Bcl-2$^+$ Tonsillar Plasma Cells Are Rescued from Apoptosis by Bone Marrow Fibroblasts

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Summary

Plasma cells represent the final stage of B lymphocyte differentiation. Most plasma cells in secondary lymphoid tissues live for a few days, whereas those in the lamina propria of mucosa and in bone marrow live for several weeks. To investigate the regulation of human plasma cell survival, plasma cells were isolated from tonsils according to high CD38 and low CD20 expression. Tonsillar plasma cells express CD9, CD19, CD24, CD37, CD40, CD74, and HLA-DR, but not CD10, HLA-DQ, CD28, CD56, and Fas/CD95. Although plasma cells express intracytoplasmic Bcl-2, they undergo swift apoptosis in vitro and do not respond to CD40 triggering. Bone marrow fibroblasts and rheumatoid synoviocytes, however, prevented plasma cells from undergoing apoptosis in a contact-dependent fashion. These data indicate that fibroblasts may form a microenvironment favorable for plasma cell survival under normal and pathological conditions.

One of the most striking facts of the immune system is the presence of several milligrams of Ig (antibody) in every milliliter of human blood, which represents 10^{16} molecules displaying 10^9 different specificities. Antibodies are secreted by plasma cells at a rate of 10^3 antibody molecules/cell per s (1, 2). Plasma cells represent the final differentiation stage of B lymphocytes after they have encountered antigens and interacted with various cell types through both membrane molecules and cytokines (for review see reference 3). To avoid overproduction of antibodies, the number of plasma cells in the body must be tightly controlled. Two basic strategies have evolved within the immune system to control plasma cell number: (*a*) during humoral immune responses, not all activated B cells undergo terminal differentiation into antibody-secreting plasma cells, as many of them differentiate into memory B cells (4); and (*b*) the life span of plasma cells is tightly regulated. It has been shown that plasma cells generated during primary humoral immune responses are mainly located within the medullary cords of lymph nodes or in the red pulp of the spleen and have a life span of only a few days (5, 6). During secondary humoral immune responses, however, some generated plasma cells will migrate from the secondary lymphoid tissues into the bone marrow (BM)[1] or into the lamina propria of mucosa (7), where they survive and secrete large amounts of antibodies for at least 3 wk (5).

Understanding the physiology of normal plasma cell survival represents an important question for both basic immunology and for pathophysiology of malignant myeloma and plasmacytoma. Although plasma cells have a distinct morphology, it has been difficult to isolate them from peripheral lymphoid tissues because of the lack of specific surface markers and their low frequency. After having isolated human B cell subsets representing naive B cells, germinal center (GC) B cells and memory B cells from tonsils (8–10), we examined the isolation and characterization of plasma cells (PC), the end point of B cell differentiation. The isolation was based on the observation that human plasma cells express high levels of CD38 and low levels of CD20 (11–13). As reported herein, human tonsillar plasma cells, which express intracellular Bcl-2 protein and lack surface Fas/CD95, die rapidly by apoptosis, a phenomenon that could not be prevented by antigen receptor or CD40 trig-

P. Merville and J. Déchanet contributed equally to this work.

[1] *Abbreviations used in this paper:* BM, bone marrow; GC, germinal center; PC, plasma cells; SAC, *Staphylococcus aureus* strain Cowan I; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP–FITC nick end labeling.

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gering. Plasma cells, however, were rescued from apoptosis when cultured in contact with bone marrow fibroblasts.

Materials and Methods

Antibodies and Reagents. The mouse mAbs used for the phenotypic studies were purchased from the following sources: FITC-conjugated anti-CD3 (IOT3), anti-CD19 (IOB4), anti-CD20 (IOB20), anti-CD37 (IOB1), anti-Fas (UB2), unconjugated anti-CD9 (IOB2), and biotinylated anti-CD24 (IOB3) (Immunotech, Marseille, France); FITC-conjugated anti-CD10 (Calla), anti-CD14 (Leu M3), anti–HLA-DR, anti–HLA-DQ, and PE-conjugated anti-CD38 (Leu 17) (Becton Dickinson Monoclonal Center, Mountain View, CA); FITC-conjugated anti-CD28 (CLB, Amsterdam, The Netherlands); FITC-conjugated anti-Bcl-2 (Dako, Glostrup, Denmark); unconjugated anti-CD56 (Coulter Immunology, Hialeah, FL); unconjugated anti-CD74 (BU45) (Binding Site, Birmingham, UK). FITC-conjugated anti-CD40 (mAb 89) was produced in the laboratory as previously described (14). Anti–IL-6 receptor gp80-blocking antibody BR6 (Innotest, Besançon, France) was used at 10 mg/ml. Anti-CD40 mAb used for cultures (G28.5) was kindly provided by Dr. E. Clark (University of Washington, Seattle, WA) (15). For ELISA, alkaline phosphatase–conjugated rabbit anti-IgA and anti-IgG antibodies were from Dako and anti-IgM antibody from Biosys (Compiègne, France). Formalinized particles of *Staphylococcus aureus* strain Cowan I (SAC) were from Calbiochem-Behring Corp. (La Jolla, CA) and were used at 0.05%.

TNF-α was purchased from Genzyme (Cambridge, MA) and was used at a final concentration of 2.5 ng/ml. IL-2 (Amgen Biologicals, Thousand Oaks, CA) was used at 10 U/ml. IL-3, IL-4, IL-6, and IL-10 (all from Schering-Plough Research Institute, Kenilworth, NJ) were used at 10 ng/ml, 50 U/ml, 40 ng/ml, and 100 ng/ml, respectively.

Isolation of Plasma Cells from Tonsils. Total tonsillar cell suspension was obtained by dilacerating tonsils with tweezers and digesting the remaining tissue twice with 1 mg/ml collagenase and 0.1 U/ml DNase (both from Sigma Chemical Co., St. Louis, MO) in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) at 37°C for 20 min. To remove small cells (red cells and small resting cells), the cellular suspension was centrifuged three or four times (10 *g*, 20 min, 4°C) on PBS 1.5% BSA (Miles Inc., Kankakee, IL). Plasma cells were enriched in the pellet. Cells were then labeled at 4°C with PE-conjugated anti-CD38 mAb either alone or in combination with FITC-conjugated anti-CD20 mAb. PC, together with GC and resting B cells used as comparison, were sorted with a FACStar Plus® (Becton Dickinson & Co., Sunnyvale, CA) equipped with a 4-W argon laser. Sorting was carried out at 4°C.

Flow Cytometric Analysis. Phenotypic analysis of plasma cells was performed by immunofluorescence flow cytometry on sorted $CD38^{high}$ PC (stained with anti-CD38-PE), using either mouse mAbs conjugated with FITC or biotinylated or unconjugated mouse mAbs followed by FITC-conjugated sheep anti–mouse IgF(ab)'2 or FITC-conjugated streptavidin. Negative controls were performed with isotype-matched, unrelated mAbs. For intracellular detection of Bcl-2 protein, cells were permeabilized by 15-min incubation at 4°C in saponin (0.5 mg/ml) before staining. Fluorescence was analyzed on a FACScan® flow cytometer (Becton Dickinson & Co.). Gating was set according to forward and right angle light scatter parameters to exclude subcellular particles from acquisition data.

Giemsa Staining and Immunoenzymatic Staining of Ig Light Chains. 5×10^4 cells from each sorted population were cytocentrifuged for 4 min at 400 rpm on slides. Some of these slides were fixed in methanol for 1 min and stained with Giemsa solution (BDH Ltd., Poole, UK) diluted 1:10 in water for 10 min. For immunoenzymatic stainings, some slides were fixed in cold acetone at −20°C for 10 min, washed in PBS, and incubated for 30 min with rabbit anti-κ and anti-λ mAbs (Dako), both diluted 1:40 in PBS. After washing, slides were incubated with biotinylated goat anti–rabbit Ig antibodies (Biosys) diluted 1:100 in PBS for 30 min, washed, and incubated with alkaline phosphatase coupled to streptavidin (Biosource International Inc., Camarillo, CA) at 1.5 mg/ml. After washing, enzymatic activity was revealed with fast red substrate (Dako), yielding a red precipitate.

Preparation of Bone Marrow Fibroblasts and Rheumatoid Synoviocytes. Human femur fragments, obtained after informed consent of adult donors undergoing orthopedic hip surgery, were dilacerated to yield a BM cell suspension. Characterization and isolation of BM fibroblasts was previously described (16). Briefly, bone fragments were decanted and cells subsequently seeded in complete MEM for 7–10 d, and nonadherent cells were removed every 3 d. Cells of hematopoietic origin were then removed by exposure of stromal cells to a cocktail of appropriate mAbs followed by depletion with immunomagnetic beads coated with anti–mouse IgG. Antibody-depleted stromal cells were seeded in 96-well plates and allowed to reach confluence, at which time stromal cell layers were fibroblast-like cells with occasional adipocytes and were used for cocultures with sorted tonsillar cells.

Synoviocytes were isolated from synovial biopsies, obtained from rheumatoid arthritis patients undergoing knee or wrist synovectomy, as previously described (17). They were a homogenous population of fibroblast-like cells, negative for the expression of CD3, CD19, CD14, and HLA-DR, and positive for the expression of CD44, CD10, and CD54, as determined by flow cytometry analysis. The human lung fibroblast cell line MRC5 (American Type Culture Collection, Rockville, MD) was established from 14 week-old male fetuses, and has been used widely as feeder cells to transform human B cells with EBV (18). This cell line expresses CD10.

Cell Cultures. After sorting, cells were cultured in RPMI 1640 supplemented with 10% FCS (GIBCO BRL), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Flow Laboratories, Inc., McLean, VA). The final volume was 100 μl (2×10^4 cells/well of flat-bottomed 96-well plates) for viable cell counting, Ig production assays, and Giemsa stainings; and 500 μl (2.5×10^5 cells/well of 24-well plates) for electron microscopy and terminal deoxynudeotidyl transferase (TdT) assays. Anti-CD40 (G28.5, 1 μg/ml) or SAC particles (0.05%) were added at the onset of the culture. For cocultures, sorted B cells were dispensed on confluent monolayers of (*a*) BM fibroblasts, (*b*) rheumatoid synoviocytes, (*c*) lung fibroblast cell line (MRC5), or (*d*) mouse L cells. IgA, IgM, and IgG levels were determined in supernatants by standard ELISA techniques, as described earlier (19). For viable cell recovery, cells were counted by trypan blue dye exclusion. In the case of the cocultures, B cells were easily distinguishable from fibroblasts by size.

Electron Microscopy. Cells were harvested and pelleted in 2% glutaraldehyde in culture medium and consecutively fixed in 2% glutaraldehyde–Na cacodylate HCl 0.1 M, pH 7.4 (15 min at 4°C). After three washes in Na cacodylate HCl 0.1 M, pH 7.4, sucrose 0.2 M (15 min at 4°C), cells were postfixed in OsO_4 1%–Na cacodylate HCl 0.15 M, pH 7.4 (15 min at 4°C), dehydrated in a graded series of ethanols, and embedded in Epon. Ultrathin

sections (60–80 nm) were obtained with an ultramicrotome (Ultracut; Reichert, Vienna, Austria) contrasted with methanolic uranyl acetate and lead citrate, and observed with a transmission electron microscope (model CM 120; Philips Technologies, Cheshire, CT).

TdT-mediated dUTP–FITC Nick End Labeling. DNA fragmentation in apoptotic cells was detected according to the method described before (20). Briefly, 5×10^5 cells obtained immediately after sorting or after 4 h of culture were fixed with 200 μl of PBS, 1% paraformaldehyde, for 10 min, then washed in PBS and permeabilized with 500 μl of 70% ethanol at −20°C overnight. After washing with PBS, the TdT-mediated dUTP–FITC nick end labeling (TUNEL) reaction was carried out by incubating cells at 37°C for 1 h with 0.3 nmol FITC-12-dUTP, 3 nmol dATP, 2 μl 25 mM $CoCl_2$, 25 U TdT, and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) in a total reaction volume of 50 μl (all reagents from Boehringer Mannheim Biochemicals, Indianapolis, IN). The reaction was stopped by adding 2 μl 0.5 M EDTA for 10 min at 4°C. After washing twice in PBS, 1% BSA, samples were analyzed by flow cytometry. Control staining was performed on aliquots of the same cells treated with the staining mixture without TdT.

Immunohistology. Tonsil pieces were snap frozen in liquid nitrogen and stored at −70°C. 5-μm frozen sections were cut and mounted on glass slides. They were dried at room temperature and fixed in cold acetone at −20°C for 15 min. Sections were washed in PBS and were incubated with peroxydase-conjugated anti-IgA (4 mg/ml; Southern Biotechnology Associates, Bir-

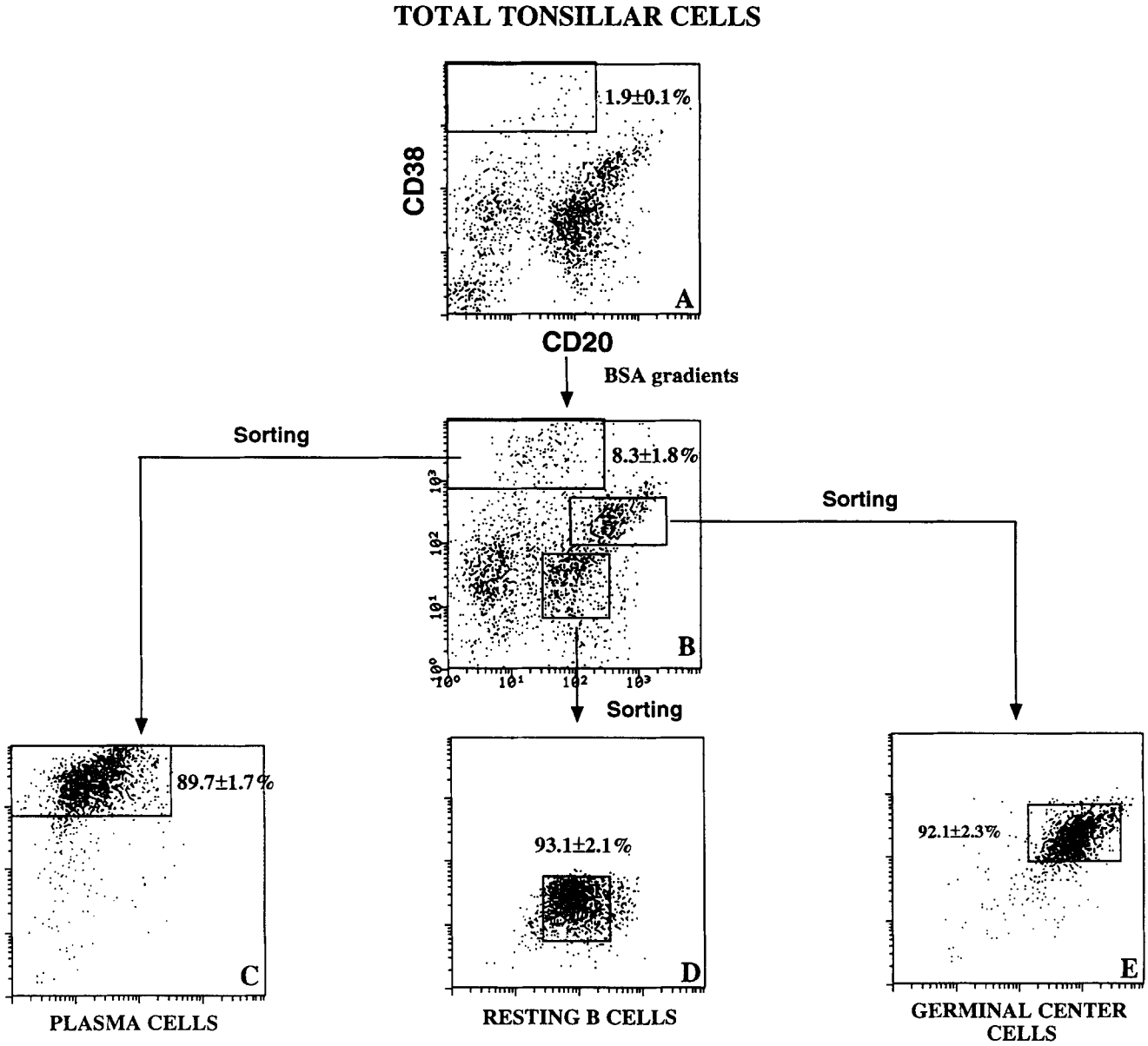

Figure 1. Isolation of the $CD38^{high}CD20^{low}$ tonsillar PC. Total tonsillar cells obtained either directly after tonsil digestion (*A*) or after three centrifugations over a 1.5% BSA solution (*B*) were stained with FITC-conjugated anti-CD20 (*horizontal axis, log scale*) and PE-conjugated anti-CD38 mAbs (*vertical axis, log scale*). $CD38^{high}CD20^{low}$ PC (*C*), $CD38^{low}CD20^{medium}$ resting B cells (*D*), and $CD38^{medium}CD20^{high}$ GC B cells (*E*) were sorted with a FACStar plus®. Numbers indicate the percentages of cells obtained in the corresponding inset (mean ± SEM of seven independent experiments).

mingham, AL) and unconjugated mouse anti-CD38 mAb (Immunotech) diluted 1:100 in PBS. After washes, slides were incubated with sheep anti–mouse Ig (Binding Site) diluted 1:20 in PBS containing 10% human serum. Slides were then washed and incubated with mouse mAbs against alkaline phosphatase and alkaline phosphatase complexes (APAAP; Dako). After a final wash, peroxidase was developed with 3-amino-9-ethylcarbazole, which gives a red color, and alkaline phosphatase was developed by fast blue substrate, which gives a blue color.

Results

The CD38^{high} CD20^{low} Fraction of Tonsillar Cells Represents Terminally Differentiated PC. Previous studies have characterized human PC as CD38^{high}- and CD20^{low}-expressing cells (11, 13). Cells with this phenotype were found to represent only 1–2% of tonsillar cells (Fig. 1 *A*). Centrifugations of the tonsil cell suspension over a 1.5% BSA solution at 10 *g* permitted us to enrich CD38^{high}CD20^{low} cells up to ~8% (Fig. 1 *B*). This enrichment step subsequently allowed us to isolate CD38^{high}CD20^{low} cells by FACS® sorting (Fig. 1 *C*). From the same tonsillar preparation (Fig. 1, *D* and *E*), CD38^{medium}CD20^{high} GC B cells and CD38^{low}-CD20^{medium} resting B cells (8, 9) were also collected and analyzed in parallel with PC.

After Giemsa staining, CD38^{high}CD20^{low} cells display a typical PC morphology, including an eccentric nucleus, a dark basophilic cytoplasm, and a pale Golgi compartment (Fig. 2 *A*). In accordance with the morphological features of PC, immunoenzymatic staining with anti-Igκ and anti-Igλ antibodies showed that the sorted CD38^{high}CD20^{low} cells contained high levels of intracytoplasmic Igs (Fig. 2 *B*), in contrast to GC B cells and resting B cells (data not shown). Electron microscopic study of sorted CD38^{high}CD20^{low} cells reveals ultrastructural characteristics of PC showing parallel arrays of rough endoplasmic reticulum, a distinct juxtanuclear Golgi area, and many mitochondrias (Fig. 2 *C*).

CD38^{high}CD20^{low} Tonsillar Plasma Cells Have a Distinct Surface Phenotype. To determine tonsillar PC surface phenotype, these cells were sorted according to their very high expression of CD38 (fluorescence intensity above log 10^3, Fig. 1) and were further stained with FITC-conjugated mAbs. PC expressed B cell markers such as CD9, CD19, CD24, CD37, CD40, CD74, and HLA-DR, but did not express CD10 nor HLA-DQ (Fig. 3). By contrast to plasmacytoma cells (21) and myeloma cells (22), CD28 and CD56 were not detectable on tonsillar PC. The absence of CD3$^+$ and CD14$^+$ cells confirmed the lack of T cells and monocytes in the PC population.

Human Tonsils Contain Many IgA-secreting PC beneath the Mucosal Epithelium. To functionally prove that the sorted CD38^{high}CD20^{low} cells are PC, they were cultured for 12 h, and the spontaneous Ig secretion into culture medium was measured by ELISA. As shown in Fig. 4, only PC were able to produce significant amounts of IgA (mean ± SEM = 214 ± 37 ng/ml, n = 3) and IgG (293 ± 89 ng/ml), but low levels of IgM (29 ± 9 ng/ml). The comparable levels of secreted IgG and IgA were surprising since the majority of tonsillar B cells express sIgG but not sIgA (10). Nevertheless, the isotype distribution in the culture supernatant correlated with that obtained after intracytoplasmic staining of sorted PC (data not shown). Furthermore, double im-

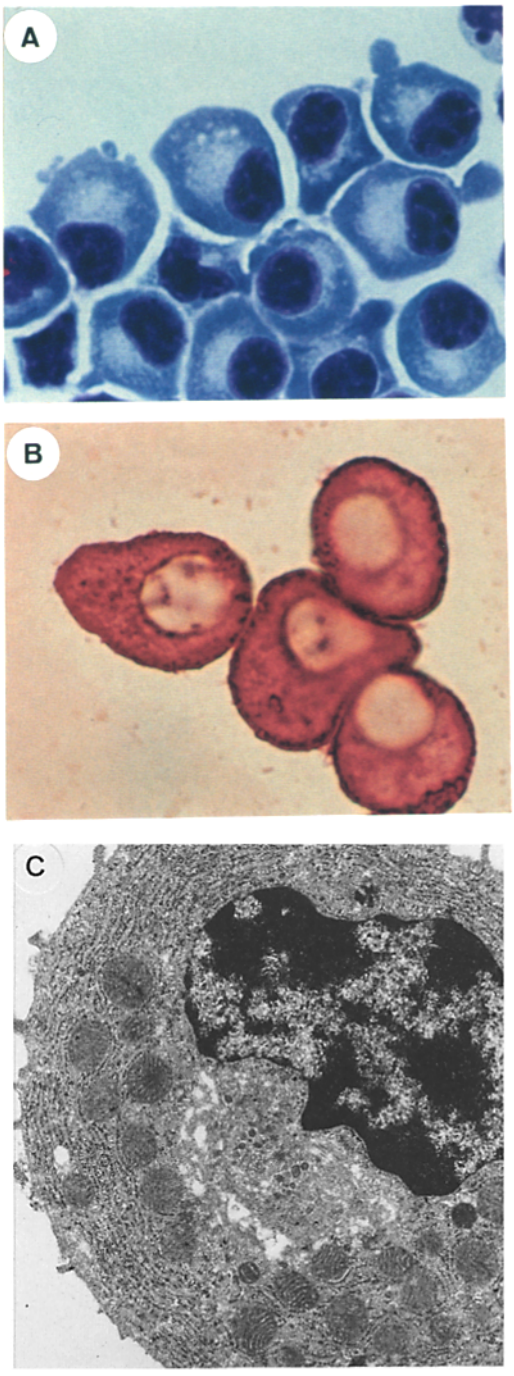

Figure 2. The CD38^{high}CD20^{low} tonsillar cells have morphological features of PC and contain high levels of Ig. (*A*) Giemsa staining of CD38^{high}CD20^{low} tonsillar PC displaying an eccentric nucleus, a dark basophilic cytoplasm, and a pale Golgi compartment. ×1,000. (*B*) Immunoenzymatic red staining with anti-κ and anti-λ antibodies showing that CD38^{high}CD20^{low} PC contain high levels of Ig. ×1,000. (*C*) Electron microscopic observation of a CD38^{high}CD20^{low} PC showing parallel arrays of endoplasmic reticulum. ×11,800.

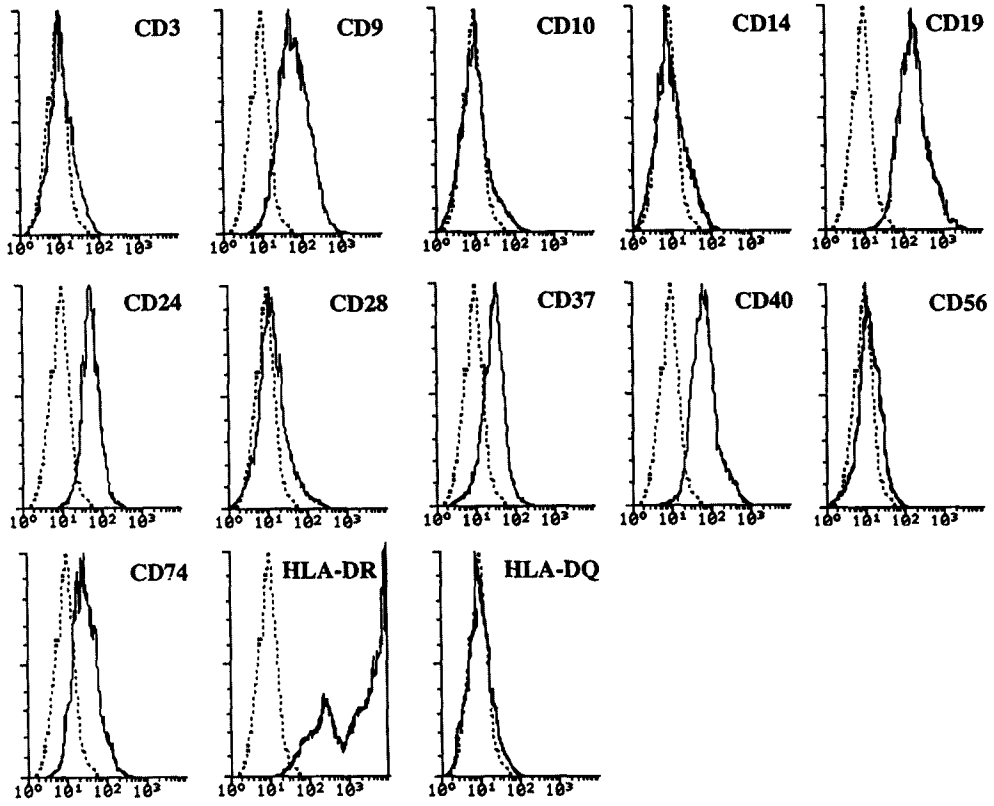

Figure 3. Surface phenotype of tonsillar PC. The surface phenotype of PC isolated by FACS® sorting according to their high CD38 expression (stained with anti–CD38-PE) was analyzed by FITC-labeled mouse mAbs. Histograms corresponding to each mAb (*solid line*) are superimposed on that of the isotype-matched unrelated mAb (*dashed line*). Horizontal and vertical axes illustrate log of fluorescence and relative cell numbers, respectively.

munohistological staining of tonsil sections with red anti-IgA and blue anti-CD38 further confirmed that ~40–50% of tonsillar CD38^{high} PC contained intracellular IgA (Fig. 5). Whereas most IgA$^+$ PC (*dark purple*) were found beneath the mucosal epithelium (Fig. 5 *A*) and occasionally in the interfollicular areas (not shown), CD38^{high} PC (*dark blue*) found in GC were IgA$^-$ (Fig. 5 *B*). The biological significance of such an anatomical distribution of PC in relation to their life span and survival is discussed later.

Tonsillar PC Die Rapidly by Apoptosis During In Vitro Culture. During our attempts to estimate survival of normal PC, we found that they died very rapidly in culture medium at 37°C. As shown in Fig. 6, PC lost their viability even more rapidly than did GC B cells, since only 22% of viable PC were recovered after 6 h and <5% after 24 h of culture. As expected (23), >60% of resting B cells remained viable after 24 h of culture. After 4 h of culture, numerous PC displayed apoptotic figures, with typical chromatin condensation and nuclear fragmentation, as revealed by Giemsa staining (Fig. 7 *A*) and electron microscopy (Fig. 7 *B*). The presence of parallel arrays of rough endoplasmic reticulum within the cytoplasm indicated that the apoptotic cells indeed derived from PC (Fig. 7 *B*). To provide molecular evidence that PC undergo apoptosis, a TUNEL method was used to detect DNA fragmentation (20). As shown in Fig. 8, only a small proportion of freshly sorted cells including resting B cells, GC B cells, and PC were labeled by dUTP-FITC. Strikingly, after only 4 h of culture, the majority of GC B cells (66.7%) and PC (80.3%) were labeled by dUTP-FITC, whereas only 12.2% of resting B cells were labeled.

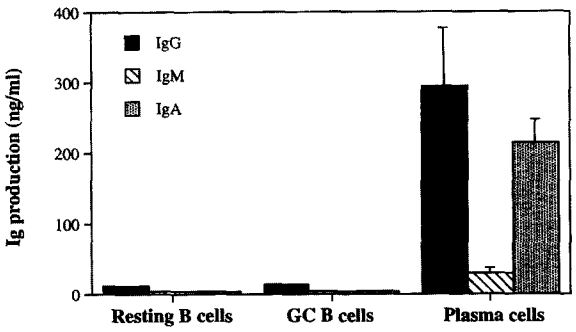

Figure 4. Tonsillar PC mainly release IgG and IgA. 2 × 10^4 sorted resting B cells, GC B cells, and PC were cultured in medium alone for 12 h, and IgA, IgM, and IgG levels were measured in supernatants by ELISA. Results are expressed as mean ± SEM of three separate experiments.

Tonsillar PC Express Bcl-2 but Not Fas/CD95. The Bcl-2 gene product (24) and Fas/CD95 (25, 26) have been documented as playing important roles in the regulation of cell survival and death. Since the rapid onset of apoptosis in GC B cells correlates with their low expression of Bcl-2 (27) and high expression of Fas/CD95 (10, 28), the expression of intracellular Bcl-2 and surface Fas/CD95 was assessed by flow cytometry in PC in parallel with that of GC B cells and resting B cells. Interestingly, a large proportion of PC expressed Bcl-2 without displaying Fas/CD95 (Fig. 9), a finding apparently in contrast with their propensity to rapidly enter apoptosis.

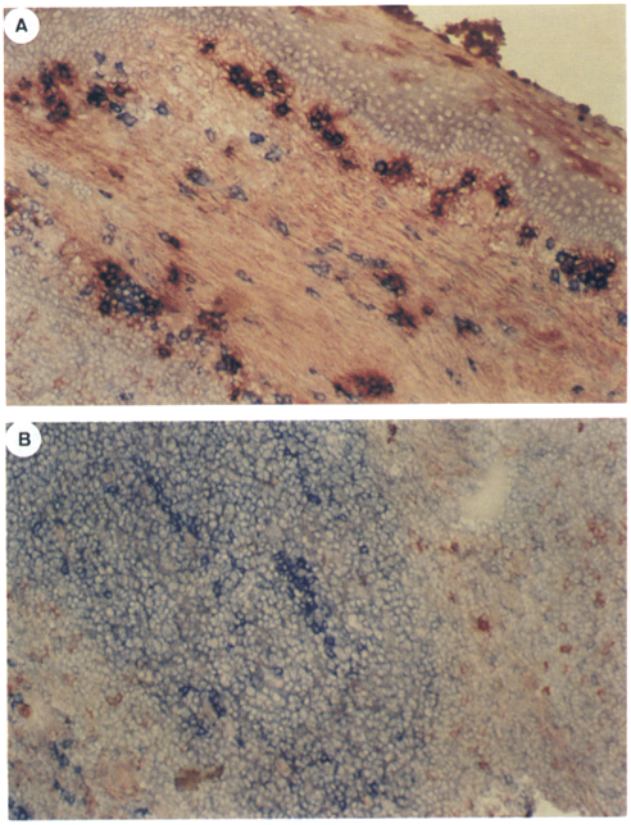

Figure 5. Anatomical localization of tonsillar IgA^+ PC and IgA^- PC. Tonsil sections were double stained with red anti-IgA and blue anti-CD38. (*A*) many double stained (*dark purple*) IgA^+CD38^{++} PC were found within the fibroblast networks beneath the basal mucosal epithelial cells. (*B*) the PC cells within a GC of the same section contain many single-stained (*dark blue*) IgA^-CD38^{++} presumptive PC. ×100.

Plasma Cells Are Rescued from Apoptosis by Contact with BM Fibroblasts but Not by Antigen Receptor or CD40 Triggering. Table 1 shows that SAC particles and anti-CD40 mAb, which markedly enhance the viability of GC B cells (23), are unable to sustain PC viability. Similarly, CD40 ligand–transfected L cells, anti-Ig antibodies, and cytokines involved in B cell activation (IL-2, IL-3, IL-4, IL-10, and TNF-α) were unable to maintain the viability of PC (data not shown). In addition, IL-6 at a concentration up to 250 ng/ml did not prevent PC death (Table 2). Together with the different patterns of Bcl-2 and Fas/CD95 expression of GC B cells and PC, these results indicate that the apoptosis/survival of both cell types is regulated through different mechanisms.

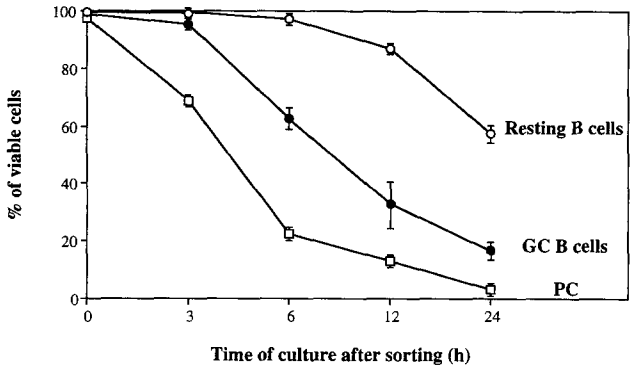

Figure 6. Tonsillar PC die rapidly in culture. 2×10^4 sorted resting GC B cells and PC were cultured in medium alone for 24 h. Percentage of viable cells was determined at various time points by trypan blue dye exclusion. Results are expressed as mean ± SD of culture triplicates.

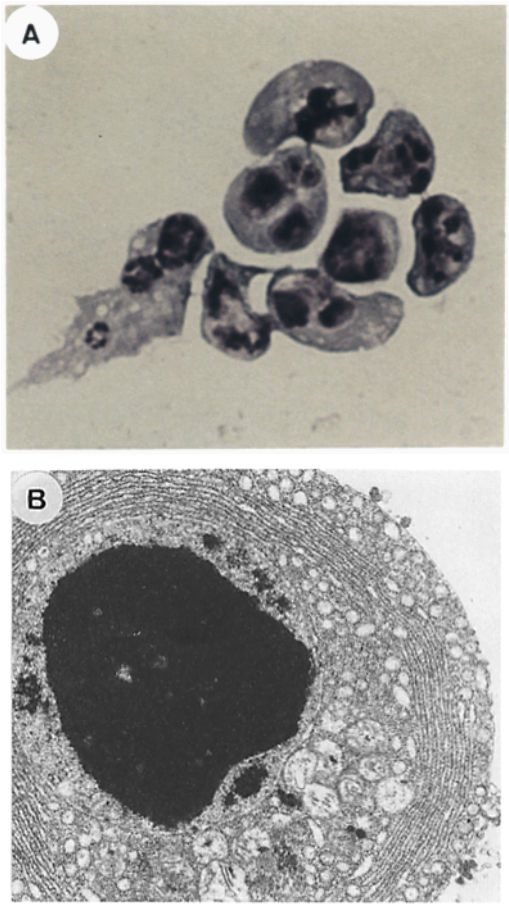

Figure 7. The dead PC show apoptotic figures. 2×10^4 tonsillar PC were cultured for 4 h in culture medium. (*A*) Giemsa staining showing apoptotic PC with nuclear condensation and fragmentation. ×1,000. (*B*) An apoptotic PC with chromatin condensation shows arrays of rough endoplasmic reticulum under the electron microscope. ×11,800.

In vivo experiments, however, have shown that a proportion of PC generated within the secondary lymphoid organs migrates into BM (29) or lamina propria of the mucosa, where they survive for 3 wk (5). This suggests that survival signals for PC may be dependent on these microenvironments. In accordance with this hypothesis, purified human BM fibroblasts greatly improved the viability of cultured plasma cells (82.7% on fibroblasts vs. 16.3% in medium alone, Table 1) as well as GC B cells (89.5% of viable cells on fibroblasts vs. 27.5% in medium alone, Table 1). Since the inflammatory rheumatoid synovium is an ectopic site for PC accumulation (30), synovial fibroblasts (synoviocytes) were compared with BM fibroblasts for their

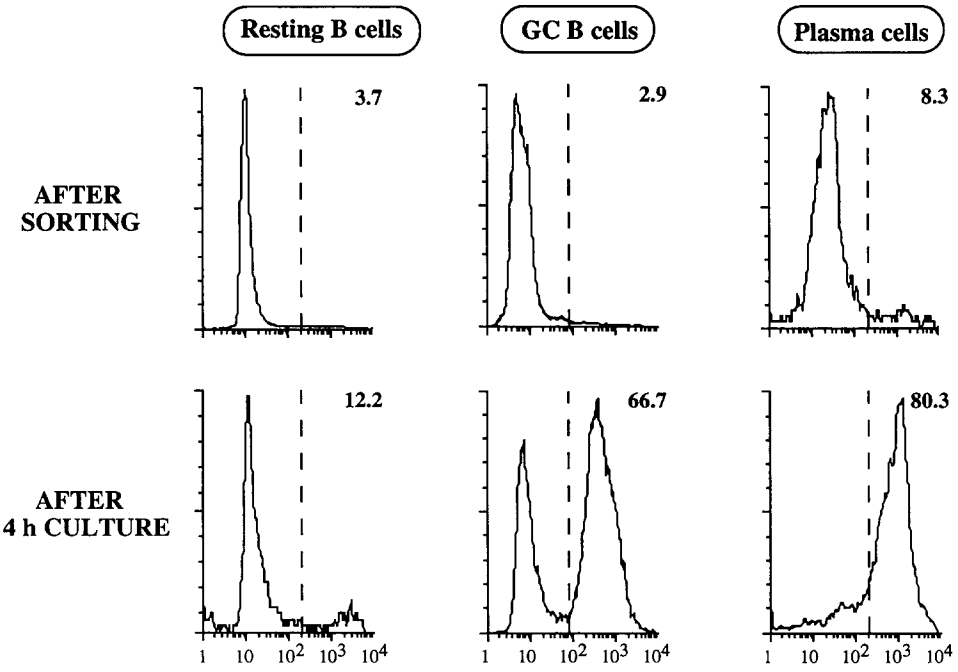

Figure 8. Detection of DNA fragmentation within apoptotic PC. PC, GC B cells, and resting B cells isolated from the same tonsils were cultured for 4 h in culture medium. The DNA fragmentation within these cells was analyzed by the TUNEL method (see Materials and Methods) before and after 4 h of culture. The maximal dUTP–FITC labeling of cells without TdT reaction are indicated by the dotted lines as negative controls. Horizontal and vertical axes illustrate log of fluorescence and relative cell number, respectively. Data shown are from one representative of three experiments.

ability to maintain PC survival. Table 1 also shows that synoviocytes were able to potently rescue GC B cells and PC from apoptosis. The maintainance of PC survival by fibroblasts was dependent upon cell–cell contact, since PC died rapidly when separated from fibroblasts by a semipermeable membrane (data not shown).

To further investigate if the fibroblast-dependent PC survival is specific to BM and synovial fibroblasts, PC were also cultured on a monolayer of human lung fibroblasts or a monolayer of murine fibroblasts (L cells) for 12 h. Table 1 shows that human lung fibroblasts significantly enhance the viability of both PC and GC B cells, whereas the murine L cell line did not. The survival effect of human lung fibroblasts on PC is, however, always lower than that of either BM fibroblasts or synoviocytes.

In contrast with the recent study showing that IL-6 protects human BM PC from apoptosis (31), IL-6 and a wide range of cytokines used in the present study (IL2, IL3, IL4, IL10, TNF-α) did not protect tonsillar PC from apoptosis. To further investigate if fibroblast-dependent PC survival was mediated by IL-6 released by fibroblasts during a close cell–cell interaction, functional blocking antibodies against the IL-6 receptor gp80 chain were added into the coculture of PC and fibroblasts. Table 2 shows that anti–IL-6 re-

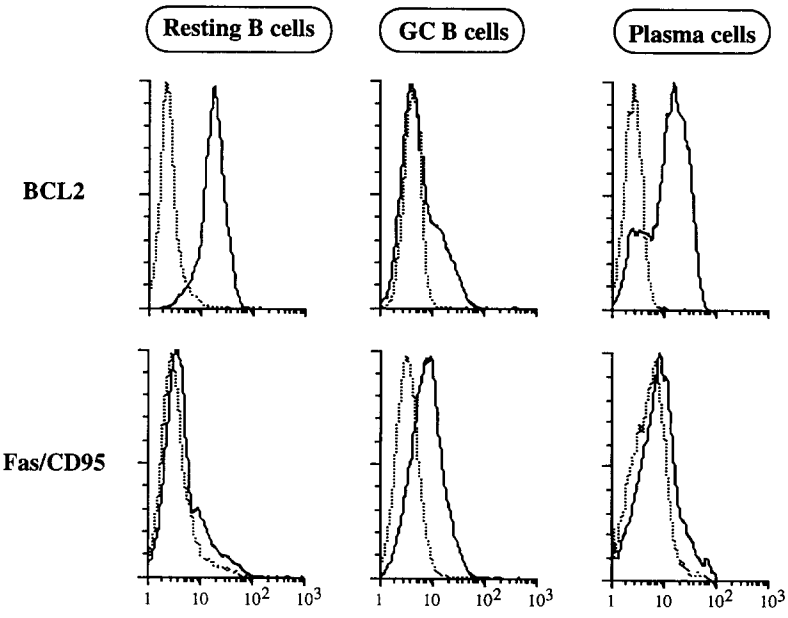

Figure 9. Tonsillar PC express intracytoplasmic Bcl-2 but not surface Fas/CD95. PC, GC B cells, and resting B cells were stained with FITC-conjugated anti-Fas/CD95 mAb or with FITC-conjugated anti–Bcl-2 mAb after permeabilization of the cells with saponin. Histograms corresponding to each mAb (*solid line*) are superimposed on that of the negative control (*dashed line*) performed with an isotype-matched unrelated mAb. Horizontal and vertical axes illustrate log of fluorescence and relative cell number, respectively.

Table 1. *PC Are Rescued from Apoptosis by Fibroblasts*

	Percentage of viable cells		
	Resting B Cells	GC B cells	PC
Medium	81.6 ± 3.8	27.5 ± 0.5	16.3 ± 0.5
SAC	70.2 ± 10.9	45.3 ± 5.2	17.7 ± 1.0
Anti-CD40	74.3 ± 6.2	44.2 ± 5.9	17.2 ± 0.2
BM fibroblasts	97.4 ± 0.9	89.5 ± 0.7	82.7 ± 5.3
Synoviocytes	97.2 ± 0.7	72.5 ± 12.2	63.4 ± 8.6
Murine fibroblasts	ND	31.2 ± 2.6	21.6 ± 1.1
Lung fibroblasts	ND	76.4 ± 1.7	51.6 ± 0.3

2×10^4 resting B cells, GC B cells, or PC were cultured in medium alone for 12 h or in the presence of SAC particles (0.05%), anti-CD40 mAbs (G28.5, 5 μg/ml), and/or a monolayer of fibroblasts. Percentages of viable cells were determined by trypan blue dye exclusion.
*Mean ± SEM of three experiments.

ceptor antibodies did not block the fibroblast-dependent PC survival, suggesting that human tonsillar PC may be different from BM PC in terms of IL-6–mediated survival.

Discussion

Differentiation of B lymphocytes into PC mostly occurs within secondary lymphoid organs, where they represent a minor population compared with naive B cells or GC B cells. Thus, our knowledge about PC is mainly derived from the phenotypic and functional studies of PC tumors, such as myelomas and plasmacytomas. Herein, we designed a procedure to purify PC from human tonsils by sorting $CD38^{high}CD20^{low}$ cells after enrichment by repeated centrifugations through a BSA gradient.

Surface phenotypic analysis shows that tonsillar PC express several pan–B cell markers such as CD19, CD37, and CD40. The expression of CD19 on PC is in contrast with the reported lack of CD19 on myeloma and plasmacytoma cell lines, which had led to a conclusion that normal PC may not express CD19 (32). CD19, however, was also recently found on normal PC isolated from BM (22, 33) and on those generated in vitro (17, 34). Thus, normal PC can be distinguished from malignant PC by their expression of CD19. In the same line, CD28 is expressed on plasmacytoma (21) and myeloma cells (33), but not on normal PC. Accordingly, CD28 may represent a potential marker for diagnosis of malignant PC tumors, and its function on malignant PC remains to be established. The expression of CD40 antigen on tonsillar PC complements the similar observation on BM PC (33) and myeloma cells (35). The function of CD40 antigen on PC remains to be determined as its ligation failed to rescue them from apoptosis (36).

Table 2. *The Fibroblast-dependent Plasma Cell Survival Is Independent of IL-6*

	Percentage of viable cells	
	PC	GC
Plastic		
Medium	23.6 ± 2.9	44.4 ± 3.7
IL-6	27.3 ± 0.2	44.2 ± 0.1
Synoviocytes		
Medium	76.5 ± 3.8	86.8 ± 2.2
Anti-IL-6 R	70.5 ± 4.1	ND

Culture conditions are described in the legend for Table 1. IL-6 was used at a final concentration of 40 ng/ml. Anti-IL-6 receptor gp80 antibody BR6 was used at 10 μg/ml. Data shown are from one representative of two independent experiments.

A major finding of this study is the propensity of PC to undergo spontaneous apoptosis. The molecular regulation of PC apoptosis/survival appears different from that of GC B cells. First, PC express the intracytoplasmic Bcl-2 protein at a level comparable to that of resting B cells, whereas GC do not. The spontaneous apoptosis of $Bcl\text{-}2^+$ PC suggests that other survival/death genes may be involved (25). The Bcl-2 gene family presently contains at least seven genes (37), which represent control elements of the first checkpoint of apoptosis (38). A second checkpoint of apoptosis has recently been identified which is controlled by members of the IL-1β–converting enzyme gene family, including ICE, ICH-1, and CPP32 (38–40). It will therefore be interesting to determine the expression of these genes in PC either undergoing apoptosis or being rescued by fibroblasts. Second, the surface triggers involved in PC apoptosis/survival are obviously different from that of GC B cells. In contrast to GC B cells, PC do not express surface Fas/CD95, which triggering has been shown to induce apoptosis of activated B cells and T cells (41–43) and accelerate spontaneous apoptosis of GC B cells (10). Signals known to rescue GC from apoptosis, such as antigen receptor and CD40 triggering, had no effect on PC. However, direct contact of PC with BM or synovial fibroblasts represents an efficient survival signal for these cells. Interestingly, human lung fibroblasts, which are efficient to maintain GC B cell survival (44), were able to enhance PC survival to a limited extent. In addition, IgA^+ PC were found mostly within the connective tissue beneath the mucosa (Fig. 5). All these data suggest that fibroblasts and/or stromal cells may provide the microenvironments beneath the mucosa, within the BM and the rheumatoid synovium, that are favorable for the survival of plasma cells (45, 46).

This finding is in accordance with a number of studies showing that GC B cells, peritoneal B cells, thymocytes, and T cells can be protected from rapid apoptosis by stromal cells or fibroblasts (18, 44, 47, 48). Although the molecular mechanism of stroma/fibroblast–mediated cell survival is currently unknown, these data suggest that PC survival depends on direct cell–cell contact, but not on cytokines such as IL-2, IL-3, IL-4, IL-6, IL-10, and TNF-α.

In conclusion, we have isolated normal tonsillar PC that

display a rapid onset of apoptosis and a unique regulation of apoptosis/survival. Analysis of the surface and intracellular molecules that control PC survival may ultimately permit us to understand the pathogenesis of myeloma and plasmacytoma.

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