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Conserved epitope on influenza-virus hemagglutinin head defined by a vaccine-induced antibody

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Circulating influenza viruses evade neutralization in their human hosts by acquiring escape mutations at epitopes of prevalent antibodies. A goal for next-generation influenza vaccines is to reduce escape likelihood by selectively eliciting antibodies recognizing conserved surfaces on the viral hemagglutinin (HA). The receptor-binding site (RBS) on the HA “head” and a region near the fusion peptide on the HA “stem” are two such sites. We describe here a human antibody clonal lineage, designated CL6649, members of which bind a third conserved site (“lateral patch”) on the side of the H1 subtype, HA head. A crystal structure of HA with bound Fab6649 shows the conserved antibody footprint. The site was invariant in isolates from 1977 (seasonal) to 2012 (pdm2009); antibodies in CL6649 recognize HAs from the entire period. In 2013, human H1 viruses acquired mutations in this epitope that were retained in subsequent seasons, prompting modification of the H1 vaccine component in 2017. The mutations inhibit Fab6649 binding. We infer from the rapid spread of these mutations in circulating H1 influenza viruses that the previously subdominant, conserved lateral patch had become immunodominant for individuals with B-cell memory imparted by earlier H1 exposure. We suggest that introduction of the pdm2009 H1 virus, to which most of the broadly prevalent, neutralizing antibodies did not bind, conferred a selective advantage in the immune systems of infected hosts to recall of memory B cells that recognized the lateral patch, the principal exposed epitope that did not change when pdm2009 displaced previous seasonal H1 viruses.

Author contributions: D.D.R., G.B., M.A.M., A.G.S., and S.C.H. designed research; D.D.R., G.B., J.F., and M.A.M. performed research; P.S., E.C.S., and S.C.H. supervised research; D.D.R., G.B., J.F., P.S., E.C.S., M.A.M., A.G.S., and S.C.H. analyzed data; and D.D.R., G.B., A.G.S., and S.C.H. wrote the paper.

Conflict of interest statement: J.F., P.S., and E.C.S. are employees of Seqirus, which has research and development programs related to influenza vaccines. The other authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. MF458174 and MF458175). The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes SW6C and SW6G).

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Significance

Antigenic variation requires frequent revision of annual influenza vaccines. Next-generation vaccine design strategies aim to elicit a broader immunity by directing the human immune response toward conserved sites on the principal viral surface protein, the hemagglutinin (HA). We describe a group of antibodies that recognize a hitherto unappreciated, conserved site on the HA of H1 subtype influenza viruses. Mutations in that site, which required a change in the H1 component of the 2017 vaccine, had not previously been observed among circulating H1 viruses. Our results encourage vaccine design strategies that resurface a protein to focus the immune response on a specific region.

influenza vaccine | hemagglutinin | B-cell memory | affinity maturation
antibodies would then have selected for fixation of the K166Q mutation (14).

We describe here an antibody clonal lineage, members of which recognize the lateral patch (Fig. 2A). We suggest that this lineage, CL6649 (named after one of its members), represents the type of response that ultimately selected for the K166Q mutation. CL6649 derives from subject 7 in a clinical trial of an adjuvanted, monovalent, A/California/07/2009-x181 (pdm2009) vaccine (15). The subject, born in 1975, had not been exposed to the 2009 pandemic strain at the time of vaccination. Antibodies from this lineage bind H1 HA from 1977 to 2009 and hence representatives of two distinct H1 pandemics (Fig. 3). Their footprint includes residues 165 and 166. The properties of CL6649 are consistent with the inferences described above concerning the K166Q mutation (14, 16). Introduction of the pdm2009 virus, to which most of the generally prevalent antibodies did not bind, would have favored recall of those rare memory B cells with BCRs that recognized the lateral patch, transforming a previously subdominant response into a dominant one.

**Results**

**Ab6649 Binds HAs Spanning 30 y of Antigenic Drift.** Antibodies in the lineage with the greatest representation among sequences from Siena subject 7 B cells, CL6615, bind the RBS; they depend on a mutation in the vaccine strain likely to have been due to egg adaptation (17). The second largest lineage from this donor included 19 paired-chain sequences (Fig. 2A). Initial analysis showed that one of its members, Ab6649, neutralized A/California/07/2009, a pdm2009 viral isolate, and A/Solomon Islands/03/2006 (Fig. 2B). Selected members of the CL6649 antibody lineage did not compete, or competed only marginally, with Ab6639 from CL6515 (Fig. 2C and Fig. S1). We analyzed the CL6649 phylogeny and inferred the unmutated common ancestor (UCA) and its intermediates (Fig. 2A). We determined the affinity of the UCA and of Ab6649 for a panel of HAs from influenza strains representing 30 y of antigenic drift and two pandemics (Fig. 3). All measurements used antigen-binding fragments (Fab) and HA1 “head” domains, to avoid complications from multivalency. The UCA bound the HA head of A/USSR/92/1977, but had no detectable affinity for any other HA tested (Fig. 3). These data suggest that exposure to an H1N1 strain circulating 2–3 y after the subject’s birth in 1975 may have elicited the UCA. Fab6649 bound HA from strains isolated from 1977 to 2009, including pdm2009 and its vaccine strain, X-181; thus, its breadth of binding included HAs from two distinct H1N1 pandemics. Neither the UCA nor 6649 bound HAs from H3, H5, H7, or B influenza viruses (Fig. 3).

**Structure of Fab6649 Bound with HA from A/Solomon Islands/03/2006.** We determined the crystal structure of Fab6649 bound with trimeric HA from A/Solomon Islands/03/2006 (Fig. 4 and Table S1). The Fab engages an epitope on the side of the HA1 head domain. It approaches HA with its long axis normal to the trimer threefold axis (Fig. 4A) and contacts the head with both heavy and the light chains. CDRs L1 and L3 bind a conserved loop (residues 125–128; H3 numbering, used here throughout), while CDRH3 and framework residues of the heavy chain contact the edge of β5, the strand farthest from the trimer threefold axis. The antigen-combining site includes 12 hydrogen bonds, six with main-chain carbonyls or amides on HA (Fig. 4B). Sequences of HAs from H1N1 influenza viruses circulating from 1977 to 2009 and mapped onto the HA structure show that the Fab6649 epitope was conserved during that entire period (Fig. 1). Conservation of the Ab footprint and dominance of main-chain interactions at the antigen–antibody interface likely account for the observed breadth.

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Structure of CL6649 UCA, the Rearranged Germ Line Precursor. We determined the crystal structure of the UCA to gain information about affinity maturation in this lineage. The structure showed that CDR loop conformations in the antigen-combining site had remained essentially invariant between the UCA and Ab6649 (Fig. 4 C). During the course of affinity maturation, however, four tyrosine residues in the UCA (Y33 and Y93 in the light chain and Y35 and Y54 in the heavy chain) had mutated to phenylalanines (Fig. 4 C), making the binding interface more hydrophobic. Light chain Y93 does not contact the antigen; the other three all have nonpolar contacts, but only light chain Y33 may have lost a hydrogen bond when losing the phenolic hydroxyl. The increased hydrophobicity of the antibody–antigen interface and the reduced requirement for hydrogen-bond complementarity appear to have contributed to the increased breadth acquired during affinity maturation.

Viral Escape. A mutation (K166Q) in the HA of new pdm2009-like H1N1 viruses (14) has been suggested as the cause of reduced vaccine efficacy for the 2013–2014 season and onward. Recent viral isolates (e.g., A/Singapore/GP1908/2015 and A/Michigan/45/2015; Global Initiative on Sharing All Influenza Data accession nos. EPI862697 and EPI830246, respectively) also include an N-linked glycosylation site at residue 165. The most recent World Health Organization vaccine recommendations include an H1N1 strain with both changes. Residues 165 and 166 are within the Fab6649 footprint. We determined whether they influence Fab6649 affinity by measuring binding with single S165N and K166Q and double S165N/K166Q pdm2009 HA head mutants (Fig. 5 A and B). The substitution K166Q substantially reduced Fab6649 affinity. The S165N mutation had no effect, but the absence of any shift in SDS/PAGE mobility suggests lack of glycosylation at the 165–167 NKS motif in the recombinant HA head (Fig. 5 C). The double mutation, S165N/K166Q, which did show a mobility shift, nearly abolished Fab6649 binding; the two mutations appeared to have additive effects. We infer, in accordance with earlier suggestions (14, 16), that recall from memory of antibodies with footprints overlapping that of Ab6649 is likely to have selected for the altered epitope in circulating pdm2009-like viruses.

Discussion
The inferred UCAs of both CL6515 and CL6649 bind the HA of H1 A/USSR/92/1977, an H1N1 isolated 2 y after the birth date of Siena subject 7, suggesting that the lineages originated in response to a 1977-like influenza infection during the donor’s childhood. The CL6649 UCA bound only the 1977 HA (of those we tested), but Ab6649 and several others from the lineage bound H1 HAs spanning more than 30 y of antigenic variation, indicating that somatic mutation had continually updated the specificity as the donor responded to subsequent exposures (from infection or vaccination). Receipt of the pdm2009 vaccine then focused the response on the most conserved sites exposed on the virion: the RBS and the lateral patch. Strong selection between 2009 and 2015 for the mutations at positions 165 and 166 indicates the development of widespread herd immunity directed at this epitope (14, 16), and thus the sequence of immune response events we have inferred for Siena subject 7 may represent that of many individuals of similar age. The lateral patch site is sufficiently large to allow antibodies with various CDR structures to bind, however, and we cannot yet judge from this single example the extent to which Ab6649 represents a
prevalent clonotype or a “typical” binding mode for the antibodies in many individuals that evidently selected for the mutations now present in most of the circulating H1N1 viruses.

Explanations for conservation of the lateral patch might be some as-yet unexplained compromise in viral fitness from changes in that region or partial occlusion by adjacent HAs tightly packed on the virion surface. Careful inspection of images from electron cryotomography suggests that the latter explanation is unlikely. Moreover, H3 viruses circulating since 1968 have a similar conserved patch on the lateral surface of the HA head, but in that case, immune escape probably appeared early, through the introduction of N-glycans at positions 165 and 245, around 1968 and 1986, respectively (Influenza Research Database; https://www.fludb.org/brc/home.spg?decorator=influenza). Thus, the lateral patch is apparently both antigenic on virions and immunogenic in infected individuals.

We have no direct evidence bearing on the possible loss of fitness of H1 viruses bearing HAs mutated at the Ab6649 site. We note that approximately 15 y after the 1918 pandemic, mutations appeared at positions 165 and 166, which reverted within a decade (Influenza Research Database; https://www.fludb.org/brc/home.spg?decorator=influenza). As these were some of the earliest mutations to be retained consistently for even a 3-y period, reversion might imply either a lack of widely distributed immune pressure or a minor disadvantage of the mutations for assembly, stability, or infectivity of the virus particle (or both).

Previous analyses of the antibody response to a first administration of a pdm2009 vaccine also found a prevalence of antibodies sensitive to changes at position 166, but without further structural information (16, 18, 19). Lineages encoded by gene segments V_{H}^{3-7} and J_{H}^{6*02}, with a CDRH3 of 18 residues. The extent of somatic hypermutation indicated that most or all of the clones were recall responses, just as we have inferred for clonal lineages CL6515 and CL6649. Another study found a similar convergence, with overrepresentation of V_{H}^{3-7} and J_{H}^{6} (18). The V_{H}^{3-7} antibodies may have had HA contacts distinct from those of the V_{H}^{4-39} and J_{H}^{5}-encoded CL6649 antibodies, although they evidently overlapped in the vicinity of residue 166. Moreover, not all antibodies sensitive to the residue at 166 restrict their contacts to the conserved, lateral patch; for example, antibody 2D1 (20) covers position 166, but

| Subtype | HA strain | UCA binding $K_{d}$ ($\mu M$) | Fab6649 binding $K_{d}$ ($\mu M$) |
|---------|-----------|-------------------------------|----------------------------------|
| H1      | A/US/90/1977 | 3.65                          | 1.12                             |
|         | A/Maryland/12/1991 | >100                         | 0.84                             |
|         | A/Solomon Islands/03/2006 | >100                       | 2.12                             |
|         | A/California/07/2009 | >100                         | 0.84                             |
| H3      | A/Victoria/3/1975 | >100                         | >100                             |
|         | A/Philippines/2/1982 | >100                         | >100                             |
|         | A/Oslo/23/1994 | >100                         | >100                             |
|         | A/Victoria/3/2011 | >100                         | >100                             |
| H5      | A/Vietnam/1/2004 | -                            | >100                             |
|         | A/gyalcan/ Washington/41088-4/2014 | -                      | >100                             |
| H7      | A/Shanghai/2/2013 | -                            | >100                             |
| Vic     | B/Malaysia/2506/2004 | -                            | >100                             |
| Yama    | B/Florida/04/2006 | -                            | >100                             |
|         | B/Plouzet/3073/2013 | -                        | >100                             |

Fig. 3. Affinity measurements of Fab6649 and UCA. Fab fragments of 6649 and UCA were screened for binding with heads of seasonal H1, H3, H5, H7, and B influenza HAs that circulated during the donor’s lifetime. Color-coding indicates the apparent $K_{d}$ measured by BLI.

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by natural infection, rather than...
for molecular replacement with Phaser (27). Density modification was performed with DM (28), and model rebuilding was done with Coot (29). Refinement was performed with BUSTER (Global Phasing) or Phenix (30, 31). Figures were prepared with the PyMOL Molecular Graphics System v 1.8.6.0 (Schrödinger). The H3 numbering scheme was used for HA.

**Bio-Layer Interferometry and Affinity Analysis.** Bio-layer interferometry (BLI) experiments were performed using the BLItz system (Pall ForteBio). Fab was immobilized on a Ni-NTA biosensor, and the cleaved HA globular head was allowed to bind until saturation. For pdm2009 mutants, the inverse setup was used, the 6×His-tagged HA head was immobilized, and tag-cleaved Fab were allowed to bind until saturation. Equilibrium dissociation constants (Kd) were obtained in GraphPad Prism version 6.0d by fitting saturation data from eight independent runs at different HA concentrations, using nonlinear least squares regression: BLI = Bmax × [HA] / (Kd + [HA]), where Bmax is the BLI signal at maximal binding and [HA] is the concentration of HA ectodomain.

**Peptide-N-Glycosidase F Treatment.** Enzymatic removal of N-linked glycans was performed with PNGase F (P0704; New England BioLabs) under native conditions. In brief, 100 μg of mammalian cell-produced HA heads at 1.25 mg/mL were incubated with 500 units of PNGase F overnight at 37 °C in 10 mM Tris·HCl and 150 mM NaCl. Deglycosylation was verified on a Coomassie blue-stained SDS/PAGE gel.

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