

# Progenitor tumours from E $\mu$ -*bcl-2*-*myc* transgenic mice have lymphomyeloid differentiation potential and reveal developmental differences in cell survival

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Mice expressing both a *bcl-2* and a *myc* transgene within the B lymphoid cell compartment invariably develop novel immature haemopoietic tumours. The likely cell of origin of these tumours was identified by a common pattern of cell surface marker expression on a subset of cells comprising ~1% of normal mouse bone marrow. The *bcl-2*-*myc* tumour cells could be induced to differentiate into either B lymphocytes or macrophages in culture with certain cytokines and feeder cells. Analysis of their progression into the B lymphoid lineage revealed that *Igk* locus transcription can precede *Igh* as well as *Igk* rearrangement. Surprisingly, the undifferentiated tumour cells died rapidly in culture, even in the presence of multiple cytokines, but they proliferated on monolayers of stromal cells derived from haemopoietic tissues. Thus, even with Bcl-2 levels that protect more differentiated cells, these immature bi-potential progenitor cells require a stromal-induced signal for survival. These results provide insight into the process of lineage commitment and suggest new levels of control of cell survival during early steps in haemopoietic development.

**Keywords:** apoptosis/*bcl-2*/haemopoiesis/*myc*

## Introduction

Cell death is an essential element of development, differentiation and tissue homeostasis, and it is now recognized that mutations disrupting apoptosis, the natural death programme, underlie several diseases, including neoplasia. Contributing to the regulation of this programme in mammalian cells is a family of genes homologous to *bcl-2*, discovered by its translocation to the *IGH* locus in human follicular B cell lymphoma (reviewed by Cory, 1995). The cytoplasmic membrane-bound protein encoded by *bcl-2* enhances cell survival, as first realized when its enforced expression was found to enable interleukin (IL)-3-dependent myeloid cells to survive cytokine deprivation (Vaux *et al.*, 1988). Since Bcl-2 protects several cell types against a diverse array of cytotoxic agents, it is believed to inhibit a biochemical process common to several pathways that evoke apoptosis.

The transforming potential of *bcl-2* for B lymphoid cells has been investigated using transgenic mice expressing *bcl-2* under the control of an *Igh* enhancer (McDonnell *et al.*, 1989; Strasser *et al.*, 1991b). The

*bcl-2* mice accumulated excess non-cycling B cells and some 10–15% developed lymphoma or plasmacytoma (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993). The plasmacytomas usually had a rearranged *myc* gene (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993), implicating collaboration between *myc* and *bcl-2* in their genesis. The presence of an 8;14 (*MYC-IGH*) chromosome translocation as well as a 14;18 translocation in certain aggressive human B lymphoid neoplasms (e.g. Mufti *et al.*, 1983) was also suggestive of a synergistic relationship between these two oncogenes.

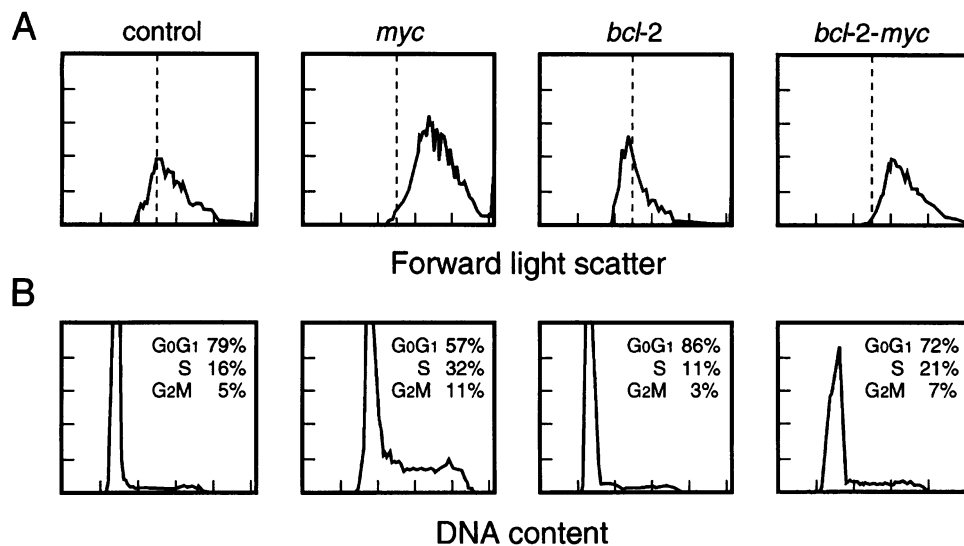
E $\mu$ -*myc* transgenic mice provided the means to test this hypothesis. Every *myc* mouse eventually develops a monoclonal pre-B or B lymphoma (Adams *et al.*, 1985; Harris *et al.*, 1988), although the copious proliferating pre-B cells present from fetal life onward are not malignant (Langdon *et al.*, 1986). Enforced expression of *bcl-2* enhanced the lymphomagenic potential of the *myc* transgene, since immortal pre-B cell lines developed from cultures of *myc* bone marrow cells infected with a *bcl-2* retrovirus (Vaux *et al.*, 1988), and introduction of an E $\mu$ -*bcl-2* transgene into the *myc* mice markedly accelerated the onset of lymphoma (Strasser *et al.*, 1990). A rationale for the synergy between *myc* and *bcl-2* was provided by the observation that cell lines which constitutively express *myc* undergo apoptosis when deprived of cytokines (Askew *et al.*, 1991; Evan *et al.*, 1992). Increased apoptosis amongst B220<sup>+</sup> cells in the bone marrow of *myc* transgenic mice (Jacobsen *et al.*, 1994) may indicate that *myc*-induced expansion of this compartment depletes the available cytokines. Hence, the *bcl-2* transgene may promote lymphomagenesis by inhibiting *myc*-induced death *in vivo*.

The lymphomas that developed in the *bcl-2*-*myc* bi-transgenic mice had an unusual phenotype suggestive of an immature stem or progenitor cell (Strasser *et al.*, 1990). We have characterized these tumours in detail and have found that they can differentiate into either B lymphoid or myeloid cells. Thus, the transformed cell is at least bi-potential, like the B lymphoid/macrophage progenitor cell characterized by Cumano and colleagues (Cumano *et al.*, 1992). These cells provide a window on very early B lymphoid differentiation and reveal that regulation of cell survival varies with differentiation stage.

## Results

### **Pre-malignant phenotype of *bcl-2*-*myc* mice**

Mice expressing both a *myc* and a *bcl-2* transgene in the B lymphoid compartment were generated by mating E $\mu$ -*myc* mice (Harris *et al.*, 1988) with E $\mu$ -*bcl-2*-22 mice (Strasser *et al.*, 1991b). Young doubly transgenic offspring had extremely high blood leucocyte levels (50- to 100-fold higher than normal), due primarily to cells that bore CD45(B220) but lacked membrane-bound immuno-



**Fig. 1.** Comparison of size and cell cycle activity of B220<sup>+</sup> B lymphoid cells sorted from bone marrow of control C57BL/6 mice and mice bearing either or both of the E $\mu$ -*myc* and E $\mu$ -*bcl-2-22* transgenes. (A) Forward light scatter distribution of live cells. (B) Cell cycle phase distribution, determined by flow cytometry of permeabilized cells stained with propidium iodide (Taylor, 1980).

globulin (sIg), and their spleen and bone marrow were also replete with pre-B cells (Strasser *et al.*, 1990). The phenotype thus resembled that of young *myc* mice prior to tumour onset (Langdon *et al.*, 1986) but was more extreme.

When B lymphoid (B220<sup>+</sup>) cells from bone marrow were analysed for forward light scatter by flow cytometry as an indicator of cell size and proliferative activity, most of those from *bcl-2-myc* animals were large, like those of *myc* littermates, in contrast to the predominantly small, quiescent cells of *bcl-2* mice and normal mice (Figure 1A). However, while almost half of the *myc* B220<sup>+</sup> cells were in the S and G<sub>2</sub>M phases of the cell cycle, most *bcl-2-myc* cells were in G<sub>1</sub> or G<sub>0</sub> (Figure 1B), presumably G<sub>1</sub> since they were larger than quiescent lymphocytes (Figure 1A). We infer that, despite constitutive synthesis of Myc, pre-B cells expressing *bcl-2* remain responsive to a G<sub>1</sub> checkpoint. It is likely that proliferation was constrained by limiting cytokine levels in the bone marrow.

#### **Tumours that develop in *bcl-2-myc* mice have a very immature phenotype**

Although the greatly expanded B lymphoid population of very young *bcl-2-myc* mice lacked malignant (i.e. transplantable) cells, by 6 weeks of age every bi-transgenic mouse had developed disseminated lymphoma (Strasser *et al.*, 1990). Whereas *myc* mice invariably yield pre-B or B cell lymphoma, all these tumours derived from a more primitive haemopoietic cell type. When the *myc* × *bcl-2* cross was performed with another *bcl-2* strain, E $\mu$ -*bcl-2-36*, essentially the same results were obtained; all of the *bcl-2-myc* bi-transgenic mice developed terminal tumours of primitive phenotype at 5 weeks of age.

The immaturity of the tumour cells was evident from the pattern of cell surface markers revealed by immunostaining and flow cytometry (Table I and Strasser *et al.*, 1990). The cells displayed B220 and class II MHC molecules, but not sIg, nor any of the pre-B cell markers PB76, BP-1, CD43 and CD25. The presence of Sca-1 (Ly-6A), CD4 and Thy-1 (low levels) might have been indicative of T lymphoid origin, but the cells were negative for other

markers of immature (Joro 37-5 and Joro 7-5) or mature T cells ( $\alpha\beta$  T cell receptors, CD3 and CD8). Similarly, expression of myeloid markers was either very low (Gr-1) or undetectable (F4/80, Mac-1 and TER 119).

The primitive phenotype was also evident from the gene expression profile (Figure 2 and Table II). The tumour cells expressed transcripts encoding two distinctive surface markers of stem and progenitor cells: CD34, a glycoprotein commonly used for enriching stem and progenitor cell populations (Berenson *et al.*, 1988; Krause *et al.*, 1994), and Flk-2/Flt-3, a tyrosine kinase cytokine receptor (Matthews *et al.*, 1991; Hirayama *et al.*, 1995). However, the tyrosine kinase receptor for stem cell factor, encoded by *c-kit* (see Witte, 1990), was not detectable either by RNA analysis or by immunofluorescence staining with the monoclonal antibody Ack-2 (Okada *et al.*, 1991). Transcripts of the Ig co-receptor gene *B29* (also known as *Ig- $\alpha$* ) (Hermanson *et al.*, 1988) hinted at an immature B lymphoid phenotype, but there was no detectable expression of the other co-receptor gene, *mb-1/Ig- $\beta$*  (Sakaguchi *et al.*, 1988). Several other genes that play a crucial role in early B lymphoid differentiation also were silent (Figure 2 and Table II): namely, the recombination activating gene, *rag-1*; the gene encoding terminal deoxynucleotidyl transferase (TdT), which introduces additional nucleotides at V/D and D/J junctions; and the genes encoding the surrogate light chains  $\lambda$ 5 and VpreB which, in association with  $\mu$  heavy chain, form the pre-B cell receptor essential for normal B cell development (Schatz *et al.*, 1992; Rolink *et al.*, 1994).

Taken together with the absence of any rearrangement of either Ig or T cell receptor (Tcr) genes (Table I and Strasser *et al.*, 1990), these observations suggested that the *bcl-2-myc* tumours derived from immature haemopoietic cells having some commitment to the B lymphoid lineage. It has been reported recently that a small population of cells comprising ~1% of normal mouse bone marrow expresses both B220 and CD4 surface markers (Rolink *et al.*, 1996). We determined the surface phenotype of these cells by three-colour immunofluorescence, assaying

**Table I.** Surface phenotype and antigen receptor gene status of Eμ-*bcl-2-myc* progenitor tumours and differentiated derivatives

Marker	Progenitor cell tumour			Normal B220 <sup>+</sup> CD4 <sup>+</sup> bone marrow cells	Differentiated BM95.1 sublines		
	BM14	BM4	BM95.1		Pre-B	B	Pro-M
B220 (CD45R)	+	+	+	+	+	+,-	-
PB-76	-	n.d.	-	-	-	-	-
CD19	-	n.d.	-	-	+	+	-
CD38	+	n.d.	+	+	+	+	-
CD25	-	n.d.	-	-	+,-	-	-
CD43	-	n.d.	-	-	+,-	-	-
BP-1	n.d.	n.d.	-	-	-	-	-
IgM	-	-	-	-	-	+	-
Class II MHC	+	+	+	+	-	+	-
Class I MHC	+	+	+	+	+	+	++
Sca-1 (Ly-6A)	+	+	+	+	-	-	lo
Thy-1	lo	lo	lo	lo	-	-	-
Joro 37-5	-	n.d.	-	n.d.	n.d.	n.d.	n.d.
Joro 75	-	n.d.	-	n.d.	n.d.	n.d.	n.d.
CD3	-	-	-	-	-	-	-
CD4	+	+	+	+	-	-	+,-
CD8	-	-	-	-	-	-	-
CD5 (Ly-1)	lo	n.d.	lo	n.d.	lo	lo	+
Mac-1	-	-	-	-	-	-	+
Gr-1	lo	n.d.	lo	n.d.	-	-	-
F4/80	-	n.d.	-	-	-	-	+
NK 1.1	-	n.d.	-	-	-	-	-
DEC-205	-	n.d.	-	n.d.	-	-	-
TER 119	-	n.d.	-	-	-	-	-
Pgp-1	+	n.d.	+	n.d.	-	-	+
HSA	-	n.d.	-	-	+	+	-
<i>J<sub>H</sub></i>	G	G	G	n.d.	R	R	G
<i>J<sub>K</sub></i>	G	G	n.d.	n.d.	R,G	R	G
<i>Tcrb</i>	G	G	n.d.	n.d.	G	G	G

Four other progenitor cell tumours (BM4, BM8, BM42, BM86) had the same phenotype as those shown here. BM95.1 was cloned by single cell sorting (see Materials and methods).

Normal B220<sup>+</sup>CD4<sup>+</sup> cells were sorted from C57BL/6 mouse bone marrow.

Lymphoid and myeloid sublines of BM95.1 were derived by *in vitro* differentiation (see text).

G, genes in the germline state, i.e. unrearranged; R, genes rearranged; lo, low levels of expression; n.d., not determined.

cells in the B220<sup>+</sup>CD4<sup>+</sup> flow cytometric window for a range of other markers. The results (Table I) showed a striking concordance between the normal cells and the *bcl-2-myc* tumour cells, indicating that the latter were probably derived by transformation of the former. The tumours maintained their immature surface phenotype and lack of antigen receptor gene rearrangement through multiple rounds of serial transplantation in mice. Indeed, the tumour followed longest (BM14) remained unchanged even after 25 such passages *in vivo*.

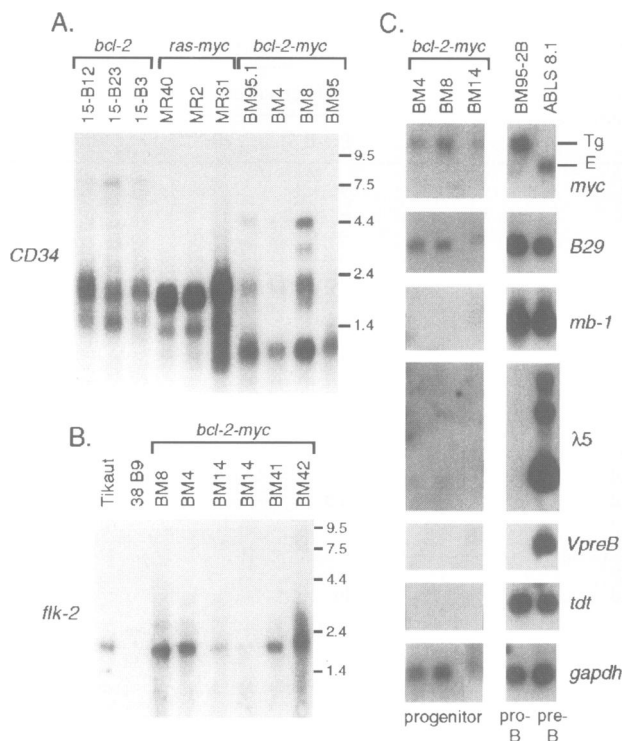
**Both *Igk* and *Igh* loci are transcriptionally active in the tumours**

The unrearranged *Igh* locus of the *bcl-2-myc* tumours proved to be transcriptionally active, a strong indication of B lymphoid differentiation potential. Northern blot analysis (Table II) revealed the small transcripts derived from the V<sub>H</sub>J558 cluster (Yancopoulos and Alt, 1985) and, as shown in Figure 3A, the so-called 'sterile' or Iμ RNAs that initiate within the intronic enhancer (Eμ) located between J<sub>H</sub> and C<sub>μ</sub> (Lennon and Perry, 1985). Such transcripts are believed to presage rearrangement of the *Igh* locus, perhaps by establishing a chromatin structure permissive for recombination (Schatz *et al.*, 1992). The Eμ enhancer contains an octamer sequence recognized by Oct-2, a POU-type homeoprotein (Herr *et al.*, 1988). The presence of *Oct-2* transcripts within the tumour cells

(Figure 3A) suggests that Oct-2 contributes to early transcriptional activity of the *Igh* locus, although the apparently normal initial phase of B cell development in mice lacking Oct-2 (Corcoran *et al.*, 1993) rules out an essential role.

Surprisingly, the unrearranged *Igk* locus within the progenitor cell tumours was also transcriptionally active. RNA species ~1.1 and 0.9 kb in size were detected with a C<sub>κ</sub> probe in all *bcl-2-myc* tumours analysed. They were also present in a similar tumour (MR31) from a mouse expressing both a *myc* and an *N-ras* transgene (Figure 3B), but not in 70Z/3 nor in two Abelson pre-B lymphoma cell lines, in agreement with previous studies (Nelson *et al.*, 1985; Schlissel and Baltimore, 1989). RT-PCR analysis of RNA from six *bcl-2-myc* tumours (see Figure 3C, and Materials and methods) indicated that the κ transcripts initiated at promoters 3.5 (P<sub>0</sub>) and 0.2 kb (P<sub>I</sub>, P<sub>II</sub>), respectively, upstream of the unrearranged J<sub>κ</sub> locus (Leclercq *et al.*, 1989; Martin and Van Ness, 1990). This conclusion was confirmed by sequencing PCR-derived products from two *bcl-2-myc* tumours (not shown). Nuclear extracts prepared from the progenitor cell tumours contained activated NF-κB/Rel complexes (R.Grumont and S.Gerondakis, personal communication), which suggests that the unexpectedly early transcription of the κ locus is determined by NF-κB/Rel.

Germline κ transcripts have been thought to be non-



**Fig. 2.** Differential patterns of gene expression in progenitor and early B lymphoid tumour cells. (A and B) Northern blot analysis of polyadenylated RNA from *bcl-2-myc* progenitor tumours (BM), a derived pro-B cell line (BM95.2B), immature B lymphoid tumours from doubly transgenic *myc-N-ras* mice (MR) (our unpublished experiments), Abelson pre-B cell lines (ABLS-8.1, 38B9), immature B lymphoid tumours from  $\epsilon\mu$ -*bcl-2-15* transgenic mice (15-B12, B23, B3) and a T cell line (Tikaut), using the indicated probes. Transgenic (Tg) and endogenous (E) *myc* RNAs are indicated in (C). Sizes are indicated in kb.

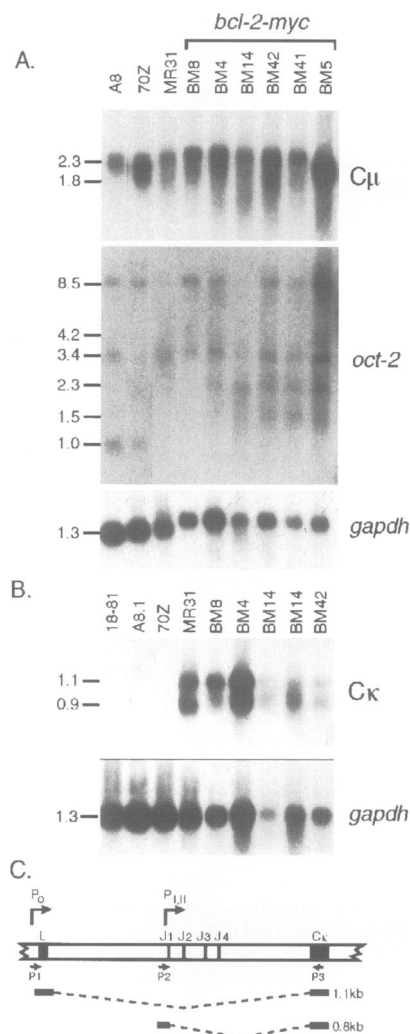
**Table II.** Patterns of gene transcription in  $\epsilon\mu$ -*bcl-2-myc* progenitor tumour cells<sup>a</sup>

Gene	Tumour line				
	BM4	BM8	BM14	BM41	BM42
<i>B29/Ig-α</i>	+	+	+	+	+
<i>mb-1/Ig-β</i>	-	-	+/-	n.d.	n.d.
$\lambda$ 5	-	-	-	n.d.	-
<i>VpreB</i>	-	-	-	-	-
<i>Tdt</i>	-	-	-	-	-
<i>rag-1</i>	-	n.d.	-	-	-
<i>CD34</i>	+	+	+	+	+
<i>c-kit</i>	-	-	-	-	-
<i>flk-2</i>	+	+	+	+	+
<i>vav</i>	+	+	+	+	+
<i>oct-2</i>	+	+	+	+	+
$C\mu^b$	+	+	+	+	+
$C\kappa^b$	+	+	+	+	+
<i>V<sub>H</sub>J558</i>	+	+	+	+	+
<i>c-fms</i>	+/-	+/-	-	+/-	+/-
<i>mac-1</i>	+/-	+/-	-	+	-

<sup>a</sup>Table indicates whether transcripts were detected by Northern blot hybridization of polyadenylated RNA with gene-specific probes.

<sup>b</sup>'Sterile' transcripts, derived from unrearranged Ig genes. n.d., not determined.

translatable because the 1.1 kb transcript lacks an open reading frame and the 0.9 kb transcript has no initiator AUG for the open reading frame encompassing  $JC_{\kappa}$

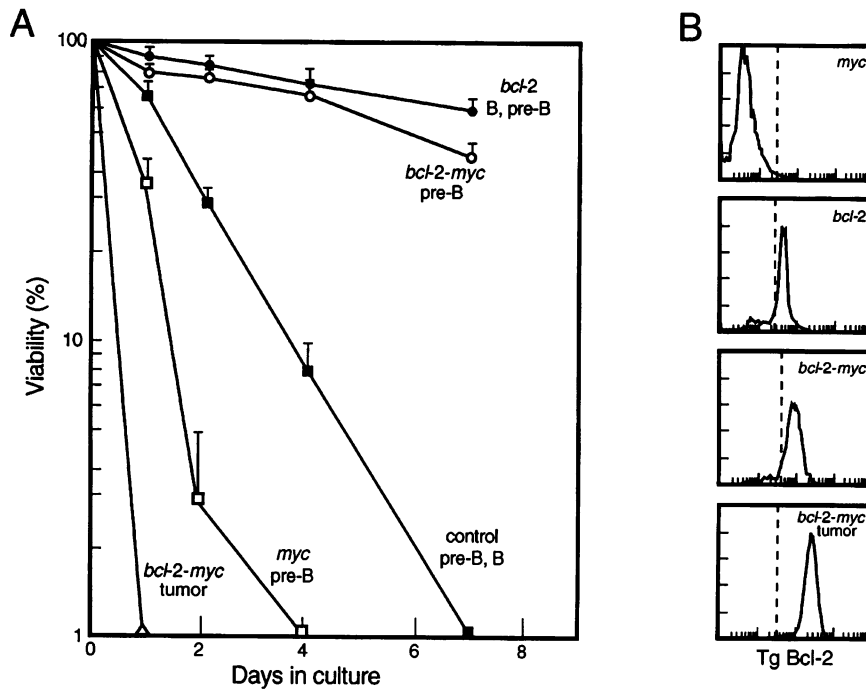


**Fig. 3.** Transcriptional activity of *Igh* and *Igk* loci in *bcl-2-myc* progenitor cell tumours. (A and B) Probes specific for  $C\mu$ ,  $C\kappa$ , *oct-2* and, as a control, *gapdh* were hybridized to Northern blots of polyadenylated RNA from progenitor tumours obtained from *bcl-2-myc* (BM), *myc-N-ras* (MR) mice and pre-B cell lines (ABLS-8, 18-81 and 70Z/3). Sizes are indicated in kb. (C) *Igh* locus, showing location of  $C\kappa$ , J elements, upstream leader (L) exon and promoters ( $P_0$ ,  $P_1$ ,  $P_{11}$ ) used for initiation of 'sterile' transcripts. The principal spliced RNA species are indicated below, as are the positions of primers used for PCR analysis (see Materials and methods).

(Martin and Van Ness, 1990). However, human B cell precursors were shown recently to express a 15 kDa  $JC_{\kappa}$  protein, presumably as a result of initiation at a non-canonical start codon, and this polypeptide can covalently associate with  $\mu$  heavy chain at the cell surface (Frances et al., 1994). In view of the strong conservation of mouse and human sequences within and immediately upstream of  $J_{\kappa}1$ , we looked for the 15 kDa  $JC_{\kappa}$  protein in the *bcl-2-myc* tumour cells. None was detected, either by immunostaining or by Western blot analysis. Thus, either the protein is unstable in the absence of  $\mu$  chain, or it is not synthesized in the mouse, raising questions about its functional relevance in the human.

**Survival of  $\epsilon\mu$ -*bcl-2-myc* tumour cells requires stromal cell support**

We placed *bcl-2-myc* tumour cells in culture to compare their ability to survive with that of non-malignant B220<sup>+</sup>



**Fig. 4.** Tumour cells from Eμ-*bcl-2-myc* mice die rapidly *in vitro*. (A) Tumour cells from *bcl-2-myc* mice and B220<sup>+</sup> cells sorted from the bone marrow of non-tumorous *bcl-2-22*, *myc* and *bcl-2-myc* transgenic mice and age-matched non-transgenic C57BL/6 mice were cultured in DME medium containing 10% FCS. Viability was assessed on days 1, 2, 4 and 7 by trypan blue exclusion. Pre-B cells predominated in the cells sorted from *myc* and *bcl-2-myc* bone marrow, and B cells predominated among *bcl-2* cells, while both pre-B and B cells were abundant among the B220<sup>+</sup> cells from normal mice. (B) Level of human [transgene (Tg)-derived] Bcl-2 protein in tumour cells from *bcl-2-myc* mice and B220<sup>+</sup> cells from bone marrow of non-tumorous *bcl-2-22* and *bcl-2-myc* mice, assessed by cytoplasmic immunofluorescence staining followed by flow cytometric analysis in the FACScan. B220<sup>+</sup> cells from *myc* mice served as a negative control.

cells isolated from the bone marrow of young *bcl-2-myc*, *myc* and *bcl-2* transgenic mice (Figure 4A). As reported previously (Langdon *et al.*, 1988), the pre-B cells from *myc* mice died rapidly in conventional tissue culture medium, presumably because they were forced into cycle by the *myc* transgene in the absence of required cytokines, such as IL-7. In contrast, pre-B cells from *bcl-2-myc* mice, like those from *bcl-2* mice, survived far longer than normal pre-B cells. Thus, Bcl-2 can counter the apoptosis of lymphocytes induced by Myc, as shown previously for fibroblasts (Fanidi *et al.*, 1992; Wagner *et al.*, 1993).

In view of this result, it was surprising to find that the tumour cells from the *bcl-2-myc* mice died very rapidly in culture (Figure 4A), even though they contained at least as much Bcl-2 protein as the *bcl-2-myc* pre-B cells (Figure 4B). The same rapid death occurred in similarly derived tumour cells co-expressing *myc* and an independent *bcl-2* transgene, *bcl-2-36* (data not shown). This unexpected difference between pre-B cells and progenitor tumour cells in survival properties may indicate that pre-B cells express a function required for realization of the survival-promoting activity of Bcl-2 while progenitor tumour cells do not. Alternatively, the progenitor tumour (but not the pre-B) cells may be susceptible to a death signal against which Bcl-2 is ineffective, like that induced by the Fas/APO-1 (CD95) and TNF-RI receptors (Strasser *et al.*, 1995). Activation of these particular receptors is not responsible for the death of the *bcl-2-myc* tumour cells since the cells died just as rapidly in medium containing soluble Fas/APO-1- or TNF-Fcγ fusion proteins, which inhibit these death pathways (data not shown).

In an attempt to establish cultured lines of the *bcl-2-*

*myc* tumour cells, we tested the effect of supplementing the medium with various cytokines (Table III). Survival was not enhanced by any of the 14 lymphokines and myeloid growth factors tested, including FL, the ligand for Flk-2/Flt-3. Over 30 combinations of these factors were also tested and found to be ineffective, as were insulin-like growth factor type I (IGF-I) and IGF-II, which promote survival of various cell types (Christofori *et al.*, 1994; Harrington *et al.*, 1994). Thus, this primitive haemopoietic cell type may well require novel growth factor(s).

Since proliferation of primitive haemopoietic cells and immature B lymphoid cells can be sustained by stromal cells (Dexter *et al.*, 1977; Hayashi *et al.*, 1990), we tested a number of fibroblast cell lines and bone marrow-derived stromal cell lines for their ability to support the *bcl-2-myc* tumour cells, and identified several that could do so (Table III). Within 4–6 h after plating on these adherent cells, the tumour cells had formed 'cobblestone' areas (Figure 5A) which subsequently expanded in size and covered most of the stromal layer within 7–10 days. Survival and proliferation of the progenitor cells apparently depended on direct contact with the monolayer, because conditioned medium from stromal cells was not an effective substitute. Once the cobblestone cultures reached confluence, they could be maintained for only 2–3 weeks; attempts to propagate them by replating after trypsinization failed.

The tumour cells within the cobblestone colonies appeared to remain undifferentiated. For all three tumours tested, CD4 expression was detectable for over a week (not shown), and Southern blot analysis of DNA showed no evidence of *Igh* (or *Tcr*) gene rearrangement. In

**Table III.** Survival of E $\mu$ -*bcl-2*-*myc* tumour cells cultured with growth factors and feeder cells

Stimulus	Tumour cell survival
<b>Soluble factors</b>	
IL-1, -2, -3, -4, -5, -6, -7, -9 <sup>a</sup>	-
M-, G-, GM-CSF <sup>a</sup>	-
LIF	-
SCF	-
SCF + IL-2, -4, -7	-
SCF + IL-2, -7	-
SCF + IL-3, -2	-
SCF + IL-3, -4	-
SCF + IL-3, -5	-
SCF + IL-3, -6	-
SCF + IL-3, -7	-
SCF + IL-3, M-CSF	-
SCF + IL-3, GM-CSF	-
SCF + IL-3, G-CSF	-
IGF-I, II	-
FL	-
FL + SCF	-
FL + IL-1, -2, -3, -4, -5, -6, -7	-
<b>Stromal cells</b>	
BAd, BMS1, NIH 3T3, Cos-1, PA6	-
S12, S17, SCL-19, NIH 3T3-L1 <sup>b</sup>	+
AC-6 <sup>c</sup>	+

<sup>a</sup>Factors were tested singly and in most combinations.

<sup>b</sup>Progenitor tumour cells apparently remained undifferentiated (see text).

<sup>c</sup>AC-6 cells promoted B lymphoid differentiation (see text).

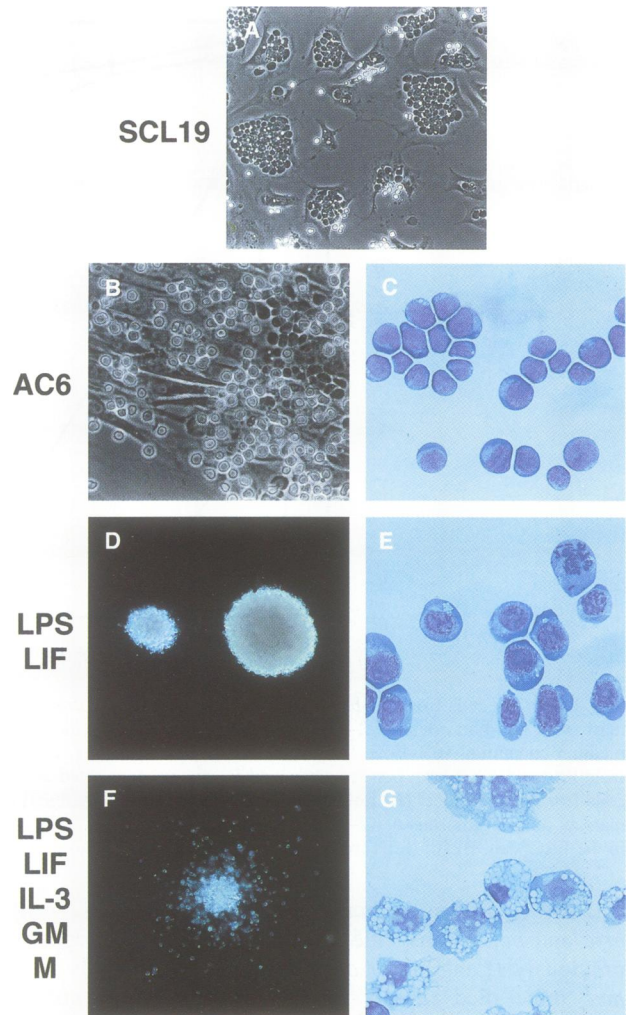
LIF, leukaemia inhibitory factor; SCF, stem cell factor, also known as Kit ligand; FL, Flk-2/Flt3 ligand.

addition, every tumour arising in animals after transplantation of cultured cells retained the same undifferentiated phenotype as the primary tumour.

### *E $\mu$ -bcl-2*-*myc* tumour cells can differentiate to B lymphoid or myeloid cells

The above data suggested that the *bcl-2*-*myc* tumours originated from B cell progenitors, which are reportedly Thy-1<sup>lo</sup>B220<sup>+</sup> (Muller-Sieburg *et al.*, 1986), although the presence of Sca-1, CD4 and low levels of Thy-1, markers of pluripotential stem cells (Spangrude *et al.*, 1988; Frederickson and Basch, 1989) and the earliest known intrathymic T cell precursors (Wu *et al.*, 1991), may have been indicative of additional potential. When we attempted to induce their maturation *in vitro*, we found that the tumours could differentiate to either B cells or macrophages, even after cloning by single cell deposition (see Materials and methods). Hence the tumours were at least bi-potential.

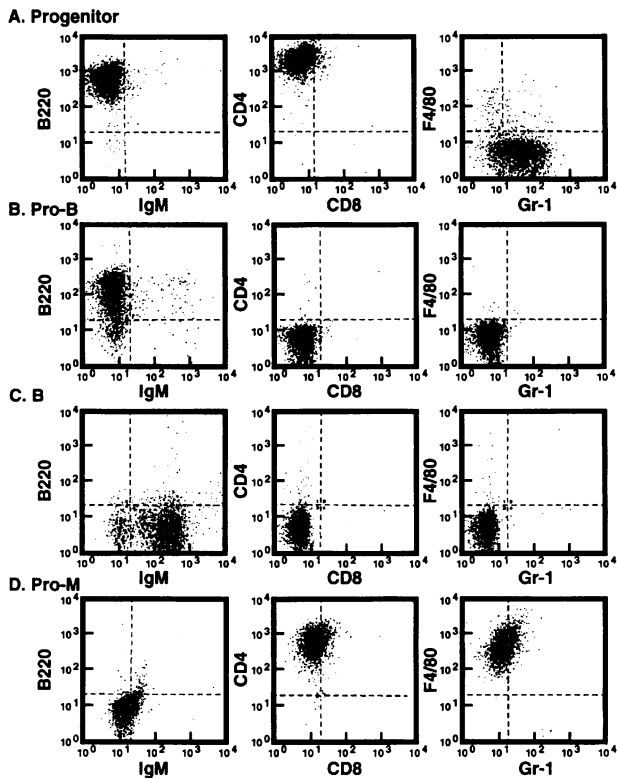
Their capacity for B lymphoid differentiation was revealed by culture at high cell density in the absence of cytokines or stromal cell support or, alternatively, by co-culture with AC-6 cells, which can nurture B lymphoid differentiation of bone marrow cells (Whitlock *et al.*, 1987). Most of the tumour cells died rapidly in the absence of stromal cells, but some survived and, after ~3 weeks, the cultures were dominated by pre-B (or less frequently, pro-B cells) that had acquired HSA but no longer displayed CD4, Thy-1, Sca-1, Gr-1 or class II MHC (Table I and Figure 6B). On monolayers of AC-6 cells, initial cell death was less pronounced, cobblestone colonies developed and



**Fig. 5.** Morphology of *bcl-2*-*myc* tumour cells and their differentiated progeny. Photomicrographs of *bcl-2*-*myc* tumour cells growing on SCL-19 (A) or AC-6 stromal cells (B). Cytocentrifuge preparations of BM14 pro-B cells (C), BM14.3 pro-M cells growing in LPS and LIF (E), and BM14.3 pro-M cells growing in LPS, LIF, IL-3, GM-CSF and M-CSF (G), stained with May-Grünwald Giemsa. Photomicrographs of BM14.3 pro-M cells growing in 0.1% agar containing LPS and LIF (D), or LPS, LIF, IL-3, GM-CSF and M-CSF (F). Original magnifications  $\times 50$  (A and B),  $\times 100$  (C, E and G),  $\times 20$  (D and F).

the abundant non-adherent cells that emerged (Figure 5B) frequently expressed sIgM, defining them as B cells, although some of these had lost the B220 marker (Figure 6C). Long-term lines were readily established and 15 independent subclones were characterized in detail. Most had undergone both *Igh* and *Igk* gene rearrangement and all expressed the *mb-1*, *Tdt* and *rag-1* genes (e.g. Table IV and Figure 7). Transcription of *CD34* was still evident and, while most of the pro-B and pre-B lines also expressed *flk-2/flt-3*, the B cell lines usually did not.

The capacity of the progenitor tumours for myeloid differentiation was revealed when cell suspensions were cultured in either lipopolysaccharide (LPS) plus leukaemia inhibitory factor (LIF), or IL-1, -3 and -6, without stromal cells. Again, most cells died soon after plating, but proliferating myeloid cells reproducibly emerged and could be cloned in soft agar. Lines derived in LPS plus LIF could be adapted to grow with either stimulus alone. The



**Fig. 6.** Cell surface phenotype of cloned *bcl-2-myc* tumour cells and their differentiated progeny. (A) BM95.1 progenitor cell tumour, cloned by single cell deposition (see Materials and methods). (B) Pro-B cell subline derived by culturing BM95.1 tumour cells at high density in DME containing 10% FCS, followed by cloning at limiting dilution. (C) B cell subline derived by culturing BM95.1 tumour cells with AC-6 stromal cells, followed by cloning at limiting dilution without AC-6 cells. Some clones, like the one shown here, lacked B220, but some others did not. (D) Pro-M subline derived by culturing BM95.1 tumour cells in LPS and LIF then cloning in soft agar. Immunofluorescence staining and flow cytometry were performed as described previously (Strasser *et al.*, 1991a).

myeloid lines comprised large immature semi-adherent cells (Figure 5E) that displayed Mac-1, F4/80, CD4, CD45 and class I MHC, but lacked class II MHC and lymphoid cell surface markers (Table I), as shown in Figure 6D for a line derived from the cloned progenitor cell tumour BM95.1. RNA analysis (Figure 7 and Table IV) revealed abundant transcripts of *mac-1* and also of *c-fms*, which encodes the macrophage colony-stimulating factor (M-CSF) receptor. (Lymphoid sublines did not express *c-fms*, and *mac-1* transcripts, present at low levels, were smaller than those in myeloid lines.) No transcripts of *mb-1/Ig-β*, *λ5*, *tdt* or *rag-1* were detectable in the myeloid lines, and the level of *B29/Ig-α* RNA was low. *Ig* (and *Tcr*) genes remained in germline configuration and, although 'sterile'  $\mu$  transcripts were apparent, 'sterile'  $\kappa$  transcripts were not (data not shown). All myeloid sublines retained expression of the  $\text{E}\mu$ -directed *myc* transgene, presumably a driving force for their proliferation. Curiously, a few sublines no longer transcribed the *bcl-2* transgene, implying that sustained Bcl-2 expression was not essential for their survival and proliferation, at least under these conditions.

To investigate the differentiation capacity of the myeloid sublines, we cultured the cells in a cocktail of myeloid cytokines [IL-3 + granulocyte-macrophage colony-stimu-

lating factor (GM-CSF) + M-CSF] as well as LPS and LIF. Numerous adherent macrophages developed (Figure 5G). When clone BM14-3M was plated in agar containing LPS and the four cytokines, 50% of the colonies exhibited a halo of differentiating macrophages (Figure 5F), while cultures containing only LPS + LIF or LPS alone (Figure 5D) contained only large compact colonies. No colonies grew in agar lacking added factors. We found no evidence of granulocytic or erythroid differentiation using cytokine cocktails that included granulocyte colony-stimulating factor (G-CSF) or erythropoietin. The myeloid lines thus appeared to be macrophage progenitors and were termed pro-M.

### ***Bi-potentiality is retained by myeloid but not B lymphoid lines***

We tested whether the differentiated sublines remained bi-potential by transplantation. Three independent pro-M clones derived from the BM14 progenitor tumour yielded tumours that were Mac-1<sup>+</sup> but otherwise identical in cell surface phenotype to the original tumours. Importantly, these transplanted tumours retained both potentialities; pre-B lymphoid lines emerged from cultures plated in the absence of cytokines, while pro-M lines arose in cultures containing LPS + LIF. In contrast, transplanted pre-B lines yielded pre-B lymphomas that did not differentiate to myeloid cells when cultured in LPS + LIF. Thus, once the progenitor cells had undertaken frank B lymphoid differentiation, they appeared to have lost their capacity for myeloid differentiation.

## **Discussion**

### ***B lymphoid/macrophage progenitor cells***

As summarized in Figure 8, the experiments described here show that the novel lymphomas induced in mice by co-expression of  $\text{E}\mu$ -*bcl-2* and  $\text{E}\mu$ -*myc* transgenes are tumours of immature progenitor cells having both B lymphoid and macrophage differentiation potential.

The *bcl-2-myc* tumour cells exhibited several features of primitive haemopoietic cells. They displayed Sca-1, CD4 and low levels of Thy-1, as do certain multipotential haemopoietic stem cells and spleen colony-forming cells (Spangrude *et al.*, 1988; Wineman *et al.*, 1992; Szilvassy and Cory, 1993). No expression of Kit, the receptor for stem cell factor, could be detected but, judging by RNA analysis, the cells expressed Flk-2/Flt3, the tyrosine kinase receptor for a ligand that stimulates proliferation of lymphohaemopoietic stem cells and B lymphoid progenitor cells (Hirayama *et al.*, 1995), as well as CD34, a marker used for isolating primitive human and mouse haemopoietic cells (Berenson *et al.*, 1988; Krause *et al.*, 1994). Apart from a low level of Gr-1, the tumour cells lacked myeloid markers and, although they displayed the general B lineage surface marker CD45R(B220), their *Ig* genes were in germline configuration and most features of immature B lymphoid cells were absent (see below). The tumours were almost certainly monoclonal since, judging by the kinetics of appearance of the malignant cells in the primary bi-transgenic host, transformation appeared to require mutation as well as constitutive expression of *bcl-2* and *myc* (Strasser *et al.*, 1990).

Although the immature phenotype was stable through

**Table IV.** Characterization of cell lines derived from E $\mu$ -*bcl-2*-*myc* progenitor tumours

Cell line	Culture conditions	Gene expression by mRNA detection <sup>a</sup>										Immunoglobulin gene rearrangement status <sup>b</sup>		
		<i>B29</i>	<i>mb-1</i>	$\lambda$ 5	<i>V<sub>H</sub>J558</i>	<i>Tdt</i>	<i>rag-1</i>	<i>CD34</i>	<i>flk-2</i>	<i>fms</i>	<i>mac-1</i>	J <sub>H</sub>	C <sub><math>\kappa</math></sub>	C <sub><math>\lambda</math></sub>
<b>B lymphoid</b>														
BM95.1 -1B	no factor	+	+	-	-	+	+	+	+	-	-	G	R1	G
-4B	no factor	+	+	-	-	+	+	+	+	-	-	R1	R2	G
-5B	no factor	+	+	-	-	+	+	+	+	-	-	G	G	G
-33B	AC6 + IL-7	+	+	+	-	+	+	+	+	-	+/- <sup>c</sup>	R2	R3	R1
-34B	AC6 + IL-7	+	+	+	n.d.	+	+	n.d.	n.d.	-	n.d.	R2	G	R1
-35B	AC6 + IL-7	+	+	+	-	+	+	+	-	-	+/- <sup>c</sup>	R2	R4	R1
-51B	AC6	+	+	-	-	+	+	+	+	-	+/- <sup>c</sup>	R2	R5	G
-52B	AC6	+	+	+/-	-	+	+	+	+	-	-	R4	R6	G
-54B	AC6	+	+	-	-	+	+	+	-	-	+/- <sup>c</sup>	R5	R6	G
<b>Myeloid</b>														
BM95.1 -3M	LPS + LIF	+	-	-	-	-	-	n.d.	-	+	+	n.d.	n.d.	n.d.
-4M	LPS + LIF	+	-	-	-	-	-	n.d.	-	+	+	G	G	G
-13M	IL-1, -3, -6	+	-	-	-	-	-	+	-	+	+	G	G	G
-14M	IL-1, -3, -6	+	-	-	-	-	-	+	-	+	+	G	G	G
BM14 -1M	LPS + LIF	+	-	-	-	-	-	+	-	n.d.	+	G	n.d.	n.d.
-3M	LPS + LIF	+	-	-	-	-	-	+	+	+	+	G	n.d.	n.d.

<sup>a</sup>From Northern blot hybridization of polyadenylated RNA with gene-specific probes.

<sup>b</sup>G, gene region in germline, unrearranged state; RV identifies individual patterns of gene rearrangement to identify clonal relationships within each group of cell lines.

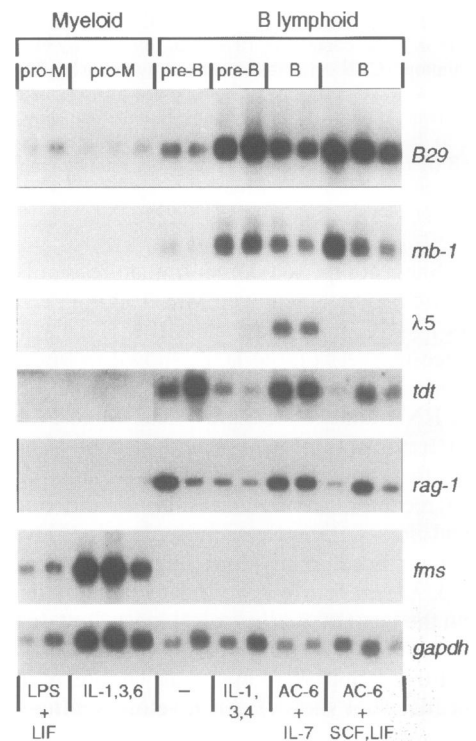
<sup>c</sup>*mac-1* transcripts were smaller than those in myeloid lines (see text).

n.d., not determined.

multiple passages *in vivo*, differentiation could be enforced *in vitro*. Pro- and pre-B cell lines emerged from cultures plated at high density in the absence of cytokines, and sIg-bearing B cell lines could be obtained by plating on AC-6 stromal cells. In the presence of LPS and LIF, however, the tumour cells acquired an immature myeloid character and, upon addition of IL-3, GM-CSF and M-CSF, differentiated into macrophages. One tumour, BM95, was demonstrated to retain the capacity for both lymphoid and myeloid differentiation after single cell cloning (Figure 6).

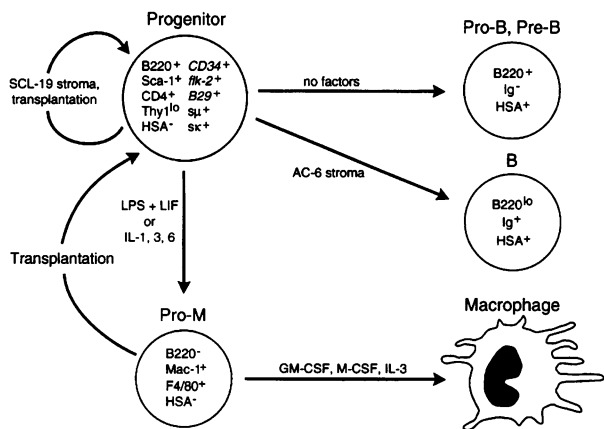
To date, we have not found conditions that induce differentiation to T lymphocytes, erythroid cells or myeloid cells other than macrophages. The tumour cells therefore appear to derive from committed progenitors rather than multipotential stem cells. A caveat is that if the transformed phenotype depends on continued expression of the *bcl-2* and/or *myc* transgenes, any cells that differentiated to a lineage in which the E $\mu$  enhancer is non-functional (e.g. erythroid or granulocytic) would probably not persist under the culture conditions used here.

Bi-potential precursors of B cells and macrophages have been identified previously in day 12 fetal mouse liver (Cumano *et al.*, 1992; Kee *et al.*, 1994). These rare cells are also Sca-1(Ly-6A)<sup>+</sup> and lack Ig gene rearrangement but, since they also lack B220, appear to represent an earlier differentiation stage than the *bcl-2*-*myc* tumour cells. Our tumours may also be more mature than the IL-3-dependent B220<sup>+</sup> pro-B cell lines isolated by Palacios and colleagues (Palacios *et al.*, 1987), which can be induced to differentiate to neutrophils, macrophages or B cells, depending on culture conditions (Lee *et al.*, 1991). By surface marker analysis (Table I), we found that the best candidate for the normal counterpart of the *bcl-2*-*myc* tumour cell is a B220<sup>+</sup>CD4<sup>+</sup> cell that constitutes ~1% of normal mouse bone marrow. This cell was



**Fig. 7.** Differential gene expression in B lymphoid and myeloid sublines derived from cloned BM95.1 progenitor cell tumour. Northern blot analysis of polyadenylated RNA isolated from cloned sublines, using the indicated probes. Myeloid sublines were derived by culturing the *bcl-2*-*myc* tumour cells in either LPS and LIF or in IL-1, -3 and -6. B lymphoid sublines were derived by culturing BM95.2 tumour cells at high density with or without AC-6 stromal cell support. Various other cytokines were sometimes included, as shown, but appeared to provide no survival or proliferative advantage.





**Fig. 8.** Relationship between *bcl-2-myc* progenitor cell tumours and their B lymphoid and myeloid derivatives. Progenitor tumour cells were maintained in an undifferentiated state by serial transplantation in mice or by short-term culture on certain stromal cell lines, e.g. SCL-19, followed by transplantation. B lymphoid differentiation was induced by culture at high cell density on AC-6 stromal cells or in the absence of cytokines. Myeloid differentiation was initiated by culture in LPS and LIF; macrophages could be generated from pro-M sublines in the presence of GM-CSF, M-CSF and IL-3. The pro-M phenotype was apparently 'reversed' back to progenitor phenotype upon transplantation (see text).

identified for the first time only recently (Rolink *et al.*, 1996) and its developmental potential is presently unknown.

Although the B lymphoid lineage is generally assumed to be developmentally closer to the T lymphoid lineage than to other haemopoietic cells, the bi-potential progenitors discovered by Cumano *et al.* (1992) and the *bcl-2-myc* tumours described here provide evidence of a close connection between B lymphoid and macrophage differentiation. Earlier evidence of this relationship has come from several transformed pre-B and B cell lines able to generate macrophages, either spontaneously (Bauer *et al.*, 1986; Davidson *et al.*, 1988; Hanecak *et al.*, 1989; Principato *et al.*, 1990) or following various treatments (Boyd and Schrader, 1982; Klinken *et al.*, 1988; Borzillo *et al.*, 1990; Martin *et al.*, 1993).

In view of these findings, it seems likely that macrophages arise by at least two distinct pathways: from granulocyte/macrophage progenitors and from B lymphoid/macrophage progenitors. Whether the resulting macrophages are functionally distinct remains to be established. In any case, our findings add weight to the view that early haemopoiesis proceeds not by unique bifurcating pathways but by redundant pathways, perhaps reflecting a stochastic basis for the steps in lineage commitment (Ogawa, 1993). Recent evidence that B lymphoid cell progenitors can switch to granulocyte production (Lindeman *et al.*, 1994) is also in accord with this conclusion.

**Early steps in B lymphoid differentiation**

The *bcl-2-myc* tumours provide a window on an earlier stage of B lymphoid differentiation than has previously been accessible. Our studies on them have revealed that transcription of the *B29* (*Ig-α*) gene precedes *Igh* gene rearrangement and transcription of either the *mb-1/Ig-β* or surrogate light chain genes. The tumour cells are thus more immature than the pre-proB (fraction A) B220<sup>+</sup>HSA<sup>-</sup>

BP-1<sup>-</sup> cells characterized by Hardy and colleagues (Li *et al.*, 1993) or the pre-B-I stage of Melchers and colleagues (Rolink *et al.*, 1994).

Transcriptional activity of unrearranged immunoglobulin genes is revealed by the tumours to be another very early sign of B lymphoid differentiation potential. Although transcription of the *Igh* locus has long been known to precede rearrangement, it was somewhat surprising to find just how early the *Igh* enhancer is active. However, it is the unexpectedly early transcriptional activation of the  $\kappa$  locus that is of particular note. The silence of unrearranged  $\kappa$  genes in Abelson pre-B cell lines (Nelson *et al.*, 1985; Schlissel and Baltimore, 1989) had led to the view that  $\kappa$  transcription does not initiate until just before the onset of  $\kappa$  gene rearrangement. Recently, however, 'sterile'  $\kappa$  transcripts have been found in normal pre-B cells, and their absence from Abelson virus-transformed pre-B lines was shown to result from inactivation of NF- $\kappa$ B/Rel by the *v-abl* gene product (Klug *et al.*, 1994). Our data establish that germline  $\kappa$  transcription is not confined to pre-B cells, which have undergone *Igh* gene rearrangement, but also takes place in progenitor cells lacking any immunoglobulin gene rearrangement.

The precocious transcriptional activation of the  $\kappa$  locus has implications for models seeking to explain the usual order of rearrangement and expression of immunoglobulin genes: first  $\mu$ , then  $\kappa$  and finally  $\lambda$ . The pre-B cell receptor complex composed of membrane-bound  $\mu$  and surrogate light chains in association with *Ig-α* and *Ig-β* has been postulated to provide the signal to rearrange the  $\kappa$  locus (Reth *et al.*, 1987; Tsubata *et al.*, 1992). However, it is now apparent that a proportion of immature B lymphoid cells can undergo light chain gene rearrangement in the absence of *Igh* rearrangement or  $\mu$  expression (Kubagawa *et al.*, 1989; Ehlich *et al.*, 1993; Grawunder *et al.*, 1993). Transcription of the unrearranged  $\kappa$  locus is probably a necessary prelude to  $V_{\kappa}-J_{\kappa}$  recombination. Our finding that transcriptional activity of the  $\kappa$  locus precedes *Igh* gene rearrangement further substantiates the view that  $\kappa$  rearrangement is not dependent on *Igh* rearrangement but must be activated by a distinct developmental signal. One intriguing possibility is that the signal involves the complex containing surrogate light chains and gp130/gp35-65 molecules found in some immature B lymphoid precursors (Rolink *et al.*, 1994).

**Two signals for survival**

Surprisingly, despite their high levels of Bcl-2 protein, the *bcl-2-myc* tumour cells died very rapidly in serum-supplemented culture medium (Figure 4). We infer that up-regulation of Bcl-2 is insufficient for survival of B lymphoid progenitor cells and that, *in vitro*, the tumour cells lack a function needed to complement Bcl-2 activity. In contrast, pre-B and B cells expressing *bcl-2* as well as the *myc* transgene had a marked survival advantage over those expressing only *myc*. This may indicate that, unlike the progenitor cells, they express a receptor for a serum factor that induces the putative complementary survival signal.

A wide range of cytokines tested both alone and in multiple combinations were unable to enhance survival of the *bcl-2-myc* tumour cells *in vitro*. The cytokines tested

included IL-3, which supports certain progenitor cells with lymphoid potential (Palacios *et al.*, 1987) and also pre-B cells (Winkler *et al.*, 1995); IL-7, which is critical for B lymphoid cell development (Grabstein *et al.*, 1993); FL, the ligand for Flk-2/Flt-3 receptors apparently present on the tumour cells (Table II) and IGF-I, which potentiates IL-7-dependent expansion of pro-B cells (Landreth *et al.*, 1992) and, like IGF-II, fosters survival of several cell types, including *myc*-expressing fibroblasts (Christofori *et al.*, 1994; Harrington *et al.*, 1994).

The *bcl-2-myc* progenitor cells were able to survive and proliferate on certain stromal cell lines, at least in short-term cultures. Survival appeared to require cell-cell contact, since medium conditioned by these stromal cells was ineffective. The supplementary survival signal may be activated by a membrane-bound cytokine expressed by the stromal cells. Alternatively, it may be transduced by an adhesion molecule. Precedence for the latter possibility has been provided by recent reports that CD44 (Pgp-1) engagement counteracts apoptosis of T cells induced by dexamethasone or T cell receptor activation (Ayroldi *et al.*, 1995) and that triggering of adhesion receptors and antigen receptor is synergistic in preventing apoptosis of germinal centre B cells (Koopman *et al.*, 1994).

In view of the rapid growth of the *bcl-2-myc* tumours in animals, the cells presumably receive supplementary survival signals *in vivo*. Curiously, however, the numbers of apoptotic cells in histological sections of *myc* and *bcl-2-myc* tumours were similar (A.W.Harris, unpublished observations). Thus, it would appear that tumour expansion depends on a shift in the balance between proliferation and death rather than a complete block of apoptosis.

Finally, these progenitor cell tumours should prove useful for investigating further the mechanisms governing the survival and differentiation of immature lymphohaemopoietic cells. They should, for example, provide an assay for identifying secreted or cell surface molecules of stromal cells that enhance cellular survival and/or proliferation. Once the viability of the undifferentiated tumour cells can be sustained in culture, they could be used as targets for insertional activation by a retroviral provirus to identify the postulated synergistic regulator that allows Bcl-2 to promote cell survival. Moreover, transfection with cDNA libraries from later stage B-lineage or myeloid cells may enable identification of transcription factors able to direct the process of lineage determination.

## Materials and methods

### Characterization of progenitor tumour cells and derived cell lines

Progenitor cell tumours originating in bi-transgenic E $\mu$ -*myc*-E $\mu$ -*bcl-2*-22 mice as described previously (Strasser *et al.*, 1990) were maintained by serial transplantation in (C57BL/6J $\times$ SJL/J)F1 hybrid mice (2 $\times$ 10<sup>6</sup> cells injected intraperitoneally and subcutaneously). Cell surface phenotyping of tumour cells was performed on transplanted tumours to avoid the excess pre-B and B cells present in lymphoid organs of primary mice. For cloning, the FACStar Plus cell sorter (Becton-Dickinson) was used to deposit single cells of two tumours (BM14 and BM95) into 96-well culture plates seeded with stromal support cells. After 7 days, ~30% of the wells contained a cobblestone area, consistent with growth from a single cell. Cells in each positive well were harvested by trypsinization and injected into a histocompatible (C57BL/6 $\times$ SJL)F1 hybrid mouse. Only one recipient developed a tumour, designated BM95.1. Presumably,

insufficient tumour cells were transplanted from the other wells to initiate tumour growth.

Most of the antibodies used to label cell surface markers are referenced elsewhere (Strasser *et al.*, 1993). Monoclonal anti-CD19 antibody was prepared from the 1D3 hybridoma (Rolink *et al.*, 1996). Flow cytometric analysis of cell surface markers was performed as described (Strasser *et al.*, 1991a) using a FACScan or a FACStar Plus (Becton-Dickinson). Cell cycle status was assessed by permeabilization and staining with propidium iodide (Taylor, 1980). Probes used for Southern and Northern blot hybridization have been detailed elsewhere (Strasser *et al.*, 1993) and were labelled with <sup>32</sup>P by random priming (Bresatec, Adelaide, South Australia).

### PCR analysis of $\kappa$ transcripts

RT-PCR analysis was performed on 0.5  $\mu$ g of polyadenylated RNA using primers P1 (5'), P2 (5') and P3 (3') derived from the C $\kappa$  locus (see Figure 3D) and having the following sequences: P1, 5'GGAAA-GGACTTGGCTTGTGC3'; P2, 5'CGCAGTACCCACTGCTCTG3'; P3, 5'ACTGGATGGTGGGAAGATGG3'. Two bands of 260 and 165 bp were detected on 2% agarose gels and each was sequenced using P1/P2 and P2/P3 primers. The 300 bp fragment proved to be derived from the 1.1 kb  $\kappa$  germline transcript that initiates 200 bp upstream from a leader sequence found 3.5 kb 5' to the J $\kappa$  cluster; this transcript is spliced to the C $\kappa$  exon from a donor site 137 kb downstream from the leader splice site (Martin and Van Ness, 1990). The 165 bp fragment derived instead from the 0.9 kb RNA initiating within the promoter region 5' to J $\kappa$ 1 and spliced from the J $\kappa$ 1 donor site to C $\kappa$  (Martin and Van Ness, 1990).

### Cell culture

All liquid cultures used the high glucose version of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50  $\mu$ M 2-mercaptoethanol and 100  $\mu$ M L-asparagine. For agar cultures of myeloid sublines, 10<sup>3</sup>-10<sup>4</sup> cells were seeded in 1 ml of 0.3% Bacto Agar (Difco) in the presence or absence of cytokines. Approximately 1% of BM14-3M cells cultured with LPS and LIF formed colonies in 7 days, while macrophage clusters grew from ~5% of cells cultured in IL-3, GM-CSF and M-CSF. In cultures containing all five stimuli, the plating efficiency was ~5%, and at least 50% of the colonies displayed features of macrophage differentiation as judged by cell migration in the agar and the morphology of fixed colonies.

Recombinant IL-1, IL-6, IL-7, SCF, LIF and FL were kindly provided by Drs M.Martin, R.Simpson, S.Gillis and N.Nicola. Supernatants from transfected X63Ag8-653 myeloma cultures (Karasuyama and Melchers, 1988) were used as a source of IL-2, -3, -4 and -5. Fas/APO-1(CD95)-Ig and TNF-R1-Ig chimeric fusion proteins were provided by Dr P.Krammer. All cytokines and fusion proteins were used at optimal concentrations. LPS (Difco) was used at 20  $\mu$ g/ml. Stromal cells were grown to 30-50% confluence before seeding with *bcl2-myc* tumour cells. For some experiments, confluent monolayers of irradiated (30 Gy) stromal cells were used instead. The stromal cell lines used were BA6 (Leung and Johnson, 1987), BMS2 (Gimble *et al.*, 1990), PA6 and ST2 (Hayashi *et al.*, 1990), SCL-19 (R.Boyd, unpublished) and AC-6 (Whitlock *et al.*, 1987).

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