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Within Germinal Centers, Isotype Switching of Immunoglobulin Genes Occurs after the Onset of Somatic Mutation

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Summary

Human tonsillar B cells were separated into naive IgD$^+$CD38$^-$CD23$^-$ (Bm1) and IgD$^+$CD38$^-$CD23$^+$ (Bm2), germinal center IgD$^-$CD38$^+$CD23$^-$ centroblasts (Bm3) and IgD$^-$CD38$^+$CD77$^-$ centrocytes (Bm4), and memory IgD$^-$CD38$^-$ (Bm5) subsets. Previous IgV$_H$ sequence analysis concluded that the triggering of somatic mutations occurs during the transition from Bm2 subset into the Bm3 subset. To determine the initiation of isotype switching, sterile transcript expression was analyzed by amplification, cloning, and sequencing. A selective sterile Iγ, Iα, and Iϵ expression was observed at centrocyte (Bm4) stage, suggesting that isotype switch is triggered within germinal centers, after somatic mutation is initiated within centroblasts (Bm3). Finally, the high level of 5′Sγ–Sμ3′ DNA switching circles observed in germinal center B cells indicates that within human tonsils, germinal center is a major location for isotype switching.

Introduction

T cell–dependent humoral immune responses are initiated by the activation of naive and memory cells in T cell–rich extrafollicular areas of the secondary lymphoid organs. The primary B cell activation in the T cell–rich areas allows the generation of short-lived plasma cells, and the recruitment of germinal center (GC) precursors into B cell follicles (Liu et al., 1991; Jacob et al., 1991a). Some B cells, however, may undergo isotype switching without somatic hypermutation (Kaartinen et al., 1983; Wysocki et al., 1986; Cumano and Rajewsky, 1985; Apel and Berek, 1990; Jacob and Kelsoe, 1992). Upon GC reaction, centroblasts proliferate and mutate in the dark zone (Berek et al., 1991; Jacob et al., 1991b; Küppers et al., 1993; McHeyzer-Williams et al., 1993; Pascual et al., 1994). Then, they progress into nonproliferating centrocytes, which undergo antigen-driven selection, and differentiate into either memory B cells or plasma cells (Kroese et al., 1990; Liu et al., 1992; MacLennan, 1994; Thorbecke et al., 1994). The observation that GC B cells essentially express immunoglobulin M (IgM) during primary immune responses and IgG during secondary responses, led Kraal et al. (1982) to suggest that GCs are also the place where isotype switching occurs. However, no molecular evidence of an active isotype-switching process in vivo has been provided. Isotype-switching recombination is a process that moves the variable heavy chain segment (VDJ) to associate with a different constant (CH) region. This is mediated (with the exception of IgD) by the junction of switch regions (S regions), which are arrays of short tandem repeats, located 5′ of each constant region (Harriman et al., 1993; Jung et al., 1993; Radbruch et al., 1986). As it has been previously demonstrated that the expression sterile transcripts is essential for the induction of isotype switching (Coffman et al., 1993; Sideras et al., 1989; Xu et al., 1993; Zhang et al., 1993), we analyzed immunoglobulin sterile transcript expression among five tonsillar B cell subsets, to investigate the triggering of isotype switching. Moreover, to provide an anatomical marker of cells that have recently undergone in vivo isotype switching via intramolecular DNA deletion, the presence of excised circular DNA was also examined.

Results

Isotype-Switching Activity, Analyzed by Sterile Immunoglobulin Transcription, Is Selectively Detected within GCs at Centrocyte Stage (Bm4)

As reported earlier, tonsillar B cells were separated into five subsets by FACS sorting (Pascual et al., 1994). As schematically depicted in Figure 1, these include the following: two naive B cell subsets, IgD$^+$ CD38$^-$ CD23$^-$ (Bm1), and IgD$^+$ CD38$^-$ CD23$^+$ (Bm2); two GC B cell subsets, IgD$^-$ CD38$^+$ CD77$^+$ centroblasts (Bm3), and IgD$^-$ CD38$^+$CD77$^-$ centrocytes (Bm4); and an IgD$^-$CD38$^-$ memory B cell subset (Bm5). Sequence analysis on the IgV$_H$ genes demonstrated that somatic mutation is initiated within the GC centroblasts (Bm3) (Pascual et al., 1994). To determine whether the initiation of isotype switching correlates with the acquisition of a GC phenotype, a reverse transcription (RT)-mediated polymerase chain reaction (PCR) assay was designed to examine the expression of sterile immunoglobulin transcripts within the purified human B cell subsets. Sterile immunoglobulin transcripts are initiated at a segment denominated as the I exon, located upstream of the S region of corresponding isotype (Figure 2A). After primary sterile transcript processing, the I exon is juxtaposed to downstream CH domains. Following FACS sorting of the five B cell subsets, the RNA of 500 cells per subset was extracted and directly reverse transcribed. The cDNA template generated by the RT reaction was amplified through 35 PCR cycles, using specific primers targeting the Iγ transcript. Figure 2B shows that by using a combination of consensus 5′ Iγ and 3′ Cγ primers, sterile Iγ transcripts of the expected molecular size (354 bp) were selectively detected within the GC Bm4 centrocyte subset. To rule out that such selective detection was the

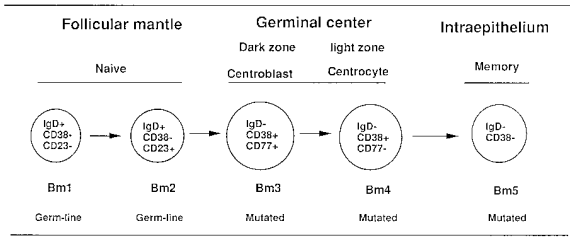

Figure 1. Peripheral B Cell Development and Diversification

Tonsillar B cells are separated into IgD^+CD38^- naive (Bm1 and Bm2), IgD^-CD38^+ GC (Bm3 and Bm4), and IgD^-CD38^- memory (Bm5) B cells (Pascual et al., 1994; Liu et al., 1995). Naive B cells are further separated into a $CD23^-$ (Bm1) and a $CD23^+$ (Bm2) subset; GC B cells are further separated into $CD77^+$ centroblast (Bm3) and $CD77^-$ centrocyte (Bm4) subsets. Previous IgV_H gene sequence analysis (Pascual et al., 1994) indicates that the molecular mechanism underlying somatic mutation is triggered at centroblast stage (Bm3). In the present study, the triggering of isotype switching was investigated, by assessing the expression of sterile transcripts in the five B cell subsets, and by determining the presence of switching DNA circles among total naive, GC, and memory populations.

result of a differential content of RT template among the five distinct Bm cell subsets, equal mRNA representation per subset was systematically controlled by an overall heavy chain (IgH) PCR amplification. Figure 2C shows the scheme of amplification, and the comparable detection of the V(D)J segment among all five Bm subsets. The equivalent levels of IgH detected in the five subsets stresses the selective expression of Iγ within the Bm4 subset. The predicted 354 bp Iγ fragment rules out amplifications originating from genomic DNA, as the intron between the immunoglobulin and Cγ exons spans several kilobases. Additionally, independent studies on the differential expression of Bcl-2 (selectively expressed by Bm1, Bm2, and Bm5), Fas (exclusively expressed by Bm3 and Bm4) and c-*myc* (selectively expressed by Bm3), whose amplifying primers span separate exons (ruling out DNA involvement), confirmed that selective gene expression can be readily determined from each Bm subset (Martinez-Valdez et al., 1996).

The Expression of Sterile Iα and Iε Confirms that Triggering of Isotype Switching within GCs Occurs at the Bm4 Centrocyte Stage

Functional IgA can be readily detected within GC B cells, albeit at lower level than IgG (Liu et al. 1995). Therefore, to investigate whether triggering of isotype switching towards IgA is also operative at the Bm4 centrocyte stage, the expression of sterile Iα was accordingly analyzed. Figure 3A shows that Iα transcripts of the expected molecular size (650 bp) were also selectively detected in the centrocyte (Bm4) population.

Although in vivo expression of Iε transcripts can be expected to be rare, in view of the relatively low IgE frequency, its detection could still be demonstrated within the Bm4 centrocyte population, provided that additional nested PCR amplification and hybridization were carried out (Figure 3B). Taken together, the selective expression of Iγ, Iα, and Iε demonstrates that the Bm4 centrocyte subset, located within the light zones

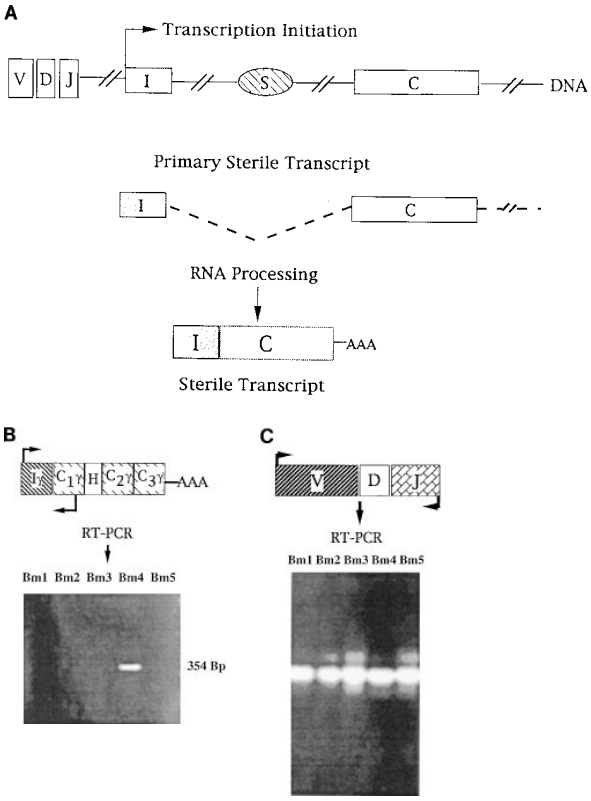

Figure 2. Expression of Sterile Immunoglobulin Transcripts in Five B Cell Subsets

(A) Schematic IgH chain gene organization, depicting VDJ domain, the I exon locus, the S region, and the constant segment. As indicated, the generation of a primary sterile transcript is initiated at the I exon, located upstream of the S region. Following processing of primary transcript, the I exon is accurately spliced to corresponding CH region.

(B) Structure of the Iγ transcript displaying the I exon, spliced to corresponding constant segments (CH1γ, Hinge, CH2γ, and CH3γ), and the location of amplifying primers (see Experimental Procedures for corresponding sequences). PCR amplification was carried out using RT cDNA corresponding to 500 cells from each subset. Lanes correspond to each of the five distinct Bm subsets. Ethidium bromide staining shows the selective detection of a sterile Iγ PCR product within the centrocytes (Bm4) subset.

(C) VDJ segment amplification, schematically shown here, was used as an internal control to evaluate equivalent mRNA input per B cell subset. The PCR products were analyzed by gel electrophoresis and ethidium bromide staining.

of GCs, represents a stage in which the machinery for isotype switching is triggered.

Nucleotide Sequencing Validates PCR-Generated Bm4 Iγ, Iα, and Iε cDNAs

To establish formally the identity of the sterile transcripts selectively detected within Bm4 centrocytes, their cDNAs were analyzed by nucleotide sequencing. RT–PCR generated products, corresponding to respective Iγ, Iα, and Iε cDNAs, were cloned into the vector pCRII and sequenced. Nucleotide sequences shown in Figures 4A, 4B, and 4C demonstrate the presence of the I segment accurately spliced to the corresponding CH domain, featuring standard donor–acceptor junctions.

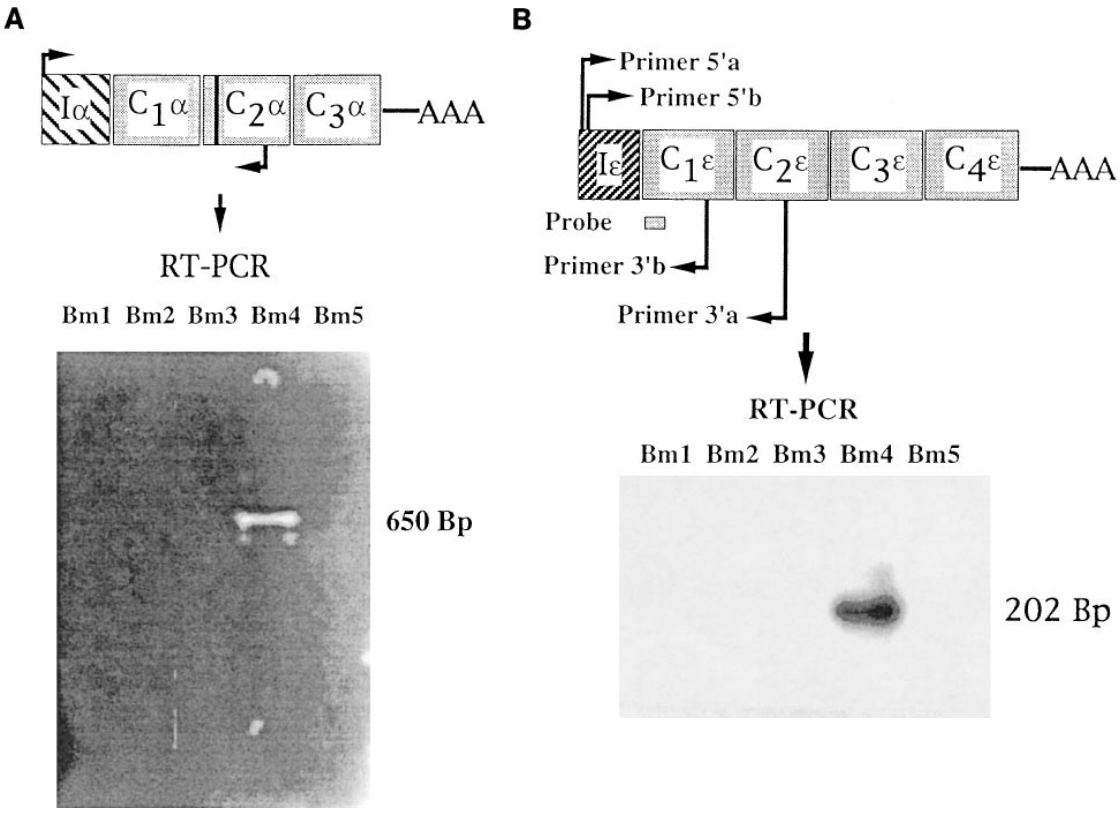

Figure 3. Expression of Sterile Iα and Iϵ Transcripts

As in Figure 2, lanes correspond to each of the five Bm subsets.

(A) The structural representation of the Iα transcript, depicting the Iα exon spliced to the constant region composed of three CH exons, including a hinge segment within the CH2 domain. Location of amplifying primers is also indicated here. Sterile Iα transcript was also selectively detected in GC centrocytes (Bm4).

(B) Scheme for the amplification of sterile Iϵ transcript, exhibiting the Iϵ and the four Cϵ exons. Primers 5′a and 3′a denote the external sense and anti-sense sequences used in the first round of PCR amplification. Primers 5′b and 3′b are nested oligonucleotides used in a second round amplification (see Experimental Procedures). Also shown here is the location of the probe used for the hybridization of the PCR products. As in previous experiments, the expression of Iϵ was selecively detected within the Bm4 subset.

Therefore, these data formally prove that the sterile transcripts expressed by the Bm4 centrocyte subset correspond to bonafide Iγ, Iα, and Iϵ messages.

Detection of Reciprocal Circular DNA Resulting from Deletional Recombination, Provides Additional Anatomical Evidence of In Vivo Isotype Switching within GCs

Several models have been proposed to explain how isotype switching occurs: intramolecular DNA deletion (Iwasato et al., 1990; Matsuoka et al., 1990; von Schwedler et al., 1990; Zhang et al., 1994), sister chromatid exchange (Obata et al., 1981), splicing of a large precursor RNA transcript (Yaoita et al., 1982), and *trans*-splicing (Shimizu et al., 1991). Although these models are not mutually exclusive, intramolecular DNA deletional recombination is the most widely accepted. During this process, schematically shown in Figure 5A, the 5′ segment of the upstream S region and the 3′ segment of the target S sequence from the recombining isotype are joined, forming a hybrid S–S junction in the chromosome. Consequently, the 3′ end sequence of the upstream S region, the 5′ end sequence of the recombining isotype, and the intervening sequences are looped out and excised within B cells undergoing switching recombination. In this context, switch circles can represent a presumptive marker of the cell population in which switch recombination occurs. Therefore, to demonstrate further that in vivo isotype switching occurs within GCs, we used a PCR strategy to determine the generation of extrachromosomal circular DNA. The basic feature of the PCR assay for the detection of switching circle fragments, relies on the fact that the intervening sequence is reciprocally looped out from the chromosome and deleted as an extrachromosomal circular DNA. Sγ–3′Sμ circles were used as a model in the present study. Since S region sequences for the four human IgG subclasses are highly homologous (F. C. M., and E. E. M., unpublished data), a consensus 5′ Sγ primer was used in combination with a 3′ Sγ primer. The orientation of these primers (Figure 5A) selectively targets on excised circular DNA resulting from isotype switching, and not from rearranged genomic DNA. To analyze semiquantitatively the intramolecular deletion of reciprocal DNA circles, it is essential to register the concentration of total DNA input per cell subpopulation tested. This restriction thus

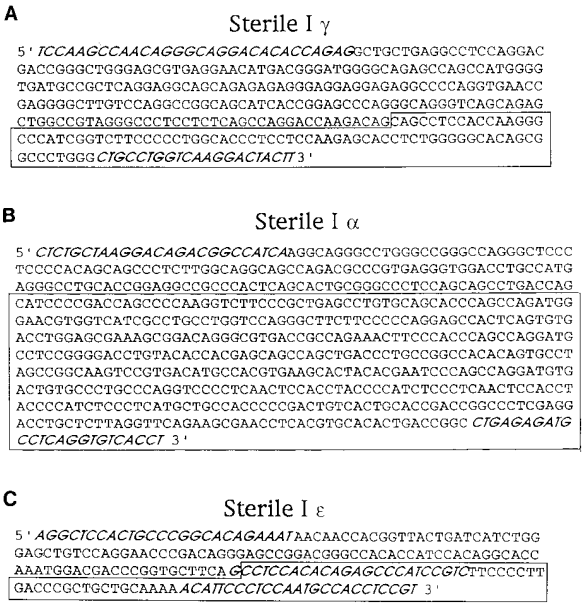

Figure 4. Nucleotide Sequence Analysis of cDNA Clones Corresponding to Iγ, Iα, and Iε Transcripts, Expressed by the Bm4 Subset

(A) Iγ transcripts.

(B) Iα transcripts.

(C) Iε transcripts.

The I exon region is displayed at the 5′ end of the sequence. The constant region for each isotype is boxed. Sequence corresponding to amplifying primers is shown in bold italics. In the case of the Iε, the primers are those used in the second round nested amplification. The probe used in the hybridization of Iε PCR products is shown in bold italics.

prevents the determination of switching circles from each of the five distinct subsets, as large number of cells are required. To circumvent this limitation, total naive (comprising the Bm1 and Bm2 subsets), GC (comprising Bm3 and Bm4 subsets), and memory populations were used for this analysis. Figure 5B shows switching circle PCR fragments of different sizes, representing distinct deletional recombination events at different breakpoints. As shown, circular DNA was most importantly detected in all of the five different DNA aliquots (A, B, C, D, and E) of GC B cells, indicating that major deletional recombination events took place within GC. In addition, lower but significant detection of switching circular DNA was readily observed within the five memory B cell subset aliquots. This can be explained by the fact that the phenotypic transition from high affinity centrocytes into memory B cells may be completed within a few hours, and the half-life of switching circular DNA might be long enough to be detected at this stage. Alternatively, a small numbers of memory B cells may undergo isotype switch upon early activation within the T cell and interdigitating cell–rich extrafollicular area. Similarly, weak sporadic circular DNA was detected within the naive B cell subset. This may either be due to contaminating GC B cells or may represent a small number of naive B cells undergoing isotype switch upon early activation within the extrafollicular area.

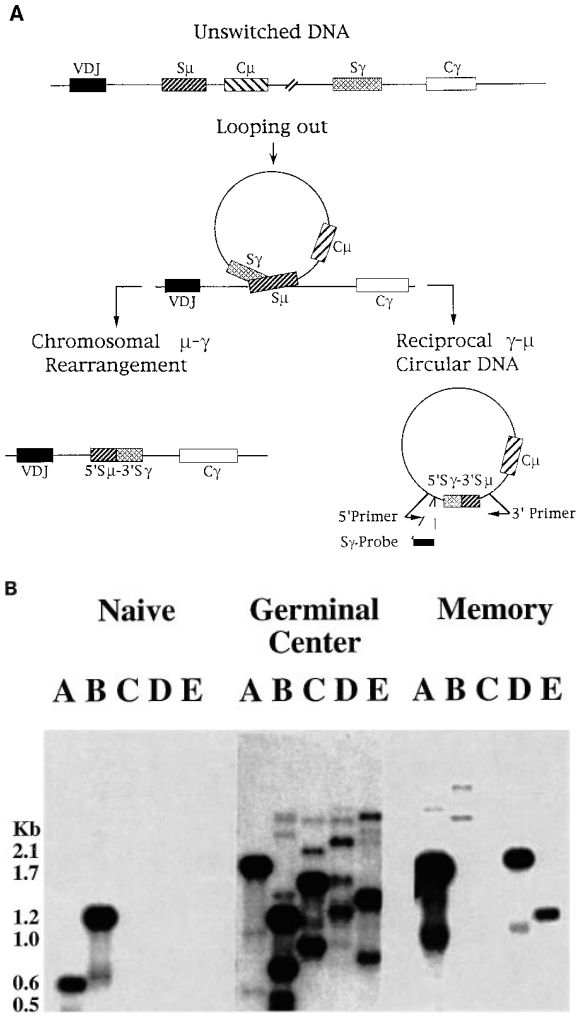

Figure 5. Detection of Extrachromosomal 5′Sγ–3′Sμ circular DNA

(A) Diagram displaying the formation and the excision of DNA circles, resulting from intramolecular deletion, during in vivo isotype-switching recombination. The scheme depicts the strategy of amplification by the location and orientation of the forward and reverse primers (see Experimental Procedures for corresponding sequences), assuring the exclusive amplification of circular DNA. The probe used for the detection of PCR-amplified switching cDNA circles is also shown.

(B) Detection of PCR amplified 5′Sγ–3′Sμ circular DNA, following Southern blotting and hybridization (see Experimental Procedures). Switching circle PCR fragments are shown, representing distinct deletional recombination events. As depicted, a minimum of five different DNA aliquots (lanes A, B, C, D, and E), corresponding to the follicular mantle (FM) (Bm1 + Bm2), GC (Bm3 + Bm4), and memory (Bm5) subsets, were examined.

Nucleotide Sequencing of Deleted Circular DNA, Reveals the Breakpoints Resulting from In Vivo Sμ–Sγ Isotype-Switching Recombination

To validate the in vivo generation of circular DNA, and to identify the breakpoints at the 5′Sγ–3′Sγ junctions, PCR-generated DNA circles were cloned and sequenced. Figure 6 shows the sequences representing two independent circular DNA events, confirming that the observed PCR products originated from 5′Sγ–3′Sμ DNA circles. Although the breakpoints of the two 5′Sγ–

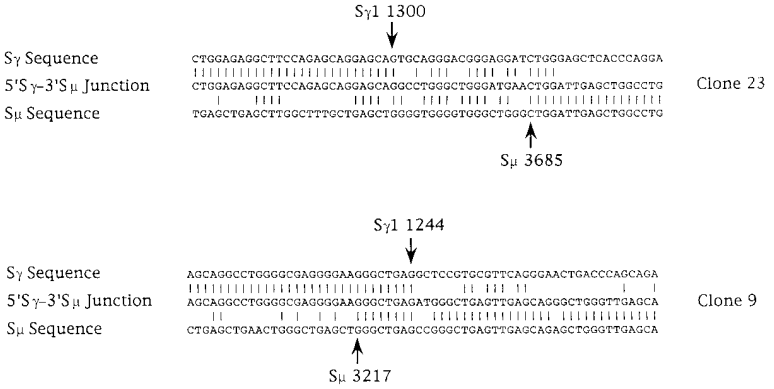

Figure 6. Nucleotide Sequences from PCR-Amplified Extrachromosomal 5′Sγ–3′Sμ Circular DNA

The sequences are those surrounding breakpoints of two independent switching fragments, derived from excised DNA circles. As shown, each circular DNA clone is aligned to germline Sμ and Sγ segments The vertical lines indicate the identity of circular DNA sequences at the breakpoint, and the homology with corresponding germline S region. Arrows indicate recombination breakpoint sites.

3′Sμ hybrids examined occur at different sites, both junctions appeared within the flanking segments of the S regions. The unique localization of breakpoints could either be the result of hot-spot recombination sites, or a bias introduced by the cloning of the PCR products, which favor smaller fragments.

Discussion

Isotype Switch and Somatic Mutation Are Two Independent Processes that Are Triggered Sequentially during GC Reaction

Our previous analysis of IgV genes demonstrated that the initiation of somatic hypermutation occurred within centroblasts (Bm3) from the GC dark zone (Pascual et al., 1994). The increase of replacement mutation versus silent mutation ratio in both IgM and IgG transcripts during the differentiation from centroblasts (Bm3) to centrocytes (Bm4) indicates an ongoing antigen-driven selection process (Pascual et al., 1994). In the present study, sterile transcripts (Iγ, Iα, and Iϵ), the initiation markers of isotype switch, were selectively detected in centrocytes from the GC light zone. Thus, within GCs, isotype switching is most likely initiated after B cells have undergone affinity maturation by somatic mutation, and positive selection. This allows the acquisition of a distinct immunoglobulin isotype, which confers effector functions, while keeping the high affinity for the selecting antigen.

Interestingly, a small number of memory cells expressing mutated IgM has been identified in both human tonsils (Pascual et al., 1994) and peripheral blood (Klein et al., 1994). It indicates that somatic mutation and isotype switch are two independent processes and that somatic mutation can occur within GCs without triggering isotype switch. The appearance of unmutated IgG expressing B cells during the first week of primary immune responses (Kaartinen et al., 1983; Wysocki et al., 1986; Cumano and Rajewsky, 1985), together with the ability of T cell–independent antigens to induce IgG antibody responses in the absence of somatic mutation (Maizels and Bothwell, 1985; Maizels et al., 1988), suggests that isotype switching occurs without somatic mutation as well. By sequencing IgV genes of antigen-specific B cells directly picked from splenic sections, Jacob and Kelsoe (1992) provide further evidence suggesting that the extrafollicular T cell–rich areas are the anatomical sites where B cells undergo isotype switch without somatic mutation at early timepoints of primary immune responses.

5′Sγ–3′Sμ circular DNA that are predominantly detected within GC cells provides a molecular signature of the cell population that recently underwent deletional recombination in vivo. The particular clustering of the breakpoints, observed in the present analysis, might indeed be related to the technique used. The smaller switching circle fragments would be more efficiently amplified and cloned. Alternatively, it could be the result of hot-spot recombination sites. The low levels of circular DNA detected in both memory B cells and naive B cells may represent exrafollicular isotype switch.

Evidence of Reëntry of IgG and IgA Memory B Cells into GCs

Although sterile transcripts were not detected within the GC centroblasts (Bm3) subset, many of them express IgG and IgA functional transcripts and surface protein and have deleted their Cμ locus (Islam et al., 1994; Pascual et al., 1994; Feuillard et al., 1995; H. M.-V. et al., unpublished data). This can be explained by two hypotheses. First, the IgG and IgA expressing centroblasts (Bm3) may be derived from B cells that had undergone isotype switch within the T cell–rich extrafollicular foci (Jacob and Kelsoe, 1992). In agreement with this, the mouse hybridoma lines derived from PNA^{hi} splenic GC B cells 10 days after primary immunization with phenyloxazolone were found to be mainly IgG lines, containing germline or low mutated IgV genes (Apel and Berek, 1990). However, this is in contrast with the preferential IgM expression of splenic PNA^{hi} GC B cells from mouse undergoing primary anti-sheep red blood cell immune response (Kraal et al., 1982). Second, the IgG and IgA expressing GC centroblasts (Bm3) may be mainly derived from isotype-switched memory B cells. In keeping with this notion, we (Pascual et al., 1994) and others (Klein et al., 1994) demonstrated that most IgG transcripts from human GC centroblasts (Bm3) were heavily mutated and had accumulated twice as many mutations as their IgM counterparts. This is obviously different from the observation in the mouse model, where unmutated IgG expressing GC B cells can be identified in the spleen at the early timepoint of primary immune responses. However, during secondary and tertiary anti-phenyloxazolone immune responses in mice, memory

B cells generated during the primary immune responses accumulate further mutations, presumably by going through GCs (Berek and Milstein, 1987). Since human tonsils represent a site of chronic antigenic stimulation, our present data support the hypothesis that memory cells recirculate through GCs, to generate even higher affinity clones and to switch to downstream isotype (Berek and Milstein, 1987; Kepler and Perelson, 1993).

Isotype Switching Occurs in the Apical Light Zone and Outer Zone of GCs where GC T Cells Are Accumulated

Human GCs have recently been divided into four distinct functional compartments according to the mitotic index, apoptotic index, and the expression of a series of molecules within the secondary lymphoid follicles (Hardie et al., 1993). Within the four GC compartments as depicted in Figure 7, a simple model shows the maturation pathway of a IgM^+ centroblast that is recently recruited from primary B cell activation in T cell–rich extrafollicular foci. This model does not consider the reëntry of isotype-switched memory B cells. The GC dark zone is devoid of CD23-expressing FDC dendritic networks (Figure 7A) and contains few T cells (Figure 7B). This area does not contain antigen–antibody immune complexes at a detectable level (Nossal et al., 1968; Tew et al., 1990). An IgM centroblast undergoes rapid clonal expansion here. Point mutations are introduced into the IgV region genes stepwise in the course of clonal proliferation of IgM-expressing centroblasts (Figure 7C). Three type of mutants are expected to be generated, including high affinity, low affinity, and autoreactive mutants. These mutants migrate into the basal light zone of GC (Figure 7C). The basal light zone is characterized by weak CD23-expressing FDC networks (Figure 7A), high apoptosis index (Hardie et al., 1993), and very few T cells (Figure 7B). The high death rate among centrocytes in the basal light zone is consistent with the concept that there is selection among the cells in this zone (Hardie et al., 1993; MacLennan, 1994). The low levels of antigen–antibody immune complexes on the follicular dendritic cells here (Y. J. L. et al., unpublished data) may facilitate the selection of high affinity mutants specific for the immunizing antigens. While low affinity mutants and autoreactive mutants die by apoptosis (Liu et al., 1989), high affinity mutants pick up antigen and process it on their migration pathway to the apical light zone and outer zone of GCs (Figure 7C), where most GC T cells are localized (Figure 7B). In contrast with outer zone, apical light zone contains strong CD23-expressing FDC networks (Figure 7A) and high level of antigen–antibody immune complexes (Nossal et al., 1968; Tew et al., 1990; Y. J. L. et al., unpublished data). Here, the selected centrocytes (Bm4) encounter and present antigen to antigen-specific GC T cells. T cells are induced to express CD40 ligand (Casamayor-Palleja et al., 1995; Lederman et al., 1992) and secrete cytokines including interleukin-4 (IL-4) (Butch et al., 1993), which are both key elements for the induction of isotype switching (Clark and Ledbetter, 1994; Kühn et al., 1991). Thus, the detection of sterile I transcripts within centrocytes (Bm4) and the colocalization of centrocytes (Bm4) and GC T cells within the apical light zone and outer zone, suggests that the cognate T–B interaction that results in the expansion and then isotype switch of high affinity centrocytes occurs here. Whether high affinity GC centrocytes undergo further mutation in the apical light zone and the outer zone is unknown. Nevertheless, early experiments have indicated that isotype switching does not terminate somatic mutation (Shan et al., 1990). Finally, the high affinity isotype-switched centrocytes differentiate into memory B cells in the presence of prolonged CD40 ligand signaling and into plasma cells when CD40 ligand signaling are removed (Arpin et al., 1995).

Experimental Procedures

Isolation of Tonsillar B Cells

Tonsil B cells were prepared as previously described (Liu et al., 1995). In brief, tonsils taken from patients during routine tonsillectomy were finely minced and the resulting cell suspension was subjected to two rounds of depletion of non-B cells: first, T cells were depleted by rosetting with sheep red blood cells; second, the residual non-B cells were depleted by T cell–specific antibodies (CD2, CD3, CD4, Immunotech, Marseille, France) followed by magnetic beads coupled with anti-mouse IgG (Dynabeads, Dynal Oslo, Norway). The resulting cells from all the experiments contain more than 98% $CD19^+$ B cells.

Separation of Tonsil B Cells into High Density and Low Density B Cells on Percoll Gradient

Some tonsil B cells were further separated into high density B cells and low density B cells by centrifugation through 15%, 60%, and 65% Percoll gradient (Pharmacia, Uppsala, Sweden). In brief, the 100% Percoll is prepared by mixing 90 ml of Percoll (Pharmacia, Uppsala, Sweden) with 10 ml of ten-times concentrated phosphate-buffered saline. The 15% Percoll solution is prepared by mixing 15 ml 100% Percoll with 85 ml PBS. The 60% and 65% Percoll solutions are prepared accordingly. The discontinuous Percoll gradient is generated by layering 3 ml 60% Percoll on top of 3 ml 65% Percoll in a 15 ml Falcon tube (Becton Dickinson). Afterwards, 2 ml 15% Percoll are deposited on top of the 60% Percoll. Then, 2 ml B cell suspension (at 2×10^8 cells/ml) are layered on to the gradient. Cells are centrifuged for 20 min at $400 \times g$ and 20°C. While dead cells remain on the surface of 15% Percoll, low density B cells are collected on the surface of 60% Percoll. High density B cells that penetrate through the 60% Percoll are recovered. The resulting total tonsil B cells, high density B cells, and low density B cells were used for isolation of B cell subsets by immunomagnetic beads.

Isolation of Bm1 and Bm2 Subsets

High density B cells are stained by goat anti-IgD–biotin (Amersham) and mouse anti-CD23–fluorescein isothiocyanate (FITC) (Serotec) for 30 min. After washing twice, IgD^+CD23^- (Bm1) and IgD^+CD23^+ (Bm2) B cells are sorted on a cell sorter.

Isolation of $CD38^+CD77^+$ Centroblasts (Bm3) and $CD38^+CD77^-$ Centrocytes (Bm4) by Cell Sorting

Low density B cells were incubated with mouse anti-human CD38–phycoerythrin (Becton-Dickinson) and rat anti-human CD77 (Immunotech) for 30 min. After washing twice, cells are incubated with sheep anti-rat IgM–FITC for 30 min. After washing, the stained cells are sorted into $CD38^+CD77^+$ Bm3 and $CD38^+CD77^-$ Bm4 subsets.

Isolation of Bm5 IgD^-CD38^- Memory B Cells by FACS Sorting

Total tonsillar B cells are incubated with goat anti-human IgD–biotin (Amersham) and mouse anti-CD38 (Immunotech) for 30 min. After washing twice, the cells were incubated with streptavidin–phycoerythrin and goat anti-mouse IgG–FITC for 30 min. After washing, the IgD^-CD38^- B cells were sorted as memory B cell subset.

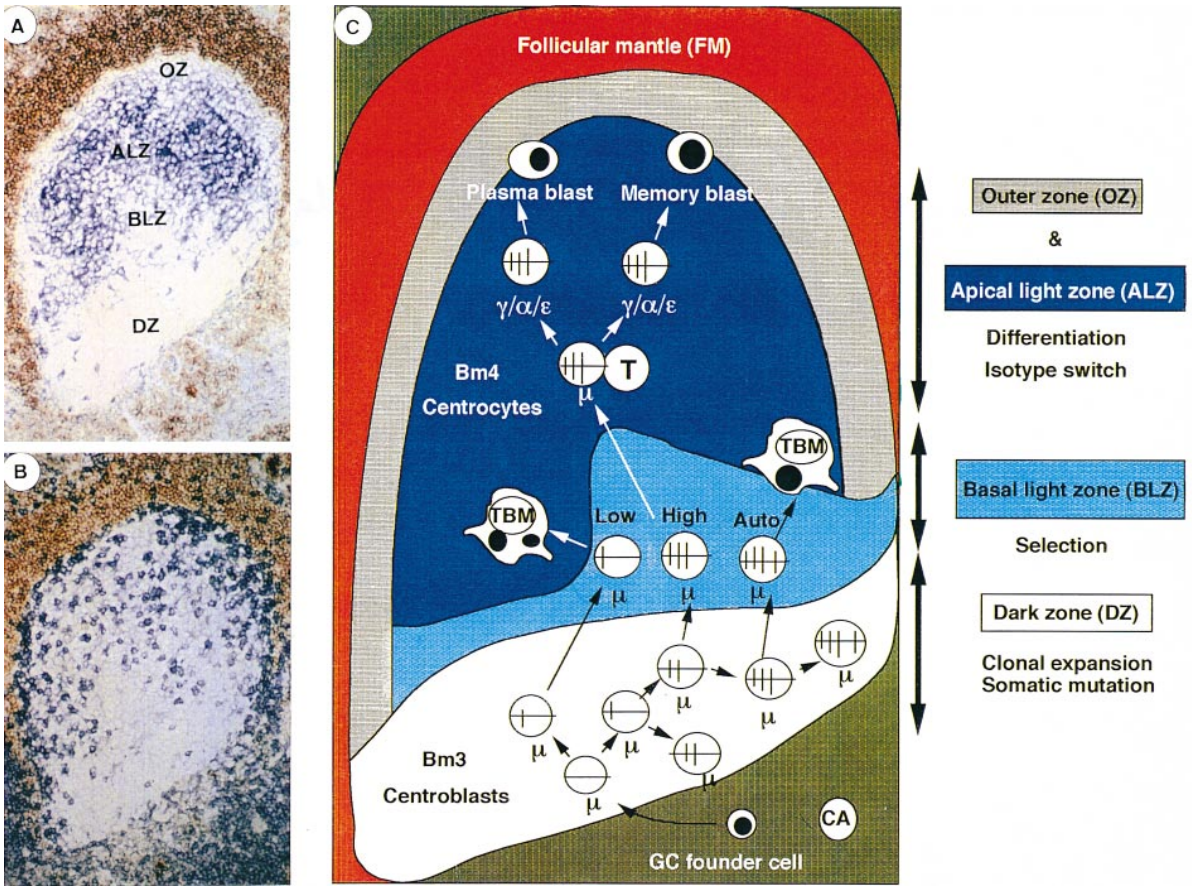

Figure 7. Isotype Switch Occurs within the T Cell–Rich GC Apical Light Zone and Outer Zone

(A) Double red anti-IgD and blue anti-CD23 immunoenzymatic staining of a secondary follicle from human tonsils. Follicular mantle (FM) contains red IgD^+ B cells. The strong blue CD23 expressing FDC networks represent the apical light zone (ALZ) (Liu et al., 1992; Hardie et al., 1993). The area in between the FM and ALZ is the outer zone (OZ). The area below the ALZ that contains very weak CD23 expressing FDC networks represent basal light zone (BLZ). The area below the BLZ is the dark zone (DZ).

(B) Double red anti-IgD and blue anti-CD3 staining of the same secondary follicle. Blue CD3-expressing T cells are mainly located within the ALZ and OZ.

(C) An IgM-expressing GC founder B cell generated during the extrafollicular activation of a naive B cell migrates into the DZ of a GC. It undergoes rapid clonal expansion and somatic hypermutation in IgV gene. Three type of mutants are generated, including high affinity, low affinity, and autoreactive mutants. These mutants migrate into the BLZ of GC. The low levels of antigen–antibody immune complexes on the follicular dendritic cells here select high affinity mutants specific for the immunizing antigens. While low affinity mutants and autoreactive mutants die by apoptosis, high affinity mutants pick up antigen and process it on their migration pathway to the ALZ and OZ of GCs. In these areas, the selected centrocytes (Bm4) encounter and present antigen to many antigen-specific GC T cells. T cells are induced to express CD40 ligand (Casamayor-Palleja et al., 1995; Lederman et al., 1992) and secrete cytokines including IL-4 (Butch et al., 1993), which are both key elements for the induction of isotype switching. This cognate T–B interaction results in the expansion and then isotype switch of high affinity centrocytes. Whether high affinity GC centrocytes undergo further mutation in the ALZ and the OZ is unknown. Finally, the high affinity isotype-switched centrocytes differentiate into memory B cells in the presence of prolonged CD40 ligand signaling and into plasma cells when CD40 ligand signaling are removed (Arpin et al., 1995).

RNA Preparation and RT

Isolation of total RNA, was performed essentially as described by Chomczynski and Sacchi (1987). Total RNA from either 500 or 1000 of each human tonsillar mature B (Bm) cell subset was converted into single-stranded cDNA by a standard reverse transcription reaction, using oligo-d(T)12–18 (Pharmacia, LKB, Uppsala, Swenden) and the kit Superscript (RNase H^- MMLV reverse GIBCO BRL, Gaithersburg, Maryland) in a final volume of 40 μl.

RT–PCR

The PCR was performed as described by Saiki et al. (1988) with minor modifications. Of the RT reaction, 5 μl was amplified in a 100 μl PCR mixture, containing 100 ng each of sense and anti-sense primers, 2.5 U of Taq polymerase (Cetus, Norwalk, Connecticut), and 5% DMSO. Other components of the reaction were exactly as those previously reported. Primers used were as follows: a consensus Iγ exon 5′ forward primer, 5′-T CCAAGCCAACAGGGCAGGACA CACCAGAG-3′ (Sideras et al., 1989), and a consensus Cγ 3′ reverse primer, 5′-AAGTAGTCCTTGACCAGGCAG-3′, spanning a region within the CH1 exon (Huck et al., 1986). Amplification was carried out through 35 cycles: 1 min denaturation at 94°C, 2 min annealing at 60°C, and 3 min primer extension at 72°C. This was followed by an additional 10 min extension of the PCR products at 72°C. PCR products were analyzed on a 1.6% agarose gel, stained with ethidium bromide. PCR analysis for Iα and Iε were as described for the Iγ. Amplifying primers for the Iα transcript were as follows: a 5′ forward primer, 5′-CTCTGCTAAGGACAGACGGCCATCAAGGC-3′, located within the Iα region recognizing both Iα1, Iα2 (Nilsson et al.,

1991) and a 3′ reverse primer 5′-AGGTGACACCTGAGGCATCTC TCAG-3′, located within the second exon of the Cα region (Flanagan et al., 1984). Iα PCR products were analyzed as above. For the detection of the Iε, two 35 cycle PCR rounds were necessary. The first round was performed using a 5′ forward primer, 5′-AGGCTCCA CTGCCCGGCACAGAAAT-3′, located between the nucleotides 7–31 of the Iε exon (Gauchat et al., 1990) in combination with a 3′ reverse Cε primer, 5′-TCGCAGGACGACTGTAAGATCTTCA-3′, spanning a sequence between codons 11–19 of the second Cε exon (Flanagan and Rabbitts, 1982). For the second amplification, a nested 5′ forward primer 5′-AGCTGTCCAGGAACCCGACAGGGAG-3′, located downstream of the first 5′ primer, was used together with another 3′ reverse primer, 5′-ACGGAGGTGGCATTGGAGGGAATGT-3′, nested upstream of the first 3′ primer, between codons 17–25 of the first Cε exon. For the amplification of overall heavy chain, a forward V_H consensus 5′-TCTGAGGTGCAGCTGGTGGAGTCTG-3′ primer, located within the frame work 1, and displaying the highest degree of conservation, was used in combination with a reverse J_H consensus 5′-TGAGGAGACGGTGACCAGGGTCCC-3′ primer.

Preparation of Circular DNA Templates

Isolation of total DNA from 10^6 each of naive, GC, and memory B cell subsets, containing both genomic and extrachromosomal circular DNA was carried out by standard procedures (Sambrook et al., 1989). In brief, following FACS sorting purification, B cells of each follicular mantle (comprising Bm1 and Bm2), GC (Bm3 + Bm4), and memory (Bm5) subset pools were lysed in 10 mM Tris–HCl, 10 mM EDTA (pH 8.0), containing 1% SDS. Lysates were then incubated in the presence of proteinase K (at a final concentration of 100 μg/ml) for several hours at 37°C. DNA was phenol/chloroform extracted, and precipitated with 2.5 vol of ethanol. After resuspending DNA in water, a combined EcoRI and RNAse digestion was carried out, overnight at 37°C, to linearize DNA and to remove RNA, respectively. Presumably, amplification of linearized DNA circles yields a more efficient PCR. As neither Sμ or Sγ exhibit EcoRI sites, digestion of circular DNA will not alter S regions. DNA was spectrophotometrically quantitated, and used for the amplification of circular DNA. Engineered 5′Sγ–3′Sμ plasmid constructs, featuring a portion of the 5′ Sγ (approximately 2 kb: Sγ1, HindIII–PstI fragment; Sγ2, HindIII–HaeIII; Sγ3, HindIII–Pst; Sγ4, HindIII–Bsu36I) ligated to the 3′ portion of Sμ (spanning a 0.9 kb segment between the Sst and EcoRI sites) were used as positive controls.

Amplification of Circular DNA

Aliquots (100 ng) of total DNA, containing excised circular DNA, or a defined quantity of the control artificial circular construct, were amplified in 25 μl PCR reactions: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 100 nM of each sense Sγ: 5′-AAGAGTCCAGGGAG GCCCAGAAAGGCCCAG-3′ (a consensus for the four subclasses) and anti-sense (Sμ) CTGAGTGCCCTCACTACTTGCGTCCCG primers, 200 nM of the four deoxynucleotide triphosphates (dNTPs), 5% DMSO, and 0.625 U of Taq polymerase (Perkin Elmer, Branchburg, New Jersey). PCR was carried out through 35 cycles: denaturation at 94°C for 1 min; annealing at 72°C for 2 min; primer extension at 72°C for 6 min. Amplified circular DNA was examined by electrophoresis, Southern blotting, hybridization, and sequencing.

Southern Transfer and Hybridization

PCR products were transferred onto nylon membranes by standard procedures (Sambrook et al., 1989) and hybridized with a specific probe. For the detection of Iε, an oligonucleotide probe was used, from a sequence located at position 36–57 of the Cε region of the ε-sterile transcript. The probe was end-labeled using the digoxygenin-dUTP kit (Boehringer-Mannheim, Federal Republic of Germany), and used according to the protocol of the supplier, including posthybridizations, washes, and development by chemiluminiscence. For the analysis of circular DNA, Sμ- and Sγ-specific probes were generated by PCR. Sμ probe was generated from a region located at its 3′ end, between positions 3711–4041 (Mills et al., 1990). Sγ probe was obtained by the amplification of a region spanning a segment between positions 1280–1546 of the Sγ flanking region (F. C. M. and E. E. M., unpublished data). A hybridization was performed overnight at 65°C, in 2× SSPE (1× SPPE= 20 mM phosphate buffer [pH 7.4], 300 mM NaCl, 2 mM EDTA), 200 μg/ml of denatured salmon sperm DNA (Sigma, St. Louis, Missouri), 1% SDS, and the Sγ PCR-generated probe labeled with ^{32}P using the random priming kit (Boehringer-Mannheim, Federal Republic of Germany). After hybridization, blots were sequentially washed: 30 min in 2× SSPE + 1% SDS at room temperature, 30 min in 2× SSPE + 1% SDS at 65°C, and 30 min in 2× SSPE + 1% SDS at 65°C. Blots were then autoradiographed from 5 hr to overnight.

Cloning

Sterile Iγ, Iα, and Iε cDNAs as well as circular DNA generated by PCR were cloned, either in a homemade pBluescript KS(+)–dd(T) vector (Stratagene, La Jolla, California) tailed with dideoxythymidine triphosphate using terminal transferase (Holton and Graham, 1990) or into the commercially prepared T vector PCRII (Invitrogen, San Diego, California). Cloned DNA was positively selected by hybridization to respective probes. For circular DNA clones, the selection was based on independent hybridizations to corresponding Sγ and Sμ cDNA probes, generated by PCR and ^{32}P-random priming, labeled as above.

DNA Sequencing

Reactions were performed using both Taq-DyeDeoxy termination, and dye-primer cycle sequencing kit (catalog number 401150, Applied Biosystems, Incorporated, Roissy, France) and analyzed on an Applied Biosystems automatic 373A DNA sequencer.

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