Prolonged Survival of B-Lineage Acute Lymphoblastic Leukemia Cells Is Accompanied by Overexpression of *bcl-2* Protein

By Dario Campana, Elaine Coustan-Smith, Atsushi Manabe, Michael Buschle, Susana C. Raimondi, Frederick G. Behm, Richard Ashmun, Maurizio Aricò, Andrea Biondi, and Ching-Hon Pui

Overexpression of *bcl-2* delays the onset of apoptosis in lymphohematopoietic cells. We measured levels of *bcl-2* protein in normal and leukemic human B-cell progenitors with a specific monoclonal antibody and flow cytometry. Normal immature B cells had low levels of *bcl-2* protein; the intensity of fluorescence, expressed as molecules of soluble fluorochrome per cell, within CD10$^+$ cells was 3,460 ± 1,050 (mean ± SD; 5 samples). In 16 cases of B-lineage acute lymphoblastic leukemia (ALL), cells had levels of *bcl-2* that were strikingly higher than those of their normal counterparts (33,560 ± 14,570; $P < .001$ by *t*-test analysis). We next investigated whether the intensity of *bcl-2* expression correlated with the resistance of immature B cells to in vitro culture. In 12 cases of B-lineage ALL, the cells recovered after 7 days of culture on allogeneic bone marrow stromal layers were 69% to 178% (median, 95.5%) of those originally seeded. Prolonged survival of leukemic cells in vitro was observed even in the absence of stromal layers in 6 of these 12 cases; the intensity of *bcl-2* protein expression in these cases was 45,000 ± 13,270, compared with 21,500 ± 7,260 in the 6 cases in which greater than 99.5% of cells rapidly died by apoptosis under the same culture conditions ($P = .003$). Five immature B-cell lines, continuously growing in the absence of stroma, had the highest *bcl-2* expression (79,400 ± 20,330). By contrast, most normal CD19$^+$, sIg$^-$ immature B cells died despite the presence of bone marrow stromal layers; 9.7% to 28.2% were recovered after 7 days of culture in three experiments. We conclude that abnormal *bcl-2* gene expression influences the survival ability of B-cell progenitors. This may contribute to leukemogenesis and explain the aptitude of leukemic lymphoblasts to expand outside the bone marrow microenvironment.

THE PRODUCT OF the human gene *bcl-2* antagonizes the triggering of programmed cell death ("apoptosis").[1-5] Thus, overexpression of *bcl-2* delays apoptosis in factor-dependent cell lines cultured under suboptimal conditions[2,3] and in cortical thymocytes exposed to glucocorticoid, radiation, and CD3 antibodies.[6]

It has been postulated that abnormally high levels of *bcl-2* at critical stages of differentiation may favor the development of neoplasia.[4,5,7-10] This concept is supported by the observation of discrepancies in *bcl-2* protein expression between follicular B-cell non-Hodgkin's lymphoma (NHL) cells, which exhibit high levels of *bcl-2,* and their normal counterparts, proliferating germinal center cells, which appear to be *bcl-2* negative and are susceptible to apoptosis during the selection processes that occur during immune response in the peripheral lymphoid organs.[11-14] Abnormally high expression of *bcl-2* by these cells may suppress apoptosis, leading to the survival of inappropriate clones. In NHL, increased *bcl-2* synthesis results from a chromosomal translocation, t(14;18), that brings the *bcl-2* gene on chromosome 18 at q21 next to the Ig heavy chain locus (14q32).[15-18] However, abnormally high levels of the *bcl-2* protein have also been observed in rare cases of NHL that lack either karyotypic or molecular evidence of *bcl-2* gene rearrangement,[19] suggesting that other mechanisms may induce overexpression of *bcl-2.*

Whether malignancies beyond NHL overexpress *bcl-2* is still unclear. Because the *bcl-2* protein is widely distributed among different normal lymphohematopoietic cells and is found in lymphoid, myeloid, and erythroid lineages,[11,12] techniques for detecting *bcl-2* expression at the single cell level are needed to assess the amounts of this protein in normal versus malignant cells. So far, attempts to quantitate *bcl-2* expression have generally relied on measurements of the abundance of messenger RNA (mRNA) transcripts, an approach that is not suited for comparative investigations with small cell subpopulations.

We used flow cytometric techniques and a monoclonal antibody (MoAb) that recognizes the human *bcl-2* protein[11] to study *bcl-2* protein expression in normal human B-cell progenitors and their neoplastic counterparts, B-lineage acute lymphoblastic leukemia (ALL) cells. Our findings indicate that normal B-cell progenitors express low levels of *bcl-2* protein, in contrast to ALL cells, which accumulate high levels of the protein despite the absence of detectable rearrangements of the *bcl-2* gene. Differences in *bcl-2* protein expression correlate with the cells' ability to survive in vitro and may confer a growth advantage to leukemic lymphoblasts.

MATERIALS AND METHODS

Cells. Bone marrow (BM) samples were taken from eight healthy BM transplantation donors, aged 2 to 23 years (median, 11 years). Normal peripheral blood (PB) samples were collected from five healthy 22- to 37-year-old volunteers. Tonsil samples were from three children undergoing routine tonsillectomies; cell suspensions were prepared with forceps and surgical blades. Leukemic marrow or PB samples (patients 1 and 6, Table 1) were collected at diagnosis from 16 patients, aged 1 to 12 years (median, 6 years), with B-lineage ALL. In all cases, greater than 90% of the blasts expressed CD22, HLA class II antigens, and terminal deoxynucleotidyl transferase (TdT)

From the Department of Hematology-Oncology and the Department of Pathology and Laboratory Medicine, St Jude Children's Research Hospital, and College of Medicine, University of Tennessee, Memphis; the Clinica Pediatrica Università, IRCCS Policlinico San Matteo, Pavia, Italy; and the Clinica Pediatrica Università di Milano, Ospedale San Gerardo, Monza, Italy.

Submitted July 24, 1992; accepted October 15, 1992.

Supported by grants from the National Cancer Institute (CA-20180 and CA-21765) and Bristol-Myers, and by the American Lebanese Syrian Associated Charities (ALSAC).

Address reprint requests to Dario Campana, MD, PhD, Division of Bone Marrow Transplantation, Department of Hematology-Oncology, St Jude Children's Research Hospital, 332 N Lauderdale, Box 318, Memphis, TN 38101.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

0006-4971/93/8104-0004$3.00/0

(Table 1). Samples from patients 2 through 4, 9, 10, and 12 were included in a previous publication.[20] The Institutional Review Board approved these studies and informed consent was obtained from patients and their guardians. Mononuclear cells were collected after centrifugation on a density gradient (Lymphoprep; Nycomed, Oslo, Norway) and washed three times in phosphate-buffered saline (PBS). The following cell lines derived from samples of B-lineage ALL were investigated: 380 and MR87 (provided by Dr H.G. Drexler, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany); SUPB15 (provided by Dr S.D. Smith, University of Chicago, Chicago, IL); and Nalm6 and REH (American Type Culture Collection, Rockville, MD).

In vitro culture experiments. Mononuclear cells from leukemic samples were cryopreserved within 4 hours of collection and used immediately after thawing. The cells' viability always exceeded 95% by trypan-blue dye exclusion. Normal $CD19^+$ BM cells were obtained by using CD19-immunomagnetic beads (Dynal, Oslo, Norway). After separation on a magnet, cells were detached from the beads using a goat antiserum to mouse Fab (DETACHaBEAD; Dynal). These procedures were performed according to the manufacturer's instructions and yielded cell populations containing 91% to 98% of $CD19^+$ cells, as assessed by flow cytometric analysis (see below).

BM stromal cells were prepared as described in Manabe et al.[20] All culture experiments were performed with AIM-V tissue culture medium (GIBCO, Grand Island, NY; cat. no. 320-2055AJ) without animal sera. Leukemic cells and normal $CD19^+$ cells were resuspended in AIM-V medium at a final concentration of 10^6/mL and placed directly in tissue culture plates or seeded onto marrow stromal cells. All cell cultures were performed in an incubator set at 37°C, 5% CO_2, and 90% humidity.

Estimate of bcl-2 *protein expression and flow cytometry.* The anti-*bcl-2* MoAb used in this study was raised by immunizing mice with a synthetic peptide corresponding to amino acids 41 to 54 of the *bcl-2* protein.[11] The antibody recognizes a 26-Kd band, identical in size to the *bcl-2α* protein, in immunoblots under both reducing and nonreducing conditions.[11] Staining with this reagent is confined to the cytoplasm. In the present study, antibody reactivity was analyzed with cytocentrifuge preparations as well as by flow cytometry using suspensions of permeabilized cells. The former were prepared with a Cytospin II Shandon centrifuge (Shandon, Runcorn, UK), air dried, fixed in acetone:methanol 1:1 for 15 minutes at 4°C, and labeled with antibodies and second-layer antisera. In these experiments, anti-

Table 2. *bcl-2* Protein Expression in Normal BM B Cells

Sample No.	*bcl-2* Expression Within		
	$CD19^+$	$CD10^+$ *	$sIgM^+$ †
1	3.8 (5.2)‡	5.0 (30.1)	NT
2	3.0 (4.8)	4.1 (9.4)	1.6 (<1)
3	2.9 (21.6)	2.6 (27.9)	2.3 (20.3)§
4	3.0 (11.6)	2.9 (11.0)	1.8 (2.7)
5	2.6 (7.4)	2.7 (9.1)	2.0 (6.8)
6	NT	NT	5.2 (46.9)§

Abbreviation: NT, not tested.

* Percentage of $CD19^+$ cells expressing CD10 in the samples studied was as follows: no. 1, 44.3%; no. 2, 56.9%; no. 3, 83.7%; no. 4, 83.8%; No. 5, 65.3%.

† Percentage of $CD19^+$ cells expressing sIgM in the samples studied was as follows: no. 2, 13.0%; no. 3, 30.9%; no. 4, 21.1%; no. 5, 18.8%.

‡ MESF $\times 10^3$ after staining with anti-*bcl-2* antibody (percentage of cells with fluorescence levels above the highest obtained with an isotype-matched, unreactive antibody).

§ Two distinct cell populations were shown by anti-*bcl-2* staining. In sample 3, 20.3% of cells had a MESF of 23×10^3, while in sample 6, 46.9% of cells had a MESF of 32×10^3. These cells are likely to be mature, recirculating B lymphocytes.

Table 1. Characteristics of B-Lineage ALL Cases Studied

Patient No.	Sex/Age	WBC ($\times 10^9$/L)	%S*	Immunophenotype†	Karyotype
1	M/6	72.7	3.0	$CD34^+$, $CD10^+$	46,XY,t(9;22)(q34;q11)
2	M/6	56.2	9.2	$CD34^-$, $CD10^+$, $c\mu^+$	47,XY,+X,del(6)(q15q25),del(11)(q23),del(16)(q22),−1,+der(1)t(1;?)(q21;?),−9,+der(9)t(9;?)(p13;?),−21,+der(21)t(21;?)(q22;?)
3	M/1	109.6	8.5	$CD34^+$, $CD10^-$	46,XY,t(4;11)(q21;q23)
4	M/10	49.2	3.3	$CD34^-$, $CD10^+$, $c\mu^+$	46,XY,del(9)(p13),−19,+der(19)t(1;19)(q23;p13)
5	M/9	372.6	3.8	$CD34^+$, $CD10^+$	46,XY,t(9;22)(q34;q11)
6	F/10	870.0	3.5	$CD34^+$, $CD10^-$	46,XX,t(4;11)(q21;q23)
7	M/6	13.8	2.7	$CD34^+$, $CD10^+$, $c\mu^+$	46,XY,del(6)(q21q25)
8	M/5	57.1	8.8	$CD34^-$, $CD10^+$	Not available (DNA index = 1.00)
9	F/5	6.0	2.3	$CD34^+$, $CD10^+$, $c\mu^+$	46,XX,del(11)(q23)
10	M/3	38.7	2.2	$CD34^+$, $CD10^+$, $c\mu^+$	46,XY,del(12)(p12)/46,XY,−C,+mar
11	M/12	225.0	1.7	$CD10^+$ ‡	Not available (DNA index = 1.00)
12	F/6	99.5	7.5	$CD34^-$, $CD10^+$	46,XX,−13,+der(13)t(13;?)(q34;?)
13	F/1	186.0	4.8	$CD34^+$, $CD10^+$	45,XX,−7,−12,del(1)(q32),+der(12)t(?7;12)(q11;p13)
14	M/5	21.0	22.6	$CD34^-$, $CD10^+$	Not available (DNA index = 1.00)
15	M/4	24.1	8.6	$CD34^+$, $CD10^+$	47,XY,+21/46,XY,del(9)(p21)
16	F/9	13.7	1.4	$CD34^+$, $CD10^+$	45,X,−X,del(12)(p11)

Abbreviation: WBC, white blood cell count at diagnosis.

* Percentage of cells in S phase at diagnosis.

† In all cases, greater than 90% of blasts expressed CD19 and/or CD22, as well as nuclear TdT.

‡ CD34 and $c\mu$ expression were not tested in this case.

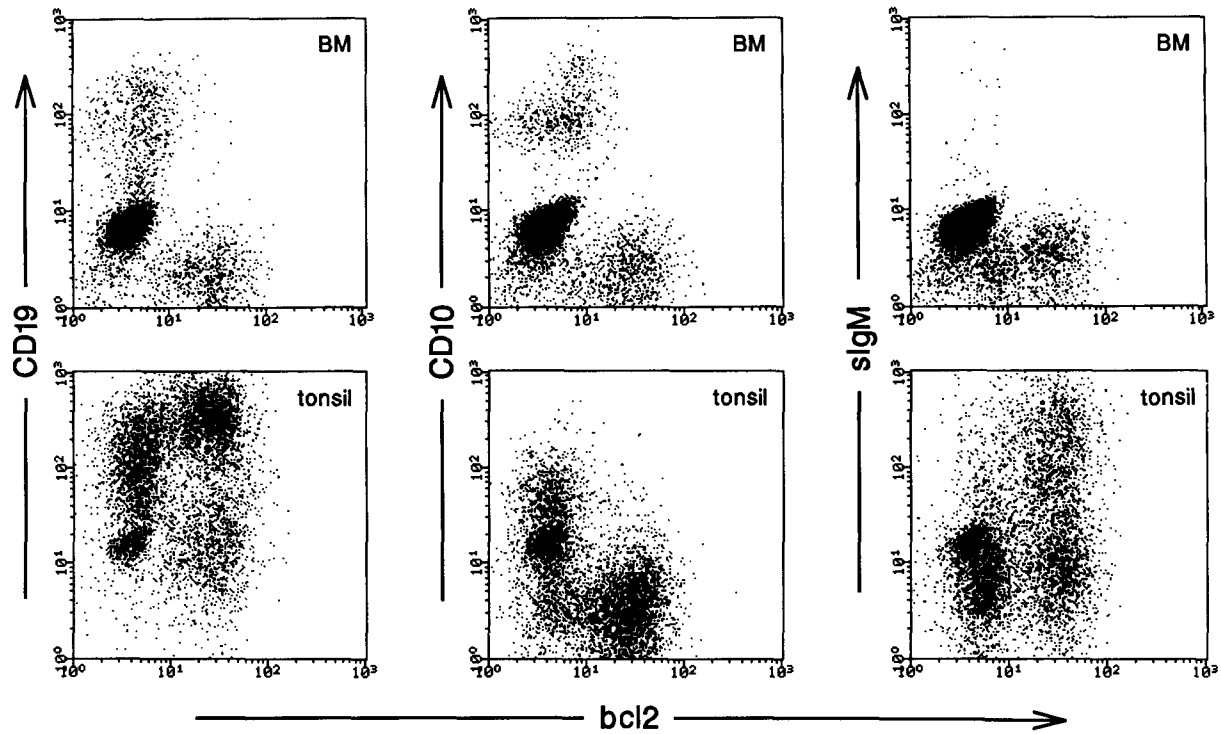

Fig 1. Flow cytometric analysis of *bcl-2* protein expression in normal B-lineage cells. Double-color immunofluorescence staining with anti-*bcl-2* MoAb in combination with CD19 (left panels), CD10 (central panels), and antihuman IgM (right panels) antibodies was performed as described in Materials and Methods. BM (top panels; sample no. 2 in Table 2) and tonsil (bottom panels) samples were studied. Antibody labeling of cells with "lymphoid" morphology is illustrated, after appropriate "gating" using the forward versus 90° light scatter dot plot. Most CD10$^+$ in BM and tonsil show weak or negative *bcl-2* staining (see text).

bcl-2 was used in conjunction with a rabbit anti-TdT antiserum (Supertech Inc, Bethesda, MD) and species-specific second-layer antisera conjugated to fluorescein isothiocyanate (FITC) and tetramethyl rhodamine (TRITC). Slides were viewed with a Zeiss fluorescence microscope equipped with blocking filters for FITC and TRITC.

For cell suspension staining, mononuclear cells were resuspended in PBS to a final concentration of 2×10^7/mL. Cell fixation and permeabilization were performed as described by Schmid et al,[21] using 0.25% para-formaldehyde and 0.2% Tween 20, respectively. Double-color immunofluorescence staining was performed by labeling unpermeabilized cells with goat antihuman-IgM conjugated to phycoerythrin (PE; Southern Biotechnology Associates [SBA], Birmingham, AL), CD19 (AB1; IgM class; obtained from the IVth Conference on Leucocyte Differentiation Antigens), or CD10 (IgM class; Clonab, Biotest Ag, Dreieich, Germany). The latter reagents were followed by goat antiserum to mouse IgM PE. After cell permeabilization, anti-*bcl-2* MoAb (IgG class) was added, followed by goat antimouse IgG FITC (both second-layer reagents were from Jackson Immunoresearch Laboratories, Inc, West Grove, PA).

To study *bcl-2* expression during the different phases of the cell cycle, we labeled BM mononuclear cells with CD10 and goat-antimouse IgM PE and permeabilized them as above. The cells were then stained with anti-*bcl-2* and goat-antimouse IgG FITC postfixed with 0.5% paraformaldehyde for 10 minutes, treated with DNAse-free RNAse (Boeringer Mannheim, Indianapolis, IN; 11.25 Kunitz units), and labeled with 7-actinomycin D (7-AAD; 25 μg/mL; Sigma, St Louis, MO), for at least 30 minutes.[21] To investigate Nalm6 cells, we omitted surface staining and labeled the DNA with propidium iodide (PI; 10 μg/mL; Calbiochem, La Jolla, CA). Antibody controls for surface antigens and *bcl-2* staining were unreactive MoAbs of IgM (Coulter Immunology, Hialeah, FL) and IgG (Becton Dickinson [BD], San Jose, CA) isotype.

Cells were analyzed with a FACScan flow cytometer with Lysis II software (BD); the CellFit software (BD) was used for cell cycle analysis. The fluorescence intensity of *bcl-2* was measured with detectors and amplifiers set on a logarithmic scale. The findings were converted into the number of molecules of equivalent soluble fluorochrome per cell (MESF) by comparing the results of anti-*bcl-2* staining with calibrated fluorescence reference standards (Quantum Series; Flow Cytometry Standards Corp [FCSC], Research Triangle Park, NC), which were run in parallel with the samples in each experiment, as previously described.[22,23] Fluorescence intensity achieved with an isotype-matched unreactive antibody was subtracted to that obtained with anti-*bcl-2* to calculate the net MESF values. The fluorescence intensities of CD22 (Leu 14; BD) followed by goat-antimouse IgG FITC and of CD19 FITC (Leu 12; BD) were measured with the same technique.

The synthetic peptide GAAPAPGIFSSQPG, analogous to that used for immunization, was synthesized with an Applied Biosystems Peptide Synthesizer 430A (Foster City, CA) with Fmoc HOBt/NMP chemistry.

Determination of DNA fragmentation and counting of nonapoptotic cells. DNA fragmentation was assessed by agarose gel electrophoresis as described in detail elsewhere.[20] Nonapoptotic cells were counted by a flow cytometric technique, as follows. Cells harvested as above after different times in culture were washed three times in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA). An aliquot of the suspension was incubated with CD19 FITC MoAb or with a combination of CD19 conjugated to peridin chlorophyl protein (Leu12 PerCP; BD), CD34 PE (BD), and a mixture of goat-

Table 3. Quantitation of *bcl-2*, CD22, and CD19 Expression in B-Lineage ALL

Sample	*bcl-2*	CD22	CD19
Patients			
1	14	26	27
2	16	14	13
3	18	15	24
4	20	25	16
5	22	9	NT
6	30	9	14
7	31	20	16
8	31	10	NT
9	32	15	17
10	34	15	8
11	36	15	NT
12	40	19	14
13	45	13	15
14	49	13	NT
15	51	16	28
16	68	14	16
Cell lines			
380	49	14	NT
Nalm6	70	19	NT
SUPB15	85	35	NT
MR87	93	32	NT
REH	100	30	NT

Values are MESF $\times 10^3$.
Abbreviation: NT, not tested.

antihuman Ig light-chain antisera conjugated to FITC (SBA). After two washes in PBSA, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan and the Lysis II software (BD). In each case, we designed "gates" around the area of the light-scatter dot plot where the vast majority of leukemic cells were found and used these to calculate the number of cells with the predetermined light-scattering properties present in the culture. These numbers were then corrected according to the percentage of cells expressing different antigens present in the sample studied.[20]

RESULTS

bcl-2 *expression during normal B-cell differentiation.* The overall expression of *bcl-2* within $CD19^+$ cells (including all B-lineage cells) in five normal BM samples was low (MESF, 2,600 to 3,800; median, 3,000; Table 2 and Fig 1). The percentage of $CD10^+$ immature B cells with levels of *bcl-2* above control ranged from 9.1% to 30.1%; the intensity of *bcl-2* expression within the entire $CD10^+$ cell population ranged from 2,600 to 5,000 (median, 2,900; Table 2 and Fig 1). The expression of *bcl-2* within BM $sIgM^+$ lymphocytes was heterogeneous (Table 2). In three of five samples, the vast majority of $sIgM^+$ cells had low *bcl-2* MESF values (1,600, 1,800, and 2,000). By contrast, in the two remaining samples, staining of $sIgM^+$ cells for *bcl-2* yielded a biphasic histogram corresponding to two separate cell populations. The MESF in the cells with low *bcl-2* expression was 1,600 in one sample and 2,000 in the other; in the cells with high *bcl-2* expression, the values were 23,000 and 32,000, respectively (Table 2). The expression of *bcl-2* in the latter cells was similar to that seen in B lymphocytes from five PB samples, in which greater than 90% of $sIgM^+$ cells expressed *bcl-2* with overall MESF values of 25,000 to 36,000 (median, 30,000). Similar observations were made when the antihuman IgM reagent was substituted with a mixture of anti-κ and anti-λ human light-chain antibodies (data not shown).

When studied by fluorescence microscopy, 42% to 65% of TdT^+ progenitor cells had detectable staining with anti-*bcl-2*. These cells were heterogeneous in size, with some appearing as small lymphoid cells and others being large blasts with nucleoli.

Expression of *bcl-2* protein was also studied in three tonsil samples, in which 16.4%, 19.1%, and 44.1% of cells were $CD10^+$; 89.5%, 72.9%, and 84.1% of these cells had weak or absent *bcl-2* expression, with overall MESF values of 5,000, 5,800, and 3,500, similar to that observed in $CD10^+$ cells in BM (Table 2 and Fig 1). Tonsillar $CD10^+$ cells include reactive germinal center cells[24] and these data are in line with the observation that most of these cells have undetectable levels of *bcl-2* on immunohistologic examination.[11,12]

Increased bcl-2 *expression in B-lineage ALL and immature B-cell lines.* Sixteen samples of B-lineage ALL and five continuously growing B-lineage cell lines were also studied. In all leukemic cases, greater than 80% of cells showed *bcl-2* staining higher than the highest intensity observed with the isotype-matched negative control antibody; the MESF ranged from 14,000 to 68,000 (Table 3). Cell staining was completely abolished when the anti-*bcl-2* antibody was preincubated with the peptide used for immunization. Thus, leukemic cells expressed strikingly higher levels of *bcl-2* than did their normal $CD10^+$ counterparts (mean MESF ± SD of 33,560 ± 14,570 *v* 3,460 ± 1,050; $P < .001$ by *t*-test). However, the intensity of *bcl-2* in cell lines (79,400 ± 20,330) was even higher than the value for the ALL cases ($P < .001$; Table 3).

Expression of *bcl-2* by leukemic cells did not correlate with the patients' clinical features at diagnosis. The initial white blood cell (WBC) count in this series ranged from 6.0 to 870 $\times 10^9$/L, without any linear relationship to *bcl-2* expression ($r = -.164$; Tables 1 and 3).

We also attempted to relate the intensity of *bcl-2* expression to the leukemic cell karyotype, which was available for 12 cases. None of the cases had metaphases with the t(14;18), the translocation found in the majority of cases of follicular B-cell NHL in adults, or abnormalities involving 18q21 (Table 1).

Table 4. Survival of Normal BM B Cells on Allogeneic Stromal Feeder Layers

Experiment No.	$CD19^+$ Cells Recovered (%)	Within $CD19^+$ Cells*		
		$CD34^+$, slg^-	$CD34^-$, slg^-	$CD34^-$, slg^+
1†	30.5‡	2.8	30.1	73.1
2	14.2	2.7	11.2	35.0
3	30.0	23.7	28.8	39.2

* Different subsets of BM B cells were defined by triple-color flow cytometric analysis (see text).

† $CD19^+$ cells were purified as described in Materials and Methods before seeding onto BM stromal layers. The percentage of $CD19^+$ cells at the beginning of the culture in the three experiments was 92, 98, and 91, respectively.

‡ Values represent the percentage of cells expressing the indicated phenotype recovered after 7 days of culture on stroma. Cell numbers were calculated by flow cytometry at day 0 and day 7 (see text).

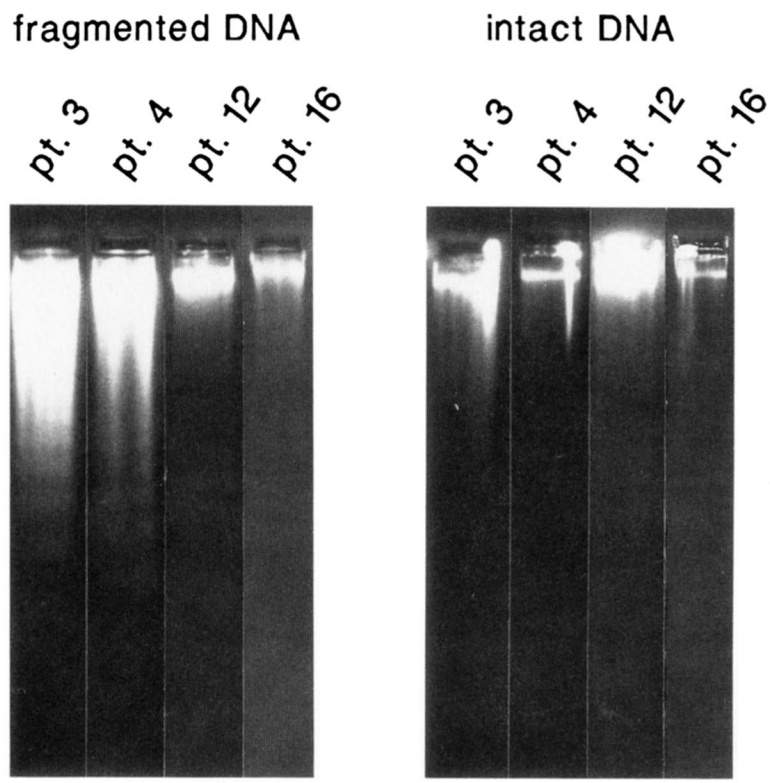

Fig 2. Onset of apoptosis is delayed in B-lineage ALL cells with higher *bcl-2* levels. Shown is the analysis of DNA fragmentation in B-lineage ALL cells after 48 hours of culture in serum-free medium alone. In this DNA fragmentation assay, intact chromatin and oligonucleosomal fragments are separated by centrifugation. Therefore, in the absence of apoptosis, the gel lane loaded with the supernatant (fragmented) DNA appears empty. By contrast, the slot containing the corresponding pelleted (intact) DNA appears extremely bright after ethidium bromide staining. Samples from patients 3 and 4 rapidly underwent massive apoptosis and less than 0.5% of cells were recovered after 7 days of culture. By contrast, no signs of apoptosis were seen in the samples from patients 12 and 16.

Leukemic lymphoblasts survive longer than normal B-cell progenitors. In a previous study, we demonstrated that allogeneic BM stromal feeder layers prevent apoptosis and support optimal survival of leukemic lymphoblasts in vitro.[20] In the present study, we compared the ability of these feeder layers to maintain normal and leukemic B-cell progenitors in vitro. Twelve of the 16 cases of B-lineage ALL included in this study were tested. Cell numbers were investigated at the beginning and after 7 days of culture and the percentage of cells recovered was 68% to 178% (median, 95.5%) of those originally seeded. By contrast, the number of normal $CD19^+$ cells considerably decreased under identical culture conditions and less than one-third were recovered after 7 days in three samples studied (Table 4). Cell loss was severe within the rapidly dividing $CD19^+$, sIg^- immature B-cell compartment. Triple-color analysis by flow cytometry showed that the most immature $CD19^+$ cells, identified by the expression of CD34, were also the cells disappearing most rapidly (Table 4).

bcl-2 *expression correlates with survival requirements of leukemic cells.* A further heterogeneity in cells life span associated with different expression of *bcl-2* protein was found within the B-lineage ALL cases. We previously observed that in some cases a substantial proportion of cells can be recovered even after prolonged periods of culture without stromal cells.[20] Phenotypic or karyotypic markers that would distinguish cases requiring stromal support from those capable of surviving in serum-free medium have not been identified. In 6 of the 12 cases included in the present study (nos. 9, 10, 12, 13, 15, and 16), no signs of apoptosis were seen after 72 hours of culture in the absence of stroma (Fig 2). The mean ± SD MESF of *bcl-2* in these cases was 45,000 ± 13,270. In the other six cases (nos. 1 through 4, 6, and 7), signs of massive apoptosis were evident within 3 days of culture in serum-free medium (Fig 2). The mean ± SD MESF of *bcl-2* in these cases was significantly lower than in the previous group (21,500 ± 7,260; $P = .003$). By contrast, the expression of

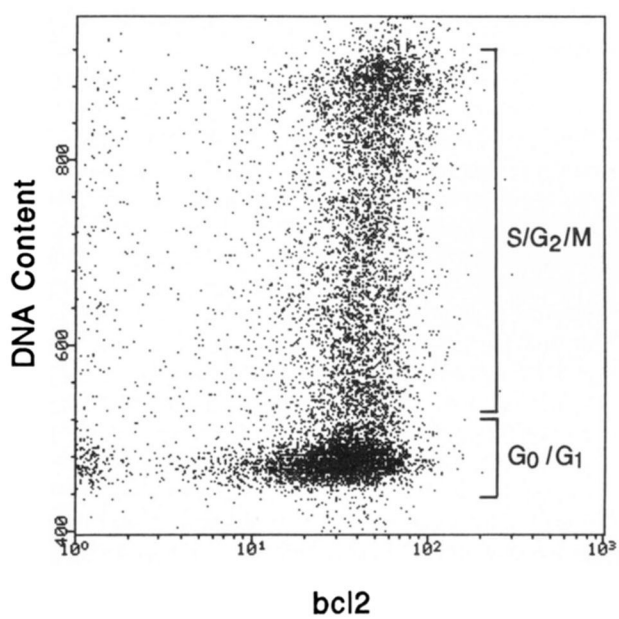

Fig 3. *bcl-2* protein expression does not change during the different phases of the cell cycle. Flow cytometric analysis of Nalm6 cells simultaneously labeled with propidium iodide and anti–*bcl-2* antibody is illustrated.

CD22 and CD19 in both groups was similar (15,330 ± 2,070 *v* 18,170 ± 6,680 for CD22; 16,330 ± 6,530 *v* 18,330 ± 5,750 for CD19). In all cases in which signs of rapid apoptosis were observed, less than 0.5% of the cells were viable after 7 days of culture. By contrast, in the cases with higher *bcl-2* protein expression, a proportion of nonapoptotic $CD19^+$ cells (20% to 95% of the original population) persisted after 7 days of culture. However, the percentage of cells recovered (95% in patient 9; 90% in patient 10; 55% in patient 12; 20% in patient 13; 32% in patient 15; 46% in patient 16) did not have a linear relation to the intensity of *bcl-2* expression, suggesting that other factors had influenced the expansion of ALL cells.

Constant bcl-2 *expression during the cell cycle.* The percentage of cells in S phase at diagnosis ranged from 1.4% to 22.6% among the 16 cases of ALL; these values did not strongly correlate with *bcl-2* expression ($r = .146$; Tables 1 and 3). To investigate whether the expression of *bcl-2* changes as cells progress through the cell cycle, we simultaneously labeled a pre-B–leukemia cell line, Nalm 6, for *bcl-2* and DNA content. Normal B-cell progenitors from two marrow samples were also labeled by this method; the immature B cells were recognized by CD10 labeling, with triple-color immunofluorescence used to study the samples. The expression of *bcl-2* remained constant in both leukemic and normal progenitor cells throughout the different phases of the cell cycle (Fig 3).

DISCUSSION

In this study of the expression of the *bcl-2* protein among normal and leukemic human B cells, we showed that *bcl-2* expression is low in immature B-cell precursors, which are, in this respect, similar to reactive germinal center cells.[11,12] Thus, during B-cell differentiation, levels of *bcl-2* protein are at their lowest in B-lineage cells that proliferate in central and peripheral lymphoid organs. It is unlikely that the fluctuations of *bcl-2* levels we observed are related to cell cycle because continuously growing cell lines had the highest levels of *bcl-2* protein and cells in S, G2, or M phase did not have lower levels of *bcl-2* expression than cells in G0 or G1. In addition, *bcl-2* expression in B-lineage ALL did not correlate with the proportion of cells in S phase.

Low expression of *bcl-2* in germinal center cells could be instrumental in allowing selection mechanisms based on affinity for antigen to operate through apoptosis.[11-14] Immature B-cell progenitors are also likely to undergo apoptosis during normal development because rearrangements of Ig genes may be unsuccessful in a large proportion of cells[25-28]; it has been postulated that less than 10% of these progenitors mature to sIg^+ B lymphocytes.[29] Low levels of *bcl-2* expression at this stage of differentiation may be advantageous, facilitating the swift demise of ineffective progenitor cells. The rapid disappearance of these cells was observed when purified normal BM B cells were cultured under conditions that can support the expansion of leukemic B cells for several months. Only a fraction of $CD19^+$, sIg^- cells could be recovered after 7 days of culture despite the presence of stromal feeder layers. It is unlikely that the disappearance of these rapidly proliferating[30] progenitor cells was entirely due to cell maturation because the numbers of sIg^+ lymphocytes in our cultures did not increase. It is possible that improved culture conditions and the addition of cytokines may allow greater numbers of normal immature B cells to survive and differentiate in culture[31,32] (A. Manabe and D. Campana, unpublished observations). However, in the culture system used in this study, they appear to be more vulnerable than their neoplastic counterparts.

High levels of *bcl-2* protein in leukemic cells may lead to a prolonged lifespan, and lend a growth advantage to these cells despite their lower proliferation rate.[30] BM-derived stromal cells support long-term culture of most ALL samples.[20,33] In the absence of stromal feeder layers, cultured cells rapidly die by apoptosis in most cases.[20,34] In this respect, these cases of ALL resemble factor-dependent hematopoietic cell lines cultured under suboptimal conditions.[1-3,35,36] However, in a proportion of ALL cases, delayed onset of apoptosis and prolonged survival of cells in vitro is seen in the absence of stromal feeder layers. In this study, we observed that these latter cases had a higher *bcl-2* expression. These results are consistent with experiments showing that transfection of *bcl-2* in cytokine-dependent cell lines retards the initiation of cell death in unfavorable culture conditions.[1-3]

Our findings support the concept that, during normal B-cell development, *bcl-2* protein levels are low at stages of differentiation when apoptosis may be physiologically triggered (eg, in cells that have failed to rearrange Ig genes, and in antigen-responding clones with low affinity for the antigen). Overexpression of *bcl-2* in cells susceptible to apoptosis is likely to favor the survival of immunologically inadequate cells.[4,5,7-10] A consequence of high *bcl-2* expression in B-cell progenitors would be the prolonged survival of cells with nonproductive Ig heavy- or light-chain gene rearrangement, a characteristic of virtually all cases of B-lineage ALL.[26,37] While in NHL, the t(14;18) abnormality leads to overexpression of *bcl-2*,[10,15-18] we could not find karyotypic evidence of *bcl-2* gene rearrangement in the B-lineage ALL cases. Structural abnormalities of chromosome 18q21 are extremely rare in childhood ALL, and only two children with NHL and the t(14;18) have been reported in the literature.[38] The mechanism for increased expression of *bcl-2* in other cases of ALL remains to be identified.

In conclusion, the results of this study suggest that regulation of *bcl-2* expression plays an important role in the development of human B cells and that abnormal gene expression may contribute to leukemogenesis by prolonging the life span of progenitor cells unable to further differentiate. In addition, overexpression of *bcl-2* may facilitate the expansion of the leukemic clones outside the BM microenvironment. This study cannot address whether the expression of *bcl-2* correlates with treatment outcome in ALL, as all patients remain in continuous remission with a relatively short follow-up (3 to 46 months; median, 9 months). Further studies may disclose the value of *bcl-2* expression as a prognostic indicator and its association with clinical features of ALL, such as occurrence of extramedullary relapse and resistance of leukemic cells to drugs.

ACKNOWLEDGMENT

We are grateful to Drs D.Y. Mason and F. Pezzella (Department of Haematology, John Radcliffe Infirmary, Oxford, UK) for providing anti–*bcl-2* antibody. We thank Dr M.E. Conley for helpful suggestions.

REFERENCES

1. Vaux DL, Cory S, Adams JM: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B-cells. Nature 335:440, 1988
2. Nuñez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ: Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 144:3602, 1990
3. Hockenbery D, Nuñez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334, 1990
4. Williams GT: Programmed cell death: Apoptosis and oncogenesis. Cell 65:1097, 1991
5. Sawyers CL, Denny CT, Witte ON: Leukemia and disruption of normal hematopoiesis. Cell 64:337, 1991
6. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ: Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879, 1991
7. Tsujimoto Y, Cossman J, Jaffe E, Croce CM: Involvement of the bcl-2 gene in human follicular lymphoma. Science 228:1440, 1985
8. Reed JC, Cuddy M, Slabiak T, Croce CM, Nowell PC: Oncogenic potential of bcl-2 demonstrated by gene transfer. Nature 336: 259, 1988
9. Bertoli LF, Kubagawa H, Borzillo GV, Burrows PD, Schreeder MT, Carroll AJ, Cooper MD: Bone marrow origin of a B-cell lymphoma. Blood 72:94, 1988
10. McDonnell TJ, Korsmeyer SJ: Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature 349:254, 1991
11. Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY: Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225, 1990
12. Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ: Bcl2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961, 1991
13. Liu Y-J, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan ICM: Mechanism of antigen-driven selection in germinal centres. Nature 342:929, 1989
14. Liu Y-J, Mason DY, Johnson GD, Abbot S, Gregory CD, Hardie DL, Gordon J, MacLennan ICM: Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. Eur J Immunol 21:1905, 1991
15. Yunis JJ, Oken MM, Theologides A, Howe RB, Kaplan ME: Recurrent chromosomal defects are found in most patients with non-Hodgkin's lymphoma. Cancer Genet Cytogenet 13:17, 1984
16. Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNA for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19, 1986
17. Tsujimoto Y, Croce CM: Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214, 1986
18. Bakhshi A, Wright JJ, Graninger W, Seto M, Owens J, Cossman J, Jensen JP, Goldman P, Korsmeyer SJ: Mechanism of the t(14;18) chromosomal translocation: Structural analysis of both derivative 14 and 18 reciprocal partners. Proc Natl Acad Sci USA 84: 2396, 1987
19. Pezzella F, Gatter KC, Mason DY, Bastard C, Duval C, Krajewski A, Turner GE, Ross FM, Clark H, Jones DB, Leroux D, LeMarc'hadour F: Bcl-2 protein expression in follicular lymphomas in absence of 14;18 translocation. Lancet 2:1510, 1990
20. Manabe A, Coustan-Smith E, Behm FG, Raimondi SC, Campana D: Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia. Blood 79:2370, 1992
21. Schmid I, Uittenbogaart CH, Giorgi J: A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. Cytometry 12:279, 1991
22. Muirhead KA, Schmitt TC, Muirhead AR: Determination of linear fluorescence intensities from flow cytometric data accumulated with logarithmic amplifiers. Cytometry 3:251, 1983
23. Ryan DH, Nuccie BL, Abboud CN, Winslow JM: Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. J Clin Invest 88:995, 1991
24. Hoffman-Fezer G, Knapp W, Thierfelder S: Anatomical distribution of CALL antigen expressing cells in normal lymphatic tissue and in lymphomas. Leuk Res 6:761, 1982
25. Blackwell TK, Alt FW: Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 264:10327, 1989
26. Greaves MF: Differentiation-linked leukemogenesis in lymphocytes. Science 234:697, 1986
27. Rolink A, Melchers F: Molecular and cellular origins of B lymphocyte diversity. Cell 66:1081, 1991
28. Jacobsen K, Osmond DG: Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. Eur J Immunol 20:2395, 1990
29. Cohen JJ, Duke RC, Fadok VA, Sellins KS: Apoptosis and programmed cell death in immunity. Ann Rev Immunol 10:267, 1992
30. Campana D, Janossy G: Proliferation of normal and malignant human immature lymphoid cells. Blood 71:1201, 1988
31. Wolf ML, Buckley JA, Goldfarb A, Law C-L, LeBien TW: Development of a bone marrow culture for maintenance and growth of normal human B cell precursors. J Immunol 147:3324, 1991
32. Sealand S, Duvert V, Pandrau D, Caux C, Durand I, Wrighton N, Wideman J, Lee F, Banchereau J: Interleukin-7 induces the proliferation of normal human B-cell precursors. Blood 78:2229, 1991
33. Novotny JR, Duehrsen U, Welch K, Layton JE, Cebon JS, Boyd AW: Cloned stromal cell lines derived from human Whitlock/Witte-type long-term bone marrow cultures. Exp Hematol 18:775, 1990
34. Baxter GD, Collins RJ, Harmon BV, Kumar S, Prentice RL, Smith PJ, Lavin MF: Cell death by apoptosis in acute leukaemia. J Pathol 158:123, 1989
35. Koury MJ, Bondurant MC: Erythopoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248:378, 1990
36. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR: Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76, 1990
37. Janossy G, Coustan-Smith E, Campana D: The reliability of cytoplasmic CD3 and CD22 antigen expression in the immunodiagnosis of acute leukemia: A study of 500 cases. Leukemia 3:170, 1989
38. Mikraki V, Jhanwar SC, Filippa DA, Wollner N, Chaganti RSK: Distinct patterns of chromosome abnormalities characterize childhood non-Hodgkin's lymphoma. Br J Haematol 80:15, 1992