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Rapid Communication

Expression of the *bcl*-2 Oncogene Protein Is Not Specific for the 14;18 Chromosomal Translocation

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It has been reported previously that the* bcl-*2 proto-oncogene protein is detectable in neoplastic cells from cases of human lymphoma in which the 14;18 chromosomal translocation is present, but not in lymphomas that lack this chromosomal rearrangement or in normal lymphoid tissue. In the present study we confirmed, by immunohistologic labeling with polyclonal and monoclonal antibodies, that* bcl-*2 protein is strongly expressed in many cases of follicular lymphoma and that these neoplastic follicles differ clearly from their nonmalignant counterpart (reactive germinal centres) in which* bcl-*2 protein is undetectable. However we also found* bcl-*2 protein in normal T- and B-lymphoid cells and in a variety of lymphoproliferative disorders in which the 14;18 translocation is not present. It is therefore concluded that expression of* bcl-*2 protein is not a specific marker for lymphomas bearing the 14;18 chromosomal translocation and that the observations of other investigators may have reflected the inadequate sensitivity of their staining procedure. (Am J Pathol 1990, 137:225–232)

A reciprocal translocation between chromosomes 14 and 18 occurs in approximately two thirds of cases of human follicular lymphoma.[1] This translocation brings part of the *bcl*-2 gene on chromosome 18 into juxtaposition with the immunoglobulin heavy chain gene locus on chromosome 14. Most breakpoints on chromosome 18 occur in the untranslated region of the *bcl*-2 gene, with the consequence that the portion of the gene encoding this protein remains intact.[2–4]

Several laboratories have demonstrated the 14;18 translocation in human lymphomas by molecular biologic techniques (ie, Southern blotting or the polymerase chain reaction) but there has been only one report on the immunohistochemical localization of *bcl*-2 protein in sections of normal and neoplastic tissue. In this study, Ngan et al[5] found that *bcl*-2 protein was not detectable in normal human tissue but that it could be found in all lymphomas in which the 14;18 translocation was present. Most other lymphomas were *bcl*-2 negative, and the authors therefore concluded that the presence of detectable *bcl*-2 protein is a specific marker for the 14;18 translocation.

Here we report an immunohistologic study based on the use of recently produced monoclonal and polyclonal antibodies against *bcl*-2 protein. Our results confirm the findings of Ngan et al[5] that neoplastic germinal centers in most follicular lymphomas express high levels of *bcl*-2 protein, whereas normal germinal centers are negative. However *bcl*-2 protein also was detectable easily in normal lymphoid cells, of both B- and T-cell types, and in a substantial number of lymphoproliferative disorders in which the 14;18 translocation is not found, eg, hairy cell leukaemia and Ki-1 lymphoma. We conclude that immunohistologic staining for *bcl*-2 protein may help to distinguish between reactive and neoplastic lymphoid follicles, but that it does not provide a means of detecting lymphomas bearing the 14;18 translocation.

Material and Methods

Tissue Samples and Cell Line

Fresh frozen and paraffin-embedded tissue, comprising biopsy samples from lymphoproliferative disorders and

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reactive lymphoid tissue, were obtained *via* the routine diagnostic histopathology laboratories at the John Radcliffe Hospital, Oxford. Histologic diagnosis was based on conventional morphologic examination of paraffin-embedded material and on immunohistologic staining of frozen sections.[6] Frozen samples were stored at −70°C until use. High-molecular-weight genomic DNA was extracted from the frozen material by conventional techniques.[7]

The cell line 380, which carries a 14;18 translocation, was provided by Dr. L. Pegoraro.[8]

*Production of Anti-*bcl-*2 Antibodies*

Mice and rabbits were immunized with synthetic peptides corresponding to amino acids 41 to 54 and amino acids 61 to 76 of the *bcl-2* protein,[2,3] both of which had a C-terminal cysteine residue added to allow protein coupling. The major part of this work was performed with monoclonal antibodies raised to the first of these two peptides, which has the sequence GAAPAPGIFSSQPG. Peptides were synthesized by solid-phase techniques on an Applied Biosystems Peptide Synthesizer (Foster City, CA) model 430A with Fmoc HOBt/NMP chemistry. After cleavage and deprotection in the presence of scavengers, peptides were purified by reverse-phase HPLC before use. Peptides were coupled to thyroglobulin using m-maleimido benzoyl-N-hydroxy sulphosuccinimide ester and to bovine serum albumin (BSA) using sulphosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate. Peptide to protein ratios were determined for all conjugates by amino acid analysis and were in the range of 10% to 20% (w/w).

The peptides used were chosen on the basis that the human sequence differs from that found in the mouse and that there is a high content of proline residues.

Mice were immunized with peptide:thyroglobulin conjugate (50 μg/injection) three times at intervals of 10 days. Their sera were screened (at dilutions from 1/50 to 1/800) on cryostat sections of human tonsil and on a follicular lymphoma known to have the 14;18 translocation. When testing on tonsil tissue, sections were analyzed for any staining of lymphoid tissue, and when screening on follicular lymphoma a supernatant was considered to be positive if neoplastic follicles were stained while intervening areas were weak or negative. Although the work of Ngan et al[5] indicated that normal lymphoid tissue does not contain detectable levels of *bcl-2* protein, we included this tissue in the initial screen because of contrary findings from other sources.[9,10] Cell fusion and hybridoma production from the mouse immunized with peptide 41-54 was performed by conventional techniques (using the same immunohistologic screening method).[11] Three monoclonal antibodies (designated 90, 100, and 124) were found to react with lymphoid tissue in an identical pattern, each antibody showing extensive labeling of lymphoid areas but not of germinal centers (see Results for more details). Each of these three antibodies also labeled neoplastic lymphoid follicles in the case of follicular lymphoma and gave identical reactions when tested by Western blotting (see Results).

Rabbits received four injections, at intervals of 2 weeks, of peptide 41-54 coupled with thyroglobulin (200 μg/injection) emulsified in Freund's complete adjuvant. They were bled 1 week after the final injection and anti-peptide antibodies were affinity purified on a Sepharose-BSA-peptide column, as described previously.[12]

Immunohistological Staining

Staining was performed on cryostat and paraffin sections by the alkaline-phosphatase:anti-alkaline-phosphatase (APAAP) procedure, as described previously.[13] The rabbit polyclonal anti-*bcl-2* peptide antibodies were used at a concentration of 20 μg/ml, the monoclonal antibodies as undiluted tissue culture supernatants, and the mice sera at dilutions of 1:100 and 1:200.

Double Immunoenzymatic Staining

Cytocentrifuged cell preparations were acetone fixed, air dried, and incubated with Ki-67 monoclonal antibody (DAKOPATTS, Glostrup, Denmark) and then with peroxidase-conjugated goat anti-mouse Ig (DAKO). The peroxidase reaction was developed using diaminobenzidine/H_2O_2 substrate containing nickel chloride (1 mg/ml).[14] After washing with TBS, *bcl-2* antibody was added and the immunostaining was completed according to the APAAP technique as described.[13]

Molecular Specificity of Antibodies

The polyclonal and monoclonal antibodies were evaluated by Western blotting on extracts of normal human spleen, of follicular lymphoma with the 14;18 translocation, and of the cell line 380. Briefly, normal spleen, lymph node, or 380 cells were homogenized in 0.02 mol/l (molar) TRIS (pH 7.5) containing 1 mmol/l (millimolar) iodoacetamide, 0.2 mmol/l phenyl methyl sulphonyl fluoride, and 0.5% sodium azide (Sigma Chemical Co., St. Louis, MO). Any large fragments of tissue were allowed to settle and 20 μl of the remaining homogenate were added to 80 μl of sample buffer (0.2 mol/l TRIS, 2% SDS and 8 mol/l urea) and heated at 95°C for 4 minutes. Samples were run on a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel slab.[15] Proteins were transferred electrophoretically over-

night onto nitrocellulose paper by Western blotting[16] using a TRANSBLOT apparatus (Biorad, Richmond, CA). The nitrocellulose paper was stained with anti-*bcl-2* monoclonal antibodies, as previously described.[17] Negative controls included the omission of *bcl-2* antibodies or the use of an irrelevant monoclonal antibody.

Detection of t(14;18)

Southern Blot Analysis

Southern blotting was performed by conventional means.[7] Filters were hybridized with radiolabeled probes: pFL-1 HindIII-EcoRI 1.5 Kb for the major breakpoint region[18] and pFL-2 EcoRI 4 Kb for the minor breakpoint region.[19] DNA was digested with HindIII for both pFL-1 and pFL-2 probes.

The Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed on genomic DNA extracted from frozen tissue according to the method described by Saiki et al[20] and the PCR products were electrophoresed in 2% agarose gel and analyzed by conventional Southern blotting, using a radiolabeled 2.8-Kb EcoRI-HindIII *bcl-2* probe[21] in pSP 65 vector for the detection of the major breakpoint, and one kinased oligoprobe MC12[22] for the minor breakpoint. A universal J_H oligonucleotide primer was used for chromosome 14.[23] For chromosome 18, a B1 primer[23] was used for the major cluster region, a 20 mer B2 primer[2] for the remaining area of the major breakpoint region, and a MC8 primer for the minor breakpoint region.[22] In control experiments a 250 bp β globin gene sequence was amplified using two previously described primers.[20]

Results

Specificity of Anti-bcl-2 Antibodies

The monoclonal anti-*bcl-2* antibodies were analyzed by Western blotting on extracts of normal human spleen and of lymph node involved by a t(14;18)-positive follicular lymphoma, and on cell line 380. Under both reducing and nonreducing conditions, all three monoclonal antibodies against peptide 41-54 and mouse antiserum to peptide 61-76 recognized a band of 26 kd in extracts of normal spleen (Figure 1) and of a t(14;18)-positive follicular lymphoma. We could see no clear difference in the intensity of the 26-kd band between the three different antigen sources and the band was not observed in negative control samples. Cytologic preparations from the 380 cell line were strongly positive after immunostaining with all three monoclonal antibodies.

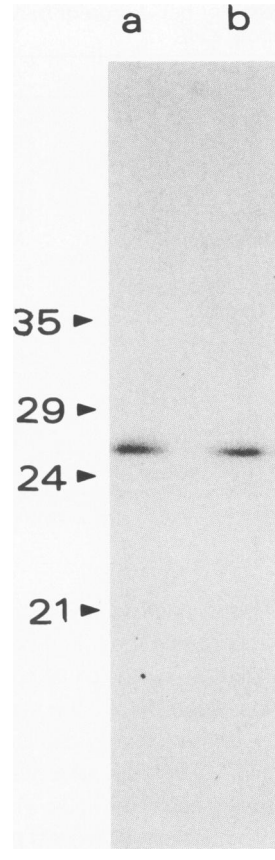

Figure 1. *Immunoblot analysis of normal spleen for* bcl-*2 protein. Monoclonal antibody anti-*bcl-*2 recognizes a 26-kd band, corresponding in size to the* bcl-*2α protein, under both* (a) *nonreducing and* (b) *reducing conditions (molecular weight standards are shown in kd).*

Immunohistologic Labeling of Human Tissues

Essentially identical staining reactions were obtained with both polyclonal and monoclonal antibodies to the 41-54 peptide, and with the polyclonal mouse serum to the 61-76 peptide, although the latter reagent gave additional staining that could be abolished largely by choice of suitable dilution.

Normal Lymphoid Cells

The staining reactions of the anti-*bcl-2* antibodies on normal tissues are summarized in Table 1. Almost all peripheral blood lymphocytes were strongly positive (Figure

Table 1. *Expression of* bcl-*2 Protein in Normal and Reactive Lymphoid Cells*

Cell	Staining
Peripheral blood	
Lymphocytes	Positive
Tonsil	
T cells	Positive
Mantle zones	Positive
Germinal centers	Negative*
Spleen	
T cells	Positive
Mantle zones	Positive
Germinal centers	Negative
Red pulp	Positive
Thymus	
Cortex	Negative†
Medulla	Positive

* Scattered positive cells were present in germinal centers.
† Many cells in the thymic cortex are unstained but scattered positive cells (ranging in intensity from weak to strong) were present.

2). In sections of lymph node and tonsil, there were many positive small lymphocytes, both in T-cell areas and in mantle zones (Figure 3), but germinal center cells were unstained, with the exception of a few scattered positive cells (Figure 3). A similar staining reaction was seen in spleen, in that both T- and B-cell areas were stained, but germinal centers were negative (Figure 4). The *bcl-2* antigen appeared to be intracytoplasmic in both cytocentrifuged cell preparations and in tissue sections.

This difference in *bcl-2* expression between germinal center cells and other lymphoid cells in reactive lymphoid tissue was confirmed by staining cytocentrifuged tonsil cells. In these preparations *bcl-2*-positive cells are mainly small lymphocytes, large cells being weakly stained or negative (Figure 5). Double staining for *bcl-2* protein and for Ki-67, a proliferation-associated antigen, confirmed that *bcl-2* protein was found in nonproliferating lymphoid cells (Figure 5).

The thymus also showed a clear distinction between resting and proliferating lymphoid cells in that cells in the medulla were stained, whereas only a few cells were strongly stained in the cortex, the majority being either weakly stained or unlabeled (Figure 6).

Neoplastic Lymphoid Samples

Immunohistologic analysis for *bcl-2* protein expression in lymphoproliferative disorders was performed on a total of 89 biopsies. The neoplastic follicles in most cases of follicular lymphoma (37 of 43 cases, 86%; Table 2) were labeled for *bcl-2* protein (Figure 7 a, b). Follicular lymphomas showed three different patterns of staining. In 20 cases the neoplastic nodules were positive and only scattered positive cells were seen in interfollicular areas. In the second pattern (17 cases), many positive cells were seen in interfollicular areas, giving a diffuse positive pattern that frequently made it impossible to distinguish the neoplastic nodules. Finally in six cases the neoplastic nodules were negative or very weakly stained, contrasting with the many strongly positive cells in the interfollicular areas (Figure 7c).

Molecular biologic analysis (with Southern blotting and/or PCR) was performed in 40 cases and demonstrated the 14;18 translocation in 23 of the 36 (64%) *bcl-2*–positive cases, whereas none of the negative cases had evidence of the translocation (Table 3).

Strong staining of neoplastic cells for *bcl-2* also was found in 31 of 38 (81%) cases of diffuse (ie, nonfollicular) lymphoma and lymphoproliferative disorders (Table 2), including chronic lymphocytic leukemia, hairy cell leukemia (Figure 8), T-cell lymphoma, and Ki-1 lymphoma (Figure 9). Reed–Sternberg and Hodgkin's cells in eight cases of Hodgkin's disease were negative for *bcl-2*, although in all samples many *bcl-2*–positive reactive lymphocytes were detected.

Discussion

The *bcl-2* gene is composed of two exons encoding two closely similar proteins, *bcl-2* α (26 kd) and *bcl-2* β (21 kd), which differ only in their carboxyl terminal portions.[3] The *bcl-2* gene has been classified as a possible proto-oncogene[3,24,25] both because of its involvement in the 14;18 translocation and because of homology with the Epstein–Barr virus BHRF1 gene.[4] Furthermore it has been shown that lymphoid and myeloid cell lines transfected with the human *bcl-2* gene acquire the ability to survive in the absence of otherwise essential growth factors.[26] Evidence has been provided that *bcl-2* α is anchored to the cell membrane, although it is probably, unlike a number of other cellular oncogene products, not a cell-surface receptor because it appears to lie within the cell cytoplasm.[4,9]

Ngan et al[5] studied the expression of *bcl-2* protein by immunohistochemical staining of lymphoid tissue specimens using a rabbit anti–*bcl-2* antiserum. They found *bcl-2*–positive neoplastic cells in all lymphomas bearing the 14;18 translocation, whereas reactive tissue and tumors without the translocation were found to be consistently negative. The only exceptions were four cases expressing *bcl-2* protein in which t(14;18) was not detectable by Southern blotting, but Ngan et al[5] suggested that the translocation in these cases involved breakpoints lying outside regions covered by the DNA probes they used. Based on their results, the authors concluded that immunohistochemically detectable *bcl-2* protein is diagnostic of the 14;18 translocation.

Here we have investigated the distribution of *bcl-2* protein in both normal and neoplastic tissue using polyclonal and monoclonal antibodies. The specificity of our antibod-

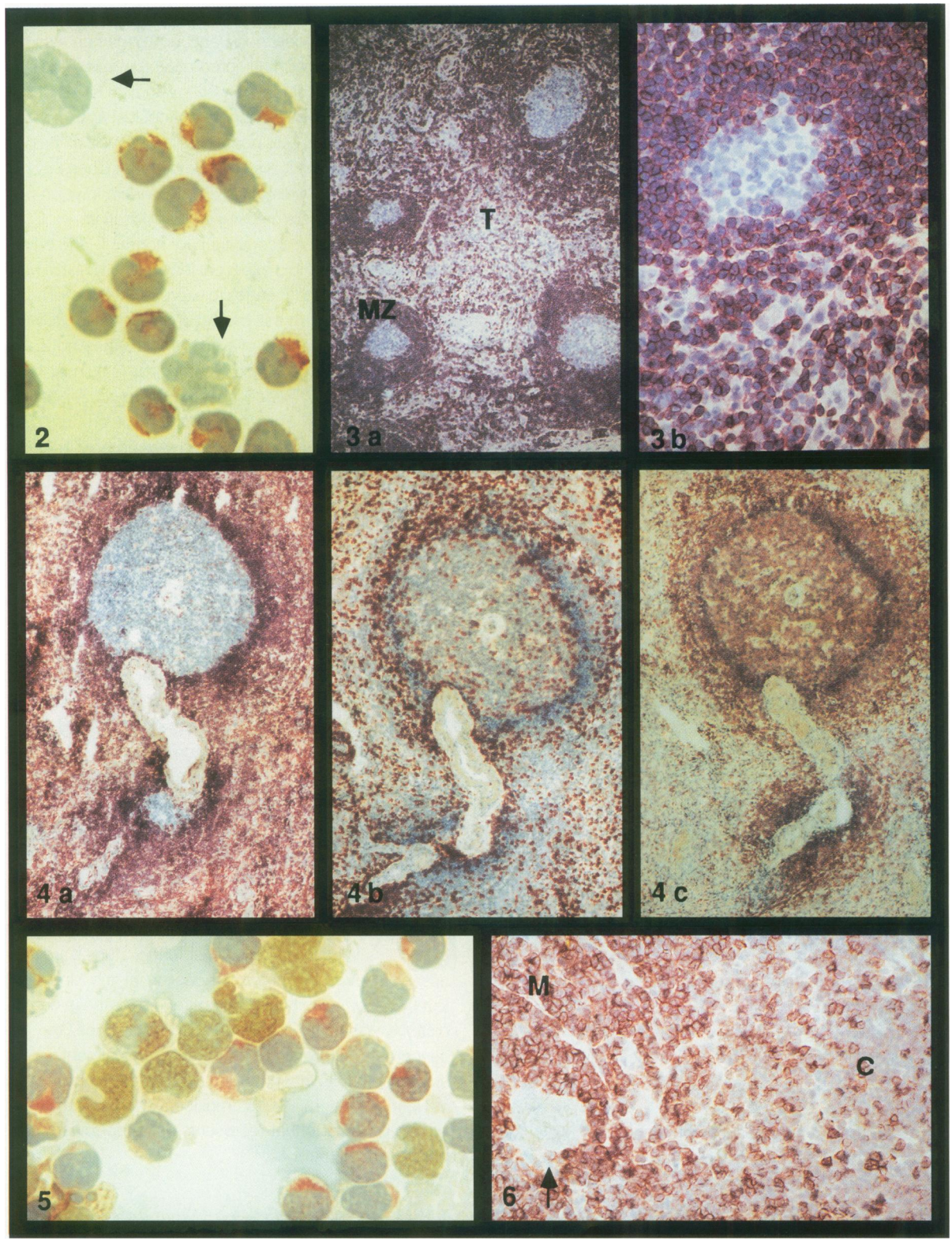

Figure 2. *Peripheral blood leukocytes. Cytoplasmic* bcl-*2 protein is seen in small lymphocytes. Note unstained nonlymphoid cells (arrows) (APAAP method).* **Figure 3.** *Lymph node with reactive follicular hyperplasia.* Bcl-*2 protein is detectable in mantle zone B cells (MZ) and in interfollicular T-cell areas (T), but is absent from germinal centers (APAAP method).* **Figure 4.** *Normal human spleen.* **(a)** *Human spleen expresses* bcl-*2 protein in both B- and T-cell areas and in the red pulp but not in germinal centers (GC). Staining of adjacent sections for* **(b)** *T cells (CD3) and* **(c)** *B cells (CD22) (APAAP method).* **Figure 5.** *Normal human tonsil (cytocentrifuged cell suspension). Double staining for* bcl-*2 and the proliferation-associated marker Ki-67. Large cells (representing germinal center cells) are Ki-67–positive (brown staining) but are negative for* bcl-*2 protein, which is confined to the cytoplasm of smaller cells (red staining) (immunoperoxidase and APAAP).* **Figure 6.** *Human thymus.* Bcl-*2 protein is present in many cells in the medulla (M), but in only scattered cells in the cortex (C). The arrow indicates a Hassall's corpuscle (APAAP method).*

Table 2. *Expression of* bcl-*2 Protein in Lymphoproliferative Disorders*

Diagnosis	Cases positive/ cases studied
Non-Hodgkin's lymphoma	
Low grade	
Follicular	37/43
Chronic lymphocytic leukemia	7/7
Hairy cell leukemia	3/3
T cell, angioimmunoblastic type	1/2
High grade	
B cell, large cell type	11/14
T cell, large cell type	5/6
B cell, lymphoblastic lymphoma	0/1
T cell, lymphoblastic lymphoma	1/2
Anaplastic large cell Ki-1 lymphoma	3/3
Hodgkin's disease	0/8*

* Refers to the staining of Reed-Sternberg and Hodgkin's cells.

ies was demonstrated by the fact that a protein band of appropriate molecular weight (26 kd) was seen on Western blotting of a B-cell line (380), which is known to carry the 14;18 translocation[8] and to express high levels of *bcl*-2 mRNA.[21] It should be noted also that we obtained essentially identical results, both in terms of Western blotting and immunohistologic reactivity, with antibodies raised against each of the two peptide sequences from the *bcl*-2 molecule.

In clear contrast to the results reported by Ngan et al,[5] we could find easily detectable levels of *bcl*-2 protein in many normal B and T lymphocytes in peripheral blood and in lymphoid tissue, indicating that the 14;18 translocation is not necessary for the production of this protein. The validity of the staining results was supported by Western blotting analysis of normal spleen, which showed that this tissue contains a protein of the same molecular weight as that seen in the 380 cell line (Figure 1). Recently other laboratories have provided evidence that cell lines, mainly of pre-B-cell origin, and normal lymphoid cells lacking the translocation may produce at least low levels of *bcl*-2 mRNA and/or protein, as demonstrated by Northern or Western blotting.[9,10,21] These results are likely to represent synthesis of *bcl*-2 protein early in lymphocyte maturation, following which the mRNA either disappears or decreases to low levels.

Why Ngan et al[5] failed to detect *bcl*-2 protein in normal lymphoid tissue is unclear, but the answer may lie in the sensitivity of their labeling technique. Ngan et al[5] used polyclonal rabbit antiserum raised against a recombinant *bcl*-2 protein that had been produced in bacteria and purified by elution from SDS polyacrylamide gels. Crude antisera inevitably carry a risk of unwanted background labeling and it is noteworthy that Ngan et al[5] reported nonspecific reactivity with their antiserum both on frozen sections, in which there was 'background staining of non-lymphoid cells . . . or intercellular staining' and on cell lines, in which 'faint background bands' were noted on Western blot analysis. Unwanted immunocytochemical reactions due to nonspecific antibodies may be avoided by using an antiserum at high dilution, but this may have led Ngan et al[5] to detect only cells with high levels of *bcl*-2 protein expression (ie, neoplastic follicles) and to obtain negative results on cells containing lower levels of the protein. In this context it may be relevant that Chen-Levy et al[9] reported that levels of *bcl*-2 protein were lower in normal cells than in cells carrying the translocation.

If *bcl*-2 protein is a constituent of normal T- and B-lymphoid cells, one would expect at least some t(14;18)-negative tumors of both lineages to express *bcl*-2 protein. Our own studies confirm this prediction in that 31 of 38 neoplasms (81%), in many of which t(14;18) has not been reported, including chronic lymphocytic leukemia, hairy cell leukemia, and T-cell lymphoma, were *bcl*-2–positive. These findings are different from the results reported by Ngan et al[5] who detected *bcl*-2 protein in only 14 of 59 (24%) diffuse lymphoproliferative neoplasms, and who argued that all of these positive cases had the 14;18 translocation.

One observation in the present paper that correlates with the study of Ngan et al[5] is that reactive germinal centers were clearly unstained for *bcl*-2 protein and thus contrasted with neoplastic follicles, which were strongly positive in most nodular lymphomas. Proliferating T cells (in the thymic cortex) also lacked *bcl*-2 protein, suggesting that both T and B lymphocytes suppress production of (or degrade) *bcl*-2 protein when they are stimulated to proliferate. Many of the proliferating cells present in germinal centers are destined for extinction, presumably because they have not encountered their respective antigen, and it may be that the absence of *bcl*-2 protein from most germinal center cells is related to their short subsequent survival. The idea that *bcl*-2 production is suppressed in proliferating T and B cells contrasts with the reported increase[10,27] in *bcl*-2 mRNA in peripheral blood lymphocytes stimulated *in vitro*. However further studies are necessary to evaluate mRNA production at the single-cell level, eg, by *in situ* hybridization, to resolve these contradictions. Whatever its physiologic significance, the absence of *bcl*-2 protein from normal germinal center cells suggests that its inappropriate expression by these cells as a result of the 14;18 translocation may provide a growth advantage that represents one step toward neoplastic transformation.[26,28,29]

Table 3. *Correlation of* bcl-*2 Protein Expression and t(14;18) in Follicular Lymphoma*

Staining pattern for *bcl*-2 protein	Cases with t(14;18)*/cases positive for *bcl*-2 protein
Positive, nodular	12/19
Positive, diffuse	11/17
Negative, nodular	0/4

* Detected by Southern blot and/or polymerase chain reaction.

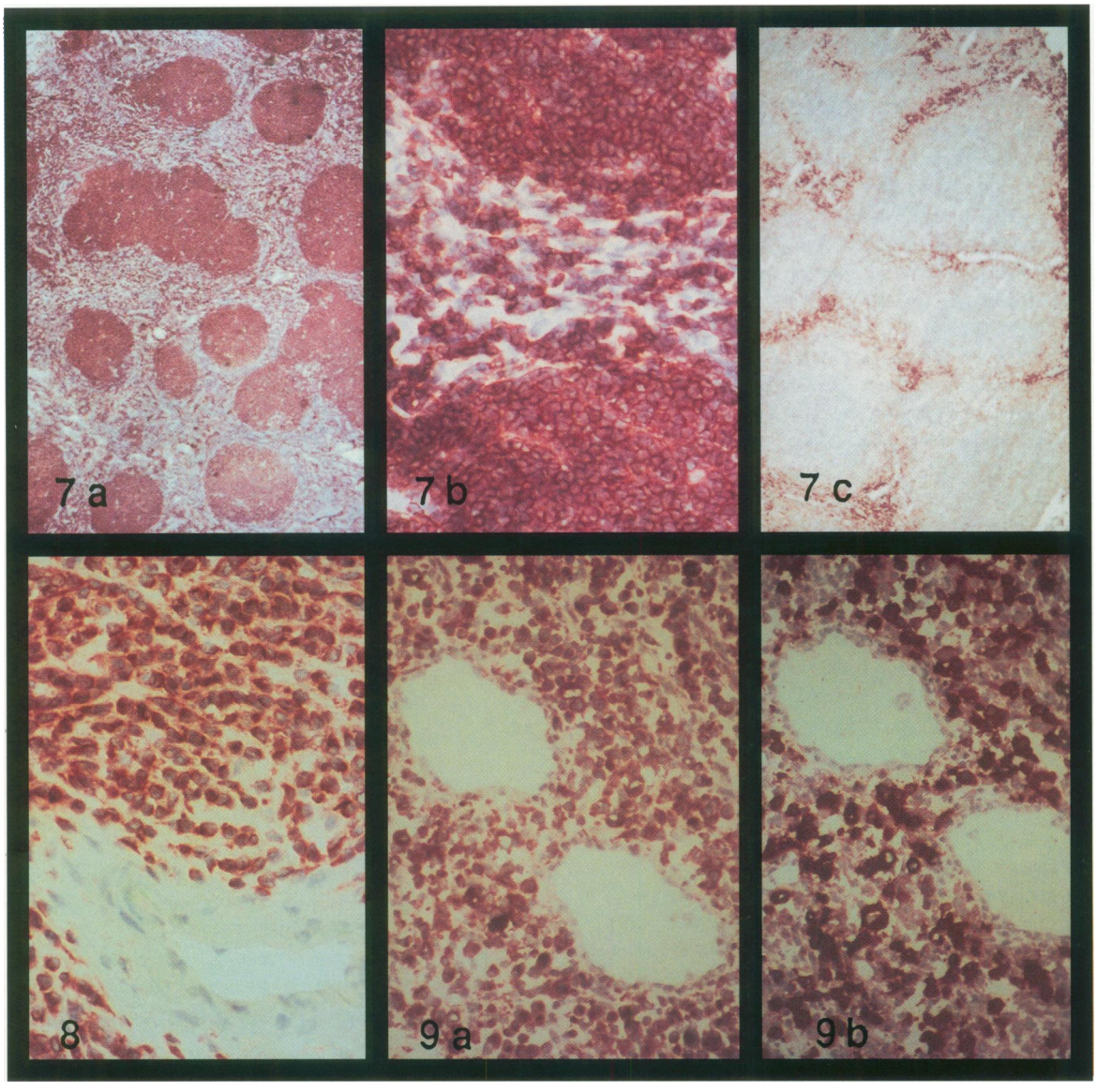

Figure 7. *Follicular lymphoma. Two cases of follicular lymphoma, one of which* **(a, b)** *is positive for* bcl-*2 protein and the other* **(c)** *is negative (APAAP method).* **Figure 8.** *Hairy cell leukemia.* Bcl-*2 protein is strongly expressed (APAAP method).* **Figure 9.** *Anaplastic large cell lymphoma (Ki-1 lymphoma).* **a:** bcl-*2 protein is expressed by this high-grade lymphoma, which is also positive* **(b)** *for CD30 (Ki-1) antigen (APAAP method).*

The present study indicates that *bcl*-2 protein is immunohistochemically detectable in normal lymphoid tissues, that it is absent from proliferating lymphoid cells, and that it is expressed in a wide range of neoplastic lymphoproliferative diseases. Consequently the immunohistologic demonstration of this molecule is not specific for the presence of the 14;18 translocation. The major diagnostic potential of staining for *bcl*-2 protein therefore would appear to lie in the distinction between reactive and neoplastic germinal centers.

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