

Human Germinal Center B Cells Express the Apoptosis-inducing Genes Fas, c-myc, P⁵³, and Bax but Not the Survival Gene bcl-2

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Summary

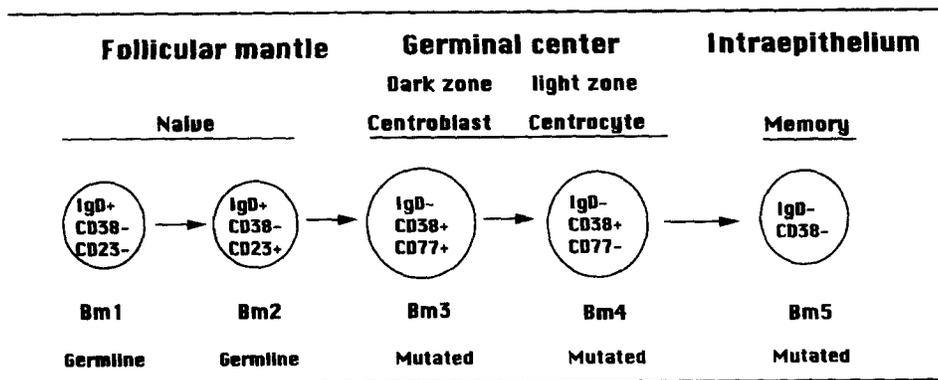
During T cell-dependent antibody responses, B cells within germinal centers (GC) alter the affinity of their antigen receptor by introducing somatic mutations into variable region of immunoglobulin (IgV) genes. During this process, GC B cells are destined to die unless positively selected by antigens and CD40-ligand. To understand survival/death control of germinal center B cell, the expression of four apoptosis-inducing genes, Fas, c-myc, Bax, and P⁵³, together with the survival gene bcl-2, has been analyzed herein among purified tonsillar naive, GC, and memory B cells. IgD⁺CD38⁻ naive B cells were separated into CD23⁻ (mature B cell [Bm] 1) subset and CD23⁺ (Bm2), IgD⁻CD38⁺ GC B cells were separated into subsets of CD77⁺ centroblasts (Bm3) and CD77⁻ centrocytes (Bm4), whereas IgD⁻CD38⁻ cells represented the Bm5 memory B cell subset. Sequence analysis of IgV region genes indicated that somatic hypermutation was triggered in the Bm3 centroblast subset. Here we show that bcl-2 is only detectable with naive (Bm1 and 2) and memory B cell (Bm5) subsets, whereas all four apoptosis-inducing genes were most significantly expressed within GC B cells. Fas was equally expressed in Bm3 centroblasts and Bm4 centrocytes, whereas Bax was most significantly expressed in Bm4 centrocytes. c-myc, a positive regulator of cell cycle, was most significantly expressed in proliferating Bm3 centroblasts, whereas P⁵³, a negative regulator of cell cycle, was most significantly expressed in nonproliferating Bm4 centrocytes. The present results indicate that the survival/death of GC B cells are regulated by the up- and downregulation of multiple genes, among which the expression of c-myc and P⁵³ in the absence of bcl-2 may prime the proliferating Bm3 centroblasts and nonproliferating Bm4 centrocytes to apoptosis.

During B lymphopoiesis in the bone marrow, the majority of cell death occurs at late pro-B to pre-B cell stage presumably because of the engagement of pre-B cell antigen receptors with autoantigens (negative selection), resulting in the elimination of autoreactive B cells (1, 2). During affinity maturation of antigen-specific B cell responses within germinal centers (GC)¹, extensive cell death occurs leading to the elimination of both low affinity and autoreactive B cells generated by somatic mutations (3–6). Remarkably, the expression of the survival protein was shown to be downregulated at these two stages of B cell development (7–10). In vitro experiments showed that whereas pre-B cells die only if their antigen receptors are triggered (11), GC B cells undergo rapid spontaneous apoptosis (12). This suggests that the diminished Bcl-2 expression in pre-B cells may facilitate apoptosis but does not directly lead to apoptosis. Thus, the mechanisms that con-

tribute to the propensity of GC B cells to undergo apoptosis remain to be determined.

Using a set of surface B cell markers (including IgD, CD23, CD38, and CD77), together with cell cycle analysis and IgV gene sequence analysis (13–15), human tonsillar B cells were separated into five B cell subsets (Fig. 1). Mature B cell development, during T cell-dependent responses, starts with sIgD⁺CD38⁻CD23⁻ resting unmutated naive B cells (mature B [Bm]1 subset). During early activation, Bm1 cells express early activation surface antigen CD23 and become IgD⁺CD38⁻CD23⁺ B cells (Bm2 subset) displaying unmutated IgV genes. Upon further T cell-dependent activation, some activated B cells migrate into B cell follicles and differentiate into IgD⁻CD38⁻CD77⁺ proliferating centroblasts (Bm3), which form the dark zone of GC where somatic mutation in IgV genes occurs. Centroblasts (Bm3) differentiate into IgD⁻CD38⁺CD77⁻ nonproliferating centrocytes (Bm4) that undergo positive selection based on the affinity of their mutated antigen receptors in the GC light zone. The positively selected high affinity

¹Abbreviations used in this paper: Bm, mature B; GC, germinal center(s); RT, reverse transcription.



centrocytes (Bm4) may undergo isotype switching (16) and differentiate into either plasma cells or into $\text{IgD}^- \text{CD38}^-$ memory B cells (Bm5) (17).

In this study, we have analyzed the apoptotic propensity of the five B cell subsets, together with the expression of four apoptotic-inducing genes (Bax, c-myc, P⁵³, and Fas) and a survival gene, bcl-2 (7–10). The Fas gene-encoded surface receptor is a member of the tumor necrosis factor receptor family. It is expressed on many activated lymphoid cells including GC B cells. Fas triggering on lymphocytes either by monoclonal antibody or by Fas-ligand induces rapid apoptosis. Natural mutation in either Fas gene or Fas-ligand gene results in abnormal lymphoproliferation in *lpr/lpr* autoimmune or in *gld/gld* mice (18, 19). The cellular protooncogene c-myc-encoded protein is a positive regulator of cell cycle progression. Expression of c-myc is required for apoptosis in many situations, including activation-induced apoptosis in T cell hybridomas and growth factor deprivation-induced apoptosis in factor-dependent cell lines (20, 21). The tumor suppressor gene P⁵³-encoded protein is a negative cell cycle regulator that arrests cells in G1. DNA damage within cells induced by ionizing radiation rapidly upregulates P⁵³ expression, which is required for the subsequent apoptosis of irradiated cells (22, 23). Bax is a member of an expanding bcl-2 gene family that has the ability to dimerize with itself or with Bcl-2 and promote apoptotic cell death (24).

Here, we present evidence that the molecular mechanism contributing to spontaneous apoptosis is turned on within GC centroblast (Bm3), possibly as a result of the high levels of expressions of c-myc, P53, Bax, and Fas in the absence of bcl-2.

Materials and Methods

Isolation of Five Tonsillar B Cell Subsets (Bm1–Bm5). Total B cells were prepared as previously described (13). Briefly, 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: (a) T cells were depleted by rosetting with sheep red blood cells; (b) the residual non-B cells were de-

pleted by T cell-specific antibodies (CD2, CD3, and 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 >98% CD19-positive B cells.

Some tonsil B cells were further separated into high density and low density B cells by centrifugation through 15, 60, and 65% Percoll gradient (Pharmacia/LKB, Uppsala, Sweden) at 400 g, 20°C for 20 min. Whereas dead cells remained on the surface of 15% Percoll, low density B cells were collected on the surface of 60% Percoll. High density B cells that penetrated through the 60% Percoll were recovered (13).

For isolation of Bm1 and Bm2 subsets, high density B cells were stained by goat anti-IgD-biotin (Sigma Chemical Co., St. Louis, MO) and by mouse anti-CD23-FITC (Serotec, Oxford, UK) for 30 min. After washing twice, $\text{IgD}^+ \text{CD23}^-$ (Bm1) and $\text{IgD}^+ \text{CD23}^+$ (Bm2) B cells were sorted on a cell sorter (Becton Dickinson, Sunnyvale, CA).

For isolation of Bm3 and Bm4, low density B cells were incubated with mouse anti-human CD38-PE (Becton Dickinson & Co., Mountain View, CA) and rat anti-human CD77 (Immunotech) for 30 min. After washing twice, cells were incubated with sheep anti-rat IgM-FITC for 30 min. The stained cells were washed and sorted into $\text{CD38}^+ \text{CD77}^+$ Bm3 and $\text{CD38}^+ \text{CD77}^-$ Bm4 subsets.

For isolation of Bm5 subset, total tonsillar B cells were incubated with goat anti-human IgD-biotin (Sigma Chemical Co.) and mouse anti-CD38 (Immunotech) for 30 min. After washing twice, the cells were incubated with streptavidin-PE and goat anti-mouse IgG-FITC for 30 min. After washing, $\text{sIgD}^- \text{CD38}^-$ Bm5 subset was sorted.

Quantitation of Spontaneous Apoptosis. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 80 $\mu\text{g}/\text{ml}$ gentamicin, and 2 mM glutamine (all from Flow Laboratories, Inc., McLean, VA) at 37°C for 16 h. 10^5 cells from each subset after culture were cytocentrifuged for 5 min at 500 rpm on a microscope slide. Slides were fixed in methanol for 5 min and then stained with Giemsa staining solution (BDH Chemicals, Ltd., Poole, UK) diluted 1:5 with distilled water (12).

Immunohistology. A frozen tonsillar section was stained with mouse anti-Ki67 proliferating antigen (Dako, Glostrup, Denmark), followed by the alkaline phosphatase-antialkaline phosphatase (APAAP) system. Two other serial sections were stained by mouse anti-Bcl-2 and mouse anti-Fas, respectively, followed

Figure 1. Tonsillar B cells are separated into $\text{IgD}^+ \text{CD38}^-$ naive B cells, $\text{IgD}^- \text{CD38}^+$ GC B cells, and memory B cells (15). Naive B cells are further separated into a CD23^- subset (Bm1) and a CD23^+ subset (Bm2). GC B cells are further separated into a CD77^+ centroblast subset (Bm3) and a CD77^- centrocyte subset (Bm4). Memory B cells represent the Bm5 subset. Previous IgV_H gene sequence analysis has indicated that the molecular mechanism underlying somatic mutation is triggered in the GC centroblast (Bm3) subset (15). Here we describe the analysis on the apoptotic propensity and the expression of survival/death genes in these five B cell subsets (Bm1–Bm5).

by sheep anti-mouse IgG1-biotin (for Bcl-2 staining) or sheep anti-mouse IgM-biotin (for Fas staining). The sections were then stained with ExtrAvidin-Peroxidase (Sigma Chemical Co.). After a final washing, peroxidase was developed by 3-amino-9-ethyl-carbazole which gives a red color, and alkaline phosphatase was developed by Fast blue substrate which gives a blue color (13).

RNA Preparation and Reverse Transcription. Isolation of total RNA was performed essentially as described by Chomczynski and Sacchi (25). For all reverse transcriptions (RT) except Bcl-2, the totality of RNA derived from 500 to 5,000 cells of each Bm subset was converted into single-stranded cDNA by a standard 40- μ l RT reaction using oligo-d(T)₁₂₋₁₈ (Pharmacia/LKB) and Superscript™ (RNaseH⁻MMLV reverse transcriptase; GIBCO BRL, Gaithersburg, MD) kit, according to the manufacturer's instructions, except that the denaturing step was changed from 70 (10 min) to 90°C (2 min). Bcl-2 mRNAs display extremely long 3' untranslated regions (21), a frequent limitation for full-length RT-primer extension. Therefore, for its amplification, oligo-d(T)₁₂₋₁₈ was substituted by the Bcl-2-specific RT-primer 5'CTTGTCGCTCAGATAGGCACCCAGGGTGATGCA3'.

RT-PCR. PCR was performed as described by Saiki et al. (26), with minor modifications. 5 μ l of the RT reaction, as described above, was amplified in a 100- μ l reaction mixture using 100 ng each of sense and antisense primers, 2.5 U of Taq Polymerase (Cetus, Norwalk, CT), and 5% DMSO. Other reaction conditions were exactly as those previously reported. The amplification primers used were as follows: (a) For Bcl-2 a sense primer 5'ATGGCGCACGCTGGGAGAACG3' was used in combination with the antisense primer 5'TTCCACAAAGGCATCCCA-GCC3'; (b) for Fas, 5'CTGCATCATGATGGCCAATTCTGC3' and 5'ATGACACTAAGTCAAGTTAAAGGC were used as the sense and antisense primers, respectively; (c) For Bax, the sense 5'ATGGACGGGTCCGGG GAGCAGCCC3' and the antisense 5'GGTGAGCACTCCCGCCACAAAGAT3' were used; (d) c-myc was amplified using the sense primer 5'GAGAGGCAG-AGGGAGCGAGCGGGC3' and the antisense primer 5'TGT-CGTTGAGAGGGTAGGGGAAGA3'; (e) P⁵³ was amplified with the sense primer 5'GACGGTGACACGCTTCCCTGGATT3' together with the antisense primer 5'GGGAACAAGAAGTGG-AGAATGTCA3'; and (f) IgH, using the consensus V_H sense primer 5'TCTGAGGTGCAGCTGGTGGAGTCTG3' in combination with a J_H antisense consensus 5'TGAGGAGACGGT-GACCAGGGTCCC3' primer. 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.

Southern Transfer and Hybridization. Alternatively, PCR products were transferred to nylon membranes and hybridized with a specific oligonucleotide probe located between the amplifying primers. The probe was labeled using the digoxigenin-dUTP kit (Boehringer Mannheim, Mannheim, Germany), and used according to the manufacturer's protocol. The probes used were: Bax, 5'GCCGTCCCAACCACCCTGGTCTTG3'; c-myc, 5'CCT-GGATGATGATGTTTTTATGA3'; P⁵³, 5'CCCTTT CTT-GCGGAGATTCTCTTC3'; and IgH, 5'AAGGCTTCTGGA-TACACCTTCACT3'.

Results

Both GC Centroblasts (Bm3) and Centrocytes (Bm4) Undergo Rapid Apoptosis In Vitro. Our previous study on five

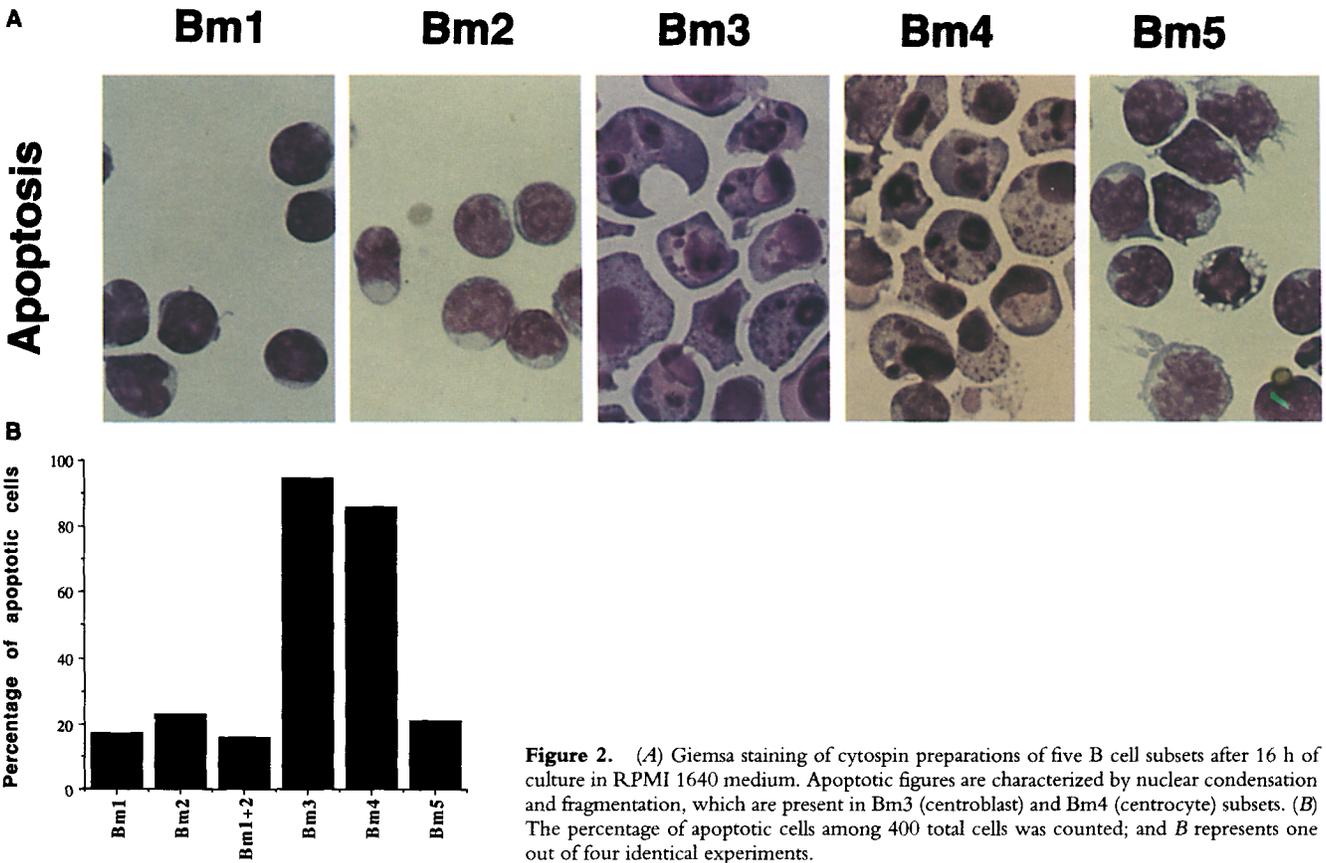
tonsillar B cell subsets showed the IgV_H genes from two naive B cell subsets Bm1 and Bm2 to be in germline configuration and the IgV_H genes from GC centroblasts Bm3, centrocytes Bm4, and memory B cells Bm5 to be mutated (15). To determine their propensity to enter into spontaneous apoptosis, B cells from each of the five subsets were cultured in medium for 16 h. Fig. 2 shows that >90% of GC centroblasts (Bm3) and centrocytes (Bm4) displayed apoptotic figures, whereas <10% of naive (Bm1, Bm2) and memory B cells (Bm5) displayed apoptotic figures after 16 h of culture. This indicates that the molecular mechanisms underlying both somatic mutation as well as spontaneous apoptosis are turned on within the GC dark zone (Bm3). In addition, spontaneous apoptosis is not terminated in the light zone (Bm4).

The Expression of bcl-2 and Fas Transcripts Correlates with the Expression of Their Proteins in GC B Cells. To determine the feasibility of analyzing the expression of survival/death-related genes by a RT-PCR, the bcl-2 and Fas transcripts were amplified among 500 B cells from each subset. Fig. 3 A shows that the Bcl-2 transcripts are selectively detected within Bm1, Bm2, and Bm5 subsets, but not within Bm3 and Bm4 subsets. In contrast, the Fas transcripts are detected within Bm3 and Bm4 subsets, but not in Bm1, Bm2, and Bm5 subsets. To control for the amplification sensitivity and intersubset purity, the mRNA for bcl-2 and Fas were also amplified from 1,000 and 5,000 cells, and the same patterns of expression were observed (data not shown). Equal amounts of RNA template per B cell subset were routinely verified by overall IgH chain RT-PCR, using consensus V_H-J_H primers (Fig. 3 B). Thus, the selective expression of Fas transcripts in GC Bm3 centroblasts, Bm4 centrocytes, and Bcl-2 transcripts in Bm1 and Bm2 naive B cells correlates with the patterns of Fas and Bcl-2 protein expression detected by immunohistology (Fig. 4) and flow cytometry (data not shown) (13). This established the validity of mRNA analysis in low numbers of cells from highly purified B cell subsets.

c-myc Is Predominantly Expressed in Bm3 Centroblasts, Whereas P⁵³ and Bax Are Predominantly Expressed in Bm4 Centrocytes. To investigate whether the expression of c-myc, P⁵³, and Bax is developmentally regulated during the differentiation pathway from naive B cells (Bm1 and 2) to GC B cells (Bm3 and 4) and then memory B cells (Bm5), their mRNA were amplified by RT-PCR, followed by Southern blot hybridization. Fig. 5 shows that c-myc is strongly expressed in GC centroblasts (Bm3) and weakly in GC centrocytes (Bm4) and memory B cells (Bm5). P⁵³ and Bax are mainly expressed in GC B cells, more importantly in centrocytes (Bm4) than in centroblasts (Bm3). Bax is also observed at low level in memory B cells (Bm5).

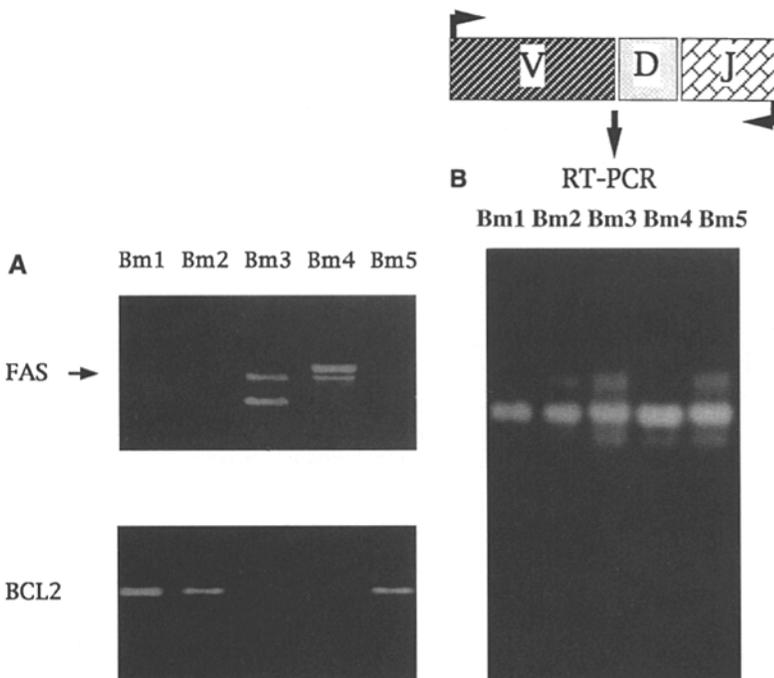
Discussion

The GC reaction is a hallmark of T cell-dependent antibody responses (3, 4, 27). It allows the rare antigen-specific B cells to expand rapidly and to increase the affinity of their antigen receptor by introducing somatic mutations into



their IgV genes, followed by affinity selection (28–31). In established GC, proliferation and somatic mutation seem to occur mainly in the GC dark zones, whereas affinity selection mainly occurs in the GC light zone (3, 4).

In agreement with these two functional compartments, *c-myc* gene, which is a positive regulator in cell cycle control (18, 19), was most significantly detected in proliferating centroblasts (Bm3) within the GC dark zone. The differen-



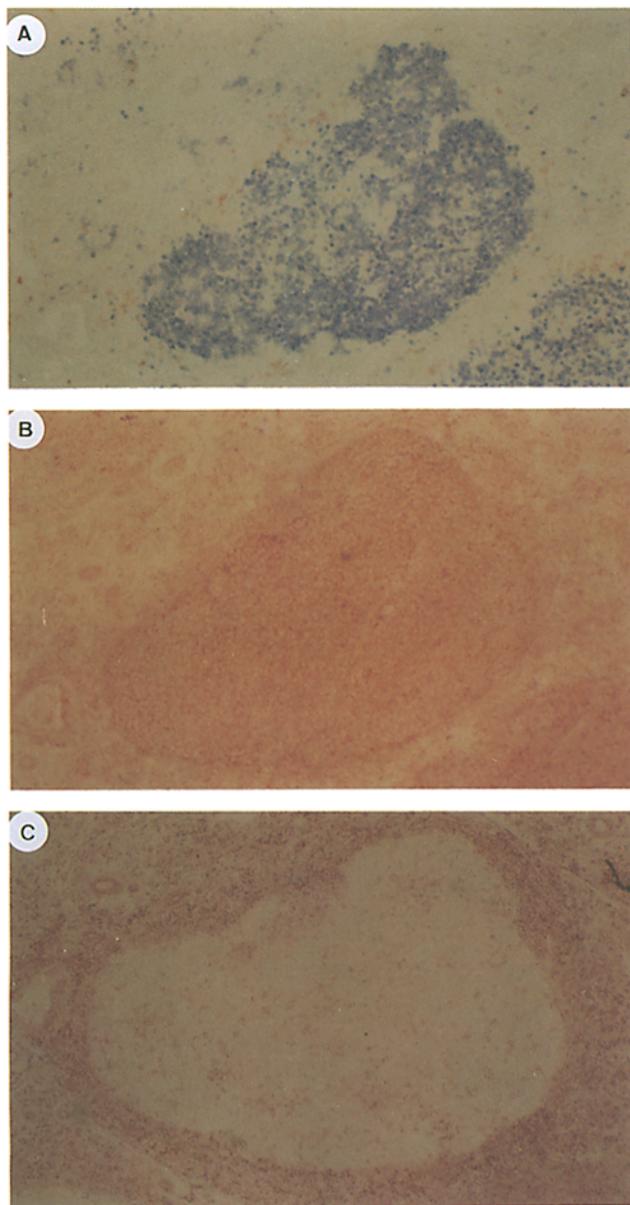


Figure 4. Expression of proliferating nuclear antigen Ki67, Fas, and Bcl-2 in situ on serial tonsil sections. (A) Blue Ki67 nuclear staining within a GC; (B) Red Fas staining within the same GC; and (C) most of the B cells within the same GC do not express Bcl-2.

tiation of proliferating centroblasts (Bm3) into nonproliferating centrocytes (Bm4) is associated with downregulation of *c-myc* gene expression. Since *c-myc* plays key roles in both proliferation and apoptotic cell death (20, 21), upregulation of *c-myc* expression in centroblasts may equally have two functional consequences: (a) it may confer the ability of centroblasts to proliferate at the rate of 6–10 h per division (3, 4); and (b) it may prime the highly proliferative centroblasts to cell death in the absence of *bcl-2* expression, to avoid the neoplastic transformation of GC centroblasts. In support of this hypothesis is the observation that trans-

fection of the *c-myc* gene into EBV-transformed B cells results in the generation of centroblast-like cells that express CD10 and CD38 but not CD23 or CD39 and become sensitive to spontaneous apoptosis (32).

An important experimental signal inducing P^{53} expression is DNA damage or single-stranded DNA generated by ionizing radiation. P^{53} induce G1 cell cycle arrest thereby allowing DNA repair or induction of cell death program (22, 23). As *c-myc* is dominant over P^{53} -mediated cell cycle arrest in mouse fibroblasts (23), the low expression of P^{53} in centroblasts (Bm3) and its further upregulation in centrocytes (Bm4) suggest that centroblasts (Bm3) may come out of cell cycle and differentiate into centrocytes (Bm4) as a result of downregulation of *c-myc* and upregulation of P^{53} . Upregulation of P^{53} in GC B cells may also directly contribute to the downregulation of *bcl-2* and upregulation of Bax within GC, because P^{53} is a direct transcriptional activator of Bax gene (33), as well as a transcriptional inhibitor of *bcl-2* gene (34).

Whereas our recent experiments showed that triggering Fas by monoclonal antibodies can accelerate the rate of spontaneous apoptosis of GC B cells (13), the function of Fas expression on GC B cells remains to be established. Fas-mediated apoptosis in both activated human and mouse B cells were shown to be prevented by antigen receptor triggering but not by T cell cytokines or CD40-ligand (35–37). Thus, Fas-ligand-expressing T cells may discriminate high affinity B cells from autoreactive or low affinity B cells by the occupancy of their antigen receptors with follicular-dendritic cell-bound antigens, and selectively kill autoreactive or low affinity B cells. In this context, upregulation of Fas on GC B cells may facilitate the rapid elimination of autoreactive or low affinity B cells generated by somatic hypermutation within GC. It will be important to determine if GC T cells or other GC cell types express Fas-ligand.

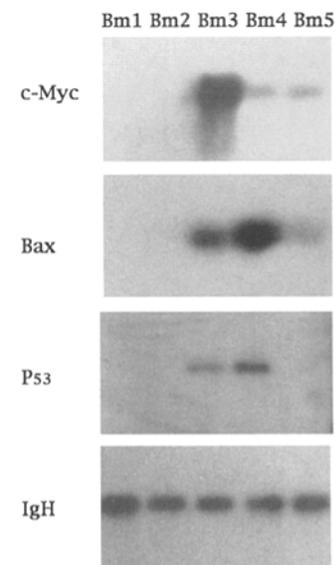


Figure 5. Bax, *c-myc*, P^{53} , and IgH RT-PCR determination on the five distinct Bm subsets. Shown here is the selective expression of Bax, *c-myc*, and P^{53} by corresponding Bm subsets, after amplification, Southern blotting, and hybridization (see Materials and Methods). Also depicted here, is the expression of overall Ig heavy chain (*IgH*), systematically used as an internal control to evaluate equivalent mRNA input per B cell subset.

In conclusion, by selective B cell subset gene expression analysis, our data show that the genes related to cell cycle, apoptosis, and DNA repair are tightly regulated during the affinity maturation of peripheral B cells. Such regulation

results in a high rate of GC B cell proliferation, which favors the introduction of point mutations into IgV region genes, and promotes the propensity of GC B cell to die, thus allowing for positive selection of high affinity mutants.

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