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Herman N. Eisen

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Affinity Enhancement of Antibodies: How Low-Affinity Antibodies Produced Early in Immune Responses Are Followed by High-Affinity Antibodies Later and in Memory B-Cell Responses

Herman N. Eisen^{1,2}

Abstract

The antibodies produced initially in response to most antigens are high molecular weight (MW) immunoglobulins (IgM) with low affinity for the antigen, while the antibodies produced later are lower MW classes (e.g., IgG and IgA) with, on average, orders of magnitude higher affinity for that antigen. These changes, often termed affinity maturation, take place largely in small B-cell clusters (germinal center; GC) in lymphoid tissues in which proliferating antigen-stimulated B cells express the highly mutagenic cytidine deaminase that mediates immunoglobulin class-switching and sequence diversification of the immunoglobulin variable domains of antigen-binding receptors on B cells (BCR). Of the large library of BCR-mutated B cells thus rapidly generated, a small minority with affinity-enhancing mutations are selected to survive and differentiate into long-lived antibody-secreting plasma cells and memory B cells. BCRs are also endocytic receptors; they internalize and cleave BCR-bound antigen, yielding peptide-MHC complexes that are recognized by follicular helper T cells. Imperfect correlation between BCR affinity for antigen and cognate T-cell engagement may account for the increasing affinity heterogeneity that accompanies the increasing average affinity of antibodies. Conservation of mechanisms underlying mutation and selection of high-affinity antibodies over the ≈ 200 million years of evolution separating bird and mammal lineages points to the crucial role of antibody affinity enhancement in adaptive immunity. *Cancer Immunol Res*; 2(5); 381–92. ©2014 AACR.

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Learning Objectives

Antibodies (Abs) are core components of adaptive immune responses, and the conservation of mechanisms underlying mutation and selection of high-affinity Abs over the ≈ 200 million years of evolution separating bird and mammal lineages points to the crucial role of Ab affinity enhancement in adaptive immunity. Understanding how antibodies with affinity-enhancing mutations are selected to survive and differentiate into long-lived Ab-secreting plasma cells and memory B cells will improve the design of immunotherapeutics. Upon completion of this activity, the participant should gain a basic knowledge of antibody affinity maturation.

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Authors' Affiliations: ¹Department of Biology, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology; and ²Ragon Institute of MGH, MIT, and Harvard, Cambridge, Massachusetts

Corresponding Author: Herman N. Eisen, Department of Biology, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139. E-mail: HNEisen@MIT.edu

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Introduction

It has been said that science is characterized by the ability to make reliable predictions. In immunology one of the few predictions that can be made with confidence is that in response to infection or immunization (vaccination), the antibodies made initially have low affinity for the inciting antigen (the immunogen) and those produced later have higher affinity for the same antigen. The change results in

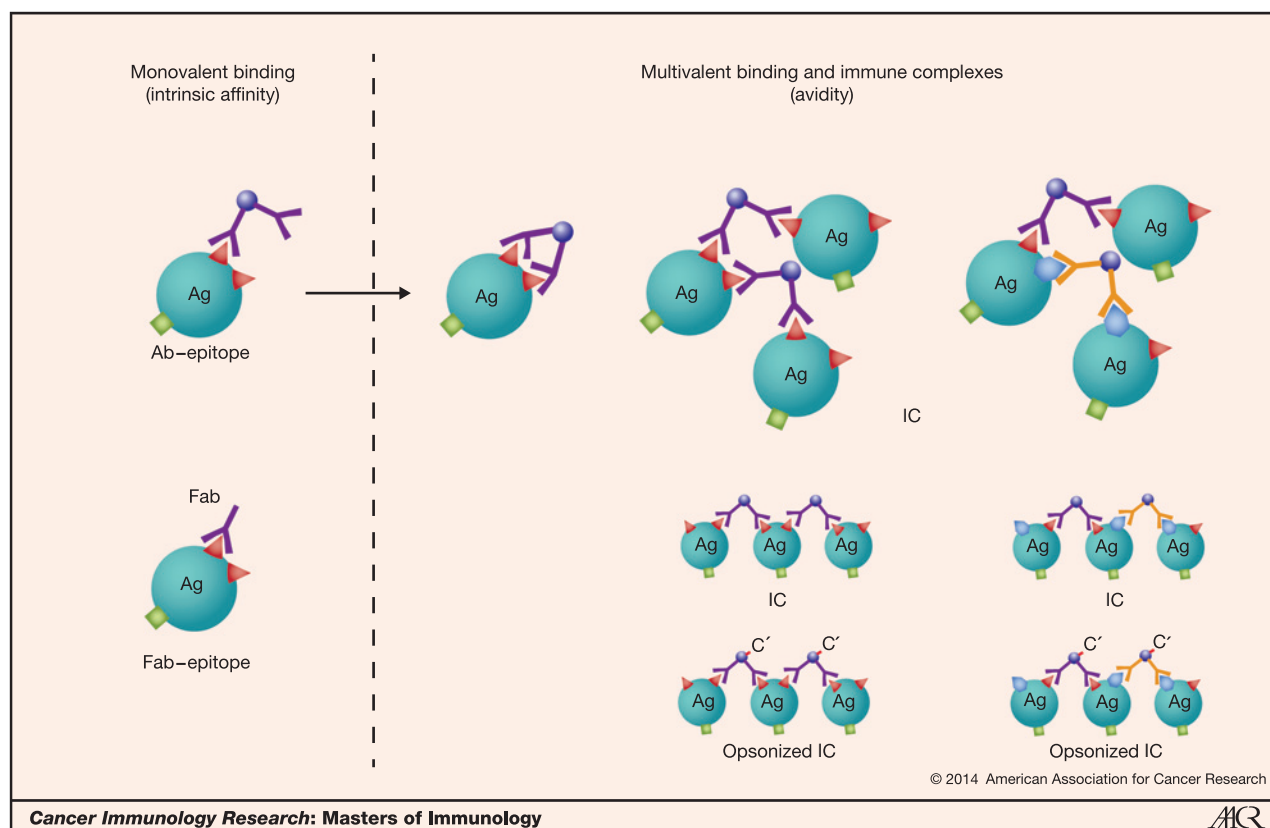


Figure 1. Diagrammatic representation of various antibody-epitope bonds. Left, monovalent binding of an epitope by an intact bivalent antibody (Ab) or an antibody fragment (Fab). Bond strength (free energy) is measured by intrinsic affinity. Right, various multivalent antibody-epitope bonds: concatenated linkages ($\dots\text{Ag-Ab-Ag-Ab}\dots$) form immune complexes (IC) involving the same or diverse epitopes. Strength of multivalent bonding ("avidity") exceeds intrinsic affinity not only because of statistical factors that lower k_{off} but also because of on-rates differences between formation of the first and subsequent bonds (2). Immune complexes with bound complement (C') fragments (opsonized immune complexes) bind especially tightly to follicular dendritic cells in the germinal centers.

the production of what are often termed affinity-matured antibodies. The several tons of monoclonal antibodies (mAb) now being produced annually worldwide are affinity matured. So are virtually all the other innumerable antibodies used as diagnostic reagents in clinical and experimental laboratories. The myeloma proteins produced by multiple myeloma, a hematologic cancer, are also affinity-matured antibodies. Here, we wish to describe some molecular and cellular mechanisms that underlie affinity maturation, and how we have come to understand them.

At the outset, it should be clear that affinity is a metaphor for the equilibrium constant (K) that characterizes the reversible binding of an antibody (Ab) to an antigenic determinant (epitope) on an antigen (Ag). Antibodies have multiple identical binding sites per molecule, and those of the most common type [immunoglobulin G (IgG)] have two sites per molecule. The equilibrium constant can thus refer to the formation of a monovalent complex (Ab-Ag), termed the intrinsic affinity, or to higher order complexes, divalent or multivalent, depending on the number of binding sites (epitopes) per antigen molecule or particle ($\dots\text{Ag-Ab-Ag}\dots$; Fig. 1). The equilibrium constants of multivalent interactions, termed avidity, usually exceed, sometimes greatly, the corre-

sponding intrinsic affinity. But for several reasons there is no consistent relationship between them. One reason, implied in Fig. 1, arises from the rebinding of dissociated Ab-epitope bonds in multivalent complexes. Another arises from differences in the formation of the first and subsequent Ab-epitope bonds in such complexes (1, 2). Thus, it is the intrinsic affinity, not avidity, that provides a unique and unambiguous basis for comparing antibodies produced against the same epitope at different times during an immune response, and also for comparing Ab-Ag reactions with other reversible binding reactions, for example, enzyme-substrate or receptor-ligand binding.

Affinity values are usually determined *in vitro* under conditions in which equilibrium can be achieved, but Ab-Ag interactions *in vivo*, like most biologic processes, are rarely, if ever, at equilibrium (3). However, the underlying rates of binding (k_{on}) and unbinding (k_{off}) take place over much shorter time scales than needed to achieve equilibrium, and it is these rates that determine interaction outcomes; they account for the distinctive value of the equilibrium constant ($k_{\text{on}}/k_{\text{off}}$ or the inverse) as a metric that measures of the free energy of antibody-epitope bonding, whether *in vitro* or *in vivo*.

Affinity Maturation of Antibodies to Simple Haptens

The finding that intrinsic affinities of serum antibodies increase over time after immunization first emerged from the use of hapten-protein conjugates as immunogens. From the serum of rabbits immunized with 2,4-dinitrophenyl groups covalently linked to proteins (DNP-proteins), the purified anti-DNP antibodies were easily isolable from serial bleeds, and their intrinsic affinity for the homologous epitope (ϵ -DNP-lysine) was determined readily by a fluorescence energy transfer assay that rapidly measures affinity over a >10,000-fold range (4, 5). The earliest antibodies examined (IgG class) had low affinity for DNP-lysine ($K_D \sim 10,000$ nmol/L). The average intrinsic affinity of the antibodies obtained later increased progressively to <1 nmol/L. The findings were subsequently confirmed with antibodies to other haptenic groups (e.g., to fluorescein, lactose, azophenyl arsonate, phenyloxazone, 4-hydroxy 3-nitro phenylacetic acid, nitrophenyl phosphonate; refs. 6–10).

Affinity Maturation of Antibodies to Protein Antigens

Most immunogens are proteins, and early efforts to account for the variations in strength of binding of anti-protein antibodies focused on antisera that neutralized diphtheria toxin. These antisera, which dramatically reduced the mortality in those infected with the diphtheria bacillus, varied considerably in neutralizing potency. Sera were termed more "avid" if smaller amounts were required to neutralize the toxin, or reacted faster with the toxin, or the toxin-antitoxin complexes were less dissociable on dilution. However, antisera considered more avid by one measure were often indistinguishable by other measures. The intense disagreements led to the recommendation "...to avoid the use of the term avidity, as it was (*and often still is*) used with different senses by different workers" by Llellyn Smith, 1938 (see ref. 11; italics added by the author).

Nevertheless, Jerne, in a classic PhD dissertation (11), concluded from "A study of avidity based on rabbit skin responses to diphtheria toxin-antitoxin mixtures" that the avidity differences all boiled down to differences in equilibrium constants for the reversible reaction between toxin (T) and antitoxin (A), that is, $T + A \rightleftharpoons TA$. The significance of that conclusion subsequently became unclear as awareness of protein structure grew. Diphtheria toxin proved to be a heterodimer, and antibodies to either subunit could block (neutralize) the inflammatory reaction due to intracutaneous injection of the toxin (Schick test). Moreover, antibodies to diverse epitopes on protein antigens usually appear at different times, resulting in the accumulation over time of increasingly complex mixtures of serum antibodies and formation of less dissociable toxin-antitoxin complexes, without necessarily involving changes in the affinity for a particular epitope (see anti-lysozyme antibodies, below).

The struggle to explain differences between early and late antisera to proteins continued even after mAbs could

be produced (see below). Consider, for example, antibodies to hen's egg lysozyme, a small single chain, structurally well-characterized monomeric protein and a potent immunogen (12). More than 60 anti-lysozyme mAbs derived from Balb/C mice have been subjected to extensive analyses. A study of 49 mAbs to lysozyme concluded that the apparent increase in affinity of serum antibodies over time was actually due to "... an increase in the number and diversity of antibodies, rather than an overall increase in the 'avidity' of individual antibodies" (13). In another study of 23 mouse anti-lysozyme mAbs that differed widely in intrinsic affinities ($K_D = 0.1$ –100 nmol/L), there was no discernible increase in the affinity over time (14); importantly, however, in the latter series, the IgM anti-lysozyme mAbs consistently had exceedingly low affinities ($\leq 10,000$ nmol/L). Nevertheless, comparison of mAbs to a serologically defined epitope of a protein (an anti-hapten mAb) was consistent with affinity maturation (15), as was X-ray crystallographic comparison of a few selected mAbs to lysozyme (16).

The difficulties in confirming the widely held belief that anti-protein antibody responses underwent the same affinity maturation process as anti-hapten antibodies arose from various sources, including

- i. the multiplicity of overlapping epitopes on proteins confounds efforts to track changes over time of antibodies to a particular epitope;
- ii. antibodies to different epitopes on the immunogen arise at different times after the initiation of the antibody response;
- iii. methods for obtaining mAbs entailed sacrificing mice, precluding longitudinal time studies of antibodies to a given epitope in an individual animal; and
- iv. the rate at which affinity maturation is observed can differ considerably in different individuals (5).

These obstacles have recently been overcome by powerful new methods for deriving mAbs from single B cells captured with biotinylated antigen from bleeds from individual human subjects (17–19). The captured cell's genes that encode its antibody's heavy (H) and light (L) chain variable (V) regions can be sequenced and expressed as a recombinant mAb. Any mutations in these variable regions [see "GC Reactions" and antigen-induced cytidine deaminase (AID) below] can be reverted back to the presumed unmutated progenitor antibody, which could then be expressed as another mAb corresponding to what was produced by the initial immunogen-activated B cell. Comparison of the reverted, initially produced, and the vaccine-elicited mAbs amounts to a virtual longitudinal study of affinity changes over time in an individual's antibody response to an epitope. In a recent study, several mAbs to influenza virus hemagglutinin (HA) were derived from the same person about a week after a flu virus vaccine was injected (20). X-ray crystallographic studies demonstrated that these antibodies recognized the same epitope of the several that have been identified on the influenza virus HA, and the few differences in their otherwise shared V_H and V_L gene sequences

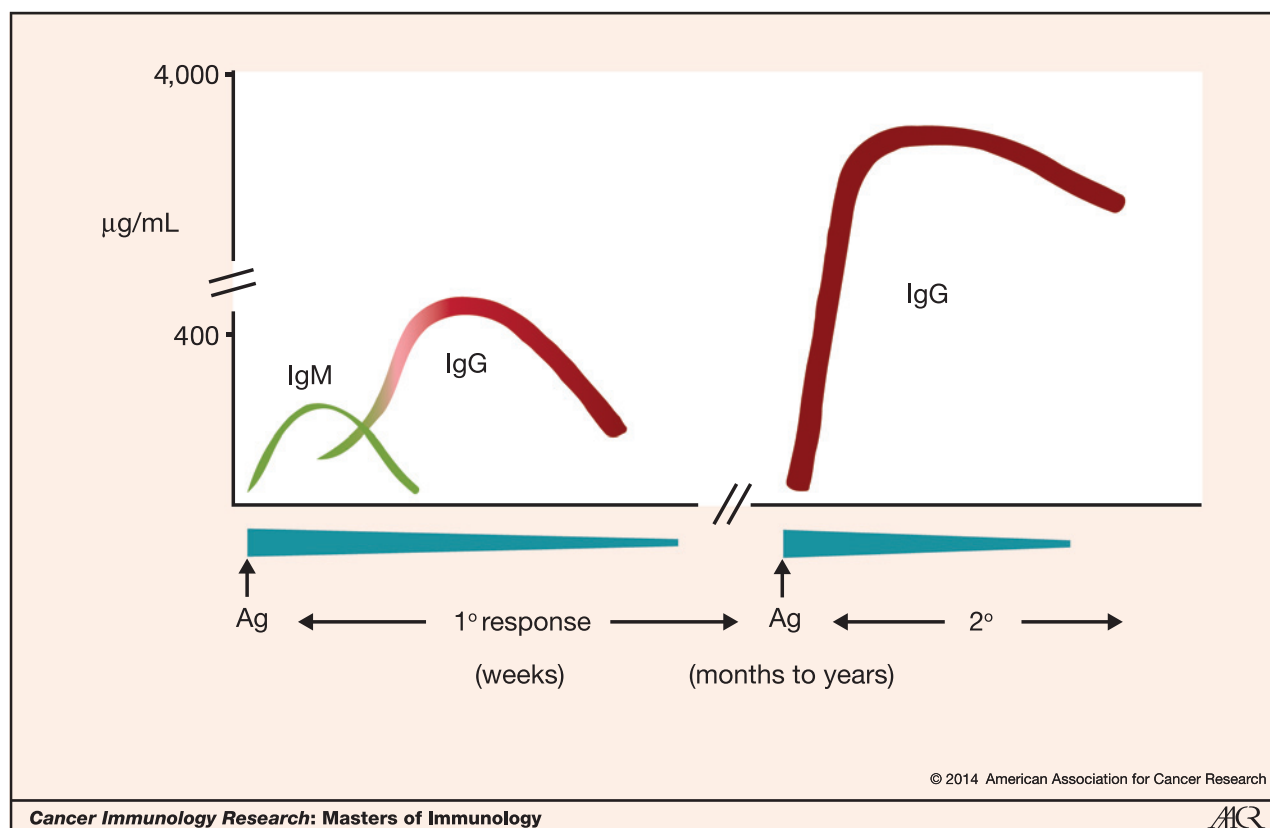


Figure 2. Changes over time in serum antibodies to a small (hapten) epitope. Y-axis values are concentration of antibodies in serum after immunizing rabbits with hapten–protein conjugates in Freund's adjuvant. Color values indicate average intrinsic affinities (green, lowest affinities; pink, higher affinities; red, highest affinities). Thickness of lines corresponds to heterogeneity with respect to intrinsic affinity. The heterogeneity was evident when the hapten–protein conjugates used as immunogen were large (with many DNP groups attached to a high MW protein, e.g., hemocyanin) or small (with a single DNP attached to a particular lysine in a low MW protein, ϵ -DNP–lysine₄₁–ribonuclease). (On the basis of refs. 2 and 75, and for 2° or memory response, refs. 7 and 76.)

indicated that they were part of a clonal lineage. The sequences of the successive antibodies in that lineage, extending back to the presumed ancestral antibody (inferred by maximum likelihood strategies), led to its expression as the initial progenitor mAb. Intrinsic affinity of the vaccine-elicited antibodies was about 300-fold higher than that of the progenitor antibody, providing strong evidence that antibodies to an extensively studied protein antigen, similar to those generated against the simple haptens, do indeed undergo affinity maturation (Fig. 2).

Mechanisms Underlying Affinity Maturation: Diversification by Mutation of Antigen-Binding Receptors on B Cells and Selection of B Cells with the Most Favorable Mutations

The mechanisms underlying affinity maturation became susceptible to analysis only after two landmark developments in the 1970s. One stemmed from the discovery that genes encoding antibody heavy and light chains were generated by rearranging different gene segments [V and J (joining) for light chains and V, D (diversity), and J for heavy chains; ref. 21]. This led to the identification and sequences of >200 Ig V gene segments for heavy and light chains in the human genome and similar numbers in the mouse genome.

The other landmark was the ability to immortalize B cells by fusing them to myeloma tumor cells and thereby generate mAbs (22). Comparisons of mAb variable region sequences with what seemed to be the corresponding germline Ig V gene sequences revealed mutations localized in the N-terminal domains, which house the antibody's binding sites (23, 24). Mutations are usually more frequent in certain variable regions [termed complementarity determining regions (CDR)] than in the intervening ones [termed framework regions (FR)]. As shown by crystallographic studies of free and antigen-bound antibodies, FRs form a sort of scaffold from which the CDRs extend as loops that constitute the binding site, where the amino acids of the antibodies contact the bound epitope of the antigen. Nevertheless, the FR sequences that are remote from the binding site of an antibody can have pronounced effects on intrinsic affinity, most likely by affecting the conformation or flexibility of the binding site (20, 25).

The cell-surface antibodies on unstimulated, "naïve" B cells are IgM molecules with their heavy chain V domains linked to the μ constant domain. Following antigen stimulation, the V-encoding genes are switched from μ to downstream exons of one of several other classes of heavy chain constant domains in the genome, eliminating the intervening sequences—a change

called class-switching (IgM $\rightarrow$ IgG or IgA or IgE, etc.). As discussed below, class-switching and variable region mutations occur in the GCs of lymphoid tissues.

Tracking early events

To appreciate the cellular and molecular events that lead to affinity-matured antibodies, it is useful to track events that begin with immunogen entry into lymphoid tissues, such as lymph nodes (LN) that drain a site of infection or vaccine injection. In LN, B cells are clustered into follicles separated by T cell-rich zones. The B-cell follicles consist mostly of "naïve" B cells, whose receptors on B cells (BCR) are integral membrane IgM molecules associated with several signaling coreceptors. These cells are activated by their BCR binding to immunogens that are presented in various ways, for example, on dendritic cells (DC), on special LN-resident macrophages, as Ab–Ag immune complexes (IC), or perhaps even certain soluble antigens. Most immunogens are "T cell-dependent antigens"; the B cells they initially stimulate migrate to the border between the B-cell follicles and the contiguous T cell-rich zones, ostensibly for further stimulation by cognate helper (CD4⁺) T cells. In some activated cells, the exon for the IgM gene's membrane-anchoring domain is spliced out, resulting in the BCR's IgM component being secreted as free IgM molecules. Many IgM antibodies (and presumably the corresponding BCR) can bind structurally diverse antigens (polyreactive). One consequence is that the number of epitopes that can activate immune responses exceeds the total number of naïve B cells in an individual. The polyreactivity of IgM molecules is supported by a considerable amount of evidence that their V domains are configurationally highly flexible (10, 26), which is most dramatically visualized in movies of molecular dynamic simulations (20). Indeed, it has been proposed that those germline Ig segments that encode the V regions with the greatest impact on antigen-binding specificity (i.e., CDR3 of heavy chains) have been selected in evolution to be "nearly optimal for conformational flexibility" (27). In binding to an antigen, the V domain becomes configurationally constrained, thereby incurring an entropic penalty that is a contributing factor to the characteristically very low intrinsic affinities of IgM Ab–Ag interactions; some are too weak to measure except by highly sensitive methods [$<10,000$ nmol/L (K_D), e.g., see ref. 14]. These features call attention to the marked similarities between antigen-binding receptors on T cells (TCR) and BCR on naïve B cells. V domains of both TCR and BCR are encoded by germline (unmutated) rearranged Ig segments; both receptors bind their respective ligands with similarly low affinities [in the range of $\approx 10^4$ – 10^6 (mol/L)⁻¹ (K_A)], and both exhibit considerable polyreactivity (28). The V domains of TCR, like those of IgM molecules, also seem to be configurationally flexible (29, 30).

In view of such weak intrinsic affinity, how can antigens initiate far-reaching responses from IgM⁺ B cells? Assuming that BCR on naïve B cells are as abundant as TCR on splenic T cells ($\sim 60,000$ molecules/cell; ref. 2) and similarly clustered in cell-surface islands (31), it is understandable that naïve B cells are usually activated by multivalent BCR interactions with immunogens that have multiple, closely

spaced epitopes, as in polysaccharide envelopes of some bacteria, glycoprotein spikes on some viruses (flu, VSV), Ab–Ag immune complexes (Fig. 1), or aggregated, rather than monomeric, proteins. In addition to binding antigens, BCRs are also endocytic receptors. The bound antigen is endocytosed and cleaved into peptides, which are displayed on the cell surface as peptide–MHC-II complexes that can be engaged by cognate Th cells.

The low probability of a productive encounter between rare T cells that can recognize the peptide–MHC complexes presented by the rare IgM⁺ B cells that responded to the immunogen is enhanced by the chemotactic migration of activated B cells toward T cells at the follicle borders (32) and by the polyreactivity of many IgM⁺ BCRs and TCRs (28). Activated IgM⁺ B cells follow one of two paths. Some become extra-follicular short-lived plasma cells that secrete IgM antibodies (or even become IgM⁺ memory B cells; ref. 33); IgM molecules are pentameric with 10 identical epitope-binding sites per molecule. Their ability to bind avidly to microbes with repeating, closely spaced, surface epitopes likely accounts for the considerable protection against lethal systemic bacterial infections conferred by normal serum IgM (34). IgM-based immune complexes are also especially effective in activating complement (C') and binding C' fragments. They thus form the opsonized immune complexes that probably bind with especially high avidity to follicular DCs (FDC) and may well provide an early source of antigen that drives GC reactions (see below).

Other activated IgM⁺ B cells proliferate rapidly in B-cell follicles and push aside other B cells to form GC, where they express the mutagenic "master catalyst" that modifies genes that encode antibody heavy and light chain variable regions (see AID, below).

GC Reactions

Histologically, GCs appear as small clusters of proliferating cells ($\approx 3,000$ cells/cluster) in LN and other secondary lymphoid structures such as Peyer's patches and spleen (Fig. 3). About 20 years ago, GCs were found to develop only after antigens had stimulated an antibody response. Although there are hints that affinity-matured antibodies might arise in GC-deficient mice (35), GCs clearly serve as the principal and certainly best-studied cellular factories in which low-affinity IgM antibodies are converted to high-affinity antibodies of other classes. By light microscopy, GCs are polarized into two sectors of roughly the same size, a dark zone with densely packed cells and a less densely packed light zone. The cells are embedded in a reticular network associated with FDCs and infiltrated by specialized follicular helper CD4⁺ T cells (Tfh).

FDCs

Although similar in appearance to the ubiquitous bone marrow-derived DCs [myeloid DC (mDC)], FDCs are different developmentally and functionally (36, 37). FDCs, which seem to arise from perivascular stromal cells, are characterized by dendrite-like processes decorated with receptors for the Ig constant (Fc) domains and for fragments of complement (C') proteins. Accordingly, Ab–Ag IC with bound C' fragments

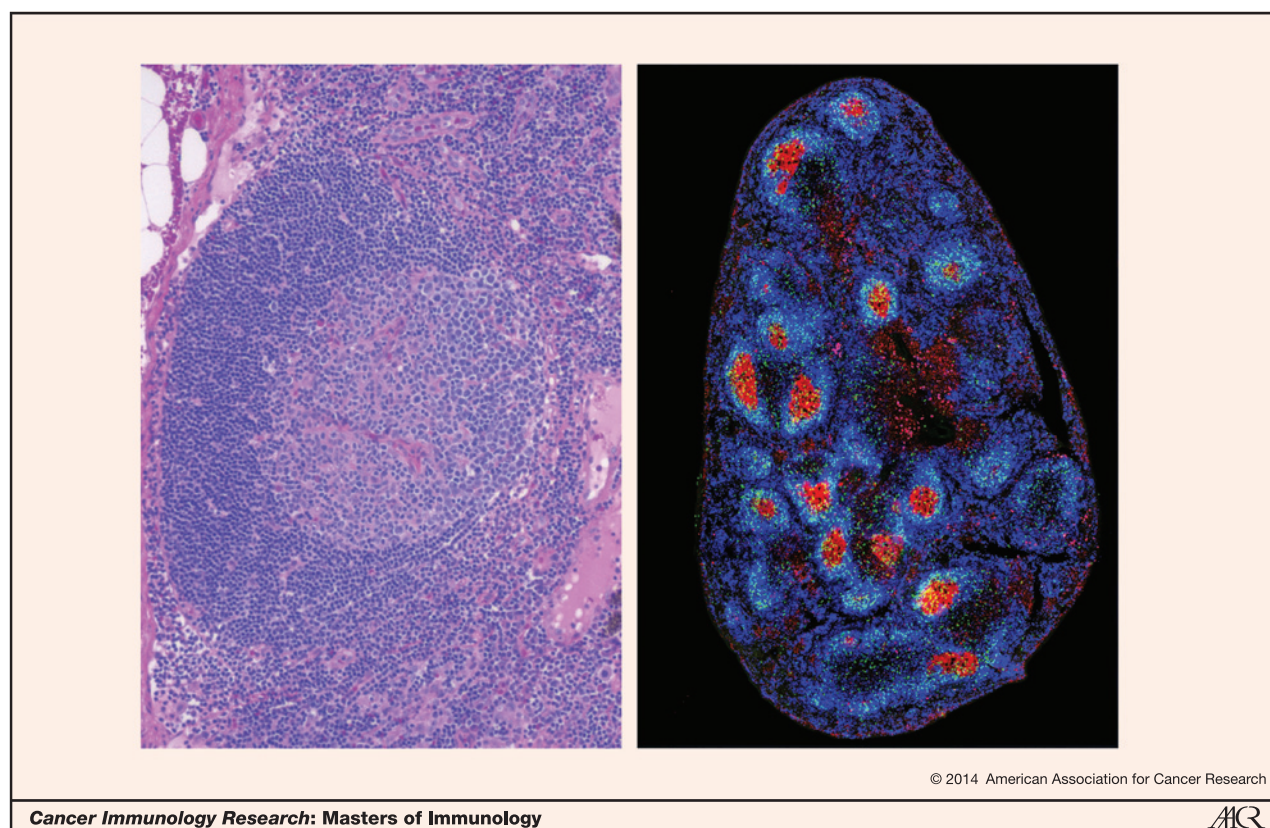


Figure 3. GCs in lymphoid tissues. Right, human LN section, stained with hematoxylin and eosin, showing a GC surrounded by the many small unstimulated B cells (likely expressing IgM BCR) that make up most of a B-cell follicle. GC B cells are larger (lymphoblasts), proliferating, and undergoing class-switching and rapid rates of mutation in heavy and light chain variable regions. (Courtesy of R.B. Colvin, Department of Pathology, Massachusetts General Hospital, Boston, MA.) Left, live cells in the spleen of an immunized mouse showing GC cells expressing the mutagenic AID (red fluorescence), and the antigen-specific Th cells (green fluorescence). Blue patches, B-cell follicles stained for IgD. (Image was generated by Jeroen Tas of G. Victora's laboratory at the Whitehead Institute, Cambridge, MA.)

(opsonized IC; Fig. 1) can bind avidly to these receptors, which internalize them into degradative or nondegradative endosomes from which the IC cycle back to the FDC dendrite surface (38). Depending upon the antigen and the immunization regimen, the Ag/IC are displayed on FDCs for variable periods, from several weeks to more than 9 months (39). The density and persistence of the Ag/IC on FDCs are likely crucial determinants for the selection of BCR-diverse B cells, but attempts to measure them seem rarely to have been undertaken. A critical role of FDCs in maintaining GCs is evident from the disappearance of GCs when FDCs are ablated (40). The strategic position and the remarkable stability of IC on FDCs may account for the unusual effectiveness of Ab–Ag complexes as immunogens. For example, in the 1920s, vaccines that effectively protected human populations from diphtheria consisted of what were doubtless immune complexes formed *in vitro* by diphtheria toxin admixed with neutralizing amounts of antitoxin antiserum.

The GC dark zone is populated largely by Ag-stimulated, rapidly proliferating B cells, some with doubling times as short as 6 to 7 hours. From the sequences of expressed variable regions (V_H and V_L) of cells isolated by laser-capture microdissection from LN GC, it seems that a typical B-cell population

in a GC consists initially of dividing cells that represent the clonally related lineages that arise from only a few (≈ 3 or 4) activated founder B cells (23, 41, 42).

The foregoing static view has been greatly enriched and substantially modified by imaging live cells in intact mouse LN, especially when enhanced with photo-activatable chromophores that tag cells and allow them to be tracked for hours in real time as they move within and between GCs (43–45). In GCs, B cells crawl extremely rapidly (about 1 cell length/min) predominantly from the dark zone to the light zone. Survival of these B cells depends upon their binding of antigen with sufficient energy (above a threshold, corresponding perhaps to an intrinsic affinity of only ≈ 0.1 mmol/L K_D). Most GC B cells evidently bind antigen too weakly to survive and die by apoptosis, the corpses appearing as "tingible bodies" in macrophages. Some survivor B cells migrate back from the light zone to dark zone for repeated rounds of proliferation, mutation, and further selection (45, 46). Other survivors leave the GCs and LN (47) to become long-lived plasma cells in the bone marrow (48) or memory B cells that are widely dispersed. Although the B cells within a GC largely represent the diversifying progeny of a few antigen-activated founder B cells, GC can also be infiltrated by antigen-reactivated memory B cells. It is thus

likely that, GCs can support further rounds of maturation (i.e., mutation and selection, see below) of memory B cells that had been affinity matured earlier in another GC in response to the same or a cross-reacting antigen ("GC reutilization"; refs. 45, 49–51).

Follicular Th cells

In addition to ligation of their BCR by immune complexes on FDC, most Ag-stimulated B cells require additional stimuli provided by cognate Th cells, for example, interactions between CD40 on the B cells with CD40L on specialized GC T cells (T_{fh}). In contrast to the strict confinement of recently activated antigen-stimulated B cells within the boundary of a GC, T_{fh} move freely through LN follicles and from one GC to another GC (Fig. 3; ref. 52), thus increasing the probability that the activated B-cell responses will be enhanced by interaction with T_{fh}. How the progeny of rapidly dividing B cells acquire BCR mutations and how BCR-mutated B cells are selected to survive and leave the LN to become long-lived antibody-secreting plasma cells or memory cells are considered in the next section.

Antigen-induced cytidine deaminase

The "master catalyst" responsible for extensive modifications of Ig genes was discovered in the course of studying a B-cell lymphoma cell line that could be induced by lipopolysaccharide (LPS, the agonist of Toll-like receptor 4; TLR4), to switch the Ig class these cells produced from IgM to IgA (53). By subtracting cDNA from class-switched cells with cDNA from unswitched cells, several novel genes were cloned. The sequence of one of them matched closely a known cytidine deaminase (APOBEC1, an RNA-editing enzyme). Called AID, the novel class switch-associated enzyme converts deoxycytidine (dC) to deoxyuridine (dU), which resembles deoxythymidine and results in dU:dG mismatches. These lesions are subjected to repair in various ways, including excision (by uracil DNA glycosylase leaving an abasic site) and a nontemplated error-prone polymerase (Pol η) to yield point mutations as well as insertions and deletions (54, 55). Linked to transcription of Ig genes and possibly physically associated with an RNA polymerase spliceosome, AID acts on single-stranded DNA (ssDNA, not double-stranded DNA), starting just downstream of the promoter and decreasing in activity to peter out before the C region. The mutation frequency [somatic hypermutation (SHM)] has been estimated to be around 10^{-2} to 10^{-4} /bp/cell division, or around a million times greater than the rate of random mutations in the genome. It has been estimated that only about 20% of these mutations affect affinity (increasing or decreasing it), $\approx$ 50% are silent and the rest ($\approx$ 30%) are lethal, that is, they decrease affinity to below the free energy threshold or hinder proper Ig folding (A.K. Chakraborty; personal communication).

In addition to point mutations, AID activity also leads to staggered double-stranded breaks (DSB) in the "switch-sequences" in introns flanking exons that encode various heavy chain constant domains. Repair of these breaks by non-homologous end joining (NHEJ) and removal of intervening introns and exons result in switching joined VDJ sequences from μ to a constant region of another downstream Ig class, with changes in the antibody Fc domain and effector functions

[e.g., C' fixation, binding to various Fc receptors on cells, and antibody-dependent cell-mediated cytotoxicity (ADCC)].

Targeting AID activity

Given the potentially damaging consequences of hypermutation, AID activity is highly regulated at multiple levels, viz., transcription, posttranslational modification (phosphorylation), and nuclear import and export. Its activity is largely confined to nuclei of rapidly proliferating GC B cells, where it is targeted to ssDNA of transcriptionally active Ig V genes and the switch-sequences that flank exons for the Ig constant regions. In other B cells, such as memory B cells and, especially in long-lived plasma cells where transcription of Ig genes is extremely active, AID is silenced. Still, some non-immunoglobulin genes, such as *c-myc*, *BCL6*, and *FAS ligand*, are also mutated, but at lower rates than V genes. As noted below, AID-mediated DSB involving proto-oncogenes can have pathologic consequences (lymphomas, below).

Conservation of AID and antibody affinity enhancement in evolution

Different strategies have evolved in different species for diversifying the V domains in BCR during B-cell development (56). In humans and mice before encountering foreign Ags, highly diverse BCR B-cell populations are generated by stochastically recombining in each developing B cell one of many V gene segments with one of several D and J gene segments ($V_H/D_H/J_H$ for heavy chains and V_L/J_L for light chains) as immature B cells develop in the bone marrow throughout life. In contrast, birds and some mammals (e.g., rabbits, sheep, etc.) have only one or very few potentially effective rearranged V_H and V_L genes that become highly diversified by serving as acceptors for small gene segments introduced from defective, upstream Ig V donor pseudogenes (defective because they lack recombination signal sequences). This "gene conversion" mechanism is also AID-dependent and leads to rapid diversification of the BCR repertoire, after birth or hatching, in gut-associated lymphoid tissues (Bursa of Fabricius in birds, appendix in rabbits, and Peyer's patches in the ileum in sheep).

Regardless of the different strategies used in various species to diversify the BCR variable regions during early B-cell development, the BCR diversification that is triggered by immunogens seems to follow the same path in all vertebrates, including chickens (57), in which Ig class-switching and V region SHM result in progressive increases in antibody affinity. Thus, as testament to the crucial role played by antibody affinity enhancement in adaptive immunity, antibody affinity maturation and heavy chain class-switching have apparently been conserved over the $\approx$ 200 million years of evolution that separate bird and mammal lineages.

Selection

Although the molecular genetics of AID-mediated diversification of BCR variable regions is coming to be understood in exquisite detail (54–56), the mechanisms responsible for the preferential survival of high-affinity variants are much less understood. In a BCR affinity-diverse B-cell population, cells with high-affinity BCR have obvious competitive advantages in

binding to antigen in an antigen-limiting environment (as presumably occurs in GC light zone). This notion is consistent with the finding that large doses of immunogen greatly reduced the rate at which the affinity of serum antibodies increased (5), as high levels of antigen on FDCs are expected to reduce the competitive advantage of high-affinity BCR. Thus, affinity was long thought of as the sole driving force behind affinity maturation. However, there were puzzling observations that at the outset called this simple, straightforward view into question. If affinity were the sole driver, it would be expected that over time an initially low-average and affinity-diverse population would, through selection of high-affinity variants, tend to become progressively more homogeneous as the average affinity increased. Instead, the progressive increase in average affinity of serum antibodies was clearly seen to be accompanied by increasing (not diminishing) heterogeneity of affinity values (Fig. 2; ref. 5). This paradox was lost sight of in the subsequent shift of focus from polyclonal serum antibodies (which typically analyze sample populations of $\approx 10^{21}$ antibody molecules, allowing the extent of heterogeneity to be measured) to mAbs of which there are usually too few in any given study to quantify diversity.

The suggestion that T-cell help also drives selection (43) has been supported strongly by recent elegant studies that take advantage of an endocytic receptor (DEC205) through which antigen can be internalized into B cells independently of the BCR (45, 52). In mice with a mixed population of DEC205^{pos} and DEC205^{neg} B cells that share the same BCR, a conjugate of antigen linked to anti-DEC205 antibody led to greater proliferation of DEC205^{pos} than DEC205^{neg} cells, as would be expected from the enhanced cognate T-cell stimulation resulting from greater display by DEC205^{pos} B cells of relevant peptide–MHC-II complexes. However, in mice with GCs populated entirely by DEC205^{pos} B cells, Ag-anti-DEC205–Ab conjugates seemed to abrogate affinity maturation, as though excessively high levels of peptide–MHC-II displayed on all B cells, reduced competition for T-cell help. Thus, in BCR-diverse B-cell populations, B cells are evidently selected for their ability to compete successfully both for antigen (on FDC) and for help from cognate Tfh.

Although antigen binding to BCR is a critical first step, and its outcome determined by on- and off-rates (affinity), ligation of the BCR leads to many critical downstream effects that are essential for effective engagement with cognate T cells. These include:

- i. reorganizing the B-cell cytoskeleton (58);
- ii. B-cell spreading over membrane-bound antigen and literally extracting it from antigen-presenting cells (59), as from FDCs in GC light zone; and
- iii. internalizing BCR-bound antigen into B cells by endocytosis, cleaving it into peptides that are displayed as peptide–MHC-II complexes, which engage specialized Tfh and thereby benefit from costimulatory interactions.

These steps are likely to be variable and contribute to determining the fate of antigen-stimulated GC B cells. However, because they are not quantifiable, as is affinity, we refer to

them collectively below as "fudge factors" (ϕ) in an effort to account for the heterogeneity with respect to affinity that has been seen to increase along with increases in average intrinsic affinity (Figs. 2 and 4).

Progressive heterogeneity of serum antibodies to the same epitope

Antibody production is elicited by immunogens that are introduced under widely varying conditions—from an injection of a flu vaccine, or of an antigen adsorbed on alum precipitates or incorporated in water-in-oil emulsions (such as complete Freund adjuvants), or naturally by an acute or a persistent infection. The results shown in Fig. 2 can be considered to approximate persistent infection. Under these conditions, the entry of antigen or immune complexes into lymphoid tissues would be prolonged with slowly decreasing and fluctuating levels on FDCs (38). The subsequent variations in persistence and in turnover (lifetimes) of GC could contribute to the affinity heterogeneity of the resulting memory B cells and long-lived plasma cells and thus to serum antibody affinities.

Another possible source for affinity heterogeneity could arise from diversity among members of a clonal lineage within a given GC. Studies have shown that individual T cells that share the same TCR (i.e., members of the same T-cell clone) can differ functionally from each other in characteristic T-cell activities (60). It is reasonable to suppose that B cells of a given GC lineage, which share the same or similar BCR, also vary in the fudge factors that, together with affinity, drive B-cell selection in GC. Thus, plasma cells that produce these antibodies could have arisen from members of a B-cell lineage that were selected primarily because of high-affinity BCR and others primarily because of favorable (ϕ) factors leading to more effective help from Tfh. Although affinity (K) and fudge factors are correlated, the correlation is likely imperfect (Fig. 4). Hence, cohorts of affinity-matured memory B cells and plasma cells that exit from the LN could consist of cells with lower-than-average K that had been selected for greater ϕ , and others with higher-than-average K but lower ϕ . Over time, with iterative rounds of mutation/selection, this diversity (measurable as a serum antibody affinity heterogeneity index) may well increase. Given the antigenic variability of many microbial pathogens, the protective advantages of increasing antibody combining site diversity, with corresponding increases in breadth of cross-reactivity, seem evident. The advantage is especially clear when considering the alternative scenario in which the end stage of affinity maturation would be one or a few mAbs, albeit with high affinity but with a limited range of cross-reactivities. The latter antibodies would be desirable for use as diagnostic reagents but not likely to afford as much protection as the former against diverse variants of microbial pathogens with high mutation rates.

Affinity ceiling

The intrinsic affinity for protein–ligand interactions can be as high as 10^{15} (mol/L)^{−1} (biotin–avidin) or 10^{14} (carboxypeptidase

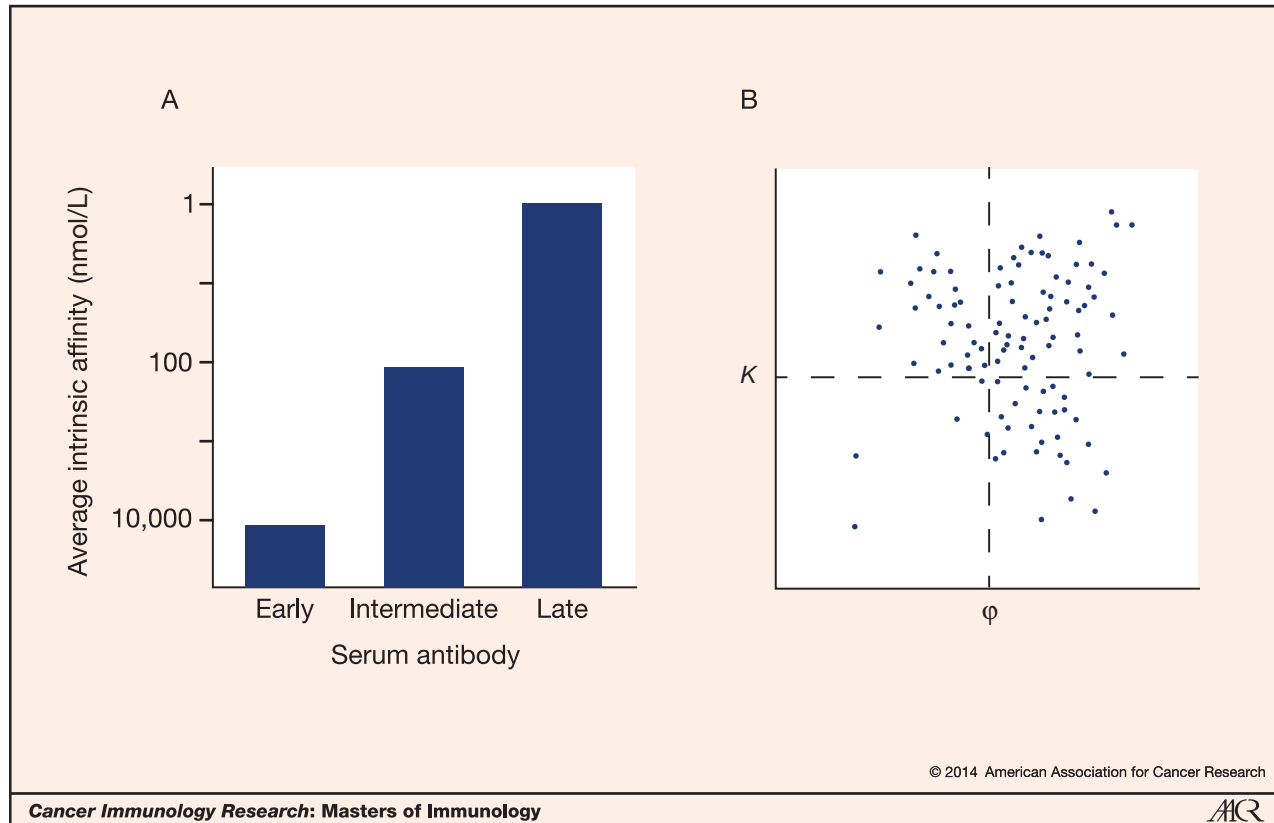


Figure 4. Hypothetical basis for the diversity of intrinsic affinities of antibodies made in an immune response to an epitope. In selecting GC B cells with mutated BCR, the correlation between affinity (K) and T-cell help (fudge factors or ϕ) is far from perfect. A, differences in average (mean) intrinsic affinity of serum antibodies (early, after ~1–2 weeks; late after ~1–2 months). Y-axis is affinity as K_D in nmol/L units. B, hypothetical distribution in late antibodies. Each dot represents a single B cell or mAb. Y-axis affinity values are K_A (reciprocal of K_D); X-axis values are entirely speculative as there is no way as yet to measure "fudge factors" underlying help from Tfh cells.

A and a tripeptide; ref. 61). By means of genetic engineering, antibodies with intrinsic affinities in this range [10^{13} (mol/L) $^{-1}$ for fluorescein] have been generated (62). For naturally occurring antibodies, however, there is an affinity ceiling of $\approx 10^{10}$ (mol/L) $^{-1}$. The ceiling derives from (i) the diffusion-limited on-rate for globular protein interactions of $\approx 10^6$ (mol/L) $^{-1}$ /sec, and (ii) an off-rate limit of $\approx 10^{-4}$ /sec beyond which longer dissociation times are not expected to confer selective advantages (63, 64). Rare antibodies of higher affinity may be found, but would not be expected to have arisen from competitive advantages in GC reactions.

AID, Affinity Maturation, and Hematologic Malignancies

AID activity is highly focused on the transcription of Ig V genes in proliferating GC B cells. It can, however, also act on other genes in these cells and initiate the development of B-cell lymphomas and plasma cell tumors (multiple myeloma). About 95% of lymphocytic neoplasia are B-cell lymphomas (65). Clinically diverse, these tumors are marked by chromosomal translocations in which DSB at an Ig locus and at a proto-oncogene on another chromosome are

joined, resulting in the immunoglobulin's promoter driving expression of an oncogene. Such aberrant DSB and translocations would be expected to trigger a DNA-damage response that results in cell senescence or apoptosis. However, with defects in the DNA-damage response due, say, to mutations in p53, B cells with the oncogenic chromosomal translocations survive and develop into lymphomas (66). The many forms of lymphoma vary clinically, depending perhaps on the particular proto-oncogene partner (e.g., c-myc, BCL1, BCL-2, and BCL-6). By gene-expression profiling, many lymphomas resemble GC B cells, especially those in the light zone (67, 68).

Malignant tumors can also arise from plasma cells in the bone marrow (multiple myeloma). These plasmacytomas express class-switched immunoglobulin (IgG and IgA) with multiple variable region mutations, indicating that they had undergone multiple rounds of SHM and selection before differentiating into long-lived plasma cells. They can persist in a relatively benign state for years in the bone marrow, revealing their presence by serum protein electrophoresis as a compact gamma globulin "monoclonal band." On becoming malignant and spreading beyond the bone marrow niche where they originate, they characteristically

secrete enormous amounts of their immunoglobulin as a homogeneous myeloma protein that can achieve levels up to about 50 mg/mL serum. Some myeloma proteins and the corresponding proteins seen in benign monoclonal gammopathy have been found to bind antigens or haptens no differently than do conventional polyclonal serum antibodies except for their homogeneity with respect to affinity (69, 70). As an indication of AID participation in generating these tumors, most plasmacytomas have chromosomal translocations that involve the heavy chain loci and a variety of different oncogenic partners on other chromosomes. The frequency of these translocation increases up to 90% in the most highly malignant multiple myelomas (71).

AID activity has also been detected in a variety of other malignancies associated with chronic infection and inflammation, including malignancies of the gastric mucosa (*Helicobacter pylori*), the liver (HCV), and EBV-infected peripheral blood B cells.

Manipulating GC reactions

FDCs express various TLRs, and the inclusion of combinations of TLR agonists in a vaccine has led to a pronounced increase in the number, size, and persistence of GC (72). Whether these intriguing effects have an impact on the rate and the extent of vaccine-elicited antibody affinity maturation remains to be seen.

At the other extreme, rapamycin, the cyclosporine-binding immunosuppressive drug, abrogated the GC response to the flu virus vaccine and, as expected, the resulting antibody response consisted predominantly of IgM antibodies (73). Although these antibodies likely had low intrinsic affinity for the viral epitopes, they would be expected to bind avidly to the many closely spaced epitopes (HA spikes) on the flu virus. Consistent with the configurational flexibility of IgM V domains (discussed above), these anti-HA IgM antibodies

were highly cross-reactive with HA variants and consequently were broadly protective against diverse flu strains in mice. These findings may influence the design of novel vaccine strategies for some pathogens.

Landsteinerian fallacy?

In an influential introduction to a Cold Spring Harbor Symposium, Landsteiner's monumental work on antisera raised against simple aromatic chemicals (haptens) was referred to as a "fallacy," presumably because it deflected attention from the immune system's main function—to protect against infectious pathogens (74). Infectious microbes are surely the key driving force underlying the evolution of adaptive immunity. However, the suggestion that responses to haptenic epitopes were misleading misses their profound importance, for they revealed the adaptive immune system's capacity to recognize virtually any organic structure—whether newly created (or still to be created) in organic chemistry laboratories or by pharmaceutical companies or yet to be encountered in nature as emerging microbial pathogens. Indeed, the discovery that intrinsic affinities of antibodies increase progressively over time after immunization would surely have been delayed by many decades had not anti-hapten antibodies elicited by hapten–protein conjugates pointed the way 50 years ago. One is left to wonder how our current understanding of antibody affinity maturation would have come about if studies of antibody responses had been limited to those elicited by conventional protein or microbial immunogens.

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