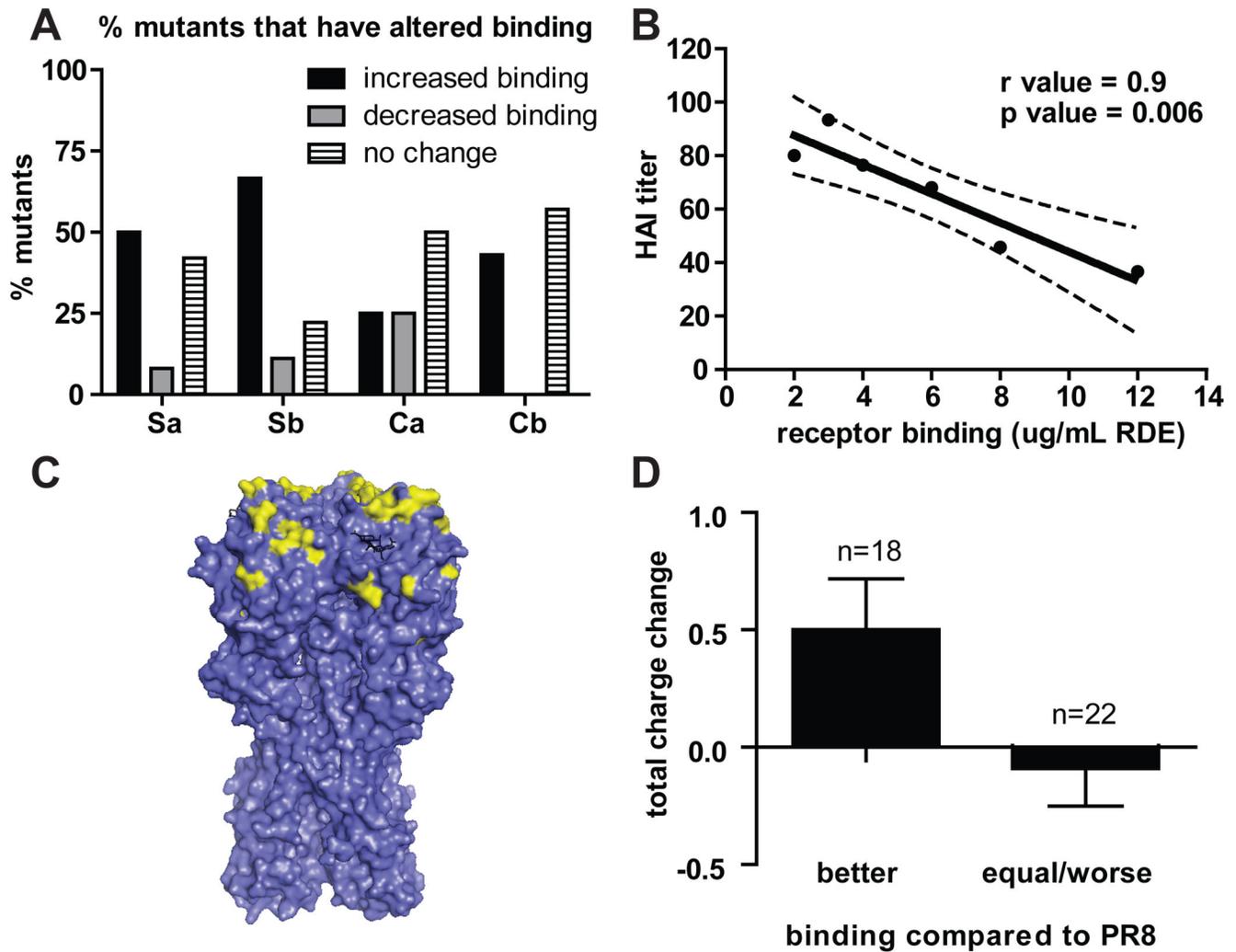


Fig. 2. Numerous HA amino acid substitutions simultaneously modulate receptor binding and escape from polyclonal antibodies

(A) Cellular avidities of 40 viruses with single HA amino acid substitutions were determined using 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 PR8 polyclonal antibodies by HAI. Data are plotted as ability to escape polyclonal antibodies vs 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 charge change of mutant viruses was determined. n = number of viruses in each group. Means \pm SEM are shown.

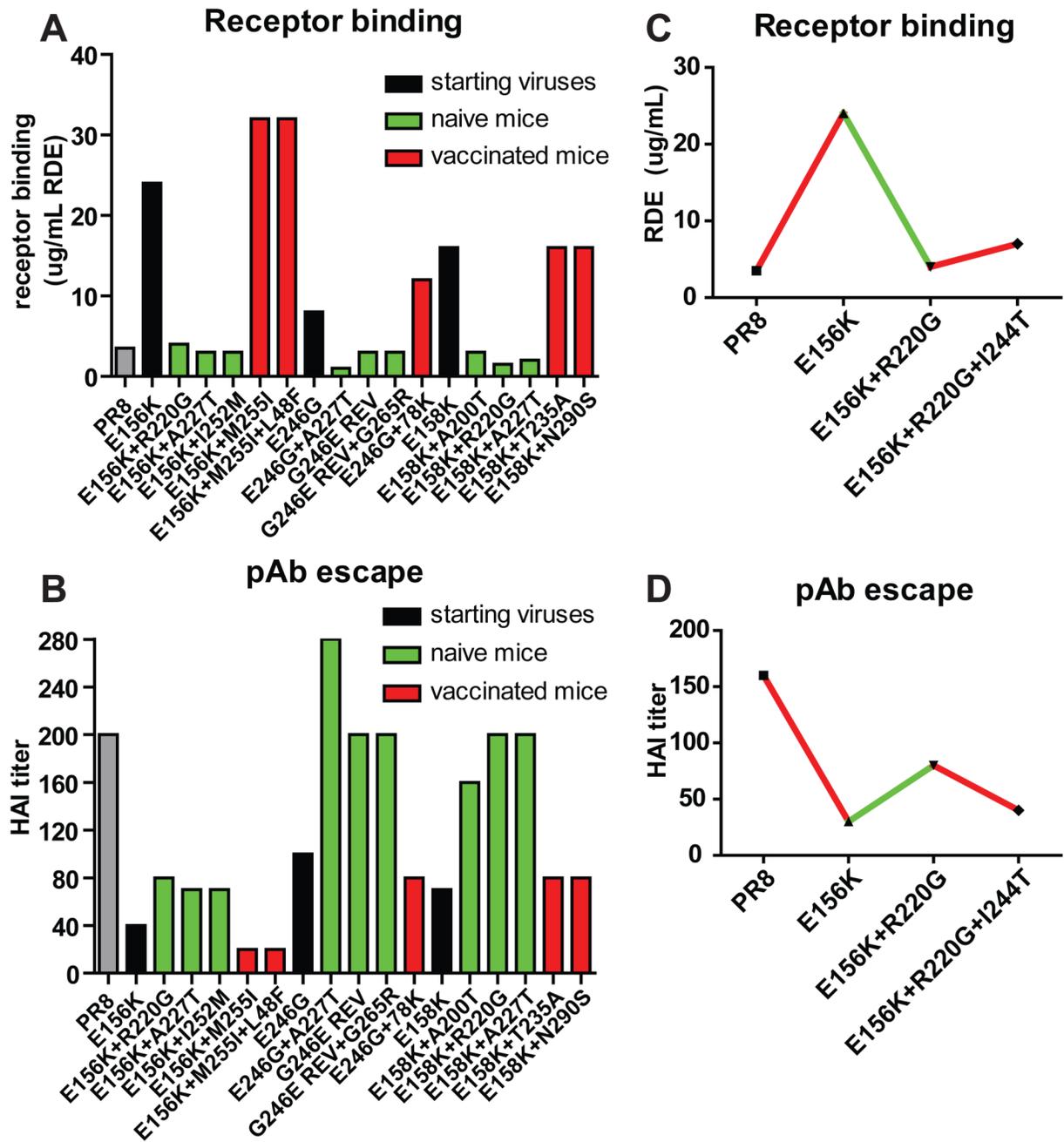


Fig. 3. Removal of polyclonal antibody selection promotes decreased receptor binding of *in vivo* selected mutants

(A) Mutant viruses were tested for agglutination of RDE-treated turkey erythrocytes.

REV=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.