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Focused antibody response to influenza linked to antigenic drift

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The selective pressure that drives antigenic changes in influenza viruses is thought to originate from the human immune response. Here, we have characterized the B cell repertoire from a previously vaccinated donor whose serum had reduced neutralizing activity against the recently evolved clade 6B H1N1pdm09 viruses. While the response was markedly polyclonal, 88% of clones failed to recognize clade 6B viruses; however, the ability to neutralize A/USSR/90/1977 influenza, to which the donor would have been exposed in childhood, was retained. In vitro selection of virus variants with representative monoclonal antibodies revealed that a single amino acid replacement at residue K163 in the Sa antigenic site, which is characteristic of the clade 6B viruses, was responsible for resistance to neutralization by multiple monoclonal antibodies and the donor serum. The K163 residue lies in a part of a conserved surface that is common to the hemagglutinins of the 1977 and 2009 H1N1 viruses. Vaccination with the 2009 hemagglutinin induced an antibody response tightly focused on this common surface that is capable of selecting current antigenic drift variants in H1N1pdm09 influenza viruses. Moreover, amino acid replacement at K163 was not highlighted by standard ferret antisera. Human monoclonal antibodies may be a useful adjunct to ferret antisera for detecting antigenic drift in influenza viruses.

Introduction

Influenza viruses circulating in humans mutate and change their antigenicity at a rate that necessitates frequent updating of subunit vaccines. This process of antigenic drift requires a global collaboration, organized through the WHO, to monitor the antigenic relatedness of new viruses and select the most appropriate for inclusion in vaccines that are updated regularly (1). The selective force driving the evolution of influenza in humans is thought to be the neutralizing antibody response. However, this raises a paradox, as the antibody response is polyclonal and in principle is capable of neutralizing the virus at multiple sites on the hemagglutinin glycoprotein (HA). As many as 12 sequential mutations in the hemagglutinin may be required to completely abrogate neutralization by a strong immune serum (2). This would require mutation at multiple positions in HA simultaneously for the virus to escape in nature, which should be a very rare event.

Two alternative mechanisms have been suggested to resolve the paradox. Adsorptive amino acid substitutions have been described that can globally reduce the neutralizing power of a serum (3). It is thought that this is mediated through the substitutions causing an increase in the binding affinity of hemagglutinin for its receptor sialic acid (4). Selection of virus variants of this type has been demonstrated in an animal model of influenza infection (4, 5) and also defined with neutralization-resistant variants selected in vitro with certain monoclonal antibodies (3, 6). However, a recent analysis of the evolution of H3N2 viruses has demonstrated that the binding affinity of H3 hemagglutinin has significantly decreased since the appearance of H3N2 viruses in humans in 1968, which suggests additional mechanisms may be at work (7).

The second possibility is that, in some humans, the antibody response can be focused on a subregion of the hemagglutinin to such an extent that it becomes functionally monoclonal and can select viruses with point mutations. Early evidence for this came from finding reduced neutralization of viruses with single-point mutations (8–12) and selective inhibition by sera of binding by characterized hemagglutinin-specific monoclonal antibody Fab fragments (13). A recent paper has identified a single amino acid substitution (K163Q in our numbering) to be responsible for reduced serologic reactivity with 2013–2014 H1N1pdm09 viruses in up to 42% of middle-aged adults born between 1965 and 1979, who would have been exposed to the H1N1 viruses that reemerged in 1977 (14).

These studies did not address the clonal basis for the focused antibody responses in humans or show that the antibodies in the sera were able to select antigenic variants of influenza viruses. We have addressed this by analysis of 157 monoclonal antibodies isolated from a donor with reduced serological reactivity to the recently evolved clade 6B H1N1pdm09 influenza viruses. We show that the response was markedly polyclonal and displayed varying cross-reactivity for recent seasonal H1N1 viruses. However, 88% of the isolated antibodies failed to recognize the clade 6B viruses, while all of these clones neutralized A/USSR/90/1977
(H1N1), which circulated during the donor’s childhood. Resistant viruses isolated in vitro with representative monoclonal antibodies selected amino acid substitutions at HA K163 that matched viruses isolated in vitro with representative monoclonal antibodies. Panels of human monoclonal antibodies may be a useful adjunct to ferret antisera in detection of antigenic drift and ferret antisera. Finally, the K163 substitutions in the clade 6B H1N1pdm09 influenza viruses. They also show that viruses circulating in the donor's childhood and the H1N1pdm09 virus (15). This suggests that antibodies primed by this common patch of surface may have been selectively recalled and expanded by exposure to the H1N1pdm09 hemagglutinin to form a focused antibody response, capable of selecting antigenic variants with substitutions at K163. Our results match the focused antibody response in a subset of humans with the recent evolution of the clade 6B H1N1pdm09 influenza viruses. They also show that selection of virus variants with human monoclonal antibodies isolated from vaccinated or infected individuals may be able to predict the sites of future antigenic drift in influenza A viruses. Finally, the K163 substitutions in the clade 6B H1N1pdm09 viruses were not flagged as significant antigenic drift by standard ferret antiserum. Panels of human monoclonal antibodies may be a useful adjunct to ferret antiserum in detection of antigenic drift and in the selection of viruses for inclusion in influenza vaccines.

Results

Influenza virus–specific antibody response after vaccination. A healthy adult volunteer (referred to as donor H) had received trivalent inactivated influenza vaccine 3 times since 2008. The vaccines contained the antigens shown in Table 1.

Figure 1 shows the neutralization titers recovered from donor H for former seasonal H1, H3, pandemic H1N1 2009, and H5 viruses. Before the first vaccination in November 2008, plasma contained low-titer antibodies to all influenza A subtypes screened. Six months after the first dose of vaccine, a response was seen to former seasonal H1 (A/Brisbane/59/2007) and H3 (A/Victoria/361/2011) viruses that did not cross-neutralize H1N1pdm09 or H5. This pattern of reactivity was maintained 2 years after the second vaccination. The third vaccination on November 10, 2011, contained H1N1pdm09 HA for the first time. One day after the third vaccination a low-level response to H1N1pdm09 virus was detected. This may have reflected an earlier exposure or a very early response to vaccination. Twelve days after vaccination a very strong response to H1N1pdm09 influenza was detected, which was associated with a small-titer increase to H3 viruses and no change in the titer to seasonal H1. The titer to an H5-pseudotyped influenza, albeit low, was boosted by the H1pdm09 virus as compared with levels before vaccination.

This serum was next tested on a selection of the recently evolved clade 6B H1N1pdm09 viruses. We found a substantial reduction in the neutralization titer of between 4- and 8-fold on the clade 6B viruses. To analyze the clonal basis for this reduced titer, we isolated monoclonal antibodies from the donor’s plasmablasts taken 7 days after vaccination.

Identification of HA-specific B cells. We have previously confirmed that influenza virus–specific plasmablasts are induced transiently in large number in the peripheral blood during the first week after vaccination with inactivated former seasonal H1N1 and H1N1pdm09 viruses and the frequency of influenza virus–specific plasmablasts significantly correlated with the level of neutralizing antibody response (16–18). At day 7 after vaccination of donor H, flow cytometry surface marker analysis revealed a distinct population of plasmablasts (defined as CD3 CD20+CD20+ CD19+CD27+CD38+ cells), which accounted for 0.9% of total peripheral lymphocytes. We next detected hemagglutinin-specific cells by ex vivo B cell ELISPOT with purified hemagglutinin and measured frequencies of H1N1pdm09 (A/California/07/2009) HA-specific and Eng195 (A/England/195/2009) HA-specific IgG plasmablasts at 425 and 315 per million peripheral blood mononuclear cells.

Binding of recombinant HA to transmembrane Ig G1. In these experiments, the HA molecules did not have any mutations to abolish sialic acid binding, as described by Whittle et al. (19). In contrast to Whittle’s method, in which the trimeric HA was combined in excess with streptavidin to form oligomers prior to staining, we stained sequentially with hemagglutinin followed by ExtrAvidin. For specificity this method relies on the high affinity of membrane-bound antibody for HA, compared with the low affinity of single HA trimers for sialic acid.

We first established that our preparations of recombinant soluble HA could detect specific transmembrane Ig expressed at the cell surface. As shown in Supplemental Figure 1A, row 4 (supplemental material available online with this article; doi:10.1172/JCI81104DS1), all of the cells transfected with the reconstructed transmembrane Ig G1 (TMigG1) clones were stained with an anti-IgG reagent. The cells were then stained with biotin-labeled recombinant H3 (A/Victoria/361/2011; Supplemental Figure 1A, row 2), H1N1pdm09 (A/England/195/2009; Supplemental Figure 1A, row 1), H5 (A/Vietnam/1203/2004; Supplemental Figure 1A, row 3), and H7 (A/Anhui/1/2013; Supplemental Figure 1A, row 5) HAs. The H3 HA stained specifically the anti-H3–transfected cells (Supplemental Figure 1A, column 4); H1N1pdm09 HA stained cells expressing TMigG1 to epitopes in the head and stem of H1 HA (Supplemental Figure 1A, columns 1–3); and the H5 protein stained only the cells expressing TMigG1 specific for the stem of H1N1pdm09 HA (Supplemental Figure 1A, column 3) that cross-reacted with the H5 HA. The H7 HA

### Table 1. Vaccination history and vaccine content

<table>
<thead>
<tr>
<th>Date</th>
<th>Vaccine composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>November 11, 2008</td>
<td>A/Brisbane/59/2007 H1N1-like virus</td>
</tr>
<tr>
<td>November 10, 2011</td>
<td>A/Brisbane/59/2007 H3N2-like virus</td>
</tr>
<tr>
<td>November 26, 2009</td>
<td>A/Brisbane/59/2007 H1N1-like virus</td>
</tr>
<tr>
<td>November 10, 2011</td>
<td>A/California/07/2009 H1N1-like virus</td>
</tr>
<tr>
<td>November 11, 2008</td>
<td>A/Brisbane/59/2007 H3N2-like virus</td>
</tr>
<tr>
<td>November 11, 2008</td>
<td>A/Perth/16/2009 H3N2-like virus</td>
</tr>
<tr>
<td>November 10, 2011</td>
<td>B/Brisbane/50/2008-like virus</td>
</tr>
<tr>
<td>November 10, 2011</td>
<td>B/Brisbane/50/2008-like virus</td>
</tr>
</tbody>
</table>
Antigen-specific sorting of plasmablasts. The biotinylated HA of A/England/195/2009 stained approximately 26% of the plasmablasts at day 7 after the vaccination of donor H during the 2011–2012 influenza season (Supplemental Figure 1B). A total of 288 cells stained with the HA were sorted singly, and 167 cells were found to be PCR positive for the heavy and light chains of Ig. These 167 cells were used to produce human monoclonal antibodies (20). Of the 167 antibodies, 157 were shown to bind HA by one or more of the following: staining of HA-transfected cells, ELISA, hemagglutination inhibition (HAI), specific neutralization, or binding in Western blots. Each of the 157 antibodies had a unique sequence, which was identified as a productive sequence by the International Immunogenetics Information System (21).

Table 2. Set of 157 HA-binding and productive sequences

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Vγ gene usage</th>
<th>No. of antibodies</th>
<th>No. of clones</th>
<th>Size of the largest clone</th>
<th>HCDR3 length</th>
<th>Vn</th>
<th>Dn</th>
<th>Jn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head S</td>
<td>3-7</td>
<td>88</td>
<td>6</td>
<td>73</td>
<td>18</td>
<td>3-7*01 or 05 or 06</td>
<td>3-16*01</td>
<td>6*02</td>
</tr>
<tr>
<td></td>
<td>3-33</td>
<td>12</td>
<td>2</td>
<td>10</td>
<td>17</td>
<td>3-33*01 or 05 or 06</td>
<td>1-26*01</td>
<td>6*02</td>
</tr>
<tr>
<td>Head CR</td>
<td>1-18</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>17</td>
<td>1-18*01 or 05 or 06</td>
<td>2-21*02</td>
<td>6*02</td>
</tr>
<tr>
<td>Stem CR</td>
<td>3-15</td>
<td>18</td>
<td>3</td>
<td>16</td>
<td>17</td>
<td>3-15*01 or 02 or 03</td>
<td>2-22*01</td>
<td>4*02</td>
</tr>
<tr>
<td></td>
<td>1-69</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>13</td>
<td>1-69<em>01 or 1-69D</em>01</td>
<td>2-22*01</td>
<td>6*03</td>
</tr>
<tr>
<td></td>
<td>3-23</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>17</td>
<td>3-23<em>01 or 3-23D</em>01</td>
<td>3-22*01</td>
<td>4*02</td>
</tr>
</tbody>
</table>

4Each of the Vγ-related sets were defined by their specific VDJ rearrangements. HCDR3, heavy chain complementarity-determining region 3; Dn, heavy chain diversity region; Jn, heavy chain joining region; Head S, head specific; Head CR, head cross reactive; Stem CR, stem cross reactive.
The 157 unique sequences of HA-binding antibodies could be divided into 11 sets defined by their use of particular heavy chain variable region (VH) genes (Table 2); 150 of 157 sequences (96%) were derived from the 6 largest VH sets (Figure 2A and Table 2). Each VH set was composed of several clones defined by specific VDJ rearrangements (Figure 2A and Table 2) in the heavy chain, and each clone was further diversified by a unique pattern and frequency of somatic mutations. In each VH-related set, one clone tended to dominate (Table 2). For example, within the VH 3-7 set, 73 of 88 antibodies belong to one clone, which diversified by somatic mutation (Supplemental Figure 2). These clones are defined by the unique identity of their VDJ segments and their reading frames. Selections of clones from each of the 6 largest VH-related sets were expanded, and their antibodies were purified and tested in a quantitative assay (see Methods) for neutralization of a variety of viruses (Figure 2).

Several different neutralization assays are described in the literature, so quantitative comparisons between data sets are difficult. For direct comparison, we have included samples of 7 published antibodies (Figure 2B) (see Methods and Acknowledgments for details). Two antibodies reacted broadly to the H1 head, 15-2A06 and 19-4G05 (22), and five antibodies were reactive to the stem of the HA, V3-2G6 (23), FI6v3 (24), SF70-1F02 (25), and 05-2G02 and 09-3A01 (22). The antibodies from donor H neutralized influenza in our quantitative assay at comparable concentrations to the reference antibodies and showed similar patterns of activities to the various virus isolates. Six percent of clones (10 of 157) were definitely defined as reacting with the HA stem on the basis of competition for binding with the canonical stem-reactive antibody C179 (26, 27), inability to block red cell agglutination, and broad neutralization, including our example of a VH 3-23 antibody (T3-5D) that recognized seasonal and pandemic H1, H5, and recent H3 HAs (Figure 2B). Three further antibodies were very closely related in sequence to definite stem antibodies and have been classified as “stem” in Table 2. Eighty-eight percent of antibodies (138 of 157) were able to inhibit hemagglutination and showed less broad reactivity, consistent with binding to the globular head domain of HA. The sequences of all antibodies named in Figure 2B are deposited in the GenBank database (accessions KP231608–KP231649).

### Table 3. MN of natural variants and in vitro antibody-selected variant viruses

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VH Specificity</th>
<th>MN of natural variants</th>
<th>MN of in vitro antibody-selected variant viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H1N1 viruses</td>
<td>Clade 6B viruses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/USSR/90/1977</td>
<td>A/Bayern/14/2003 (K163E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/Bayern/7/1995</td>
<td>A/Bayern/6/2009 (G155E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/New Caledonia/20/1999</td>
<td>H/Serbia/NS 601/2013 (K163E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/Faribard/55/2007</td>
<td>A/Serbia/NS 601/2013 (K163E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XT179 (A/Kalifornia/07/20109)</td>
<td>A/Serbia/NS 601/2013 (K163E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/Brisbane/14/2003 (K163E)</td>
<td>A/England/62/2013 (K163E)</td>
</tr>
</tbody>
</table>

*MN EC50 values: –, negative; +, 2–20 μg/ml; ++, 0.2–2 μg/ml; ++++, 20–200 ng/ml; ++++, 0–20 ng/ml. BSera were obtained on day 7 and 12 after 2011–2012 influenza vaccination.
Figure 2. The 6 largest V_{H} related sets of HA-binding antibodies derived from day 7 Eng195 HA-specific B cells. (A) The average number of V_{H}, nucleotide and amino acid replacements in the 6 largest sets of HA-binding antibodies. The representative HAI and MN results of head-specific (Head S), head cross-reactive (Head CR), and stem cross-reactive (Stem CR) antibodies are summarized in the figure. –, negative; +, EC_{50} ~366 ng/ml; ++, EC_{50} ~70 ng/ml; ++++, EC_{50} ~7 ng/ml for neutralization of X179A virus (A/California/07/2009 H1pdm09). (B) Neutralization titers of head-specific, head cross-reactive, and stem cross-reactive antibodies for former H1, H3, H1pdm09, and H5 viruses. Two H1 head-specific, 15-2A06 and 19-4G05 (22), and 5 stem-reactive, FI6v3 (24), V3-2G6 (23), SF70-1F02 (25), and 05-2G02 and 09-3A01 (22), reference antibodies were included. Each symbol represents an independent measurement. Some measurements overlap. MN titers were assayed at least twice for each antibody. The mean EC_{50} for neutralization for the 6 largest sets of HA-binding antibodies in the MN assay was analyzed by 1-way ANOVA, and a P value less than 0.05 was considered significant.
H1N1pdm09 HA–specific clones that do not recognize recent seasonal H1N1 viruses. Fifty-six percent (88 of 157) of the sequences were derived from a dominant VH-related set, in which the Ig variable region was encoded by VH 3-7, associated most frequently with JH 6*02/DH 3-16*01 and Vλ 1-40*01/Jλ 2*01 (Table 2 and Supple- mental Figure 2). At least 6 clones could be identified within this set based on their unique VDJ heavy chain gene rearrangements, all of which resulted in a CDR3 length of 18 amino acids (Table 2). One dominant clone made up 73 of 88 sequences. An amino acid sequence dendrogram of the VDJ regions (Supplemental Figure 2) revealed the diversity in these sequences generated by V region somatic mutations (which induced an average of 7–amino acid changes per sequence) and clonal D-J combinations. This result is very similar to findings by Krause et al. (28) and Jackson et al. (29), who identified very similar sets of clones based on VH 3-7/JH 6 rearrangements in individuals responding to the Sa site of H1N1pdm09 HA and discussed the evidence for intraclonal divergence and interclonal convergence of these sequences.

Initial screening showed that clones from the VH 3-7 set inhibited hemagglutination by H1N1pdm09 (X179A A/California/07/2009) virus. Three examples were expanded, and purified IgG1 was titrated against various viruses in HAI and micro-neutralization (MN) assays. The antibodies from these clones were the most potent of all those isolated, with an EC50 for neutralizing H1N1pdm09 virus at concentrations of approximately 7 ng/ml (~5 × 10−11 M), but they failed to react by HAI or MN with recent seasonal H1 viruses (Figure 2 and Table 3).

To identify the site recognized by these VH 3-7*01 clones, variants of the vaccine virus X179A (containing A/California/07/2009 HA) were selected with the VH 3-7*01 antibody T2-6A (see Methods). We found a single mutation encoding the K163E substitution in the HA in the Sa antigenic site to be associated with loss of neutralization and binding by this antibody and related members of the VH 3-7 set (Figure 3 and Table 3). The K163E substitution corresponds to K166E isolated by Krause et al. (28), with their VH 3-7*01 clone 4K8 in the context of A/California/04/2009 HA (our sequence numbering is as used in the WHO annual reports) (30).

Two further VH-related sets of sequences characterized by rearrangements of VH 3-33 (12 of 157) and VH 1-18 (20 of 157) had similar properties to the VH 3-7 family. They also were polyclonal (Table 2) and specific for H1N1pdm09 (Figure 2A) but tended to require higher concentrations of antibody for neutralization (Fig-
ure 2B) and contained more somatic mutations (Figure 2A; 21 ± 2 mutations for VH 3-33 and 20 ± 3 mutations for VH 1-18). Remarkably, neutralization and binding by representatives of both sets of antibodies were lost for the same K163E variant, selected by the VH 3-7*01 clone (Figure 3 and Table 3).

Antibodies to H1 HA head that cross-react on former seasonal H1N1 strains. A fourth dominant set of clones (18 of 157, 11%) (Table 2), which cross-reacted almost equally between seasonal (A/Brisbane/59/2007) and H1N1pdm09 (A/California/07/2009) viruses in HAI and MN tests, was characterized by rearrangements of VH 3-15 (Figure 2 and Table 3). Further analysis revealed neutralization of a broad range of former seasonal H1N1 viruses isolated between 1995 and 2007 (Table 3). All of the VH 3-15 clones inhibited hemagglutination and were not able to compete for binding by stem-specific antibody C179 (26). This suggests that the VH 3-15 antibodies bind to an epitope in the head of H1 HA conserved between recent former seasonal and H1N1pdm09 viruses (27). The mean EC50 for neutralization for a selection of these VH 3-15 antibodies in the MN assay was approximately 70 ng/ml for X179A (Figure 2B), significantly higher than that for the VH 3-7 family (ANOVA, P < 0.001). The average number of somatic mutations in the 18 VH 3-15 sequences was 25 ± 4 (Figure 2A), which was also significantly higher than that for the VH 3-7 H1N1pdm09-specific clones (ANOVA, P < 0.001).

To identify the site recognized by these more broadly reactive antibodies, we selected mutants of X179A with a representative VH 3-15*01 antibody, T3-4B. It selected a mutation at exactly the same position, K163Q, as the VH 3-7*01 clone T2-6A, which selected K163E.

Antibodies to the globular head focused on K163. We then tested a selection of antibodies from the 4 VH-related sets, representing 138 of 157 of the antibodies isolated from donor H, for binding and neutralization of viruses that contained K163E and K163Q substitutions (Figure 3 and Table 3 summarize the results). The K163E and K163Q substitutions abolished neutralization (Table 3) and binding (Figure 3) by all 4 antibody sets (VH 3-7, 3-33, 1-18, and 3-15) recognizing the globular head. Both mutations also prevented neutralization by the 2 broadly reactive control antibodies recognizing the globular head, 15-2A06 and 19-4G05 (22), which are encoded by different VH genes (VH 1-2 and 3-74, respectively), implying that these 2 antibodies bind to closely related epitopes. The K163Q substitution only partially inhibited binding by the 19-4G05 control antibody, but this was sufficient to prevent neutralization (Figure 3 and Table 3).

By contrast, a clone from a different individual, 2-12C (VH 5-51), was not affected by the substitutions at K163E/Q in neutralization or binding assays (Figure 3 and Table 3). The 2-12C antibody selected a variant virus with HA substitutions K130E and G170R. This variant resulted in loss of neutralization and binding by the selecting antibody but was still susceptible to antibodies from donor H that were sensitive to changes at K163, showing that the binding sites of the 2 antibodies were functionally independent. Subsequently,
we found that an antigenic variant (A/Bayern/69/2009) with a single HA1 G155E substitution (WHO Influenza Centre February 2010 report; ref. 30) showed greatly reduced recognition by 2-12C antibody but was susceptible to antibodies from donor H (Table 3). The substitutions K130E and G170R also prevented neutralization by another control antibody, Q11A6 (V_{3-15} 3-21) from a different donor, with a pattern of reactivity similar that of to 2-12C (Table 3). Binding of 2-12C to cells infected with K163E/Q variant viruses was retained in full (Figure 3) but was abolished for cells infected with its own K130E/G170R variant. Binding by Q11A6 was reduced on the K130E/G170R variant (Figure 3), but this was associated with complete loss of neutralization (Table 3). As an additional positive control, a typical V_{3-7} 1-69 HA stem-reactive antibody produced by donor H reacted with all of the selected variant viruses in the infected cell–binding assays (Figure 3).

Mapping the substitutions onto a HA 3D structure (15) showed that positions 130 and 155, recognized by clone 2-12C, lie close together, bordering the sialic acid–binding site (shown in red in Figure 4). By contrast, K163, recognized by representatives of the 4 V_{3-15}–related sets of receptor-blocking antibodies from donor H, and the 2 broadly reactive control antibodies, 15-2A06 and 19-4G05 (21), map into the Sa antigenic site on the far side of the globular head domain but in reasonable proximity to the receptor-binding site (shown in blue in Figure 4). This physical separation is consistent with independent binding sites for the respective antibodies. The G170R amino acid substitution coselected by the control antibody 2-12C is likely to be coincidentally associated with K130E, as it is widely separated from K130 in the structure and was absent in the antigenic drift variant A/Bayern/69/2009, which also showed reduced neutralization by 2-12C.

As shown in Table 3, within the set of V_{3-15} sequences were members sharing identical VDJ rearrangements and differing only in somatic mutations that could distinguish between drifted viruses of former seasonal H1 viruses in the MN assay. For example, 3 of 6 V_{3-15} antibodies lost reactivity to A/Beijing/262/1995, while retaining reactivity with A/Bayern/7/1995. These two viruses differ at several positions, including K163N.

Reactivity with natural antigenic drift variants of H1N1pdm09 virus. Since 2009, the H1N1pdm09 viruses isolated in various centers around the globe have acquired a significant number of mutations, leading to HA amino acid substitutions, but have not undergone major antigenic drift, as determined by ferret antisera after infection in the HAI assay (WHO Influenza Centre February 2015 report; refs. 30, 31). It was of interest to assess the reactivity of the antibodies from donor H with recent H1N1pdm09 viruses.

The most informative comparisons are shown in Table 3. The EC_{50} for neutralization by the donor H serum harvested 7 days after 2011–2012 influenza vaccination was reduced from 1:2,560 on X179A to 1:480–1:640 on a selection of clade 6B viruses isolated in 2013 to 2014. Consistent with this, the head-specific monoclonal antibodies derived from the 4 most abundant VH–related sets (V_{3-7}, 3-33, 1-18, and 3-15), representing 138 of 157 (88%) of the clone sequences from donor H, lost the ability to neutralize clade 6B viruses (Table 3). HA K163Q is one of the substitutions that defines the 6B clade, but it is absent in A/California/07/2009 (WHO Influenza Centre September 2014 report; ref. 30). By contrast, antisera from ferrets infected with A/California/07/2009 are known to focus on the Sa site around positions 153–155, which is conserved in the clade 6B viruses (32). The control antibody from a different donor, 2-12C, which mapped to positions 130 and 155, like the ferret antisera, retained the capacity to neutralize clade 6B viruses at <10 ng/ml, similar to its activity on the A/California/07/2009 virus (Table 3). The amino acid sequence between 128 and 159 is identical for A/California/07/2009 and
the clade 6B viruses, which is consistent with retention of the binding sites for the standard ferret antisera and antibody 2-12C. The Q11A6 control showed similar recognition to 2-12C and also retained neutralization of clade 6B viruses.

Cross-inhibition by antibodies to H1N1pdm09 HA. We looked for cross-inhibition of binding to purified HA by the various sets of antibodies isolated from donor H that are sensitive to K163E/Q substitution and compared them to both the receptor-blocking antibody 2-12C (VH 5-51*01 recognizing K130/G155) isolated from a different donor and a typical VH 1-69 stem-reactive antibody. We tested 3 sets of receptor-blocking antibodies from donor H based on VH 3-7*01, 1-18*01, and 3-15*01 and found they were all able to cross-inhibit each other in an ELISA but were not inhibited by the typical VH 1-69 stem-reactive antibody (Supplemental Figure 3). By contrast, none of these antibodies from donor H could compete for binding with the H1N1pdm09 HA-specific antibody 2-12C, which was mapped adjacent to the receptor-binding site by the substitutions K130E and G155E. As shown by Supplemental Figure 3, the 2-12C antibody could inhibit the binding of the VH 3-15 antibody, T3-4B. This unidirectional competition suggests overlap in the binding footprints of these two antibodies.

The antibody response from donor H is focused. Taken together, these results suggest that approximately 88% of HA-reactive monoclonal antibodies derived from day 7 plasmablasts produced by donor H in response to vaccination with H1N1pdm09 HA from A/California/07/2009 were focused on a limited region of the HA head domain and shared an overlapping footprint that included position K163, which was substituted in the clade 6B viruses that have evolved since 2013 (WHO Influenza Centre September 2014 report; ref. 30).

Figure 6. Neutralizing activities by sera and monoclonal antibodies from vaccinated individuals against the variant viruses. (A) Sera from recently vaccinated individuals born between 1943 and 1982 (n = 28) and 1983 and 1988 (n = 13) were tested for neutralization of X179A and HA variants carrying single amino acid substitutions. Sera from two individuals (donor H and TIV-20) failed to neutralize the K163E variant of X179A, and their response was focused on the Sa site. Sera from two individuals born between 1983 and 1988 were focused on K130 (Ca2 site). Mean and 95% confidence interval are shown. (B) Serum after vaccination and the VH 3-7 antibody T2-6A from donor H, together with serum after vaccination from another donor (TIV-20), neutralized X179A strongly but failed to neutralize the K163E virus variant. Their neutralizing activity was unaffected by the K130E substitution. T1-3B and control serum had similar neutralization titers to all the viruses. In our MN assays, the OD450 readout was dependent on NP expression following viral entry (65). The MN assays were performed in duplicate.
Reactivity with A/USSR/90/1977. The high frequency of somatic mutations in the 4 sets of monoclonal antibodies from donor H that recognize K163 suggested that they may have been selected from the memory B cell pool (33). It is well known that the first exposure to influenza virus induces a repertoire of B cells that remain for life, from which subsequent exposures to influenza can select, if cross-reactive (34–37). The H1N1 viruses related to A/USSR/90/1977 reappeared in humans in 1977 after being absent since 1957. As donor H was a child in 1977, it is highly likely that the first exposure to H1N1 viruses was to a virus related to A/USSR/90/1977.

A selection of monoclonal antibodies from the 4 V\(_{H}\)-related sets (3-7, 1-18, 3-33, and 3-15), which were dependent on residue K163, were tested for neutralization of A/USSR/90/1977. All of these antibodies neutralized the 1977 virus strongly, suggesting that they may have been primed by this virus (Table 3). The amino acid residues surrounding K163, when mapped onto the HA structure, form a patch of conserved surface shared between the 1977 and 2009 HAs, consistent with the cross-reactivity shown by these antibodies (Figure 5).

A second donor with a similar pattern of reactivity to that of donor H. To assess the frequency of individuals that can make a highly focused antibody response to the region around K163, we compared sera for reactivity with A/California/07/2009 and variant viruses carrying substitutions at K163. Of the 41 sera samples screened after vaccination (MN titer >1:40), we found a second example in which the sera lost the ability to neutralize H1N1 viruses with K163 substitutions (Figure 6). These findings with sera are consistent with those of the survey of Linderman et al. (14), who found up to 42% of sera from donors born before 1979 showed reduced reactivity with clade 6B H1N1pdm09 isolates. Both our sera were from donors born before 1982 (2 of 28).

Broadly reactive antibodies to the H1 HA stem. Not all of the antibodies isolated from the day 7 plasmablasts from donor H were dependent on residue K163. We identified 10 of 157 (6%) definite stem-reactive antibodies (and 3 additional closely related sequences) by a combination of 4 assays: inhibition of C179 antibody binding to purified HA in ELISA (26, 27), broad neutralization (Figure 2), lack of HAI activity, and lack of reactivity in Western blots (data not shown). Six antibodies were encoded by V\(_{H}\) 1-69, 3 by V\(_{H}\) 3-23, and 1 by V\(_{H}\) 4-39 (Figure 2B and Table 2). These antibodies required a significantly higher concentration (mean 366 ng/ml) that was coated such that each of the 157 sequences was unique. The frequency of somatic mutations in each set is shown in Figure 2A. The high level of neutralization of the H5-pseudotyped influenza virus (EC\(_{50}\) ~1 ng/ml) that was coated in HA from A/Vietnam/1203/2004 (Figure 2B). This proportion of stem-reactive antibodies is consistent with the low level of neutralization of the H5-pseudotyped influenza by 12 days after vaccination in serum from donor H (Figure 1).

Therapeutic potential. To determine whether representatives of the 3 major sets of antibodies isolated from donor H, studied in detail here, were protective in vivo, we treated DBA/2 mice 24 hours after intrapulmonary infection with X179A virus (that contained HA from A/California/07/2009) with a single dose of 10 mg/kg of each antibody. The DBA/2 mouse strain is extremely sensitive to influenza infection (38), and we used an inoculum of virus approximately 150 times that required to cause 20% weight loss in at least 50% of the recipients. As shown in Supplemental Figure 4, antibodies with the 3 major specificities (head-specific V\(_{H}\) 3-7, head cross-reactive V\(_{H}\) 3-15, and stem cross-reactive V\(_{H}\) 1-69) were able to reverse weight loss after a 24- to 48-hour delay. By contrast, animals treated with PBS, or an H3 HA–specific antibody, failed to respond.

Discussion

Our study has confirmed that staining and sorting peripheral B cells with soluble HA can greatly enrich for virus HA-specific clones (19, 23). This enabled us to analyze a large sample of monoclonal antibodies from a single individual, for which heavy and light chains and function could be assigned. However, we acknowledge that this method may not sample the complete repertoire of B cells specific for HA. Plasmablasts that have lost expression of surface Ig would be missed, and the PCR strategy used selected for IgG-expressing clones. In addition, the plasmablasts taken at day 7 may not sample the complete hemagglutinin-reactive B cell repertoire present in the pool of memory B cells.

Sequence analysis of the 157 HA-reactive antibodies clearly showed that the B cell response in donor H was dominated by relatively few V\(_{H}\) and V\(_{L}\) genes and was focused around residue K163 (Table 2 and Supplemental Table 1). This selection was unlikely to have been an artifact of the sorting procedure with HA because (a) we established that our HA preparation presented epitopes from head and stem to membrane-bound Ig (Supplemental Figure 1A); (b) in ELISA (data not shown), our HA preparation was bound by H1 HA–specific antibodies recognizing multiple epitopes in the head and stem (antibodies were from our own laboratory and the laboratories of Ahmed, Schrader, and Lanzavecchia); (c) the same method of production has been used to obtain crystals of correctly folded protein (39); and (d) the dominant effect of K163 on antibody binding was seen in the polyclonal sera as well as the selected monoclonal antibodies (Figure 6 and Table 3), as expected from the high proportion of the monoclonal antibodies that recognized this residue.

Eleven V\(_{H}\) gene-related sets, diversified by variations in J and D rearrangements, accounted for the 157 unique antibody sequences isolated. Six of the ten V\(_{H}\)-related sets provided 96% of the antibody sequences, and the majority of these sets, while polyclonal, were dominated by a single expanded clone, defined by a unique pattern of gene rearrangements in heavy and light chains (Table 2). Each V\(_{H}\) set was further diversified by extensive somatic mutation, such that each of the 157 sequences was unique. The frequency of somatic mutations in each set is shown in Figure 2A. The high level of neutralization and accumulation of somatic mutations indicated that they may have been derived from preexisting memory B cells (16, 23, 27, 33, 40).

The limited V\(_{H}\) gene usage we have found in donor H is similar in extent to the response detected to vaccination by Moody et al. (41), with up to 12 V\(_{H}\)-related sets in any one individual; however, our data contrast with data from Okada et al. (42, 43), who isolated 221 unique V\(_{H}\) sequences that reacted with H3 HA by phage display from a single individual, who, unlike our donor,
was hyperimmunized by multiple exposures and vaccinations. 

*V*<sub>H</sub> gene usage was considerably more diverse in their collection, and antibodies to all 5 antigenic sites in H3 HA were identified. Similarly, the majority of serum samples obtained after infection in 1978, 10 years after the introduction of H3 viruses into humans, contained antibodies to more than one site on the HA (13). However, of samples obtained within 3 years of H3 introduction, 1969–1971, 17% recognized a single site (13). This suggests that multiple exposures to influenza may induce a broader range of antibodies encoded by a larger selection of *V*<sub>H</sub> genes than we have detected in donor H, who had probably been exposed to H1N1pdm09 for the first time (44).

Of the 157 clones from donor H, 138 (88%) were derived from 4 *V*<sub>H</sub>-related sets of antibodies targeting the globular head of HA that cross-reacted between A/USSR/90/1977 and the recent H1N1pdm09 virus. At least 10 antibodies (6%) from 3 *V*<sub>H</sub>-related sets were specific for the HA stem. In addition, within the HA head-specific antibodies, a subgroup of 18 antibodies encoded by *V*<sub>H</sub> 3-15 were broadly reactive against former seasonal H1 viruses, whereas the others did not recognize those viruses isolated from at least 1995 to 2007. These patterns and degrees of cross-reactivity between various A viruses and human monoclonal antibodies generated against influenza HA are comparable those found by others (22, 23, 25, 27, 28, 45, 46).

The dominance of HA head-specific *V*<sub>H</sub> 3-7*01/*J*<sub>H</sub> 6*02 sequences that we found in the largest *V*<sub>H</sub>-related set has been seen in other individual responses following exposure to H1N1pdm09 (28, 29). For example, the *V*<sub>H</sub> 3-7/*J*<sub>H</sub> 6 antibodies rearranged with a different group of 4 D segments (3-16*02, 3-22*01, 6-13*01, and 4-17*01) from those from our donor (3-16*01, 3-3*01 in reading frame 2 and 3, and 3-3*02) (28). However, all of these D segments are closely related and share an amino acid sequence motif, DTY, at the D-J junction. In addition an antibody from a representative of our *V*<sub>H</sub> 3-7*01 clones selected the identical K163E substitution as clone 4K8 isolated by Krause et al. (28). This result suggests that both sets of *V*<sub>H</sub> 3-7*01 clones bind the same site on the HA. Krause et al. also showed that their antibodies, while failing to cross-react with recent former seasonal H1 viruses (including A/Brisbane/59/2007), recognized the HAs shared in 1995 (Table 3). For example, clones T2-5D and T1-9B share the same gene rearrangements in H and L chains but differ in somatic mutations, and this is associated with a difference in their ability to neutralize A/Beijing/262/1995 (Table 3). This reactivity of naturally produced human monoclonal antibodies to HA selected through natural antigenic drift mirrors the classic studies on clonal antibody specificity in individual BALB/c mice, tested for binding variant viruses selected in vitro, which demonstrated a similar restricted *V* gene usage with polyclonality (48) and a clearly defined role for somatic mutation influencing the fine specificity for the HA Sb site (49, 50).

Both *V*<sub>H</sub> 3-7 and broadly reactive *V*<sub>H</sub> 3-15 antibodies to the globular head as well as a *V*<sub>H</sub> 1-69 clone specific for the HA stem were therapeutic in DBA/2 mice at a dose of 10 mg/kg (Supplemental Figure 4), despite their widely differing neutralization titers in vitro (Figure 2B), as seen for murine monoclonal antibodies (51). These results emphasize that the antibodies we have studied are functional. It is noteworthy that, although the *V*<sub>H</sub> 3-15 antibodies would now be redundant as therapeutic agents because of the recent expansion of the clade 6B viruses, they would have been useful for at least 14 years (1995–2009), during which their neutralizing epitope was conserved.

In view of our evidence for diversity and redundancy in the human antibody response to individual antigenic sites on the HA, it is surprising that all 4 receptor-blocking *V*<sub>H</sub>-related sets of antibodies from donor H, representing 138 of 157 (88%) of the total rescued clones, focus on a limited overlapping region on the HA head, involving residue K163. The site recognized was shown to be distinct from the site occupied by the 2-12C receptor-blocking antibody from another individual (Figure 4 and Table 3) and reference ferret antisera after H1N1pdm09 virus infection, which do not detect a major change in antigenicity in the clade 6B viruses (WHO Influenza Centre September 2014 report; ref. 30). This conclusion was based on loss of neutralization of the recently evolved clade 6B viruses (that all contain mutations at K163) by all 4 sets of antibodies (Table 3), selection of substitutions at the same site (K163) by *V*<sub>H</sub> 3-7*01 and *V*<sub>H</sub> 3-15*01 clones, loss of recognition of K163E/Q variant viruses by all 4 sets (Figure 3), and cross-inhibition between the 4 sets of antibodies for binding to HA (Supplemental Figure 3).

Focusing of the polyclonal antibody response in donor H was confirmed by the loss of neutralizing activity for viruses selected for
the K163E/Q substitution, compared with A/California/07/2009, in the sera obtained 12 days after vaccination (Table 3). In addition, a screen of 41 immune sera revealed a second vaccinated donor whose serum was unable to neutralize viruses with the K163E/Q substitutions (Figure 6), and two of the control human antibodies that cross-react between former seasonal and H1N1pdm09 viruses described previously (22), provided by Li et al., also recognized K163 (Table 3).

The results of our detailed clonal analysis extend two recent serological studies of the human antibody response to the H1N1pdm09 virus (14, 37). Li et al. have shown that human individuals born between 1983 and 1996 (after donor H), who were exposed in early life to former seasonal H1 viruses that retained K130, narrowly focused their antibody response on this region on exposure to H1N1pdm09 (37). This is similar to the site defined by our antibody, 2-12C, derived from a control individual, which selected for the K130E substitution, and we also detected 2 sera after vaccination with reduced neutralization of viruses carrying the K130 substitution (Figure 6A), confirming the observations of Li et al. However, mutations at K130 have not yet as appeared as new variant hemagglutinins isolated from infected humans (WHO Influenza Centre September 2014 report; ref. 30).

Linderman et al. noted that severe influenza infection was occurring with higher frequency in middle-aged individuals in the 2013–2014 season that was related to the appearance of clade 6B H1N1pdm09 viruses and showed that, in the cohort born between 1965 and 1979 (which would have included our donor), 42% of serum samples had reduced titer in HAI and MN assays on viruses with K166E/Q substitutions (K163 in our HA numbering) (14). Our paper extends these results by showing that multiple monoclonal antibodies from such individuals can select substitutions at this same site, which suggests that selection is occurring in nature. This link between the focused antibody response in humans and selection of substitutions in the HA from influenza viruses found in nature has long been suspected but not directly demonstrated (4, 5, 8–13, 48, 52–55).

The mechanism by which this focusing of the antibody response occurs is not clear from these data, but three possibilities include (a) a limited response in the first exposure to a new virus (13), (b) selection of cross-reacting B cells from the available repertoire induced by earlier exposure to related influenza viruses (15, 16, 25, 34–37, 41, 44, 47, 56, 57), or (c) the selection and expansion of B cells making antibodies that influence antigen processing and presentation to T cells in such a way that optimizes T cell help, “T-B reciprocity” (33, 58–62). These mechanisms are not mutually exclusive and could act together.

The region of the H1 HA surface that immediately surrounds residue K163 substitutions, which were selected for by the V_H residues K163 substitutions, which were selected for by the V_H not mutually exclusive and could act together.

The clade 6B variants are considered to be A/California/07/2009-like antigenically by the WHO. Antigenic similarity has been ascribed based on HAI reactivity with panels of primary ferret antisera after infection raised against a range of H1N1pdm09 viruses, including A/California/07/2009 (WHO Influenza Centre February 2015 report; ref. 30). Mutagenesis studies have shown that the ferret HAI response to A/California/07/2009 is tightly focused on residues 153–155 in the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32). The single HA G155E substitution in A/California/07/2009 defines this, and, with K130, G155 helps form the Sa antigenic site (32).
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(14, 37). The importance of this distinction lies in the fact that the 2013–2014 H1N1 viruses, which were not detected as drifted in the standard analysis with ferret sera, have been associated with severe infections in middle-aged and elderly humans previously shown to be relatively protected (63).

Our detailed clonal analysis extends the serological survey from Linderman et al. (14) and the deep-sequencing study of Jackson et al. after vaccination (29) by showing that the antibodies detected in these studies can select virus variants in vitro that match the dominant natural clade 6B variants that have arisen since 2013. Our results show that assessing antigenic relatedness of evolving influenza viruses with panels of defined, renewable, and human monoclonal antibodies could be a very useful supplement to traditional ferret antisera and may also be used to predict antigenic drift. In addition, the production of therapeutic monoclonal antibodies should be considered as part of national planning for pandemic influenza. This is particularly relevant to the H7N9 threat, for which virus isolates with the NA-R292K substitution show resistance to the neuraminidase inhibitors but retain their virulence and transmissibility (64).

Methods

Secreted HA of influenza A/England/195/2009 virus. cDNA corresponding to codons 1 to 520 of the ectodomain of HA from influenza A/England/195/2009 virus was amplified and purified as previously described (39, 65). Briefly, the construct incorporated at the C-terminus included a thrombin cleavage site, a trimerization sequence, a histidine-tag region for protein purification, and the BirA recognition sequence LNDIFEAQKIEW for site-specific biotinylation. The cDNA sequence was codon optimized for eukaryotic expression in 293T cells. Biotinylation was performed with BirA enzyme from Avidity.

Staining and sorting of plasmablasts. The identification of plasmablasts by flow cytometry was based on the methods of Smith et al. (20) with minor modifications. A week after vaccination, fresh peripheral blood mononuclear cells were suspended in staining buffer. A mix of surface antibodies, including Pacific Blue anti-CD3 (BD; catalog 558117), FITC anti-CD19 (BD; catalog 555412), Allophycocyanin-H7 anti-CD20 (BD; catalog 641396), PE-Cyanine 7 anti-CD27 (BD; catalog 560609), and PE-Cyanine 5 anti-CD38 (BD; catalog 555461), were added to the aliquot of peripheral blood mononuclear cells with 10 μg/ml biotinylated Eng195 HA and incubated for 30 minutes on ice. After washing with staining buffer, cells were further incubated with ExtrAvidin-PE (Sigma-Aldrich) for 30 minutes on ice. Cells were washed and resuspended in PBS and passed through a cell strainer. The MoFlo cell sorter (DakoCytomation) was used for sorting. After applying a gate on CD3 CD20 CD20 CD19 CD27 CD38 B+IgM+ cells, single plasmablasts were sorted into 96-well plates containing 10 μl RNase-inhibiting RT-PCR catch buffer (20).

Generation of monoclonal antibodies. Human monoclonal antibody was produced from single B cells as described previously (20). In brief, single B cells were sorted directly to RT-PCR buffer, and the variable region genes from each cell were amplified in a One-Step RT-PCR reaction (Qiagen), using a cocktail of primers specific for the leader region and antisense primers to the C′ constant region for heavy chain and Χ and C′d for light chain as described previously (20). The RT-PCR products were amplified in separate PCR reactions for the individual heavy and light chain gene families using nested primers to incorporate restriction sites at the ends of the variable gene as described previously (20). These variable genes were then cloned into expression vectors for the heavy and light chains as described previously (20). Plasmids were transfected into the 293T cell line for expression of recombinant human monoclonal antibodies.

Construction of transmembrane forms of human monoclonal antibodies. The transmembrane-spanning domain of human IgG1 (66) was cloned from sorted human plasmablasts between unique SmaI and HindIII sites, with the oligonucleotides 5′ GCCCCCATCCCGGATGAGCT and ATGGTCAGGCT TACATGCCCCTGTTCCGATCAT. Full-length TMigG1 cDNAs encoding antibodies that we isolated and characterized were reconstructed by replacing the sequence between the unique SmaI and HindIII sites in the IgG1 expression vector of Smith et al. (20) (GenBank FJ517647) and transfected into 293T cells for staining with preparations of labeled recombinant HAs. Three antibodies were converted to the TM form: one specific for the head of seasonal H3 HA (NZB10, Vα 4-61′O) as well as one for the head (T2-9A, Vα 3-7′O) and one for the stem (T1-3B, Vα 1-69′O) of H1N1pdm09 and H5 HAs.

Enzyme-linked immunosorbent, indirect immunofluorescent, virus titration, HAI, and MN assays. Assays were performed using standard procedures as described previously (65). The ELISA or MN titers were expressed as half maximal effective concentrations (EC50; midpoint between negative and plateau positive controls) derived by linear interpolation from neighboring points in the titration curve. The MN assay detects inhibition of virus entry into MDCK-SIAT-1 cells in the absence of trypsin (67), with antibody present throughout the assay (23). MN assays with clade 6B viruses incorporated a replication step in the presence of trypsin for 48 hours, which gave lower background staining with the developing antibody to NP AASH (65). Serum and plasma samples were heat inactivated at 56°C without RDE treatment, and monoclonal antibodies were untreated. Serum and plasma samples from donor H taken on the same day did not give significantly different titers in the MN assay. Seven published antibodies were included: 15A206, 19-4G05, 05-2G02, and 09-3A01 (22) as well as SF70-1F02 (25) were gifts from Rafi Ahmed (Emory University, Atlanta, Georgia, USA); V3-2G6 (23) was a gift from John Schrader (University of British Columbia, Vancouver, British Columbia, Canada); and FI6v3 (24) was a gift from Antonio Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland). Antibody competition was assessed by ELISAs performed with birin-labeled antibodies in the presence of excess unlabeled blocking antibodies. Detection was performed with HRP-labeled ExtrAvidin (Sigma-Aldrich).

Animal studies. DBA/2 (DBA/2J-Tyr^+^) H2^+^ mice were purchased from Harlan and housed in individually vented cages. X179A vaccine virus (provided by the National Institute for Biological Standards and Control, South Mimms, United Kingdom) had a minimum dose which caused at least 20% weight loss in 50% of the mice (MD50) of 32 tissue culture 50% infectious dose (TCID50). Mice were anesthetized with isoflurane (Abbot) and were intranasally infected with 4.6 × 104 TCID50 X179A (~150× MD50), followed 24 hours later by i.p. injection of 500 μl antibody at a dose of 10 mg/kg into anesthetized animals. Mice were weighed and scored according to previously described criteria (65), and mice with 20% weight loss or morbid clinical scores were humanely killed.
Selection of influenza variants with monoclonal antibodies. Approximately 10^10 TCID<sub>50</sub> X179A virus was mixed with antibody at 20 μg/ml and incubated at 37°C for 1 hour. The mixture was then diluted to 10 ml (2–4 μg/ml antibody) with viral growth medium (DMEM, 0.1% BSA, 10 mM HEPES, pH 7.0, penicillin and streptomycin) (65), and 100 μl was added to 80 wells of a flat-bottomed 96-well microtiter plate containing confluent monolayers of MDCK-SIAT-1 cells (68) plated the previous day. The remaining wells contained medium without virus for comparison. The mix was incubated for a further hour at 37°C, and then 100 μl viral growth medium was added, containing trypsin at 2 μg/ml. The mix was incubated at 37°C for 48 hours and then the medium from each well was removed and stored. The monolayers were stained for NP expression as described previously (65), and viruses from wells showing replication in the presence of antibody were cloned by limiting dilution in the presence of antibody at a final concentration of 1 μg/ml (or a known saturating concentration). Viruses from single clones were expanded in 3-mL cultures for extraction of RNA, PCR amplification of HA and neuraminidase, and sequencing of the PCR products.

Hemagglutinin sequence numbering. Sequence numbering throughout the manuscript refers to the mature H1 HA polypeptide, i.e., with the signal sequence removed, as used in annual and interim reports from the WHO Collaborating Centre for Reference and Research on Influenza, the Crick Worldwide Influenza Centre, and summaries of the WHO Vaccine Composition Meetings (1, 30).

Statistics. Graphs were generated using GraphPad Prism software (version 5) and Microsoft Excel 2010. Statistical analysis was done by GraphPad Prism and SPSS. P values of less than 0.05 were considered significant.

Study approval. This study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Protocols and informed consent were reviewed and approved by the Oxford Tropical Research Ethics Committee and the Weatherall Institute of Molecular Medicine. Signed informed consent was obtained from each individual donor who supplied a blood sample. All animal procedures were approved by an internal University of Oxford Ethics Committee and the United Kingdom Home Office.

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