Antigen Receptor Engagement Turns off the V(D)J Recombination Machinery in Human Tonsil B Cells

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Summary

The germinal center (GC) is an anatomic compartment found in peripheral lymphoid organs, wherein B cells undergo clonal expansion, somatic mutation, switch recombination, and reactivate immunoglobulin gene V(D)J recombination. As a result of somatic mutation, some GC B cells develop higher affinity antibodies, whereas others suffer mutations that decrease affinity, and still others may become self-reactive. It has been proposed that secondary V(D)J rearrangements in GCs might rescue B cells whose receptors are damaged by somatic mutations. Here we present evidence that mature human tonsil B cells coexpress conventional light chains and recombination associated genes, and that they extinguish recombination activating gene and terminal deoxynucleotidyl transferase expression when their receptors are cross-linked. Thus, the response of the recombinase to receptor engagement in peripheral B cells is the opposite of the response in developing B cells to the same stimulus. These observations suggest that receptor revision is a mechanism for receptor diversification that is turned off when antigen receptors are cross-linked by the cognate antigen.

Key words: secondary V(D)J recombination • germinal center • recombination activating gene • surrogate light chain • terminal deoxynucleotidyl transferase

B lymphocytes develop clonally restricted antigen specific receptors by randomly joining Ig variable (V), diversity (D), and joining (J) gene segments by V(D)J recombination (1). V(D)J recombination is a highly regulated process that requires coordinate expression of a series of lymphoid-specific and non-lymphoid-specific genes (2). Among the lymphoid-restricted components, only recombination activating genes RAG1 and RAG2 are essential for V(D)J recombination (3-6). Together, these two proteins form a complex that initiates Ig genes rearrangements by recognizing the recombination signal sequences (RSSs) and cleaving DNA (7-13). There are three distinct waves of RAG expression in the bone marrow (14, 15). These waves of RAG expression correspond to heavy and light chain gene rearrangements and receptor editing (16-18).

In addition to RAG1 and RAG2, B cell progenitors also express a series of other proteins that are developmentally restricted, B cell specific, and required for efficient antibody gene assembly (19, 20). These include the terminal deoxynucleotidyl transferase (TdT) and murine λ5 or human λ-like, and V-preB proteins. TdT increases antibody diversity by adding nontemplated nucleotides to V(D)J junctions (21, 22) whereas λ5 and V-preB associate with each other to form the surrogate light chain (ΨL) (23-26). The ΨL is believed to enhance the efficiency of B cell development by pairing with heavy chains before a conventional light chain is assembled. In the absence of λ5, B cell development is severely impaired at the pro-B cell to pre-B...
Secondary V(D)J recombination in Human Tonsil B Cells

Material and Methods

Human Cell Isolation and Fractionation. Tonsil B cells and B cell subsets were prepared as previously described (38). In brief, after depletion of non-B cells, tonsil mononuclear cells were stained with biotin-labeled goat antimouse IgD (Amersham Pharmacia Biotech, Piscataway, N.J.), which was visualized with streptavidin–FITC (Immunotech, Westbrook, ME), and PE-labeled mouse anti-human CD38 (Becton Dickinson, San Jose, CA). Cells were then sorted into the following fractions: (a) IgD+CD38–follicular mantle (FM) B cells; (b) IgD+CD38–GM B cells and (c) IgD–CD38– memory B cells. Alternatively, tonsil mononuclear cells were stained with PE-labeled mouse anti-human CD38 (Becton Dickinson) and rat anti-human CD77 (Immunotech) that was visualized with FITC-labeled sheep anti-rat IgM (Seren, Ltd., Kidlington, UK) and sorted into three fractions: (a) CD38–CD77+ centroblasts; (b) CD38+CD77– centrocytes; and (c) a CD38–CD77– fraction that contained both naive FM B cells and memory B cells. To analyze for V-preB expression, tonsil cells were stained with tricolor anti-CD38 (Caltag Labs, San Francisco, CA), PE-labeled IgG1 monoclonal anti–V-preB anti-body (4G7) (Schild, C., manuscript in preparation), and FITC labeled anti-IgM (DAKO Corp., Carpenteria, CA), or anti-j (DAKO Corp.) and/or anti- (Ortho Diagnostic Systems, Raritan, NJ) mAb.

Cell Culture. Human FM and GC B cells were maintained in tissue culture as previously reported (38). In brief, 5 × 10^5 cells were cocultured for 3 days with 40% transfectant L cells and stimulated with: IL-2 (10 U/ml), or IL-4 (50 U/ml), or IL-10 (100 ng/ml) or anti- , or anti- , or both, or Fab2 anti- + anti- , or irrelevant control IgG, at the indicated concentrations in micrograms per milliliter (Kallestad Lab., Inc., Austin, TX).

Linker-ligation PCR. DNA was extracted from 2 × 10^5 cells in agarose plugs (35) and ligated to the BW linker at 20 pM as previously described (39). PCR was performed using AmpliTaq Gold Taq polymerase (PE Applied Biosystems, Foster City, CA). In the first round of PCR, we used linker primer BW-1 or BW-1H (39) and either 5'-GCTGGTACTCTTCTCGCCGCTTC3' or 5'-TCAATTC3' and either BW-1 or the closely related BW-1H linker primers (39). The expected size for the specific J, or J, S RSS signal break products are 322 and 249 bp, respectively. Control PCR assays used the J, R primer specific for the human J, coding exon (5'-GTTTATCCTACGTGGTCCCTCGTTG-3') and J, 5'-GGGGTGACCTCTCTCCGCTTC3' or 5'-ATTAATG3' combined with J, 5'-5'-TTGAAGATCGCAG- TAACTGAGGATTCTCAATTCGACG3'.

Cloning and Sequencing. PCR products were gel purified with the Qiaquick kit (QIAGEN Inc., Chatsworth, CA) and cloned using the TA cloning system (Invitrogen Corp., Carlsbad, CA). Double-stranded DNA sequences were obtained using T7 and M13 primers with a Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The sequencing reactions were run and analyzed on a genetic analyzer (model 310; PE Applied Biosystems).

RNA Preparation and Reverse Transcriptase PCR. Total RNA was extracted from 2 × 10^6 cells using TRIzol Reagent (GIBCO BRL) and reverse transcribed in 20 µl with Superscript II (GIBCO BRL). For reverse transcription (RT)-PCR reactions, 2 µl of cDNA were amplified for 26, 28, or 30 cycles (J, J, and J preB) or 36, 38, or 40 cycles (J, J, and J) cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C with a final 10-min extension step at 72°C using Platinum Taq DNA polymerase (GIBCO BRL) and the following primers: J, sense 5'-TGGAGACATCCTAACATTTTGG3' and antisense 5'-ACATCTGGCTTTACCACGTACC3'; J, sense 5'-AGCAG- CCCCTTGGCCCTTTG3' and J, sense 5'-ATGGCCAGGCTGGCGTTGTCTC3' or 5'-GGGGTGACCTCTCTCCGCTTC3'; J, sense 5'-ATGGCCAGGCTGGCGTTGTCTC3'.

Phospho-MAPK ELISA was performed on samples amplified in the linear range. Analysis of M. m. cells. 4- to 6-wk-old X5 targeted mice (41) and C57BL/6 controls were bred and maintained under specific pathogen-free conditions. Mice were immunized with 50 µg of NC-P-CGG in alum 10 d before analysis. Spleen cells from immunized mice were stained with aliphophycocyanin-labeled anti-B220, PE-labeled anti- , PE-labeled anti- , and FITC-labeled anti-GL7 (PharMingen, San Diego, CA), and biotin-labeled...
specific intermediate products of the V(D)J recombination analyzed because both are at the 3′ ends of their respective J regions and are least likely to be deleted during V(D)J recombination in the bone marrow. Using this assay PCR products corresponding to the expected 5′ phosphorylated human Jκ,6 and Jκ,5 signal ends were readily detected in human bone marrow, and were verified as specific by cloning and sequencing (Fig. 1 b, and data not shown).

Having established the validity of the assay we examined B cells from tonsils fractionated into IgD+CD38− naive FM B cells, and IgD−CD38− GC cells. We found no RSS signal breaks in the naive FM B cells (Fig. 1 b). In contrast, GC B cells displayed Igκ but no Igμ RSS signal breaks. The number of Igκ RSS breaks in GC B cells could not be determined precisely because the RSS break assay involves a less than stoichiometric ligation reaction and two rounds of amplification. Nevertheless, the amount DNA breaking detected in GC samples was always 5–10-fold lower than in bone marrow as determined by dilution. We conclude that human GC B cells are actively undergoing V(D)J recombination at the Igκ locus.

The absence of Igμ RSS breaks in GC B cells may be due to the 12/23 rule. Dμ segments are 12/23 compatible with both VH and JH gene segments, but all Dμ segments are deleted by any V to DJH joining event. VH to JH joining is prohibited in the absence of Dμ segments by 12/23 incompatible RSSs (1). All mature B cells carry at least one productive VDJH on one allele and in addition many B cells have a nonproductive VDJH on the second allele (44). Thus, there is a relatively dearth of potential 12/23 compatible targets for recombination at the heavy chain locus. Consistent with this idea, heavy chain RSS breaks can be found in activated B cells when Dμ segments are artificially preserved by allelic exclusion in mice that carry targeted VH genes (35). VH gene recombination using internal cryptic enhancers as signals for recombination has been documented but would not be detected by our assay (45, 46).

Immunohistochemistry revealed that mouse RAG1 protein expression is most prominent in the GC light zone, suggesting that recombination is activated in centrocytes (33). To determine whether expression of other recombinase components is restricted to a specific subset of mature B cells, we separated human tonsil B lymphocytes into five fractions by cell sorting: (a) FM cells IgD+CD38−; (b) mixed FM cells and memory B cells, CD38−CD77−; (c) total GC B cells, IgD−CD38−; (d) GC centroblasts CD38+CD77−; and (e) GC centrocytes, CD38−CD77− (38). Centroblasts are rapidly dividing early GC cells that initiate somatic mutation, whereas switch recombination occurs primarily in the centrocyte fraction that is derived from the centroblasts (38, 47). RAG1, RAG2, TdT, λ-like, and V-preB were not expressed in resting FM B cells or in mixtures of FM B cells and post-GC memory B cells (Fig. 2). In contrast, all of these mRNAs were found in the GC fraction and RAG1, RAG2, TdT, λ-like, and V-preB were expressed in fractions of FM B cells and post-GC memory B cells (Fig. 2). Although the PCR assay we used is only semiquantitative, it is in the linear range and shows that the levels of these mRNAs are comparable to the levels of these mRNAs found in unfractionated adult bone marrow samples as

![Figure 1](image-url)

Figure 1. Ligation-mediated (LM)-PCR assay for detecting signal breaks in human GC B cells. (a) Scheme for LM-PCR strategy to detect RSS DNA breaks within the human Igκ light chain locus. A primary VμJμ1 rearrangement is represented at the κ locus, and after V(D)J recombinase activation, downstream J segments may be chosen for a secondary rearrangement; J5 is depicted. Blunt signal ends at the nonamer/heptamer sequences (triangles) are ligated to BW linkers. Linker-ligated DNA molecules are then amplified by PCR using two sets of specific primers (see Materials and Methods). (b) J5 signal breaks in human GC B cells. LM-PCR for Jκ6 (top) and Jκ5 (middle) signal breaks in human monoclonal Igκ B cells (right). PCR products were visualized with a gel stain specific for PCR products. PCR from a region overlapping the germline Jκ5 segment was used as a control for DNA loading (bottom).
measured by phosphorimaging. Low levels of RAG1 were also found in centroblasts (20% of the levels in centrocytes by phosphorimaging), but RAG2 was not detected in these cells (Fig. 2). In accordance with the RAG mRNA expression data, Igκ R SS breaks were found in centrocytes and not in centroblasts (Fig. 1 b). Thus receptor revision by secondary V(D)J recombination is found in B cells that have already passed through the centroblast stage where somatic mutation is initiated (38).

In developing B cells in the bone marrow, λ-like and V-preB proteins associate with each other as ΨLs that combine with nascent heavy chains to form the pre-B cell receptor (42, 48). The number of human bone marrow cells that express surface ΨL is very small (1–3% of B cells; references 40, 48, 49). Nevertheless, in both mouse and human, B cell development is inefficient in the absence of the ΨL (41, 50), presumably due to loss of B cells that express a heavy chain but no light chain. ΨL may play a similar role in the GC B cell. According to this hypothesis, ΨL would combine with heavy chains to rescue GC B cells that have lost conventional light chain expression (33, 34, 37). To characterize the GC B cells that reexpress ΨL, we stained human tonsil lymphocytes with anti-V-preB antibodies in conjunction with anti-CD38, anti-Igκ, and anti-Igλ antibodies (Fig. 3 a). Surface V-preB expression was found on 0.85–11% of CD38+ GC B cells in six independent samples (both ends of the spectrum are shown in Fig. 3 a), whereas CD38− cells did not express V-preB (Fig. 3 a). In all cases, GC cells that expressed V-preB co-expressed either Igκ or Igλ (Fig. 3 a and data not shown). The low number of ΨL-positive cells in most of the human GC samples is reminiscent of the low levels of ΨL on the surface of human bone marrow cells (40, 48, 49). In contrast to the human, ΨL are readily detected on the surface of mouse B cells in the bone marrow (42). To confirm our findings in human cells, we examined mouse spleen B cells for λ5 expression using two different anti-λ5 antibodies (reference 42; Fig. 3 b). In immunized mice, 15–20% of B220−GL7+ B cells expressed surface λ5 (five consecutive experiments), and, as in humans, these cells co-expressed either κ or λ light chains (Fig. 3 b). The relative absence of variability in ΨL expression on mouse peripheral B cells as compared with human may be due to controlled immunizations and the use of inbred mouse strains. Control B220−GL7− non-GC B cells did not express surface ΨLs and neither did B220−GL7+ B cells from λ5 targeted mice (Fig. 3 b). Furthermore, ΨLs are coexpressed with RAGs in B220−GL7+ B cells as determined by cell sorting and RT-PCR analysis (data not shown). We conclude that ΨLs are coexpressed with conventional light chains in GL7+ mature spleen B cells in mice and human, and that loss of conventional light chains is not a prerequisite for activation of ΨL expression in these cells.

GC B cells may not be the only peripheral B cells that are CD38− IgD− (38). To directly confirm that ΨLs are expressed in GC B cells, we stained human tonsil tissue sections with anti-CD21 and anti-V-preB antibodies. Consistent with the cell fractionation experiments, ΨL expression was found in GCs (Fig. 4). In addition, occasional ΨL-positive B cells were found in the mantle and T cell zones. These occasional ΨL-positive cells represent CD38− IgD− non-GC B cells because isolated CD38− IgD− FM B cells are
always CL negative as measured by immunofluorescence (data not shown). CL expressing non-GC CD38⁺ IgD⁻ B cells may be recent bone marrow immigrants that are CD38⁺ IgM⁺ IgD⁻. We conclude that CLs are expressed in human GC B cells but are also expressed in other tonsillar B cells.

To define the requirements for RAG induction in peripheral B cells, we cocultured purified IgD⁺ CD38⁻ FM and IgD⁺ CD38⁺ GC B cells with fibroblasts that express CD40L and ILs (51). After 3 d of culture with CD40L, resting FM B cells were induced to express high levels of TdT mRNA, but did not express RAG1, RAG2, λ-like, or V-preB (Fig. 5a). IgD⁺ CD38⁺ B cells differ from FM B cells in that they express RAG1, RAG2, λ-like, and V-preB, and all of these mRNAs are maintained in cocultures of GC B cells and CD40L-expressing fibroblasts (Figs. 2 and 5, a and b). Consistent with the results obtained with FM cells, only TdT mRNA expression is upregulated in the GC B cells in culture (Fig. 5a). Addition of IL-2, IL-4, or IL-10 was not sufficient for RAG induction in the resting FM B cells, and did not alter the levels of RAGs or TdT expressed by GC B cells (Fig. 5a). These results are in contrast to experiments with mouse spleen B cells that appeared to be induced to express RAGs in response to CD40L plus IL-4 or LPS plus IL-4 (35, 36). We conclude that reinduction of TdT in mature human B cells is mediated through CD40, and that distinct pathways are required to activate RAG expression.

Immature autoreactive IgM⁺ IgD⁻ bone marrow B cells can be induced to reexpress RAGs when their receptors are cross-linked by self-antigens (15, 52). Reexpression of RAGs in these self-reactive B cells has been shown to delete the auto-reactive receptors, and this process is referred to as receptor editing (16–18). To determine whether RAG expression in peripheral B cells could also be modulated by receptor cross-linking, we added anti-κ and anti-λ (anti-κ+λ) antibodies to cultured IgD⁺ CD38⁻ and IgD⁺ CD38⁺ B cells (Fig. 5, a and b). In contrast to the activation of RAG expression in bone marrow B cells, addition of anti-B cell receptor (BCR) antibodies to cultured peripheral B cells inhibited the expression of RAG1, RAG2, and TdT (Fig. 5, a and b). Anti-κ+λ antibodies also downregulated CD40L-induced TdT expression in FM cells (Fig. 5a). To further

Figure 4. CL expression in human tonsil B cells. Immunohistochemical staining of human tonsil sections was performed using anti-V-preB (left), anti-CD21 (middle), or both (right). GCs, FM, and the T cell zones (T zone) are indicated.

Figure 5. Regulated expression of RAG, TdT, λ-like, and V-preB genes. (a) Human tonsil CD38⁺ IgD⁻ FM or CD38⁺ IgD⁻ GC B cells were sorted (d0) and cultured on CD40L-transfected fibroblasts with or without anti-κ+λ (κ Ig) at 10 μg/ml, or IL-2, IL-4, or IL-10 for 3 d (d3). Gene expression was analyzed by RT-PCR as described above. (b) CD38⁺ IgD⁻ GC B cells were cultured with CD40L alone or with CD40L and an irrelevant IgG control (Control Ig), or with intact anti-κ (Ig κ-κ) or anti-λ (Ig α-λ) or both (Ig κ-κ + α-λ) or with Fab2 anti-κ or anti-λ (Fab2 κ-κ + α-λ). Antibody concentrations are indicated in micrograms per milliliter. (c) GC cells reanalyzed by flow cytometry using anti-CD38 and anti-V-preB mAbs after 3 d of culture with CD40L and the indicated antibodies. GC B cells cultured with Ig κ-κ + α-λ or Fab2 κ-κ + α-λ antibodies contained 1.5–2-fold more B cells than those with cultured with control antibody, and the percentage of dead cells was 40–50% in all of cultures as determined by trypan blue exclusion.
characterize the suppressive effect of anti-BCR antibodies on RAG expression, we performed dose–response experiments with both intact anti-κ + λ antibodies and Fab'2 fragments as well as control nonspecific antibody preparations (Fig. 5 b). mRNA levels were measured by semi-quantitative RT-PCR and phosphorimaging. RAG1 and RAG2 downregulation was observed with 10 μg/ml of intact anti-κ + λ and as little as 2 μg/ml of Fab'2 fragments of anti-κ + λ antibody but was difficult to detect with 10 μg/ml of intact anti-κ or anti-λ alone (RAG1 expression was decreased by a factor of 6–8 after treatment with 10 μg/ml of intact anti-κ + λ antibody, and up to 20× after treatment with 10 μg/ml of Fab'2 fragments of anti-κ + λ; Fig. 5 b). Direct measurements of cell viability showed that addition of either intact or Fab'2 fragments of anti-κ + λ antibodies to the cultures did not result in increased cell death (see legend to Fig. 5). More importantly, FACS analysis showed that the number of cells undergoing “neoteny” (33) did not change after anti-BCR cross-linking as determined by anti-V-preB staining and confirmed by RT-PCR analysis of V-preB mRNA levels (Fig. 5, a–c). Thus, the effects of anti-BCR antibodies on RAG expression cannot be explained by preferential killing of the specific subset of GC B cells that are undergoing receptor revision (36). We conclude that the effect of receptor cross-linking on recombination-associated gene expression in peripheral B cells is the opposite of the effect of cross-linking the same receptor on immature IgM + IgD- bone marrow B cells (15).

What is the function of receptor revision in the periphery? Our experiments indicate that new antigen receptor assembly does not require light chain inactivation and is not likely to be a mechanism for removing autoreactive receptors (Figs. 3 and 5). On the contrary, activation of recombination in cells that fail to bind antigen suggests that secondary recombination may participate in repertoire diversification by improving very low affinity receptors (35, 36). Expression of TdT lends further support to the notion that new receptor diversification by gene recombination occurs in mature B cells in the periphery since the only known function for TdT is to increase the diversity in the repertoire by adding N nucleotides to V(D)J junctions (21, 22).

The idea that specific antibodies might be induced during an immune response is difficult to accept because it is directly contradictory to current interpretations of the clonal selection theory (53, 54). Nevertheless, receptor assembly in GC B cells is already a well-established mechanism for antibody diversification in avian immune systems (55). In the chicken, Ig gene conversion is a prominent feature of the GC reaction, resulting in groups of interrelated clones of B cells (55). In mice and humans, Ig recombination in GC B cells may be limited to the light chain genes. Since much of the antibody-binding pocket is shaped by the heavy chain, changing just the light chain could result in a group of related B cells with antibodies that have a spectrum of antigen binding affinities. Our findings are consistent with the idea that secondary recombination is terminated when antigen receptors are cross-linked and suggest a mechanism for positive selection of B cells that produce high affinity receptors by receptor revision in the periphery.

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