Epitope-Specific Human Influenza Antibody Repertoires Diversify by B Cell Intraclonal Sequence Divergence and Interclonal Convergence

Jens C. Krause, Tshidi Tsibane, Terrence M. Tumpey, Chelsey J. Huffman, Bryan S. Briney, Scott A. Smith, Christopher F. Basler and James E. Crowe, Jr.

*J Immunol* 2011; 187:3704-3711; Prepublished online 31 August 2011;
doi: 10.4049/jimmunol.1101823
http://www.jimmunol.org/content/187/7/3704

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/08/29/jimmunol.1101823.DC1

References
This article cites 42 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/187/7/3704.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Epitope-Specific Human Influenza Antibody Repertoires Diversify by B Cell Intraclonal Sequence Divergence and Interclonal Convergence

Jens C. Krause,* Tshidi Tsibane,† Terrence M. Tumpey,‡ Chelsey J. Huffman,* Bryan S. Briney,* Scott A. Smith,§ Christopher F. Basler, † and James E. Crowe, Jr.*‡§

We generated from a single blood sample five independent human mAbs that recognized the Sa antigenic site on the head of influenza hemagglutinin and exhibited inhibitory activity against a broad panel of H1N1 strains. All five Abs used the VH3-7 and JH6 gene segments, but at least four independent clones were identified by junctional analysis. High-throughput sequence analysis of circulating B cells revealed that each of the independent clones were members of complex phylogenetic lineages that had diversified widely using a pattern of progressive diversification through somatic mutation. Unexpectedly, B cells encoding multiple diverging lineages of these clones, including many containing very few mutations in the Ab genes, persisted in the circulation. Conversely, we noted frequent instances of amino acid sequence convergence in the Ag combining sites exhibited by members of independent clones, suggesting a strong selection for optimal binding sites. We suggest that maintenance in circulation of a wide diversity of somatic variants of dominant clones may facilitate recognition of drift variant virus epitopes that occur in rapidly mutating virus Ags, such as influenza hemagglutinin. In fact, these Ab clones recognize an epitope that acquired three glycosylation sites mediating escape from previously isolated human Abs. The Journal of Immunology, 2011, 187: 3704–3711.

I
duction and maintenance of a diversity of broadly neutralizing Abs against viruses is desirable for immunity against reinfection, but the molecular features of human Ab repertoires specific for particular agents or epitopes are poorly understood. Isolation of limited panels of epitope-specific human mAbs to viruses has suggested that the circulating human B cell response often is dominated by major clonal populations. In vivo selection in germinal centers of particular B cell clones using BCRs with high-affinity binding to virus epitopes likely leads to expansion of dominant clonal populations. The extent to which a dominant clone of B cells responding to a viral epitope represents a single BCR with an optimal affinity for binding, versus a family of related somatic variants, has not been determined in the past because of the difficulty in generating large numbers of human Abs.

The 2009 H1N1 influenza pandemic was the first influenza pandemic in >40 y. Pediatric death rates were 10 times the rates for seasonal influenza in previous years (1). Elderly people had pre-existing cross-reactive Abs against this 2009 H1N1 virus (2–4). Preserved epitopes within H1N1 hemagglutinin (HA) were the likely structural correlate for this cross-reactivity, particularly the Sa antigenic site on the globular head (5–7). We had shown previously that the Sa site-specific Ab 2D1 that was cloned from a survivor of the 1918 pandemic potently neutralized 2009 pandemic virus (5, 8). We elucidated the crystal structure of Ab 2D1 in complex with 1918 HA (9). Ab 2D1 uses the VH3-7/2-7 germline gene with a unique insertion close to CDR H2 that enhances the function of the Ab (8, 10).

In this study, we describe a panel of H1N1-specific Abs that was cloned from a 47-y-old healthy woman after the pandemic. Like Ab 2D1, Abs from this new panel bound the Sa site, but they shared VH3-7/JH6 germline gene usage and had hemagglutination activation inhibition (HAI) activity against a broader panel of H1N1 strains than 2D1, including viruses with glycosylation sites in the Sa site. These VH3-7/JH6 Abs belonged to four different clones that arose independently, yet converged toward similar amino acid sequences. Ultra-deep sequencing has been used previously to determine the combinatorial diversity of Abs (11–14). We used this technology to elucidate the VH3 repertoire of this donor to find related Ab sequences using the VH3-7/JH6 H chain gene segments to more fully define the molecular diversity of an epitope-specific human Ab repertoire. The data revealed unexpected features of the evolution of Ab repertoires and the persistence of corresponding B cells in the peripheral blood.

Materials and Methods
Hybridoma generation and recombinant Ab expression
Acquisition of human blood samples was approved by the Vanderbilt University Institutional Review Board. The animal studies were approved by the Institutional Review Boards of the Centers for Disease Control. PBMCs were
isolated from a 47-yr-old healthy female donor with Histopaque-1077 (Sigma-Aldrich), EBV transformed in 384-well plates (Nunc) in the presence of 2.5 μg/ml CpG ODN 2006 (Invivogen), 10 μM Chl2 inhibitor II (C3742; Sigma-Aldrich), and 1 μg/ml cyclosporine A (Sigma-Aldrich), essentially as described previously (10, 15). Supematants from wells containing EBV-transformed lymphoblastoid cell lines were screened for binding activity by ELISA against a panel of recombinant soluble HA proteins. Positive wells were fused with HMA2.5 myeloma cells and cloned molecularly using previously described primer sets (16) into pGEM-T Easy vector (Promega) and eventually into pEE12.4pEE6.4 mammalian expression vectors (Lonza), from where they were expressed (9) and purified on a protein G column using an AKTA chromatography instrument (GE Healthcare). All following studies were performed using recombinant Abs. We used Kabat numbering as determined using the Abnum server (17) for the Abs and an H3 numbering scheme (18) for HA. Ab clonality was determined strictly by shared Vγ gene, shared VDJ junction, and a sequence of shared somatic mutations.

**Generation and purification of recombinant soluble HA molecules**

The 1918 or 2009 influenza HA constructs were ordered sequence-optimized for expression in human cells (GenScript) based on the extracellular domain of the respective HA, a thrombin recognition cleavage site, a fibrin trimerization domain, and a 6X His-tag (19). The constructs were cloned into pcDNA3.1(+) (Invitrogen), expressed in 293F cells (Invitrogen), purified over nickel columns using an AKTA chromatography instrument (GE Healthcare), and concentrated with Amicon Ultra centrifugal filters with a 30-kDa molecular mass cutoff (Millipore).

**ELISA**

The 384-well clear plates (242757; Nunc) were coated with HA at 1 μg/ml in Dulbecco’s PBS overnight, blocked with 0.5% cow milk, 0.2% goat serum, and 0.5% Tween 20 (Sigma-Aldrich) in Dulbecco’s PBS. Five microliters hybridoma supernatant per well was transferred to 25 mL blocking solution and incubated on ice for 30–60 min. After another wash, phosphatase substrate (S0942; Sigma-Aldrich) was dissolved in substrate buffer per the instructions of the manufacturer and dispensed onto the plates. The plates were read at 405 nm on a PowerWave HT (BioTek).

**Virus-like particles expression and HAI assays**

Expression plasmids encoding the parental or mutated 1918 HA were coexpressed with N1 neuraminidase to produce virus-like particles (VLPs) in 293T cells (10, 19). Two days posttransfection, supernatants were collected. HAI assays were performed as described (20) using VLPs (for 1918 H1N1 or H2) or live virus (all other influenza strains). Briefly, serially diluted Abs were preincubated with eight hemagglutinating units of virus (H1N1 or H2) or live virus (all other influenza strains) for 1 h. After the addition of 0.5% chicken RBCs for 1 h, absorbance at 405 nm was measured.

**Table I. Specific HAI activity of human Abs against influenza viruses or VLPs**

<table>
<thead>
<tr>
<th>Influenza A Strain</th>
<th>4A10</th>
<th>2010</th>
<th>4K8</th>
<th>6D9</th>
<th>2K11</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/South Carolina/1/1918 wt (VLP)</td>
<td>0.08</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.2</td>
<td>0.16</td>
</tr>
<tr>
<td>A/South Carolina/1/1918 K166E (VLP)</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/South Carolina/1/1918 K166N (VLP)</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/South Carolina/1/1918 K166Q (VLP)</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/South Carolina/1/1918 K166R (VLP)</td>
<td>10</td>
<td>&gt;</td>
<td>&gt;</td>
<td>20</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/swine/iowa/15/1930</td>
<td>0.63</td>
<td>0.08</td>
<td>&lt;0.01</td>
<td>0.4</td>
<td>0.16</td>
</tr>
<tr>
<td>A/Weiss/1943</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/L3/47</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/New Jersey/11/1976</td>
<td>2.5</td>
<td>2.5</td>
<td>0.16</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>A/USSR/92/1977</td>
<td>0.32</td>
<td>2.5</td>
<td>0.32</td>
<td>2.5</td>
<td>0.32</td>
</tr>
<tr>
<td>A/New Caledonia/20/1999</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/Brasilia/59/2007</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>A/California/04/2009</td>
<td>0.16</td>
<td>0.32</td>
<td>&lt;0.01</td>
<td>0.7</td>
<td>0.63</td>
</tr>
</tbody>
</table>

* Activity was not detected at the highest concentration tested, 20 μg/mL.

**Results**

Hybridoma generation, Ab reactivity, and animal studies

We isolated a panel of five human mAbs named 4A10, 2010, 4K8, 6D9, and 2K11 from a single blood sample of a human subject. All Abs showed HAI activity against both 1918 and 2009 H1N1 pandemic viruses, the related swine influenza viruses from 1930 and 1976, and the H1N1 virus from 1977, but not against strains from the 1940s or H1N1 strains after 1977 (Table I). Ab 6D9 tested negative for functional activity against representative H2N2, H3N2, or H5N1 viruses by HAI (data not shown). Generally, Ab 4K8 was the most potent Ab, with an HAI activity <0.01 μg/ml against pandemic H1N1 viruses. We selected this Ab for testing in a lethal mouse model of 1918 influenza virus infection (Fig. 1, Table I). Ab 4K8 protected six out of six animals from death at...
both the highest and the intermediate dose levels and two out of six animals at the lowest dose (Fig. 1A). Ab 4K8 reduced lung virus titers as compared with the human IgG control by 3.1 log_{10} PFU/ml at the 200 µg dose, 2.6 log_{10} PFU/ml at the 20 µg dose, and still 1.3 log_{10} PFU/ml at the 2 µg dose (Table II).

**Generation and validation of escape mutations in epitopes recognized by these five Abs**

Ab 4K8 selected for a K166R mutation in the A/USSR/97/1977 context and a K166E mutation in the A/California/04/2009 H1N1 context. To confirm that these mutations alone were sufficient to confer escape, we produced 1918 VLPs with mutant HA. The entire panel of Abs was unable to inhibit hemagglutination of VLPs with K166E, K166N, or K166Q mutations (Table I). Abs 4A10 and 6D9 showed modest HAI activity against VLPs with the K166R mutation, but not 2O10, 4K8, or 2K11 (Table I). These data suggested that all five Abs recognized a common epitope, possibly with minor differences in the mode of binding among those five Abs.

**Sequence analysis of the Ab variable gene sequences in the hybridomas**

Next, we determined whether shared germline genes were the basis for the recognition of this epitope. Nucleotide sequence determination and sequence analysis of variable gene sequences (Supplemental Fig. 1, Table III) using IMGT (24) revealed that all of the five Abs shared usage of the V_{H}3-7*01 gene and the J_{H}6*02 gene; furthermore, 4A10, 2O10, and 2K11 used the same V_{L}1-40*01 gene. Also, 4K8 and 6D9 used both V_{L}3-20*01 and J_{L}2. The D genes of these five Abs were predicted to be of different gene; furthermore, 4A10, 2O10, and 2K11 used the same VL1-40*01 gene. Also, 4K8 and 6D9 used both VK3-20*01 and JK2.

Despite four different clonal origins, the CDR H3s of these Abs were remarkably similar (Fig. 2); for instance, the CDR H3 length of 18 aa was identical across this panel. The amino acids in positions 93–95, 96, 100, 100A, 100B, 100D, 100F, and 100H–103 were fully conserved. Interestingly, somatic mutations were shared between the Abs: for example, the tyrosine to histidine mutation in position 100E of 2K11 and 4A10 or the glycine to alanine mutation in position 100G of 2010 and 4K8 (Fig. 2). Also, several common mutated amino acid residues were found despite differing sequences in the inferred germline origin sequence: the glycine in position 96 was encoded entirely by the N1 segment (2K11, 2010), by the D segment (4A10), or by both (4K8/6D9). The S97 was encoded by the D segment alone (4A10, 4K8/6D9) or by both N1 and D1 (2K11). An aspartic acid was found in position 100 of 2K11 and 2010 because of their germline; clones 4A10 and 4K8/6D9 acquired this aspartic acid through a somatic mutation, suggesting that this panel converged toward a consensus sequence (Fig. 2). Position 100A was predicted by IMGT to be encoded by the N2 segment alone (2K11, 4K8/6D9), by the D chain and the N2 segment (2010), or by the D and the J chain (4A10; Fig. 2). No matter the origin, a threonine was found in all five CDR H3s in this position (Fig. 2). Residues from 100B onward were encoded by the J_{H}6 gene in all clones; four to six successive tyrosine residues are typically encoded by that germline gene segment (25), although somatic mutations were found in positions 100C (2K11) and 100E (4A10, 2K11).

We reviewed the variable gene encoded N-terminal part of H chain clones for common somatic mutations: 4A10 and 2K11 shared a threonine to serine mutation in position H28. All five Abs shared a threonine instead of the germline serine in position H35 (Fig. 3). Abs 4A10 and 4K8/6D9 acquired this threonine to alanine mutation in position 100G of 2010 and 4K8 (Fig. 2). Also, several common mutated amino acid residues were found despite differing sequences in the inferred germline origin sequence: the glycine in position 96 was encoded entirely by the N1 segment (2K11, 2010), by the D segment (4A10), or by both (4K8/6D9). The S97 was encoded by the D segment alone (4A10, 4K8/6D9) or by both N1 and D1 (2K11). An aspartic acid was found in position 100 of 2K11 and 2010 because of their germline; clones 4A10 and 4K8/6D9 acquired this aspartic acid through a somatic mutation, suggesting that this panel converged toward a consensus sequence (Fig. 2). Position 100A was predicted by IMGT to be encoded by the N2 segment alone (2K11, 4K8/6D9), by the D chain and the N2 segment (2010), or by the D and the J chain (4A10; Fig. 2). No matter the origin, a threonine was found in all five CDR H3s in this position (Fig. 2). Residues from 100B onward were encoded by the J_{H}6 gene in all clones; four to six successive tyrosine residues are typically encoded by that germline gene segment (25), although somatic mutations were found in positions 100C (2K11) and 100E (4A10, 2K11).

We reviewed the variable gene encoded N-terminal part of H chain clones for common somatic mutations: 4A10 and 2K11 shared a threonine to serine mutation in position H28. All five Abs shared a threonine instead of the germline serine in position H35 (Fig. 3). Abs 2010 and 4K8 shared a threonine to serine mutation in position H28. All five Abs shared a threonine instead of the germline serine in position H35 (Fig. 3). Abs 2010 and 4K8, and clonally related, whereas 4A10, 2010, and 2K11 were derived independently of each other and of the 4K8/6D9 clone (Fig. 2).

**Therapeutic efficacy of Ab 4K8 against virus replication in mice inoculated with 1918 influenza A virus**

Four mice were inoculated intranasally with 5× LD_{50} and administered 4K8 Ab or human IgG i.p. 24 h later. Mice were euthanized on day 4 after inoculation for the determination of lung titers.

At the α = 0.025 level, controlling the overall type I error at 7.5%, the lung homogenates of the 4K8 groups had lower virus titers than the human IgG control groups for all dose levels by the Wilcoxon rank sum test (p = 0.01429 for each level).

**Table II. Therapeutic efficacy of Ab 4K8 against virus replication in mice inoculated with 1918 influenza A virus**

<table>
<thead>
<tr>
<th>Ab</th>
<th>Dose (µg/Mouse)</th>
<th>Mean Lung Virus Titer* (log_{10} PFU/ml ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab 4K8</td>
<td>200</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Human IgG control</td>
<td>200</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.9 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.6 ± 0.4</td>
</tr>
</tbody>
</table>

**Table III. Genetic features of H1N1-specific human mAbs**

<table>
<thead>
<tr>
<th>Ab</th>
<th>H Chain Genes</th>
<th>L Chain Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A10</td>
<td>3-7*01</td>
<td>3-16*02</td>
</tr>
<tr>
<td>2O10</td>
<td>3-7*01</td>
<td>3-16*02</td>
</tr>
<tr>
<td>4K8</td>
<td>3-7*01</td>
<td>3-20*01</td>
</tr>
<tr>
<td>6D9</td>
<td>3-7*01</td>
<td>3-20*01</td>
</tr>
<tr>
<td>2K11</td>
<td>3-7*01</td>
<td>4-17*01</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Therapeutic efficacy of Ab 4K8 against disease caused by the 1918 A(H1N1) virus in mice. Mice were inoculated on day 0 and treated on day 1 with the indicated Ab and dose. In each group, six mice were monitored for survival every day (A) and weight every other day (B). At all dose levels, the differences in survival distribution between the 4K8 and the human IgG control groups were significant by log-rank test (p < 0.001 for the high-dose level, p < 0.001 for the medium-dose level, p < 0.01 for the low-dose level).
2K11 were found to have a valine instead of an alanine in position H84 (Fig. 3). An aspartic acid took the place of a glutamic acid in position H85 of Abs 4A10 and 4K8. Finally, both Abs 4A10 and 2K11 have a histidine to tyrosine mutation in position L34 of the λ chains in common (Supplemental Fig. 1).

Deep sequencing of the V_{H}3 gene-encoded repertoire of this donor

The response of this donor toward 2009 H1N1 was dominated by Abs encoded by the V_{H}3-7/J_{H}6 gene segments. We hypothesized that this limited panel of five clones might represent only a small portion of the circulating antigenic site Sa-specific repertoire in this individual. To test this idea, we extracted mRNA from total PBMCs from this donor 6 mo after hybridoma generation and isolated Ab genes using an RT-PCR amplification specific for VH3. We used pyrosequencing to delineate the entire V_{H}3 repertoire. We identified sister clones of the above Abs based on shared V/J usage and identical VDJ junctions to more fully define the genetic diversity within these clones in circulating cells. We obtained 26.2 megabases of data including 80,687 sequences that passed filter reads. The sequences had an average length of 325 bp after removal of primer sequences. Analysis with IMGT identified 60,484 of those sequences as productive; 60,447 belonged to the 4A10 lineage and 60,447 to the 2O10 lineage. Of those sequences, 138 were nonredundant on the protein level belonging to the 4A10 lineage (5 to clone 2O10, 4K8, 6D9, and 2K11), meaning that every single clone was still found in the peripheral blood of the individual 6 mo after the initial blood draw for hybridoma generation. Because residues DTY at positions 100-100B were completely conserved across all Abs, we screened the remaining clones for the presence of this motif, but none of them shared this DTY motif in CDR H3, suggesting that we likely had identified most of the clones of the V_{H}3-7/J_{H}6 gene segment-encoded Abs that recognized the influenza HA Sa site.

Intraclonal sequence divergence of the 2K11 clone

Although we identified sequences highly related to those of Abs 4A10, 2010, or 4K8/6D9, none of the sequences from the high throughput analysis was completely identical to those in the original clones. Remarkably, we were able to recover a sequence that was virtually identical to the 2K11 hybridoma cell IgH sequence in the high-throughput sequence BQ2Y7. This occurrence may have been linked to the fact that sequences recovered that were related to Ab 2K11 represented by far the biggest clonal family identified and thus provided a more comprehensive snapshot of the evolution of this Ab. Within the panel related to Ab 2K11, the AE441 and AIF9Z sequences embodied essentially a germline state with just a single, nonsilent, somatic mutation in the variable gene-encoded amino acid sequence, the valine in position 84. In contrast, the Ab 2K11 sequence itself and related sequences were highly mutated, particularly in the CDR H1, with up to five changes in amino acid sequence. Although both germline states and highly mutated states were present simultaneously in the peripheral blood, sequences representing many of the intermediate predicted steps were not detected. For example, it was not clear in what order the mutations within CDR H1 of the hybridoma 2K11 occurred except that S31N probably occurred first because it was present in A32OY by itself. Also, the S30I mutation probably occurred last within CDR H1 because AUOJR contained all of the other mutations except the aforementioned one. Because the other three clones had fewer representatives than the 2K11 clone, the order of mutations would be more difficult to establish for those clones. Nevertheless, sequence divergence from the germline sequence was readily apparent. Notably, insertion/deletion events seemed to play a minor role in this Ab panel with only sequence BQVKK within the 2K11 clone bearing evidence of a three base pair deletion, leading to the loss of a single amino acid residue within CDR H1.

FIGURE 2. Comparison of Ab gene junctional sequences reveals four independent clones. The IgH gene segment junctions of the five V_{H}3-7/J_{H}6 Abs 4A10, 2010, 4K8, 6D9, and 2K11 are shown in amino acid and DNA sequence. Mutated amino acids and nucleotides are underlined. The amino acid residues are color-coded per standard IMGT color scheme based on chemical properties (44). Briefly, aliphatic (A, I, L, V) residues are dark blue, phenylalanine light blue, sulfur (C, M) residues cyan, glycine dark green, residues with hydroxyl groups (S, T) medium green, tryptophan purple, tyrosine light green, proline yellow, acidic (D, E) residues light orange, amide (N, Q) residues dark orange, and basic (H, K, R) residues red. Kabat numbering for amino acids is used instead of IMGT numbering and is shown at the top level; the CDR H3 margins are denoted in red. The contributions of the VH, D, and JH genes are shown in light gray (V), medium gray (D), and dark gray (J).
Interclonal sequence convergence

We defined convergence as the same altered amino acid introduced by somatic mutation present in two or more independent clones. Despite sequence divergence within the individual clones, sequence comparison revealed many further examples of interclonal sequence convergence not evident in the hybridoma sequences such as valine in H23 (members of clones 4A10/2K11) or threonine in the same position (2O10, 4K8/6D9, 2K11), leucine in H29 (4A10/2K11), asparagine in H31 (4A10/2K11), glutamine in H46 (4A10/2K11), asparagine in H58 (2O10/4K8/6D9), histidine in H59 (4A10, 2K11), and several others (Fig. 3). Overall, there were 20 positions within the VH protein sequence with evidence of convergence. Only 8 of those 20 positions were found within CDR H1, H2, or the V-GENE–encoded portion of H3, so 12 conver-
gence positions were located in the framework regions. Remarkably, a subclone each within two different clones converged toward a set of similar somatic mutations within the CDR H1 (amino acids SLIN in positions H28-31 in the 2K11 clones and amino acids SLKN in the same positions of the 4A10 lineage). An analysis of silent versus nonsilent mutations by framework and CDR is presented in Supplemental Table I.

Discussion
Glycosylation within site Sa does not always confer escape from neutralization

We describe a panel of four independent clones of human Abs. Like Ab 2D1, they were Sa site Abs based on the selection of escape mutations in position K166 and that showed potent HAI activity against pandemic H1N1 influenza (5, 9, 10). Unlike Ab 2D1, however, this panel had HAI activity against USSR/77 H1N1 as well. This finding is striking because the USSR virus possesses three predicted N-linked glycosylation sites within its Sa antigenic site (9). Glycosylation at these sites is thought to shield HA from neutralization (7, 9). In support of this model, experimental introduction of glycosylation sites into the 1918 or 2009 HAs conferred resistance to neutralization by antisera elicited by the uncleaved glycosylated HAs (7). The fact that neither the 1918 virus nor the 2009 pandemic virus possess glycosylation sites within the Sa antigenic site, whereas human H1N1 viruses acquired N-linked glycosylation over the course of the 20th century, likely explains, at least in part, why elderly people had pre-existing cross-reactive humoral immunity against the 2009 pandemic virus (7, 9). The fact that VH3-7/JH6 Abs inhibited USSR virus, despite the presence of the glycosylation sites, demonstrated that the presence of glycosylation sites within site Sa did not always confer escape from Sa-specific Abs.

Redundancy of the immune response

Interestingly, this VH3-7/JH6 panel of four independent clones was derived from a single donor; her response to pandemic 2009 H1N1 may have been VH3-7/JH6 dominant and oligoclonal because we were only able to generate another H1N1 globular head Ab, Ab 5J8, from this donor that selected for escape mutations along the receptor-binding pocket (26). The redundancy of this response may ensure adequate neutralization of a given pathogen such as influenza A virus. This redundancy may be quite common, but may only be detected more frequently through advances in Ab engineering and sequencing technology.

Same genes suggest a common epitope

Wrammert et al. (27) described cross-reactive human Abs in subjects convalescing from infection with 2009 H1N1 pandemic virus; this work did not feature neutralizing VH3-7/JH6 Abs, potentially due to a limited number of Abs. Although a similar genetic makeup (like VH3-7/JH6) suggests a common epitope, the Sa antigenic site can likely be reached from a variety of different germline configurations such as VH3-7/JH6 or the VH3-7-70 of the 2D1 Ab (5, 8, 10).

The VH3-7/JH6 Abs described in this study showed strongest HAI activity against 1918 VLPs of a virus that circulated well before the birth of this donor and thus could not have served as the inciting agent. In fact, given that this donor was born between 1957 and 1977 when H1N1 did not circulate in humans, subtype H1N1 infection was unlikely to have been the cause of her first exposure to influenza or original antigenic sin (28). These Abs could have been created in response to vaccination with the 1976 swine influenza virus (29) or vaccination or infection with a USSR/77-like virus or the 2009 H1N1 virus. Because these Abs in general seemed to neutralize 2009 H1N1 best among those three candidates, we hypothesized that these Abs were created in response to the 2009 virus and that the process of several Abs independently hitting the same epitope with a similar genetic makeup was entirely stochastic.

Intraclass sequence divergence and interclonal sequence convergence

The ultra-deep sequencing in this study revealed that the VH3-7/JH6 clones represented large circulating clonal populations. Because pyrosequencing was performed on PBMCs isolated 6 mo after the initial hybridoma generation, the phylogenies seemed to persist for at least several months in the peripheral blood. B cells that are not stimulated because they do not express high-affinity Ab are supposed not to undergo further proliferation (30). Still, wide divergence of sequences from essentially unmutated germline states to extensively mutated sequences was found within clones in the peripheral blood of this donor. Persistent low-affinity memory populations may aid in the immunologic response toward a related HA Ag that the individual subsequently encounters (31, 32).

B cell development has been traced by molecular analysis of single cells picked from histological sections in human germinal centers (33, 34) and spleen (34, 35) showing the presence of both naïve and memory B cells. An alternative method to track the development of single memory-lineage B cells has been the use of a specific anti-idiotypic Ab E4, which recognizes a canonical V region (36). In the mouse study by Liu et al. (36), this E4+ pool is derived from fewer than five canonical precursors. A lack of shared somatic mutations across clonally related cells by day 13 indicates that the selective expansion of mutant subclones typical of memory responses did not yet occur as it did in our panel of Abs. The strength of our study was the combination of functional data from human hybridoma technology with ultra-deep sequencing. A limitation of the deep sequencing was that we were not able to document the concurrent evolution of the corresponding L chains, although the hybridoma technology allowed us to find at least one hybridoma per clone with a matching H and L chain, adding further evidence that these hybridomas represented four distinct clones.

Despite different clonal origins within the VDJ junction, these four independent clones converged toward common amino acid residues within this junction and throughout the shared VH3-7 gene. This suggested a strong selection for optimal binding sites across clones. Because three clones also share the VH1-40 gene, but different J genes, CDRs H1, H2, H3, L1, and L2 would be expected to make contacts with the HA. Given the frequency of mutations within the CDR H1 segment and the conservation of the typically very variable CDR H3, we hypothesize that those two loops would be especially critical in the Ab–Ag interaction. The majority of the convergence positions are within framework regions; these residues can be at the surface, the core, or the H chain–L chain interface of the Ab. Core mutations partially determine stability (35) and may change the conformation of adjacent and distant CDR loops (8).

Sequence convergence has been found in a variety of proteins across individuals and species

Abs of the human VH1-69 germline from diverse phage display libraries, plasmablasts, and EBV-transformed B cells neutralize the stem of H1N1 and H5N1 influenza viruses (27, 38–41). Likewise, the phage-derived HIV Abs Fab 8066 and D5 from entirely different Ab libraries and panning procedures show convergence in their sequences and also in the conformation of the CDR H2 loop (42). Indeed, pyrosequencing of zebrafish Abs

Downloaded from http://www.jimmunol.org/ by guest on April 12, 2022
showed evidence of convergence, in which different individuals made the same Ab (12), but this was not correlated with functional data. Sequence convergence is not only seen in Abs across individuals of the same species (or, as demonstrated in this study, within a single individual), but also even in proteins that serve a similar purpose across distinct mammalian species (43). With continued progress in B cell technology, there will likely be further discoveries of converging sequence-related Abs from one or multiple individuals that share common epitopes. Once common germline gene responses to defined epitopes of pathogens are better understood, it might be possible to diagnose an infection of an individual based on the immune repertoire documented by ultra-deep sequencing.

Acknowledgments
We thank the anonymous blood donor, Jose A. Archuleta, Jr., and Cheryl Kinnard of the Vanderbilt Clinical Trials Center, M. Pooner and L. Cavacini for the HMMA2.5 cell line, Kimberly S. Crimin for statistical help, and Kinnard of the Vanderbilt Clinical Trials Center, M. Posner and L. Cavacini.

We thank the anonymous blood donor, Jose A. Archuleta, Jr., and Cheryl Kinnard of the Vanderbilt Clinical Trials Center, M. Pooner and L. Cavacini.

We thank the anonymous blood donor, Jose A. Archuleta, Jr., and Cheryl Kinnard of the Vanderbilt Clinical Trials Center, M. Pooner and L. Cavacini.

References


