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**Citation:** Eisen, H. N., and A. K. Chakraborty. "Immunopaleontology reveals how affinity enhancement is achieved during affinity maturation of antibodies to influenza virus." Proceedings of the National Academy of Sciences (January 1, 2013). Copyright © 2013 National Academy of Sciences

**As Published:** <http://dx.doi.org/10.1073/pnas.1219396110>

**Publisher:** National Academy of Sciences (U.S.)

**Persistent URL:** <http://hdl.handle.net/1721.1/79777>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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# Immunopaleontology reveals how affinity enhancement is achieved during affinity maturation of antibodies to influenza virus

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The Abs made by B lymphocytes on first encountering an antigen bind it with low intrinsic affinity, and, over time, the average affinity of the Abs made against that antigen gradually increases. These changes, known as affinity maturation, were found initially for serum antibodies that recognized small, chemically well-defined epitopes (e.g., 2,4-dinitrophenyl). That similar affinity increases occur in responses to protein antigens, which elicit most immune responses to infections or vaccination, has been generally assumed but difficult to prove (1) largely because of the complexity and multiplicity of epitopes (antigenic determinants) on even small, single-chain proteins (2). Though some protein epitopes are linear stretches of amino acids, they usually are configurational clusters of noncontiguous residues best delineated crystallographically in antibody–antigen complexes (3); and more often than not, the diverse antibodies elicited to a protein antigen bind to different epitopes on that protein (4), confounding efforts to convincingly demonstrate affinity maturation. There are at least five epitopes on the influenza virus hemagglutinin (HA) that binds the virions to host cells (5). By comparing the binding properties and structures of affinity matured Abs to one of the HA epitopes with those of their progenitor Abs, the elegant study by Schmidt et al. reported in PNAS (6) provides clear evidence for affinity maturation of Abs. The study is notable, moreover, for its focus on the human immune response to influenza virus vaccination with a conventional influenza seasonal vaccine (FLUZON flu shot). By bringing together diverse approaches (crystallography, molecular dynamics simulations, and kinetic studies), the authors shed intriguing light on how the affinity enhancement of affinity matured antibodies is achieved.

Affinity maturation of Abs was originally established by measuring changes in a test animal of the serum Abs isolated from sequential bleedings taken over many weeks and months following antigen injection of a test animal (7, 8). In contrast to this longitudinal approach, the approach used by Schmidt et al. could be

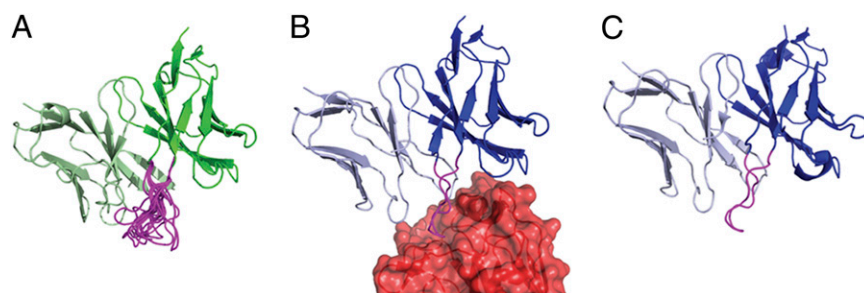


Fig. 1. CDR H3 (magenta) is conformationally diverse in the UCA antibody (A) but more rigid in the affinity-matured antibody CH67, whether bound to influenza virus HA (red; B) or not bound (C). With permission from S. C. Harrison and A. G. Schmidt.

characterized as “immunopaleontology.” From a single bleeding drawn from a volunteer 1 wk after receiving a conventional seasonal “flu shot” injection—Fluzone, a mixture of three inactivated strains of the virus—mature B lymphocytes were isolated by cell sorting and individually screened for secretion of virus-neutralizing Ab. Then, using recently developed powerful methods for rapid cloning of Ig VH and VL genes from individual B cells (9–12) and determining their sequences, three Abs (called CH65, CH66, and CH67), each from a different B cell (i.e., plasmablast), were found to have similar sequences and to constitute a clonal lineage from which antecedent Abs could be inferred. These antecedents included an unmutated common ancestor (UCA) and an intermediate (I-2) of a clonal tree of which CH65, CH66, and CH67 constitute the terminal twigs. Expressed as recombinant mAbs, they all became available in essentially unlimited amounts for the remarkably comprehensive analyses by Schmidt et al. (6).

The variable domain sequences of CH65 [previously described (13)] and CH66 were nearly identical, whereas the slightly different sequences of CH67 place it on another branch of the lineage. Crystal structures of the complexes formed by the HA antigen with the Abs’ antigen-binding domains (i.e., Fab) showed that all three Abs recognized the same epitope. This epitope was located in the HA global “head” at the site where HA binds

sialic acids on host cells, a binding reaction essential for virus attachment to host cells and infection. Crystal structures of complexes of the HA global head with the Fab domain of each of these Abs showed the same protrusion of the Ab H chain’s complementarity determining region (CDR H3) loop into the HA epitope (Fig. 1). This mimics sialic acid and accounts for the ability of these Abs to block virion binding to cells, and thus broadly neutralize infectivity of many H1N1-type influenza virus strains (30 of 36 isolates obtained at various times over 30 y).

The CH65 and CH67 Abs had essentially the same intrinsic affinity for the HA epitope ( $K_a = 2\text{--}2.8 \times 10^6 \text{ M}^{-1}$ ) whereas the UCA and the I-2 intermediate bound approximately 300 times more weakly ( $K_a = 0.7\text{--}0.8 \times 10^4 \text{ M}^{-1}$ ). The matured Abs bound HA with association rates that were approximately 40 times faster and dissociation rates that were slower than were found for UCA or the I-2 Ab. That matured high-affinity Abs have faster on-rates than lower affinity Abs made earlier in response to the same epitope (a low molecular weight hapten) was seen previously by Foote and Milstein (8), who speculated that

Author contributions: H.N.E. and A.K.C. wrote the paper.

The authors declare no conflict of interest.

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selection of high-affinity Ab-producing B cells might be driven by faster binding of antigen to cell-surface Ab.

The mechanisms for obtaining faster on-rates by affinity maturation have, however, remained obscure. Schmidt et al. (6) now provide a clear example of a pathway by which this is achieved. The conformations adopted by the Ab heavy-chain third CDR (CDR H3) loops of the free matured Abs are very similar to that in the Ab-antigen complexes, with their CDR H3 loop rigidly extended in a form able to insert into the sialic acid binding site of the HA (Fig. 1). In contrast, their UCA and I-2 predecessors exhibit far more CDR H3 conformational heterogeneity. This is in accord with the flexibility of early response Abs of the IgM class (14, 15, 16). The CDR H3 loop of UCA—despite having the same sequence as in CH65 and CH66 and virtually the same as CH67—was disordered or constrained, as was that of the I-2 Ab, in conformations that differed from that of matured Abs. As Schmidt et al. (6) suggest, the latter's conformational rigidity derives from mutations outside the binding loop. The differences found in crystal structures are supported—and, indeed, extended—by truly long molecular dynamic simulations of free Abs and their (Fab domain) binding to HA. Together, the crystal structures and molecular dynamic simulations show that the more rigid CDR H3 conformation preconfigured in the unbound mature antibodies results in a lower entropic penalty upon binding, thus leading to their enhanced on-rates.

The affinity differences between the B cell-derived Abs and the inferred pre-

decessors in the lineage seems much too great to have developed in the 7 d between vaccination and sampling blood B cells if the initial responders to the vaccine were antigen-naïve B cells. Likewise, the number of replacement mutations (at least 12 in heavy chain and six in light chain) by which the B cell-derived Abs differed from the UCA seems far too many to have been generated in 7 d. It is likely therefore that, as surmised previously (13), the injected vaccine stimulated memory B cells that had resulted from prior exposures to the virus. That surmise is in accord with evidence that antigen-stimulated memory cells promptly produce the high-affinity matured Abs they were making at the end of a previous response (17).

The use of monoclonal Abs, especially those derived from isolated individual B cells, has enormously extended our understanding of the mechanisms underlying affinity maturation. Although early studies of affinity maturation with polyclonal antisera were limited, they revealed that, as the average affinity of serum Abs increases over time, so does heterogeneity with respect to affinity (7). Long overlooked, this heterogeneity implies that some low-affinity Ab-producing B cells, as well as the high-affinity producers, are selectively stimulated by antigen. Indeed, in serum Ab populations with very high average intrinsic (nM) affinity, subsets of low-affinity Abs to the same (i.e., 2,4-dinitrophenyl) epitope are readily demonstrable (18); similarly, among the high-affinity monoclonal Abs harvested late in the response to another epitope (phenyloxazone), there were some low-affinity mAbs (8). Thus, other factors

besides affinity are involved in the selective stimulation of B cells by antigen. Whether it is the rate of antigen binding to B cells, or endocytosis of bound antigen followed by B-cell display of antigen-derived peptides as peptide-MHC complexes that engage helper T cells, or dynamics of B-cell circulation through germinal centers (19) remains to be seen.

The broadly neutralizing activity of CH65, 66, and 67 against H1N1-type flu viruses suggests their inclusion in therapeutic mixtures (“cocktails”) of several human mAbs that, by virtue of binding an epitope in the HA stalk region, blocks fusion of virus with host cell membranes and virus entry into cells (20–23). Used therapeutically, such mAb mixtures are likely to reduce mortality from life-threatening flu virus infections, just as, before the advent of antibiotics, polyclonal Abs in horse and rabbit antisera to pneumococcal polysaccharides decreased mortality from pneumococcal pneumonia. Notably, a synthetic peptide that corresponds to conserved 55 residues in the HA stalk region has been found to be a promising “universal” vaccine that protects mice against a variety of influenza subtypes (24). The design of other immunogens, aimed at maximizing the immunogenicity of the HA head epitope recognized by Abs CH65, 66, and 67 poses a challenge that, if overcome, would bring closer another universal vaccine that elicits broadly neutralizing Abs. These could better protect the world than current virus strain-specific vaccines against annual visitations of flu virus epidemics and their potential to occasionally develop into devastating pandemics.

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