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Monoclonal Antibodies PG-B6a and PG-B6p Recognize, Respectively, a Highly Conserved and a Formol-Resistant Epitope on the Human BCL-6 Protein Amino-Terminal Region

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The human BCL-6 gene, which is rearranged in approximately 30% of diffuse large B cell lymphomas, encodes a 706-amino-acid nuclear protein of the Kruppel-type zinc finger transcription factors mainly expressed in normal germinal center B cells and related lymphomas. Four monoclonal antibodies (PG-B6, PG-B6a, PG-B6p, and PG-B6m), specifically directed against the human BCL-6 protein, were generated by immunizing BALB/c mice with a recombinant protein corresponding to the BCL-6 amino-terminal region (amino acids 3 to 484). The PG-B6 monoclonal antibody reacted with a BCL-6 epitope sensitive to fixatives and preserved in all mammalian species. PG-B6a (a is for avian) recognized the most evolutionarily conserved BCL-6 epitope (expressed in all animal species including avian). PG-B6p (p is for paraffin) recognized a fixative-resistant epitope of BCL-6 that was detectable on paraffin sections after microwave heating in 1 mmol/L EDTA buffer. PG-B6m (m is for mantle) was the least specific monoclonal antibody as, in addition to BCL-6, it reacted with a yet undefined antigen selectively located in the cytoplasm of mantle and marginal zone B cells. All monoclonal antibodies detected strong nuclear expression of BCL-6 in follicular lymphomas, diffuse large B cell lymphomas, Burkitt's lymphomas, and nodular, lymphocyte-predominance Hodgkin's disease. In diffuse large B cell lymphomas, BCL-6 expression was independent of BCL-6 gene rearrangements and did not correlate with expression of other markers or the proliferation index. BCL-6 was not expressed in B-CLL, hairy cell leukemia, mantle-cell- and marginal-zone-derived lymphomas. Labeling of paraffin sections with PG-B6p proved useful for differentiating proliferation centers in B-CLL (BCl-2^+/BCL-6^-) from trapped germinal centers in mantle cell lymphomas (BCL-2^-/BCL-6^+) and for identifying neoplastic cells in cases of nodular, lymphocyte-predominance Hodgkin's disease. Because of their high specificity, wide reactivity in humans and animal species including avians (PG-B6a), and suitability for labeling routine paraffin sections (PG-B6p), the reagents described in this paper should prove valuable in both research and diagnostics. (Am J Pathol 1996, 148:1543–1555)

Reciprocal translocations involving band 3q27 and several other chromosomal sites have been de-

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scribed in 8 to 12% of non-Hodgkin's lymphomas.[1] A breakpoint cluster region at 3q27 that involves a gene designated BCL-6 or LAZ3,[2–6] which is rearranged in approximately 30% of diffuse large B cell lymphomas (DLCL-B) and a small fraction of follicular lymphomas,[7–9] has recently been identified. An association between BCL-6 rearrangements, extranodal involvement by DLCL-B, and favorable clinical outcome has been reported by Offit et al.[7]

The wild-type BCL-6 gene encodes a 95-kd protein of 706 amino acids.[10] The amino and carboxy termini of BCL-6 share homologies with members of the Kruppel-like subfamily of zinc finger proteins, many of which have been implicated in developmental regulation.[11] We recently reported that a specific monoclonal antibody (MAb), PG-B6, detects nuclear expression of the BCL-6 protein in normal germinal center B cells and related lymphomas.[12] Identical results were obtained by others using polyclonal antibodies directed against the BCL-6 protein.[13,14] These immunohistological findings and the previous experimental evidence that BCL-6 is mainly expressed in mature B cell lines,[10] strongly suggest that BCL-6 is implicated in regulating B cell differentiation within germinal centers of secondary follicles.[12,13] Deregulated BCL-6 expression caused by chromosomal rearrangements may contribute to lymphomagenesis.[12,13]

This paper describes three new anti-BCL-6 MAbs (PG-B6a, PG-B6p, PG-B6m) that, in addition to the distinctive and previously described[12] immunoreactive features of the prototype PG-B6 MAb (eg, nuclear labeling of human germinal center B cells), show unique staining characteristics that may reflect targeting of different epitopes on the amino-terminal region of BCL-6. Such reagents were applied to the study of a large number of normal and pathological tissues. This panel of highly specific antibodies should represent an effective tool in both research and diagnostics.

Materials and Methods

Production of MAbs against the BCL-6 Protein

Eight copies of two synthetic peptides corresponding to amino acids 207 to 216 (H-SLLFSDEEFRDVR) and 300 to 310 (SKEEERPSSED) of the human BCL-6 protein were synthesized on a branched lysine (MAP carrier core; Research Genetics, Huntsville, AL). A glutathione S-transferase (GST)-BCL-6 recombinant protein corresponding to the amino-terminal region (amino acids 3 to 484) of the human BCL-6 protein was produced as previously described.[13]

Twelve BALB/c mice were injected intraperitoneally three times with 200-μg aliquots of the MAP-BCL-6 or the GST-BCL-6 recombinant protein emulsified in complete Freund's adjuvant at 10-day intervals. Booster was performed by intravenous injection of 300 μg of the MAP-BCL-6 peptides or the GST-BCL-6 protein and the fusion between mice spleen cells and the NS-O myeloma cell line carried out 3 days later, as described previously.[15] Hybridoma supernatants were screened by the immunoalkaline phosphatase (APAAP) technique[16] on cryostat sections of human tonsil. Cloning of the selected hybridomas was carried out by a limiting dilution technique in flat-bottom 96-well plates.

Tissue reactivity of the four selected murine hybridomas PG-B6, PG-B6a, PG-B6p, and PG-B6m (see Results) was compared with that of two rabbit polyclonal antibodies (N-70–6 and N-3) directed against the BCL-6 gene product. The antibody N-70–6 was raised in one of the authors' (R. Dalla-Favera) laboratories by immunizing rabbits with a synthetic peptide corresponding to the carboxy terminus (KVQYRVSATDLPPEL) of the human BCL-6 protein. The characteristics of this antibody have been described previously.[13] N-3 (Santa Cruz Biotechnology, Santa Cruz, CA) is an affinity-purified rabbit polyclonal antibody raised against a GST-tagged fusion protein containing sequences corresponding to amino acids 3 to 484 mapping within the amino-terminal two-thirds of BCL-6 of human origin.

Molecular Specificity of the Anti-BCL-6 MAbs

Expression of BCL-6 cDNA in EB3 Cells

The generation and characterization of the BCL-6 and control-transfected EB3 cell lines has been reported elsewhere.[13] Briefly, the Burkitt lymphoma cell line EB3 (not expressing the BCL-6 mRNA and protein) was stably transfected with an episomally replicating plasmid vector PHeBo-CMV-BCL-6 expressing 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. After transfection, cells were characterized for BCL-6 RNA and protein expression by Northern and Western blot analysis.[13]

Control- and BCL-6-transfected EB3 cells were grown in Iscove's modified Dulbecco's medium containing 10% fetal calf serum, penicillin (100 IU/ml),

streptomycin (100 μg/ml), and G418 (1.4 mg/ml). Cytospins were prepared from exponentially growing cells, air dried overnight, fixed in acetone for 10 minutes, and stained by the APAAP technique.[16]

Immunoprecipitation and Western Blot Analysis

Rd (a human B cell line that does not express BCL-6 mRNA), Bjab (a human Burkitt cell line expressing both BCL-6 mRNA and protein), and control- and BCL-6-transfected EB3 cells (see above) were lysed with 1X loading buffer or single-detergent lysis buffer (250 mmol/L NaCl, 50 mmol/L Hepes, pH 7.0, 0.1% Nonidet P-40, 5 mmol/L EDTA, 1 mmol/L dithiothreitol) containing a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride), sonicated, and immunoprecipitated with supernatants (1:2 dilution) of the PG-B6, PG-B6a, PG-B6p, and PG-B6m MAbs. The total lysates and the MAb immunoprecipitates were loaded onto an 8% sodium dodecyl sulfate (SDS)-acrylamide gel and electrotransferred to nitrocellulose sheets. Nitrocellulose sheets were blocked in Tris-buffered saline with Tween-20 plus 5% bovine serum albumin and then incubated overnight at 4°C with a polyclonal antibody (1:1000 dilution) directed against the carboxy terminus of the BCL-6 protein (C19, Santa Cruz Biotechnology). After extensive washing in Tris-buffered saline with Tween-20, the blots were incubated for 1 hour with an alkaline-phosphatase-conjugated goat anti-rabbit antibody and then stained by using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL).

Reactivity of Anti-BCL-6 MAbs with Normal Human and Animal Tissues

The reactivity of the four MAbs was tested on a variety of normal human tissues including tonsils (n = 10), reactive lymph nodes (n = 10), spleen (n = 5), bone marrow (n = 5), thymus (n = 2), skin (n = 5), liver (n = 5), lung (n = 2), thyroid (n = 4), striated muscle (n = 2), and kidney (n = 5). Tonsils were obtained from children with recurrent tonsillitis who underwent tonsillectomy at the Ear-Nose-Throat Department, Perugia University Hospital. Normal liver, lymph node, and spleen samples were from patients who underwent exploratory laparatomy for Hodgkin's disease and were found not to be involved by the disease. Normal thymuses were from children who had open heart surgery. Other normal tissues were removed for diagnostic or therapeutic purposes or were obtained at the time of autopsy.

BCL-6 expression was also studied on normal tissues from calves, lambs, pigs, rabbits, rats, chickens, and pigeons obtained from the Department of Veterinary Pathology, Perugia University.

Reactivity of Anti-BCL-6 MAbs with Pathological Lymphoid Samples

The four MAbs were used to investigate BCL-6 expression on a variety of reactive lymphadenitis including follicular hyperplasia (n = 10), toxoplasmic lymphadenitis (n = 4), tubercular lymphadenitis (n = 3), cat scratch disease (n = 2), sarcoidosis (n = 2), and reactive T immunoblastic proliferations (n = 2).

A total of 173 non-Hodgkin's lymphomas and 12 cases of Hodgkin's disease were also studied. The selected cases were representative of all subtypes of lymphomas, as defined by the Kiel[17] and the revised European-American lymphoma[18] classifications.

Tissue Processing for Immunohistochemistry

Normal and pathological samples were fixed in formalin and embedded in paraffin according to standard methods. Paraffin sections, 3 to 5 μm thick, were mounted on silane-coated slides, dried overnight at 37°C, dewaxed in xylene, and rehydrated through gradient alcohols. Slides were then placed in covered glass Coplin jars filled with 0.01 mol/L sodium citrate buffer, pH 6.0,[19] or 1 mmol/L EDTA buffer, pH 8.0, as previously described.[20] The jars were incubated three times for 4 minutes at 700 W in a De Longhi microwave oven. During microwave processing, care was taken that sections were submerged in buffer. After microwave irradiation, the sections were allowed to cool to room temperature for approximately 20 minutes, washed with Tris-buffered saline, and immunostained.

A portion of the specimens was snap-frozen in liquid nitrogen and cut at 5 μm in a cryostat. Frozen sections were air dried overnight, fixed in acetone for 10 minutes, and subjected to immunostaining, as previously described.[16,21]

Single and Double Immunoenzymatic Labeling

All specimens were stained by the APAAP technique, as previously described.[12,16] Cross-reactivity

of the rabbit anti-mouse Ig with immunoglobulins from the various animal species was inhibited by adding 10% normal serum from each of the species to the rabbit anti-mouse Ig preparation.[22] This step was omitted when immunostaining rabbit tissues. APAAP staining with MAbs directed against human antigens other than BCL-6 (CD20 and CD45) served as negative control for immunostaining of animal tissues. The alkaline phospatase reaction was revealed by the new Fuchsin substrate.[16] Endogenous alkaline phosphatase was blocked by adding levamisole to the substrate solution at a final concentration of 1 mmol/L.[23] Slides were then counterstained for 5 minutes in Gill's hematoxylin and mounted in Kaiser gelatin.

Tonsil frozen sections were double stained for the following pairs of antigens: BCL-6/IgD, BCL-6/IgM, BCL-6/CD23, BCL-6/CD38, BCL-6/CD3, BCL-6/CD8, BCL-6/CD4, and BCL-6/CD68 (PG-M1).[24] Briefly, slides were incubated with the PG-B6p MAb followed by a rabbit anti-mouse Ig and mouse PAP complexes (both purchased from Dako, Glostrup, Denmark).[25,26] The peroxidase reaction was developed using a diaminobenzidine/H_2O_2 substrate. After three washes in Tris-buffered saline, slides were incubated with the second pair of monoclonal reagents and developed by the APAAP technique.[16,25] Slides were then counterstained for 30 seconds in Gill's hematoxylin and mounted in Kaiser's gelatin.

Southern Blot Analysis

For detection of BCL-6 rearrangements, high-molecular-weight genomic DNA was extracted from 21 DLCL-B samples, digested with *Bam*HI and *Xba*I, and subjected to Southern blot analysis using a 4-kb *Sac*I-*Sac*I fragment of BCL-6 gene as probe.[7]

Results

Production and Specificity of the Anti-BCL-6 MAbs

No anti-BCL-6-reacting hybridomas were generated from BALB/c mice immunized with the two MAP-BCL-6 peptides. At APAAP immunoscreening of tonsil cryostat sections, 4 out of 1000 hybridoma supernatants from mice immunized with the GST-BCL-6 protein reacted strongly with the nuclei of germinal center B cells. Corresponding hybridoma cells were cloned by a limiting dilution technique to produce the clones PG-B6, PG-B6a, PG-B6m, and PG-B6p that were used in subsequent studies.

The specificity of the four MAbs for the BCL-6 protein was evaluated by Western blotting and immunoprecipitation experiments on the cell lines Rd, Bjab, EB3-BCL-6 (a lymphoblastoid cell line stably transfected with a BCL-6 expression vector; see above), and control-transfected EB3. No specific polypeptides reacting with the four MAbs were detected in the total lysates from any of these cell lines by Western blotting (data not shown), suggesting that the PG-B6, PG-B6a, PG-B6m, and PG-B6p MAbs do not react with the denatured BCL-6 protein. Therefore, lysates from the same cell lines were immunoprecipitated with PG-B6, PG-B6a, PG-B6m, and PG-B6p resolved by SDS-polyacrylamide gel electrophoresis and blotted with a commercially available anti-BCL-6 polyclonal antibody (C19, Santa Cruz Biotechnology). An anti-PG-B6a- and anti-C19-immunoreactive polypeptide of approximately 95 kd was detected in the lysates of Bjab and EB3-BCL-6 cells but not in those of Rd and control EB3 cells (Figure 1A). The same immunoprecitation band was obtained with the MAbs PG-B6, PG-B6m, and PG-B6p (Figure 1B).

All MAbs strongly stained the nuclei of BCL-6-transfected EB3 cells but not those of control-transfected EB3 cells (data not shown). Similar results were obtained with the polyclonal antibodies N-70-6 and N-3.

Taken together, the above data confirm that the PG-B6, PG-B6a, PG-B6m, and PG-B6p MAbs have specificity against the BCL-6 protein.

Reactivity of Anti-BCL-6 MAbs with Normal Human Tissues

All MAbs gave optimal labeling of tonsil frozen sections, but only one (PG-B6p) recognized BCL-6 on microwave-heated paraffin sections (Table 1 and Figure 2). Among anti-BCL-6 polyclonal antibodies, only N-3 reacted on paraffin sections, but the intensity of staining was less than with PG-B6p and the background was higher (data not shown).

In normal tonsil, both monoclonal and polyclonal anti-BCL-6 antibodies strongly stained the nuclei of centroblasts (Ki-67$^+$/CD19$^+$/CD20$^+$) in the dark zone and centrocytes in the basal and apical light zones of germinal centers (Figure 3A). Nuclear positivity was diffuse/microgranular, nucleoli being consistently negative (Figure 3A, inset). Intense cytoplasmic staining unassociated with metaphase chromosomes was noted only in mitotic figures (not shown).

None of the four MAbs reacted with any other germinal center cell constituent, eg, plasma cells

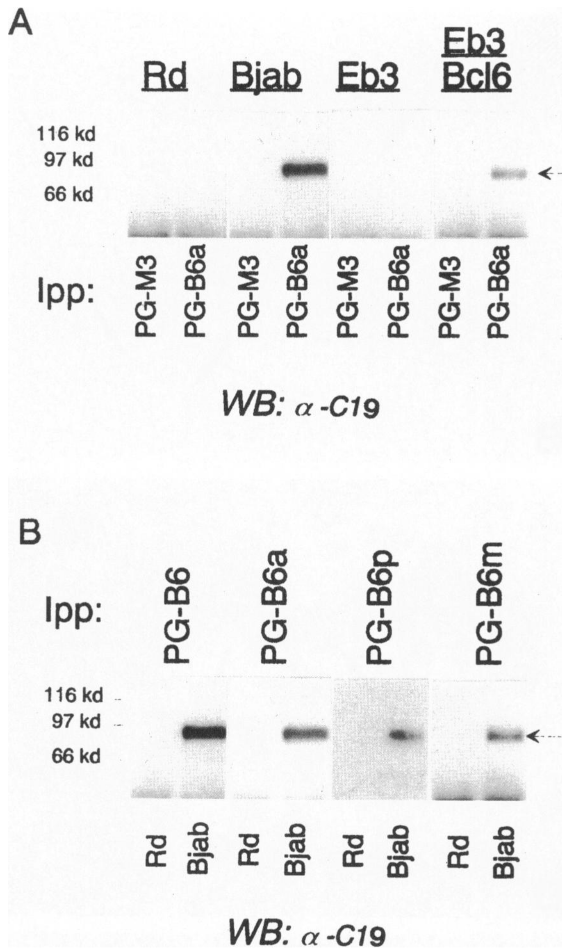

Figure 1. A: *PG-B6a immunoprecipitates from the indicated cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and blotted with the polyclonal anti-BCL-6 antibody C19 (Santa Cruz Biotechnology). A 95-kd band corresponding to the size of BCL-6 is seen in lanes corresponding to Bjab and BCL-6-transfected EB3 cells. No bands are seen in lanes corresponding to Rd and control-transfected EB3 cells. Identical results were obtained with the PG-B6, PG-B6p, and PG-B6m MAbs (data not shown). The MAb PG-M3 (anti-PML) served as negative control.* **B:** *The same method as in* **A**. *All MAbs (PG-B6, PG-B6a, PG-B6p, and PG-B6m) recognize the same 95-kd band in Bjab but not in Rd cells.*

(BCL-6$^-$/CD38$^+$), macrophages (BCL-6$^-$/CD68$^+$), and follicular dendritic cells (BCL-6$^-$/CD23$^+$). Although most germinal center T cells were BCL-6$^-$, a few (approximately 10%) were BCL-6$^+$ (data not shown).

IgM$^+$/IgD$^+$ follicular mantle lymphocytes did not react with the MAbs PG-B6, PG-B6a, and PG-B6p but exhibited intense cytoplasmic labeling with PG-B6m (Table 1 and Figure 3, C and D). T cells in the interfollicular areas were not stained by the MAbs with the exception of a small percentage (<0.5%) of CD3$^+$/CD4$^+$ elements (data not shown).

The staining pattern of normal spleen B and T cell compartments was similar to that of the tonsil; splenic marginal zone B cells were PG-B6$^-$/PG-

Table 1. *MAbs Directed against the BCL-6 Protein*

Tissues	Reactivity*			
	PG-B6	PG-B6a	PG-B6p	PG-B6m
Human tonsil & spleen				
GC B-cells	+	+	+(p)†	+
Mantle B cells	−	−	−	+(c)
Marginal zone B cells	−	−	−	+(c)
Bursa of Fabricius‡				
Cortex	−	+	−	−
Medulla	−	−	−	−

GC, Germinal center; p, paraffin sections; c, cytoplasmic positivity

*Unless specified, refers to nuclear positivity on frozen sections. For comparison with anti-BCL-6 polyclonal antibodies, see Results.

†After microwave heating in 1 mmol/L EDTA buffer, pH 8.0.

‡In chicken and pigeon.

B6a$^-$/PG-B6p$^-$ but strongly PG-B6m$^+$ (cytoplasmic-restricted positivity; Table 1). Although most cortical and medullary thymocytes were negative, a few scattered BCL-6$^+$ lymphoid-like cells of undefined phenotype were present.

Extra-lymphoid expression of BCL-6 included a faint nuclear positivity of squamous epithelia in the tonsil, thymus, and skin. There was no reactivity with

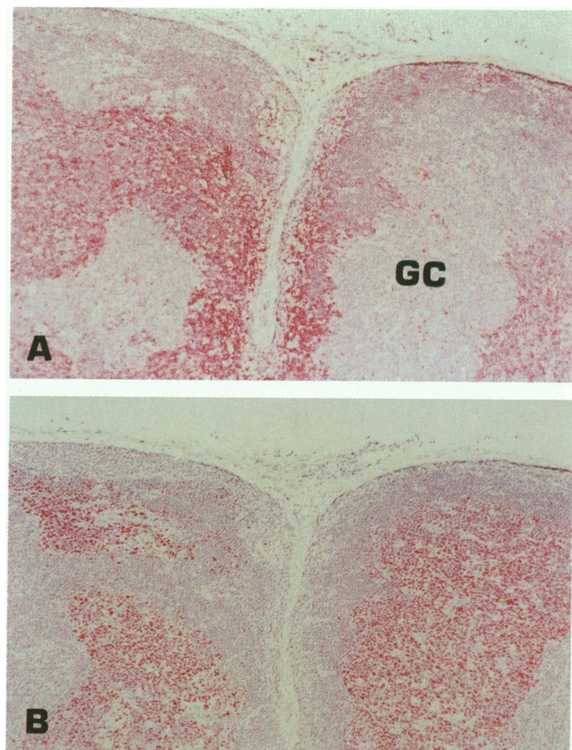

Figure 2. *Microwave-heated, lymph node paraffin sections from reactive follicular hyperplasia stained with MAbs against BCL-2* (**A**) *and BCL-6 (PG-B6p;* **B**). *B cells within the germinal centers (GC) are characteristically BCL-2$^-$* (**A**) *and BCL-6$^+$* (**B**). *APAAP technique; hematoxylin counterstain; magnification, ×125.*

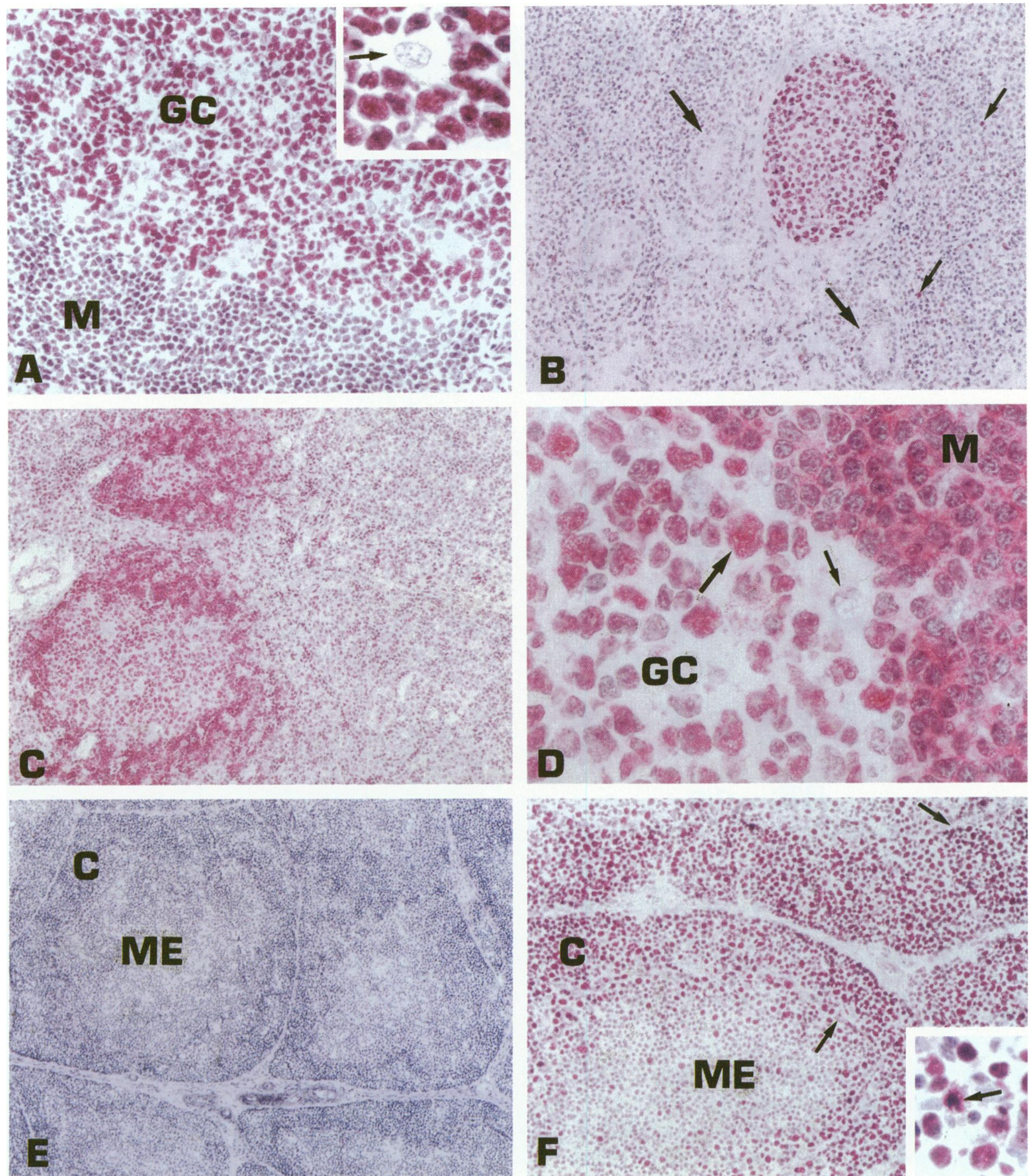

Figure 3. A: *The BCL-6 protein is strongly expressed in germinal center (GC) B cells of normal human tonsil. Mantle B cells (M) are BCL-6⁻ (frozen section stained with PG-B6a; magnification, ×250). The* **A, inset:** *Higher magnification (×800) of the strong, diffuse nuclear positivity for BCL-6 of centroblasts and a BCL-6⁻ tingible body macrophage* (**arrow**). **B:** *Chicken cecal tonsil (frozen section stained with PG-B6a; ×125). The staining pattern is identical to that observed in normal human tonsil shown in* **A**. *The* **long arrows** *point to BCL-6⁻ colon epithelium; the* **short arrows** *indicate a few BCL-6⁺ cells in the T cell area (possibly avian T cells).* **C:** *The PG-B6m MAb stains the nuclei of germinal center B cells and the cytoplasm of mantle B cells (human tonsil frozen section; ×200).* **D:** *The same field as* **C** *at higher magnification (×800); the* **long arrow** *points to a BCL-6⁺ centroblast, and the* **short arrow** *to a BCL-6⁻ macrophage.* **E** *to* **F:** *Immunolocalization of BCL-6 in the chicken bursa of Fabricius (frozen sections).* **E:** *No staining is observed with the PG-B6p MAb (×125).* **F:** *The PG-B6a MAb stains selectively the nuclei of B cells located in the cortex (C) of the bursa, whereas the medulla (ME) is PG-B6a⁻ (×250). The* **arrows** *point to BCL-6⁻ epithelial basal membrane at the cortico-medullary junction. The nuclear staining pattern of PG-B6a⁺ bursal B cells (shown at higher magnification, ×800, in the* **inset**) *is identical to that observed in human germinal center B cells (compare with* **A**). *The* **arrow** *in the* **inset** *points to a BCL-6⁺ mitotic figure. APAAP technique; hematoxylin counterstain.*

Table 2. *Reactivity of Anti-BCL-6 Antibodies with Different Species**

Species	PG-B6	PG-B6p*	PG-B6a	PG-B6m	N-70-6†	N-3†
Human	+	+	+	+	+	+
Bovine	+	+	+	+	−	ND
Ovine	+	+	+	+	−/+	ND
Swine	+	+	+	+	−	ND
Rabbit	+	+	+	+	ND	ND
Rat	+	+	+	+	−	ND
Chicken	−	−	+	−	−	−
Pigeon	−	−	+	−	−	−

ND, not done.
*Also suitable for staining of paraffin sections.
†Polyclonal antibodies. For specificity, see Materials and Methods.

any of the four MAbs in liver, thyroid, striated muscle, or kidney tissue sections.

Reactivity of Anti-BCL-6 MAbs with Normal Animal Tissues

All MAbs reacted strongly with the nuclei of germinal center B cells in peripheral lymphoid organs from calf, lamb, pig, rabbit, and rat (Table 2). As in humans, positivity was confined to nuclei of centroblasts and centrocytes. Mantle and marginal zone B cells were BCL-6$^-$. Most thymocytes were negative, but a few BCL-6$^+$ cells of undefined phenotype were present in the cortex and medulla.

Only PG-B6a reacted with avian (chicken and pigeon) tissues (Tables 1 and 2; Figure 3, B, E, and F, and Figure 4). Germinal center B cells of chicken cecal tonsil stained strongly for PG-B6a with a pattern identical to that of the mammalian species (Figure 3B). Notably, in the bursa of Fabricius from adult chicken, BCL-6 expression was topographically restricted to the cortex, the medulla being consistently negative (Figure 3F and Figure 4). None of the anti-BCL-6 polyclonal antibodies reacted with avian tissues. No reactivity was observed in animal tissues stained with MAbs against the human antigen CD20 and CD45 used as negative controls.

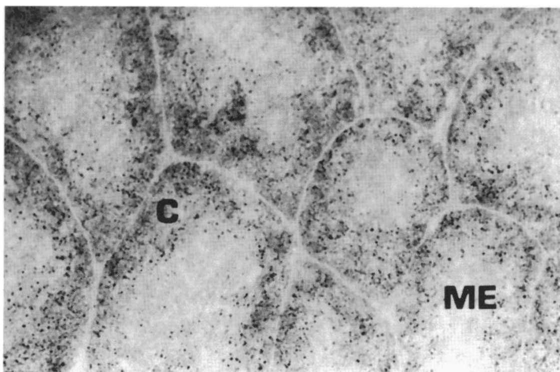

Figure 4. *Frozen section from the bursa of Fabricius of a 2-month-old chicken stained with the PG-B6a MAb. Reactivity is restricted to the nuclei of B cells located in the cortex (C), whereas B cells in the medulla (ME) are negative. APAAP technique; hematoxylin counterstain; magnification, ×125.*

Reactivity of Anti-BCL-6 MAbs with Reactive Lymphoid Tissues

The staining pattern of the four MAbs with reactive lymphoid tissues was similar to that in the tonsil. Hyperplastic perisinusoidal monocytoid B cells in cases of toxoplasmic lymphadenitis were BCL-6$^-$ (data not shown). Different histiocyte populations, including tingible-body and sinus macrophages in reactive lymphadenitis, epithelioid histiocytes in toxoplasmosis and sarcoidosis, and multinucleated giant cells in cat-scratch disease and tubercolosis, were consistently BCL-6$^-$.

Reactivity of the Anti-BCL-6 MAbs with Human Lymphomas

The immunostaining results on 173 cases of human lymphomas are summarized in Table 3. Both the predominant neoplastic cell population of small B lymphocytes and the larger lymphoid cells (prolymphocytes and paraimmunoblasts) clustered in proliferation centers (pseudofollicles) of B-cell chronic lymphocytic leukemia (B-CLL) were characterized by the absence of nuclear BCL-6 protein (PG-B6$^-$, PG-B6a$^-$, PG-B6p$^-$). However, approximately 50% of cases showed weak to moderate cytoplasmic reactivity with the PG-B6m antibody.

BCL-6 expression in follicular lymphomas paralleled that observed in normal germinal centers. Neoplastic centroblasts and centrocytes were strongly BCL-6$^+$, whereas neoplastic cells in cases of mantle cell and marginal zone lymphomas were usually BCL-6$^-$. We studied 30 cases of DLCL-B, which included 5 cases of T-cell-rich B cell lymphomas and 3 cases of primary mediastinal (thymic) B cell lym-

Table 3. *BCL-6 Expression in Human Lymphoid Neoplasms*

Lymphoma/Leukemia*	Number of cases	BCL-6 expression by tumor cells†
Non-Hodgkin's lymphomas		
B cell derived		
Pre-B acute lymphoblastic leukemia	7	0/7
Small lymphocytic/B-CLL	16	0/16‡
Hairy cell leukemia	6	0/6
Mantle cell leukemia	22	0/22‡
Marginal zone lymphoma	14	0/14‡
Follicle center lymphoma	24	24/24
Diffuse large cell lymphoma	30	29/30§
Burkitt's lymphoma	13	13/13
T cell derived		
Acute lymphoblastic leukemia T	10	0/10
Mycosis fungoides	3	0/3
Peripheral T cell lymphoma	6	0/6
Peripheral T cell lymphoma, AILD-like	2	0/2
Anaplastic large cell lymphoma	8	4/8
Hodgkin's disease		
Nodular, lymphocyte predominance	5	5/5
Nodular sclerosis	4	1/4
Mixed cellularity	3	1/3

*Categorized according to the revised European-American lymphoma classification.
†By labeling of frozen sections with the PG-B6, PG-B6a, and PG-B6p MAbs. PG-B6p gave identical results in paraffin sections.
‡A faint nuclear staining might be seen in occasional cases.
§The percentage of positive neoplastic cells ranged from 10 to 100%. The phenotype of the BCL-6$^-$ case is shown in Table 4 (case 4).

phomas, for BCL-6 expression (Table 3). In 21 of 30 cases we also investigated the following: 1) the reactivity pattern of PG-B6, PG-B6a, PG-B6p, and PG-B6m MAbs, 2) the correlation between expression of BCL-6 and other markers, and 3) the correlation between expression and rearrangement of the BCL-6 gene (Table 4). Strong nuclear expression of BCL-6 was observed in 29 of 30 DLCL-B cases. The percentage of BCL-6$^+$ neoplastic cells in these cases ranged from 10 to 100% (Table 4 and Figure 5, A and B), and the staining intensity varied from cell to cell. Case 4 in Table 4 displayed an unusual phenotype in that it was PG-B6$^-$/PG-B6a$^-$/PG-B6p$^-$/N-70-6$^-$/N-3$^-$ (Figure 5C) but showed strong cytoplasmic positivity for the PG-B6m antibody (Table 4 and Figure 5D). The BCL-6 gene was in germline configuration and all cells had an unexpectedly low proliferative index for a DLCL-B (<25% Ki-67$^+$ tumor cells; Table 4). The BCL-6 staining pattern of T-cell-rich B cell lymphomas (Figure 5, E and F) and primary mediastinal B cell lymphomas (Figure 6) was not unlike that of classical DLCL-B. There was no correlation between the expression of BCL-6 and other markers in DLCL-B (Table 4). Moreover, BCL-6 expression was independent of BCL-6 gene rearrangements (Table 4).

Strong nuclear positivity for BCL-6 was detected in the neoplastic (L&H) cells from all five cases of nodular, lymphocyte-predominance Hodgkin's disease (NLPHD) studied (Figure 5, G and H, and Figure 7). In contrast, only a small percentage of Hodgkin and Reed-Sternberg cells in two of the seven mixed-cellularity and nodular sclerosis Hodgkin's disease cases investigated expressed BCL-6.

Discussion

We have described the characteristics of four MAbs (PG-B6, PG-B6a, PG-B6p, and PG-B6m) raised against a recombinant GST-BCL-6 protein corresponding to the amino-terminal region (amino acids 3 to 484) of human BCL-6 and extended our previous studies[12,13] on BCL-6 expression in normal and neoplastic lymphoid tissues.

The specificity of our antibodies for BCL-6 is supported by immunoprecipitation studies and immunocytochemical analysis of EB3 cells transfected with a human BCL-6 expression vector. Moreover, the cell/tissue reactivity pattern of the four MAbs was identical to that we have previously reported to be specific for the BCL-6 protein, ie, nuclear expression by germinal center B cells.[12,13] Nuclear location of the BCL-6 protein is in keeping with the nature of this molecule, which is a member of the Kruppel-like subfamily of zinc finger transcription factors.[10,11] The nuclear staining pattern of BCL-6 (diffuse/microgranular) clearly differs from the speckled one associated with nuclear bodies that is typical of proteins belonging to other families of zinc finger proteins (eg, PML, a member the C3HC4 RING finger).[27,28] Moreover, the tissue distribution of BCL-6 is unlike that of PML, which is absent or weakly expressed in germinal center B cells (strongly BCL-6$^+$) but overexpressed in activated macrophages (consistently BCL-6$^-$).[28]

The reactivity of the four MAbs with tissues of various mammalian species was identical to that observed in human tissues, ie, strong nuclear positivity of germinal center B cells. These findings strongly suggest that our antibodies recognize epitope(s) shared by homologue(s) to human BCL-6 in various species and that the protein may play a universal role in B cell maturation processes that occur within the germinal centers.[29,30]

As, in addition to this common reactivity, each of the four MAbs manifest unique features, they may

Table 4. *BCL-6 Expression/Rearrangements and Immunophenotype in DLCL-B*

Case	Type	BCL-6*	BCL-2	CD10	CD20	CD30	CD38	Ki-67
1	Ib-B	75% (G)	75%	0%	100%	0%	0%	75%
2	CB	>90% (G)	0%	0%	100%	20%	0%	>90%
3	CBp	30% (G)	>90%	0%	100%	50%	0%	75%
4	CBp	0% (G)†	100%	0%	100%	0%	0%	<25%
5	CBp	>90% (G)	>90%	0%	100%	0%	0%	>90%
6	CBp	>90% (G)	ND	ND	100%	ND	ND	ND
7	CBp	90% (G)	ND	0%	100%	0%	0%	50%
8	CBp	>90% (G)	ND	0%	100%	100%	0%	50%
9	DLCL-B	10% (G)	75%	ND	100%	0%	ND	>50%
10	CBm	>75% (G)	ND	0%	100%	0%	0%	<25%
11	Ib-B	>75% (G)	ND	0%	100%	0%	0%	>75%
12	CB	>90% (R)	>90%	0%	100%	0%	0%	75%
13	MLCL-Bs	40% (R)	ND	0%	100%	0%	ND	>50%
14	CB	50% (R)	ND	0%	100%	0%	0%	50%
15	CBp	>90% (R)	0%	0%	100%	0%	>90%	>90%
16	CBp	>90% (R)	>90%	0%	100%	30%	0%	>50%
17	CBp	50% (R)	ND	0%	100%	0%	0%	>50%
18	CB	>75% (R)	ND	0%	100%	0%	0%	>75%
19	CBp	50% (R)	ND	0%	100%	0%	0%	>50%
20	CBp	75% (R)	ND	0%	100%	100%	0%	50%
21	CB	100% (R)	ND	0%	100%	NE	0%	50%

G, germline; R, rearranged; Ib-B, immunoblastic B; CBp, centroblastic polymorphic; CBm, centroblastic multilobated; MLCL-Bs, mediastinal large B cell lymphoma with sclerosis; ND, not done; NE, not evaluable.

*As defined by immunostaining of frozen sections with the four mAbs. Unless specified, it refers to nuclear labeling.

†The cytoplasm of neoplastic cells was strongly stained by PG-B6m.

recognize different epitopes on the amino-terminal portion of the BCL-6 protein. The characteristics of the PG-B6 MAb have already been described.[12] Briefly, this antibody recognizes a fixative-sensitive epitope of BCL-6 that is conserved through all mammalian species.

PG-B6a (a for avian) was the only reagent to recognize an epitope conserved in avian species, and this allowed BCL-6 expression to be analyzed in chicken lymphohemopoietic tissues, including the bursa of Fabricius, the organ responsible for generation of the B cell repertoire in avians.[31,32] Our immunohistological findings provide a strong argument for the existence of a putative avian homologue of human BCL-6, the expression of which appears to be topographically restricted to germinal center B cells of peripheral lymphoid organs and the cortical area of the bursa of Fabricius. Studies are in progress to correlate immunohistological findings with specific bursal functions and to analyze BCL-6 expression in the bursa during embryogenesis (B. Falini et al, manuscript in preparation).

The PG-B6p MAb recognizes an epitope of BCL-6 that is fixative resistant and, therefore, detectable in routine biopsies. Retrieval of the antigenic epitope recognized by PG-B6p was strictly dependent upon microwave heating in the presence of 1 mmol/L EDTA buffer, pH 8.0 (instead of citrate buffer, pH 6.0). EDTA may act by chelating calcium ions that are possibly responsible for masking the BCL-6 epitope, as reported for other nuclear antigens (eg, Ki-67).[20] PG-B6p was especially valuable for assessing BCL-6 expression in pathological conditions such as lymphocyte-predominance Hodgkin's disease and T-cell-rich B cell lymphoma,[33,34] which contain a low percentage of tumor cells that are often difficult to recognize with certainty in frozen sections.

PG-B6m was the least specific antibody as, in addition to BCL-6, it reacted with a yet uncharacterized antigen selectively expressed in the cytoplasm of mantle and marginal zone B cells.[35] This makes PG-B6m a useful marker for diagnosis of mantle cell and marginal zone lymphomas, as it concomitantly highlights the neoplastic component (labeling of the BCL-6-unrelated cytoplasmic component) and the germinal centers of residual entrapped follicles (nuclear labeling of BCL-6).

We used these antibodies to extend our previous studies on BCL-6 expression in human lymphomas. None of the acute lymphoblastic leukemia cases exhibited nuclear positivity for BCL-6, which is in keeping with the *in vitro* observation that immature B cell lines are usually BCL-6$^-$.[10] Future studies should be aimed at investigating whether BCL-6 may help in the differential diagnosis between pre-B and mature B (Burkitt-type) lymphoblastic leukemias.

B-CLL, as well as other categories of low grade B cell lymphomas (mantle cell- and marginal zone-derived) were characterized by the absence of BCL-6. Thus, BCL-6 expression in these lymphoma

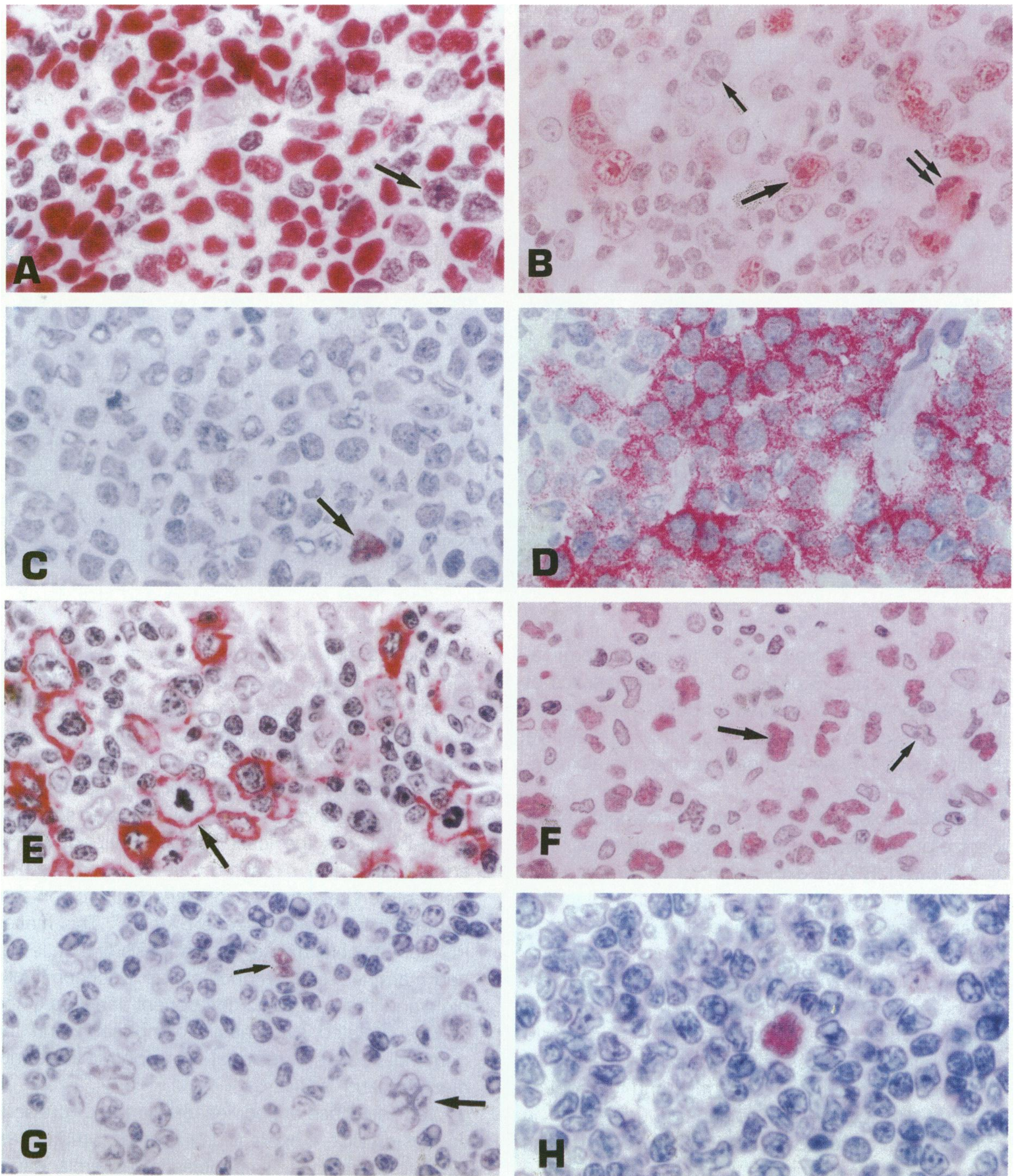

Figure 5. A: *DLCL-B (lymph node frozen section stained with PG-B6a). The nuclei of the majority of tumor cells show a strong and diffuse positivity for BCL-6. The* arrow *points to a BCL-6⁻ tumor cell.* B: *DLCL-B (lymph node paraffin section; microwave heated and stained with PG-B6p). Notice the variable expression of BCL-6 in tumor cells. The* long *and* short arrows *point to a BCL-6⁺ and BCL-6⁻ tumor cell, respectively;* double arrows *point to a neoplastic cell in mitosis showing cytoplasmic positivity for BCL-6.* C *and* D: *DLCL-B (lymph node frozen sections from case 4, Table 4). No nuclear reactivity of neoplastic cells with MAbs PG-B6, PG-B6a, or PG-B6p is observed in* C *(the* arrow *points to a residual BCL-6⁺ cell). The cytoplasm of tumor cells is strongly stained by PG-B6m in* D. E *and* F: *T-cell-rich B-cell lymphoma (lymph node paraffin sections; microwave heated). Tumor cells show strong membrane positivity for CD20 (L26;* E*); the* arrow *points to a CD20⁺ tumor cell in mitosis.* F: *The PG-B6p MAb labels the nuclei of tumor cells* (long arrow), *whereas epithelioid histiocytes are BCL-6⁻* (short arrow). G *and* H: *Lymph node paraffin sections from NLPHD stained for the zinc-finger proteins PML* (G) *and BCL-6* (H). G: *L&H cells of NLPHD* (long arrow) *are PML⁻; the* short arrow *in* G *points to a PML⁺ endothelial cell (positive internal control).* H: *The nucleus of an L&H cell is strongly BCL-6⁺ (PG-B6p MAb). The background of reactive IgD⁺/IgM⁺ small B lymphocytes are BCL-6⁻. APAAP technique; hematoxylin counterstain; magnification, ×800.*

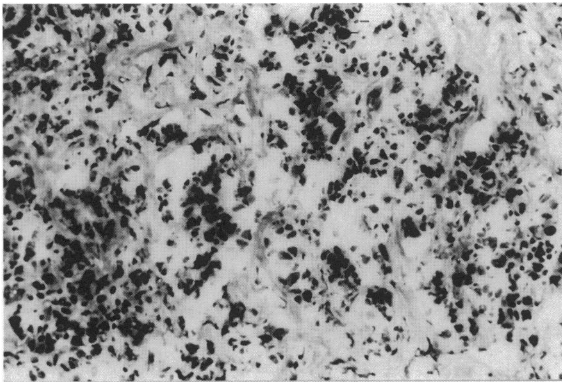

Figure 6. *Frozen section from primary mediastinal large B-cell lymphoma stained with the PG-B6a MAb. Most tumor cells surrounded by fibrous bands show strong BCL-6 nuclear positivity. APAAP technique; hematoxylin counterstain; magnification, ×800.*

subtypes parallels that of their putative normal counterparts, eg, $CD5^+$ peripheral B cells of the inner follicle mantle[18,36] and marginal zone B cells.[18,35] It has been recently suggested that the proliferation centers[18,37] or pseudofollicles[38] of B-CLL share several properties (including a delicate follicular dendritic cell network) with reactive B cell follicles[39,40] and that they may be centers of antigen-driven proliferation.[40] Our finding that B-CLL proliferation centers consistently fail to express BCL-6 would, rather, suggest that the B cell maturation events occurring in these structures are somewhat different from those observed in germinal centers of secondary follicles, possibly reflecting different functional properties. From a diagnostic point of view, detection of BCL-6 could be of value in differentiating B-CLL and mantle cell lymphoma cases with overlapping features. In fact, B-CLL proliferation centers are consistently $BCL\text{-}2^+/BCL\text{-}6^-$, whereas an opposite phenotype is observed in trapped germinal centers of mantle cell lymphomas.

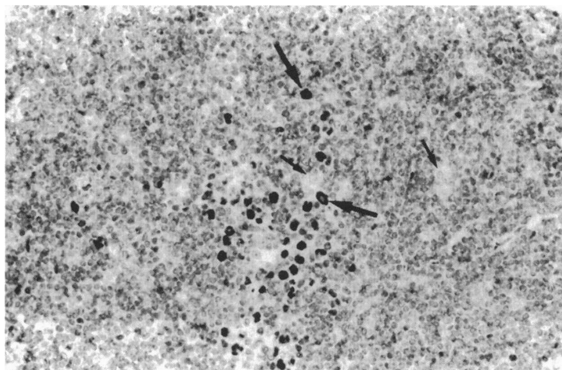

Figure 7. *NLPHD (microwave-heated paraffin section from lymph node biopsy). The PG-B6p MAb strongly labels the nuclei of tumor (L&H) cells* (long arrows) *against a background of $BCL\text{-}6^-/IgD^+/IgM^+$ B lymphocytes; the* short arrows *point to $BCL\text{-}6^-$ epithelioid histiocytes. APAAP technique; hematoxylin counterstain; magnification, ×125.*

Most DLCL-B displayed nuclear expression of BCL-6. The percentage of $BCL\text{-}6^+$ neoplastic cells varied greatly from case to case (range, 10 to 100%), and there was no correlation between BCL-6 gene rearrangement and the phenotype of lymphoma cells. The unusual phenotype of case 4 (germline BCL-6 gene, $PG\text{-}B6^-/PG\text{-}B6a^-/PG\text{-}B6p^-$, cytoplasmic positivity for PG-B6m, and <25% $Ki\text{-}67^+$ tumor cells) remains to be clarified. The staining pattern of BCL-6 in T-cell/histiocyte-rich B cell lymphomas[33,34] was comparable to that observed in classical DLCL-B. This immunohistological finding further supports the inclusion of this pathological entity into the category of DLCL-B.[18] Diagnostically, the labeling of paraffin sections with the PG-B6p MAb proved valuable for picking up the small percentage of $BCL\text{-}6^+$ large neoplastic B cells in the context of the $BCL\text{-}6^-$ background of T lymphocytes and epithelioid histiocytes, especially when combined with markers like CD20[41] and Ki-67. BCL-6 expression was also found in another variant of DLCL-B, the so-called primary mediastinal (thymic) B cell lymphomas.[42] Expression of BCL-6 was independent of rearrangements of the BCL-6 gene, which in this subtype of lymphoma occur at low frequency (approximately 6%) compared with other DLCL-B (approximately 30%; D. Knowles, personal communication).

Neoplastic (L&H) cells from all three cases of NLPHD showed strong nuclear expression of the BCL-6 protein, which agrees with the proposed relationships between NLPHD and germinal centers.[43]

In conclusion, because of their high specificity, broad reactivity with human and animal species including avians (MAb PG-B6a), and suitability for labeling paraffin sections (MAb PG-B6p), the reagents described in this paper may be regarded as an effective tool for both research and diagnostics.

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