RAPID COMMUNICATION

BCL-6 Protein Is Expressed in Germinal-Center B Cells

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Structural alterations of the 5' noncoding region of the BCL-6 gene have been found in 40% of diffuse large cell lymphoma (DLCL) and 5% to 10% of follicular lymphomas (FL), suggesting that deregulated BCL-6 expression may play a role in lymphomagenesis. Nucleotide sequencing of BCL-6 cDNA predicted a protein containing six zinc-finger domains, suggesting that it may function as a transcription factor. Using antisera raised against N- and C-terminal BCL-6 synthetic oligopeptides in immunoprecipitation, immunoblot, and immunocytochemical assays, this study identifies the BCL-6 gene product as a 95-kD nuclear protein. Western blot analysis of human tumor cell lines representative of various hematopoietic lineages/stages of differentiation showed that the BCL-6 protein is predominantly expressed in the B-cell lineage where it was found in mature B cells. Immunohistochemical analysis of normal human lymphoid tissues indi-

cated that BCL-6 expression is topographically restricted to germinal centers including all centroblasts and centrocytes. The BCL-6 protein was also detectable in inter- and intrafollicular CD4+ T cells, but not in other follicular components including mantle-zone B cells, plasma cells, dendritic cells, and macrophages. Immunohistochemical analysis of DLCL and FL biopsy samples showed that the BCL-6 protein is detectable in these tumors independent of the presence of BCL-6 gene rearrangements. These results indicate that the expression of the BCL-6 gene is specifically regulated during B-cell differentiation and suggest a role for BCL-6 in germinal center development or function. Because DLCL derive from germinal-center B cells, deregulated BCL-6 expression may contribute to lymphomagenesis by preventing postgerminal center differentiation.

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THE BCL-6 GENE has been identified by virtue of its involvement in chromosomal translocations affecting chromosome 3q27 in diffuse large cell lymphoma (DLCL).[1-4] The same gene was then found to be rearranged in ≈40% of DLCL and 5% to 10% of follicular lymphomas (FL), including cases with cytogenetically normal 3q27.[5-7] The rearrangement breakpoints cluster within a 4-kb region spanning the BCL-6 promoter sequences and the first noncoding exon, and result in the fusion of BCL-6 coding sequences (exons 2-10) to heterologous promoters from other chromosomes,[5,8,9] presumably leading to the deregulated expression of the BCL-6 protein.

Analysis of BCL-6 cDNA sequences predicted a 706-amino acid protein with six C-terminal zinc-finger motifs similar to those of Krüppel-type zinc finger transcription factors.[10] Its N-terminus contains a POZ domain found in other zinc-finger transcription factors including the Drosophila developmental regulator protein Tramtrack (ttk) and Broad-complex (Br-c),[11,12] the human KUP, PZLF, ZID proteins,[13] as well as in proteins (eg, VA55R) of the poxvirus family.[14] In the ZID protein, this domain has been shown to act as a specific protein-protein interaction domain capable of regulating DNA binding by the zinc-finger domain.[15] Based on the presence of these functional domains and on the preferential expression of BCL-6 RNA in the transformed cell lines derived from the B-cell lineage, it has been suggested that BCL-6 may function as a DNA-binding transcription factor involved in the regulation of B-cell proliferation and/or differentiation.

This study explores the function of BCL-6 in B-cell development and lymphomagenesis by using specific antisera to identify the BCL-6 protein and investigate its pattern of expression in normal and neoplastic lymphoid tissues. The results indicate that BCL-6 is a nuclear protein selectively expressed in mature B cells within normal germinal centers as well as in their transformed counterparts in DLCL and FL biopsies.

MATERIALS AND METHODS

Tissues and cell lines. Lymph nodes and tonsils, obtained during elective surgery for nonneoplastic diseases, were half snap-frozen

in precooled isopentane (Fisher, Pittsburgh, PA), and the remaining half fixed in buffered 10% formalin (Fisher) and embedded in paraffin. Bone marrow smears and cell suspensions free from hematopoietic diseases and solid tumor infiltration were obtained during periodic monitoring or at initial diagnostic evaluation of four patients. Cell lines, purchased from ATCC (American Type Culture Collection, Rockville, MD) and cultivated in 5% CO_2 at 37°C in exponential growth, were cytocentrifuged on clean glass slides. Frozen tissue samples and air-tight plastic foil-wrapped cytospins and smears were stored at −80°C. Non-Hodgkin's lymphoma (NHL) biopsy samples were obtained from the Department of Pathology of the Memorial Sloan-Kettering Cancer Center. NHL were classified according to the Working Formulation. The molecular and cytogenetic characterization of these cases were previously reported.[16]

Two cell lines (the BCL-6 RNA-negative 293 epithelial cell line and the Burkitt lymphoma cell line EB3, which express traces of BCL-6 RNA), were used for transfections. The 293 cell line was transiently transfected by electroporation with the pMT2T-BCL-6 plasmid, which was constructed by inserting the full-length coding

domain of a BCL-6 cDNA in the pMT2T vector.[17] The EB3 cell line was stably transfected either with the episomally replicating plasmid pHeBo-CMV-BCL-6, which expresses the full-length coding region of a BCL-6 cDNA under the control of a cytomegalovirus enhancer/promoter element, or with a control-plasmid lacking BCL-6 sequences. Control and BCL-6–transfected EB3 cells were grown in Iscove's modified Dulbecco's medium containing 10% fetal calf serum (FCS), penicillin (100 IU/mL), streptomycin (100 μg/mL), and G418 (1.4 mg/mL). After transfection, cells were characterized for BCL-6 RNA and protein expression by Northern and Western blot analysis, respectively.

Northern blot analysis. RNA from fresh tissues and cell suspensions was extracted by the guanidine isothiocyanate method.[18] RNA was then electrophoresed through 0.9% agarose-2.2 mol/L formaldehyde gels and transferred to nitrocellulose filters. Hybridization was performed in 50% formamide, 3× standard saline citrate (SSC), 10% dextran sulfate, 5× Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS) at 37°C for 16 hours. BCL-6 cDNA[5] that had been ^{32}P-labeled by the random priming technique[19] was used as a probe. Filters were washed in 0.2× SSC-0.5% SDS at 60°C for 2 hours and autoradiographed for 24 to 48 hours at −80°C using intensifying screens.

Western blot and immunoprecipitation analysis. Proteins were extracted from exponentially growing cell lines, subjected to gel electrophoresis, transferred to nitrocellulose filters, and immunostained according to published methods.[19,20]

Immunohistochemistry. Cryostat sections and cytospins were processed as previously described[21] with modifications. Briefly, air-dried slides were fixed in acetone (Fisher; 10 minutes at room temperature [RT]), dried, fixed in buffered 10% formalin (10 minutes at RT), rinsed in 0.05 mol/L phosphate-buffered saline (PBS), pH 7.5, and fixed in cold methanol (Fisher; 10 minutes at −20°C). The slides were then washed in 0.05 mol/L Tris-buffered saline, pH 7.5, 0.01% Tween-20 (TBS) before incubation with a blocking 3% human AB serum followed by overnight exposure to the appropriate primary antibody. Slides were then washed twice in TBS, incubated with a biotin-labeled goat-antirabbit antibody (Dako, Carpintera, CA; 1:300) for 45 minutes, washed twice in TBS, and then overlayed with horseradish peroxidase-conjugated avidin (Dako; 1:300, 20 minutes) and washed again. The slides were developed in aminoethyl carbazole (Sigma, St Louis, MO) and counterstained with hematoxylin. Alternative fixation methods (acetone 10 minutes at RT; buffered 10% formalin 10 minutes at RT; cold methanol at −20°C for 10 minutes; acetone followed by methanol) were compared with the above-described acetone-formaline-methanol fixation. Double-staining of sections and cytospins was performed by applying the primary mouse monoclonal antibody after the formalin fixation step (45 minutes at RT). The slides were then washed, fixed in methanol, and incubated with the rabbit antibody. Two-color immunohistochemistry was performed as published.[21] Antisera and cell lines were coded and the results were scored in blind.

Antibodies. Two synthetic peptides, composed of 15 amino acids and corresponding to the COOH terminus (KVQYRVSATDLPPEL) and the N-terminus (ASPADSCIQFTRHAK) of the BCL-6 protein, were synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits. A 1:1,000 working dilution in 0.05 mol/L PBS (pH 7.5), 1% bovine serum albumin, was found to be optimal for these antisera. Absorption of the antiserum with the corresponding peptide was performed by overnight incubation of the antiserum at 1:1,000 working dilution with 100 mmol/L peptide solution.

The following monoclonal antibodies (MoAbs) (CD number/clone name) were also used: CD1a/L404, CD3/UCHT1, CD68/KiM6, KiM4b,[22,23] CD2/MT910, CD20/L26, CD23/MHM6, bcl-2/124, CD30/BerH2 (Dako), CD21/HB5, CD38/HB7, CD69/L78, IgD/ TA4.1 (Becton Dickinson, Rutherford, NJ), CD34/QBEnd10, CD70/

HNE51 (Immunotech, Marseille, France), CD79/mb-1 (D.Y. Mason, Oxford, UK),[24] MIB 1 (J. Gerdes, Borstel, Germany).[25]

The method used for the production of the MoAb PG-B6 and its characterization are reported elsewhere.[26]

RESULTS

Identification of the BCL-6 protein by specific antisera. Two anti-BCL-6 antisera, N-71-6 (anti–N-terminus) and C-73-6 (anti–C-terminus), were tested for their ability to recognize the BCL-6 protein in: (1) cell lines expressing (Ly-1, BJAB) or lacking (RD) BCL-6 RNA; and (2) in a cell line (EB-3) which originally expressed only traces of BCL-6 RNA and was stably transfected with a vector containing a full-length BCL-6 cDNA (EB-3-CMV-BCL-6) or a control plasmid vector (EB-3-CMV). Figure 1, A and B, shows that a 95-kD protein was detectable by immunoblot analysis using N-70-6 only in cells expressing BCL-6 RNA or transfected with the BCL-6 expressing vector. Similar results were obtained with C-73-6 (not shown), although this antiserum was significantly less effective in Western blot assays. Figure 1C shows that the same 95-kD protein is detected by both antisera by immunoprecipitation-PAGE analysis of Ly-1 cells. The reactivity of these two antisera was considered specific because it could be inhibited by preabsorbtion with their respective cognate peptides.

The same antisera were then used in the immunocyto-chemistry analysis of BJAB, Ly-1, RD cells, and 293 cells transiently transfected with a control- (pMT2T) or a BCL-6–expressing (pMT2T-BCL-6) vector. Figure 2 shows that, using C-73-6, a clear nuclear staining was obtained only in BCL-6–expressing (Fig 2A) or BCL-6–transfected cells (Fig 2C), but not in RD or control-vector–transfected cells (Fig 2, B and D). No staining was seen by using a pre-immune serum (C-73-0; not shown) or when C-73-6 was preabsorbed with the cognate peptide (not shown). Fixation in formalin followed by methanol (see Materials and Methods) was found to be necessary for optimal detection of the antigen by C-73-6, whereas other fixation methods gave a decreased immunoreactivity and a higher background staining. Analogous patterns of staining were obtained using: (1) N-70-6; (2) a commercially available antiserum (C19; Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide partially overlapping the one used to generate C-73-6; or (3) an MoAb raised against the N-terminal half of the BCL-6 protein (MoAb PG-B6; see Materials and Methods). The N-70-6 and the commercial antiserum were less specific than both C-73-6 and the MoAb, because they both displayed additional nonspecific staining in the cytoplasm and in the nucleus (not shown).

Figure 2 also shows that BCL-6 is localized exclusively in the nucleus except in mitotic cells, where the chromosomes were unstained and the immunoreactivity was restricted to the cytoplasm in a pulverulent fashion (Fig 2A). The nuclear staining pattern consisted in a multitude (>50) of fine granules in a delicate mesh of interlacing fibrils distributed throughout the entire nucleus, but sparing the nucleoli. The granularity of the staining ranged from very fine to coarse varying from cell to cell and among cell lines, correlating with variations in the chromatin pattern.
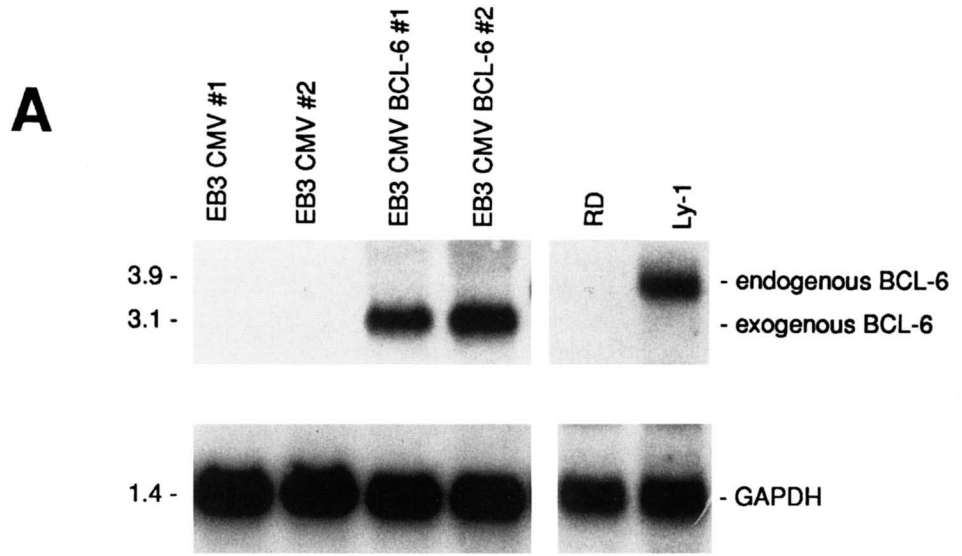
Fig 1. Identification of the BCL-6 gene protein product by specific antisera. (A) Northern blot analysis of RNA extracted from the RD (Epstein-Barr virus [EBV]-immortalized lymphoblastoid), Ly-1 (DLCL) cell lines, or from the EB3 cell line stably transfected with the control vector (EB3 CMV#1, EB3 CMV#2), or with a vector expressing a full-length BCL-6 cDNA (EB3 CMV BCL-6 #1, EB3 CMV BCL-6 #2). A 3.9-kb band, representing endogenous BCL-6 transcripts, can be seen in the Ly-1 cell line. In EB3 cells transfected with the BCL-6 vector, the 3.1-kb mRNA species corresponding to the size of the transfected cDNA transcript is also detectable. Hybridization of the same filter to a GAPDH probe is shown at the bottom as a control for RNA loading. (B) Western blot analysis of the same cell lines using the N-70-6 antiserum. A 95-kD band corresponding to the BCL-6 protein is detectable in lysate from Ly-1 and BCL-6 transfected EB3 cells, but not in RD and control vector-transfected EB3 cells. The bands below 66 kD are caused by nonspecific reactivity of the secondary antiserum. (C) Immunoprecipitation-polyacrylamide gel electrophoresis analysis of Ly-1 cell lysates using the N-70-6 and C-73-6 antisera (lanes +). Cognate peptide competition shows the specificity of the reaction (lane −).
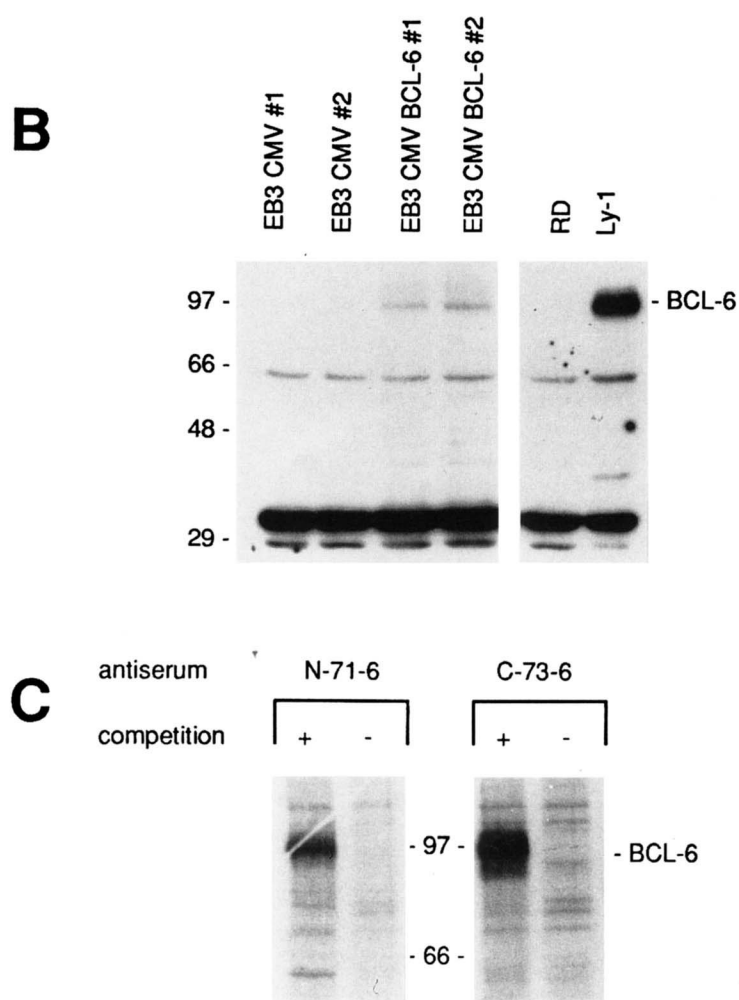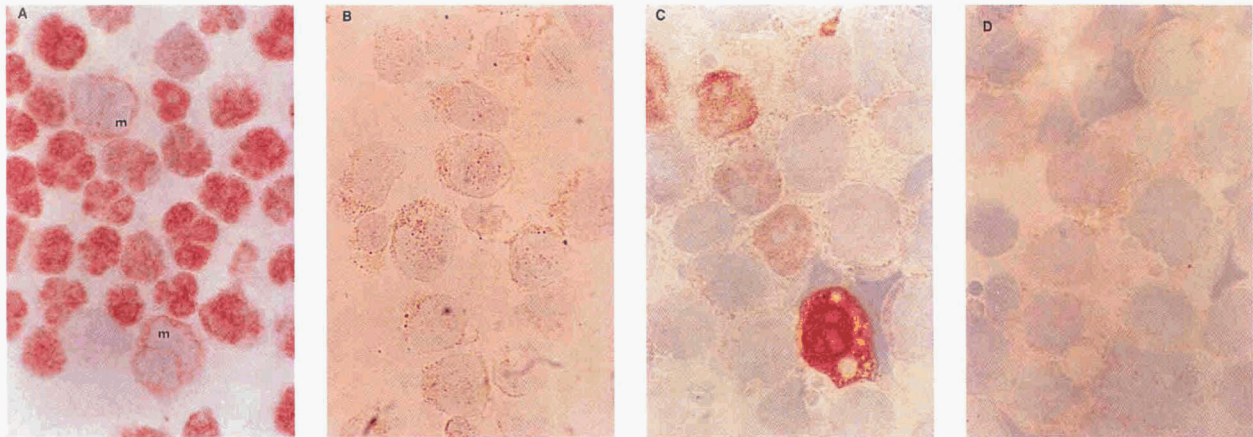
Fig 2.   Immunocytochemical analysis of the BCL-6 protein. (A) Cytocentrifuged BJAB cells stained by the C-73-6 antiserum. Nuclear staining is detectable in most cells, except for mitotic cells (m), which show cytoplasmic staining. (B) RD cells, which lack BCL-6 RNA (Fig 1A), are not stained by the C-73-6 antiserum. (C) 293 cells transiently transfected with the pMT2T-BCL-6 vector are stained by the C-73-6 antiserum. Reactivity is detectable only in the minority of cells because of the low efficiency of transfection of this cell line. (D) 293 cells line transfected with the pMT2T control vector are not stained by the C-73-6 antiserum. In all panels amino-ethyl-carbazol (AEC) immunostain was used with light hematoxylin counterstain. Original magnification × 1,630.
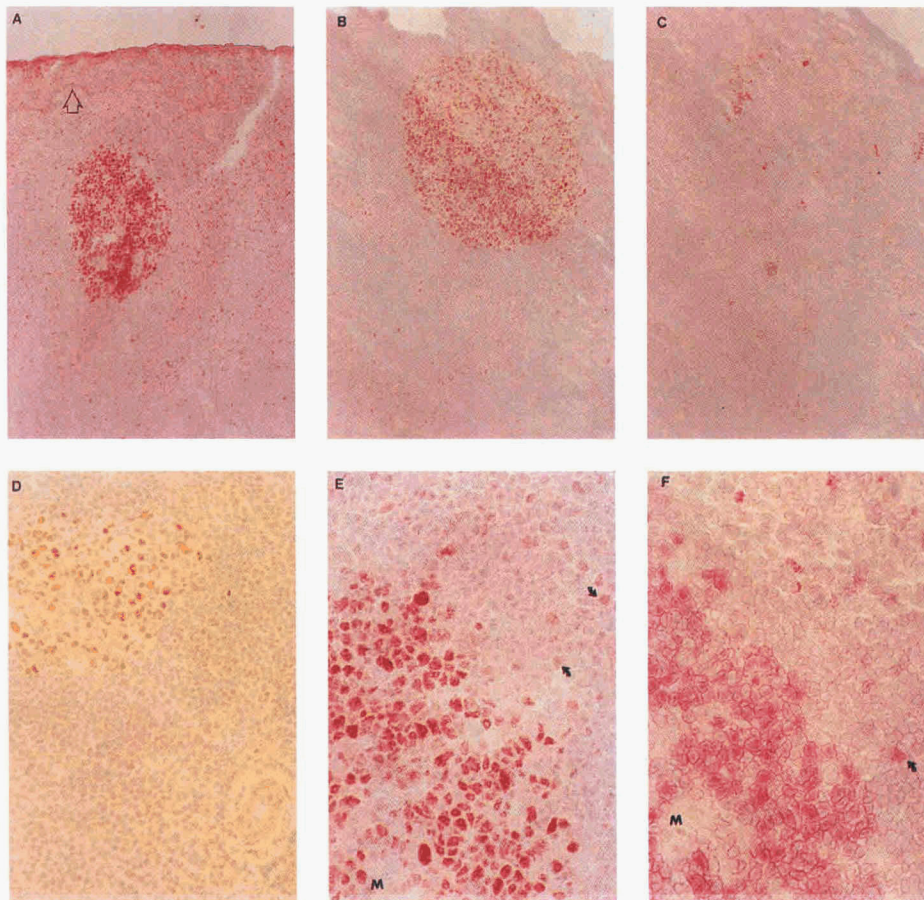

Fig 4.   Immunohistochemical analysis of BCL-6 expression in normal lymphoid tissues (cryostat sections). (A) Tonsil section stained by the C-73-6 antiserum shows staining in a germinal center. The mantle zone is mostly unstained; rare cells are stained in the paracortex. Note the nuclear staining of cell within in the squamous epithelium (arrow). (B) Lymph node section showing staining in a germinal center. (C) A section of the same lymph node shown in (B) stained with cognate-peptide pre-absorbed C-73-6 antiserum shows no specific staining. (D) Spleen section stained by the C-73-6 antibody. (E and F) Two adjacent tonsil sections stained by the C-73-6 antibody (E) or by the mouse anti–BCL-6 MoAb PG-B6 (F). Both antibodies detect large centroblasts and smaller centrocytes in the germinal center. Macrophages (m) are unstained. Mantle cells are largely unstained, with the exception of rare small lymphoid cells (arrows). (A through E) AEC immunostain with light hematoxylin counterstain; (F) alkaline phosphatase–anti-alkaline phosphatase immunostain with light hematoxylin counterstain. Original magnifications: (A) × 164; (B through D) × 409; (E and F) × 655.

Fig 3. Western blot analysis of BCL-6 expression in hematopoietic cell lines. The same immunoblot has been serially analyzed using the N-70-6 antiserum (top panel) or an anti–β-tubulin antiserum (bottom panel) as a control for protein amounts. A 95-kD band is detectable in Bjab and Ramos cells. See Table 1 for a summary of the phenotypic features of each cell line.
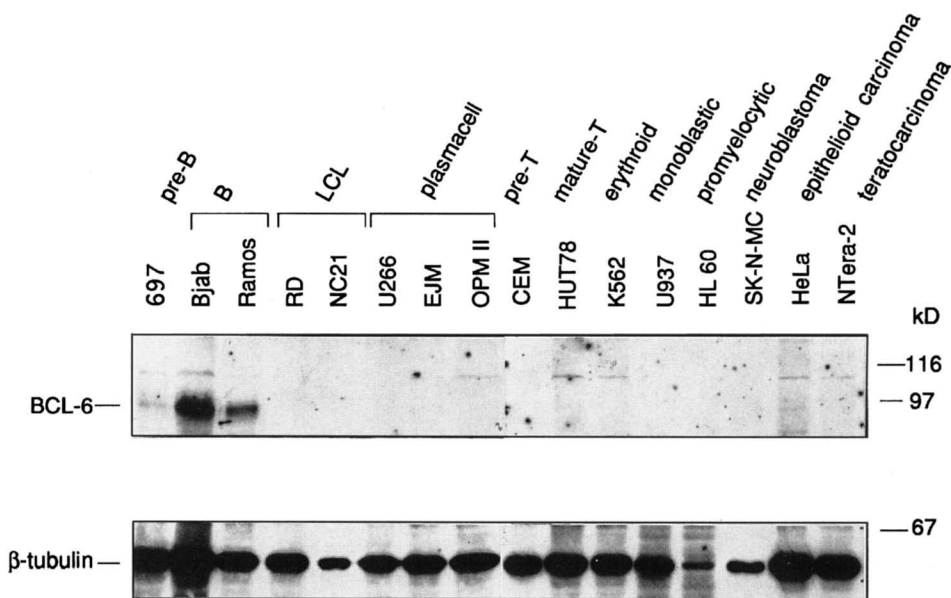
In conclusion, these results show that the product of the BCL-6 gene is a 96-kD nuclear protein. This protein can be specifically detected by both the antisera, although with different efficiency in different assays. While N-70-6 appeared more effective in Western blot and immunoprecipitation assays, C-73-6 appeared particularly suitable for the immunohistochemical detection of the protein.

Analysis of BCL-6 protein expression in hematopoietic cell lines. To further investigate the pattern of BCL-6 expression and to confirm previous results based on RNA analysis, we performed parallel Western blot (using N-70-6; Fig 3) and immunohistochemical (C-73-6; not shown, results summarized in Table 1) analysis of a panel of cell lines representative of discrete stages of B-cell differentiation as well as of other hematopoietic cell lineages (Table 1). The results show a complete concordance between RNA and protein expression detected by either antiserum. Both lines of evidence show that BCL-6 is selectively expressed in cells displaying a mature B-cell phenotype (DLCL and BL), but not in transformed derivatives of pre-B cells (acute lymphoblastic leukemia [ALL]), or more differentiated elements such as immunoblasts (lymphoblastoid cell line [LCL]) or plasma cells (multiple myeloma [MM]).

Analysis of BCL-6 expression in normal lymphoid tissues. Frozen sections of lymphoid tissues (3 tonsils, 3 lymph nodes, 2 spleens) and other organs containing lymphoid cells (1 appendix, 1 colon, 1 small bowel) as well as smears from 4 bone marrow aspirates, were examined by immunohistochemical analysis using C-73-6.

In all lymphoid tissue samples tested, a strong and specific reactivity was detectable that uniformly stained the germinal centers (GC) (Fig 4, A through D). Within GC, BCL-6 was detectable in both centroblasts and centrocytes (Fig 4E). The mantle and paracortical zones were mostly negative with the exception of a minority of small lymphoid cells staining moderately, and rare isolated large cells staining strongly.

These results were confirmed using an MoAb, PG-B6 (see Materials and Methods) specific for the N-terminus of BCL-6 (Fig 4F). GC macrophages, tissue histiocytes, and monocytes were unstained. Lymphoid cells in the submucosa of the digestive epithelium and the respiratory tract lacked BCL-6 expression. Consistent with the results obtained in transformed cell lines (Table 1), disease-free bone marrow smears and cytospins, which contain hematopoietic precursors including pre-B cells, also lacked BCL-6 expression.

Immunophenotypic characterization of BCL-6-positive cells. Double immunostaining for BCL-6 and pan-B (CD20, not shown; CD79, Fig 5B) or GC-restricted (CD38, Fig 5D) B-cell markers showed that virtually all B cells within GC express BCL-6. Mantle-zone–specific B-cell markers (CD21, CD23, and BCL-2, not shown; IgD, Fig 5F) did not colocalize with BCL-6, except for a few IgD-positive small B lymphocytes in the mantle and marginal zones (Fig 5F).

To determine whether the GC B cells expressing BCL-6 belong to the proliferative compartment, we performed a double staining for BCL-6 and the proliferation-associated Ki-67/MIB-1 antigen. Figure 5E shows that the majority of large- (centroblasts) and a portion of the intermediate-size cells (centrocytes) were coexpressing BCL-6 and Ki-67 and therefore were proliferating. However, BCL-6 was also detected in Ki-67–negative quiescent cells resembling centrocytes, suggesting that BCL-6 expression is not strictly associated with proliferation.

Because, in addition to B cells, GC contain other cell types including dendritic reticulum cells (DRC) and T cells, we analyzed whether these cell types expressed BCL-6 by double-staining with C-73-6 and antibodies recognizing DRC-associated antigens (KiM4b, CD23) or T-cell–associated markers (CD2, CD3, CD4, CD8). The results indicated that DRC do not express BCL-6, but they are interspersed and in close contact with B cells expressing BCL-6 within

Table 1. BCL-6 RNA and Protein Expression in Hematopoietic Cell Lines

Cell Line	Tumor	Phenotype	BCL-6 RNA	BCL-6 Protein	
				WB	IHC
697	c-ALL	Pre-B	−	±	±
Ly-1	DLCL	Mature B	+	+	+
Ly-8	DLCL	Mature B	+	+	+
Ramos	BL	Mature B	+	+	+
EB3	BL	Mature B	±	−	−
P3RH1	BL	Mature B	+	+	+
Daudi	BL	Mature B	+	+	+
ST486	BL	Mature B	+	±	±
AC8	LCL	Pre-B	ND	ND	−
CB6	LCL	Pre-B	ND	ND	±
RD	LCL	Immunoblast	−	−	−
CB33	LCL	Immunoblast	−	−	−
NC21	LCL	Immunoblast	−	−	−
U266	MM	Plasma cell	−	−	−
RPMI8226	MM	Plasma cell	−	ND	−
EJM	MM	Plasma cell	−	−	−
CCRF-CEM	T-ALL	Thymocyte	−	−	ND
JURKAT	T-ALL	Thymocyte	ND	ND	−
HUT 78	T-ALL	Mature T	−	−	ND
K562	CML-BC	Erythroblast	−	−	−
U937	H-NHL	Monoblast	−	−	−
HL-60	AML	Myeloblast	−	−	−

Abbreviations: c-ALL, common acute lymphoblastic leukemia; LCL, Epstein-Barr virus-transformed lymphoblastoid cell line; T-ALL; T-cell acute lymphoblastic leukemia; CML-BC, chronic myeloid leukemia blastic crisis; AML M2, acute myeloid leukemia M2 FAB type; H-HNL, histiocytic lymphoma; +, positive band on Northern and Western blots or strong nuclear staining; ±, faint bands or weak staining; −, absence of bands or no nuclear staining; WB, Western blot; IHC, immunohistochemistry.

the GC (Fig 5G). Conversely, T cells were found to express BCL-6 in the mantle (24% to 26%) and paracortical zones (1% to 2%) as well as within GC (10% to 16%) (Fig 5C). Double-staining for BCL-6 and CD4 (Fig 5H) or CD8 (not

shown) indicated that these T cells belong exclusively to the CD4 subset.

Double-staining with BCL-6 and the activation-associated markers CD30, CD69, and CD70 showed coexpression in rare lymphoid cells located in the interfollicular areas. In particular, rare CD30^{+}, BCL-6^{+} large cells were consistently seen at the edge of the GC (not shown). Finally, double-staining for BCL-2 and BCL-6 show that, as expected, most of the BCL-6^{+} cells within the GC do not express BCL-2[27]; coexpression was detectable in the rare BCL-6 expressing cells in the interfollicular zone, most likely representing T cells.

These results showed that in all lymphoid organs studied, BCL-6 expression was topographically restricted to: (1) all B cells within the GC; (2) rare B cells in the interfollicular zone; and (3) CD4^{+} T cells both within and outside the GC.

BCL-6 expression in lymphoma samples. The BCL-6 gene is rearranged in 30% to 40% of DLCL and in 5% to 10% of FL.[6] To determine whether the BCL-6 protein was expressed in these tumors, a panel of nine cases including DLCL and FL biopsy samples representative of cases carrying normal or altered BCL-6 genes was analyzed by immunocytochemistry using the C-73-6 antiserum. The results (Fig 6; summary in Table 2) indicated that comparable levels of BCL-6 expression were detectable in most tumor cells of all cases studied independent of histotype (DLCL or FL) and genotype (BCL-6 gene). Nuclear staining in lymphoma sections was heterogeneous both as individual cell staining intensity and as topographic variations. Section areas with a morphologic follicular pattern showed a more intense staining pattern than the surrounding neoplastic tissue.

DISCUSSION

Following recent reports on the identification of the BCL-6 gene and its structural alteration in a large fraction of DLCL, this study attempted to identify the BCL-6 protein and to determine its pattern of expression in normal and neoplastic lymphoid tissues. The results show that the product of the BCL-6 gene is a 95-kD nuclear protein expressed in mature B cells within GC and in a small subpopulation of T cells. These findings provide insights into the normal

Fig 5. Phenotypic analysis of BCL-6 expressing cells in normal lymphoid tissues by double-staining using antibodies for lineage- and differentiation-specific markers. Tonsil frozen sections. (A) Negative controls for mouse and rabbit antibodies. (B) CD79 B-cell specific antibody (blue) labels the majority of C-73-6–stained, BCL-6^{+} (brown) germinal center cells. In the germinal center, CD79^{−} macrophages and CD79^{+} plasma cells are BCL-6^{−}. CD79 strongly labels mantle-zone B cells (lower half), containing rare BCL-6^{+} mantle B cells (solid arrow). CD79^{−} cells resembling T cells are also identifiable (empty arrow). (C) A section adjacent to the one shown in (B) stained by the T-cell–specific CD2 antibody (blue) and C-73-6 (brown). Numerous BCL-6^{+} T cells are seen in the germinal center (upper half) and in the mantle zone (lower half). (D) CD38 antibody (blue) stains BCL-6^{+} germinal center cells (brown: upper right corner) and BCL-6^{−} plasma cells (center). A portion of tonsil squamous epithelium is seen in the lower left corner, containing BCL-6^{+} epithelial nuclei (arrow). (E) Double-staining for BCL-6 (blue) and the MIB 1/Ki-67 proliferation-associated antigen (red) shows double-stained centroblasts and BCL-6^{+}/Ki-67^{−} centrocytes (arrows). The mantle zone at the left is largely unstained by both antibodies. (F) Double staining by anti-IgD (blue) and anti–BCL-6 (brown) antibodies shows nonoverlapping lymphocyte subsets in the mantle-zone. An isolated BCL-6^{+} IgD[+] cell is shown (arrow). (G) Double-staining by the DRC-specific Kim-4b antibody (blue) and by C-73-6 (brown) shows BCL-6^{−} DRC cells interspersed with BCL-6^{+} GC B cells. (H) CD4^{+} T cells (blue) are stained by the anti–BCL-6 antibody (brown) both in the germinal center (arrows) and in the mantle and marginal zone (lower half). Original magnifications: (A through F, H) × 655; (G) × 409.

Fig 6. Immunohistochemical analysis of BCL-6 expression in lymphoma (cryostat sections). (A) DLCL (case no. 534), carrying a rearranged BCL-6 gene. (B) FM (1311). (C) DLCL (885). (D) D-MIX (1139). Original magnifications × 655. See Table 2 for abbreviations and genetic features of each case.

function of BCL-6 and have pathogenetic and diagnostic implications for DLCL.

Consistent with its structure containing zinc-finger domains typical of transcription factors, the BCL-6 protein was localized in the cell nucleus. This finding was confirmed by a more detailed biochemical characterization of the protein by immunoprecipitation analysis of fractionated nuclear and cytoplasmic components.[28] Both in cell lines and in normal tissues, immunocytochemical staining of the BCL-6 protein indicated a distinct microgranular distribution within the nucleus. In the case of other nuclear proteins such as cdc2,[29] SC35,[30] PCNA, DNA polymerase α,[31] and PML,[32,33] nonuniform patterns of staining have been correlated with particular subnuclear localizations and, eventually, with the function
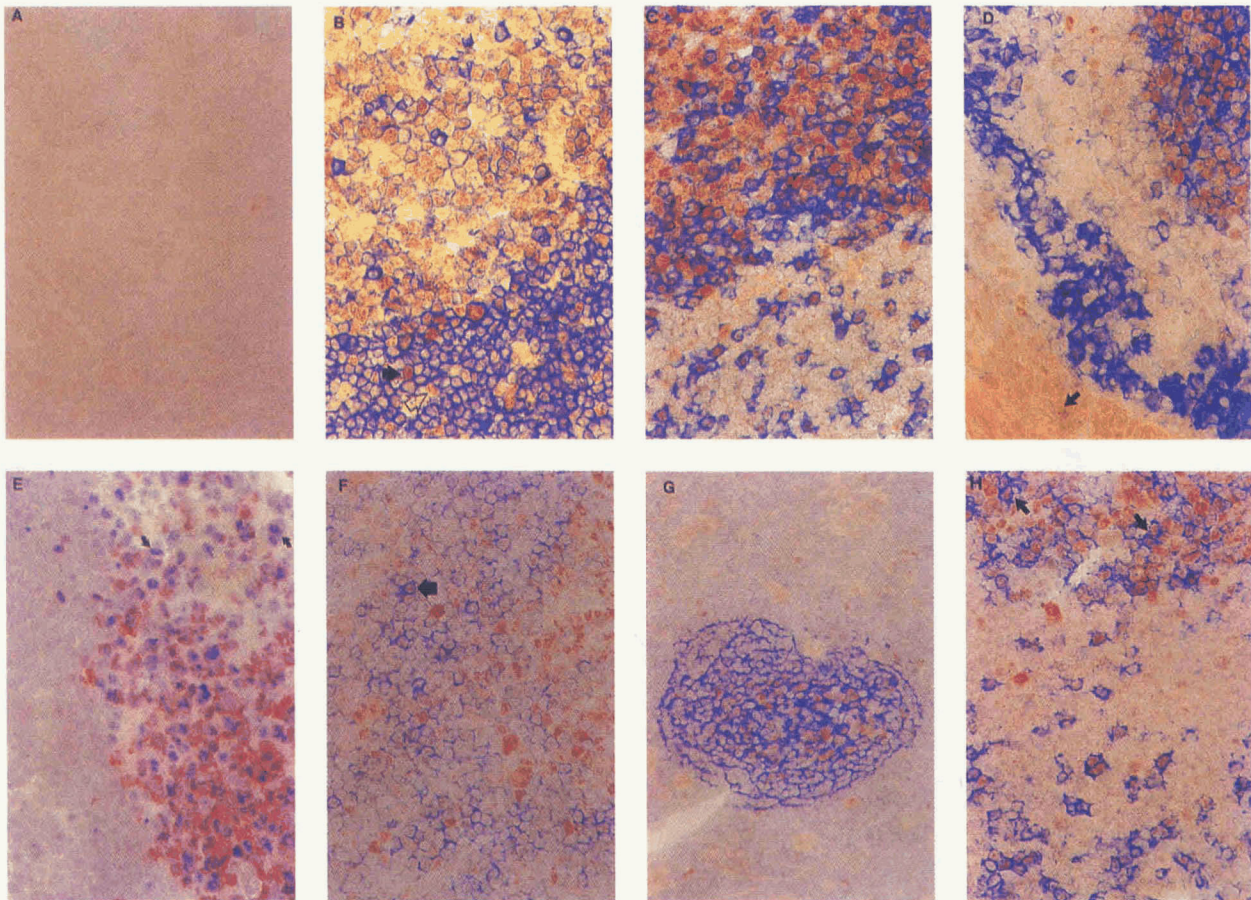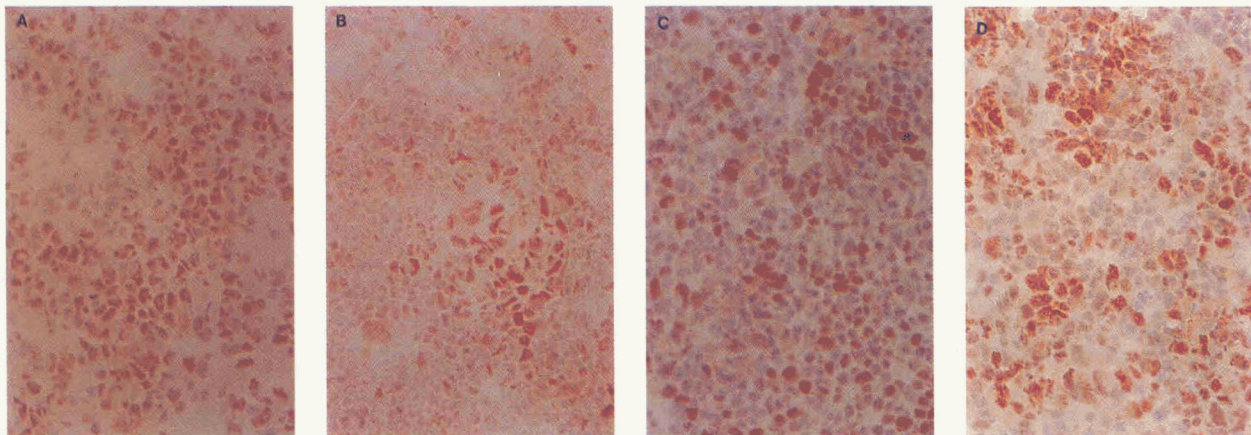

Fig 5.

Fig 6.

Table 2. Histologic, Cytogenetic, and Genotypic (BCL-6) Features of Tumor Cases Analyzed for BCL-6 Protein Expression

Tumor	Histology*	3q27	BCL-6 Gene	BCL-6 Protein
1311	F-MIX	N	G	+
534	DLCL	A	R	+
1139	D-MIX	N	G	+
885	DLCL	N	G	+
1338	DLCL	A	G	+
1668	F-MIX	A	G	+
810	F + D-MIX	A	G	+
1351	F-MIX	A	R	+
1616	F + D-LCL	A	G	+

Abbreviations: A, altered; N, normal; ND, not determined; G, germline; R, rearranged; +, present.

* Histology codes (according to the Working Formulation): F-MIX, follicular mixed; DLCL, diffuse large cell; D-MIX, diffuse mixed; F-MIX, follicular mixed; F + D-MIX, follicular and diffuse mixed; F + D-LCL, follicular and diffuse, large cell.

of each protein. In our preliminary observation, the microgranular staining pattern of BCL-6 is significantly finer than the one observed for PML,[32,33] and similar to the patterns observed for p53, PCNA, and EBNA-2. Further studies involving high-sensitivity imaging of the BCL-6 protein are necessary to provide further information on the possible specific compartmentalization of this protein in the nucleus.

Results obtained in cell lines as well as in normal tissues indicate that the expression of the BCL-6 gene is specifically regulated during B-cell differentiation, being expressed in mature B-cell subsets, but not in immature bone marrow precursors or mature progenies including immunoblasts and plasma cells. In addition, within the mature B-cell compartment, BCL-6 expression is topographically restricted to GC where virtually all B cells, including centroblasts and centrocytes, express BCL-6. GC are dynamic structures in which antigen-primed B cells undergo a complex functional transformation, including a rapid antigen-driven proliferative expansion, hypermutation of Ig V region genes, and Ig isotype-switching.[34] During these processes, the B cell will be programmed to follow one of three main pathways: apoptosis, memory B cell, or immunoblastic/plasma cell differentiation.[34] Because B cells appear to express BCL-6 within the GC, but not before entrance (mantle zone) or after exit (immunoblasts, plasma cells), one simple interpretation of the findings is that, consistent with the role of related POZ/zinc-finger proteins as developmental regulators,[10-12] BCL-6 may be involved in the induction of GC-associated functions and that its downregulation may be necessary for further B-cell differentiation or apoptosis. Our results tend to exclude the possibility that BCL-6 may be involved in GC-associated proliferation because the protein was found in both proliferating and quiescent GC cells (Fig 5E). It is conceivable that BCL-6 may be involved in the response to specific signals provided by antigens, GC cells (DRC and T cells), or cytokines (IL-4) within the GC.[34]

The significance of BCL-6 expression in cells other than GC B cells remains unclear. This study did not include the analysis of nonlymphoid tissues, although preliminary analysis of various tissues indicated that several of them, namely

skeletal muscle and skin epithelium, may express BCL-6 although at levels significantly lower than observed in B cells. A comprehensive analysis of the pattern of expression of BCL-6 in the T-cell lineage could not be performed because normal thymus biopsy samples were not available at the time of this study. However, in other lymphoid organs BCL-6 expression appears to be restricted to mature CD4$^+$ elements in the GC and in the parafollicular areas, whereas circulating T cells in nonhematopoietic organs do not express BCL-6 (not shown). Both in parafollicular areas and in the GC, CD4$^+$ T cells are known to help B cell survival via specific cell-cell interaction (eg, CD40-CD40 ligand pathway[35]) and cytokine production. These observations suggest that BCL-6 expression in T cells may also be associated with some GC-related function.

Structural alterations of the regulatory region of the BCL-6 gene are associated with DLCL which, based on morphologic criteria, may originate from GC B cells.[36] Thus, our results suggest that BCL-6 may be normally expressed in the cells that represent the normal counterparts of DLCL, further supporting the hypothesis[9] that deregulation, rather than ectopic expression, of BCL-6 may be the critical consequence of BCL-6 gene rearrangements in DLCL. In particular, because BCL-6 gene rearrangements lead to the expression of a normal protein under the control of heterologous promoters,[9] these alterations may prevent the switch-off of BCL-6 expression, which appears to be associated with B-cell differentiation in cell lines (Fig 3) and normal B cells (Fig 5).

Finally, one aim of this study was to determine the diagnostic significance of BCL-6 expression and, in particular, whether BCL-6 expression could distinguish DLCL cases carrying normal or altered BCL-6 genes. Because BCL-6 rearrangements identify a subset of DLCL characterized by more favorable prognostic features,[16] the possibility of the detecting these alterations by a rapid and practical immunohistochemical assay would have clinical relevance. However, the results presented here suggest that BCL-6 expression cannot discriminate neither between normal and neoplastic GC-derived B cells, nor between DLCL carrying a normal or a rearranged BCL-6 gene. It remains to be tested whether BCL-6 expression can be clinically useful in determining the GC-origin of neoplastic cells and therefore in helping the differential diagnosis of lymphoma.

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