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Neoteny in Lymphocytes: Rag1 and Rag2 Expression in Germinal Center B Cells

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The products of the Rag1 and Rag2 genes drive genomic V(D)J rearrangements that assemble functional immunoglobulin and T cell antigen receptor genes. Expression of the Rag genes has been thought to be limited to developmentally immature lymphocyte populations that in normal adult animals are primarily restricted to the bone marrow and thymus. Abundant Rag1 and Rag2 protein and messenger RNA was detected in the activated B cells that populate murine spleen and Peyer’s patch germinal centers. Germinal center B cells thus share fundamental characteristics of immature lymphocytes, raising the possibility that antigen-independent secondary VDJ rearrangements modify the peripheral antibody repertoire.

Periodic expression of the recombination-activating genes Rag1 and Rag2 controls the assembly of immunoglobulin (Ig) genes and defines the principal stages of B lymphopoiesis in the bone marrow (1). Transcription of the Rag genes ends with the expression of competent Ig on the surface of immature B cells, precluding further VDJ recombination in the mature lymphocyte pool (2). However, we and others have found that lymphocytes in germinal centers (GCs) exhibit features of immature T and B cells, including the expression of membrane markers typically present on developing lymphocytes (3) and exquisite sensitivity to activation-induced apoptosis that is independent of the Fas molecule (4). Perhaps most remarkable is the similar spectrum of nucleotide exchanges introduced during antigen-driven VDJ hypermutation in murine GCs and thrice developmentally regulated generation of point mutations in the Ig genes of B cells of neonatal mice (5).

Germinal centers are sites of antigen- and T cell–dependent cellular reactions that develop in secondary lymphoid tissues. Germinal centers are necessary for immunological memory in the B cell compartment (6, 7) and are the site of VDJ hypermutation and selection that is required for affinity maturation of antibody responses (8). Two populations of B lymphocytes, the mitotically active Ig− centroblasts and the nondividing Ig+ centrocytes, make up the majority of GC cells; centrocytes arise from centroblasts, and in turn, some centrocytes reenter the proliferating pool (3, 9). Evidence suggests that the centrocyte population is subject to selective apoptosis (4). Splenic GCs first appear 4 to 5 days after primary immunization and may be identified by their distinctive ability to bind peanut agglutinin (PNA) and the monoclonal antibody GL-7 (GL-7+) (3). The GC reaction is transient, peaking by day 12 of the response and waning after 3 weeks (9). In contrast, GCs are constitutively present in murine PPs, being chronically stimulated by food antigens and the gut flora (10).

To determine if the immature character of GC B cells extended to the level of Rag expression, we used affinity-purified antibodies specific for active Rag1 and Rag2 proteins (11) to label histologic sections of spleen and PPs from immunized and normal mice (12). Mature GCs, those present in spleen 16 days after immunization (Fig. 1), contain PNA+, GL-7− B cells that express substantial amounts of immunoreactive Rag1 and Rag2 protein. The distribution of labeled cells coincided with the development of B7−2 expression, suggesting that RAG proteins are predominantly expressed in the centrocytes of the GC light zone (7). Virtually identical staining patterns for immunoreactive RAG1 and RAG2 protein. The distribution of labeled cells coincided with the development of B7−2 expression, suggesting that RAG proteins are predominantly expressed in the centrocytes of the GC light zone (7). Virtually identical staining patterns for immunoreactive RAG1 and RAG2 protein.
The presence of immunoreactive RAG1 and RAG2 in GC B cells was further supported by using the reverse transcriptase–dependent polymerase chain reaction (RT-PCR) assay to detect the presence of RAG1, RAG2, and hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (14) in small numbers (5 × 10³ to 2 × 10⁴ cells) of GC (GL-7⁺ B220⁺) and follicular (GL-7⁻ B220⁻) B cells purified by fluorescence-activated cell sorting (15) from spleens of immunized mice. Comparable numbers of immature, CD4⁺CD8⁻ (double-positive) thymocytes were similarly prepared to serve as controls for the RT-PCR assay. RAG1 and RAG2 message was readily detected in double-positive thymocytes and in as few as 5 × 10⁴ GC B cells (Fig. 3). Re-analysis of sorted GC B lymphocytes indicated enrichment of GL-7⁺ B220⁺ cells to only ~35% compared with ≥96% for follicular B cells and double-positive thymocytes. In contrast, neither RAG1 nor RAG2 message could be detected in even larger numbers (2 × 10⁵ cells) of follicular B cells or lipopolysaccharide-activated B cell blasts (Fig. 3). Approximately equivalent amounts of HPRT mRNA were present in all cell cohorts, indicating generally equivalent recoveries of intact RNA (Fig. 3). Sequence analysis of RAG RT-PCR products from GC B cells confirmed these to be RAG1 and RAG2.

GC B cells have been proposed to represent a distinct lineage of B lymphocytes (16). Do the B cells that migrate into nascent GCs already carry the RAG proteins or is their expression induced by the GC microenvironment? In contrast to mature GCs, only about half of newly developed splenic GCs contain B cells that express detectable amounts of RAG proteins. The number of RAG⁺ centrocytes in GCs and the intensity of their labeling increases during the GC reaction, suggesting that events within the GC microenvironment up-regulate RAG1 and RAG2 expression (Fig. 4). This pattern of expression mirrors the onset of μ → γ1 Ig class switching and the accumulation of point mutations in the Ig heavy chain genes of GC B cells (17). However, Ig class switching simultaneously occurs in RAG⁻ B cells located within extrafollicular foci of antibody-secreting cells (18), and immunization with pneumococcal vaccine, a type-II T cell–independent antigen (19), induces RAG1⁺ GCs in the absence of significant levels of V(D)J hypermutation (20). These observations imply that RAG proteins are not necessary for Ig class switching nor sufficient for V(D)J hypermutation. Indeed, extensively mutated Ig light chain transgenes have been recovered from Ig transgenic Rag1⁻/⁻ mice reconstituted with specific T helper cells and antigen (21). By day 19 after immunization, a fraction of B cells within GCs had lost the ability to bind PNA or GL-7 but remained positive for immunoreactive RAG1 and RAG2. RAG2 protein also persists in newly generated B cells beyond the cessation of transcription (2), suggesting that the V(D)J recombinase may be briefly present in B cells that have exited GCs.

Expression of RAG1 and RAG2 in GCs reveals the GC microenvironment as a site that supports a population of peripheral B cells profoundly similar to pre-B cells in the bone marrow. The many phenotypic characters shared by developing B cells and those in GCs (3) extend to reactivation of the V(D)J recombinase. The antigen-dependent GCs of mice may represent evolutionary homologs of gut-associated tissues that drive developmentally regulated diver-

Fig. 1. Immunohistological staining of a single GC in serial splenic sections. Adjacent, 6-µm sections (A to E) through the spleen of a C57BL/6 mouse immunized with NP-CGG 16 days earlier were stained with (A) peanut agglutinin (PNA) (red), (B) rabbit antibody to RAG1 (anti-RAG1) (blue), (C) rabbit anti-RAG2 (blue), (D) normal rabbit Ig (blue), and (E) GL-7 antibody (blue). The GC structure and adjacent splenic architecture are diagrammatically illustrated. GL-7 and PNA label centroblasts and centrocytes to define the location of the GC through the intervening sections; note that the GC column rotates clockwise as it follows the parieto-artrial lymphoid sheath and central arteriole through the splenic white pulp, LZ, light zone; DZ, dark zone; ca, central arteriole; pals, parieto-artrial lymphoid sheath, the splenic T cell zone. Magnification ×90.

Fig. 2. Expression of RAG1 and RAG2 in PP germinal centers. Adjacent sections of PP from naïve C57BL/6 mice were labeled with (A) rabbit anti-RAG1 (blue) and PNA-HRP (red), (B) anti-RAG2 (blue) and PNA-HRP (red), and (C) normal rabbit Ig (blue) plus PNA-HRP, as described (12). Doubly labeled cells appear black. LZ, light zone; DZ, dark zone; ser, serosal surface of the PP. Magnification ×208.
sification of Ig after rearrangement in other vertebrate species (3, 5, 22). However, in mice these properties are present in both the intestinal PPs and splenic GCs (Figs. 1 and 2). Induction of an immature-like state in GC lymphocytes may reflect a mechanism to remove autoreactive cells that arise by mutation (4, 9). The physiologic state that permits this selective apoptosis may coordinate reactivation RAG1 and RAG2.

The availability of V(D)J recombinase in GC B cells also suggests several possibilities for the diversification of Ig genes in centrocytes. RAG1 and RAG2 could mediate secondary V(D)J rearrangements leading to light chain replacement or the introduction of new V, G segments by means of cryptic recombination signals present near their 3' termini (23). Light chain receptor editing is commonly observed in autoreactive immature B cells driven to initiate apoptosis by Ig engagement—a scenario not unlike the fate of self-reactive centrocytes (4). In fact, a significant fraction of human B cells that express the λ light chain carry productively rearranged κ light chain genes that have been inactivated by somatic mutation (24). Other evidence consistent with light chain replacement comes from genetic analysis of follicular lymphomas, tumors that exhibit many characteristics of GC lymphocytes including V(D)J hypermutation (25). Sklar et al. (26) have reported one tumor composed of two clonal lymphomas related by a common Ig heavy chain rearrangement but distinct by virtue of dissimilar light chain genes. It may be significant also that the t(14;18) chromosomal translocation present in most follicular lymphomas is thought to arise as an error of V(D)J recombination (27). Documentation of secondary V(D)J rearrangements in GCs would constitute a striking exception to one of immunology’s fundamental tenets: that antigen does not elicit the formation of novel receptors.

Note added in proof: Messenger RNA specific for the X component of the pre-B cell receptor complex (2) can be readily detected by a specific RT-PCR assay in as few as 5 × 10^5 GC (GL-7^B220^+) B cells. In contrast, X5 message was not found in larger numbers (5 × 10^6) of follicular B cells (GL-7^B220^). These findings further substantiate the immature character of B lymphocytes in GCs.

**REFERENCES AND NOTES**

12. C57BL/6 mice (female, 6 to 8 weeks old) were immunized intraperitoneally with 100 μg of alun-precipitated (4-hydroxy-3-nitrophénylacetyl) coupled to chicken γ-globulin (NP-CGG). Serial, 6-μm-thick sections of spleens from immune mice and PPs from naïve mice were stained with a murine monoclonal antibody specific for RAG1 or affinity-purified rabbit antibodies to RAG2 or RAG3 (13). Normal rabbit Ig (Sigma) was used as a negative control. The rabbit antibodies were incubated with tissue sections at 4°C overnight, followed by incubation with goat F(ab')2 anti-bodies to rabbit Ig conjugated to biotin (Southern Biotechnology). Biotinylated antibody was then detected with streptavidin-alkaline phosphatase (SA-AP); the same or adjacent sections were stained with PNA coupled to horseradish peroxidase (HRP) (E-Y Laboratories, San Ma teo, CA) or biotinylated GL-7 antibody followed by
Enhancement of Class II–Restricted T cell Responses by Costimulatory NK Receptors for Class I MHC Proteins

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An important feature of the human immune system is the ability of T cells to respond to small quantities of antigen. Class II major histocompatibility complex (MHC)–restricted T cells that expressed a costimulatory natural killer (NK) cell receptor for class I MHC proteins were cloned. In the presence of low doses of superantigen, the proliferative response of these T cell clones was three- to ninefold greater when the T cells were costimulated by way of the NK receptor. Thus, the action of costimulatory NK receptors on T cells may play a significant role in initiating and sustaining immune responses.

T cells recognize antigens through contacts made between the T cell receptor (TCR) and peptides presented in association with specific MHC proteins on an antigen-presenting cell (APC). However, the T lymphocyte response is also shaped by many other interactions between cell-surface molecules on T cells and APCs, as well as by the action of cytokines (1). Although no distinct “antigen receptor” analogous to the TCR has been found on NK cells, NK cell–mediated lysis can be inhibited by NK receptors that also bind to class I MHC proteins (2, 3). In particular, lysis by NK1 and NK2 cells is inhibited by target cells expressing human leukocyte antigen (HLA)–Cw2, Cw4, Cw5, or Cw6 and HLA-Cw1, Cw3, Cw7, or Cw8, respectively (4, 5). Also, lysis by NKBI* NK3 cells is inhibited by target cells expressing an HLA-B allele containing the Bw4 epitope at residues 77 to 83 (6). Such inhibition is initiated by the recruitment of protein tyrosine phosphatases on the cytoplasmic tail of the NK receptor (7). T cells share with NK cells a common lineage and many phenotypic markers (8) including NK inhibitory receptors. Indeed, ~28% of cytotoxic T cells express p58 NK inhibitory receptors (9), and the p70 NK inhibitory receptor, NKBI, is expressed on ~0.2 to 15% of T cells (10). The action of these NK inhibitory receptors can affect T cell function because NKBI* T cell clones that kill superantigen-coated target cells cannot kill the same target cells transfected with class I MHC alleles expressing the Bw4 epitope (10).

Recently, an isoform of NK inhibitory receptors was described. These p50 NK receptors share similar extracellular sequences with the p58 receptors but have altered transmembrane regions, including the addition of a charged lysine residue, and truncated cytoplasmic tails (3, 11), so that they lack the YXXL sequences (L; Leu; Y; Tyr; X, any amino acid) to which protein tyrosine phosphatases can bind. Such short-tailed NK receptors do not mediate inhibition of class I cell–mediated lysis but instead activate or coactivate NK clones (11). To investigate the possible effect of NK activating receptors on T cell function, we studied T cell clones isolated as by-products in NK cell cloning (12).

Two of these clones, TANK-1 and TANK-2, were prepared from a donor whose HLA type is HLA-A1, -A2, -B7,
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