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Life and Death in Germinal Centers (Redux)

Minireview

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Curiously enough, B cell responses to thymus-dependent antigens begin in the T cell zones of secondary lymphoid tissues, where T and B cells initiate antigen- and costimulus-dependent proliferation. These initial cognate interactions are essential for humoral immunity, but alone result only transient low affinity antibody responses (Klaus et al., 1980; Coico et al., 1983; Han et al., 1995a). It is a subsequent set of cellular encounters, collectively known as the germinal center (GC) reaction, that drives affinity maturation by V(D)J hypermutation, B cell memory, and the continued self-tolerance of lymphocytes bearing mutated antigen receptor molecules.

The GC is a complex cellular microenvironment that supports and directs post-V(D)J diversification and selection. In the GC, T and B lymphocytes balance precariously between receptor-driven activation and apoptotic death, processes common in primary lymphoid tissues. Increasingly, evidence suggests that the developmentally regulated proliferation and selection of newly generated lymphocytes in bone marrow and thymus may be mirrored in GCs. Here, I shall briefly describe the GC reaction in mice, focusing on the possibility and significance of T cell hypermutation, cellular selection, and the maintenance of self-tolerance in the periphery. I also note characteristics of the GC reaction that appear analogous to events in thymus, bone marrow, and the gut-associated follicles that support ontogenic diversification of rearranged immunoglobulin genes.

The GC Reaction

Histologists prosper by finding and naming novel microanatomies: the relationship is not unlike that between biochemists and tyrosine kinases. Unfortunately, this process has resulted in an unwieldy jargon that can confuse the uninitiated. In this outline of the genesis and loss of splenic GCs (Figure 1), I provide only the names of prominent and important histologic structures. Those wishing for a fuller naming of parts are referred to recent fulsome reviews on the subject (MacLennan, 1994; Kelsoe, 1995).

After their activation in T cell areas (in the splenic white pulp this is the periarteriolar lymphoid sheath [PALS]), selected T and B lymphocytes migrate into the lymphoid, or primary, follicles. There, they accumulate within the extensive processes of follicular dendritic cells (FDCs) that form a scattered network within the B cell–rich follicle. FDCs retain antigen–antibody or antigen–complement complexes on their surface for long periods and act as depots for the antigen that sustains the GC reaction (Nossal et al., 1964; Tew et al., 1980; Schriever and Nadler, 1992). The immigrant lymphocytes rapidly proliferate, filling the FDC reticulum and acquiring novel phenotypic characteristics. For example, GC B cells avidly bind peanut agglutinin (PNA) and become positive for the activation marker recognized by the GL-7 monoclonal antibody (Miller et al., 1994), and most GC T cells down-regulate expression of Thy-1 (Harriman et al., 1990). This early phase of the GC reaction compresses the surrounding uninvolved follicular cells to form a mantle zone about the new GC, or secondary follicle.

Shortly after this initial period of expansion, the GC polarizes to form a dark zone (DZ) proximal to the T cell area that contains rapidly dividing immunoglobulin-negative B cells called centroblasts and a distal light zone (LZ) that contains nondividing immunoglobulin-positive centrocytes, the bulk of the FDC network, and most of the helper T cells present in GCs. Cell labeling studies suggest that centroblasts proliferate rapidly, dividing every 6–7 hr and demonstrate that the centrocyte population is continuously derived from cells in the DZ (Liu et al., 1991). Recent evidence (Han et al., 1995b) suggests that, in turn, LZ centrocytes reenter the DZ, join the centroblast population, and reinitiate proliferation. Interestingly, these cyclic migrations may help explain the rapid tempo of selection for high affinity B cells into the memory compartment (Kepler and Perelson, 1994).

Importantly, newly formed GCs represent oligoclonal B cell populations (Jacob et al., 1991a, 1991b; Liu et al., 1991). On average, each mature GC is derived from only 1–3 B cell clones that survive a dramatic reduction in clonal diversity that precedes the onset of significant V(D)J hypermutation (Jacob et al., 1993; McHeyzer-Williams et al., 1993). The GC reaction reaches its maximum by day 10–12 of primary responses, accounting for as much as 1%–3% of the total splenic volume. Without further antigenic stimulation, GCs wane by 21 days post-immunization, losing volume and avidity for PNA (Liu et al., 1991). By 32 days after immunization, GC residua occupy <5% of their peak volume and appear as infrequent collections of a few antigen-binding blast cells in association with FDCs.

V(D)J Hypermutation and Selection in GC Lymphocytes

Outside its coterie of enthusiasts, the GC is best known as the site of antigen-driven V(D)J hypermutation and selection (Jacob et al., 1991b; Berek et al., 1991). Within GCs, antigen-specific B cells acquire (mostly) point mutations in the V regions of transcriptionally active rearranged immunoglobulin genes. Mutated immunoglobulin genes are first observed on day 7–10 of primary responses, coincident with the polarization of the GC and the expression of CD86 on centrocytes (Han et al., 1995a). Mutations accumulate steadily at least until day 18 of the response by the step-wise introduction of 1–3 nucleotide substitutions, resulting in clonal genealogies that recapitulate the repeated rounds of intraclonal mutation, selection, and proliferation that take place in GCs (Clarke et al., 1985; Jacob et al., 1991b, 1993; McHeyzer-Williams et al., 1993). Clonal evolution proceeds independently in each GC, as there is little or no B cell trafficking between GCs (Jacob and Kelsoe, 1992), and

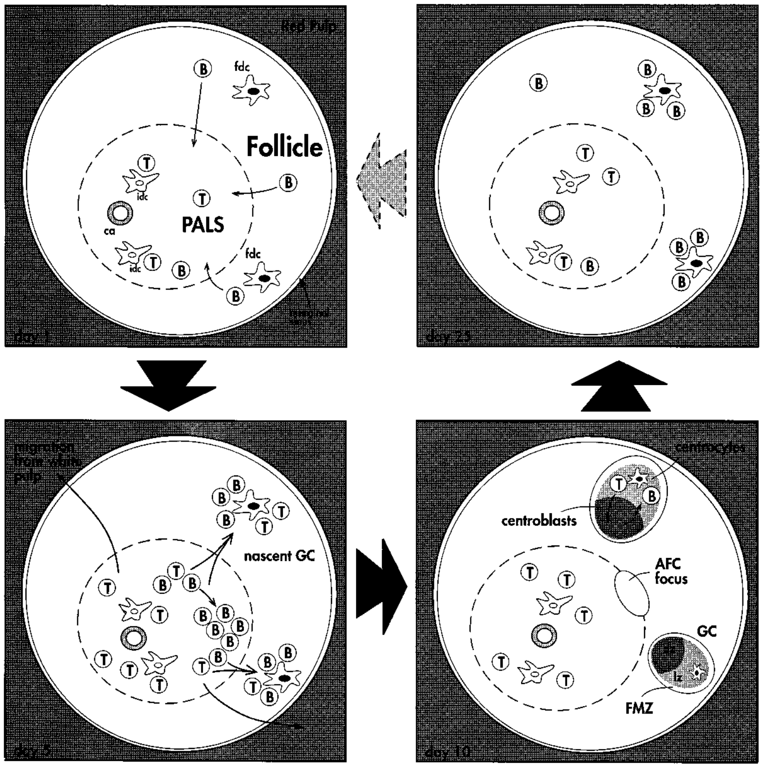

Figure 1. The Natural History of a Primary GC Reaction in the Spleen

In the spleen, the white pulp lymphocytes are segregated from the red pulp by a marginal sinus. The white pulp is organized about a central arteriole (ca) and may be divided into T and B cell zones, the PALS and lymphoid follicle, respectively. Two distinct cells that share dendritic morphology are also present in the white pulp. Interdigitating dendritic cells (idc) are potent antigen-presenting cells, especially for naive T lymphocytes, that are found in the PALS; follicular dendritic cells (fdc) retain native unprocessed antigen as immune complexes on their surface. Within hours after immunization, antigen-specific B cells enter the outer PALS and initiate costimulus-dependent interactions with specific T helper cells (upper left). Antigen-driven T cell proliferation in the PALS reaches its peak by day 5 postimmunization and is followed by emigration to the lymphoid follicle and other peripheral sites. B cells also proliferate in the outer PALS and then return to the lymphoid follicle to establish GCs or remain in the PALS to differentiate into foci of extrafollicylar plasmacytes (lower left). The GC reaction is fully developed by day 10 of the response; GCs surrounded by follicular mantle zone (FMZ) are polarized into dark zones (DZ) containing rapidly dividing immunoglobulin-negative centroblasts and light zones (LZ) filled with immunoglobulin-positive centrocytes, FDC, and CD4$^+$ T cells. T–B collaboration in the LZ is necessary to maintain the GC reaction and it is likely that selected B cells in the LZ return to the DZ to proliferate. PALS-associated plasmacytic foci are rapidly lost after day 10, as serum antibody titers are maintained by the migration of antibody-secreting cells into the bone marrow (lower right). By 21–28 days after immunization, the GC reaction wanes and only occasional collections of PNA$^+$ B cell blasts may be found in association with FDC (upper right).

in the absence of significant convergent selection driven by circulating antibody (Vora and Manser, 1995). Thus, each GC represents a local fitness optimum, one island in an archipelago of selected clones. Where and when interclonal competition among memory B lymphocytes takes place remains an important unanswered question.

The mechanism of V(D)J hypermutation is unknown but it introduces a distinctive pattern of nucleotide misincorporations. Characteristically, hypermutation favors transition mutations and exhibits biased nucleotide exchange and strand polarity (Golding et al., 1987; Both et al., 1990; Weber et al., 1991; Gearhart and Levy, 1991; Jacob et al., 1993; Betz et al., 1993a, 1993b; Pascual et al., 1994). Also, short sequence motifs have been identified that are intrinsic mutational hotspots within both human and murine V_H and V_L exons (Rogozin and Kolchanov, 1992; Betz et al., 1993a).

Surprisingly, recent transgenic "switch-and-bait" experiments (Azuma et al., 1993; Betz et al., 1994; Yélamos et al., 1995) have revealed that hypermutation can act on a variety of DNA substrates, including prokaryotic genes and Vκ genes driven by nonimmunoglobulin promoters. In fact, the only *cis* element required for V(D)J mutation in the well-studied Lκ transgene was the intronic enhancer/matrix attachment region (κi/MAR). Deletion of κi/MAR abrogated mutation in Peyer's patch B cells even though the expression of the transgene remained high in hybridomas, owing to the presence of the κ 3′ enhancer (Betz et al., 1994).

Mounting evidence indicates an important, or even necessary, role for transcription and/or transcription-linked DNA repair in V(D)J hypermutation (Storb, 1996). For example, in contrast with V_H and Vκ, unrearranged Vλ gene segments can undergo hypermutation (Weiss and Wu, 1987; Motoyama et al., 1991; Selsing and Storb, 1981; Gorski, 1983); unrearranged Vλ genes are transcribed in B cells, while Vκ genes are not (Picard and Schaffner, 1984; Mather and Perry, 1981). Likewise, the characteristics of V(D)J hypermutation, including strand bias, absence of mutation upstream of the transcriptional start site (Both et al., 1990; Gearhart and Levy, 1991; Rada et al., 1994; Rogerson, 1994), and dependence upon transcriptional enhancers (Betz et al., 1994), suggest an association between hypermutation and transcription. Perhaps the strongest evidence for transcription-linked V(D)J hypermutation comes from a recent study (Peters and Storb, 1996) demonstrating that introduction of a redundant transcriptional promoter into the J–C intron of a κ transgene promoted a sharp increase in the frequency of mutations in Cκ. Thus, the modified transgene supported two distinct tracts of mutation, one focused over VJ and the other over the C region; the origins of both tracts were precisely coincident with transcription initiation.

As the immune response progresses, evidence for phenotypic selection in GC B cells becomes increasingly obvious. Early GCs contain as many as 10 unique VDJ joint sequences, indicating colonization by at least 10

B cell clones; by day 8 of the response, CDR3 diversity in GCs falls to only 1.5 distinct VDJ sequences in each GC. This reduction in diversity is largely due to the loss of B cells expressing rearrangements that encode antigen-specific but low affinity antibody.

Selection in GCs can also be inferred from the V(D)J mutations within GC B cell populations. Initially, mutations are uniformly distributed within $V_{H/L}$ exons but with time become focused within the complementarity-determining regions (CDRs). The proportion of VDJ rearrangements containing crippling mutations, e.g., misincorporations leading to termination codons or replacements at invariant amino acid residues, can be as high as 33% of total mutations on day 8 of a primary response but falls to less than 3% by day 14 (Jacob et al., 1993). Ratios of replacement:silent (R:S) mutations initially approximate random values but become biased towards S mutations in framework regions and R mutations in CDRs. The frequency of mutations that confer high affinity becomes enriched in GCs, presumably reflecting their selection (reviewed by Berek and Ziegner, 1993).

Hypermutation in GC T cells

Although the GC is widely accepted as the histologic site for immunoglobulin hypermutation, a sequence analysis of $V\alpha$ rearrangements indicates that the T cell receptor (TCR) may also mutate there (Zheng et al., 1994). It must be noted (and has been, sometimes pointedly) that this finding is controversial (Bachl and Wabl, 1995; McHeyzer-Williams and Davis, 1995). T cell responses to pigeon cytochrome c (PCC) are dominated by clones bearing $V\alpha11^+$ $V\beta3^+$ TCR in mice expressing I-E^k (Winoto et al., 1986). After intraperitoneal immunization with haptenated PCC, the number of $CD4^+$ $V\alpha11^+$ $V\beta3^+$ cells increases dramatically in the spleen, first in the PALS and later in GCs. GC T cells probably represent immigrants from the PALS, as both populations often share complex junctional sequences. It is considerably surprising that $V\alpha11$, but not $V\beta3$, rearrangements recovered from $CD4^+$ GC cells contained mutations far in excess of that expected from PCR errors (Zheng et al., 1994; Kelsoe et al., 1995). Mutations were not observed in the nearby clonally related PALS T cells; TCR mutations in GC T cells were confined to the V region of the α chain, exhibited the biased nucleotide substitutions and DNA strand polarity characteristic of immunoglobulin hypermutation, and while the observed mutations differed significantly from that expected for unselected meiotic point mutations, they were indistinguishable from mutations flanking hypermutated immunoglobulin genes (Zheng et al., 1994). In contrast with immunoglobulin hypermutation, the majority of TCR mutations were recovered in out-of-frame rearrangements. This finding was interpreted as evidence for strong negative selection against GC T cells expressing mutant TCRs. Indeed, $CD4^+$ cells containing the fragmented DNA characteristic of apoptosis ($TUNEL^+$) (Gavrieli et al., 1992) are frequent in GCs. Further, recovery of TCR α rearrangements from single GC T cells is consistent with the notion of selection against TCR mutants; more than one-half of 16 $V\alpha J\alpha$ rearrangements from $TUNEL^+$ $CD4^+$ cells dissected from GCs contained point mutations; most (≈90%), associated with in-frame VJ joints. In contrast, mutations observed in single $TUNEL^-$ $V\alpha11^+$ cells from the same sites ($n = 25$) were less common (≈10% of sequences) and equally distributed among productive and out-of-frame rearrangements. This selection and confinement of TCR mutations to the small pool of GC T cells may explain why a recent flow cytometric study (McHeyzer-Williams and Davis, 1995) found no mutated $V\alpha11$ rearrangements in PCC-specific T cells recovered 6 days after primary ($n = 9$) and secondary ($n = 9$) immunizations. Nonetheless, these observations are likely to prove unconvincing if TCR mutations can not be recovered from antigen-specific T cell lines or hybridomas.

Maintenance of Self-Tolerance in GCs

Mutational diversification of V(D)J genes within GCs must occasionally produce antigen-receptors that acquire new specificities; a fraction of these may be autoreactive and potentially detrimental (Diamond and Scharff, 1984; Shlomchik et al., 1991). Diversification of TCRs in the absence of thymic censoring would be especially problematic. However, the GC may maintain tolerance to self by the elimination of autoreactive lymphocytes. Several groups have developed experimental models to study the fate of GC B cells that acquire specificity for self-antigens present in the GC (Pulendran et al., 1995; Shokat and Goodnow, 1995; Han et al., 1995b). Injection of soluble antigen into recently immunized mice induces massive and rapid apoptosis in GCs but not elsewhere in the spleen, including extrafollicular sites of antigen-specific T and B cell proliferation. Cell death peaks by 5–8 hr postinjection and apoptosis is most obvious in the GC LZ, suggesting that the immunoglobulin-negative centroblasts are unaffected. Apoptosis is antigen specific, dose dependent, and sensitive to hapten density, suggesting that cell death may be mediated directly by surface immunoglobulin engagement. This conclusion is supported by V_H sequence analysis of GC B cells that resist prolonged administration of soluble antigen. These B cells contain typically mutated V_H rearrangements but with a distinctive spectrum of R mutations that indicates selection for decreased affinity for antigen (Han et al., 1995b).

The effects of soluble antigen do not stem from interference in cognate T–B collaboration nor are they the product of costimulatory disruption. Immune mice injected with anti-CD40L antibody instead of soluble antigen exhibited GC apoptosis only slightly above background levels (Han et al., 1995b). Analogous experiments performed in C57BL/6 mice congenic for the *lpr* locus demonstrated that antigen-induced GC apoptosis was independent of the Fas pathway of programmed cell death (Watanabe-Futunaga et al., 1992; Adachi, 1993). Taken together, these observations support the notion that antigen causes GC B cell death directly by cross-linking surface immunoglobulin or by preventing centrocytes from interacting with the FDC.

Other recent work on GCs (Nossal et al., 1993; Pulendran et al., 1994) has demonstrated another form of immunological tolerance mediated through the absence of T cell help. Interestingly, although this tolerance mechanism is clearly T dependent and distinct from that described above, it also affects only follicular responses, i.e., GCs but not PALS-associated plasmacytes. Thus, it is possible that GC cells are uniquely sensitive to tolerance induction, as if recapitulating that susceptibility observed in primary lymphopoiesis. Indeed, GC centrocytes express a collection of surface markers typically absent or weakly expressed on mature peripheral B lymphocytes but abundant on the immature/transitional B cells in bone marrow. For example, HSA, Fas, and GL-7 are commonly expressed on bone marrow B cell populations, but in the periphery are abundant only in GCs. Reciprocally, Bcl-2 is present in follicular lymphocytes but not in GC cells or immature B cells.

Origins of the GC Reaction

In certain species, ontogenic diversification of immunoglobulin V region genes continues after V(D)J recombination. In chicken (Reynaud et al., 1985), rabbits (Becker and Knight, 1990; Weinstein et al., 1994), and sheep (Reynaud et al., 1991), B cells migrate from regions of primary lymphopoiesis, colonize epithelial crypts along gut mucosae, and proliferate to form prominent lymphoid follicles that share many histologic features of GCs. Here, B cells undergo postrearrangement V(D)J diversification by gene conversion (chickens), hypermutation (sheep), or both (rabbits). Even though birds, rabbits, and sheep represent widely divergent taxa, the many conserved features of this process imply a single underlying mechanism. It is unlikely that such a complex developmental pathway for postrearrangement diversification of the immunoglobulin repertoire arose independently three times during evolution (Kelsoe, 1995; Maizels, 1995).

Reynaud and colleagues (1995) have demonstrated that the spectrum of mutations introduced into the V regions of Peyer's patch B cells in fetal lambs is similar to the mutations observed in passenger κ transgenes in mice (Betz et al., 1993a, 1993b). This finding links antigen-driven and developmental V(D)J diversification, raising the possibility that the GC is homologous to the gut-associated follicular microenvironments. In contrast with V(D)J hypermutation (Wilson et al., 1992; Hinds-Frey et al., 1993; Greenberg et al., 1995), the GC reaction is not thought to be present in cold-blooded vertebrates (Kroese et al., 1985). Wilson et al. (1992) note that affinity maturation is also absent in lower vertebrates and have suggested that GCs may represent an evolutionary adaptation to link V(D)J hypermutation to antigen-specific immune responses. If so, hypermutation might not require the GC microenvironment but be catalyzed by it. Could the mammalian GC reaction have evolved from the earlier gut-associated follicles? It may be time for renewed interest in immunology's zoo.

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