Immunogenic Stimulus for Germline Precursors of Antibodies that Engage the Influenza Hemagglutinin Receptor-Binding Site

Authors
Aaron G. Schmidt, Khoi T. Do, Kevin R. McCarthy, ..., M. Anthony Moody, Barton F. Haynes, Stephen C. Harrison

Correspondence
harrison@crystal.harvard.edu

In Brief
Schmidt et al. use an approach they call “immuno-viral archaeology” to probe the history of influenza exposure and antibody response in a single individual. They find that viruses, circulating during a donor’s infancy, bind tightly to the germline precursors of six distinct lineages of antibodies targeting the hemagglutinin receptor binding site.

Highlights
- UCAs of RBS-directed lineages bind viruses circulating during a donor’s infancy
- H1 viruses circulating after 1995 have escaped binding by lineage UCAs
- Vaccination recalled lineages, with further maturation and increased breadth
- Imprinting by H1 influenza early in life may direct later B cell responses
Immunogenic Stimulus for Germline Precursors of Antibodies that Engage the Influenza Hemagglutinin Receptor-Binding Site

Aaron G. Schmidt,1 Khoi T. Do,1 Kevin R. McCarthy,1 Thomas B. Kepler,2 Hua-Xin Liao,3,4 M. Anthony Moody,3,5,6 Barton F. Haynes,3,4,6 and Stephen C. Harrison1,7
1Laboratory of Molecular Medicine, Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA
2Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, USA
3Duke Human Vaccine Institute
4Department of Medicine
5Department of Pediatrics
6Department of Immunology
Duke University Medical School, Durham, NC 27710, USA
7Howard Hughes Medical Institute, Boston, MA 02115, USA
*Correspondence: harrison@crystal.harvard.edu
http://dx.doi.org/10.1016/j.celrep.2015.11.063
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

Influenza-virus antigenicity evolves to escape host immune protection. Antibody lineages within individuals evolve in turn to increase affinity and hence potency. Strategies for a “universal” influenza vaccine to elicit lineages that escape this evolutionary arms race and protect against seasonal variation and novel, pandemic viruses will require directing B cell ontogeny to focus the humoral response on conserved epitopes on the viral hemagglutinin (HA). The unmutated common ancestors (UCAs) of six distinct, broadly neutralizing antibody lineages from one individual bind the HA of a virus circulating at the time the participant was born. HAs of viruses circulating more than 5 years later no longer bind the UCAs, but mature antibodies in the lineages bind strains from the entire 18-year lifetime of the participant. The analysis shows how immunological memory shaped the response to subsequent influenza exposures and suggests that early imprinting by a suitable influenza antigen may enhance likelihood of later breadth.

INTRODUCTION

Influenza virus in humans evolves in response to pressure from immunity in the susceptible population, leading to progressive variation of viral antigenicity. Introduction of a new strain of influenza A from birds or swine (“antigenic shift”) initiates a cycle of antibody generation and viral escape (“antigenic drift”), the latter largely through mutation of surface residues on the viral hemagglutinin (HA) but secondarily through variation of antigenic determinants on the neuraminidase (NA). Detailed antigenic analysis of annual HA variation in H1 and H3 subtypes shows a punctuated evolutionary trajectory, with a shift in “antigenic cluster” (defined by reactivity with standard panels of ferret immune sera) every few years (Smith et al., 2004; Fonville et al., 2014). Strong selective pressure from widespread immunity in the human population thus appears to require more than one seasonal cycle.

The humoral response within individuals also evolves, through immune memory and B cell affinity maturation. When stimulated by a new exposure (infection or vaccination), memory cells can re-enter germinal centers and undergo new rounds of somatic hypermutation and selection (Vicenza and Nussenzweig, 2012; De Silva and Klein, 2015). The net effect of this on-going selection across the entire population exposed to the virus is a virus-immunity “arms race.” Mutated HA with reduced affinity for a particular antibody can, in principle, select for mutations in the latter that restore strong binding. We can study this evolutionary process by detecting B cells descended from the same common ancestor and determining the sequences of their rearranged variable-domain genes (Moody et al., 2011).

Antigenic variation requires an annual revision of vaccine components. A more effective vaccine strategy would protect against many rounds of this seasonal variation and ideally against introduction of new serotypes from viruses circulating in animal reservoirs (a so-called “universal” influenza vaccine; Burton et al., 2012; Krammer and Palese, 2015). Broad protection will probably come from a humoral response to conserved sites on the viral HA. The two relatively invariant epitopes so far recognized are the receptor binding site (RBS) on the HA “head” and a surface along the HA “stem” (Knossow et al., 2002; Ekiert et al., 2009; Sui et al., 2009; Corti et al., 2011; Whittle et al., 2011; Corti and Lanzavecchia, 2013). Study of over 100 influenza (subtype H1) RBS-directed antibodies from three individuals, all of whom received the trivalent influenza vaccine in 2008 (Moody et al., 2011), has shown that antibodies engage the RBS through contacts that recapitulate many of those made by the viral receptor, sialic acid (Weis et al., 1988; Whittle et al., 2011; Schmidt et al., 2015). The key interactions come from a critical dipeptide (valine-aspartic acid or a related...
sequence) at the tip of the third heavy-chain complementarity determining loop (CDR H3). This class of antibodies is nearly unrestricted in V\textsubscript{H} and V\textsubscript{L} gene usage; moreover, the lineages show that distinct affinity maturation pathways can lead from a single germline precursor (the unmutated common ancestor [UCA]) to functionally similar outcomes. Many of these antibodies came from one individual (designated TIV01); they defined various clonal lineages, each with a unique germline precursor. A suitable set of three or four such antibodies would have in common only contacts with conserved, receptor-interacting amino acid residues. We proposed that this sort of polyclonal response would approximate the broad immunity to H1 subtypes that a universal vaccine should elicit.

We have chosen six lineages of H1 RBS-directed antibodies from TIV01 and studied the binding of their UCAs and intermediates from H1 strains circulating in various years since 1990 and examined their affinities for the UCAs of antibodies in six lineages of RBS-directed antibodies (Figures 1A and S1). The average V\textsubscript{H} gene mutational frequency of all the RBS-directed antibody lineages isolated from TIV01 was ~5%, greater than the likely level of somatic hypermutation during a primary response (Figures S2A and S2B). We infer that the antibodies identified in the day 7 samples represent a recall response. Mutation could have occurred because of an influenza virus infection sometime between 1990 and 2006 and also during the response to the vaccine. The initial screen had no bias for HA binding: of the 404 paired sequences recovered from the entire cohort of five individuals at day 7, 252 were positive (by ELISA) for at least one HA in the panel tested (Moody et al., 2011), and Figures S2A and S2B show that at least 90% of the 174 HA-positive antibodies that came from TIV01 had somatic mutation levels greater than 2.5%. Thus, not only were the lineages we analyzed part of a secondary response but also the vast majority of all antibodies with detectable HA affinity. A similar analysis of lineages from other individuals in the same cohort also shows an almost exclusively secondary response (Figure S2C). The six chosen lineages represent three different V\textsubscript{H} genes and five different V\textsubscript{L} genes (three V\textsubscript{L} and two V\textsubscript{L}5) and a total of 54 mature antibodies (Figure 1B). Among the lineages we analyzed is the broadly neutralizing antibody lineage 860, which includes the well-characterized antibodies, CH65 and CH67 (Whittle et al., 2011; Schmidt et al., 2013, 2015; Xu et al., 2015).

**Germline Precursors Bind an Early Seasonal Influenza**

All six UCAs bound A/Massachusetts/1/1990, which was isolated in the year TIV01 was born (Figure 1B). They also bound A/Florida/2/1993, but most had low affinities for HAs from viruses isolated after 1995, and all failed to bind HAs from 1999 onward. In no case did the UCA of the lineages bind the H1 A/Solomon Islands/03/2006 included in the vaccine. The loss of affinity corresponds accurately to the “antigenic cartography” of H1N1 strains (Bedford et al., 2014). Viruses between 1986 and

---

**Figure 1. Timeline of Seasonal H1 Influenzas and Lineage UCA Reactivity**

(A) H1 influenza strains from 1977 to 2009 used in this study. TIV01 birth and administration of the vaccine are marked.

(B) Six RBS-directed lineages (Lin) with their V\textsubscript{H} and V\textsubscript{L} genes listed. The seasonal strains and year of isolation (in parentheses) are on the left. K\textsubscript{D} values for the Fab binding to monomeric HA heads are in μM. The “heatmap” color scheme is arbitrary, as an aid in visualization; warm colors are high-affinity interactions (e.g., values <1 μM—15 μM in red) and cool colors are lower-affinity interactions (e.g., values 60–75 μM in blue); color changes represent 15 μM intervals; affinities marked as >100 μM are those beyond the limit of detection.

See also Figures S1 and S3.

---

**RESULTS**

**TIV01 Immune History and RBS-Directed Lineages**

TIV01 was 18 at the time of the 2008 study and reported no previous vaccination history. We prepared a panel of HA\textsubscript{1} head domains from H1 strains circulating in various years since 1990 and examined their affinities for the UCAs of antibodies in six lineages of RBS-directed antibodies (Figures 1A and S1). The average V\textsubscript{H} gene mutational frequency of all the RBS-directed antibody lineages isolated from TIV01 was ~5%, greater than the likely level of somatic hypermutation during a primary response (Figures S2A and S2B). We infer that the antibodies identified in the day 7 samples represent a recall response. Mutation could have occurred because of an influenza virus infection sometime between 1990 and 2006 and also during the response to the vaccine. The initial screen had no bias for HA binding: of the 404 paired sequences recovered from the entire cohort of five individuals at day 7, 252 were positive (by ELISA) for at least one HA in the panel tested (Moody et al., 2011), and Figures S2A and S2B show that at least 90% of the 174 HA-positive antibodies that came from TIV01 had somatic mutation levels greater than 2.5%. Thus, not only were the lineages we analyzed part of a secondary response but also the vast majority of all antibodies with detectable HA affinity. A similar analysis of lineages from other individuals in the same cohort also shows an almost exclusively secondary response (Figure S2C). The six chosen lineages represent three different V\textsubscript{H} genes and five different V\textsubscript{L} genes (three V\textsubscript{L} and two V\textsubscript{L}5) and a total of 54 mature antibodies (Figure 1B). Among the lineages we analyzed is the broadly neutralizing antibody lineage 860, which includes the well-characterized antibodies, CH65 and CH67 (Whittle et al., 2011; Schmidt et al., 2013, 2015; Xu et al., 2015).

**Germline Precursors Bind an Early Seasonal Influenza**

All six UCAs bound A/Massachusetts/1/1990, which was isolated in the year TIV01 was born (Figure 1B). They also bound A/Florida/2/1993, but most had low affinities for HAs from viruses isolated after 1995, and all failed to bind HAs from 1999 onward. In no case did the UCA of the lineages bind the H1 A/Solomon Islands/03/2006 included in the vaccine. The loss of affinity corresponds accurately to the “antigenic cartography” of H1N1 strains (Bedford et al., 2014). Viruses between 1986 and
1995 belonged to the same antigenic cluster; A/Beijing/262/1995 was a transition to the 1999–2005 cluster that included A/New Caledonia/20/1999. The concordance suggests that, during the course of the decade, seasonal influenza viruses had evolved resistance to similar antibodies present in the general population. This conclusion is consistent with an analysis of H3N2 antigenicity from 1968 to 2003, which showed that substitutions around the rim of the RBS determined the principal antigenic changes during that time period (Koel et al., 2013) and hence that antibodies binding in or near the RBS were widespread enough to exert strong selective pressure.

Viral Resistance to RBS-Directed Germline Precursors
The one difference between all HAs that bind these UCAs and those that do not is the loss, in 1995, of residue K133a at the edge of the receptor-binding pocket. Deletion of K133a from A/Massachusetts/1/1990 eliminates detectable binding by all six UCAs; insertion of K133a into A/Solomon Islands/03/2006 generates moderate-to-high affinity for four of the six (Figure S3). We conclude that viruses that had lost K133a escaped infectivity neutralization by antibodies such as those studied here and that the presence of this residue was a principal determinant of neutralization susceptibility.

The germline origins of the six antibody lineages have in common only Jμ6, which encodes a string of tyrosines immediately C-terminal to the critical dipeptide at the tip of CDR H3. Hydrogen bonds between K133a and the phenolic hydroxyls are probably present in many of the UCA-bound complexes. Whereas the UCAs appear dependent on K133a, subsequent affinity maturation to an immunogen during a secondary exposure has apparently made the antibody response independent of its presence.

Affinity Maturation of RBS-Directed Clonal Lineages
We examined initial steps of affinity maturation in three of the lineages (Figures 2, 3, and 4) by measuring binding of early intermediates to the 1990 A/Massachusetts and 1993 A/Florida HAs (Figure 5). In lineage 860, somatic mutations encoding three amino acid residues, all in the heavy chain, accumulated between the germline response and intermediate antibody I-2. Because neither Fab had detectable affinity for A/Solomon Islands/03/2006, we concluded in a previous paper that fixation of the three mutations must have come from enhanced affinity of I-2 for the virus that induced the primary response (Schmidt et al., 2013). Indeed, the data in Figure 5 show that I-2 binds the putative primary antigen (i.e., some strain between 1990 and 1993) substantially more tightly than does the UCA. The limited number of mutations between UCA and I-2 did not, however, impart increased breadth, which presumably came from mutations fixed by exposure to later strains (Figure S4).

In lineage 1277, increased affinity for A/Massachusetts/1/1990 occurred at the first assignable intermediate, with little affinity gain through two additional intermediates to the contemporary antibody (Figures 3 and 5). Those later intermediates did have successively greater affinities for A/Solomon Islands/03/2006, however, perhaps driven by a post-1995 strain that had lost K133a. The earliest intermediates in lineages 1277 and 687 both bind A/Solomon Islands/03/2006 with moderate affinity, and hence, each lineage has acquired some degree of breadth even with only four- and three-amino-acid residue changes, respectively (Figures 3 and 4). We have drawn similar conclusions from the more detailed structural and biophysical analyses we have carried out on lineages 641, 643, and 652, which will be the subjects of a separate paper.

The UCAs of the six lineages studied had no detectable affinity for the year 2008 trivalent inactivated vaccine HA immunogens, but they all bound tightly with HAs likely to be close relatives of the eliciting viral antigen. Thus, the affinity increase inferred from binding measurements with only the vaccine immunogen overestimates the gain in response to any single exposure, when the course of affinity maturation has involved succession.
of mutated immunogens. We note that the affinities measured here are for monomeric Fabs and that dimeric IgGs (or IgMs) will show stronger differential effects.

Breadth of RBS-Directed Clonal Lineages

The UCAs of the six lineages bind HAs only from H1 strains isolated around 1990, but a representative, affinity-matured antibody (or late intermediate) from each of the six lineages binds with high affinity to nearly all seasonal H1 HAs in a panel covering the period from 1977 to 2008 (Figure 6). These lineages illustrate how an initially restricted response can broaden after encountering an antigenically distinct HA, if there is a shared, conserved epitope such as the RBS. The enhanced breadth extends even to H1 viruses circulating before 1990: matured antibodies in all six

Figure 3. Properties of Clonal Lineage 1277

(A) Phylogenetic tree of lineage 1277.
(B) Sequence alignment of V_H and V_L domains for the clonal members. CDR 1 (blue), 2 (pink), and 3 (green) are highlighted; (.) denotes conservation in reference to the UCA.
(C) Summary of nt and aa changes in V_H and V_L. The percent SHM for the mature antibodies is listed.

See also Figure S2.

Figure 4. Properties of Clonal Lineage 687

(A) Phylogenetic tree of lineage 687.
(B) Sequence alignment of V_H and V_L domains for clonal members. CDR 1 (blue), 2 (pink), and 3 (green) are highlighted; (.) denotes conservation in reference to the UCA.
(C) Summary of nt and aa changes in V_H and V_L. The percent SHM for the mature antibodies is listed.

See also Figure S2.
DISCUSSION

The changing antigenicity of circulating influenza viruses and the spectra of human serological responses have been the subjects of an extensive literature since the 1930s (Davenport et al., 1953; Jensen et al., 1956; Kim et al., 2009; Li et al., 2012, 2013; Hensley, 2014). By 1960, T. Francis, Jr. could write, based on serological observations, that “the antibody which is first established continues to characterize...[an age] cohort of the population throughout its life” and designated the phenomenon by the colorful phrase “original antigenic sin” (OAS). Subsequent observations of a strong serum response to the most-recent exposure appeared at odds with this interpretation, but a contemporary analysis has shown that the highest serological titers in a large, unvaccinated, Vietnamese cohort were indeed for viruses that circulated when the person was young (mean age about 6 years), the likely time frame of a first infection, but that the increases in antibody titers immediately following a new infection were for recent strains (Fonville et al., 2014). Thus, early exposure dominated the steady-state profile, whereas recent infection determined the immediate response.

Lineage analysis, structures, and affinities of clonally related human antibodies, lineage intermediates, and their UCAs with HAs of viruses circulating during the lifetime of the donor have now allowed us to relate the dynamics of memory, recall, and somatic hypermutation to the previous serological characterizations. The results of this “immuno-viral archeology” show that RBS-directed antibodies from TIV01 lineages amplified by the short-term (7-day) response to vaccination bound strongly to HAs from most of the potential previous exposures but that their UCAs dated to the earliest exposure and lost affinity as the virus mutated. That is, even the immediate post-exposure response, although it had highest affinity for the most-recent strains, descended from the first encounter. A large majority of the antibodies from the TIV cohort, including antibodies with epitopes other than the RBS, had somatic mutation levels high enough to indicate a recall rather than primary response (Figures S2B and S2C), consistent with the published results of several related studies (Wrammert et al., 2011; Jackson et al., 2014; Tan et al., 2014). The antigenic distance, irrespective of epitope, between influenza virus isolates from well-separated time points thus appears to be within a range that strongly favors recall, consistent with less direct inference from serological data (Fonville et al., 2014). Thus, successive influenza virus infections or vaccinations “update” an existing repertoire, at least for relatively conserved epitopes such as the RBS, but do not appear to add many new components.

Figure 5. Affinity Maturation of Clonal Lineages

Biolayer interferometric analysis of binding by members of lineage 860 (A), lineage 1277 (B), and lineage 687 (C) to three H1 seasonal isolates—Massachusetts (MA) (1990), Florida (FL) (1993), and Solomon Islands (SI) (2006). $K_D$ values are highlighted in gray; immediately below are the $k_a$ ($10^3 M^{-1} ms^{-1}$) and $k_{off}$ ($10^{-2} s^{-1}$) values, respectively, that we used to obtain $K_D$.

See also Figure S3.

lineages bind more tightly to the 1977 and 1986 strains than do their respective UCAs.
studies of the arms race between influenza virus and the collective human humoral immunity co-evolution, which proceeds by episodic exposure (from vaccination or occasional infection) of a single person (Kuraoka et al., unpublished data). We recognized that the extent of somatic hypermutation indicated a recall response, but we have not had until now an estimate for the HA affinity required to initiate a germinal center reaction. The dissociation constants for the Fab-HA head interactions reported here correspond, of course, to lower affinities than those expected for a trimeric HA with a dimeric B cell receptor (IgM or IgG). For example, in cases for which we can compare the dissociation constant ($K_d$) for Fab-head binding with an avidity estimate from ELISA measurements for the corresponding IgGs and immobilized HAs, we find half-maximal binding from the latter experiments that are lower than the monomer-monomer $K_d$s by between two and three orders of magnitude. The binding of a Fab to a monomeric head is a direct way to relate affinity changes to the structural consequences of mutations in the Fab, because avidity effects, which can depend on the details of the measurement, are difficult to normalize. We have in practice found an excellent correlation between avidity, as measured by ELISA with HA or with head, and affinity of Fab for head, as determined here (Kuraoka, A.G.S., T. Nojima, D. Kitamura, S.C.H., T.B.K., and G. Kelsoe, unpublished data).

Somatic hypermutation and fixation of mutations over a period of 18 years, presumably by exposure to viruses that had evolved resistance to one or more of the earlier antibodies in the lineages, follows the course expected for co-evolution of influenza virus and responding human antibodies. Determination of single-genome HIV glycoprotein sequences from infected individuals participating in longitudinal studies and concomitant B cell lineage analyses have enabled studies of the HIV antibody arms race as it plays out in a single person (Liao et al., 2013; Fera et al., 2014). A more-complex relationship dictates influenza virus and human humoral immunity co-evolution, which proceeds by episodic exposure (from vaccination or occasional infection) of an individual immune system to antigen and continuous exposure of the circulating virus to herd immunity (Figure 7). Nonetheless, our results suggest that it should be possible to carry out studies of the arms race between influenza virus and the collective immune response of the sampled population.

Exposure of TIV01 to an H1 influenza virus circulating during infancy (1990–1993) elicited B cell memory that dominated the response to a trivalent vaccine administered in 2008 (Moody et al., 2011). More than 60% of the heavy-chain variable regions cloned from sorted B cells taken 7 days post-vaccination have the sequence hallmarks of RBS-directed antibodies, and 50% belong to one of the six lineages studied here. The large number of distinct lineages of RBS-directed antibodies found in TIV01 is unusual, however. Whereas RBS-directed antibodies may be relatively common (Schmidt et al., 2015), other individuals probably have at most one or two, making antigenic drift toward resistance possible. Indeed, the data in Figure 6 show that, by 2008, a circulating virus (A/North Carolina/AF1292/2008) no longer bound antibodies from four of the six lineages. One objective for an improved influenza vaccine might be to increase the frequency of multiple RBS-directed antibodies in a large enough fraction of a population to hinder accumulation of such mutant viruses.

The observations reported here are consistent with the notion that viral antigens seen very early in life, as an infant’s immunity develops, pre-condition subsequent responses by biasing the memory repertoire more strongly than do antigens first seen at later times. This outcome would be similar to imprinting of the B cell response to bacterial antigens by the early intestinal microbiome, as seen in experiments with mice (Hooper et al., 2012). In a recently published study of a donor who received the 2011 trivalent vaccine, four different day 7 antibodies that neutralized the A/California/07/2009 component of the vaccine also neutralized the A/USSR/90/1977 isolate, suggesting priming by a strain closely related to the 1977 virus (Huang et al., 2015). The epitope recognized by those antibodies is on the head but in a surface patch well separated from the RBS. The A/USSR/90/1977 virus, which represents influenza viruses circulating during the donor’s childhood, was also the first reappearance of a seasonal H1 after its displacement by H2 in 1957. It would have been the earliest H1 strain that the donor could have seen. The published study did not include analysis of the properties of the UCAs of the day 7 antibodies, as we have done here for the six RBS-directed lineages from TIV01, and any inference that the germline precursors of the antibodies in that work would recognize the 1977 strain is indirect. Nonetheless, its results suggest that our findings from TIV01 apply more broadly, as they come from an individual over a decade older, who received a different trivalent vaccine, and they involve a different epitope.

The concept of “B cell lineage vaccine design” underlies much of current research on vaccines for HIV, influenza, and other rapidly evolving pathogens (Haynes et al., 2012; Jardine et al., 2015). This approach assumes (1) that dominant responses to exposed, variable epitopes outcompete broadly neutralizing, subdominant responses to more cryptic, conserved epitopes and (2) that within the naive B cell repertoires of most individuals in a population are cells that can respond to those conserved epitopes when appropriately stimulated. A stimulus that might, in principle, increase the likelihood of eliciting an otherwise subdominant response is a modified immunogen that binds with high affinity to the UCA of a lineage with broadly neutralizing potential. In the lineages studied here, the apparent priming stimulus was infection with a seasonal strain during the year after the person was born, with potential boosts from subsequent

**Figure 6. Breadth in RBS-Directed Clonal Lineages**

Antibodies from clonal lineages assayed for binding to H1 influenza viruses. $K_d$ values are for Fabs and expressed in μM. The heatmap color scheme is arbitrary; see legend to Figure 1.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>H1 Strain</th>
<th>H1220</th>
<th>H1222</th>
</tr>
</thead>
<tbody>
<tr>
<td>California (69)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>North Carolina (68)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>North Carolina (97)</td>
<td>2.9</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>North Carolina (03)</td>
<td>2.6</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>New California (99)</td>
<td>3.4</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Beijing (95)</td>
<td>1.1</td>
<td>12.4</td>
<td>0.41</td>
</tr>
<tr>
<td>Florida (93)</td>
<td>0.54</td>
<td>1.0</td>
<td>0.96</td>
</tr>
<tr>
<td>Wellington (92)</td>
<td>0.76</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Massachusetts (90)</td>
<td>0.34</td>
<td>0.38</td>
<td>0.53</td>
</tr>
<tr>
<td>Kawasaki (82)</td>
<td>0.19</td>
<td>0.55</td>
<td>0.72</td>
</tr>
<tr>
<td>USSR (77)</td>
<td>16.7</td>
<td>54.5</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Nonetheless, the data in Figure 6 show that, by 2008, a circulating virus (A/North Carolina/AF1292/2008) no longer bound antibodies from four of the six lineages. One objective for an improved influenza vaccine might be to increase the frequency of multiple RBS-directed antibodies in a large enough fraction of a population to hinder accumulation of such mutant viruses. The observations reported here are consistent with the notion that viral antigens seen very early in life, as an infant’s immunity develops, pre-condition subsequent responses by biasing the memory repertoire more strongly than do antigens first seen at later times. This outcome would be similar to imprinting of the B cell response to bacterial antigens by the early intestinal microbiome, as seen in experiments with mice (Hooper et al., 2012). In a recently published study of a donor who received the 2011 trivalent vaccine, four different day 7 antibodies that neutralized the A/California/07/2009 component of the vaccine also neutralized the A/USSR/90/1977 isolate, suggesting priming by a strain closely related to the 1977 virus (Huang et al., 2015). The epitope recognized by those antibodies is on the head but in a surface patch well separated from the RBS. The A/USSR/90/1977 virus, which represents influenza viruses circulating during the donor’s childhood, was also the first reappearance of a seasonal H1 after its displacement by H2 in 1957. It would have been the earliest H1 strain that the donor could have seen. The published study did not include analysis of the properties of the UCAs of the day 7 antibodies, as we have done here for the six RBS-directed lineages from TIV01, and any inference that the germline precursors of the antibodies in that work would recognize the 1977 strain is indirect. Nonetheless, its results suggest that our findings from TIV01 apply more broadly, as they come from an individual over a decade older, who received a different trivalent vaccine, and they involve a different epitope.

The concept of “B cell lineage vaccine design” underlies much of current research on vaccines for HIV, influenza, and other rapidly evolving pathogens (Haynes et al., 2012; Jardine et al., 2015). This approach assumes (1) that dominant responses to exposed, variable epitopes outcompete broadly neutralizing, subdominant responses to more cryptic, conserved epitopes and (2) that within the naive B cell repertoires of most individuals in a population are cells that can respond to those conserved epitopes when appropriately stimulated. A stimulus that might, in principle, increase the likelihood of eliciting an otherwise subdominant response is a modified immunogen that binds with high affinity to the UCA of a lineage with broadly neutralizing potential. In the lineages studied here, the apparent priming stimulus was infection with a seasonal strain during the year after the person was born, with potential boosts from subsequent
exposures between 1993 and 2008 and a known one from the 2008 vaccine. The boost(s) led to further affinity maturation, with development of increased breadth; analysis of the intermediates in certain lineages suggests where affinity maturation to the primary exposure ceased and where the secondary exposure(s) took over. Our observation, that early exposure appears to have biased immunological memory in this individual, suggests that vaccination of infants with HA immunogens selectively exposing conserved epitopes might be a constructive step toward a vaccine that elicits long-lasting immunity.

**EXPERIMENTAL PROCEDURES**

**Subjects**

TIV01 was recruited at Duke University and given the trivalent inactivated seasonal influenza vaccine (TIV) 2007–2008 containing A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004, as described previously (Liao et al., 2009; Moody et al., 2011).

**Lineage Analysis**

The lineages analyzed here have been described and characterized previously (Moody et al., 2011; Schmidt et al., 2015). The phylogenetic tree for each clone was estimated using the dnaml program from Phylip 3.69 package, and the UCA was obtained by aligning the inferred sequence at the root to germline gene libraries. The tree was then recomputed with the UCA at the root, and the sequences of intermediates at branch points in that tree were inferred as described (Keppler, 2013). The degree of somatic hypermutation was such that we found no ambiguity in assigning from the IMGT database the alleles and sequences for heavy-chain V, D, and J and light-chain V and J. The only potential uncertainties were at the V-D and D-J boundaries in the heavy chain, where residues encoded by potential n nucleotides cannot be derived from genome sequences. Five of the six clones had so few mutations in CDR H3 that the inferred UCA sequences were unambiguous; for the sixth (641), we followed the analysis in Moody et al. (2011). If the UCA sequence (the root of the tree) and sequences of the day 7 antibodies (the twigs of the tree) are known, then the inferred sequences of intermediates are essentially unambiguous. Paired-chain sequence used in the original study ensures correct assignment of the heavy- and light-chain combination.

**Expression and Purification of HA**

All rHA “head” constructs were cloned into pFastBac vector for insect cell expression. All contained a cleavable C-terminal His6X tag and were purified from supernatants by passage over Co-NTA agarose (Clontech) followed by gel filtration chromatography on Superdex 200 (GE Healthcare) in 10 mM Tris-HCl, 150 mM NaCl at pH 7.5. The tag was removed using PreScission protease (MolBioTech; ThermoScientific) and the protein repurified using Co-NTA agarose to remove the protease, tag, and non-cleaved protein. All mutations were made using QuikChange Mutagenesis (Agilent).
Fab Expression and Purification

The genes for the heavy- and light-chain variable domains were synthesized and codon optimized by Integrated DNA Technologies or GenScript and subcloned into protein expression vectors containing human heavy- and light-chain constant domains. All heavy-chain constructs contained a non-cleavable His6 or 6XHis tag. Fabs were produced by transient transfection in HEK293T cells using Lipofectamine 2000 (Invitrogen). Supernatants were harvested 5 days later, clarified by centrifugation, and the Fabs were purified using Co-NTA agarose (Clontech) followed by gel filtration chromatography on Superdex 200 (GE Healthcare). The buffer purification buffer was 10 mM Tris-HCl, 150 mM NaCl at pH 7.5. All constructs were confirmed by DNA sequencing at the DNA Sequencing Core Facility at Dana Farber Cancer Institute.

Interferometry and Binding Experiments

Interferometry experiments were performed using a BLItz instrument (forte-BIO; Pall). Purified Fab was immobilized on a Ni-NTA biosensor, and cleaved HLA heads were titrated to obtain binding kinetics and affinities. $K_D$ values were obtained by applying a 1:1 binding isotherm using vendor-supplied software under the “Advanced Kinetics” program. UCA binding was tested at an initially obtained 5 days later, clarified by centrifugation, and the Fabs were purified using Co-NTA agarose (Clontech) followed by gel filtration chromatography on Superdex 200 (GE Healthcare). The buffer purification buffer was 10 mM Tris-HCl, 150 mM NaCl at pH 7.5. All constructs were confirmed by DNA sequencing at the DNA Sequencing Core Facility at Dana Farber Cancer Institute.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2015.11.083.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank members of the S.C.H. laboratory for discussions. The work at Harvard Medical School and Boston Children’s Hospital was supported by NIH grant P01-A1089618. S.C.H. is an investigator of the Howard Hughes Medical Institute.

REFERENCES


Immunogenic Stimulus for Germline Precursors of Antibodies that Engage the Influenza Hemagglutinin Receptor-Binding Site

<table>
<thead>
<tr>
<th>Country</th>
<th>Date</th>
<th>Accession</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>9/1977</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD 101</td>
</tr>
<tr>
<td>Kawasaki</td>
<td>6/1986</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>Massachusetts</td>
<td>1/1990</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>Wellington</td>
<td>4/1992</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>Florida</td>
<td>2/1993</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>Beijing</td>
<td>2/1995</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>New Caledonia</td>
<td>20/1999</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>3/2003</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2006</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2007</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>A/Solomon Islands</td>
<td>03/2006</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>A/Florida</td>
<td>2/1993</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>A/Wellington</td>
<td>4/1992</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>A/Beijing</td>
<td>6/1986</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2007</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2007</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2007</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
<tr>
<td>North Carolina</td>
<td>1/2007</td>
<td>A/LILNA/1/77</td>
<td>APLQGLKCN1AG9ILNGFECSE5LF6K6S5A1ETPTNENGTCYPYFAD</td>
</tr>
</tbody>
</table>

Figure S1. Sequence alignment of H1 influenza virus HA1s from 1977-2009 used in this study, Related to Figure 1. Residues of the RBS are highlighted in yellow with other key residues highlighted and marked. See also Figure 1.
Figure S2. Mutational frequency of isolated TIV antibodies, Related to Figure 2, Figure 3 and Figure 4. (A) Average $V_{H}$ mutational frequency of RBS-directed lineages. For reference, the approximate mutational frequencies expected for naïve, primary and secondary responses are listed. (B) Average $V_{H}$ mutational frequency of all HA-specific antibodies isolated from TIV01 day 7 plasmablasts. (C) Average $V_{H}$ mutational frequency of all HA-specific antibodies isolated from additional TIV cohort (Moody et al, 2011). See also Figure 2, Figure 3 and Figure 4.
Figure S3. Dependence of UCA binding on residue K133a, Related to Figure 1 and Figure 5. Affinities (in μM) for UCAs with deletion (Δ) in H1 Massachusetts (1990) or insertion (+) in H1 Solomon Islands (2006). See also Figure 1 and Figure 5.

<table>
<thead>
<tr>
<th></th>
<th>Massachusetts</th>
<th>Solomon Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin 641 UCA</td>
<td>&gt;100</td>
<td>1.9</td>
</tr>
<tr>
<td>Lin 643 UCA</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Lin 652 UCA</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Lin 687 UCA</td>
<td>&gt;100</td>
<td>3.6</td>
</tr>
<tr>
<td>Lin 860 UCA</td>
<td>&gt;100</td>
<td>59</td>
</tr>
<tr>
<td>Lin 1277 UCA</td>
<td>&gt;100</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Figure S4. Related to Figure 2 and Figure 6. Lineage 860 UCA and I-2 HA-reactivity. (A) Lineage 860 clonal tree; (B) data for UCA and I-2 binding to members of the panel of H1 HA1s. $K_D$ values, expressed in mM, are for interaction of monomeric Fab and HA head. See also Figure 2 and Figure 6.