A Specific Monoclonal Antibody (PG-B6) Detects Expression of the BCL-6 Protein in Germinal Center B Cells

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The BCL-6 gene is frequently involved in translocations occurring at the 3q27 locus and is rearranged in approximately 30% of diffuse large cell lymphomas and in a small fraction of follicular lymphomas. The BCL-6 gene encodes for a Krueppel-type zinc-finger protein, the cell/tissue expression and function of which is unknown. In this study, we describe a new monoclonal antibody (PG-B6) that is specifically directed against a fixative-sensitive epitope on the amino-terminal region of the BCL-6 protein. By immunocytochemical analysis, BCL-6 localizes in the nucleus where PG-B6 staining gives a microgranular/diffuse pattern with exclusion of the nucleoli. The main reactivity of PG-B6 in tonsil and spleen is with the nuclei of germinal center B cells, whereas B cells within the mantle and marginal zones do not express BCL-6. No other lymphoid cells in the tonsil express BCL-6 except for a subset of CD3+CD4+ intrafollicular and interfollicular T cells. A few lymphoid cells of unknown phenotype express BCL-6 in the thymus. Extra-lymphoid BCL-6 expression includes a weak nuclear positivity of epithelia. In non-Hodgkin’s lymphomas, BCL-6 expression parallels that observed in normal lymphoid compartments, eg, expression in germinal center-derived tumors (follicular and diffuse large cell lymphomas), but not in mantle cell and marginal zone lymphomas. In most diffuse large cell lymphomas, the BCL-6 protein is expressed at high levels in cases with or without BCL-6 gene rearrangements. These findings indicate that BCL-6 expression is specifically regulated during B lymphocyte development and suggest that BCL-6 may play a role during B cell differentiation in the germinal center. (Am J Pathol 1995, 147: 405-411)

Reciprocal translocations involving band 3q27 and several other chromosomal sites have been described in 8 to 12% of non-Hodgkin’s lymphomas. A breakpoint cluster region at 3q27 has been recently identified involving a gene named BCL-6 or LAZ3.1-6 Rearrangements of the BCL-6 gene have been detected in approximately 30% of non-Hodgkin’s lymphomas of diffuse large B cell type (DLCL)7-9 and are frequently associated with extranodal involvement and favorable clinical outcome.7

The BCL-6 gene encodes a protein of 706 amino acids with a predicted molecular weight of 79 kd.10 The NH₂ and COOH termini of BCL-6 share homologies with members of the Krueppel-like subfamily of zinc-finger proteins, many of which have been implicated in developmental regulation.10,11 By Northern blot analysis, expression of BCL-6 mRNA is found only in cell lines derived from mature B cells,10 suggesting that the BCL-6 protein may play a role in the control of normal B cell differentiation.10

To gain further insight into the function of BCL-6, we produced a monoclonal antibody (MAb), PG-B6, spe-

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cifically directed against the amino-terminal portion of the BCL-6 protein. The antibody was used to study (1) the cellular and tissue distribution of the BCL-6 protein and (2) the correlation between BCL-6 expression and rearrangement in DLCLs.

Materials and Methods

Production of a MAb (PG-B6) Against BCL-6

BALB/c mice were injected intraperitoneally with 200 μg of a glutathione S-transferase-BCL-6 fusion protein (amino acids 3-484) plus Freund's adjuvant twice at 10-day intervals. A booster was performed by intravenous injection of 200 μg of glutathione S-transferase-BCL-6 protein, and the fusion was carried out 3 days later, as described previously.12 Hybridoma supernatants were screened by the immunoalkaline phosphatase (APAAP) technique13 on cryostat sections of tonsil. Cloning was performed by a limiting dilution technique in flat-bottom 96-well plates.

Specificity of the PG-B6 Antibody

For Western blotting and immunoprecipitation analysis, RD (a B cell line that does not express BCL-6 mRNA), Bjab (a Burkitt cell line expressing BCL-6 RNA), control, and BCL-6-transfected EB3 cells (see below) were lysed with 1X loading buffer or single detergent lysis buffer (250 mM NaCl, 50 mM HEPES, pH 7.0, 0.1% NP-40, 5 mM EDTA, 1 mM dithiothreitol (DTT) containing a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride), sonicated, and immuno precipitated with the PG-B6 antibody supernatant diluted 1:2. The total lysates and the PG-B6 immune precipitates were loaded onto an 8% sodium dodecyl sulfate acrylamide gel and electrophoresed to nitrocellulose sheets.14 Nitrocellulose sheets were blocked in Tris buffered saline-Tween (TBS-T) buffer plus 5% bovine serum albumin and then incubated overnight at 4°C with a polyclonal antibody (1:500 dilution) directed against the carboxy terminus of the BCL-6 protein (C19, Santa Cruz, Biotechnology, Santa Cruz, CA). After extensive washing in TBS-T buffer, the blots were incubated for 1 hour with a horseradish peroxidase-conjugated goat anti-rabbit secondary antiserum followed by enhanced chemiluminescence reaction (Amersham, Arlington Heights, IL).

For expression of BCL-6 mRNA in EB3 cells, the generation and characterization of the BCL-6- and control-transfected EB3 cell lines are reported elsewhere.15 Briefly, the Burkitt lymphoma cell line EB3, which does not express BCL-6 RNA, 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.15 Control and BCL-6-transfected EB3 cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and G418 (1.4 mg/ml). Cytosines were prepared from exponentially growing cells, air dried overnight, fixed in acetone for 10 minutes, and stained by the APAAP technique.13

Tissue Specimens

BCL-6 expression was studied in normal lymphoid tissues and in the following B cell lymphoma cases: follicular lymphomas (n = 10), mantle cell lymphomas (n = 5), marginal zone lymphomas (n = 3), B-CLL (n = 5), and DLCLs (n = 10). The 10 DLCL cases were selected from a series of 40 DLCLs studied for rearrangement of the BCL-6 gene.

A portion of the samples 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 (see below). A piece of the samples was used for DNA extraction.

Single and Double Immunoenzymatic Labeling

All specimens were stained by the APAAP technique,13 counterstained for 5 minutes in Gill's hematoxylin, and mounted in Kaiser's 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/CD68.16 Detection of BCL-6 was by a three-stage mouse peroxidase anti-peroxidase (PAP) technique17 and that of the second pair of antigens by the APAAP procedure.13,18 Slides were then counterstained for 30 seconds in Gill's hematoxylin and mounted in Kaiser's gelatin.

Southern Blot Analysis

High molecular weight genomic DNA was extracted from non-Hodgkin's lymphoma samples by conventional techniques.15 For detection of BCL-6 rear-
rangements, DNA from each case was digested with BamHI and XbaI and subjected to Southern blot analysis with a genomic 4-kb SalI-SalI BCL-6 fragment as a probe.

**Results**

**Production and Specificity of the PG-B6 MAb**

Of 500 hybridoma supernatants (clone PG-B6), 1 showed strong and restricted reactivity with the nuclei of germinal center B cells on tonsil cryostat sections. The specificity of the PG-B6 MAb was evaluated by Western blotting and immunoprecipitation experiments with a variety of different cell lines: RD, a B cell line negative for BCL-6 RNA expression; Bjab, a cell line expressing BCL-6; EB3-BCL-6, a lymphoblastoid cell line stably transfected with a BCL-6 expression vector, and the control-transfected EB3 cell line. No specific anti-PG-B6 immunoreactive polypeptides were detected in the total lysates from any of the above mentioned cell lines by Western blotting (data not shown), suggesting that the PG-B6 MAb does not react against the denatured BCL-6 protein. Immunoprecipitates obtained from the same cell lines with PG-B6 were, therefore, resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted with a commercially available anti-BCL-6 polyclonal antibody (C19, Santa Cruz Biotechnology). As shown in Figure 1, an anti-PG-B6 and anti-C19 immunoreactive polypeptide of approximately 95 kd was detected in the Bjab and EB3-BCL-6 cell lysates, but not in any of the cell lines lacking BCL-6 RNA. The 95-kd protein corresponded to the BCL-6 gene product identified by in vitro transcription/translation of a BCL-6 cDNA clone (not shown) or transfection of the same clone into BCL-6-negative cells followed by immunoblot analysis. Immuno-}

**Expression of the BCL-6 Protein in Normal Human Tissues**

In normal tonsil, expression of the BCL-6 protein was mainly confined to the nuclei of centroblasts (Ki-67+/CD19+/CD20+) in the dark zone and of centrocytes in the basal and apical light zones of germinal centers (Figure 2a). Nuclear positivity for PG-B6 was diffuse/microgranular with exclusion of nucleoli (Figure 2, b and c). Strong cytoplasmic staining was observed only in mitotic figures and was not associated with metaphase chromosomes (not shown). No expression of BCL-6 was seen in other cell constituents of germinal centers, e.g., plasma cells (BCL-6+/CD38+), macrophages (BCL-6+/CD68+) (Figure 2b), and follicular dendritic cells (BCL-6+/CD23+). Most T cells within the germinal centers were BCL-6− (Figures 2c and 3), but some of them (5 to 10%) did express the protein (data not shown).

IgM+/IgD+ follicular mantle lymphocytes were negative for BCL-6, although rare BCL-6-positive cells could be occasionally seen in the follicular mantles. Some of these cells were T lymphocytes (BCL-6+/CD3+), whereas others were unidentified CD20+/IgD- elements, perhaps representing B cells escaping from germinal centers. T cells in the interfollicular area were PG-B6-negative except for a small percentage (<0.5%) of CD3+/CD4+ elements.

B and T cell compartments in normal spleen exhibited a staining pattern similar to that observed in the tonsil; notably, splenic marginal zone B cells were BCL-6 negative.

Cortical and medullary thymocytes were usually negative, with the exception of a few scattered cells resembling lymphoid cells, but of undefined phenotype. Extra-lymphoid expression of BCL-6 included a faint nuclear positivity of squamous epithelia in the
tonsil and thymus. No BCL-positive cells could be detected in normal liver.

**BCL-6 Expression in Non-Hodgkin’s Lymphomas**

BCL-6 expression in follicular lymphomas paralleled that observed in germinal center B cells of normal tonsil and reactive lymph nodes. In contrast, cases of mantle cell and marginal zone lymphomas as well as B-CLL were consistently negative. The results of PG-B6 immunostaining in 10 selected cases of DLCL are summarized in Table 1. Of 10 cases, 9, including 4 displaying BCL-6 gene rearrangements, showed a diffuse/microgranular positivity for BCL-6 that was strictly confined to the nucleus (Figure 4). The percentage of positive neoplastic cells in these cases ranged from 20 to 100% and the intensity of staining varied among neoplastic cells. One case, germinal for BCL-6, did not show any expression of the BCL-6 protein and this result was confirmed by using an antiserum directed against the carboxy terminus of the protein. In conclusion, our data indicate that BCL-6 is expressed in DLCLs regardless of the status (germ-line versus rearranged) of the BCL-6 gene.

**Discussion**

In this study, we describe the characteristics of a new murine MAb (PG-B6) specifically directed against the amino-terminal portion of the BCL-6 protein. The epitope recognized by PG-B6 is fixative sensitive and appears to be conserved through several mammalian species (data not shown), where it produces the same staining pattern as in human tissues, eg, strong nuclear labeling of germinal center B cells. The specificity of the PG-B6 MAb for BCL-6 is supported by immunoprecipitation studies and immunostaining of EB3 cells transfected with a BCL-6 expression vector.

Immunostaining with the PG-B6 MAb provides important information about the cell compartmentalization and tissue distribution of the BCL-6 protein. Both in cell lines (data not shown) and in primary tissues, BCL-6 appears to be strictly confined to the nucleus, where PG-B6 produces a typical diffuse/microgranular positivity sparing the nucleoli. The nuclear location of BCL-6 was expected as this protein is a member of the Kruppel-like subfamily of zinc-finger transcription factors. However, the nuclear staining pattern of BCL-6 clearly differs from the typical nuclear-body-associated, speckled pattern (eg, 10 to 15 discrete dots per nucleus) of promyelocytic leukemia.
proteins. Colocalization and immunoelectron microscope studies should provide additional insights concerning the topographical and functional compartmentalization of the BCL-6 protein in the nucleus and its relationship with other nuclear proteins.

Although present in a few other cell types, BCL-6 appears to be mainly expressed in B cells. The most striking characteristic of antibody PG-B6 is its strong reactivity with the nuclei of germinal center B cells; in contrast, IgD+/IgM+ virgin B lymphocytes of the follicle mantle, marginal zone B cells, and plasma cells do not react with PG-B6. Despite their physical proximity, mantle and germinal center B cells differ in their state of differentiation, circulation, and proliferative capacity and express a number of different function-associated antigens. Thus, the difference in BCL-6 expression among these B cell populations may reflect a role of BCL-6 in regulating germinal center-related B cell differentiation.

A small percentage of CD3+/CD4+ T cells in the intrafollicular and interfollicular areas of the tonsil was found to express BCL-6; whether these elements represent a subset of T lymphocytes with particular function needs to be further investigated.

BCL-6 expression in non-Hodgkin’s lymphomas parallels that observed in their normal counterparts, e.g., strong positivity of germinal center-derived B cell tumors and negativity of mantle cell and marginal zone lymphomas. In DLCLs and follicular lymphomas, BCL-6 gene rearrangements juxtapose heterologous promoters from different chromosomes to the BCL-6 coding region (exons 2–10), presumably leading to its deregulated expression. This deregulation may prevent the down-regulation of BCL-6 expression, which appears to be associated with differentiation and/or exit from the germinal center.

Consistent with this model, variable expression of

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* Categorized according to criteria given in Reference 24.
† Rearranged (R) or germline (G) BCL-6 gene.
‡ As defined by immunostaining with the PG-B6 mAb; the numbers given in parentheses refer to the percentage of neoplastic cells showing nuclear positivity.

![Figure 3](image3.png) Normal tonsil (cryostat section). Double staining for CD3 (brown) and BCL-6 (red). Large BCL-6+ B cells (long arrows) are predominantly detectable within the germinal center, with a few also present in the inner part of the mantle zone (M); double arrows point to a BCL-6-negative macrophage in the germinal center. IgD+/IgM+ mantle B cells do not express BCL-6; the short arrow points to a CD3+/BCL-6+ T cell in the mantle (M). Hematoxylin counterstain; ×800.

![Figure 4](image4.png) BCL-6 protein expression in a DLCL-B carrying a rearranged BCL-6 gene. Tumor cells show membrane positivity for CD20 (a, paraffin section) and strong nuclear positivity (diffuse/microgranular) for BCL-6 (b, cryostat section). The long arrow in a points to a mitotic figure showing diffuse BCL-6 cytoplasmic positivity with exclusion of chromosomes; the short arrows indicate BCL-6-negative epithelioid blastocytes. APAAP technique; hematoxylin counterstain; ×800.
BCL-6 is observed in DLCL independent of BCL-6 gene rearrangements. The significance of the finding of one DLCL case lacking BCL-6 expression (Table 1) is presently unclear, although it is conceivable that some tumors or some tumor subpopulations (see heterogeneity of expression in positive cases) may express BCL-6 at levels below those detectable by immunohistochemical analysis. Taken together, the results suggest that the analysis of BCL-6 protein expression may be of limited value in the diagnosis of DLCL and follicular lymphoma or in determining which cases carry lesions of the BCL-6 gene.

Acknowledgments

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