The role of the Bcl-2 family of proteins in the pathogenesis of B-cell chronic lymphocytic leukaemia

By

Andrew Kennedy

This thesis is submitted in part fulfilment of the degree of Master of Science in the University of Glasgow.

Department of Biochemistry and Molecular Biology
Faculty of Science
University of Glasgow
Glasgow.

February 2000

Synopsis.

B-cell chronic lymphocytic leukaemia (B-CLL) is an acquired neoplastic disease characterised by a clonal accumulation of long-lived, functionally immature and $CD5^+$ B-lymphocytes, which particularly accumulate in the lymphatic system, peripheral blood, bone marrow, spleen and liver. Symptoms include lymphocytosis, immune system dysfunction and autoimmune disease, but transformation to more aggressive forms of neoplastic disease occur and development of a second malignancy is not uncommon. The disease is one of later years being unusual before 50 years of age, the rates of incidence vary on a racial basis, and it has a highly variable prognosis. Some patients die within months of diagnosis despite intensive treatment, whilst others survive for 30-plus years without any form of medical intervention and die of unrelated causes. The principal causes of death in patients whose deaths are directly related to the disease are opportunistic infection due to the impairment of immune system function and bleeding disorders. No treatment has been shown to cure the disease or consistently extend life expectancy.

It has been recognised for more than 30 years that the accumulation of malignant cells in B-CLL is at least as important in the pathogenesis of the disease as their neoplastic proliferation. With the discoveries that Bcl-2 extended the life of follicular lymphoma cells by conferring resistance to apoptosis and was commonly expressed in B-CLL cells, it was extrapolated that Bcl-2 might play a similar role in the development of the disease by extending the life span of B-CLL cells. Bcl-2 has frequently been shown to be over expressed in B-CLL cells and genetic translocations and/or malfunction of Bcl-2 family regulating molecular entities may play a part in this. However, since its discovery, Bcl-2 has been shown to be part of a large family of genes which is highly and evolutionarily conserved. Members of the *bcl-2* family are defined by sequence homology in four Bcl-2 homology (BH) regions and a hydrophobic membrane-spanning domain, with the possession of specific BH domains determining whether individual proteins have pro- or anti-apoptotic activity. Family members such as Bcl-2 and Bcl-x_L extend the life span of cells, whilst others such as Bax and Bak shorten it.

Oltvai, Milliman and Korsmeyer have proposed a general model of apoptosis, in which the cell's apoptotic fate is determined by the cellular balance between pro- and anti-apoptotic *bcl-2* family members.

The effect of unregulated expression of Bcl-2 family members in B-CLL cells conforms to this paradigm and resistance to apoptosis appears to be conferred through a cellular imbalance of power between pro- and anti-apoptotic *bcl-2* family members, particularly Bcl-2 and Bax, which is tilted in favour of cell survival. However, the apoptotic fate of B-CLL cells, and hence the neoplasm, may be influenced by other family members, with Mcl-1, Bcl-x_L, Bak, with the non-family but Bcl-2-associated protein, Bag-1, also found expressed in B-CLL cells.

Similarities between the structure of the more conserved family members and other pore-forming proteins, along with the ability of Bcl-2, Bcl-x_L, and Bax to form pores in synthetic membranes, suggest that they may exert their influence through pore-forming activities in intracellular membranes, particularly mitochondrial membranes. Bcl-2 family members may regulate apoptosis by changing the permeability of membranes to ions and apoptosis-inducing molecules, and physical interactions between Bcl-2 family proteins mediated by the BH domains may be important in both pore-forming and pore-inhibiting activities.

Research findings suggest that the levels of Bcl-2 family members in B-CLL cells may be modulated by a wide range of largely extracellular influences, including the cytokines interleukin-4 (IL-4), IL-8, IL-10, interferon-α (IFN-α), IFN-γ, and basic fibroblast growth factor (bFGF). Levels of Bcl-2 family members may also be modulated by contact between B-CLL cells and bone marrow (BM) stromal cells, activation of IgM, CD95, CD40 or CD6, the *p53* gene product, and co-cultivation with CDw32-transfected murine fibroblasts. Such modulation may offer some insight into the pathogenesis of the disease, an explanation for the higher level expression of Bcl-2 family members in B-CLL, and an explanation for the highly variable prognosis. Additionally, if Bcl-2 family members can be shown unequivocally to be controlled by any of these molecular entities, the existence of these influences may offer the opportunity to reduce the neoplastic cells' apoptotic threshold by manipulating the relative levels of pro- and anti-apoptotic Bcl-2 family members as a treatment regime, or prior to more conventional treatment regimes.

for my wife Sue

Table of Contents.

Acknowledgements.

My thanks to my supervisor Dr. William Cushley for his advice and encouragement, to Mr. Joseph Russell for advice and assistance with illustrations, and to my wife Sue for her patience and support, help with word processing, illustrations, and proof reading.

Abbreviations.

A	alanine (Ala)
AIF	apoptosis-inducing factor
AIHA	autoimmune haemolytic anaemia
aka	also known as
ATP	adenosine triphosphate
bad	*bcl-x*$_L$*/bcl-2*-associated death promoter
bag-1	*bcl-2*-associated athanogene 1
bak	*bcl-2*-homologous antagonist/killer
Bax	*bcl-2*-associated X protein
BaxΔC	Bax truncated at the C-terminus
bcl-2	B-cell lymphoma/leukaemia 2
B-CLL	B-cell chronic lymphocytic leukaemia
B-CLL/PLL	B-cell chronic lymphocytic leukaemia/prolymphocytic leukaemia
BCP	B-cell precursor
BCR	B-cell receptor
bFGF	basis fibroblast growth factor
bfl-1	*bcl-2*-related gene expressed in foetal liver
BH	Bcl-2 homology
bid	BH3 interacting domain death agonist
bik	*bcl-2*-interacting killer
BM	bone marrow
bok	*bcl-2*-related ovarian killer
B-PLL	B-cell prolymphocytic leukaemia
brag-1	brain-related apoptosis gene
C	cysteine (Cys)
CD40L	CD40 ligand
CDR3	complementary determinant region 3
C. elegans	*Caenorhabtidis elegans*
CSF	colony-stimulating factor
CVID	common variable immune deficiency
cyc c	cytochrome c
D	aspartic acid (Asp) or diversity
$\Delta\Psi_m$	mitochondrial inner membrane potential
E	glutamic acid (Glu)
EBV	Epstein-Barr virus

ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
F	phenylalanine (Phe)
FACS	fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain (aka MORT1)
FAra	9-β-D-arabinosyl-2-fluoroadenine (fludarabine des-phosphate)
FasL	Fas ligand
FDC	follicular dendritic cells
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
G	glycine (Gly)
GC	germinal centre
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
grs	Glasgow rearranged sequence
H	histidine (His) or heavy
HC	hydrocortisone
^{3}H-TdR	^{3}H-thymidine
I	isoleucine (Iso)
ICE	interleukin-converting enzyme
IFN-	interferon-
Ig	immunoglobulin
IL-	interleukin-
IL-7R	interleukin-7 receptor
ISR	isotype switch recombination
J	joining
K	lysine (Lys)
ksbcl-2	Kaposi sarcoma *bcl-2*
L	leucine (Leu)
LFA-	leukocyte functional antigen
LPS	lipopolysaccharide
LSC	lymphoid stem cell
M	methionine (Met)
mbr	minor breakpoint region
mcr	major cluster region
M-CSF	macrophage colony-stimulating factor
MESF	mean equivalent of soluble fluorochrome

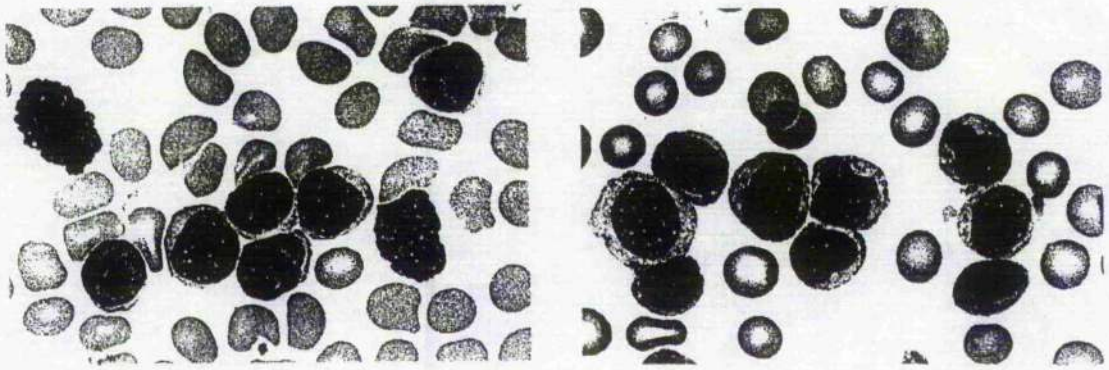
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MRBC-R	mouse red blood cell receptors
multi-CSF	multilineage colony-stimulating factor
N	asparagine (Asn)
NMR	nuclear magnetic resonance
NZB	New Zealand bald
Oct-2	octamer binding site protein 2
ORF	open reading frame
P	proline (Pro)
PBMC	peripheral blood mononuclear cells
PBR	peripheral benzodiazipine receptor
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PLL	prolymphocytic leukaemia
PMA	phorbol myristic acetate
PSC	pluripotent stem cell
PT	permeability transition
Q	Glutamine (Gln)
R	arginine (Arg)
rIFN-	recombinant interferon
rIL-	recombinant interleukin-
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
S	serine (Ser)
SAC	*Staphylococcus aureus* Cowan
SHC	surrogate heavy chain
SHM	somatic hypermutation
sIg	surface immunoglobulin
SLC	surrogate light chain
STAT	signal transducer and activators of transcription
T	threonine (Thr)
TdT	terminal deoxynucleotidyltransferase
TGF-	transforming growth factor-
TM	transmembrane
TNF-	tumour necrosis factor-
TNFR	tumour necrosis factor receptor

TPA	12-0-tetradecanoylphorbol-13-acetate
V	valine (Val)
VCAM	vascular cell adhesion molecule
VH	variable chain heavy region
vcr	variant cluster region
VLA	very late antigen
W	tryptophan (Trp)
wt	wild-type
Y	tyrosine (Tyr)
zVAD-fmk	benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(0-methyl)-fluoromethylketone

List of illustrations and tables.

1 Introduction (the disease).

B-cell chronic lymphocytic leukaemia is an acquired neoplastic disease characterised by a clonal accumulation of long-lived, functionally immature lymphocytes which proliferate in the lymphatic system. Neoplastic B-cells accumulate particularly in the peripheral blood, bone marrow, lymph nodes, spleen and liver, but in later stage disease may infiltrate the skin, tonsils, lung, pleura, gastrointestinal tract, prostrate and central nervous system. B-CLL commonly presents with cells of a marginally mature morphology and immunophenotype typical of early virgin B-lymphocytes, but prolymphocytes and lymphocytes of a more variable morphology may be present. The majority of peripheral blood cells are small, apparently homogeneous cells, with a rounded shape and a diameter larger than an erythrocyte. Cytoplasm is scanty, usually visible around half the circumference of the cell, with a centrally placed nucleus containing heavily condensed chromatin clumped in blocks and surrounded by paler areas. Typically there is little organelle development, particularly of the endoplasmic reticulum and golgi apparatus. Intracytoplasmic inclusions, globular and κ-chain associated, or crystalline and λ-chain associated, are visible in 5-10% of cases. Examples of a B-PLL cell and a B-CLL cell showing an intracytoplasmic inclusion, follow in Figure 1.



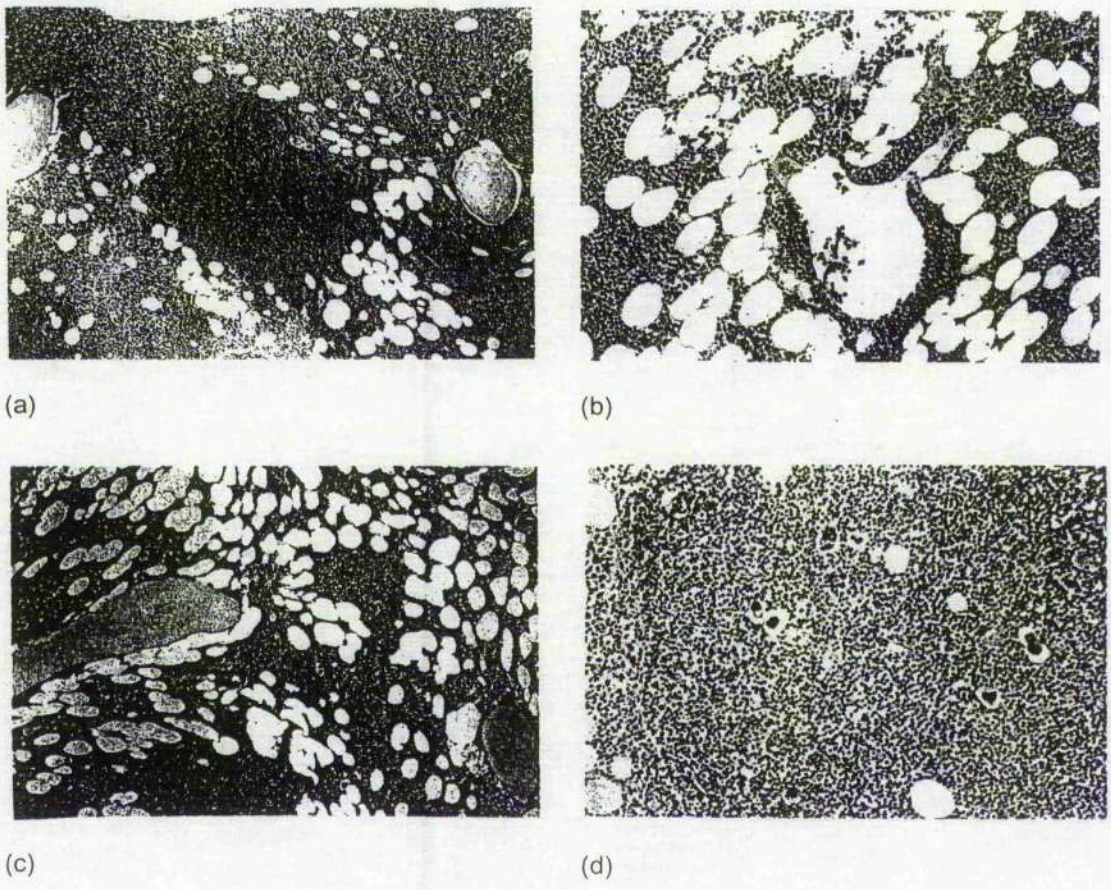
(a) (b)

Figure 1. Examples of B-CLL (a) and B-PLL (b) cells in blood films. Reprinted, by permission, from Ghulam J. Mufti *et. al.*, An atlas of malignant haematology, Figures 4.1 & 4.2. © Martin Dunitz Limited 1996.

A persistent clonal lymphocytosis of $\geq 5 \times 10^9\ l^{-1}$ of peripheral blood (normal value 1.5 - $3.5 \times 10^9\ l^{-1}$), with bone marrow infiltration representing at least 25% of the nucleated cells, is a fundamental requirement of diagnosis.

However, more typically lymphocytosis ranges from 20-250 x 10^9 l^{-1} and may be as high as 1000 x 10^9 l^{-1}. The accumulation of B-CLL cells is commonly described as relentless, but the rate of accumulation varies enormously and in some patients it may be very slow or arrested. The neoplastic B-cells in the peripheral blood are generally regarded as being frozen at G_0/G_1 of the cell cycle and are thought to be non- or very slowly-dividing. The relative contributions made to the clonal accumulation by the propagation of the neoplasm in the secondary lymphoid organs, and/or by the longevity of the cells, remains unclear.

Immune system dysfunction is caused by the progressive infiltration of the BM by neoplastic lymphocytes leading to the displacement of normal bone marrow elements. The four stages of infiltration, in order of progression and illustrated in Figure 2, are described as nodular, interstitial, mixed (nodular and interstitial) and diffuse.



(a) (b)

(c) (d)

Figure 2. Examples of bone marrow biopsies (a) nodular, (b) interstitial, (c) mixed, and (d) diffuse. Reprinted, by permission, from Ghulam J. Mufti *et. al.*, An atlas of malignant haematology, Figures 4.12, 4.13, 4.15, & 4.16. © Martin Dunitz Limited 1996.

Resulting bone marrow failure may produce non-haemolytic anaemia, neutropenia, hypergammaglobulinaemia (typically IgM and IgG, but occasionally including IgA), hypogammaglobulinaemia, and a more generalised dysfunction of the immune system. Serum Igs typically show a progressive fall with advancing disease, with IgA generally reduced more than IgG or IgM, but with a fall in serum IgG usually providing the first sign of a failing immune system. T-cell function and cellular immunity are relatively normal in early stage disease, but BM failure leads to the loss of cellular immunity, as measured with recall antigens, in progressive disease. An increase in absolute numbers of $CD4^+$ and $CD8^+$ T-cells, but with a relative reduction in $CD4^+$ and a relative increase in $CD8^+$ cells to the point where the normal $CD4^+$:$CD8^+$ ratio is inverted, is common. It is not clear to what degree the impairment of the immune function is due to the change in the balance between $CD4^+$ and $CD8^+$ T-cells, the effective dilution of T-cells as the ratio of T:B cells in peripheral blood changes from the normal 3 to 1 to between 1 to 20-50 with increasing lymphocytosis, or the absence of normally functioning B lymphocytes, but the resulting failure provides an opening for opportunistic infections.

Autoimmune events are common in B-CLL, but generally restricted to haemato-poiesis and reported, depending on the nature of the event, in between 2-36% of cases. Coombs-positive autoimmune haemolytic anaemia (AIHA) is most common, with immune thrombocytopenia and pure red blood cell aplasia running, respectively, a poor second and third. B-CLL patients do not appear to have a higher rate of incidence of other autoimmune phenomena such as systemic lupus erythematosus, polyarteritis nodosa, pernicious anaemia and autoimmune thyroiditis, although occasional non-haematological autoimmune disease is reported (Diele, 1998; Efremov, 1996; Hamblin, 1986; Jonsson, 1996; Keating, 1996; Pritsch, 1998). The source and the mechanics of induction of haematopoietic autoantibodies in B-CLL remains unclear. B-CLL clones, which are overwhelmingly positive for the CD5 antigen normally found on a $CD5^+$ B-1 subset of B-cells associated with autoantibody production, are the natural suspect. However, support for the non-leukaemic source of autoantibodies comes from a study showing the autoantibodies to be polyclonal IgG displaying activity against monomorphic elements of the rhesus system and not the product of a neoplastic clone (Dighiero, 1988). In contrast, a more recent investigation of the neoplastic cells of patients with B-CLL-associated AIHA found the preferential expression of two heavy chain variable region (VH) gene segments and the complementary determinant region 3 (CDR3). Expression of such a limited VH region repertoire would suggest that the leukaemic cells are also directly involved in the pathogenesis of the AIHA (Efremov, 1996).

A third, and not necessarily mutually incompatible possibility, is that the emergence of autoimmune events in B-CLL are linked to the initiation of radiation or chemical therapy between which a close temporal relationship is often observed (Keating, 1996). This view is supported by two studies suggesting that the onset of autoimmune phenomena is triggered by treatment with fludarabine (Bastion, 1992; Di Raimondo, 1993).

Transformation of B-CLL to more aggressive forms of B-lineage neoplastic disease occurs in ~5-10% of cases. In the prolymphocytoid transformation, characterised by a significant increase in the cells with a prolymphocytic appearance from the 2-5% of neoplastic cells typical of earlier stage disease, B-CLL is transformed into B-prolymphocytic leukaemia (B-PLL) or B-CLL/PLL. Little is known about the mechanics of the transformation, but cells from the same clone as B-CLL exhibit a relatively immature morphology juxtaposed with an immunophenotype more indicative of a later stage of development by virtue of profusely expressed Ig. The disease is more refractory to treatment than B-CLL, with a median survival of 2-3 years from diagnosis. Transformation to Richter's syndrome, a diffuse large cell lymphoma (DLL) with pleomorphic and giant multinucleate cells, occurs in at least 3% of B-CLL cases, but the actual rate may be higher as occult transformations may go undetected by autopsy. 30-50% of the transformations have received no previous treatment and in some cases cells stem from the same clone (as defined by rearrangement of the Ig-heavy chain (H) gene and particularly the CDR3 region of the IgH gene), suggesting that Richter's syndrome is a natural progression in B-CLL. However, some studies have shown that Richter's Syndrome is not a clonal extension of B-CLL using the CDR3 and Ig V_HDJ_H (variable heavy, diversity and joining heavy) sequences as markers of clonality. The transformation to Richter's Syndrome has also been linked, sometimes very tenuously, to Epstein-Barr virus (EBV), trisomy 12, trisomy 7, an exon 7 missense point mutation of the *p53* gene, as well as, paradoxically, arising *de novo*. No satisfactory treatment is currently available and most B-CLL patients die within six months of the diagnosis of Richter's syndrome (Ansell, 1999; Byrnes, 1995; Cuneo, 1996; Giles, 1998; Matolcsy, 1994, 1995(1 & 2); Ohno, 1998; Petrella, 1997). Blast cell transformation in the terminal phase of B-CLL is rare and in approximately half of these the blast cells are myeloid in origin, most frequently myeloblastic, myelomonoblastic or acute erythroleukaemic. It is unclear whether the transformation to blast cells, myeloid or otherwise, is a natural progression of the disease or an artefact of treatment, e.g., with alkylating agents such as chlorambucil (Keating, 1996).

Spontaneous regression is relatively rare in B-CLL with studies suggesting that the rate is between 1.8-3%, but interestingly the evidence suggests that in some cases the remission coincided with the onset of viral infections (varicella-zoster and bilateral orchitis), small pox vaccinations, and with the development of a second malignancy (Han, 1987; Ribera, 1987).

B-CLL is a disease of later years. Its occurrence is rare under the age of 30, unusual under the age of 50, and rates grow exponentially with age until the 6th. decade. It is commoner in men than in women with the male:female ratio reported as being between 1.5 - 2.0. In the USA the disease is common in those of European and African ancestry, but uncommon in Asians, American Indians, those of Filipino origin and Los Angeles' Hispanics. In Israel the disease is very common amongst Ashkenazi Jews compared to non-Ashkenazi Jews, with a ratio of 4:1. B-CLL represents ≈30% of all leukaemia diagnosed in the Western World, but 38% in Denmark and only 2.5-5.0% in the Orient and Philippines. However, the lower rates of incidence in some Asian countries may be a reflection of shorter life-expectancy or a lower frequency of routine blood tests producing a lower rate of diagnosis. Demographic differences in the incidence of B-CLL are unlikely to be explained by environmental factors, as Japanese and Filipino Americans retain a rate of incidence of B-CLL comparable to the incidence of B-CLL in their countries of origin, suggesting that the differential incidence is due to genetic factors. B-CLL has a familial incidence with rates of disease being demonstrably higher in first degree relatives. Although environmental causes also cannot be absolutely excluded as the cause of this, the absence of any convincing indication of external cause, along with the different incidence of disease in ethnic groups regardless of where they are living, would suggest the most likely underlying cause is a genetic predisposition to develop the disease (Bartal, 1978; Brincker, 1982; Cuttner, 1992; Keating, 1996; Linet, 1989).

No treatment regime has been shown to consistently produce long term complete remissions or survival benefit. There is a general consensus that patients in Binet A-B (Rai 0-II), which are illustrated in Table 1, should only be treated in the event of disease progression typically indicated by a consistent decrease in the peripheral blood lymphocyte doubling time, lymphocytosis at unacceptable levels (≈ 150 x 10^9 l^{-1}), BM dysfunction, impairment of immune function, or by significant enlargement of organs. The cytotoxic drug of first resort is chlorambucil, which is sometimes used in combination with the corticosteroid prednisone, but more complex combination chemotherapy, including drugs such as vincristine sulphate, cyclophosphamide, doxorubicin, cytosine arabinoside, and etoposide, is commonly used in later stage or refractory disease.

	Rai		Binet
Stage		Stage	
0	Absolute lymphocytosis > 15 x $10^9 l^{-1}$	A	0 - 2 areas of organ enlargement†
I	As stage 0 + enlarged lymph nodes (adenopathy)		
II	As stage 0 + enlarged liver and/or spleen ± adenopathy	B	3 - 5 areas of organ enlargement Hb ≥ 10 g dl^{-} and platelets ≥ 100 x $10^9 l^{-1}$
III	As stage 0 + anaemia (Hb < 10.0 g dl^{-1})* ± adenopathy ± organomegaly		
IV	As stage 0 + thrombocytopenia (platelets < 100 x $10^9 l^{-1}$)* ± adenopathy ± organomegaly	C	Organ enlargement not considered Hb < 10 gdl^{-} and/or platelets < 100 x $10^9 l^{-1}$

Table 1. Comparison of Rai and Binet stages of B-CLL.
* Secondary causes of anaemia (e.g. iron deficiency) or autoimmune haemolytic anaemia or thrombocytopenia must be treated before staging.
† Organ enlargement comprises enlarged spleen or liver, and lymph nodes > 1 cm in neck, axillae or groin each represent one area of enlargement.

Newer drugs include the purine analogues fludarabine monophosphate, 2-chlorodeoxy-adenosine, and deoxycoformycin, which appear to have significant cytotoxic activity in B-CLL. Fludarabine has been shown to be superior to the cyclophosphamide, doxorubicin & prednisone regime in a multicentre randomised trial of previously treated and untreated patients and has been shown to produce partial and complete remissions in refractory B-CLL. However, time is needed to determine if using fludarabine monophosphate translates into better survival (Brittinger, 1997; Dighiero, 1996; Johnson, 1996; Montserrat, 1996, 1997; Sorensen, 1997). Other options seen as holding out the hope of a cure for B-CLL include treatment with monoclonal antibodies and/or stem cell rescue. The anti-CD52 antibody (Campath-1H) has been used to purge residual disease in patients where drugs have failed to displace persistent leukaemic infiltration of blood and BM, before successful autologous stem cell transplantation. Allogeneic and autologous stem cell transplants probably offer the best opportunity of curing B-CLL. A retrospective study spanning 1984-1992 involving patients with an age range of 21-58 in all stages of disease, showed mixed results. More recent studies have concentrated on patients with late stage/refractory disease. Three of these have shown more encouraging results with 89%, 59% and 87% of the patients reported as displaying complete remission.

These results are particularly hopeful as these patients had largely exhausted conventional treatment regimes and, interestingly, one of the studies showed little difference in the complete remission success rate between patients given allogeneic (6/11) and those given autologous or syngeneic BM transplants (7/11). However, stem cell rescue is still in the experimental stage and it remains to be seen to what extent this treatment translates into improved survival and/or a cure (Dyer, 1997; Khouri, 1994, 1997; Michallet, 1996; Rabinowe, 1993).

The principal cause of death in patients whose deaths are directly related to the disease is opportunistic infection due to impairment of immune function. However, death as a result of bleeding disorders, other malignancies and transformation to more aggressive neoplasias, is also common. Survival time varies enormously, as indicated in Figs 3 and 4, with some patients surviving untreated for 30+ years, whilst others die within months of diagnosis. B-CLL is normally categorised by Rai or Binet stage, which are compared above, and Figures 3 and 4 show that prognosis appears to be directly related to both the Binet stage and age at diagnosis (a similar picture emerges when survival is analysed by Rai stage). It is not immediately clear if these phenomena are due simply to "lesser disease" and disease occurrence at an age when general health and resilience may be expected to be better, or whether the different survival curves hint that there may be subtypes of B-CLL with different underlying biologies and prognoses. Factors that have been linked to a poor prognosis or progressive disease include trisomy 12, aberrant overexpression of the mutant form of p53 recognised by the monoclonal antibody (MoAb) PAb240, a higher Bcl-2/Bax_{α} ratio, and a higher expression of Bcl-2 alone. In contrast some research has failed to link p53 mutants to the stage of disease and deletions of 13q have been linked to good prognosis . Unfortunately no clear explanation has emerged that might explain such distinct survival curves (Aguilar-Santelises, 1994, 1996; Gaidano, 1994; Juliusson, 1993; Robertson, 1996).

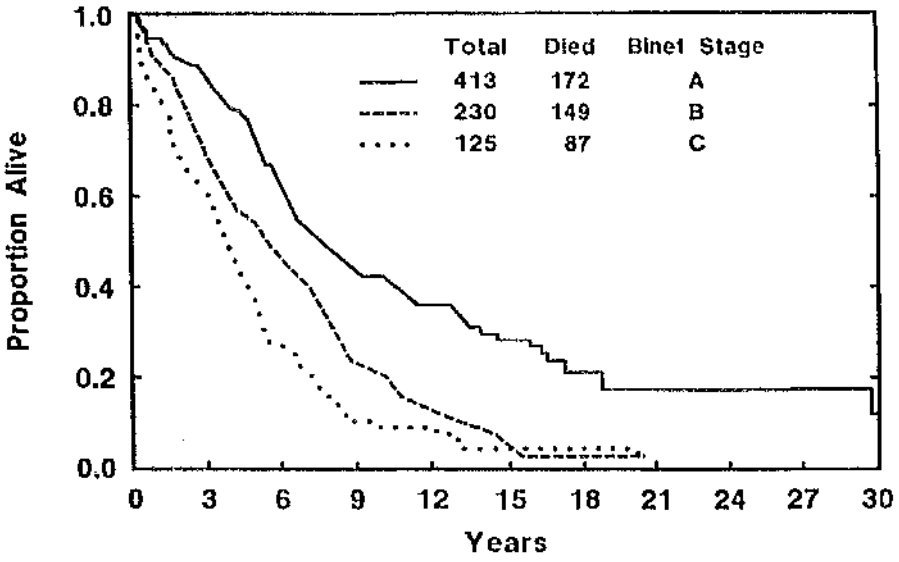


Figure 3. Survival of untreated B-CLL patients by Binet stage. Reprinted, by permission, from Keating, M. J., Chronic Lymphocytic Leukemia, Figure 3. © Marcel Dekker Inc 1993.

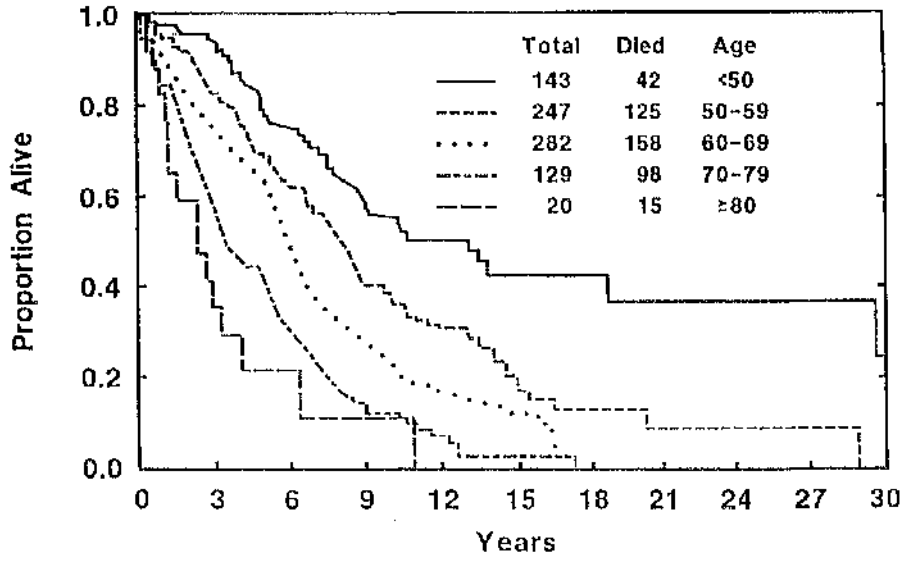


Figure 4. Survival of untreated B-CLL patients by age at diagnosis. Reprinted, by permission, from Keating, M. J., Chronic Lymphocytic Leukemia, Figure 4. © Marcel Dekker Inc 1993.

2 B-cell lymphopoiesis.

2.1 Preamble.

Most adult human B-cells originate from a small population of self-renewing pluripotent stem cells (PSC) in the BM by what is essentially a two phase process. During the primary antigen-independent phase of differentiation and proliferation in the BM microenvironment, PSCs differentiate through a lymphoid stem cell (LSC) stage, pre-B-cell stages defined by the absence of membrane Ig, to become immature IgM^+ IgD^- virgin B-cells. The post-LSC, pre-B-cell stages are generally referred to as pro- and pre-B-cell stages or as the pre-B-I and pre-B-II cell stages, with an equivalence between pro-B-cell and pre-B-I stages, and between pre-B-cell and pre-B-II cell stages. In the second, generally antigen-dependent phase, virgin B-cells which have migrated via the BM sinuses and peripheral blood to the secondary lymphoid tissues, are activated by a cognate encounter with antigen. Once activated and with appropriate T-cell support, these develop through centroblast and centrocyte stages to become Ig-secreting plasma or long-lived memory cells. The development process is summarised in Figures 5 & 6.

Differentiation and proliferation is orchestrated by unique endogenous patterns of gene expression, by cell-cell, cell-BM matrix and cytokine cues. The process is characterised to some extent by changes in morphology, but is particularly characterised by the expression of molecules of the immunoglobulin rearrangement machinery, the rearrangement states of the Ig heavy (H)- and Ig light chain genes, the state of the pre- and B-cell-receptor complexes, and the expression of membrane-associated antigens.

2.2 Antigen-independent development.

In the primary antigen-independent phase illustrated in Figure 5, the earliest marker of B-lineage commitment was traditionally regarded as being CD19, the principal phenotypic marker of human B-lineage, but earlier stages exist. A $CD10^+$, $CD19^-$ common lymphoid progenitor of T-, B-, natural killer, and dendritic cells has been identified in humans (Galy, 1995). More recently murine $CD19^-$ cells purified into three separate fractions by multiparameter flow cytometry appeared to show a linear development to B-committed cells, but with the earliest fraction not expressing genes associated with B-lineage commitment. Fraction A_0 ($CD4^{low}$, AA4.1$^+$ (stem cell antigen), $B220^-$) expressed low or undetectable levels of germline $IgH\mu$, recombination-activating gene (*rag-1*), *rag-2*, *Igα*, or *Igβ* gene transcripts.

Fraction A_1 ($CD4^{low}$, $AA4.1^+$, $B220^+$) expressed germline IgHμ, along with low levels of *rag-1*, *rag-2*, *Igα*, and *Igβ*. Fraction A_2 ($CD4^-$, $AA4.1^+$, $B220^+$) expressed germline IgHμ, *rag-1*, *rag-2*, *Igα*, *Igβ*, and *λ5* (Li, Y.S., 1996). Given the similarities between B-lymphopoiesis in mouse and man, this A_0 fraction may be the equivalent of the $CD10^+$, $CD19^-$ common lymphoid progenitor in man and offer insights into the earliest stages of B-cell differentiation in humans.

The earliest indication of B-lineage commitment is found in a small compartment of BM cells which are reported to be $CD34^+$ $CD10^-$ $CD19^-$, although the fact that both LSCs and all of the early stages of B-lymphopoiesis are $CD10^+$ inevitably brings into question the CD10-negativity of these early B-cells. However, some of these also express the α-chain of the interleukin-7 receptor (IL-7Rα) and have been reported as expressing *vpreB*, a gene encoding one of the two components of surrogate light chains (SLC), germline IgHμ and D_HQ52 transcripts, terminal deoxynucleotidyl transferase (TdT), the Ig β signalling molecule, Rag-1 and Pax-5 (a B-cell specific transcription factor critical to B-cell development) mRNA, as well as DJ rearrangements in both adult and foetal cells. CD19 is expressed in all but the very earliest stages of B-lymphopoiesis, as is CD10 which is expressed from a very early stage up to the IgM^+ immature B-cell stage (Bertrand, 1997; Billips, 1995; Davi, 1997; Loken, 1987; Ryan, 1997). In the circumstances CD19 appears more of a manifestation of B-lineage commitment and not the potential effector of B-cell lineage as it has traditionally been seen. Human $CD19^+$, $CD10^+$, $CD34^+$ pre-B-I cells also express TdT and Rag-1 in their nucleus from a very early stage. At this point the majority of pre-B-I cells are DJ_H rearranged with Ig light chain genes in germline configuration (Ghia, 1996, 1998). Additional early B-lymphopoietic antigens include CD45 (B220), IL-7R, and CD43, the latter of which subsequently shows a marked decrease in the pre-B-II cell stage (Candeias, 1997; Hardy, 1991; Karasuyama, 1994; Li, Y.S. 1996; Melchers, 1995).

The pre-B-I cell stage is also marked by expression of SLC (a dimer consisting of VpreB and λ5) in the absence of membrane IgHμ, but apparently in association with several other molecules of varying molecular weight including the possibility of a molecular structure described as a surrogate heavy chain (SHC) (Ghia, 1996; Grawunder, 1995; Karasuyama, 1993; Meffre, 1996; Sanz, 1996). Also expressed in the pre-B-I cell stage are Igα and Igβ. Together these form a signalling dimer and are an integral part of both the B-cell receptor complex (BCR), which consists of IgHμ, rearranged light chain, and Igα /Igβ, and the pre-BCR, in which SLC takes the place of rearranged light chain (Rajewsky, 1996).

The pre-B-II cell stage is marked by the expression of high levels of cytoplasmic IgHμ, the expression of which requires a functional V_HDJ_H rearrangement, but the absence of TdT expression and CD34, with a recent study suggesting that cytoplasmic IgHμ is responsible for the downregulation of TdT (Ghia, 1998; Wasserman, 1997). The expression of a functionally rearranged IgHμ,allows it to associate with SLC and Igα/Igβ on the membrane, forming the pre-BCR which appears to be a *sine qua non* for progression to subsequent stages of differentiation (Grawunder, 1995). For example, in both mouse and man expression of the λ5 gene appears not to be essential for successful progression to the pre-B-I cell stage, up to the emergence of a rearranged IgHμ chain. However, λ5 defective mice produced by a targeted mutation, do not form a pre-BCR despite production of an IgHμ chain and are significantly B-cell deficient, suggesting that expression of λ5 and the pre-BCR are essential for a progression to the large proliferative pre-B-II cell stage and beyond (Ehlich, 1993; Karasuyama, 1994; Kitamura, 1992; Minegishi, 1998; Rolink, 1993). Similarly mice harbouring mutations of the IgHμ or Igβ genes exhibit severe disruption of B-cell development (Gong, 1996; Kitamura, 1991; Torres, 1996). A similar picture emerges from studies of λ5 expression in common variable immune deficiency (CVID) in humans. Differentiation to the pre-B-I cell stage is unaffected by the condition, but there is a significant reduction in the pre-B-II cell population in patients with CVID and peripheral cells are IgM^{LO} and $CD38^{LO}$ (Minegishi, 1998).

Expression of rearranged IgHμ and the pre-BCR coincides with the downregulation of Rag-1 and Rag-2 at both the protein and mRNA level (Grawunder, 1995) and this may be part of the mechanism by which allelic exclusion prevents productive V_H to DJ_H rearrangements of the second H-chain allele. Activation of the rearrangement machinery appears to be a bimodal event in both mice and humans, with the *rag* genes expressed as both mRNA and protein during the first wave of rearrangement of the H-chain locus at the $CD34^+$ $CD10^+$ pre-B-I cell stage. However, the *rag* genes are downregulated at the large cycling $CD10^+$ $cIgH\mu^+$ pre-B-II cell stage, but are again detectable during the $VpreB^+$ $CD10^+$ $cIgH\mu^+$ small pre-B-II cell stage where, on the basis that V_LJ_L rearrangements are detectable, a second wave of rearrangement appears to occur. *rag-1 & -2* genes continue to be expressed throughout the pre-B-II cell and in the immature IgM^+ stages, suggesting that rearrangement of the L-chain loci continues throughout these development stages (Ghia, 1995, 1996, 1998; Grawunder, 1995; Li, Y.S. 1993; Li, Z. 1996; Lin, W.C. 1993; Ma, 1992). Immature IgM^+-expressing B-cells continue to express $CD10^+$, which is subsequently downregulated on mature B-cells, low levels of *rag* mRNA, and with Rag-2 protein levels as high as those in small pre-B-II cells.

The continued expression of *rag genes* is in keeping with the recent findings that a proportion of B-cells undergo a secondary rearrangement of the L-chain, changing the specificity of the BCR and testing it on the membrane (Ghia, 1995; Harada, 1991; Prak, 1995; Rolink, 1993).

This microenvironment in which the primary antigen-independent stage of B-lymphopoiesis occurs, consists of other lymphohaematopoietic cells, a complex framework of non-lymphohaematopoietic stromal cells and the extracellular matrix. The stromal cells, including fat cells, endothelial cells, fibroblasts and macrophages, provide an haematopoiesis-inducing environment where membrane-bound antigens or diffusible growth factors are crucial for the proliferation and differentiation of PSCs into LSCs and subsequently into lineage-committed progenitor lymphocytes and later stages of B-cell development (Pribyl, 1996; Rawlings, 1995). Cell membrane molecules such as the integrins, that mediate cell-cell and cell-substratum interactions with stromal cells, appear to play a critical role in regulating the growth and differentiation of B lymphocytes. For example, the very late antigen (VLA) integrin subfamily of surface antigens can bind to receptor molecules on other cells and to components of the extracellular matrix such as fibronectin, various collagens and laminin. B-cell precursors (BCP) express two members of the VLA family designated VLA-4 and VLA-5. The receptors for VLA-4 include the vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily, and the CS-1 domain of fibronectin. The receptor for VLA-5 is the central cell-binding domain of fibronectin. Human bone marrow cells that support the growth and differentiation of BCPs constitutively express VCAM-1 in contrast to vascular endothelial cells where it appears to be cytokine-regulated. BCPs adhere to stromal cells via a VLA-4/VCAM-1 interaction and this, along with the VLA-5/fibronectin interaction, is thought to be of central importance in B-lymphopoiesis (LeBien, 1996; Oostendorp, 1997).

When infection is present the major source of the growth factors acting on the BM lymphohaematopoietic microenvironment are the haematopoietic cytokines produced by activated macrophages and helper T-cells, in comparison to the situation which prevails in the absence of infection, when the stromal cells are the major source of cytokines which induce homeostatic haematopoiesis. Proliferation and development of different lineages can be altered by either a change in the local concentrations of particular growth factors, or by altered expression of the membrane receptors/adhesion molecules. The stage of differentiation and the state of activation influence both the type and quantity of receptors/adhesion proteins expressed.

These may be influenced by the presence and concentration of specific cytokines, so the regulatory system is a complex and dynamic one consisting of controls within controls which responds to the changing needs of the immune system. No definitive picture has emerged of the regulation of lymphohaematopoiesis by cytokines, but in vitro studies on human and murine cells have shown a number of growth factors to affect the proliferation and/or differentiation of lymphocyte progenitors. Multilineage colony-stimulating factor (multi-CSF also known as (aka) IL-3), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-11, transforming growth factor-beta (TGF-β), IFN-γ, tumour necrosis factor-alpha (TNF-α), IFN-α, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) have all been shown to influence some aspect of haematopoiesis in culture (Dorshkind, 1991; Hirayama, 1994; Kincade, 1989; LeBien, (1996); Lee, 1993; Maraskovsky, 1998; Nilsson, (1993); Witte, 1993; Yamada, 1996). IL-7 and IL-7R have both been implicated in murine B-lymphopoiesis. Mice deficient in IL-7 production or the IL-7Rα/γ chains exhibit a severe disruption of B-cell development, but with deficiencies of the receptor causing more severe effects than deficiency of IL-7 (Stephan, 1997, 1998; von Freeden-Jeffry, 1995). One explanation put forward for this, proposes that the IL-7R has an as yet unidentified ligand other than IL-7 which is involved in B-lymphopoietic signalling during B-cell development. It remains to be seen if IL-7 plays a similar role in human B-lymphopoiesis, but one *in vitro* study found that addition of IL-7 or goat anti-IL-7 antibody to the culture conditions made no significant difference to the tempo and phenotype of B-cell development (Pribyl, 1996). The full range of influences of cytokines, either singly or in combination *in vivo*, and particularly at different stages of differentiation, remains to be elucidated.

During their development, immature B cells acquire the degree of flexibility that allows them to pass through the sinusoidal wall into the sinuses of the bone marrow, from where they enter the peripheral blood circulation and home to the secondary lymphoid tissues where the maturation process continues with the expression of IgD (Rajewsky, 1996) and the antigen-dependent phase of B-cell development occurs. Homing by blood- borne lymphocytes to the secondary lymphoid tissues is mediated by multiple cell surface adhesion molecules including the β1 (VLA), β2, and particularly by the selectins. For example leukocyte selectin, CD62L, is expressed at relatively low levels during the early stages of B-cell ontogeny, but increases as B-cells mature in the bone marrow. Such differential expression of adhesion molecules is wholly consistent with a requirement for homing to secondary lymphoid tissues.

In this context, selectin is thought to mediate adhesion to activated epithelium using GlyCAM-1 (a major L-selectin ligand), MAdCAM-1 (mucosal addressin cell adhesion molecule-1), and CD34 ligands (LeBien, 1996).

2.3 Secondary B-cell development.

Secondary B-cell development is largely antigen-dependent, but multimeric antigen can also stimulate B-cells, predominantly though not exclusively B-1 cells, to proliferate and differentiate into plasma cells (Forster, 1987; Mond, 1995; Rajewsky, 1996). Like the primary antigen-independent phase of B-cell development, the continued development of B-cells in lymphoid tissues is also characterised by changes of morphology, by changed patterns of gene expression, by changes in immunophenotype, and is summarised in Figure 6.

In antigen-driven development and differentiation, naive B-cells in a primary follicle or in the circulation are activated by a cognate encounter between antigen and the BCR. Activation results in the formation of a germinal centre (GC), in which the activated cells undergo a complex development process including cytokine stimulation by IL-1 and IL-4, and dependent on association with T_H- and follicular dendritic cells (FDCs). This process begins with a rapid clonal expansion, during which rapidly proliferating B-cells have their apoptotic potential increased by the expression of *c-myc* mRNA and protein. The expanding clone is then subject to somatic hypermutation (SHM), during which the V regions of Ig genes are subject to a high rate of point mutation, before isotype switch recombination (ISR), and receptor editing of the Ig L-chain genes (Cutrona, 1997; Han, S. 1995; Kuppers, 1993; Liu, Y. J. 1996(1-3)). Centroblasts, which are subject to several rounds of SHM, ISR, and selection whilst cycling between the dark and light zones of the GCs, migrate to the light zone where they are subject to positive selection for high-affinity antibody and negative selection for low-affinity or autoreactive antibody (Kelsoe, 1995, 1996(1 & 2); MacLennan, 1994). After undergoing successful rounds of SHM, receptor editing, ISR and selection, B-cells, in the form of plasmablasts, begin to proliferate before leaving the GC and differentiating into either long-lived memory cells or plasma cells (Henderson, 1998).

The initial activating encounter with antigen results in the upregulation of cell membrane molecules involved in B-T-cell interactions and the expression of membrane antigens involved in the formation of GCs.

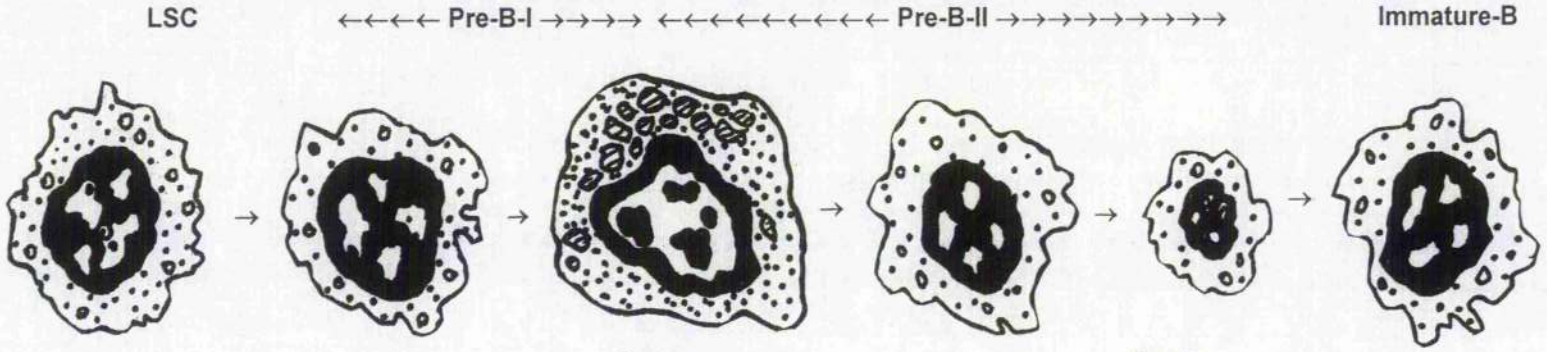
These include class II major histocompatibility (MHCII) proteins, B7-1, B7-2, CD40, CD19, and the tumour necrosis factor receptor type I (TNFR-1) (Clark, 1989, 1991; Glimcher, 1992; Han, S. 1995; Lenschow, 1996; Matsumoto, 1996; Rickert, 1995). Additionally, expression of NF-IL-6 (nuclear factor-IL-6), Oct-2 (octamer binding site protein 2), STAT1 (signal transducer and activator of transcription 1) and Jaks (Janus kinases) are increased in activated B-cells. In keeping with the recombination that occurs in GC centroblasts for the purpose of generating high-affinity antibody, the *rag-1* and *rag-2* genes are reexpressed at this stage. Bcl-6 is also increased but expression of IgM is reduced (Han, S. 1996; Henderson, 1998).

In the light zone B-cells interact with T-cells and FDCs via CD20, LFA-1 and VLA-4 (Holder, 1995; Pound, 1997). In addition centrocytes continue to express CD40, B7-1, B7-2, CD23, but they also express IgM without IgD (Liu, Y. J. 1996c). Centrocytes also express genes concerned with apoptosis including *fas* (CD95), *c-myc*, *bax*, *p53*, but with only low levels of *bcl-2* expression (Lagresle, 1995; Martinez-Valdez, 1996; Smith, 1995). On exiting the GC, differentiating into plasma or memory cells coincides with the down-regulation of *fas* expression, but with an increase in the expression of Bcl-2, Bcl-x_L, and the cyclin-dependent kinases 4 (Cdk4) and 6(Cdk6) and the IL-6 receptor (Choe, 1996; Ishida, 1995; Koopman, 1997). Additionally, expression of the transcription factors jun, NF-κB, and NF-AT is increased (Berberich, 1994; Choi, 1994; Li, Y. Y. 1996).

The mechanisms by which the fate of the B-cells exiting the GC is determined remains imperfectly understood, but human memory cells are $CD38^-$, $CD20^+$, $CD39^+$, and can be stimulated to proliferate in response to CD40L (Arpin, 1995). Outside the GC, plasmablasts become plasma cells which are stimulated by increased expression of IL-6 to induce Ig, and Oct-2 expression (Henderson, 1998; Natkunam, 1994). Plasma cells have superabundant levels of Ig and J chain mRNA and inducement of the transcription factor Blimp-1 coincides with differentiation into plasma cells (Turner, 1994). Differentiation into plasma cells also results in the reduction of expression of many genes including those expressing CD19, CD23, CD22, class II MHC, c-Myc, BSAP, early B-cell factor (EBF), and CIITA (Hagman, 1993; Henderson, 1998; Lin, 1997; Rinkenberger, 1996; Ruzek, 1995; Scheuermann, 1995; Silacci, 1994; Tedder, 1997). Memory cells can be distinguished from GC B-cells and plasma cells on the basis of CD38 and CD20 expression. GC B-cells are $CD38^+$ $CD20^+$, whereas plasma cells are $CD38^+$ $CD20^-$, and memory cells are $CD38^-$ $CD20^+$. Additionally memory cells are reported to be HSA^{LO} and $CD44^+$ (Arpin, 1995; Sprent, 1994).

Although normal B-cells can also be involved in T-cell-independent activation, a particular exception to the normal two-phase process of lymphopoiesis described in the preceding paragraphs involves a subset of B-cells which have been mainly studied in mice but which are also present in humans. These cells are designated B-1a if they are CD5(Ly-1)$^+$, and B-1b if CD5(Ly-1)$^-$ and in adults they are most commonly found in the pleural and peritoneal cavities where they participate in gut- and lung-associated immune responses. Studies in chickens, sheep and mice suggest that they are produced in the early stages of life and unlike normal B-cells, may subsequently have their origins founded in self-replication rather than in PSCs. Furthermore, multimeric antigens on cell surfaces can stimulate T-independent proliferation into plasma cells via efficient cross linking of BCR, resulting particularly in the production of IgM and IgG3 antibodies. B-1 cells may be either CD5$^+$ (expression of which is increased upon activation) or CD5$^-$, CD23$^-$, B220$^+$, IL-5R$^+$, CD72$^+$, are typically IgM^{Hi}, express variable amounts of IgD, and are CD11b$^+$ in the peritoneal cavity but CD11b$^-$ in the spleen (Henderson, 1998; Kantor, 1993; Rajewsky, 1996). Development and differentiation of B-1 cells is less well characterised than the lymphopoiesis of normal B-cells and is not illustrated in Figures 5 & 6.

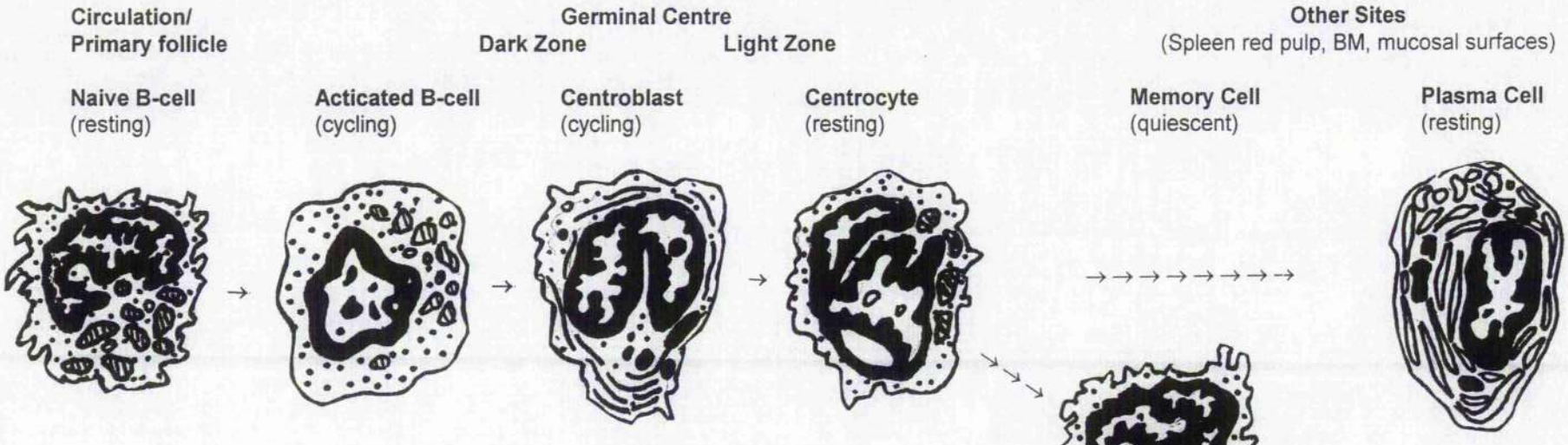
Antigen-independent primary B-Lymphopoiesis in bone marrow, summary of antigen, gene expression & Ig rearrangement status.



	$CD10^+$ $CD19^-$	$CD34^+$ $CD10^+$ $CD19^+$ $CD45^+$ IL-7R Igα/β SLC TdT c$Ig H\mu^-$	$CD34^-$ $CD10^+$ $CD19^+$ c$Ig H\mu^+$ TdT^- pBCR	$CD10^+$ $CD19^+$	cIgM^+ $CD10^+$ $CD19^+$	sIgM^+ $CD10^+$ $CD19^+$
		↑*rag-1* ↑*rag-2* ↑*pax-5*	↓*rag-1* ↓*rag-2*	↑*rag-1* ↑*rag-2*		
IgH	germline	DJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H
IgL	germline	germline	germline	germline	V_LJ_L	V_LJ_L

Figure 5. Summary of the major indicators of antigen-independent B-lymphpoiesis.
Key s = surface and c = cytoplasmic.

Secondary B-cell development, summary of membrane antigen and gene expression.



IgM/D

↑MHCII
↑CD40
↑CD19
↑B7-1/2
↑TNFR-1
↑C-myc
↑NF-IL-6
↑Oct-2
↑STAT1
↑Jaks

↑Rag-1
↑Rag-2
↑Bcl-6

↓IgM

CD20
CD40
CD23
CD95(Fas)
LFA-1
VLA-4
B7-1/2
IgM
C-myc
Bax
P53
Bcl-2^{LO}

$CD38^{-}$
CD20
CD39
CD44
HSA^{LO}

sIgG/A/M
CD38
$CD20^{-}$
cJ chain

↑Bcl-2
↑Bcl-x_L
↑Cdk4/6
↑IL-6R
↑Jun
↑NF-κB
↑NF-AT
↑Blimp-1

↓CD19
↓CD23
↓CD22
↓MHCII
↓C-myc
↓BSAP
↓EBF
↓CIITA
↓CD95

Figure 6. Summary of the major indicators of T-cell- & antigen-dependent B-cell development.
Key s = soluble and c = cytoplasmic.

3 B-CLL Ontogeny.

The origins of the progenitors of the neoplastic clones found in B-CLL remains unclear. Proliferation is believed to occur in the lymphatic system, but the ambiguity posed by a relentless and often aggressive accumulation of apparently non- or slowly-dividing neoplastic cells in peripheral blood, bone marrow and lymphoid tissues, has yet to be resolved by the unequivocal identification of the neoplastic progenitor and the proliferative compartment.

Speculation about the identity of the normal counterpart of the putative neoplastic parent is based to a very limited extent on the morphology of B-CLL cells, but mainly on a comparison of the immunophenotype of the typical neoplastic B-CLL cell with the various subsets/developmental stages of normal B-cells. A simplified version of the latter is illustrated in Figure 7, which includes, as well as the antigens and developmental stage molecular markers described in previous paragraphs, other membrane antigens that have been reported in studies of B-cells (Freedman, 1993; Keating, 1996; LeBien, 1996; Nilsson, 1993). Most B-CLL clones appear to have a morphology equivalent to those of small recirculating B lymphocytes resting at G_0 of the cell cycle and the immunophenotype is strongly suggestive of B cells that are marginally immature, e.g. the limited surface expression of IgM along with high levels of cIgM. However, markers suggesting a relatively earlier stage of development may include CD9, CD10, mouse red blood cell receptors (MRBC-R) and the presence on cell membranes of fewer receptors for ABO blood group antigens, for "i" antigen and for sialic acid residues.

Figure 8 represents, as far as it is possible to do so in a condition which is notoriously heterogeneous, the immunophenotype found on B-CLL cells grouped by the developmental stage/lineage which they are normally thought to represent (Freedman, 1993; Keating, 1996; Nilssson, 1993).

Of particular interest in typical B-CLL cells, when Figures 7 & 8 are compared, is the juxtaposition of antigens normally associated with normal resting G_0 B-cells (cIgM, sIgM, CD11a/CD18, CD44, L-Selectin, CD22, CD21 and CD35), antigens more typical of activated B-cells (B5, Blast-1, CD23 (low-affinity Fcε receptor), CD25, BB-1/B7, B8.7, CD54, CD71, CD77, and 4F2), antigens normally regarded as being representative of myelomonocytic lineage, with CD5, a pan-T antigen expressed on both a subset of B-cells and on B-cells during activation.

Antigenic map of common B-cell populations.

LSC	Pre-B-I	Pre-B-II	Immature-B	Mature Resting-B	Activated B-cells	Memory Cells	Plasma Cells
←←←←←←←←←← Pan-B MHC II (Ia), CD19, CD20, CD24, CD29/49d, CD40, CD45R (unless otherwise indicated) →→→→→→→→→→							
CD10 CD19$^-$	CD10 CD22 CD34 CD38 CD45 CD72 IL-7R Igα,β SLC (VpreB+λ5)	CD9 CD10 CD34$^-$ CD38 CD45 CD72 pre-BCR (μ, Igα,β SLC)	IgM CD9 CD10 CD21 CD22 CD38 CD72	IgM/D CD1 (a, b or c) CD9$^-$ CD10$^-$ CD11a/CD18 CD21 CD22 CD35 CD37 CD39 CD44 CD72 CD73 CD74 CDw75 CDw76 CD78 L-Selectin	IgM/D CD9 CD21 CD22 CD37 CD39 CD72 CD73 CD74 CDw75 CD76 CDw78 *CD5*(1) *CD23* *CD25* *CD54* *CD71* *4F2* *Blast-1* *B7/BB-1* *CD77* *B5* *Bac-1*	CD20 CD38$^-$ CD39 CD44 HSA^{LO}	secretedIgG/A/M CD20$^-$ CD24$^-$ CD38 CD40$^-$ IL-6R ↓CD19 ↓CD22 ↓CD23 ↓MHCII

Figure 7. Simplified antigen map representing common B-cell subpopulations in adult bone marrow, blood and lymphoid organs.

(1) Antigens in italics are not restricted to B-cells and are transiently expressed with peak expression at 72 hrs and absent after 120 hrs.

Setting aside the possibility that B-CLL may be as heterogeneous in its neoplastic origins, as it is in almost every other respect, the similarities between B-CLL lymphocytes and $CD5^+$ B-cells seem to raise three possibilities.

Antigens common to B-CLL cells by lineage/stage

Pan-B antigens	Pre-B	Immature/ Mature B-cell antigens	Activation Antigens	Myelo/ monocytic	Other
MHC II (Ia)	CD9(20%)	**c/sIgM/D**	**B5**	CD11b/18	**CD5**
CD19	CD10	CD1c	Blast-1(50%)	CD11c/18	**MRBC-R**
CD20		CD11a/18	CD23	CD14	DCA-1
CD24		**CD21**	CD25(50%)	CD15	CD38
CD40		CD22(25%)			
CD45R		CD35	*BB-1/B7*		
		CD44(76%)	*CD54*		
		L-Selectin	*CD71*		
			CD77		
			B8.7		
			4F2		

Figure 8. Antigens typically found expressed in B-CLL with the fingerprint immunophenotype highlighted in bold, the less commonly expressed activation antigens highlighted with *italics,* and reported percentage occurrence in brackets.

First, the $CD5^+$ subset of B-lymphocytes, which are found in foetal lymph node and spleen, in adult peripheral blood, the periphery of adult lymph nodes, adult tonsils, after allogenic transplantation and in autoimmune disease such as rheumatoid arthritis or systemic lupus erythematosis (Freedman, 1993), is a genuine and distinct population of B-cells and that B-CLL is derived from it. This hypothesis is supported by similarities between B-CLL cells and those of the normal small subpopulation of $CD5^+$ B-lymphocytes which are quite striking. Within generally similar immunophenotypes, 95% of B-CLL cells and this putative subpopulation of B-cells are positive for the almost-pan-T CD5 antigen, both weakly express sIgM, both express MRBC-Rs and have a long survival in vitro (Kipps, 1989). In common with B-CLL cells, normal $CD5^+$ cells express myelomonocytic antigens as part of their normal repertoire, although it is uncertain if the expression of the myelomonocytic antigens CD11b/CD18, CD11c/CD18, CD14 and CD15 on $CD5^+$ B-CLL cells (Nilsson, 1993) represents a neoplasia-induced lineage infidelity, or if it offers further support to the theory that B-CLL is derived from the normal population of $CD5^+$ cells. In addition they also share the frequent production of polyreactive autoreactive antibodies, the ability to cap sIg and the expression of low levels of CD20 (Caligaris-Cappio, 1993).

Furthermore, the fact that some of these B-1 cells are apparently $CD5^-$, offers a ready-made explanation for the much rarer and less-studied $CD5^-$ B-CLL clones.

Secondly, the suggestion that B-CLL is derived from a subtype of normal activated B-cells and that expression of CD5 is representative of a particular activation stage is supported by the fact that many clones are known to express antigens normally associated with the activated B-cell phenotype. B5, Blast-1, CD23 and CD25 are expressed with a variable frequency while BB-1/B7, CD54, CD71 and CD77 are less commonly expressed and/or weakly expressed. Although many of the stimuli that activate B-cells, e.g. anti-Ig, EBV, the anti-CD20 monoclonal antibody 1F5, recombinant interleukin-1 (rIL-1), rIL-2, rIL-4, recombinant interferon-γ (rIFN-γ) and B-cell growth factor do not induce the expression of CD5, CD5 expression has been induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) which was found to induce the expression of CD5 on splenic B-cells between 48 and 96 hours after activation. In the case of TPA, the parallels between activated splenic B-cells and B-CLL were supported by the expression of B5, CD23 and CD25 on the splenic B-cells activated by this phorbol ester (Freedman, 1989).

Although the balance appears to be tilted in favour of B-CLL originating from a cell from the subpopulation of B-1 $CD5^+$ B-lymphocytes, the phenotypic heterogeneity of B-CLL clones, particularly the $CD5^-$ nature of some, the lack of knowledge of the extent, if any, to which expression of a B-CLL marker is caused by the malignancy, and the currently limited knowledge of the normal $CD5^+$ population of B-cells, prevents the putative precursor from being identified with any certainty. In such circumstances the third possibility remains that B-CLL is derived from an ordinary B-lymphocyte and that the juxtaposition of CD5, resting, myelomonocytic and activation antigens on the cell membrane is the genetic consequence of the neoplastic event(s).

4 Apoptosis.

4.1 Preamble.

B-CLL is characterised by the enigmatic and often relentless clonal accumulation of functionally immature B-lymphocytes juxtaposed with a non-existent or low proliferation coefficient. There is a general consensus, traceable to Dameshek's comments in 1967, that chronic lymphocytic leukaemia was a lymphoaccumulative disease of immunologically immature lymphocytes, and those of Kerr, Wyllie and Currie in 1972, concerning the role that apoptosis might play in the spontaneous and therapeutically-induced regression of malignant neoplasms, that the apoptosis-resistant nature of the neoplastic B-cells is the source of the enigma and a major contributor to tumourigenesis in B-CLL.

Apoptotic cell death is a fundamental biological mechanism involved in, for example, embryogenesis, morphogenesis, tissue homeostasis and defence against pathogens. Disturbed apoptosis is a major contributor to autoimmune disease and malignancy, although the genes involved in tumourigenesis, e.g. the Myc proteins, in deregulating the cell cycle, appear to prime the cell for apoptosis at the same time. However, of some relevance to B-CLL is the critical role apoptosis plays in B-cell homeostasis and in the negative and positive selection of lymphoid cells (Adams, 1998; Evan, 1998; Osario, 1998).

A proportion of cells are thought to die apoptotically at the pre-B-cell stage of development as a result of their failure to rearrange their Ig genes and express functional receptors. Mice harbouring mutations of the $IgH\mu$ or $Ig\beta$ genes exhibit severe disruption of B-cell development (Gong, 1996; Kitamura, 1991; Torres, 1996). Furthermore, B-cells devoid of surface Ig (sIg) are seldom observed in the peripheral immune system and Bcl-2 is downregulated in small pre-B and immature B-cells consistent with the belief that these cells are deleted apoptotically unless rescued by a BCR-mediated signal (Rajewsky, 1996). Similarly, virgin B-cells that fail to be activated by antigen are thought to meet a similar fate. In a recent review of B-cell life span in mice, it was suggested that approximately 15% of peripheral blood B-cells are short-lived immature cells (B220 $(CD45RA)^{L_o}$) with a life-span of several days. These newly generated cells populate the B-cell follicles where they are lost apoptotically by natural attrition if they fail to be activated (Fulcher, 1997).

Apoptotic deletion of immature B-cells is believed to occur in the BM when B-cells expressing receptors for self-antigen are eliminated (Rajewsky, 1996). Studies in mice indicated that this occurred in two separate and sequential stages. The binding of self-antigen appears to block the developmental acquisition of adhesion molecules and receptors which are important for migration and activation (i.e. increased expression of IgM and expression of CD45R and IgD), prior to the B-cell's apoptotic deletion within the bone marrow where their median life-span was 15 hr. Expression of transgenic Bcl-2 extends the longevity of the cells resulting in the escape of large numbers of self-reactive B-cells from the BM, but fails to overcome the developmental arrest. This can be reversed by the removal of self-antigen, when development recommences and proceeds normally (Hartley, 1993).

Centrocytes which have undergone a process of hypermutation of the variable area of their Ig genes in GCs appear to be selected on the basis of their resulting changed affinity for antigen. In vitro tests on centrocytes isolated from human tonsillar tissue indicated that a minority of centrocytes could be rescued from apoptosis by either interaction with antigen-presenting FDCs via CD11a (LFA-1α) and CD49d (VLA-4α) or by cross-linking the B-cell antigen receptors, while the other unsupported centrocytes died by apoptosis. Apoptotic death in these circumstances is apparently mediated by a pre-existing Zn^{2+}-dependent 18 kDa murine NUC-18-like endonuclease and interestingly rescue from apoptosis correlates with increasing expression of Bcl-2 (Lindhout, 1993, 1995; Liu, 1989, 1991). Similarly, GC B-cells can be rescued from CD95-induced apoptosis by ligation of CD40 or by attachment via LFA-1 and VLA-4 on B-cells to their counter receptors, ICAM-1 and VCAM-1, on FDCs (Koopman, 1997).

The morphology of apoptosis is well characterised. Its morphological hallmark is the condensation of the nucleus whilst other organelles remain relatively normal, but other visible changes in the ultrastructure include compaction of the nuclear chromatin, condensation of the cytoplasm accompanied by an increase in density, and mild convolution of the nuclear envelope and cell membrane. The nuclear chromatin typically condenses into sharply delineated and uniformly dense masses that lie against the inside face of the nuclear envelope, the cytoplasm condenses without any obvious deterioration of organelles and both the cell membrane and the nuclear envelope adopt increasingly convoluted shapes. As apoptosis progresses compaction of organelles and dilation of the endoplasmic reticulum occurs, the nuclear envelope becomes convoluted to the point where it breaks up into discrete fragments which may contain pieces of compacted chromatin, and the cell membrane adopts a shape displaying many protruding lobes.

In the final stage of ultrastructural decay the lobes break away forming membrane-bound apoptotic bodies which contain small amounts of cytoplasm and which may also contain discrete parts of the nucleus and/or organelles. Apoptotic bodies of lymphocytes are phagocytosed by macrophages and apoptotic bodies from other types of cell in solid tissues may be phagocytosed by surrounding cells, both without any indication of the inflammation which accompanies necrotic cell death (Cohen, 1993; Kerr, 1991; Kroemer, 1997). The ultrastructural deterioration may only take minutes to complete from the point where change first becomes apparent and is illustrated below in Figure 9.

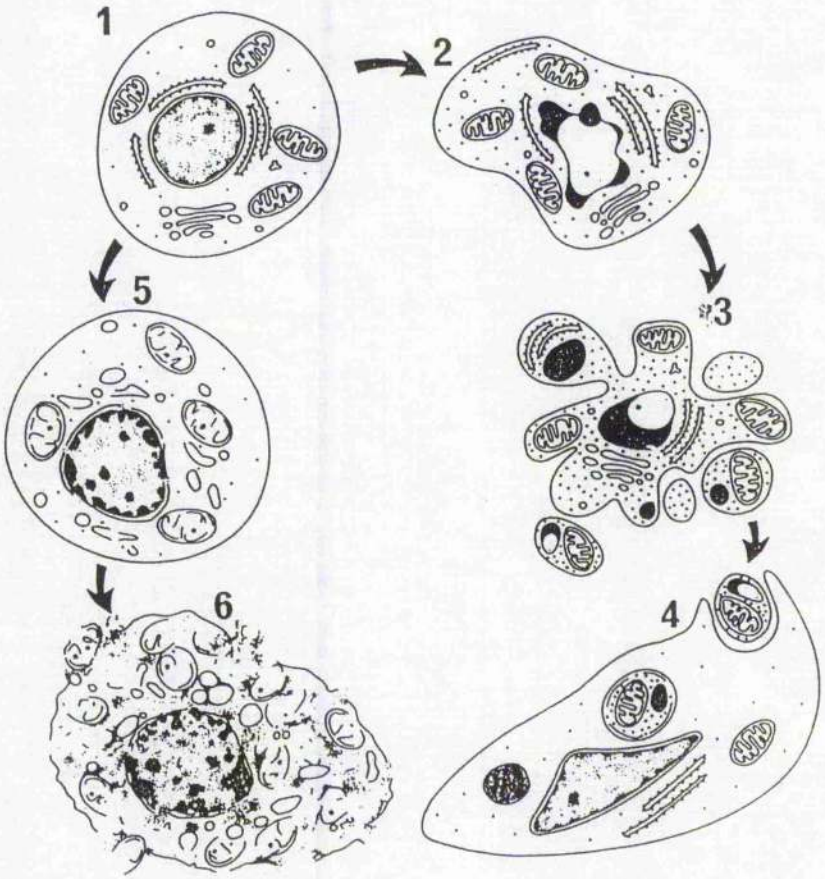


Figure 9. Ultrastructural changes in apoptosis (right) compared to necrosis (left).

Reprinted, by permission, from L. D. Tomei & F. O. Cope (eds), Apoptosis: The molecular basis of cell death, Figure 1. © Cold Spring Harbor Laboratory Press 1991.

Apoptosis is a phenomenon that is believed to occur in all metazoans, albeit with apparently much greater complexity in mammals, and the extent to which it is evolutionarily-conserved is, for example, obvious from the structural and functional similarities between mammalian genes/proteins and those of the nematode worm *Caenorhabtidis elegans (C. elegans)*. The current three-phase model of apoptosis, although incompletely understood, is increasingly well characterised in terms of the functions of the molecules involved. The paradigm includes an induction phase in which heterogeneous signals including DNA damage from ionising radiation or chemotherapeutic agents, deprivation of growth factors or metabolites, and ligation of cell death signal-transmitting receptors such as CD95, initiate the apoptotic process by separate molecular pathways.

This initiation stage is followed by a regulation phase, in which apoptosis-inducing and apoptosis-inhibiting members of the Bcl-2 family of homologues compete to determine if the cell is deleted apoptotically or remains viable. If a point-of-no-return is passed, the execution phase of apoptosis is activated and a cascade of caspases complete the process by effectively dismantling the cell from within (Adams, 1998; Kroemer, 1997). The apoptotic process is illustrated in Figure 11.

4.2 Apoptosis initiation.

Apoptosis may be initiated by a wide range of events and agents, including oncogenic events, heat shock, hypoxia, IFN, TNF, protein synthesis inhibitors, cytokine deprivation, glucorcorticoids, staurosporine, γ- and uv-irradiation, and cytotoxic drugs. However, as well as the different stimuli acting through different receptors/cellular entities, there are clearly at least two distinct paths that activate apoptosis. Bcl-2 and Bcl-w expression can protect cells from many of the diverse cellular insults listed, but apoptosis initiated by ligation of CD95 (APO-1/Fas) is apparently unaffected by these anti-apoptotic proteins (Adams, 1998; Ashkenazi, 1998; Evan, 1998; Gibson, 1996). Bcl-2's inability to inhibit apoptosis caused by activation of CD95 may be explained by the fact that, in lymphoid cells at least, CD95 triggers apoptosis through activation of procaspase-8 rather than the more common activation of procaspase-9 (Green, 1998; Thornberry, 1998). The extent of the differences between apoptosis induced by CD95 and by other means, is highlighted by the effect of caspase inhibitors. Caspase inhibitors such as benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(0-methyl)-fluoromethylketone (zVAD-fmk) and the caspase-inhibiting protein X-IAP have no effect on the release of cytochrome c (cyt c) from mitochondria or apoptosis induced by over expression of Bax.

In contrast, the release of cyt c induced by CD95 ligation is affected by caspase inhibitors, which particularly appear to inhibit apoptosis initiated by caspase-8. However, where apoptosis is instigated by caspase-8, subsequent release of cyt c can contribute to the caspase cascade by amplifying the effect of caspases downstream of caspase-8, presumably through caspase-9 (Jurgensmeier, 1998; Vander Heiden, 1997). The ability of caspase-inhibitors to influence the release of cyt c in CD95-induced apoptosis suggests that the difference between this route and the other route(s) is so great that the apparent chain of events may be reversed to some extent.

4.3 Apoptosis regulation.

The biochemical and molecular events that have been detected in the regulatory phase of apoptosis include the opening of the mitochondria permeability transition (PT) pore (aka megachannel), the collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$), and the subsequent disruption of electron transport and energy metabolism. Additionally the holochrome c form of cyt c, apoptosis-inducing factor (AIF), reactive oxygen species (ROS), and pro-caspase-3 are released from mitochondria.

Opening of PT pores and loss of $\Delta\Psi_m$ is detectable at an early stage of apoptosis before cells exhibit the classic gel electrophoretic DNA fragmentation patterns and phosphatidylserine residues are aberrantly expressed on the outer leaflet of the cell membrane. Opening of the PT pores allows molecules of $\leq$ 1.5 kDa to pass into the mitochondrial matrix, e.g. protons, calcium, glutathione etc. The resulting hyper osmolality causes a volume disregulation which results in the rupture of the membrane and release of molecules including cyt c, AIF, procaspase-3, and Ca^{2+}, into the cytosol. Cells with a low $\Delta\psi_m$ rapidly proceed to DNA fragmentation even after the apoptosis-inducing stimulus is withdrawn, suggesting that loss of $\Delta\psi_m$ is irreversible and constitutes a point of no return in the apoptotic process (Bernardi, 1996; Castedo, 1996; Green, 1998; Mancini, 1998; Zamzami, 1995(1 & 2); Zoratti, 1995).

Both procaspase-3 and the ~50 kDa protein AIF are mediators of apoptosis, the latter causing isolated nuclei to display characteristics of apoptosis such as chromatin condensation and DNA fragmentation in a cell free system. AIF appears to be stored in mitochondria and Bcl-2 inhibits its release in association with maintenance of $\Delta\psi_m$, but is unable to offset its apoptotic effect once released from mitochondria. AIF and procaspase-3 appear to be associated, with the former causing the zVAD-fmk-inhibitable proteolytic activation of the latter, suggesting that AIF is, or acts through, a protease.

Interestingly Bcl-2 does not inhibit apoptosis induced by interleukin converting enzyme (ICE), but in this case apoptosis is induced through the activation of caspase-3, rather than caspase-8 or -9 (Susin, 1996, 1997).

Inhibitors of PT, including cyclosporins and bongkrekic acid, appear to block apoptosis in a number of experimental systems, providing support for the idea that the permeability transition in mitochondria is pivotal to the apoptotic process. Bcl-2 can prevent the PT, whilst Bax and the adenine nucleotide transporter (a component of the PT pore) activator, atracytloside, induce both PT and apoptosis. Additionally, transfection-induced hyperexpression of Bcl-2 inhibits both the normal $\Delta\psi_m$ reduction and apoptosis caused by DNA damage, glucorcorticoids and ceramide (although not by anti-CD3-induced $\Delta\psi_m$ and apoptosis). This would appear to support the view that PT is a *sine qua non* for apoptosis and that it is at this juncture in the apoptotic pathway that the Bcl-2 family of proteins exert their influence. However, the evidence is not entirely unanimous and one study which induced apoptosis in CEM and HeLa cells using uv irradiation and staurosporine, found that the reduction in $\Delta\psi_m$ occurred considerably later than cyt c release and caspase activation. Furthermore, a reduction in $\Delta\psi_m$ was not apparently necessary for DNA fragmentation (Bossy-Wetzel, 1998; Xiang, 1996; Zamzami, 1995(1 & 2), 1996(1 & 2)).

Other stimuli that act directly on the PT pore, such as oxidants and pathological levels of cytosolic Ca^{2+}, can induce a rupture of mitochondria and release of caspase-activating proteins and alternative major initiators of the execution phase of apoptosis that have been suggested, including disruption of energy metabolism and export of superoxide anion (O_2^-). However, given that a fall in adenosine triphosphate (ATP) production is often seen late in the apoptotic process and that ATP appears to be a cofactor in the execution phase, disruption of electron transport and energy metabolism seems more likely to be an effect of the phenomenon rather than the cause. Similarly, given that some proapoptotic stimuli function in the absence or virtual absence of oxygen, production of superoxides may also be a consequence of the phenomenon rather than a *sine qua non* of apoptosis (Bossy-Wetzel, 1998; Bredesen, 1995; Eguchi, 1997; Green, 1998; Jacobson, 1995; Liu, X., 1996; Shimizu, 1995; Susin, 1996, 1997).

Once the apoptotic process has been initiated, members of the Bcl-2 family of proteins function as regulators, but it remains unclear if pro-survival Bcl-2 and Bcl-x_L prevent apoptosis by inhibiting Ced-4-like molecules from activating the caspases, or if they operate by maintaining organelle integrity, particularly that of mitochondria.

Bcl-x_L can bind to Ced-4 and the Ced-4-like portion of its mammalian counterpart, Apaf-1. This interaction, which is illustrated in Figure 10, has been suggested to inhibit a further interaction between Apaf-1 and procaspase-9, leading to the latter's activation and the caspase cascade that effects the apoptotic dismantling of the cell. However, Bcl-2, which is located to the outer mitochondrial membrane by its transmembrane domain and has a potential pore-forming structure, prevents the release of cyt c, which appears to be an effector of the terminal stage of apoptosis in certain cell types. Cyt c also appears to be important in combination with deoxyadenosine triphosphate in facilitating the conformational change in Apaf-1 that allows it to activate procaspase-9. To confuse the issue further, Bcl-2 is apparently able to continue exerting its anti-apoptotic influence even after significant amounts of cyt c have been released, and the inability of cyt c to induce apoptosis in certain cell types in certain circumstances inevitably poses questions about the mechanism(s) by which Bcl-2-like proteins inhibit apoptosis. However, it is particularly interesting to note that the initiation of apoptosis through CD95 seems able to bypass the step controlled by Bcl-2-like proteins. Bcl-2's apparent inability to inhibit CD95-induced apoptosis may be explained by the fact that in lymphoid cells at least, CD95 initiates apoptosis through a pathway that activates caspase-8 rather than caspase-9 (Adams, 1998; Ashkenazi, 1998; Green, 1998; Kroemer, 1997; li, P. 1997; Thornberry, 1998; Zamzami, 1998; Zou, 1997).

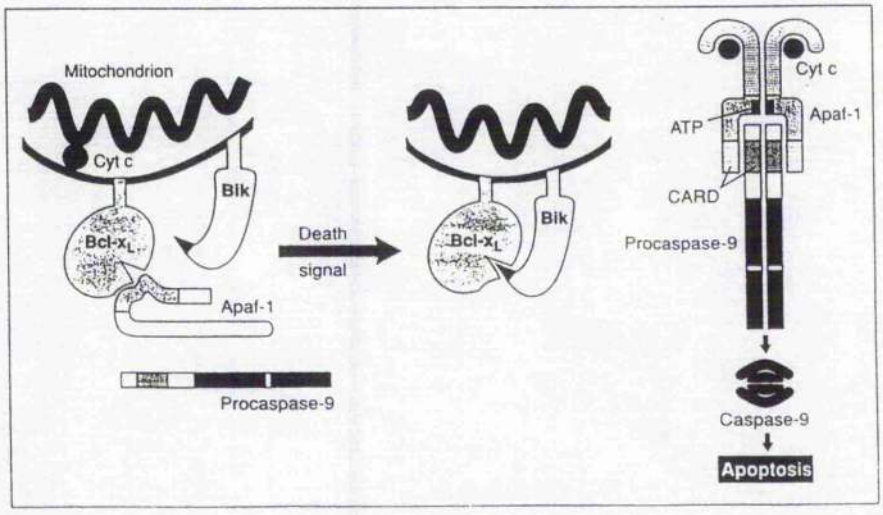


Figure 10. Proposed model of regulation of Apaf-1 by Bcl-2 family proteins. Reprinted, by permission, from J. M. Adams & S. Cory, The Bcl-2 Family: Arbiters of Cell Survival, Science 281-1324 Figure 4 (right) © The American Association for the Advancement of Science 1998.

4.4 **The Permeability Transition Pore.**

In regulating apoptosis, Bcl-2's association with the mitochondrial membrane where it is in contact with the inner membrane (Zamzami, 1998), and the protein's pore-forming potential cannot be overlooked. In both B- and T-cell subsets, the expression of Bcl-2 correlates stoichiometrically with the peripheral benzodiazipine receptor (PBR). The location of the PBR in the outer mitochondrial membrane and its putative role, along with other molecules such as porin, the adenine nucleotide transporter and the voltage-dependent anion channel, in forming the PT pore, suggests that Bcl-2 may associate with the PBR or an PBR-associated protein. Single-channel measurements of excised mitochondrial patches and reconstituted purified ADP/ATP carrier (AAC), have suggested that when converted into a large unselective channel, the AAC is a key component of the PT pore. This channel is low cation selective, is pH sensitive, closing completely at pH 5.2, and its conductance has multiple sublevels varying from 300-600 pS (Brustovetsky, 1996; Carayon, 1996; Green, 1998). A number of studies suggest that Bcl-x_L, Bcl-2 and Bax are capable of forming pores in lipid vesicles and planar lipid bilayers in experimental conditions, supporting the view that these proteins may function by forming/inhibiting pore formation in the intracellular membranes, particularly the mitochondrial membrane.

The ability of Bcl-x_L lacking a TM domain (ΔTM) to form pores in KCl-loaded synthetic lipid vesicles is dependent upon vesicles consisting of at least 10% negatively charged lipids, implying that the ability of Bcl-x_LΔTM to permeabilise the vesicles relies on electrostatic interactions between it and membrane lipids. Channels formed by Bcl-x_LΔTM in planar lipid bilayers are pH-sensitive and formed at pHs as low as 4.0. These channels display multiple discrete conductance states ranging from 80 - 301 pS depending on the electrolyte, or mixtures of electrolytes used. The predominant conductance state of 276 pS is induced by pH 7.2, with a 150:15 mM cis:trans KCl gradient with Bcl-x_LΔTM added to the cis side. The channels exhibited identical ion selectivity, but become cation-selective at physiological pH when the ion selectivity is K^+ = $Na^+ > Ca^{2+} > Cl^-$ (Minn, 1997).

Bcl-2ΔTM, as might be expected from the structural similarities between it and Bcl-x_L, also forms conductance channels in lipid vesicles and planar lipid bilayers, having a similar requirement for acidic lipid membranes and low pH. The most frequent conductance observed using efflux of K^+ or Cl^- was 18±2 pS in 0.5 M KCl at pH 7.4.

On the basis of synthetic amphipathic helices that assemble into tetrameric four-helix bundles in membranes and create a channel with a conductance of ~20 pS, this may result from a four-helix channel formed by a Bcl-2 dimer. Larger individual channel conductances of ~40 pS and ~90 pS were detected with progressively lesser frequency, suggesting that channels of progressively higher conductances may be formed by Bcl-2 oligomers of progressively higher numbers of Bcl-2 monomers. The essential nature of Bcl-2's hydrophobic α-helices 5-6 for channel formation was confirmed by a Bcl-2ΔTM mutant lacking them, which failed to form conductance channels (Schendel, 1997).

Although both BaxΔTM and Bcl-2ΔTM can induce dye efflux from carboxy-fluorescein-loaded liposomes, they do not have the same effect on cellular integrity, and in contrast to Bcl-2ΔTM, Bax induces lysis in sympathetic neurons and sheep erythrocytes at concentrations between 5-10 μM at pH 7.4. This may be caused by conductance channel formation in the cells' membranes as indicated by these protein's release of dye from carboxyfluorescein-loaded liposomes, with the different effects explained by the two proteins pH operating ranges. At pH 7.0, Bax ΔTM induces the release of carboxy-fluorescein from liposomes in a concentration-dependent manner with maximal effect at 120 nM. In contrast Bcl-2 failed to trigger carboxyfluorescein efflux in identical conditions at concentrations up to 360 nM. In common with Bcl-x_L, release of carboxyfluorescein triggered by Bax is pH-dependent and 8 times greater at pH 4.0 than at pH 7.5. Although Bcl-2 was as efficient at initiating carboxyfluorescein release as Bax at pH 4.0, its optimum pH range is different, with its channel-forming properties diminished at pH 5.0 and absent at pH 6.0. In keeping with its effect in protecting cells from apoptosis, Bcl-2ΔTM antagonises BaxΔTM-induced release of carboxyfluorescein from liposomes at physiological pH. At a Bax:Bcl-2 ratio of 1:1 the efflux was reduced by 50% and at 1:10 almost completely inhibited, in contrast to the effect of Bax alone. Conductance channels formed in planar lipid bilayers by BaxΔTM are both voltage- and pH-dependent, with conductances at pH 7.0 ranging from 5.6±0.2 pS to 2 nS, but with conductances of 26 pS, 80 pS, 180 pS also detected. The progression to conductances of 2 nS was in multiples of ~450 pS and was particularly evident with the passage of time and at higher voltages. At pH 4.0 the Bax channel-forming activity was characterised by shorter-lived conductances about threefold lower than at pH 7.0 (Antonsson, 1997).

Comparison of the ion channel characteristics of Bax and Bcl-2 using KCl-loaded lipid vesicles, insertion of Bcl-2 and Bax into planar lipid bilayers, and the fusion of Bax- and Bcl-2-containing vesicles with planar lipid bilayers produced results both confirming and extending similar studies.

As in the study by Antonsson, both BaxΔTM and Bcl-2ΔTM were found to release ions from liposomes in a pH-dependent manner, but with Bax functioning within a broader pH spectrum than Bcl-2. This phenomenon may be explained by the higher Bax α5-helix pI of 10.64, in comparison to 4.55 for the equivalent Bcl-2 helix. Like Bcl-x_L, channels formed by Bcl-2 exhibited a mild cation selectivity in contrast to Bax which displayed a constant anion selectivity. In planar lipid bilayers at low pH, the initial conductances detected for Bax- and Bcl-2-formed channels were 22 and 80 pS respectively. At pH 4.0 the Bax channel progressed from the initial small Cl^--selective channel with a conductance of 22 pS, through a transition phase with multiple conductance levels and moderate Cl^--selectivity, and finally to an apparently stable channel of 0.731 nS that is continuously open and only mildly Cl^--selective. In contrast, at pH 4.0 the Bcl-2-formed pore was K^+-selective and progressed from a small channel with a conductance of ~80 pS that opened and closed spontaneously, to a large open stable pore with a conductance of ~1.90 nS. Of note and difficult to reconcile with the observations by Antonsson *et. al.*, shifting the pH from 4.0 to 7.0 in planar lipid bilayers alters the conductance and selectivity of Bax, but leaves Bcl-2 channels virtually unchanged (Schlesinger, 1997).

Whilst the conditions in vesicles and planar lipid bilayers may bear very little similarity to the conditions prevailing in the mitochondrial membranes at the contact sites between the inner and outer membranes where Bcl-2-family proteins are known to insert, the ability of Bcl-2, Bcl-x_L, and Bax to form conductance channels in these synthetic membranes offers the opportunity of speculating about the manner in which the Bcl-2 family may adjust the apoptotic threshold of the cell. In this respect it is particularly noteworthy that the ability of Bcl-2, Bcl-x_L, and Bax to form conductance channels in synthetic membranes is linked to pH and voltage, that the channels they form are of different and varying conductance states, that they display a degree of ion-selectivity, that the α-helices 5 and 6 in Bcl-2 (and by inference their equivalents in the other family members that possess them) are particular important to pore forming function, that there is some suggestion of conductance being linked to oligomerisation of these proteins, and that Bcl-2 appears to be capable of inhibiting the channel-forming activity of Bax in lipid vesicles.

4.5 Apoptosis execution.

Caspases are the protease executioners of apoptosis in the largely common degradation phase and are synthesised as precursors with little, if any, catalytic activity.

They are usually converted to the active enzyme by autocatalysis or by another protease, e.g. caspase-9. Their specificity is conferred by an absolute requirement for cleavage after aspartic acid and recognition of at least four amino acids NH_2-terminal to the cleavage site, which vary from caspase to caspase. Caspases function by cutting off the cell's contacts with adjacent cells, disassembling cell structures, inactivating inhibitors of apoptosis, cleaving proteins involved in cytoskeleton regulation, inactivating or deregulating proteins involved in the repair and replication of DNA, destroying DNA, disrupting the nuclear structure, prompting the cell to display signals that single it out for phagocytosis, and causing the cell to disintegrate into apoptotic bodies (Thornberry, 1998).

The three caspases which appear to initiate the execution phase of apoptosis are caspase-3, -8 and -9, but caspases-2 and -10 may have both initiator and effector potential. Caspase-3, -6, and -7 are activated by caspase-9 and involved in the down-stream cascade which effects apoptosis. Caspase-9 has been shown to be involved in initiating the caspase cascade in cells treated with cytotoxic agents, whilst caspase-8 is particularly associated with CD95-induced apoptosis and activation may involve recruitment to the CD95(Fas) receptor complex. In contrast to the requirement of the apoptosome, consisting of Apaf-1, cyt c and deoxyadenosine triphosphate, for caspase-9 activation, which was described in an earlier paragraph, activation of procaspase-8 requires association with its cofactor FADD (Fas-associated protein with death domain aka MORT1) via its death effector domain (Boldin, 1996; Deveraux, 1998; Muzio, 1996; Thornberry, 1998)

The targets for caspases include Bcl-2 which is cleaved at Asp34 by caspase-3 in *in vitro* apoptosis induced by CD95 ligation and IL-3 withdrawal. The carboxyl-terminal Bcl-2 cleavage product is itself apoptosis-inducing and accelerates Sindbis-virus-induced apoptosis. A similar mechanism is found in *C. elegans*, where the Ced-3 caspase cleaves the *C. elegans* equivalent of Bcl-2, Ced-9, at two separate sites, one of which is important to Ced-9's anti-apoptotic function (Cheng, 1997b; Xue, 1997). Other targets for caspases include the nuclear lamina which is involved in chromatin organisation and which consist of head-to-tail polymers of lamins. During apoptosis, these are cleaved at a single site, causing them to collapse and contributing to chromatin condensation (Orth, 1996; Takahashi, 1996).

Proteins involved in cytoskeleton regulation are also targets for apoptotic caspases and include gelsolin, focal adhesion kinase and p21-activated kinase 2.

Gelsolin, a protein that severs actin filaments in a regulated manner, is changed to a constitutively active state by caspase cleavage. Focal adhesion kinase is involved in the suppression of apoptosis in diverse cells types and is activated by interactions between integrins and the extracellular matrix. However, focal adhesion kinase appears to be cleaved early in apoptosis and may contribute to the cell's apoptotic disintegration. Similarly the caspase-mediated proteolytic cleavage of p21-activated kinase 2, generating a constitutively active protein fragment, may contribute to membrane and morphological changes observed in apoptotic Jurkat T-cells (Kothakota, 1997; Rudel, 1997; Wen, 1997).

Additionally, caspases attack the viability of the cell by inactivating proteins involved in maintaining the integrity of DNA/RNA such as DNA-PK_{cs} which is involved in DNA repair, U1-70K, which is involved in mRNA splicing, and replication factor C, which is involved in DNA replication. Precisely how these fit into the larger apoptotic picture is not yet understood, but clearly anything which makes a cell less able to resist the apoptotic process serves a purpose in these circumstances (Cryns, 1998; Rheaume, 1997).

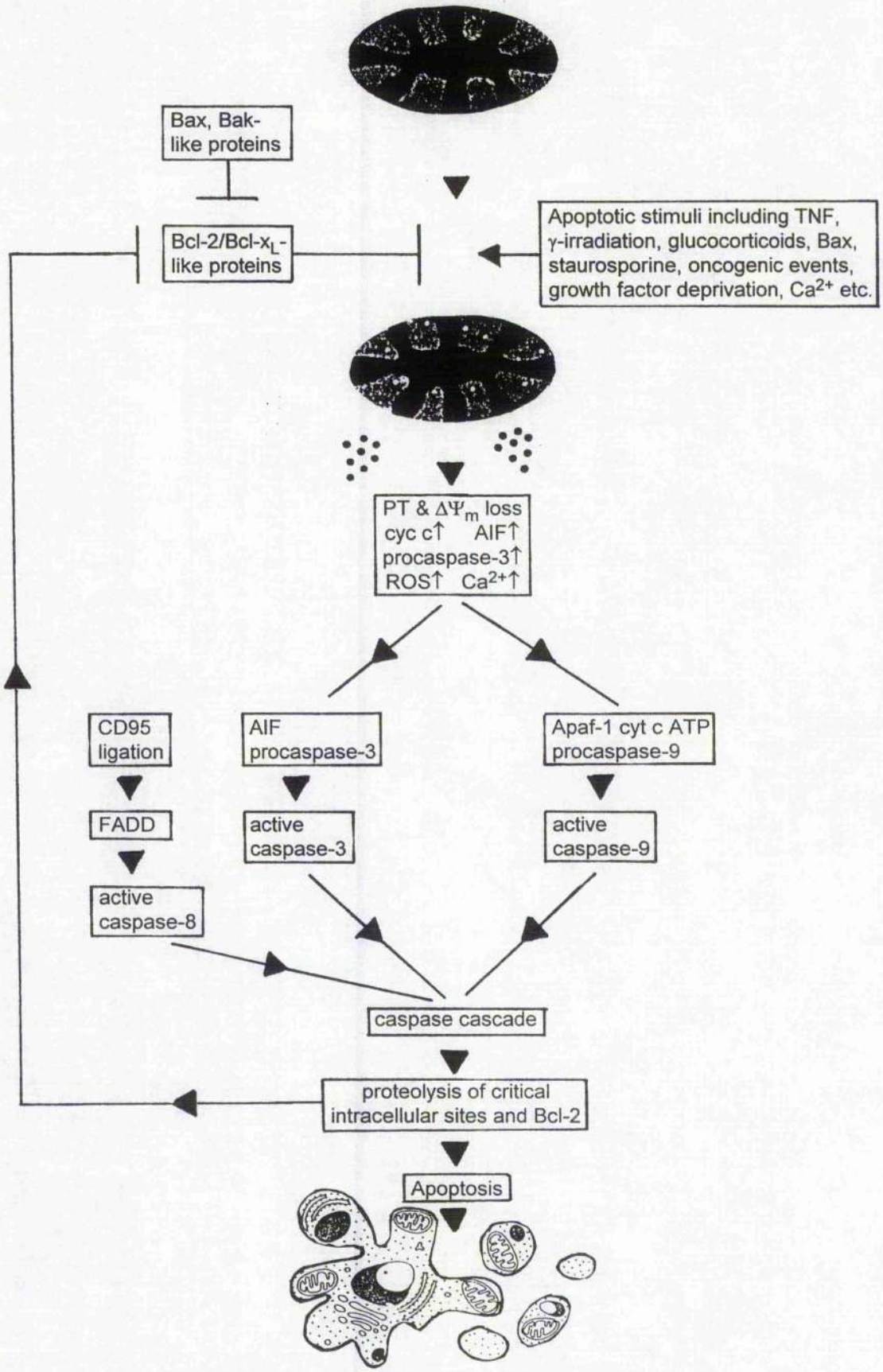


Figure 11. A generalised view of the apoptotic process.

5 The *bcl-2* family of genes/proteins.

5.1 Introduction.

bcl-2 (B-cell lymphoma/leukaemia 2), the founding member of the *bcl-2* family of genes/proteins, is a proto-oncogene which was first identified in 1984 in follicular lymphoma cells using a probe obtained from an acute B-cell leukaemia cell line. The *bcl-2* gene is normally located at band q21.3 of chromosome 18, but a high proportion of cases of follicular lymphoma show a t(14;18)(q32;q21) translocation which juxtaposes the intact *bcl-2* gene with an enhancer region in the Ig heavy chain locus on chromosome 14, leading to the constitutive expression of the protein (Bakshi, 1985; Cleary, 1985; Pegoraro, 1984; Tsujimoto, 1984, 1985). The t(14;18) translocation is generally reported as being present in only ~10% of B-CLL cases examined using Southern blotting analysis (Merup, 1996; Schena, 1992), but more recent research using the fluorescence in situ hybridisation (FISH) technique, which does not rely on the production of metaphases, has suggested that the percentage of translocations may be higher than reported, as it appears to detect them in B-CLL cells where Southern blotting does not (Amiel, 1994; Lishner, 1995).

The human *bcl-2* gene at 18q21.3 is an extremely large one due to a 225 kb intron and contains three exons (Cory, 1995; Silverman, 1990). Termination within the large intron results in an alternative transcript for *bcl-2*β, which is identical to the predominant form, *bcl-2*α, except at the carboxy terminus. The predominant form, normally referred to without the suffix as *bcl-2*, encodes a protein of 239 amino acids with a molecular weight of 26 kDa (Tsujimoto, 1986). This has been extensively implicated in the suppression of apoptosis in B-CLL over the past 10^{+} years, although it is increasingly clear that apoptosis-resistance is not as simply related to cellular concentrations of Bcl-2 as suggested by earlier *in vitro* studies (Panayiotidis, 1993, 1994) and that Bax may play a pivotal role along with other family members such as Mcl-1 (Kitada, 1998; Pepper, 1996, 1998; Thomas, 1996).

X-ray and NMR analysis of the structure of Bcl-X_L suggests that Bcl-X_L and Bcl-2 in particular are composed of two central, primarily hydrophobic α-helices, surrounded by 5 amphipathic α-helices as illustrated in Figures 12 & 13. The arrangement of the α-helices is reminiscent of the membrane translocation domain of bacterial toxins such as diphtheria toxin and the colicins.

Amino Acid Structure of Bcl-2 and Bcl-x_L Showing Homology & α-Helix Structure

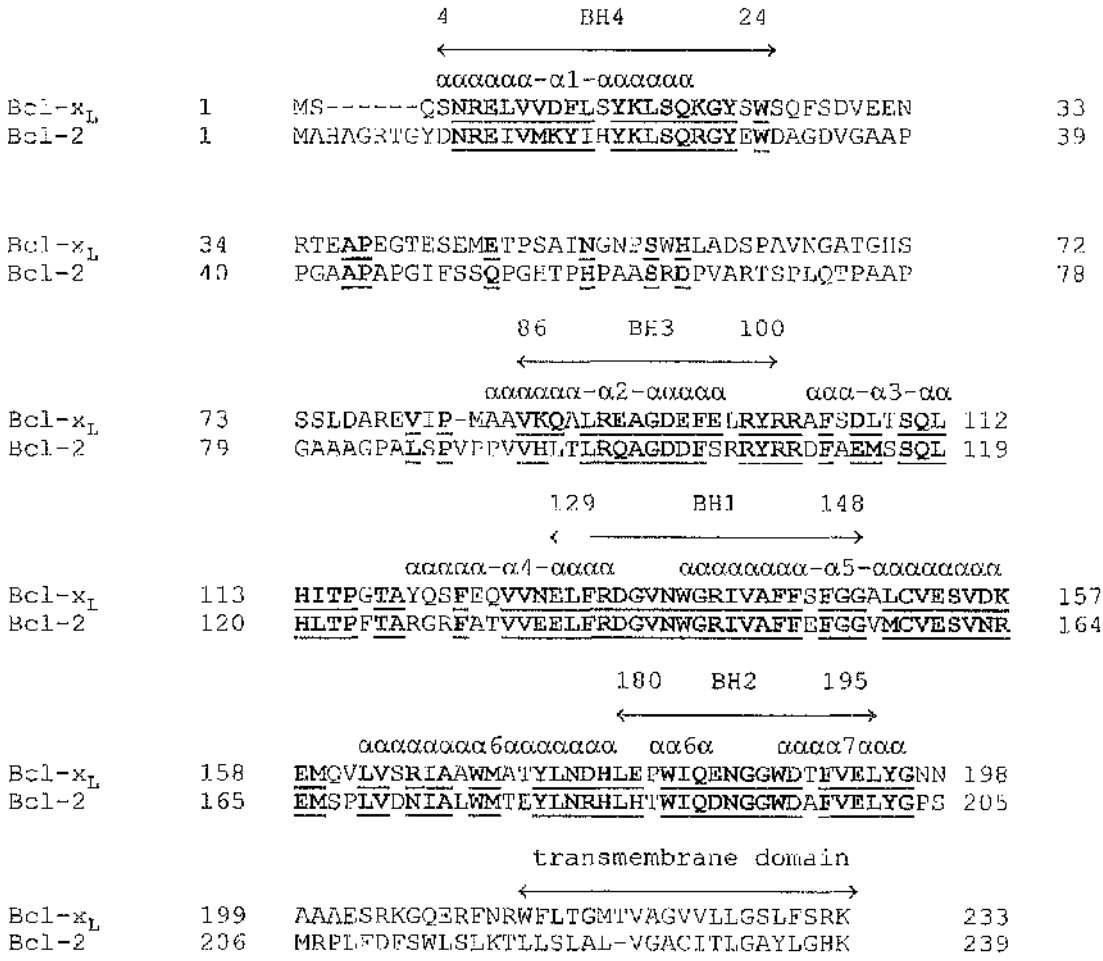


Figure 12. Illustration of the homology between Bcl-x_L and Bcl-2 relative to the Bcl-2 homology domains, transmembrane domain and α–helices (Muchmore, 1996), who consider L, I, F, V, M, and Y to be similar, N, D, K, R, Q, E, and H to be similar, with G, A, P, T, S, C, and W having no equivalents. αnα indicates numbered Bcl-x_L α-helices, ⟷ indicates the labelled Bcl-2 homology domain where the numbers refer to Bcl-x_L amino acid residues. Identical or similar amino acid residues are emboldened and underlined.

If function follows form then Bcl-X_L and Bcl-2 may act through pore forming/inhibiting activity and their location in the outer membrane of mitochondria, at the point where the outer and inner membranes are in contact, is certainly in keeping with such a theory (de Jong, 1994; Krajewski, 1993; Muchmore, 1996; Parker, 1993; Reed, 1997).

There appears to be no universal acceptance of what constitutes a member of the *bcl-2* family of genes/proteins. In the classic sense the family is defined by sequence homology in the *bcl-2* homology region 1 (BH1) and BH2 domains (Boyd, 1995), but some groups define the Bcl-2 family by a requirement for 30% homology in the BH domains (Akahani, 1997). However, as the two further *bcl-2* homology regions, BH3 and BH4, were discovered, the term *bcl-2* family has been widened to apply to those genes/proteins which show sequence homology in the BH1, BH2, BH3, or BH4 regions and in the Bcl-2 COOH-terminus membrane-spanning signal-anchor sequence. It is this latter wider definition that I have adopted. The locations of the Bcl-2 homology domains (Reed, 1997) are illustrated in Figure 13 and in Figures 14 & 15 following the descriptions of individual members. The *bcl-2* homology domains have previously been referred to as BDs (*bcl-2 domains*) or S homology regions, where BDA and S1 refer to BH4 (aka A-box), BDB or S2 refer to BH1 and BDC or S3 refer to BH2 (Cory, 1995; Hanada, 1995; Wang, H-G. 1996).

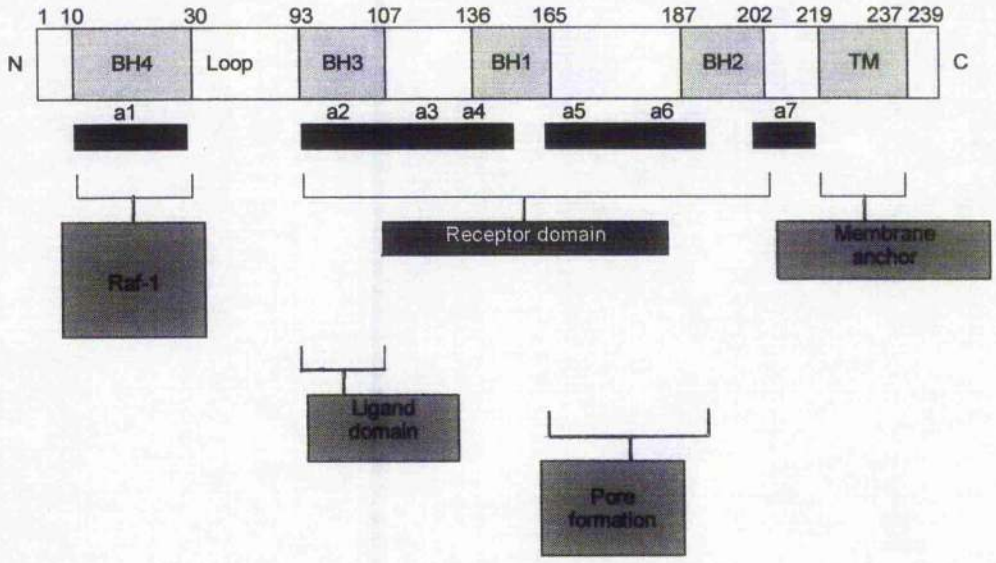


Figure 13. The structure of the human Bcl-2 protein. Adapted by permission from J.C. Reed, Double identity for proteins of the Bcl-2 family, Nature 387 – 775, © MacMillan Magazines Ltd. 1997.

The *bcl-2* family of genes is a highly conserved one as indicated by the range of organisms in which they are found and the degree of homology between species. The Bcl-2 family of proteins consist of two subgroups, one of which is involved in promoting cell survival and the other in promoting cell death. In the all-encompassing sense described above, the cell survival-promoting members include Bcl-2, Bcl-x_L, Bcl-w, Ced-9, Bhrf-1, Bfl-1, Grs, Al, Mcl-1, Brag-1, Orf16, Ksbcl-2, Gal-3, χr-1, χr-11, and Nr-13. The cell-death promoting members include Bcl-x_S, Bok, Bad, Bak, Bax, Bik/Nbk, Bim_{EL}, Hrk, Bar, and Bid. The homology of the Bcl-2 family is illustrated in Figures 14 and 15, following a brief description of the individual family members.

5.2 Bcl-2 family members.

The human form of **Bcl-x** was originally isolated using avian Bcl-x which had been isolated from chicken lymphoid cells using a murine *bcl-2* cDNA probe. The *bcl-x* gene is situated on chromosome 20 (Gibson, 1996) and contains an open reading frame (ORF) encoding a protein of 233 amino acid residues. This is designated Bcl-x_L(large), has BH1-4 and transmembrane (TM) domains similar to Bcl-2, and a similar anti-apoptotic function. Alternative splicing gives rise to the apoptosis-inducing Bcl-x_S (small). This 170 amino acid protein differs from Bcl-x_L by not possessing the 63 Bcl-x_L amino acids from 133-195 inclusive. Bcl-x_S does not contain the Bcl-2 region of highest homology, containing BH1 and BH2, but does contain BH3, BH4 and the TM region (Muchmore, 1996). Bcl-x_S, unlike Bcl-x_L, is apoptosis-inducing. The highest levels of Bcl-x are found in the lymphoid and central nervous systems (Boise, 1993).

Bcl-w is a family member with particular sequence homology to Bcl-2 and Bcl-x_L, which has been isolated from both human and murine tissue and similarly can protect cells from apoptosis-inducing IL-3 withdrawal, γ-irradiation and dexamethasone, but not from ligation of CD95 (APO-1/Fas) with MoAb. The locus of *bcl-w* is in the central region of mouse chromosome 14 and at 14q11.2 in humans. It encodes a protein of 193 amino acid residues with a molecular mass of ~22 kDa. The protein shows clear sequence homology in all four domains and possesses a C-terminus TM domain. The authors have noted conserved sequences in the TM domain of Bcl-w, which has a similar cytoplasmic distribution to Bcl-2, and suggested that these may associate with a specific protein in the membranes in which they are found (Gibson, 1996).

ced-9 (cell death defective-9) is a nematode gene that was first isolated in *C. elegans* and maps to a bicistronic locus on the right arm of chromosome III.

It was isolated as both a bicistronic 2.1 kb and a 1.3 kb *ced-9* mRNA, the bicistronic mRNA also encoding the *cyt-1* message which produces a protein similar to the cytochrome b_{560} of complex II of the mitochondrial respiratory chain. Ced-9 consists of 280 amino acid residues with a predicted molecular weight of 32 kDa. Ced-9 is homologous to Bcl-2 with which it shows 23% sequence homology. It possesses a hydrophobic TM domain, but shows less homology in BH3 than in BH1, BH2 and BH4. Over expression of Ced-9 prevents the cell deaths that occur by apoptosis in *C. elegans* during normal development, a phenomenon that can also be produced by substituting Bcl-2 overexpression (Hengartner, 1994).

mcl-1 is an early induction gene that was isolated from the human ML-1 myeloid leukaemia cell line and which parallels the action of Bcl-2 in promoting cell viability. The isolated clones encoded a 350 amino acid residue protein with a predicted molecular mass of 37.3 kDa. Mcl-1 shows considerable sequence homology to other members of the family in all four BH domains plus a C-terminus TM domain (Kozopas, 1993; Muchmore, 1996).

a1 is an early-response gene that is transiently and rapidly induced in mouse BM by GM-CSF and lipopolysaccharide (LPS). A1 expression is apparently limited to haematopoietic cells including T_H-lymphocytes, macrophages and neutrophils, but was not found in the B-lymphoid, erythroid or nonhaematopoietic cell lines examined. The cloned gene of 729 bp with a terminal polyA sequence has an ORF that encodes a potential protein of 172 amino acid residues with an expected M_r of 20,024 kDa. There is considerable sequence homology in domains BH1-3, but no obvious C-terminal TM domain (Cory, 1995; Lin, E. Y. 1993).

Bhrf-1, a viral homologue of Bcl-2, is a component of the restricted early antigen complex of the EBV lytic cycle. The *bhrf-1* ORF is 675 bp in length and encodes a protein of 191 amino acid residues with a molecular mass of 17 kDa. The main sequence homology between Bhrf-1 and other family members is in the BH1 and BH2 regions and like others possessing these conserved sequences, the protein confers anti-apoptotic properties on cells in which it is expressed. Bhrf-1 also possesses the C-terminal TM domain, but the cellular distribution of the protein varies from that of Bcl-2. Although a significant proportion localises to mitochondria with weaker staining of the endoplasmic reticulum (ER), strong staining is also observed in the perinuclear region of the cell (Khanim, 1997).

orf16 (open reading frame 16) is a functional homologue of the *bcl-2* gene encoded by the Herpesvirus Saimiri, whose expression inhibits apoptosis. *orf16* encodes a protein of 160 amino acid residues which shares sequence homology with *bcl-2* family members in BH1 and BH2, displays poor conservation in BH3, but possesses a COOH-terminus TM domain. Overall amino acid sequence identity varies between 17-26% with other family members (Nava, 1997).

ksbcl-2 (Kaposi sarcoma *bcl-2*) is a gene encoded by the Kaposi sarcoma-associated Herpesvirus aka human Herpesvirus 8. The gene encodes a protein of 174 amino acid residues which is a homologue of Bcl-2, shows significant homology only in the BH1 and BH2 domains, and possesses a COOH-terminal TM domain. When transfected into cells Ksbcl-2 inhibits cell death at concentrations similar to Bcl-2 and Bcl-x_L, but shows greater amino acid identity to Bhrf-1 (19%) and Orf16 (20%), than to Bcl-2 (15%) or Bcl-x_L(17%) (Cheng, 1997a).

brag-1 (brain-related apoptosis gene) is a family member that was isolated from a human glioma. The gene is expressed as a 4.5 kb transcript in normal human brain tissue, but is expressed as a 1.8 kb message in the glioma suggesting it may have been rearranged. The 1.8 kb message carries an ORF of 861 bp potentially encoding a protein of 286 amino acid residues with a predicted molecular mass of 31 kDa. The protein shares significant sequence homology in BH1 and BH2 and reacts with a Bcl-2 monoclonal antibody (MoAb) (Das, 1996).

bfl-1 (*bcl-2* related gene expressed in foetal liver) was isolated from human foetal liver (22 weeks gestation). It displays a particular degree of homology to A1 and Grs in the BH1-2 domains and to a lesser degree in BH3 and BH4. The cDNA includes an open reading frame encoding a protein of 175 amino acid residues with an M_r of ~20,000. Although the COOH-terminus TM domain contains 3 charged residues in addition to the hydrophobic residues, Bfl-1 appears to localise to subcellular membrane components with a distribution similar to Bcl-2. Bfl-1 appears to be highly expressed in bone marrow, in the Raji and HL60 haematopoietic cell lines, and expression was found to be dramatically increased in the metastatic and tumourous tissue of six patients with stomach cancer (Choi, 1995; D'Sa-Eipper, 1996).

grs (Glasgow rearranged sequence) was first identified as a result of its rearrangement with the fibroblast growth factor 4 gene in a case of chronic myeloid leukaemia, but a cDNA clone was subsequently isolated from a human T-cell library.

The *grs* gene is located at 15q24-25 adjacent to the t(15;17) region of the translocation associated with acute promyelocytic leukaemia. The full-length cDNA contains a 528 bp coding region (103-630) encoding a 175 amino acid residue protein. Both the gene and the protein show a close similarity to *a1* and *bfl-1*. *grs* shows a 77% nucleotide sequence identity to the murine haematopoietic-specific *a1* and approximately 71% amino acid sequence identity to A1, suggesting, particularly as *grs* appears to be selectively expressed in adults in haematopoietic tissues, that it may be the human equivalent of *a1*. The amino acid residue sequence of Bfl-1 and Grs differ only at amino acids nos. 72 and 107, suggesting that they may be the same protein (Kenny, 1997)

galectin-3 (a β-galactoside-binding protein) is a member of the family of β-Galactoside-binding lectins that is upregulated in proliferating cells and which also appears to be a member of the *bcl-2* family of genes. The gene encodes an anti-apoptotic protein of 249 amino acid residues with a molecular mass of 31 kDa. The protein shows homology in BH1 including the NWGR motif and possesses a COOH-terminal carbohydrate-binding domain. Galectin-3 shares 28% identity and 48% similarity in protein sequence with Bcl-2, in comparison to Bhrf-1 which, respectively, demonstrates only 24% and 41%. Galectin-3 has been implicated in neck, thyroid, gastric and colon cancer (Akahani, 1997; Barondes, 1994; Yang, 1996.)

χrI and *χrII* are *bcl-2* family members that were isolated from a Xenopus laevis stage 28-30 embryonic head library, respectively as 2.0 and 1.1 kb cDNA clones, and comparison of the predicted amino acid residue sequences showed significant sequence homology in the BH1, BH2 and COOH-terminus TM domains. Despite widespread expression of *χrI* mRNA, its failure to translate *in vitro* casts doubt on its apoptosis-associated role, but the *χrII* ORF encodes a 204 amino acid residue protein of ~22.5 kDa and rat fibroblasts transfected with sense cDNA demonstrated significant resistance to apoptosis induced by staurosporine, cycloheximide, serum deprivation and deregulation of *c-myc* expression (Cruz-Reyes, 1995).

nr-13 (neurotina-13) is an member of the *bcl-2* family which is activated in avian (quail embryo) cells transformed by the Rous sarcoma virus. The *nr-13* gene carries an ORF of 531 bp encoding a protein of 177 amino acid residues with an estimated molecular mass of 18-19 kDa. This protein shares significant sequence homology with the Bcl-2 family in the BH1 and BH2 domains and is thought to have an apoptosis-inhibiting effect on cells expressing it.

Unlike other members of the Bcl-2 family, Nr-13 localises to the plasma membrane where the hydrophobic region from residues 159-177 may constitute a membrane-spanning domain and serve as a signal-anchor sequence. Nr-13 and chicken Bcl-2 share 25% identity and 47% conservation in the amino acid residue sequence, in comparison to Nr-13 and Bax, where the respective figures are 28% and 54% (Gillet, 1995).

bak (Bcl-2-homologous antagonist/killer) is a primarily pro-apoptotic gene that was found to be present in significant quantities as a 2.4 kb mRNA transcript in a wide range of human tissues. The ORF encodes a protein of 211 amino acids and a M_r of 23,400. The protein possesses a COOH-terminus TM domain and shows sequence homology in all four BH domains, especially in BH1 and BH2 where it shares 53% sequence identity with Bcl-2. The *bak* gene spans 6 kb, contains at least three exons and is located on chromosome 6. Copies of the gene also exist on chromosome 20 without introns and on chromosome 11 as a processed pseudogene (Kiefer, 1995).

bik/nbk (Bcl-2 interacting killer) is a pro-apoptotic gene that was isolated from a human B-cell library as a 956 bp cDNA and is expressed in adult tissues and established cell lines as a 1 kb mRNA. The gene encodes a 160 amino acid protein with a calculated molecular mass of 18 kDa, but *in vitro* translated Bik migrates at 26 kDa. Bik contains a COOH-terminus TM domain and shows clear sequence homology to other members of the family in BH3. The protein is localised around the nuclear envelope and cytoplasmic membrane structures with a similar distribution to Bcl-2, adenovirus E1b 19 kDa, Bhrf-1, and Nip1-3. It forms complexes with Bcl-2, Bcl-x_L, Bhrf-1, and adenovirus E1b 19 kDa (Boyd, 1995; Elangovan, 1997).

Bax (Bcl-2-associated X protein) was first identified in coimmunoprecipitation experiments using the human B-cell line RL-7, when it precipitated as a 21 kDa entity. Subsequent cloning and sequencing experiments identified an ORF of 576 bp encoding a 192 amino acid protein with a predicted molecular mass of 21.4 kDa. *bax* consists of 6 exons within a 4.5 kb region, all of which contribute to the amino acid sequence, but alternative splicing can produce a β species of 1.5 kb and 218 amino acids with a molecular mass of 24 kDa, or a γ species of 1.0 kb, 41 amino acids and 4.5 kDa. The most highly conserved areas between Bax and Bcl-2 are in BH1 and BH2 and the protein possesses the COOH-terminus TM domain. Bax accelerates cell death and counters the effect of Bcl-2 (Krajewski, 1994; Oltvai, 1993).

Bad (*bcl-2*-associated death promoter) was identified by screening a mouse embryo day 14.5 cDNA fusion library. The gene encodes a protein of 204 amino acids with a predicted molecular mass of 22.1 kDa. It displays limited homology in the BH1 and BH2 domains with other family members, but lacks a C-terminal TM domain. Bad selectively dimerises with Bcl-x_L and Bcl-2, but it binds to Bcl-x_L with greater affinity and, unlike its action with Bcl-2, opposes the apoptosis-inhibiting activity of Bcl-x_L (Yang, 1995).

bok (*bcl-2*- related ovarian killer) is a pro-apoptotic gene isolated initially from a rat ovarian fusion cDNA library, although high levels of a ~1.5 kb transcript were found in the ovary, testis, uterus and the granulosa cells of ovarian follicles that undergo apoptosis during follicle degeneration. The *bok* ORF encodes a protein with 213 amino acid residues and a predicted molecular mass of 23.5 kDa, which shows extensive sequence homology to other *bcl-2* family members in the BH1-3 domains, possesses a COOH-terminus TM domain, but lacks the BH4 domain. In its homology it is particularly similar to Bax and Bak (Hsu, 1997).

bim was originally identified from a murine T-lymphoma cell line cDNA library as three isoforms designated bim_{EL}, bim_L, and bim_S. These appear to arise through alternative splicing and encode respectively, proteins of 196, 140 and 110 amino acid residues. The human equivalents of bim_{EL} and bim_L encode proteins of 198 and 138 amino acid residues which are respectively 89 and 85 % identical to their murine counterparts. The human and murine forms of Bim have sequence homology in the BH3 region, through which they appear to mediate their apoptosis-inducing function, and all possess a C-terminus TM domain. The Bim protein localises independently to intracytoplasmic membranes similar to the distribution reported for Bcl-2. In its role as an apoptosis-inducing protein Bim_S antagonises Bcl-2 more effectively than Bim_L, with Bim_{EL} the least effective (O'Connor, 1998).

Bid (BH3 interacting domain death agonist) was originally isolated from an expression library constructed from the murine T-cell hybridoma line, 2B4, using both *bcl-2* and *bax* probes, and only subsequently from human tissue. Both murine and human genes encode 195 amino acid residue proteins with predicted molecular weights of 21.95 kDa, which show extensive sequence conservation in the BH3 domain only. The protein also lacks the membrane-spanning TM domain. The protein interacts with Bcl-2, Bcl-x_L, and Bax and promotes cell death (Wang, K. 1996).

hrk (*harikiri*) is a cell death-inducing gene that was initially identified by screening a HeLa cDNA library and a 9-week old human embryo cDNA library. It is expressed as a 0.7 kb transcript encoding a 91 amino acid protein in adult human BM, spleen and pancreas. The Hrk protein shows homology to BH3 only in amino acids 37-44, but a region of 16 amino acid residues including the BH3 domain is essential for its death-inducing function. Hrk has a region of 28 hydrophobic residues at its COOH-terminus that may act as a membrane-spanning domain and shows a granular and extranuclear distribution pattern consistent with being confined to membranes of intracellular organelles similar to Bcl-2 and Bcl-x_L (Inohara, 1997).

Although not a member of the Bcl-2 protein family, **Bag-1** (Bcl-2-associated athanogene 1) is worth inclusion because of its Bcl-2 binding capacity and its ability to enhance cell survival. The *bag-1* cDNA was isolated from a mouse embryo cDNA library and carries a 630 bp coding region which encodes a 219 amino acid residue protein. Bag-1's potential acidity, arising from multiple glutamic acid residues, may explain its migration at ~29-30 kDa as opposed to the predicted ~24.5 kDA. It has no sequence homology with Bcl-2 family members, but shows as much as 50% amino acid residue sequence identity in one region (residues (37-73) with several ubiquitin and ubiquitin-like proteins. 3TS fibroblasts and the human lymphoid cell line Jurkat showed increased resistance to induced apoptosis when transfected with Bag-1, but Jurkat in particular showed much greater resistance when transfected with both Bcl-2 and Bag-1 as opposed to when transfected with either of these proteins alone (Takayama, 1995).

Amino Acid Sequence Homology in the Region of the BH3 & BH4 Domains of the Bcl-2 Family of Proteins.

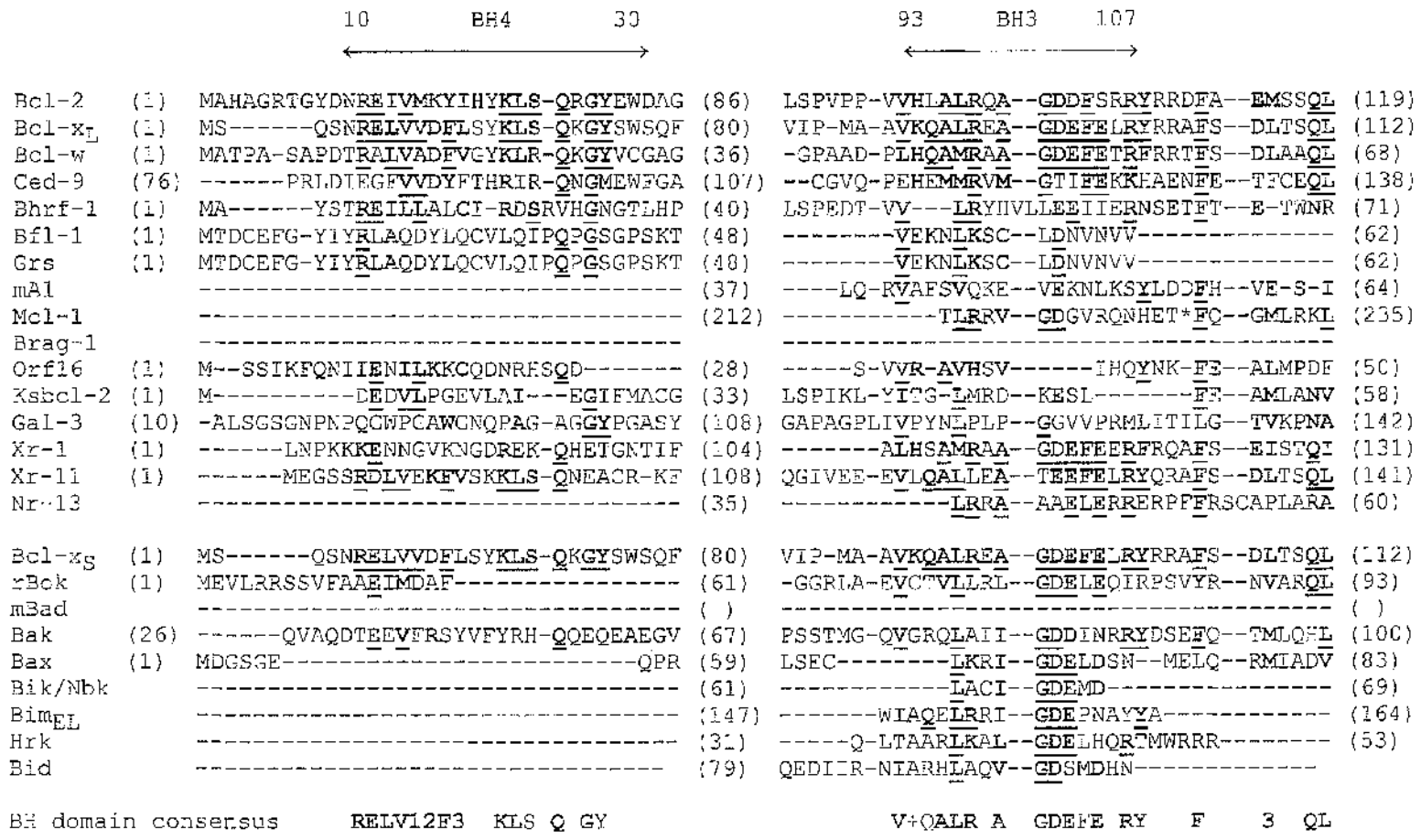


Figure 14. Sequence homology in members of the Bcl-2 family in the region of the BH3 and BH4 domains. BH domain residue numbers relate to Bcl-2 (Reed, 1997). Sequence identity is indicated by emboldening and underling, similarity is indicated by emboldening alone and homology of ≥ 50% is shown by emboldening the consensus sequence. Similarity is (with noted exceptions) defined by group; large aromatics tryptophan (W), tyrosine (Y), phenylalanine (F); hydrophobics leucine (L), valine (V), isoleucine (I), methionine (M), cysteine (C), alanine (A); hydrophilics asparagine (N), glutamine (Q); negatives glutamic acid (E), aspartic acid (D); and positives lysine (K), histidine (H), arginine (R). Helix breakers and bend inducers proline (P), glycine (G), serine (S) and threonine (T) are (with one noted exception) considered to have no equivalents. Unless otherwise stated the protein sequences listed are human. Key; mouse A1 (mA1), harikiri (Hrk), Galectin-3 (Gal-3), mouse Bad (mBad), rat Bok (rBok), 1 = V or L, 2 = ÷ or -, 3 = hydrophobic, 4 = polar (S, T, N, Q, D, E, R, H & K).

Amino Acid Sequence Homology in the region of the BH1 & BH-2 domains and Transmembrane protein sequences of Bcl-2 Family Members.

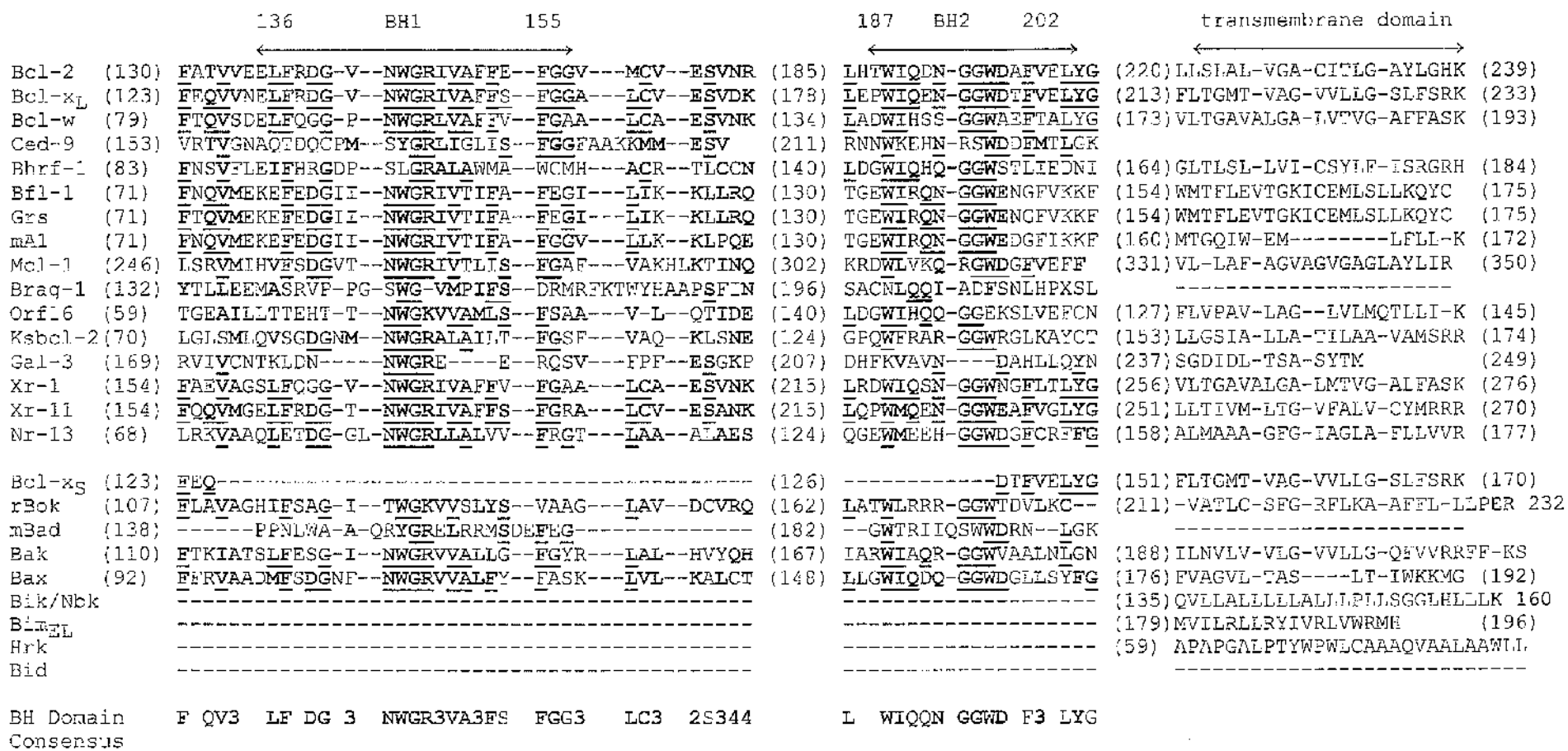


Figure 15. Sequence homology in members of the Bcl-2 family in the region of the BH1 and BH2 domains. BH domain residue numbers relate to Bcl-2 (Reed, 1997). Sequence identity is indicated by emboldening and underling, similarity is indicated by emboldening alone and homology of ≥ 50% is shown by emboldening the consensus sequence. Similarity is (with noted exceptions) defined by group; large aromatics W, Y, F; hydrophobics L, V, I, M, C, A; hydrophilics N, Q; negatives E, D; and positives K, H, R. Helix breakers and bend inducers P, G, S and T are (with one noted exception) considered to have no equivalents. Unless otherwise stated the protein sequences listed are human. Key; mouse A1 (mA1), harikiri (Hrk), Galectin-3 (Gal-3), mouse Bad (mBad), rat Bok (rBok), 1 = V or L, 2 = + or -, 3 = hydrophobic, 4 = polar (S, T, N, Q, D, E, R, H & K).

6 Bcl-2 Homology domains.

6.1 Introduction.

Any attempt to show the extent of sequence conservation amongst the members of the Bcl-2 family of proteins, as illustrated in Figures 14 - 15, inevitably involves a number of arbitrary choices, e.g. the number and nature of the family members to be included in the calculations, the extent to which sequence conservation in the BH1, BH2, and BH4 domains of anti-apoptotic members, where they appear to have greater significance, is allowed to influence the view of whole family-sequence conservation, the extent to which sequence conservation in the BH3 domain of pro-apoptotic family members is similarly allowed to influence the view of whole family-homology, and whether the same number of relatively rare residues, e.g. C, G, Q, S & Y (for amino acid key please see Figure 14), is required at a particular position before sequence conservation is assumed to have been preserved, as would be required for more commonly encountered residues, e.g. A, E & L.

The purpose of Figures 14 and 15 is to give an accurate impression of sequence conservation rather than an absolute picture and to this end a relatively strict convention of amino acid similarity has been applied. However, as the object of the exercise is to highlight areas of conservation that may be important, some flexibility and overlap has been allowed. Where it appears that a particular residue is conserved as a charged residue, regardless of the type of charge, overlap has been allowed between negative and positive groups and the homology is indicated as being "similar". Similarly, where there is no clear conservation of a particular residue, but the residues are largely hydrophobic, the residues in that position are also shown as being "similar". Where the conserved residue is a relatively rarely encountered type, a smaller number of residues would generally be taken to indicate conservation, than would be the case for more common residues. No weighting is given to the contribution made to the homology of particular BH domains by either anti- or pro-apoptotic family members, except for BH4, where conservation is determined solely on the basis of the contribution made by anti-apoptotic family members. On the basis that its sequence is a duplicate of part of Bcl-x_L, any contribution to homology from Bcl-x_S is ignored, but its sequence is included for completeness.

The analysis of conserved Bcl-2 sequence homology represented in Figures 14 and 15 reveals a number of potentially significant features of conservation, which include:

Conservation of sequence in the BH1-3 domains extends beyond the currently defined boundaries of these domains, particularly in the case of BH1. Conservation in the region of BH1 extends from Bcl-2 residues 130 to 164, conservation in the BH2 region extends from 185 to 203 and for BH3 the relevant residues extend from 93 to 109. Conversely when BH4 sequence conservation is analysed on the basis of the anti-apoptotic family members listed, conservation appears to encompass only the Bcl-2 residues from 12 to 28.

BH1 and BH2 are present and extensively conserved in all of the anti- and a subset of pro-apoptotic family members, but there is a second group of pro-apoptotic family members (Bik, Bim_{EL}, Hrk and Bid) which show no conservation in BH1-2.

BH3 is extensively conserved in both types of family members including those with no conservation in BH1-2, and is the only conserved BH domain in some family members.

Ignoring Bcl-x_S on the basis that its sequence is a duplicate of part of the Bcl-x_L sequence, BH4 is only significantly conserved in anti-apoptotic family members.

The specific features of sequence conservation which appear to be particularly noteworthy include;

- The highly conserved V at Bcl-2 position 133 near BH1 shows, on the basis of the family members listed, 81% sequence identity and 94% sequence similarity in anti-apoptotic proteins.

- Sequence homology near BH1 is especially conserved in the motifs F-DG, NWGR$_{hydrophobic}$V, FG, LC, and the hydrophobic-polar-polar group at Bcl-2 positions 162-164. These latter two highly conserved polar residues show 83% and 94% conservation, respectively.

- The extensively conserved BH2 motifs of WI followed by three positions with an exceptionally high number of the relatively rare hydrophilic Q and N residues and GGW.

- The conserved F at Bcl-2 position 198 in BH2, appears not to be conserved in pro-apoptotic family members.

- The most highly conserved motif in BH4 is the Q-G motif starting at Bcl-2 position 25.

- The most highly conserved residues in the region of BH3 are the V at Bcl-2 position 93, LR-AGDE from positions 97-103, RY at positions 107-8, F at position 112, the conserved hydrophobic residue at position 115 and the QL motif at positions 118-119.

If function follows form in the Bcl-2 family, then the clearly defined conserved sequence homology in the BH1-4 domains of all of the anti-apoptotic members of the Bcl-2 family, suggests that these domains play an important part in the apoptosis-inhibiting role of these proteins, as do the BH1-3 domains in the pro-apoptotic members. The absence of any significant conserved sequence homology in the BH4 domain of the pro-apoptotic proteins suggests that its function is restricted to apoptosis-inhibition. However, the absence of conservation in the BH1-2 domains of a group of pro-apoptotic Bcl-2 family members suggests that either these domains are not required for the pro-apoptotic function, or in apoptosis promotion there are at the very least, two variations on a theme.

Studies employing three different treeing methods, distance matrix, maximum likelihood and parsimony, suggest that the evolution of human Bax and Bcl-2 diverged a considerable time ago (Evans, 1995). As Bax is one of the pro-apoptotic proteins in which BH1 and BH2 are particularly well conserved and conservation is unlikely to occur unless it serves a useful purpose, this would suggest that BH1 and BH2 play significant roles in the function of Bax. However, given that other BH3-only members of the family can induce apoptosis it suggests quite clearly that there are two separate routes to do so. It is interesting to note that the GDE motif at residues 101-3 of BH3 in Bcl-2 is more highly conserved in pro-apoptotic family members, whilst the phenylalanine residue at position 112 is more highly conserved in anti-apoptotic members, raising the possibility that this variation has a relevance to the potential pro- and anti-apoptotic function of this domain.

A wide range of studies have sought to determine the functions/importance of BH domains and conserved residues with regard to apoptosis and interaction between Bcl-2 family members. Given the wide range and nature of the techniques used to investigate the properties and functions of the Bcl-2 family, the range of viral, mammalian, and recombinant proteins members used, many of which have been modified, particularly by removal of the TM domain, no definitive picture of the functioning of these domains in humans and particularly in B-CLL has emerged.

However, the role of these domains is becoming clearer and this is discussed by putative function rather than domain.

6.2 Homodimerisation.

Studies concerned with the structural requirements of homodimerisation in members of the Bcl-2 family of proteins have concentrated mainly on Bcl-2, Bax and to a much lesser degree, Bcl- x_L. These suggest that the structural requirements in terms of BH and TM domains varies significantly between Bcl-2/Bcl- x_L and Bax.

Use of Bcl-2 deletion mutants suggests that homodimerisation of the proteins involves two distinct regions. Bcl-2 deletion mutants consisting of residues 83-218, containing only BH1-3 and part of the TM domain, do not homodimerise with each other in a yeast two-hybrid system, but do homodimerise with another Bcl-2 mutant consisting of residues 1-81 which contains the BH4 domain. These results suggest that Bcl-2's BH4 and BH1-3 regions are involved in a complementary interaction in Bcl-2 homodimerisation. Serial deletions of BH1, BH2 and BH4 in a yeast two-hybrid assay confirm that these domains are not essential for homodimerisation with human Bcl-2, but that one Bcl-2 molecule must possess a functional BH4 domain and its partner, functional BH1 and BH2 domains (Sato, 1994). Additionally, experiments using human Bcl-2β (1-205), which does not homodimerise with some deletion mutants that homodimerise with Bcl-2 (1-218), suggests that sequences located between BH2 and the TM domain also appear to be important for Bcl-2 homodimerisation (Hanada, 1995). In keeping with the reported need for only one partner to have a functional BH1 domain for Bcl-2 homodimerisation, yeast two-hybrid studies indicate that the BH1 substitutions in Bcl-2, WGR144-6AAA, WGR144-6WAR and WGR144-6WER, do not interfere with homodimerisation of mutant Bcl-2 and wt Bcl-2. (Sedlak, 1995). Similarly the BH1 FRDG138-41AAAA and G145E substitution mutants, along with the BH2 W188A and QDN190-2LAA substitution mutants, are found to homodimerise as strongly with unmutated Bcl-2 (Yin, 1994).

The extent to which Bcl-x_L homodimerises is uncertain. In immunoprecipitation studies human Bcl-x_L did not homodimerise with haemagglutinin-tagged Bcl-x_L (Minn, 1996), and in a different series of similar studies a Bcl-x_L-derived BH3 peptide bound to Bcl-x_L with the lowest affinity of all of the BH3-derived peptides tested. I.e. a Bcl-x_L-derived BH3 peptide bound to Bcl-x_L with an affinity of 325 μM in contrast to one derived from Bak which showed an affinity of 0.20 μM in binding to Bcl-x_L (Sattler, 1997).

In contrast, human Bcl-x_L is reported to homodimerise in a yeast two-hybrid assay, although the same report reported Bax as not homodimerising in the same system, in contrast to the numerous reports indicating it homodimerises readily (Zhang, 1995). However, it is also reported that Bcl-x_L homodimerises in plate binding assays. The authors acknowledge that binding to plastic may change the conformation of the protein, but the doubts about the legitimacy of the homodimerisation process does not entirely negate the fact that the study indicated that a 16 amino acid peptide derived from residues 72-87 of the BH3 domain of Bak, GQVGRQLAIIGDDINR, and a 21 amino acid residue from residues 52-72 in BH3 of Bax, QDASTKKLSECLKRIGDELDS, are sufficient to inhibit the formation of both Bcl-2 and Bcl-x_L homodimers (Diaz, 1997).

The NWGR motif in BH1 of human Bcl-2 seems to have particular significance for homodimerisation. Bcl-2 pairs in which only one partner possesses a functional BH1 domain, cease to homodimerise when the NWGR motif is deleted. E.g. deletion of NWGR in Bcl-2 (83-218) abolishes homodimerisation activity with Bcl-2 (1-81) (Hanada, 1995).

In contrast to Bcl-2, homodimerisation in Bax appears to require a functional BH3 domain in one partner and BH1 and BH3 in the other. The sufficiency of the BH3 domain alone in one partner in Bax homodimerisation is quite well established. Yeast two-hybrid studies show that in human Bax a region of 41 amino acids from 47-87, encompassing the BH3 domain, is all that is required for Bax to interact with full length Bax. Furthermore, Bax truncations lacking the BH1-2 domains not only continue to interact with full length Bax, but do so more strongly, possibly reflecting the need for a conformational change in Bax that is necessary to effect homodimerisation of wild-type (wt) proteins (Simonen, 1997). Similarly, murine Bax proteins do not require the BH1, BH2, or the first 58 residues containing BH4 at the NH_2-terminus to form homodimers with wt murine Bax, and the region of Bax that is essential for homodimerisation mapped to the region containing BH3 from residues 59-101. Deletion of this region abolishes the ability of Bax to homodimerise with wt Bax (Zha, H. 1996). The importance of BH3 to Bax homodimerisation is also confirmed by immunoprecipitation studies which indicate that substitution of GD67-8AA or deletion of residues 63-71 in BH3, prevent homodimerisation with wt Bax and that BH1 and BH2 are unnecessary for homodimerisation with wt Bax. Substitution of G108A and deletion of residues 102-112 in BH1, as well as substitution of W158A and deletion of residues 151-159, in BH2, failed to prevent Bax homodimerisation, although deletion of BH2 residues 151-159 reduced the ability of the protein to homodimerise (Simonian, 1996b).

The BH domains needed to complement a BH3-possessing Bax monomer are not so obvious. Bax truncation mutants in a yeast two-hybrid assay show that the minimal requirements for human Bax homodimerisation to a partner with the BH3 domain alone is minimally the possession of the BH1 and BH3 domains (Simonen, 1997). These findings are directly contradicted by studies using murine Bax in another yeast two-hybrid system. These confirmed that BH1 and BH2 are not needed for homodimerisation between mutant and wt Bax, but also found that Bax mutants with deletions of BH1 or BH2 homodimerised successfully with themselves and each other, suggesting that BH1 and BH2 are unnecessary for Bax homodimerisation (Zha, H. 1996). Additionally, the need for BH3 in homodimerisation is supported by Bad which does not possess the BH3 domain and does not homodimerise (Zha, J. 1996). Of interest with respect to Bax homodimerisation in yeast two-hybrid studies is that although Bax heterodimerises with Bcl-2, the competing Bax homodimerisation reaction is stronger (Sedlak, 1995).

These results indicate that Bcl-2 requires BH1 and BH2 in one homodimerisation partner and BH4 in the other, and support the view that Bcl-2 homodimerises in a head-to-tail fashion. However, the results are not as certain with regard to BH3 being essential for Bcl-2 homodimerisation. The study by Sato (1994) indicates that the BH1-3 region is necessary as an entity for homodimerisation without offering any specific evidence that BH3 is involved. Similarly, the study by Diaz (1996) which suggests that Bak- and Bax-derived BH3 peptides can prevent homodimerisation and heterodimerisation does not offer proof that BH3 is involved in the homodimerisation process, or that homodimerisation and heterodimerisation, as Diaz suggests, involve a common binding site that mediates heterodimerisation and homodimerisation of Bcl-2 family members. These BH3 peptides may bind to the same site, but Bcl-2 homodimerisation could just as easily be prevented by steric hindrance or a conformational change in one Bcl-2 partner induced by binding the peptide. A study in the nature of Sato's, that deletes the BH3 domain in the partner that possesses the BH1-3 domains, would go some way to establishing the importance of BH3 to homodimerisation. The evidence for Bcl-x_L is tenuous to the point where it is not possible to draw any meaningful conclusions from it.

Whilst it is clear that BH3 is also essential in one partner in Bax homodimerisation, the requirements in the complementary partner are less clear and likely to remain so pending further studies.

6.3 Heterodimerisation.

Heterodimerisation occurs essentially between the pro- and anti-apoptotic members of the Bcl-2 family, although exceptions to this rule include heterodimerisation between Mcl-1 and Bcl-2 and Mcl-1 and Bcl-x_L (Sato, 1994). The process appears to involve the BH3 domain of the pro-apoptotic member binding to the BH1, BH2 and BH3 domains of the anti-apoptotic member. BH4 appears not to be required for heterodimerisation and deletion of BH4 in Bcl-2, either through deletion of residues 1-82 or deletion of residues 11-33, has no discernible effect on heterodimerisation between Bcl-2 and Bax *in vitro* (Hanada, 1995).

X-ray and nuclear magnetic resonance (NMR) analysis of the structure of Bcl-x_L, and by inference the similarly-sequenced Bcl-2, suggests that in the quaternary structure of the proteins, BH1, BH2 and BH3 are in close spatial proximity to each other forming a hydrophobic cleft that provides a binding site for other members of the Bcl-2 family (Muchmore, 1996). A 16 amino acid residue peptide derived from the BH3 region of Bak consisting of residues 72-87 is the minimal region required to bind to Bcl-x_L, and in solution this Bak peptide adopts an amphipathic α-helix form which binds to the cleft formed by the BH1-3 domains of Bcl-x_L. In this relationship specific amino acids appear to be important. Substitution of L78A, D83A, or R76A in the Bak peptide reduces binding to Bcl-x_L. D83 of Bak is thought to interact with R139 of Bcl-x_L and when the substitution R139Q is applied to Bcl-x_L, its binding to Bax is inhibited. Interestingly the Bak-derived peptide binds to Bcl-x_L with greater affinity than similar peptides derived from Bcl-2, Bax, Bik and Bcl-x_L, and in keeping with Bcl-x_L's reported failure to homodimerise, the Bcl-x_L-derived peptide bound to wt Bcl-x_L with the lowest affinity (Sattler, 1997). This relationship is also very much in keeping with sequence conservation amongst family members and is well supported by studies using a wide range of pro- and anti-apoptotic members, particularly the need for BH3 in the pro-apoptotic partner.

Peptides consisting of 16 and 21 amino acid residues derived, respectively, from residues 72-87 of the BH3 domain of Bak and positions 52-72 of BH3 in Bax, are sufficient to inhibit the formation of Bcl-2/Bax and Bcl-x_L/Bax heterodimers in plate binding assays, suggesting that this peptide occupies the site normally occupied by the pro-apoptotic partner (Diaz, 1997). Similarly, the limited sequence in Bax from residues 50-78 which encompasses BH3 is necessary and sufficient for heterodimerisation with E1B 19kDa (Han, J. 1995).

Bax truncations show that a region of 41 amino acids from 47-87, also encompassing the BH3 domain, is all that is required for Bax to interact with full length Bax, Bcl-2, and Bcl-x_L (Simonen, 1997), and a short sequence in Bad from 141-172 containing Bad's BH3 domain (151-9), is both sufficient and essential for heterodimerisation with Bcl-2 (Zha, 1997).

The importance of BH3 to heterodimerisation in pro-apoptotic Bcl-2 family members is supported by both deletions of BH3 and other Bcl-2 homology domains. Deletion of the BH3 domain in Bax and Bip1 indicate that it is critical for heterodimerising with both Bcl-2 and Bcl-x_L (Chittenden, 1995). For example, deletion of the sequence LRRIGELD, at residues 63-71 of Bax abolished Bax's ability to bind Bcl-x_L (Simonian, 1996a), deletion of the BH3 domain residues in Bik from 61-69 (LACIGDEMD) suppresses interactions between Bik and the apoptosis-inhibiting members of the family (Boyd, 1995), and deletion of residues 59-101 containing the BH3 region of Bax abolishes the protein's ability to heterodimerise with Bcl-2 (Zha, H. 1996). In contrast, deletion of other homology domains appears to have no impact on the ability of pro-apoptotic family members to heterodimerise and emphasises the singular importance of BH3 in this respect. Deletion of residues 102-112 in BH1 or deletion of residues 151-159 in BH2 in Bax did not prevent it from heterodimerising with Bcl-x_L (Simonian, 1996a). Likewise heterodimerisation between Bax and Bcl-2 occurs without the NH_2-terminus domain of Bax (Zha, H. 1996) and deletion of BH2 in Bad has virtually no effect on heterodimerisation with Bcl-2 and Bcl-x_L (Zha, 1997).

Particular residues in BH3 appear important for heterodimerisation and offer a possible explanation of the greater affinity found between some family members. Substitutions in BH3 of Bad indicate that the heterodimerisation process varies between Bcl-2 and Bcl-x_L. Bad L151A reduces binding to both Bcl-2 and Bcl-x_L by >90% and appears to be virtually unable to bind Bcl-x_L, in contrast Bad G148A reduced binding to Bcl-2 by ~50%, but had only a minimal effect on Bcl-x_L. Additionally, in contrast to the other Bak and Bax BH3-derived peptides described earlier which prevented hetero-dimerisation between Bcl-2/Bax and Bcl-x_L/Bax, a BH3 derived peptide containing a G67R substitution failed to prevent heterodimerisation between Bcl-2, Bcl-x_L and Bax, suggesting that this particular residue has some significance in this respect (Diaz, 1997).

In line with Muchmore's (1996) and Sattler's (1997) discoveries that the BH1-3 domains form a hydrophobic cleft in the tertiary structure of Bcl-x_L, BH1-3 appear to be of equal importance in the anti-apoptotic partner in heterodimerisation.

For example, deletion of BH1 or BH2 in Bcl-2 removes the ability of the protein to heterodimerise with Bax (Hanada, 1995), but much of the evidence for the importance of these domains comes from substitution mutants.

Substitution of FRDG138-41AAAA and WGR144-6AAA in BH1 of human Bcl-2 are reported to markedly reduce Bcl-2's ability to heterodimerise with Bax, in contrast to deletion of NWGR and substitution of WGR144-6AAA, WGR144-6WAR, and WGR144-6WER in BH1, which are reported to prevent Bcl-2 heterodimerising with Bax (Hanada, 1995; Sedlak, 1995; Yin, 1994). Substitution of the highly conserved G145 residue in BH1 of Bcl-2 and the equivalent residue in other Bcl-2 homologues has significant, but variable effect. Substitution of G145A in BH1 of Bcl-2 and substitution of G138A of BCL-x_L, completely abrogated the protein's ability to heterodimerise with Bax (Diaz, 1997; Yin, 1994). Similarly, substitution of G87A in BH1 of E1B 19kDa prevents the protein heterodimerising with Bax (Han, 1995). The variable effect of this substitution is illustrated by substitutions in Bid and Bad. *In vitro* binding assays indicate that Bid forms heterodimers with both Bcl-2 and Bax, but this interaction is prevented when the substitutions G145A in BH1 of Bcl-2 and G108A in BH1 of Bax are applied (Wang, K. 1996). In contrast, substitution of G145A in BH1 of Bcl-2 reduces heterodimerisation between Bcl-2 and Bad only slightly (Zha, 1997).

A conserved tryptophan residue at position 188 of Bcl-2 appears particularly important for heterodimerisation, and substitution of W188A in BH2 completely abrogated Bcl-2's ability to heterodimerise with Bax in one study and significantly reduced Bcl-2's ability to heterodimerise with Bad in another. Likewise substitution of QDN190-2LAA and E200A in BH2 of Bcl-2, markedly reduces its ability to heterodimerise with Bax (Yin, 1994; Zha, 1997).

This importance of BH1 and BH2 in the anti-apoptotic heterodimerisation partner is underlined by the failure of Bcl-x_S, which lacks BH1 and most of the BH2 region found in Bcl-x_L, to bind to Bax in the yeast two hybrid assay, in contrast to Bcl-2, Bcl-x_L, and Mcl-1 which do have BH1 and BH2 domains (Sato, 1994). However, the need for BH1 and BH2 in heterodimerisation may not be universal as Bcl-x_S forms heterodimers with Bik. In keeping with a form of heterodimerisation that clearly differs, for example, from the heterodimerisation between Bcl-2 and Bax, a yeast two-hybrid study suggested that the Bcl-2 residues from positions 43-48 and the homologous residues from positions 90-96 of E1B 19kDa are required for interaction between Bcl-2 and Bik (Boyd, 1995).

Evidence for the importance of BH3 in the heterodimerisation function of the anti-apoptotic family members comes from the evolutionarily diverse E1B 19kDa, Bcl-x_L and Bcl-2. Deletion of GDE at positions 94-6 of Bcl-x_L eliminated the protein's ability to bind Bak and the minimal region of E1B 19kDa necessary to interact with a sequence containing a BH3 sequence from Bax maps to the conserved region including BH1-3. The importance of BH3 in this centrally conserved region is emphasised by the substitution of F51S in BH3 of E1B 19kDa, which abrogates heterodimerisation with Bax. Similarly, heterodimerisation of Bcl-2 and Bad is completely abolished when the L97A substitution is applied to the BH3 domain of Bcl-2 (Chittenden, 1995; Han, J. 1995; Zha, 1997).

Given the alternate splicing origin of the protein, heterodimerisations involving Bcl-x_S are clearly a special case. Bcl-x_S does not heterodimerise with Bax or with Bad (Sato, 1994; Sedlak, 1995; Yang, 1995), but it is found to heterodimerise with Bik in yeast two-hybrid studies (Boyd, 1995). Bcl-x_S's association with Bcl-x_L, Bcl-2 and Bax is not detected in vitro and *in vivo* Bcl-x_L shows greater affinity for Bax by co-precipitating with Bax significantly better than Bcl-x_S. However, Bcl-x_S is found to heterodimerise with Bcl-2 in the yeast two-hybrid assay (Zhang, 1995).

The general lack of action or lesser affinity between Bcl-x_S and other family members suggests that it reduces the cellular apoptotic threshold by a means that is different to other pro-apoptotic members with a wider range of binding ability. For Bcl-x_S to bind Bcl-x_L the first 25 residues containing BH4 must be present in Bcl-x_S, but removal of the equivalent residues from Bcl-x_L enhances the binding affinity between the two proteins. Consistent with this, deletion of the NH_2-terminus of Bcl-x_S prevents it from interacting with Bcl-x_L. The authors suggest that the NH_2-terminus of Bcl-x_L interacts with its own BH1-2 region during homodimerisation and that deletion of Bcl-x_L's NH_2-terminus allows the same region of Bcl-x_S to react more readily with the BH1-2 region of Bcl-x_L (Minn, 1996; Sato, 1994). In contrast, the BH1 domain and the region containing the TM domain of Bcl-2 are required for heterodimerisation between Bcl-2 and Bcl-x_S, as are residues 24-78 of the NH_2-terminus of Bcl-x_S (Zhang, 1995).

The most widely observed form of dimerisation clearly involves the BH3 domain in the pro-apoptotic partner, binding to the BH1-3 domains of the anti-apoptotic partner, but clearly other forms of interaction are possible. The observed hetero-dimerisation between Bik whose conserved homology is in BH3 only, and Bcl-x_S which lacks BH1 and most of the BH2 domain, indicates other possibilities which may apply equally well to other family members.

The heterodimerisation between Bcl-x_S and Bcl-x_L shows distinct parallels with the head-to-tail Bcl-2 homodimerisation and may be explained in this way, but the heterodimerisation of Bcl-2 and Bcl-x_S shows nothing in common with other forms of heterodimerisation and hence serves only to emphasise that other possibilities may apply to other family members.

6.4 Apoptosis inhibition.

Extensive studies leave little room for doubt that the possession of the BH4, BH1 and BH2 regions confer the ability to inhibit apoptosis on the anti-apoptotic members of the Bcl-2 family. Additionally some evidence attributes anti-apoptotic function to the TM domain and sequences between it and BH2.

Truncation and deletion mutations in murine Bcl-2 indicate that BH1, BH2 and BH4 are essential to the suppression of nerve growth factor withdrawal-induced apoptosis in sympathetic neurons isolated from the superior cervical ganglia of new-born rats. Deletion of Bcl-2 residues 4-29 (BH4), residues 127-191 (BH1 and part of BH2), residues 187-207 (BH2) as well as any C-terminal truncation earlier than residue 196, abrogate the protein's anti-apoptotic function. Truncation of Bcl-2 at residue 196 reduces anti-apoptotic activity significantly, but truncations at 201, 202, 203 retain significant anti-apoptotic activity despite the absent TM domain. However the addition of a short hydrophobic peptide sequence to the truncation at residue 203 to produce Bcl-$2_{(1\text{-}203)}$+ HLEGPIL, produces an anti-apoptotic protein with significantly more effect that Bcl-$2_{(1\text{-}203)}$ alone. This would suggest that the TM locating sequence of Bcl-2 is important, but has limited specificity to anti-apoptotic function (Borner, 1994).

Support for the importance of BH1-2, BH4 and sequences downstream of BH2 come from yeast two-hybrid, transient transfection studies and mutagenesis of the BH4 domain. Bcl-2 deletion mutants lacking BH4, BH1, BH2 or the NWGR motif are ineffective at blocking Bax-mediated apoptosis in yeast. Bcl-2β is also unable to block Bax-induced apoptosis, suggesting that sequences between BH2 and the TM domain are important for blocking Bax's function. Consistently, a Bcl-2 deletion mutant consisting only of residues 1-196 also failed to prevent Bax-mediated apoptosis in yeast (Hanada, 1995). Similarly, transient transfection studies using staurosporine-induced apoptosis in fibroblasts and steroid-induced apoptosis in T-lymphoid cells, suggest that protection from apoptosis is dependent on both the region of Bcl-2 from residues 90-203 encompassing BH1 and BH2 and the region from 6-31 encompassing BH4 (Hunter, 1996).

In particular, the BH4 region appears to be essential for Bcl-2's anti-apoptotic activity and systematic mutagenesis indicates that 5 hydrophobic and aromatic residues, I14, V15, Y18, I19 and L23, are critical to its apoptosis-inhibiting function, and individual mutation to glycine in any one of these residues profoundly inhibits Bcl-2 activity in a transient transfection assay (Lee, 1996). Similar effects are found in studies in a series of Bcl-2 deletion mutants used to challenge staurosporine-induced apoptosis in a transient transfection assay. Deletion of residues 6-31 encompassing the BH4 region imparted a negative phenotype to Bcl-2 which promoted rather than prevented apoptosis. Likewise deletion of BH4 in Bcl-2 converted the protein to a pro-apoptotic function in baby hamster kidney cells (Hunter, 1996; Shibasaki, 1997).

Furthermore, substitution studies in human and murine Bcl-2 emphasise the importance of BH1 and BH2 to apoptosis. Substitution of FRDG138-41AAAA and WGR144-6AAA in the BH1 domain of human Bcl-2, along with BH2 substitutions QDN190-2LAA and E200A, markedly reduced the apoptosis-inhibiting activity of the protein. In contrast, substitutions of G145A in BH1 and W188A in BH2 completely abolished Bcl-2's ability to prevent IL-3-deprivation-, γ-irradiation- and glucocorticoid-induced apoptosis (Yin, 1994). Likewise deletion of the XFXFG motif in BH1 at residues 150-154 of murine Bcl-2 reduces the proteins anti-apoptotic effectiveness in counteracting the impact of nerve growth factor withdrawal-induced apoptosis in sympathetic neurons isolated from the superior cervical ganglia of new born rats (Borner, 1994).

Site-specific mutagenesis of Bcl-X_L supports the evidence gained from Bcl-2, indicating that BH1 and BH2 are essential to the anti-apoptotic function, but that residues critical to Bcl-X_L function are different from those required by Bcl-2. Substitution of VNW135-7AIL or GRI138-40ELN in BH1 of human Bcl-X_L abolishes its apoptosis-inhibiting activity in Sindbis virus-induced apoptosis in baby hamster kidney cells. Substitution of VV for any two Fs in the FFSFG motif of BH1 significantly reduces Bcl-X_L's activity, in contrast to single and double substitutions between residues 129-134 of BH1 which had only minimal effect. Substitution FRD131-3VRA also had very little effect on Bcl-X_L, in contrast to the same substitution in Bcl-2 which totally abrogated its anti-apoptotic activity in the same virus vector system. Single and double substitutions in BH2 of Bcl-X_L have only very limited effects. Substitution of WD188-9GA reduced Bcl-X_L's activity by approximately half, but substituting the highly conserved tryptophan at position 181 had little effect (Cheng, 1996). This latter substitution is in direct contrast to the effect on Bcl-2, where substituting tryptophan 181 had a significant impact (Yin, 1994).

Additionally, substitution of G159A in BH1 of Bcl-x_L abolished the protein's ability to repress apoptosis in FL5.12 cells deprived of IL-3 (Sedlak, 1995).

Evidence of the apparent biological universality of the need for the BH1 domain in Bcl-2 homologues comes from substitution mutants of the E1B 19kDa protein and the African Swine fever virus protein A179L. Substitution of the E1B 19kDa conserved glycine residue G87A in BH1, abolishes the protein's ability to inhibit puromycin- or staurosporine-induced apoptosis (Chen, 1996) and substitution of G85A in BH1 of A179L also abolishes its ability to protect K562 cells from apoptosis (Revilla, 1997).

6.4.7 Clearly the evidence supports not only the necessity of BH4, BH1 and BH2 to Bcl-2 and Bcl-x_L's anti-apoptotic function, but also attributes some importance to the TM domain and a sequence between it and the BH2 domain in this respect. Indeed on the basis of these studies there is significant justification for viewing BH4 as the anti-apoptotic domain. In particular the G145, NWGR motif generally, and the FRD motif in BH1 of Bcl-2, as well as the W188 in BH2 of Bcl-2 and the VNWGRI motif and G159A in BH1 of Bcl-x_L, seem to be particularly important to anti-apoptotic function. There is also some evidence to suggest that BH3 is functionally important in apoptosis inhibition and an unspecified substitution of residues 97-98 abrogated Bcl-2's and Bcl-x_L's anti-apoptotic function, and deletion of GDE at positions 94-6 of Bcl-x_L, eliminated both its ability to bind Bak and to suppress cell death (Chittenden, 1995).

6.5 Apoptosis induction.

BH1 and BH2 appear to have no function in inducing apoptosis in comparison to BH3. However, despite extensive studies using deletions and substitutions in a wide-range of pro-apoptotic Bcl-2 family members lending support to the proposition that BH3 is the pro-apoptotic domain, it remains unclear to what extent this is true.

A range of deletions and substitutions in BH1 and BH2 of Bax, e.g. deletions of residues 102-112 in BH1 and 151-159 in BH2, had no effect on the protein's ability to accelerate taxol-, vincristine-, cisplatin- or VP-16-induced apoptosis in FL5.12 cells (Simonian, 1996(1 & 2)). BH1-2 were found to be unnecessary to promote apoptosis, in contrast to BH3 which was found to be of central importance for Bak-promoted apoptosis in transient transfection assays in Rat-1 cells. Deletions in Bak identified the BH3-containing residues 67-94 as being uniquely important to accelerate apoptosis and truncated forms of Bak containing BH3 retain the protein's cytotoxic function.

Support for the importance of BH3 cytotoxic function also comes from Bip1. This distant family member shows distinct similarity to BH3 of Bax and Bak in its amino acid sequence from 57-69, LALRLACIGDEMD, and deletion of the BH3 domain substantially impairs the protein's ability to accelerate apoptosis in Rat-1 fibroblasts (Chittenden, 1995).

Similarly, BH3 appears to be the apoptosis-promoting domain in other pro-apoptotic proteins including Bik and Bax. Bik is an apoptosis-promoting member of the Bcl-2 family whose activities can be mitigated by the co-expression of Bcl-2, Bcl-x_L, Bhrf-1 and E1B 19kDa proteins, and deletion of the BH3 domain residues in Bik from 61-69 (LACIGDEMD), suppresses the ability of the protein to induce apoptosis in Rat-1 fibroblasts (Boyd, 1995).

Unfortunately the situation with Bax is not so cut and dried. Bax truncations containing only the BH3-encompassing 41 amino acids from residues 47-87, were as efficient as Bax truncations lacking only the TM domain at promoting apoptosis in REF52 rat fibroblasts (Simonen, 1997). However, the BH3 domain of murine Bax has been shown to be ultimately unnecessary to promote apoptosis in transfection studies using FL5.12 cells. Substitution of GD67-8AA and deletion of residues 63-71 in Bax did not abolish the protein's ability to promote apoptosis in FL5.12 cells and to counter the effects of Bcl-x_L (Simonian, 1996(1 & 2)). Likewise deletion of the BH3 domain in Bax reduces but does not entirely eliminate the protein's ability to promote apoptosis in Rat-1 fibroblasts (Chittenden, 1995).

There seems little doubt that the BH3 domain is, as it were, the pro-apoptotic domain, but clearly the situation is not that simple with pro-apoptotic members of the Bcl-2 family able to promote apoptosis after the BH3 domain has been deleted or elements of it substituted. However, it remains unclear to what extent this is a genuine reflection of how the pro-apoptotic proteins perform *in vivo*. In particular it is unclear to what extent the common removal of the TM domain in studies affects the functioning of these proteins and it remains possible that the experimental conditions are so different from the conditions prevailing naturally in the cell as to call many of the conclusions derived from these experiments into question. In the context of BH3 it is interesting to note that deletion of murine Bcl-2 residues 4-29 that converts Bcl-2 into a BH3-possessing Bax lookalike and deletion of residues 127-191 that converts Bcl-2 to a BH3-possessing Bcl-x_S-like form, does not confer Bax and Bcl-x_S's pro-apoptotic functions on either (Borner, 1994).

6.6 Intracellular localisation of family members.

The majority of the anti-apoptotic and pro-apoptotic members of the Bcl-2 family possess a C-terminal membrane-spanning domain that appears to enhance function in both types of family member.

The TM domain of Bcl-2 appears to confer anti-apoptotic status on the protein and the Bcl-2β protein which arises from alternative splicing of the *bcl-2* gene and is identical to Bcl-2α except that it lacks the latter's last 34 residues including the TM domain (Tsujimoto, 1986), is ineffective in prolonging the life of the IL-3-dependent haematopoietic cell line 32D in comparison to the full-length Bcl-2α. However, a chimaeric protein consisting of Bcl-2β and the TM and cytosolic domains of the IL-2 receptor αprotein expressed in the 32D cell line resulted in the prolongation of cell life. When 32D cells were subsequently fractionated much of the Bcl-2β protein appeared to reside in the cystol, in comparison to the chimaeric protein which was found in fractions associated with the mitochondrial enzyme F1-β-ATPase (Tanaka, 1993). Such results would appear to suggest that localisation to mitochondria via a TM domain is important to Bcl-2 anti-apoptotic function, but that specific conservation of sequence in the Bcl-2 family TM domain is not.

In contrast to these findings other studies suggest that only some anti-apoptotic activity is vested in the TM domain. Bcl-2α truncation mutants lacking the normal TM domain as a result of truncation after residues 196, 201, 202 and 203, continue to inhibit the induction of apoptosis in sympathetic neurons by removal of nerve growth factor. Confocal microscopy indicates that the Bcl-2α truncation mutant (1-203) is located in the extremities of neurites in neurons and filopodias in fibroblasts, suggesting that the normal membrane localisation of Bcl-2 is unnecessary to enable it to perform its anti-apoptotic function. However, fusion of a truncation mutant consisting of Bcl-2α residues 1-203 to a peptide sequence consisting of HLEGPIL restored full wt Bcl-2 anti-apoptotic activity in L929 fibroblasts as well as sympathetic neurons, confirming the apparent non-specific nature of the Bcl-2 TM domain (Borner, 1994). This latter study is supported by transient transfection studies using staurosporine-induced apoptosis in fibroblasts and steroid-induced apoptosis in T-lymphoid cells, which suggest that protection from apoptosis is only partially reduced after deletion of the Bcl-2 TM domain, and indeed a Bcl-2 155-residue lacking also most of the region from residues 30-91 and the TM domain, exhibited a significant protective effect (Hunter, 1996).

The TM domain also appears to have functional significance in pro-apoptotic family members. Microinjection of the pcDNA3 expression vector, into which full-length Bax has been cloned, into REF52 rat fibroblasts, induced apoptosis in 33% of them within 24 hrs. In contrast, cloning a version of Bax from which the TM domain coding has been deleted into the same expression vector, results in apoptosis being induced in only 8% of the cells after the same time (Simonen, 1997), suggesting that localisation to intracellular membranes via the TM plays a significant role in inducing apoptosis. These results are supported by similar results which tested the ability of Bak mutants to induce apoptosis in transient transfection assays in Rat-1 cells. Bak mutants lacking the carboxy-terminal hydrophobic TM domain had a diminished cytotoxic function (Chittenden, 1995). Of particular interest is the fact that at high levels of expression, haemagglutinin-tagged Bax possessing a TM domain shows a diffuse cytoplasmic and nuclear distribution in baby hamster kidney cells, in contrast to the normal membrane distribution found at low levels of expression. However, this changes dramatically in response to co-expression of Bcl-2, when it localises to the cytoplasmic membranes with a staining pattern similar to Bcl-2 (Shibasaki, 1997). These results appear to suggest that in some circumstances at least, Bax is targeted to the mitochondrial, ER and perinuclear membranes by Bcl-2 and not by its own TM domain.

Not enough studies have been carried out to form a definitive conclusion, but the evidence points to the TM domain as enhancing the function of both anti- and pro-apoptotic family members, which inevitably casts doubt over the results obtained from the many studies in which the TM domain of Bcl-2 family members have been removed as part of the experimental procedures.

6.7 Interaction between family members.

The model proposed by Oltvai, Korsmeyer, and Milliman (Korsmeyer, 1995; Oltvai, 1993; Oltvai & Korsmeyer, 1994) envisages that the apoptotic fate of the cell is determined by the equilibrium between the anti-apoptotic Bcl-2 and the pro-apoptotic Bax, with the former acting to raise the apoptotic threshold of the cell and the latter acting to lower it. This paradigm has grown to incorporate other family members, e.g. Bcl-x_L, and Bak, but it remains unclear if the pro-apoptotic Bcl-2 family members are the active entities regulated by the anti-apoptotic members or vice versa. However, it is proposed that family members with opposing functions regulate each other by heterodimerising and neutralising the opposing function. Additionally, it is thought that as part of their function both pro- and anti-apoptotic members of the Bcl-2 family may need to homodimerise.

Evidence in support of this model comes mainly from studies showing a correlation between the expression of pro- and anti-apoptotic members and the cell's fate, with supporting evidence indicating that substitutions which affect the ability of the two family subtypes to bind to each other also affect apoptosis induction or inhibition. However, the evidence is not entirely one-sided and significant evidence exists to suggest that pro-apoptotic proteins especially can induce apoptosis without binding opposing family members, and that monomeric forms of pro-apoptotic proteins can promote apoptosis.

The FRDG138-41AAAA, WGR144-6AAA, QDN190-2LAA, and E200A substitutions in human Bcl-2 markedly reduce, and the G145A and W188A substitutions completely abrogate, Bcl-2's ability to prevent IL-3-deprivation-, γ-irradiation- and glucocorticoid-induced apoptosis accelerated by Bax. These mutations show a striking correlation between Bcl-2's ability to repress apoptosis and its ability to bind Bax, with those mutations which bind Bax less avidly also being less effective at apoptosis inhibition, and those substitutions which abrogate Bcl-2/Bax heterodimerisation also abrogating Bcl-2's apoptosis-inhibiting function (Yin, 1994). This study suggests that both BH1 and BH2 are involved in heterodimerising with Bax and offers support to the model envisaging that Bcl-2 must bind to Bax to exert its death-repressing function.

Likewise, studies using yeast two-hybrid assays with human Bcl-2 and mouse Bax, indicate that deletions in Bcl-2 that abolish or lessen the protein's ability to inhibit apoptosis, similarly affect Bcl-2's ability to bind Bax (Hanada, 1995). These findings are further supported by a study showing that the adenovirus Bcl-2 homologue, E1B 19kDa, confers protection against apoptosis in Chinese hamster ovary cells that is virtually indistinguishable from the protection conferred by Bcl-2. When a G87A substitution is applied to BH1 of E1B 19kDa, the protein loses its ability to inhibit puromycin- and staurosporine-induced apoptosis in a manner that directly corresponds to the proteins ability to bind Bax (Chen, 1996).

Studies which create substitution mutants in the pro-apoptotic family members Bik, Bax, and Bak, also suggest that apoptosis induction or inhibition is associated with molecular interactions, include those involving Bcl-2 and Bcl-x_L. The apoptosis-promoting function of Bik is opposed by the co-expression of Bcl-2, Bcl-x_L, Bhrf-1 and E1B 19kDa proteins, and deletion of residues 61-69 (LACIGDEMD) in BH3 of Bik simultaneously suppresses both apoptosis-induction and interactions between Bik and the apoptosis-inhibiting members of the family (Boyd, 1995).

The BH3 domain seems to be of particular importance in this respect and substitution and deletion of residues in this domain in both pro- and anti-apoptotic members highlights the parallel function of binding and inhibition of apoptosis. A series of deletion mutants in Bak indicate that both the interaction with Bcl-x_L and the protein's apoptosis-inducing function depend on the residues 73-94 encompassing BH3. Similarly, BH3 appears to be functionally important in Bcl-x_L with deletion of GDE at positions 94-6 of Bcl-x_L eliminating both its ability to bind Bak and suppress cell death. Furthermore, deletion of the BH3 domain in Bax confirms that it is critical to the ability of the protein to promote apoptosis and to bind to both Bcl-2 and Bcl- x_L (Chittenden, 1995).

A particularly convincing demonstration of the association between apoptosis inhibition by Bcl-2 and heterodimerisation was contrived using a substitution mutant of Bcl-2 and a truncation mutant of Bax. Bcl-2 G145A does not bind wt Bax and does not protect mammalian 293 cells from Bax-induced apoptosis. However, Bcl-2 G145A does bind to a version of Bax truncated at the C-terminus (BaxΔC) which retains its pro-apoptotic function. When Bcl-2 G145A is co-transfected with BaxΔC the Bcl-2 mutant is as effective at inhibiting apoptosis as wt Bcl-2, suggesting that Bcl-2 exerts its protective effect via heterodimerisation with Bax (Ottilie, 1997; Yin, 1994). Studies involving chicken embryo neurotrophic factor-deprived neurons also offer some support for Oltvai, Korsmeyer and Milliman's model, although here the normally accepted role of Bax may apparently be reversed. When either a Bcl-2 or Bax expression vector was injected to produce a Bcl-2:Bax ratio of >2:1 or <1:2, many more neurons were protected from apoptosis in comparison to control cultures. However, when expression vectors were injected to produce equal concentrations of Bcl-2 and Bax, the number of surviving neurons was no greater than control injected neurons. Although this study raises questions about the anomalous role of Bax when its concentration is at least twice that of Bcl-2, the results are consistent with Bcl-2 and Bax expressing a mutual antagonism through heterodimerisation (Middleton, 1996).

There is also evidence to suggest that binding of the pro-and anti-apoptotic family members is not essential to the promotion or inhibition of apoptosis. Results from substitution mutations involving both BH1 and BH2 domains suggest that Bcl-x_L does not have to bind Bax to inhibit apoptosis. Substitution of FRD131-3VRA or G148E and G187A in Bcl-x_L abolishes the interaction between Bcl-x_L and Bax, whilst preserving 70-80% of Bcl-x_L's wt activity in inhibiting Sindbis virus-induced apoptosis (Cheng, 1996). Likewise, deletion of residues 63-71 (LRRIGELD) in BH3 in Bax abrogated its ability to bind Bcl-x_L, but not its ability to counter the effects of Bcl-x_L (Simonian, 1996a).

Studies using *Vaccinia* virus-induced transient transfection assays and *in vitro* binding assays indicate that Bid heterodimerises with Bcl-x_L and Bcl-2 via its BH3 domain. Bid promotes apoptosis without a specific apoptotic stimulus, but different Bid BH3 mutants can be generated which bind Bcl-2 without countering its anti-apoptotic effect and which do not bind Bcl-2 but which still retain an ability to promote apoptosis (Wang, K. 1996). Interestingly as well as heterodimerisation being unnecessary in some studies, homodimerisation of pro-apoptotic proteins also appears unnecessary for promotion of apoptosis. Bax proteins retain their ability to accelerate the rate of taxol- and vincristine-induced apoptosis in FL5.12 cells after deletion of residues 63-71 in BH3 although this deletion disrupted Bax homodimerisation, indicating that monomeric forms of Bax remain active in inducing apoptosis (Simonian, 1996b).

Although the weight of evidence appears to be balanced in favour of heterodimerisation between pro- and anti-apoptotic members being necessary to induce/inhibit apoptosis, it does not seem possible to form any definitive conclusion about the coupling between heterodimerisation and induction or inhibition of apoptosis, when evidence exists to the contrary. However, it has to be borne in mind that the majority of testing methods bear very little resemblance to the circumstances that prevail naturally in the cell. In particular many of the Bcl-2 family members have their TM domains removed before testing and/or are bound to expression vectors in a manner that may affect their conformation. This compares with the situation thought to prevail in the cell where many of these proteins are thought to function embedded in mitochondrial, endoplasmic reticular, or perinuclear membranes. This difference between the natural and testing methods may offer some explanation for the contradictory nature of these results, but it may also be that control of apoptosis by the Bcl-2 family is not as simple as originally envisaged. Support for the view that the results of many Bcl-2 family interaction studies should be viewed with caution comes from a study involving Bcl-2, Bcl-x_L, and Bax. This suggested that Bax homo- and heterodimerisation may be determined by detergent-induced conformations in Bax, that vary depending on the detergent used (Hsu, 1998). The ability of pro-apoptotic Bax to protect cells from apoptosis when expressed in excess (Middleton, 1996) also suggests that control of apoptosis by the Bcl-2 family requires a more detailed model. Furthermore, Bad heterodimerises with both Bcl-x_L and Bcl-2, binding more strongly to the former in mammalian cells, but countering only the anti-apoptotic activity of Bcl-x_L. In heterodimerising with Bcl-x_L, Bad appears to displace Bax and when approximately half of the cellular Bax is heterodimerised, apoptosis is inhibited (Yang, 1995).

This suggests that the control of apoptosis by the Bcl-2 family may be a multi-layered system of greater complexity than that envisaged by the Oltvai, Korsmeyer and Milliman paradigm.

Despite being investigated over a considerable number of years, the precise function of the Bcl-2 homology domains remains to be defined and many of the anomalies that the studies have raised remain to be resolved. However, a general picture is beginning to emerge that is largely in keeping with the conservation of sequence homology observed in family members. This appears to indicate that the BH4 domain is the anti-apoptosis domain, BH3 is the pro-apoptotic domain, the major role of BH1 and BH2 may be in interactions between family members and Bcl-2 family-associated proteins, and that the role of the TM domain includes functionally essential location of family members to the appropriate intracellular membranes as well as apparently having an essential role in the interactions of some family members. The heterodimerisation and homodimerisation functions seem to be radically different, with Bcl-2 and Bcl-x_L homodimers adopting a head-to-tail interaction while heterodimers appear to adopt a tail-to-tail conformation.

6.8 Interactions with Bcl-2 family-associated proteins.

It is clear that the Bcl-2 family does not exert its control over apoptosis in isolation from other non-family molecules. Bcl-2 associates with two specific molecules, Bag-1 and Raf-1, which in turn also interact with each other. Both Bag-1 and Raf-1 have been shown to interact with Bcl-2 in an apparently synergistic relationship which confers a greater survival advantage in the variety of cells in which they have been transgenically expressed. However, whilst a certain amount of information has emerged about the individual interactions, the mechanism (s) by which this trinity of Bcl-2, Bag-1 and Raf-1 exerts its effect, remains essentially unclear.

The Bag-1 protein shows no homology with Bcl-2 family members, but binds to Bcl-2 and the synergistic effect has been demonstrated in several cell types. For example, co-expression by gene transfer of Bag-1 and Bcl-2 in the human lymphoid cell line Jurkat, markedly increases the protection offered from apoptosis induced by a range of apoptotic stimuli, in comparison to the degree of protection offered when levels of Bag-1 or Bcl-2 were elevated in isolation from each other (Takayama, 1995). Similar results were obtained using hybridoma 2E3-0 cells transfected with *bcl-2* and *bag-1*.

These exhibited improved survival over a longer period when treated with thymidine in excess (30 mM) or when subjected to nutritional limitation, in comparison to mock transfectant cells or cells transfected with *bcl-2* alone (Terada, 1997). Likewise, co-expression of Bcl-2 and Bag-1 in neuronally differentiated PC12 cells, which are subject to synchronous apoptosis when deprived of nerve growth factor, are protected more by the expression of Bcl-2 and Bag-1 than by Bcl-2 alone (Schulz, 1997). These results are further supported by a study where the overexpression of Bag-1 in the IL-3-dependent B-cell line Ba/F3, generated an IL-3-independent culture with minimal apoptosis observed in the absence of the cytokine (Clevenger, 1997).

Bcl-2 has also be shown to interact with Raf-1. Bcl-2 co-immunoprecipitates with Raf-1 in a mammalian haematopoietic cell line 32D.3, a diploid murine myeloid cell clone that is IL-3 dependent, and when Bcl-2 and Raf-1 are expressed in Sf9 insect cells using recombinant baculoviruses. When expressed together, Bcl-2 and Raf-1 also exhibit a functional synergy in 32D.3 cells in protecting the cells from apoptosis induced by means of IL-3 deprivation. Bcl-2 binds to a both the wild-type Raf-1 and to a truncated form containing the catalytic domain. However, Raf-1 does not appear to phosphorylate Bcl-2 in either 32D.3 or Sf9 insect cells, suggesting that the relationship is not one of enzyme and substrate (Wang, H-G., 1994).

Bcl-2, in binding to Raf-1, appears to target the protein kinase to mitochondria. Bcl-2 and Raf-1 interact via the BH4 domain of the former and the catalytic domain of the latter, as indicated by its reaction with the BH4-containing Bcl-x_S, and its failure to react with Bax in which BH4 is absent. Furthermore, only wild-type Bcl-2 possessing BH4 targets Raf-1 fused to a green fluorescent protein to mitochondria in a punctate fashion, in contrast to a deletion mutant of Bcl-2 lacking BH4, which produces a diffuse cellular distribution pattern. Raf-1 targeted to mitochondria via Bcl-2 enhances the ability of 32D.3 cells to resist apoptosis induced by IL-3 withdrawal and staurosporine, whereas interfering with the action of endogenous Raf-1 at mitochondria by mutating the functionally essential lysine 375 to tryptophan, has precisely the opposite effect. Whereas Raf-1 does not appear to phosphorylate Bcl-2, Bcl-x_L, Bcl-x_S, Mcl-1, Bax, or Bak, it does appear to phosphorylate Bad. This phosphorylation of Bad was detected both *in vitro* and *in vivo*, in cells expressing Raf-1 targeted at mitochondria but not Raf-1 targeted at the plasma membrane (Wang, H-G., 1996a). The authors speculate that phosphorylating Bad may displace it from Bcl-2, allowing Bcl-2 to fulfil its anti-apoptotic function.

Bag-1 also binds and activates the serine/threonine specific kinase, Raf-1, although in this particular study the Raf-1 protein was not purified creating the possibility that the activation of the enzyme occurrs via an intermediary. However, in *in vitro* binding assays and yeast two-hybrid assays, Bag-1 was found to interact with Raf-1 via its catalytic domain in the C-terminal portion of the protein and to increase its kinase activity ~3-5-fold without phosphorylating the Bag-1 protein. Interestingly, Bag-1, Raf-1 and Bcl-2 formed tri-molecular complexes, although the stoichiometry was only ~1% and probably too low to represent an active part of the anti-apoptotic mechanism (Wang, H-G., 1996b).

According to studies using the haematopoietic cell line, BAF-B03 F7, Bag-1 expression can be modulated by IL-2. When BAF-B03 F7 cells expressing the gene transfer-mediated IL-2Rα chain, are exposed to *in vitro* IL-2, Bag-1 expression is upregulated (Adachi, 1996). However, any attempt to understand Bag-1's role in the apoptotic process is confused by its interaction with other cellular entities. Gene transfer-mediated expression of Bag-1 in the MKN74 human gastric cancer cell line results, apparently by means of Bag-1's association with cytokeratin and actin filaments, in increased cell migration through increased cell motility (Nashiro, 1999).

Bag-1 also appears to be a cofactor of the heat shock cognate Hsc70 protein, where it is apparently involved in regulating its chaperone function. Bag-1 binds to the ATPase domain of the Hsc70 protein and is instrumental in substantially accelerating the release of ADP from Hsc70, by stimulating ATP hydrolysis (Hohfeld, 1997, 1998). However, whether Bag-1's involvement in the cellular chaperone machinery and in cell motility reflects the complexity of the apoptotic pathways or the extent of Bag-1's multiple functions, remains to be seen.

7 Levels of expression of Bcl-2 family members in B-CLL and correlation with sensitivity to induced apoptosis.

7.1 Introduction.

The founding member of the *bcl-2* family of genes, *bcl-2*, is a proto-oncogene which was first identified in 1984 in follicular lymphoma cells using a probe obtained from an acute B-cell leukaemia cell line. The *bcl-2* gene is normally located at band q21.3 of chromosome 18, but a high proportion of cases of follicular lymphoma show a t(14;18)(q32;q21) translocation which juxtaposes the intact *bcl-2* gene with an enhancer region in the Ig H-chain locus on chromosome 14, leading to the constitutive expression of the protein (Bakshi, 1985; Cleary, 1985; Pegoraro, 1984; Tsujimoto, 1984, 1985).

Attempts to detect and quantify the involvement of the *bcl-2* family of genes in B-CLL have concentrated largely on *bcl-2*, but expression of *mcl-1, bak, bag-1, bax*, *bcl-*x_L, and *bcl-*x_S have also been detected in the disease in varying amounts (Aguilar-Santileses, 1996; Gottardi, 1996; Kitada, 1998). Bcl-2 is therefore not the only family member that may play a role in the pathogenesis of the disease by influencing the life span of B-CLL leukaemic cells, but abnormal expression of the *bcl-2* gene, and its very close homologue *bcl-*x_L in particular, has been detected too consistently in the disease for there not to be a presumption that their expression plays some part in the B-CLL neoplastic process. For example, in an analysis of peripheral blood cells from 58 previously untreated B-CLL patients, Bcl-2, Mcl-1, Bag-1, Bax, and Bak were commonly but variably found using immunoblot assays and MoAbs specific for each protein. On the basis of densitometry scores, these proteins were found to be expressed in quantities equal to or greater than the amount of Bcl-2 expressed by the RS11846 t(14;18)-containing non-Hodgkins lymphoma cell line. Bcl-2 was expressed in quantity in 34/57 cases (60%), Mcl-1 in 24/55 (44%), Bax in 27/57 (47%), Bak in 27/51 (53%), and Bag-1 in 7/51 (14%). The authors do not give specific figures for the extent of the overlap of different Bcl-2 family members in individual patients, but both the above statistics and one of the authors' illustrations (Figure 1) indicates that both Bcl-2 and Mcl-1 are jointly expressed in the B-CLL cells of some patients. However, no clear correlation could be drawn between the expression of these family members and *in vitro* chemosensitivity to fludarabine-induced apoptosis. In contrast to those listed above, Bcl-x_L, and Bad proteins were not present at detectable levels in any of the samples tested (Kitada, 1998).

These findings are strongly supported by a study of $CD5^+$ B-cells from 23 B-CLL patients,

This investigation showed not only high levels of Bcl-2 and *bcl-2* mRNA, but also strong expression of Bax and, in contrast to the findings reported by Kitada, strong expression of Bcl-x_L (although only trace quantities of Bcl-x_S were detected). Northern blotting and fluorescence-activated cell sorting (FACS) using an anti-Bcl-2 monoclonal antibody, respectively revealed both high levels of *bcl-2* mRNA and Bcl-2 in all 23 of the B-CLL samples studied. Likewise, reverse transcriptase polymerase chain reaction (RT-PCR) confirmed by FACS, showed strong expression of *bax* mRNA in 20/23 cases studied. RT-PCR also showed that high levels of *bcl-x_L* mRNA was present in 16/23 of the samples tested, although *bcl-x_S* mRNA was detectable in only trace amounts in 13/23. No relationship was observed between the expression of these family members or the clinical stage of the disease, but there appears to be a clear overlap in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L in the cells of some patients. Bcl-2 and *bcl-2* mRNA were found in all 23 samples tested so there must be an overlap in expression in the 16 samples in which *bcl-x_L* mRNA was also detected (Gottardi, 1996).

Although the levels of expression did not appear to be as high as the levels reported by Kitada *et. al.*, and referred to in a previous paragraph, a study aimed at linking Bcl-2, Bax and P53 expression to in *vitro survival* and clinical progression, similarly suggests that *bcl-2* mRNA is aberrantly expressed in B-CLL. A study of 38 B-CLL patients defined as having progressive or non-progressive disease showed significantly higher levels of *bcl-2* mRNA, as measured by competitive polymerase chain reaction (PCR), in comparison to normal values. mRNA values expressed as moles per mole of glucose-3-phosphate dehydrogenase mRNA ranged from 0.01 to 9.00 and this degree of variability was reflected in protein levels. When measured as specific mean fluorescence intensity, Bcl-2 levels were found to vary from 0.79 to 31.73 with a mean of 17.20 compared to 0.11 for a control null-*bcl-2* Daudi cell line and 67.46 for the t(14;18)-containing RL-7 cell line. However, again no obvious relationship between Bcl-2 levels and disease progression could be detected (Aguilar-Santileses, 1996).

Two further small studies confirm that the rate of expression of Bcl-2 in B-CLL is commonly higher than normal, with one of them being out-of-step by suggesting that there is a link between Bcl-2 levels and *in vitro* survival. When the expression of *bcl-2* in cells from 9 B-CLL patients was investigated, *bcl-2* mRNA transcripts were detected in 9/9 samples analysed, with the high molecular weight 8.5 kb transcript being the most abundant transcript detected. Expression was heterogeneous, with levels comparable to those found in the Karpass 422 cell line carrying a *bcl-2* rearrangement.

However, no rearrangement was detected in any of the 9 samples using either a major cluster region (mcr) or a 5' breakpoint region probe to explain the higher rate of expression of *bcl-2* mRNA. In contrast *bcl-2* mRNA was not detected in the normal $CD5^+$ B-cells with which B-CLL cells were compared and the level of *bcl-2* mRNA in the neoplastic cells showed no apparent relationship to either the patients' clinical stage or white blood cell count (Schena, 1992). Similarly a more recent study measured Bcl-2 levels in the leukaemic B-cells from 8 B-CLL patients using a flow cytometer calibrated using fluorescein isothiocyanate (FITC)-labelled micro-beads and an FITC-labelled mouse anti-human-Bcl-2 mononclonal antibody on permeabilised cells. More than 90% of the cells from all 8 patients expressed Bcl-2 at high levels measured as $39\pm10 \times 10^3$ mean equivalent of soluble fluorochrome (MESF) units before culture. This fell to $26\pm4 \times 10^3$ after 7 days culture in medium alone, but with the percentage of $Bcl\text{-}2^+$ cells remaining constant. However, when B-CLL cells were cultured on a layer of BM stromal cells the MESF was measured as 44±9 after 7 days, suggesting that the Bcl-2 levels are maintained or enhanced in the presence of BM stromal cells (Lagneaux, 1998).

Although many of the studies do not involve the size of cohort that might resolve the question at a stroke, the extent to which anti-apoptotic proteins, particularly Bcl-2, are so consistently expressed in an apparently aberrant manner at both message and protein level, lends considerable weight to the belief that Bcl-2 and other anti-apoptotic family members are involved in the pathogenesis of B-CLL. Similarly and although the evidence is not unanimous, it is clear that there is no simple cause and effect relationship between absolute levels of Bcl-2 and disease progression, or clinical stage. However, perhaps the most interesting fact to emerge from the studies referred to in the preceding paragraphs, is the extent of the overlapping over expression of anti-apoptotic members of the family which was detected, i.e. Bcl-2 and Mcl-1 in one investigation, and Bcl-2 and $Bcl\text{-}x_L$ in another.

7.2 **Bcl-2 levels and sensitivity of B-CLL cells to apoptosis.**

The apparently inconsistent pattern of Bcl-2 expression in B-CLL in relation to clinical stage or disease progression, is reflected in a similar inconsistency between the Bcl-2 levels detected and the degree of sensitivity of B-CLL cells to induced or spontaneous apoptosis *in vitro*.

Investigations that have suggested a link between Bcl-2 levels and the apoptotic threshold of B-CLL cells include simple observations of the link between Bcl-2 and spontaneous apoptosis, those which link stimulated expression of Bcl-2 to reduced rates of apoptosis, and those where apoptosis induced by cytotoxic agents has been shown to be mediated by reduced levels of Bcl-2.

An example of the former suggests that when B-CLL cells are cultured *in vitro*, the rate of spontaneous apoptosis is influenced by Bcl-2 levels. In a study of cells from 5 B-CLL patients, >85% of the cells were found to express Bcl-2 using an anti-Bcl-2 antibody and FITC-conjugated sheep anti-mouse Ig. During *in vitro* culture the mean fluorescence of the Bcl-2-expressing cells was downregulated, resulting in the appearance of cells with a Bcl-2^{low} phenotype after 48 hours. The percentage of cells downregulating Bcl-2 expression continued to increase so that after 8 days of culture the majority of cells displayed a Bcl-2^{low} phenotype. The reduction in expression of Bcl-2 was found to correlate with the rate of apoptosis, insofar as the percentage of Bcl-2^{high} cells was similar at all time points to the remaining percentage of viable cells for all 5 cell populations (Tangye, 1996).

In keeping with these results, culture of B-CLL cells with IFN-α, IL-4, bFGF, CDw32-transfected cells, or BM stromal cells, appears to reduce the extent of *in vitro* apoptosis in a manner that is linked to increased or preserved Bcl-2 levels. For example, during *in vitro* culture, Bcl-2 levels were measured by Western blotting and found to be preserved in those cells resistant to apoptosis and reduced in susceptible cells, but exposure to IFN-α and IL-4 appears to increase survival of B-CLL cells by preserving Bcl-2 levels. In an analysis of the cells from 8 B-CLL patients, Bcl-2 levels were found to be preserved in cells from 4 patients resistant to apoptosis for up to 30 days of culture in medium alone, but levels of Bcl-2 were rapidly reduced in cells from 4 patients sensitive to apoptosis. However, the rate of *in vitro* apoptosis could be reduced by culturing the cells with both IFN-α and IL-4 (which is discussed in greater detail in a subsequent chapter concerned with the modulation of levels of Bcl-2 family members), both of which reduced the rate of apoptosis in a manner that appeared to be associated with preservation of pre-existing Bcl-2 levels (Panayiotidis, 1993, 1994).

IL-4 has also been shown to increase the survival of B-CLL cells through Bcl-2 levels in a series of tests on B-CLL cells from 17 previously untreated patients. In comparison to control cultures, there was a 56% reduction in the number of cells undergoing apoptosis in B-CLL cells cultured with IL-4 for 4 days.

In the three samples of B-CLL cells tested, the prolongation of survival was accompanied by a partial preservation of Bcl-2 levels (Buske, 1997). Additionally, IL-4 has been shown to improve viability in B-CLL cells treated with hydrocortisone (HC) through its influence on Bcl-2 levels. B-CLL cells isolated from 12 patients were assessed for viability in the presence of medium alone, medium plus rIL-4 at 10ng/ml, medium supplemented with HC at 10^{-5} M, or medium and rIL-4 at 10ng/ml plus HC at 10^{-5} M. Cells cultured with HC showed the highest level of apoptosis, and cells cultured in medium plus rIL-4 showed a greater degree of viability than those cultured in medium alone. However, cells cultured with HC plus rIL-4 showed lower rates of apoptosis than cells cultured in medium alone. This lower rate of apoptosis appeared to be associated with Bcl-2 expression, with IL-4-treated cells expressing significantly more Bcl-2 than unstimulated, HC-treated, or freshly isolated B-CLL cells. Furthermore, the *in vitro* effect of IL-4 was found *in vivo* in 1 patient, with subcutaneous administration of IL-4 at 100μg/m^2 increasing Bcl-2 expression in B-CLL cells (Danescu, 1992).

bFGF also appears to exert its influence on B-CLL cells by modulating Bcl-2 levels and at a concentration of 100 μM, was found to protect cells from the EBV-transformed cell line, 183CLL, from apoptosis induced by 100 μM fludarabine. Compared to control cultures, addition of bFGF to the culture conditions resulted in both a delay in the onset of, and reduction in the percentage of cells undergoing apoptosis. Protection from apoptosis appears to be associated with upregulation of *bcl-2* mRNA expression and the 183CLL cell line, which expresses low amounts of steady-state *bcl-2* mRNA, showed a significant upregulation of the 8.5 kb transcript after stimulation with 10 ng/ml bFGF. A significant accumulation of the transcript was detected after 2 hours, with maximal response at 24 hours, and increased expression of Bcl-2 was detected in 183CLL cells with densitometric analysis of protein bands indicating that bFGF had induced a 1.7-fold increase in Bcl-2 levels (König, 1997b).

Evidence in support of the view that *in vitro* viability of B-CLL cells is linked to expression of Bcl-2 comes from studies in which Bcl-2 levels were manipulated using culture with BM stromal cells and CDw32-transfected murine fibroblasts. Cells from 8 B-CLL patients cultured with medium alone were 22±3% apoptotic after 48 hours, in contrast to cells cultured on a layer of human BM stromal cells which were only 8±2%. Bcl-2 levels in the B-cells from 8 B-CLL patients, measured using flow cytometry and an FITC-labelled mouse anti-human-Bcl-2 monoclonal antibody on permeabilised cells, showed more than 90% of the cells from all patients expressing Bcl-2 at high levels, measured as 39±10 x 10^3 mean equivalent of soluble fluorochrome (MESF) units.

At this level of fluorescence the rate of *in vitro* apoptosis was very low, but after 7 days' culture in medium alone, the level of Bcl-2 had been downregulated to 26±4 x 10^3 MESF units, with the percentage of Bcl-2 positive cells remaining constant. This downregulation of Bcl-2 levels showed significant correlation with the increased rate of apoptosis in these cultured cells. In contrast, B-CLL cells cultured on BM stromal cells showed improved viability with the level of Bcl-2 measured after 7 days as 44±9 x 10^3 MESF units (Lagneaux, 1998).

Similarly, when BM- and peripheral blood-derived B-CLL cells from 17 previously untreated patients were analysed for survival and apoptosis in relation to Bcl-2 expression after culture with various soluble ligands, the most efficient stimulus to *in vitro* survival was co-cultivation with CDw32-transfected murine fibroblasts with fibroblasts in direct contact with the leukaemic cells. In comparison to control cultures there was a reduction in the number of cells undergoing apoptosis of 90% as assessed by trypan blue exclusion. The prolongation of survival by CDw32-transfected murine fibroblasts was accompanied by a reduction in apoptosis which was associated with the partial prevention of the loss of Bcl-2 (Buske, 1997).

As increased viability of B-CLL cells appears to be mediated by increased expression of *bcl-2*, induction of *in vitro* apoptosis by cytotoxic agents appears to be mediated by reduced Bcl-2 levels. In a study using melarsoprol, an organic arsenical used in the treatment of trypanosomiasis, the compound was found to induce apoptosis in the non-EBV-transformed B-CLL cell line, WSU-CLL and the EBV-transformed B-CLL cell line, 183CLL, as well as lymphocytes from 5 B-CLL patients in a dose- and time-dependent manner. Of particular interest was the concentration-dependent downregulation of *bcl-2* mRNA and reduced expression of Bcl-2 protein that was observed in both the WSU-CLL and 183CLL cell lines, which accompanied the decreased viability of these cell lines after 24 hours exposure to melarsoprol (König, 1997a).

The results of these studies suggest that Bcl-2 levels and the rate of apoptosis appear to be inversely related, but unfortunately a significant number of other studies have produced quite contradictory results. In contrast to the results obtained using CDw32-transfected murine fibroblasts and IL-4, neither cultivation with soluble recombinant human CD40 nor cultivation with two different anti-CD40 antibodies, were found to prolong cell survival. Bcl-2 was found to be highest in freshly isolated cells and culture with soluble CD40 ligand resulted in the highest Bcl-2 levels being maintained.

However, this occurred without any detectable increase in survival rate, suggesting that survival is not simply a matter of maintaining Bcl-2 levels (Buske, 1997).

A number of recent studies have also contradicted the mechanism by which IL-4 increases the cell viability of B-CLL cells (Buske, 1997; Danescu, 1992; Panayiotidis, 1993, 1994). Comparison of the response of B-CLL cells from 11 previously treated patients (chlorambucil, prednisone, fludarabine, vincristine and cytoxan), with the cells from 14 untreated patients, showed that IL-4 protected the cells from previously treated patients against apoptosis more effectively that cells from the untreated patients. However, when the IL-4-sensitive cells from 5 treated patients were analysed using anti-Bcl-2 MoAb indirect immunofluorescence staining, FITC-labelled anti-mouse Ig, and flow cytometry, no difference was detected between the Bcl-2 levels of cells cultured with or without IL-4 (Frankfurt, 1997).

Likewise, IL-4, with an optimal concentration of 50 U/ml, was found to significantly enhance the viability of B-CLL cells in culture for periods up to 96 hours independently of Bcl-2 levels, in comparison to control cultures. Furthermore, given the consistent observation that IL-4 enhances cell viability in culture, it was paradoxically found to increase the sensitivity of B-CLL cells to melphalan (L-phenylalanine mustard)-induced apoptosis. The increased rate of apoptosis was accompanied by interstrand-DNA cross-linking in B-CLL cells following short term exposure to the compound (Pu, 1997).

The effect of IL-4 on Bcl-2 levels has also been called into question indirectly by a study that shows IL-5 is able to increase the rate of spontaneous apoptosis in B-CLL cells independently of Bcl-2 levels, but which can be antagonised by IL-4. When the cells from 13 B-CLL patients were incubated in medium supplemented with 20 U/ml of IL-5, spontaneous apoptosis increased by a mean percentage of 53% after 2 days of culture and by 37% after 3 days, in contrast to culture in medium alone. The apoptotic activity of IL-5 was dose-dependent and in half the cases studied the increase in apoptosis was as great as that induced by dexamethasone at 25 μM/L. Bcl-2 expression was quantified using unlabelled mouse anti-human Bcl-2 MoAb, FITC-labelled $F(ab)'_2$ fragments of rabbit anti-mouse Ig, and the cell-associated fluorescence with an FITC-labelled microbead-calibrated flow cytometer. In the five cases examined, Bcl-2 expression was not found to be significantly different in cells incubated with IL-5 compared to those incubated in medium alone. However, in all of these cases, IL-4 partially protected cells against IL-5-induced apoptosis in a manner that could be inhibited by anti-IL-4 MoAb (Mainou-Fowler, 1994).

IL-10 has also been shown to improve the *in vitro* cell viability of B-CLL cells independently of Bcl-2 levels. Further-unpurified peripheral blood mononuclear cells (PBMC) from 4 B-CLL patients, purified by Ficoll/Hypaque density gradient centrifugation (only 3 of which were $CD5^{+}$), incubated with IL-10 at concentrations between 1.0-50 ng/ml for between 10-21 days, were found to reduce the degree of apoptosis. However, when Bcl-2 expression was examined by flow cytometry and Western blotting, no difference was detected in its expression in the PBMC cultured with and without IL-10 (Kitabayashi, 1995). The absence of further purification of PBMC may raise doubts about the effect of IL-10, but does not change the fact that survival was improved independently of Bcl-2.

Many of the studies quoted involve questionably small cohorts, but a larger than average investigation of 42 B-CLL patients, although confirming previous observations that the propensity of B-CLL cells to undergo apoptotic death is greater in cells isolated from patients with untreated early stage disease, failed to detect any connection between Bcl-2 levels and cell survival. Bcl-2 expression was detected in 42/42 samples of leukaemic cells from B-CLL patients by Western blotting, with levels ranging from 0.07 to 3.34 arbitrary units when normalised to a t(14;18)-containing lymphoma cell line (Johnson). 9/42 samples showed higher levels of Bcl-2 expression than that found in the Johnson cell line and a further 19/42 had Bcl-2 levels ≤ levels found in B-cells from normal peripheral blood. However, no correlation between the level of Bcl-2 and clinical stage, prior treatment status, or refractory disease was detected. In particular, there was no correlation between Bcl-2 levels and spontaneous or fludarabine-induced apoptosis, as measured by DNA fragmentation. Similarly, assessment of B-CLL cell viability over 72 hours using a trypan blue assay did not appear to correlate with Bcl-2 expression (Robertson, 1996).

Whilst Bcl-2 levels do appear to have some bearing on the fate of B-CLL cells *in vitro*, the contradictory nature of the evidence available makes it is difficult to accept that the sensitivity of B-CLL cells to apoptosis is determined by Bcl-2 levels alone. In such circumstances, the cellular levels of pro-apoptotic Bcl-2 family members, particularly in relation to Bcl-2 levels, need to be considered.

7.3 Bcl-2:Bax ratios.

Although once again the evidence is not entirely unanimous, some explanation for the absence of any apparent correlation between Bcl-2 or Bcl-x_L levels in B-CLL and either disease progression or *in vitro* survival rates, may be offered by differences in the cellular Bcl-2:Bax and Bcl-x_L:Bax ratios. Higher levels of these appear to be related to progressive disease, previous treatment status, and relative insensitivity to the induction of apoptosis in a way that absolute levels of Bcl-2 cannot be linked.

For example, the study involving cells from 38 patients with B-CLL, referred to in a previous paragraph, showed no relationship between Bcl-2 levels and disease progression or *in vitro* survival, but higher levels of bax_α were detected in the non-progressive patients resulting in significantly lower *bcl-2*:bax_α mRNA ratios compared to cells from patients with progressive disease (Aguilar-Santileses, 1996). This is certainly in keeping with the relationship between Bcl-2:Bax ratios which Oltvai, Korsmeyer, and Milliman (1993, 1994) have suggested is a determining factor in regulating apoptosis, and is well-supported by other studies which have looked at the ratio of pro- and anti-apoptotic members of the Bcl-2 family in the context of *in vitro* life span, disease progression, and resistance to chemotherapeutic treatment.

These studies include a study of B-cell isolates from 24 B-CLL patients. Comparison of the 4/24 patient isolates which subsequently proved most sensitive to *in vitro* apoptosis induction with methylprednisolone or fludarabine plus mitoxantrone, with the 4/24 least sensitive, revealed that Bcl-2 levels were four-fold higher in the latter and were accompanied by only a modest increase in Bax. When isolates from all groups were incubated *in vitro*, Bcl-2 expression fell in comparison to freshly isolated controls, while Bax fell in only the apoptosis-resistant samples. This supports the theory that Bcl-2:Bax ratios are more important than absolute Bcl-2 levels in protecting the leukaemic cells from apoptosis, but of particular interest is the difference in endogenous endonuclease activity between the apoptosis-resistant and apoptosis-sensitive isolates. Nuclei from apoptosis-sensitive B-CLL cells expressed abundant levels of endogenous endonuclease activity, in comparison to the apoptosis-resistant cells, where endonuclease activity was not detected. This study involves a very small sample, but if it proved eventually to be representative of B-CLL cells with lower Bcl-2:Bax ratios, it would suggest that they are primed and ready for the apoptotic cascade (McConkey, 1996).

The Bcl-2:Bax ratio also appeared to correlate with previous cytotoxic treatment status when the leukaemic cells from 22 patients with B-CLL were assessed by quantitative immunofluorescence analysis using flow cytometry to determine Bcl-2 and Bax expression. Increased Bcl-2:Bax ratios were observed in all 22 samples compared to those detected in normal peripheral blood B lymphocytes used as controls, and previously untreated patients had lower Bcl-2:Bax ratios than previously treated patients. Additionally the higher Bcl-2:Bax ratios were particularly pronounced in those previously treated patients who were found to be clinically unresponsive to therapy. The *in vitro* response to chlorambucil-induced apoptosis correlates with the Bcl-2:Bax ratio and there appears to be a trend towards an apoptosis-resistant phenotype with treatment (Pepper, 1996, 1997). This apoptosis-resistant phenotype may be due to a degree of variation in the Bcl-2:Bax ratio within the clone and the selection of a resistant population with higher Bcl-2:Bax ratios, but the possibility that it involves the induction of drug resistance cannot be excluded.

Similarly, Western blot analysis and quantification using laser densitometry of Bcl-2 and Bax expression revealed a correlation between 9-amino-20(s)-campothecin- and fludarabine-induced apoptosis and the endogenous ratios of Bcl-2:Bax. B-CLL cells from 21 patients with low Bcl-2:Bax ratios were generally found to be drug-sensitive, compared to those with intermediate-high Bcl-2:Bax ratios, which were resistant to the induction of apoptosis *in vitro*. Prior to the induction of apoptosis, Bax was found to migrate as a single 21 kDa species, but expression was upregulated after drug-induced apoptosis when Bax migrated as complexes of 36-42 kDa. These were disrupted using two-dimensional gel electrophoresis in reducing conditions to reveal that they were comprised of Bax monomers of 18 and 21 kDa. The need for reducing conditions would suggest that disulphide bonds were involved in the formation of the dimers, but the significance of this *de novo* appearance of an 18 kDa Bax species and its increased expression in B-CLL cells undergoing drug-induced apoptosis, remains unclear (Thomas, 1996).

Particularly compelling evidence for the role of the Bcl-2:Bax ratio in determining the cell's sensitivity to apoptosis comes from an analysis of cells from a total of 26 B-CLL patients using antagonistic monoclonal antibodies, the anti-CD6 MoAb, IOR-T1, and anti-IgM. Cells from 10 patients showed a significant increase in the rate of *in vitro* apoptosis from a mean of 13.2% to 39.7% when cultured with anti-IgM MoAb, but the rate of apoptosis was reduced in 8/10 populations co-cultured with anti-IgM and IOR-T1. The induction of apoptosis by anti-IgM in B-CLL cells and the protection offered against it by IOR-T1 appears to be mediated by changes in *bcl-2* and bax_{α} mRNA levels.

In 5 cultures anti-IgM was found to reduce the *bcl-2*/*bax*$_\alpha$ mRNA ratio through a reduction in *bcl-2* mRNA and particularly by an upregulation of *bax*$_\alpha$ mRNA. Simultaneous culture with anti-IgM and IOR-T1 in 4 cases resulted in a significant reduction in *bax*$_\alpha$ mRNA without any change in *bcl-2* mRNA, raising the *bcl-2*/*bax*$_\alpha$ mRNA ratio in a manner that correlated with IOR-T1-mediated protection of anti-IgM-treated cells. In B-CLL cells from 3 patients co-cultured with anti-IgM and IOR-T1, the unchanged *bcl-2* mRNA and reduced *bax*$_\alpha$ mRNA levels were reflected in unchanged Bcl-2 and reduced Bax$_\alpha$ levels. In two further cell cultures, Bax$_\alpha$ levels were higher after culture with anti-IgM than after culture with medium alone (Osorio, 1997).

Further support for the view that Bcl-2:Bax ratios determine the fate of B-CLL cells, comes from studies using B-1 cells derived from New Zealand bald (NZB) mice which serve as a murine model for studying B-CLL. NZB mice cell lines show close parallels to B-CLL in an age-dependant onset of the clonal expansion of malignant B-1 cells, the indolent disease course, increased numbers of lymphocytes and smudge (smear) cells in peripheral blood, chromosomal abnormalities, and a similar pathology with neoplastic cells infiltrating spleen, lymph nodes and peritoneum. Although the B-1 cell line used, LNC, was resistant to the *in vitro* induction of apoptosis with fludarabine at concentrations up to 48 μM, an increase in apoptosis, detected by propidium iodide staining and DNA fragmentation gel electrophoresis, was induced by the mitotic spindle inhibitors colcemid and nocodazole. In the case of colcemid this was preceded by arrest at G2-M phase of the cell cycle. A reduction in the amount of B-cell-specific-activator-protein (BSAP) mRNA (which is constitutively expressed in malignant B-1 cells), accompanied unchanged constitutive expression of *bcl-2* mRNA, but increased levels of expression of *bax* mRNA. This suggests that colcemid- and nocodazole-induced apoptosis is also executed by changing the balance between Bcl-2 and Bax, but the significance of the G2-M arrest and BSAP remains unclear (Zhang, 1998).

Unfortunately not all of the results are consistent with the Oltvai, Milliman and Korsmeyer paradigm. An investigation of 41 cases of B-CLL based on densitometry immunoblot scores, showed that the Bcl-2:Bax ratios were skewed very clearly to ratios of 1 and above and failed to correspond with rates of *in vitro* spontaneous apoptosis. Additionally when the samples were separated into high and low Bcl-2:Bax ratio groups, an unexpected correlation between lower ratios and a failure to achieve complete remission was observed (Kitada, 1998).

If the life span of B-CLL cells is determined by a relatively simple mechanism involving the relationship between the apoptosis-inhibiting Bcl-2 and the apoptosis-inducing Bax, then precisely the opposite relationship might be expected.

However, on balance the evidence is very strongly supportive of the view that higher Bcl-2:Bax ratios are related to progressive disease, previous treatment status, and relative insensitivity to the induction of apoptosis in a way that offers a highly plausible explanation for the difficulty in linking these variables to Bcl-2 levels alone.

8 Causes of the higher level of expression of *bcl-2* mRNA and Bcl-2 in B-CLL.

Aside from attempts to explain the often higher expression of Bcl-2 in B-CLL through stimulation of the cells, attempts to explain the higher level of expression of both *bcl-2* mRNA and Bcl-2 in B-CLL have concentrated most entirely on detecting deregulating translocations of the *bcl-2* gene. The fact that levels of Bcl-2 are clearly modulated in normal cells, e.g. during B-lymphopoiesis, and that a malfunctioning of any of the modulatory mechanisms could just as easily result in pathological expression of the protein, has been largely ignored, as has overexpression of many other Bcl-2 family members. However, although the sample was very small and involved cells from only four patients in which a translocation had been detected, interestingly one study showed that expression of Bcl-2 was stronger in those cells with a translocation, than in those without (Merup, 1996). The commonest technique used to detect translocations is Southern blotting and investigations of the incidence of translocation of the *bcl-2* gene in B-CLL using it have produced contradictory results. A variable degree of rearrangement involving the immunoglobulin heavy chain locus, and light chain κ and λ loci, ranging from 0-12% has been detected, but the extent and importance of translocation in B-CLL remains unclear.

A large study investigated $CD5^+$, $CD19^+$ and $CD23^+$ neoplastic B-cells from 100 B-CLL patients in Rai stages 0-IV for oncogene rearrangements including *bcl-2*. Probes were used for the three known *bcl-2* translocation clusters (Cleary, 1986; Tsujimoto, 1985), the major cluster region (mcr), the minor breakpoint region (mbr) and the cluster region located in, and adjacent to, the 5' region of the gene. Using the probes pFL-1 and pFL-2 on *Bam*HI digests and pB16 on *Hin*dIII digests, no translocations of the *bcl-2* gene were detected, although in view of the possible connection between Bcl-2:Bax ratios and *p53*, it is interesting to note that *p53* mutations were detected in 10/100 samples representing all stages of disease (Gaidano, 1994; Miyashita, 1994).

Several other studies have also failed to detect rearrangements of the *bcl-2* gene in B-CLL. Two separate studies, in which pB3 and p18-4 probes aimed at the mcr and pFL-2 probes aimed at the mbr were used, failed to detect any occurrences of the t(14;18) translocation typical of follicular lymphoma in samples of neoplastic cells from a total of 48 (32+16) B-CLL patients (Adachi, 1990, Hanada, 1993). In another series of experiments the cells from 4 B-CLL patients, in which Bcl-2 levels were found to be preserved and which were resistant to *in vitro* apoptosis, were compared with the cells from 4 patients in which levels of Bcl-2 were rapidly reduced and which were sensitive to *in vitro* apoptosis.

Southern blotting analysis failed to detect any rearrangement of the major breakpoint cluster region in either group. Furthermore, analysis using PCR amplification failed to detect any evidence of rearrangement of the major or minor breakpoint cluster regions (Panayiotidis, 1994). Similarly an earlier study of 38 B-CLL patients also failed to identify any rearrangements of the *bcl-2* gene (Rechavi, 1989).

In keeping with the absence of the t(14;18) translocation in B-CLL shown in the studies referred to above, investigations using Southern blotting which have demonstrated evidence of *bcl-2* rearrangements, have shown only a relatively low incidence ranging from <2%-4%. Analysis of 44 patients with B-CLL using probes for the Ig genes, as well as probes for the mbr, mcr and 5' breakpoint regions associated with the *bcl-2* gene, showed a t(14;18)(q32;q21) rate of 1/44 (~2%) (Raghoebier, 1991). Likewise a very large study of the cells from 170 patients with B-CLL, showed a rate of 3/170 (~2%) with translocation of the *bcl-2* gene to the H-chain locus on chromosome 14, where all three cases had breakpoints in the 3' region of *bcl-2* mapping to the region between the mbr and mcr (Dyer, 1994). The highest detected rate for the t(14;18) translocation using a 1.5 kb pFL-1 probe for the mcr, detected rearrangements in 3/71 (4%) of the B-CLL cells tested, although in 2/3 of these a rearrangement was also detected in the variant cluster region (vcr) flanking the 5' end of the *bcl-2* gene using the 1.2 kb probe pB16 (Merup, 1996).

Higher rates of rearrangement of the *bcl-2* gene are found in relation to the 5' vcr region, which is particularly associated with the t(18;22)(q21.3;q11) and t(2;18)(p11;q21.3) translocations, which translocate the *bcl-2* gene to the Igλ and Igκ loci, respectively. The pB16 probe obtained from the first exon of *bcl-2* detected reciprocal translocations between the *bcl-2* gene and the immunoglobulin light chain loci in 3 of the 32 samples (~10%) obtained from B-CLL patients (although there is a question mark over this statistic as it appears that one of the samples was known to carry the Igλ translocation before the study was carried out, so a more representative occurrence would be 2/31 or ~6%). In two cases the reciprocal translocation involved the Ig λ locus and in the third case the Ig κ locus, with the *bcl-2* gene transposed in a head-to-head configuration. In all three cases the breakpoints on chromosome 18 were clustered in the region flanking the 5' of the *bcl-2* gene between 1100-2200 bp upstream, suggesting the existence of a hotspot for rearrangements in B-CLL in this region (Adachi, 1989a, 1989b, 1990). A slightly higher percentage of rearrangement was detected with the pB16 probe in a larger than average study, involving a total of 96 patients with B-CLL.

Of the 67 patients tested, 8 showed a rearrangement with this vcr probe (12%), including four who showed rearrangement associated with the Ig κ gene (4%) (Merup, 1996). Similarly, probes for the Ig genes and 5' vcr regions, showed gene alteration events in the cells 2/44 (5%) B-CLL patients, with rearrangements of the *bcl-2* 5'-breakpoint region without translocation to the Ig light gene loci (Raghoebier, 1991). Additionally, both 5' and 3' region *bcl-2* probes showed one example of each of the t(18;22)(q21.3;q11) and t(2;18)(p11;q21.3) translocations in 2/170 cases of B-CLL (1%) (Dyer, 1994). Not all studies aimed at the vcr adjacent to the 5' end of the gene show rearrangement of this part of chromosome 18 and a 3.85 kb p18-21H probe failed to demonstrate any evidence of this particular rearrangement in a study of the cells from 16 B-CLL patients (Hanada, 1993).

On the basis of Southern blotting these studies suggest that the incidence of *bcl-2* rearrangement in B-CLL is much lower than that encountered in follicular lymphoma, with a preferential translocation to the Ig L-chain loci, rather than the Ig H-chain locus. However, other techniques such as pulsed-field gel electrophoresis (PFGE) and fluorescence in situ hybridisation (FISH) suggest that these results may not reflect the true incidence or nature of *bcl-2* translocation in B-CLL.

In particular, one investigation found a 100% incidence rate of rearrangements of the *bcl-2* gene in neoplastic cells from 9 B-CLL patients and 2 cell lines, using PFGE to probe fragments varying in length from >50 to 10,000 kb, where no rearrangements had been discovered using Southern blotting. In all 11 samples the 1.6 kb pB16 probe, an *Eco*RI fragment containing the *bcl-2* cDNA that corresponds to the 3' part of the 5' end of the *bcl-2* gene, failed to detect any rearrangement of the *bcl-2* gene in *Hind*III-digested DNA from the B-CLL patients' neoplastic cells using Southern blotting. The same probe also failed to detect any rearrangement in B-CLL cell lines, or lymphoblastoid cell lines (LCLs) established from the B-CLL patients' normal peripheral blood lymphocytes. In contrast, when the pB16 cDNA was used in PGFE to probe large fragments of DNA produced by *Kpn*I or *Sal*I digestion, it showed *bcl-2* rearrangements in fragments of DNA 245-600 kb in length from all 9 of the neoplastic cell samples from the B-CLL patients, as well as the two B-CLL cell lines. Only the normal germ line pattern was detected in *Kpn*I- or *Sal*I-digested DNA from normal controls or the paired normal LCLs of the B-CLL patients, thereby excluding restriction fragment length polymorphisms as the cause of these results (Laytragoon-Lewin, 1998).

Additionally, a study using FISH to detect rearrangement of the *bcl-1*, *bcl-2*, and *bcl-3* genes, along with trisomy 12, showed a higher rate of rearrangement of the *bcl-2* gene with translocation to the Ig heavy chain locus in B-CLL cells, than had been detected by other means. Using a 3.5 kb *Eco*RI, *Hind*III fragment in a pYT13 plasmid as a probe for the JH region, a 4.2 kb *Hind*III fragment in a pYT13 plasmid as a probe for *bcl-2*, and α -satellite D12Z3 as a probe for trisomy 12, showed an incidence of 3/20 (15%) in B-CLL cells for the t(14;18)(q32;q21) translocation, where two of the translocations were also accompanied by trisomy 12 (Lishner, 1995).

Given the restricted ability of Southern blotting to detect gene rearrangements over long distances and the difficulty in obtaining adequate numbers of metaphases from the many clones of B-CLL cells that are unreactive to polyclonal activators such as SAC strain 1 plus IL-2 and TPA, it is not unreasonable to question the ability of this technique to detect all *bcl-2* rearrangements in B-CLL. For example, in a study involving the cells from 30 B-CLL patients, SAC and IL-2 were found to cause proliferation in only the B-CLL cells from the 15 patients with progressive disease and had only a marginal proliferative effect on 1 of 15 patients with non-progressive disease (Aguilar-Santileses, 1994). The ability of Southern blotting to detect *bcl-2* rearrangements is indirectly called into question by the results obtained using FISH (Lishner, 1995), and particularly the results obtained in detecting *bcl-2* rearrangements using PFGE (Laytagoon-Lewin, 1998). The 15% incidence of the t(14;18) translocation demonstrated by FISH is the highest reported occurrence of this translocation in B-CLL. Although the study by Lishner *et al* did not apparently attempt to detect any of 3/20 t(14;18) translocations by Southern blotting, their demonstration that *bcl-1* rearrangements that could not be detected by Southern blotting could be detected by FISH, lends credence to their claim that FISH is a more sensitive technique for detecting this translocation in B-CLL and that, by inference, Southern blotting underestimates the rate of rearrangement of the *bcl-2* gene in B-CLL. This suggestion is particularly well supported by the study by Laytragoon-Lewin *et al* demonstrating *bcl-2* gene rearrangement in 11 B-CLL samples where Southern blotting had failed to detect rearrangements. That PFGE is well-suited to detect *bcl-2* gene rearrangements is also supported by a study using this technique to detect the t(14;18) translocation in follicular and diffuse lymphoma. Using PFGE, Southern blotting, polymerase chain reaction, and cytogenetic analysis, this study showed that this technique was the most effective at detecting the *bcl-2* rearrangement in follicular lymphoma in a series of 40 patients, being the only technique to detect this translocation in one case.

In addition, in a series of seven cases with progression from follicular to diffuse lymphoma, PFGE detected the translocation in all seven cases compared to a detection rate of only 3/7 for Southern blotting (Zelenetz, 1991).

The impression given by studies using Southern blotting, that the *bcl-2* gene rearrangement occurs in only a very small minority of cases of B-CLL and that where it occurs, the *bcl-2* gene is preferentially rearranged to the Igκ and Igλ loci, appears uncertain at best and further large scale studies using the range of techniques available would be needed to resolve the issue. It remains possible that the *bcl-2* gene is rearranged in a much greater number of cases of B-CLL than previously thought and that the rearrangement may not necessarily involve translocation to another chromosome, but a study showing that expression of *bcl-2* can be modulated in B-CLL lines is a reminder that Bcl-2 overexpression in B-CLL may not be entirely due to translocation.

The investigation in question explored the growth- and differentiation-associated expression of *bcl-2* in two B-CLL cell lines, 173 and 183, using different activation protocols. When 173 B-CLL cells were advanced from G_0 to G_1 by TPA and differentiated into IgM-secreting lymphoblasts and plasmablasts, in the absence of DNA synthesis, the steady-state level of *bcl-2* mRNA was rapidly downregulated. Reductions in *bcl-2* mRNA levels were observed within an hour of treatment with maximal response after 3 hours in comparison to steady-state levels. Levels fell to ~35% of control levels, before rebounding to 4-fold control levels at 12 and 72 hours post treatment. Similarly, when the 183B-CLL cell line was subjected to mitogenic protocols including treatment with various combinations of TPA, SAC, IL-2, TNF-α, IL-4 and B-cell stimulatory factor from the T-cell hybridoma, MP6, (BSF-MP6), reductions in both *bcl-2* mRNA and Bcl-2 were observed. This was particularly true where high DNA synthesis was observed, suggesting that *bcl-2* expression was inversely related to proliferation in this 183 cell line (Schena, 1992). It is difficult to envisage how a rearranged *bcl-2* gene could be modulated in this way during growth or differentiation, suggesting that in some B-CLL cells at least, a mechanism other than deregulation of the gene as a result of rearrangement may be responsible for the aberrant expression of *bcl-2*.

One mechanism suggested to explain the high levels of expression of Bcl-2 in B-CLL without apparent rearrangement of the gene, involves its hypomethylation. This was investigated in a study that analysed the structure, methylation status, and expression of the *bcl-2* gene in 20 patients with a lymphoproliferative neoplastic disease, including 17 diagnosed as having B-CLL.

Twelve of the cases of B-CLL showed expression of p26-Bcl-2 protein that was roughly equal to, or greater than the level of Bcl-2 protein expressed by the t(14;18)-containing RS11846 B-lymphoma cell line. There was no evidence in any of the 17 B-CLL samples of rearrangement of the *bcl-2* gene using a 3.85 kb *Xho*I to *Hind*III fragment from p18-21H for the 5' breakpoint region, a 4.4 kb *Hind*III fragment from p18-4 for the 3' mcr, a 4.5 *Eco*RI fragment from pFL-2 for the mbr, or a 2.8 kb *Mbo*I fragment from pKB-Mbol 2.8 for human mitochondrial DNA. However, investigation of the methylation status of the *bcl-2* gene using a probe corresponding to the first exon of the gene and the isoschizomers, *Msp* I and *Hpa* II, demonstrated a complete absence of methylation in the region of the *bcl-2* gene corresponding to the 2.4 kb *Msp* I fragment. This applied to all 20 samples including the cells from the 17 B-CLL patients. In contrast, the results from 6/6 cells from B-cell lines that display a t(14;18) translocation suggested hypomethylation of only one allele and neither copy of the *bcl-2* allele was hypomethylated in the region corresponding to *Msp* I in 5/5 lymphoid cell lines that lack the t(14;18) translocation (Hanada, 1993). Whilst such results are not conclusive they do open up the possibility of trans-acting mechanisms causing elevated levels of transcription of the gene through a mechanism that involves hypomethylation, as opposed to the cis-regulating mechanisms that are thought to apply in *bcl-2* gene rearrangements.

9 Modulation of the level of Bcl-2 family members by extra- and intracellular influences.

9.1 Introduction.

When the cells from B-CLL patients are cultured *in vitro*, they are generally found to die apoptotically (Collins, 1989; Buschle, 1993), suggesting that culture systems lack essential survival factors that are present *in vivo*. Over a significant number of years the factors that have been shown to influence the life span of B-CLL cells *in vitro* include soluble and cell membrane bound factors as well as P53. These include the cytokines IL-4, IL-5, IL-8, IL-10, IFN-α, IFN-γ, and bFGF, as well as CDw32, BM stromal cells, CD95/APO-1/Fas, CD40, and CD6.

9.2 IL-4

IL-4 in particular appears very effective in protecting B-CLL cells from *in vitro* apoptosis, but not all studies agree as to its effect or that it does so by modulating the level of Bcl-2. For example, B-CLL cells isolated from 12 patients and assessed for apoptosis by trypan blue exclusion, were found to be protected from spontaneous and hydrocortisone-induced apoptosis when rIL-4 at 10 ng/ml was added to the culture conditions. A measure of the degree of protection is indicated by the fact that cells cultured with HC showed the highest rates of apoptosis, but culture with HC and rIL-4 produced lower rates of apoptosis than when B-CLL cells were cultured in medium alone. The rate of apoptosis appears to be associated with Bcl-2 expression, which is upregulated in those cells treated with rIL-4, with treated cells expressing significantly more protein than unstimulated, HC-treated or freshly isolated B-CLL cells. Furthermore the effect of rIL-4 is supported by an experiment in 1 patient where subcutaneous administration of IL-4 at 100μg/m^2 increased Bcl-2 expression in B-CLL cells *in vivo* (Danescu, 1992).

Support for the view that IL-4 improves viability of B-CLL cells *in vitro* by increasing Bcl-2 levels, comes from a study involving 24 B-CLL patients. B-CLL cells were ~33% apoptotic after 26-30 hours in *in vitro* culture, which increased to 90-100% after 8-10 days. At concentrations of 0.5-5 ng/ml, human recombinant IL-4 reduced the rate of apoptosis without stimulating cell proliferation and viable cells could be maintained in culture for up to 3 weeks, e.g., at 5 ng/ml, IL-4 reduced apoptotic DNA fragmentation to a mean of 14.9% after 26-30 hours.

All 11 patients analysed by Western blotting showed expression of Bcl-2, but during culture this was only preserved in cells resistant to apoptosis and reduced in susceptible cells. Bcl-2 loss was inhibited in cells cultured with IL-4, suggesting once more that this cytokine may play a part in the extended survival of B-CLL cells (Panayiotidis, 1993).

Although theophylline and chlorambucil apparently induce *in vitro* apoptosis in B-CLL cells by different pathways/mechanisms, they appear to do so by reducing expression of *bcl-2* mRNA and Bcl-2, which can be reversed by culturing the cells with IL-4. Theophylline -induced apoptosis involves an accumulation of cyclic adenosine monophosphate, in contrast to the Ca^{2+} mobilisation and influx induced by chlorambucil. However, both compounds induce apoptosis and their actions are synergistic allowing fourfold lower concentrations to be used in combination. Significant amounts of *bcl-2* mRNA and Bcl-2 were expressed in the 15 B-CLL patients tested and incubation with theophylline and/or chlorambucil reduced expression of *bcl-2* mRNA and Bcl-2. However, the effect of these cytotoxic drugs on *bcl-2* expression is partially reversed by IL-4 at 10 ng/ml (Mentz, 1996).

The effect of IL-4 on the rate of apoptosis in B-CLL cells appears to be in keeping with the expression of IL-4 receptors. Using flow cytometry, expression of IL-4 receptors on cells from 4 B-CLL patients was found to be uniform and significantly increased over background fluorescence, in comparison to IL-4 expression in 3 samples of normal $CD19^{+}$ peripheral blood B-cells, where fluorescence was minimal. In keeping with this difference in IL-4 receptor expression, culturing B-CLL cells with 10 ng/ml of IL-4 significantly reduced the amount of apoptosis in 9/11 samples after culture for 48 hours from ~34% in medium alone to ~10%. In contrast, IL-4 at 10 ng/ml had only a marginal effect on normal cells, reducing apoptosis from a mean of 24±4% to 18±3% in the 7 samples tested (Douglas, 1997).

Evidence for IL-4 exerting its anti-apoptotic effect via Bcl-2 is not unanimous. As might be expected, given that treatment appears to induce an apoptosis-resistant phenotype or drug resistance (Pepper, 1996, 1997), IL-4 at 0.01, 0.1 and 1.0 ng/ml protected B-CLL cells from 11 patients who had previously been treated with cytotoxic drugs, e.g. chlorambucil, from apoptosis more effectively than B-CLL cells from 14 untreated patients. Rates of *in vitro* apoptosis were comparable in the two groups cultured in medium alone, but IL-4 concentration had to be increased from 0.01 ng/ml in the treated patients to near 1.0 ng/ml in untreated patients to achieve the same protection.

In this study IL-4 did not appear to modulate sensitivity to apoptosis through Bcl-2 levels. Bcl-2 levels measured by indirect immunofluorescence staining using an anti-Bcl-2 MoAb, FITC-labelled anti-mouse Ig, and flow cytometry in 5 patients whose B-CLL cells were sensitive to the anti-apoptotic effect of IL-4, showed no difference when cells were cultured in the presence or absence of IL-4 (Frankfurt, 1997).

Paradoxically, IL-4 has been associated with an increase in chemically-induced apoptosis in one study, where IL-4 was found to significantly enhance the viability of B-CLL cells in culture for periods up to 96 hours compared to control cultures, with an optimal concentration of 50 U/ml. However, it was found to increase the sensitivity of B-CLL cells to melphalan (L-phenylalanine mustard)-induced apoptosis. The increased rate of apoptosis was accompanied by interstrand-DNA cross-linking in B-CLL cells following short term exposure to the compound. It remains unclear if this suggests that IL-4 does not modulate Bcl-2 levels or whether, by a separate pathway, apoptosis can be induced independently of Bcl-2 levels (Pu, 1997). The separate pathway explanation is certainly plausible as apoptosis induced in B-lymphoid cell lines through CD95 ligation and the FADD/caspase-8 pathway appears to be relatively immune to the expression of Bcl-2 (Adams, 1998), but it remains to be seen if melphalan induces this pathway.

A study to determine the effect of IL-5 on the rate of *in vitro* apoptosis in B-CLL cell cultures, casts further doubt on whether IL-4 exerts its anti-apoptotic effect on B-CLL cells through a simple variance of Bcl-2 levels, since IL-5 was found to increase the rate of apoptosis in B-CLL cells in a manner that does not involve modulation of the Bcl-2 level, but which is partially reversible by IL-4. When purified B-CLL cells from 13 patients were incubated in medium supplemented with 20 U/ml of IL-5, spontaneous apoptosis increased by 53% after 2 days of culture and 37% after 3 days in comparison to cells cultured in medium alone. In half the cases studied the increase in apoptosis, which was dose-dependent, was as great as that induced by dexamethasone of 25 μM. In the five cases examined, Bcl-2 expression quantified by flow cytometry was not found to be significantly influenced by incubation with IL-5. IL-4 partially protected cells against IL-5-induced apoptosis in a manner that could be inhibited by anti-IL-4 MoAb, and such protection suggests that IL-4's protection of B-CLL cells is not via a simple link with Bcl-2 levels (Mainou-Fowler, 1994).

9.3 IL-8

IL-8 also appears to be capable of prolonging the *in vitro* life of B-CLL cells and to do so in an autocrine fashion. When the leukaemic cells from 16 B-CLL patients were cultured with IL-8 at concentrations of 5 & 50 ng/ml, 11/16 showed a reduced rate of apoptosis as measured by propidium iodide staining and flow cytometry. Simultaneous incubation with an anti-IL-8 MoAb reversed the apoptosis-inhibiting effect of the cytokine. In the remaining 5 cases the percentage of surviving cells was so spontaneously high (90.6%) after 2 days that the effect of IL-8 could not be tested. Incubation of the cells from these 5 patients for 2 days with 10^{-4} mol/L of hydrocortisone reduced the percentage of surviving cells to 55%, but addition of IL-8 at 5 ng/ml to the culture conditions was able to effect a significant rescue of the cells. IL-8 failed to induce any *in vitro* proliferative effect as measured by ^{3}H-thymidine (^{3}H-TdR) incorporation and there appeared to be no synergy between IL-8 and IL-4 or IFN-α in preventing apoptosis. Of particular interest, all of the 9 B-CLL samples tested constitutively expressed *IL-8* mRNA and released between 0.3 and 6.5 ng/ml. The presence of the 439 bp IL-8 receptor mRNA was detected in 7/9 cases and B-CLL cells maintained stable expression of this after 2-3 days of culture. Additionally, the presence of the IL-8 receptor on the cell surface was found to be positive on 10/10 samples tested over 6 days of culture, using cytofluorometric detection of phycoerythrin-conjugated IL-8. Perhaps most interesting of all was the increase in cellular IL-8 mRNA in 10/12 B-CLL samples when the cells were cultured in 5 ng/ml of IL-8. Constitutive expression of *bcl-2* mRNA was detected in 7/7 cases tested using RT-PCR and incubation of the cells with IL-8 resulted in an increase in both *bcl-2* mRNA and Bcl-2 protein (Francia di Celle, 1996). Given that the dose range of IL-8 capable of producing the prolonged survival of B-CLL cells is comparable with the levels of IL-8 released constitutively by these cells, it appears possible that IL-8 may play a significant role in prolonging the life span of B-CLL cells in an autocrine fashion and may do so by increasing levels of Bcl-2.

9.4 IL-10

The effect of culturing of B-CLL cells with IL-10 is unclear, with studies showing widely conflicting results. For example, culture of 5 B-CLL samples with IL-10 increased the rate at which cells were lost in a dose-dependent manner, with all samples showing a 2- to 4-fold increase in apoptotic cells, measured using Hoechst 33342, propidium iodide and flow cytometry. After 1 week B-CLL populations cultured with IL-10 were lost, whilst those cultured without it survived.

Treatment with IL-10 also inhibits spontaneous DNA synthesis in B-CLL cells. In the 4/12 cells that spontaneously incorporated significant amounts of ^{3}H-TdR (2,800-5,700 cpm), incorporation was reduced by 54-96% after 3 days of culture in a dose-dependent manner. The IL-10-induced increase in apoptosis appears to correlate with Bcl-2 levels, which freshly isolated B-CLL cells were found to express at high levels in a unimodal fashion. After four days of culture in medium this changed to a bimodal pattern, with 74% of the cells continuing to express Bcl-2 at high levels and 26% expressing low levels. A culture repeated with medium plus IL-10 resulted in a decrease in Bcl-2 expression, with 83% of cells expressing low levels and only 17% expressing high levels. The addition of a neutralising anti-IL-10 monoclonal antibody, IL-2, IL-4, IFN-γ and anti-CD40 monoclonal antibody, was able to counteract the apoptosis-inducing effect of IL-10 (Fluckiger, 1994).

However, further experiments failed to support these findings. B-CLL cells from 9 patients were found to express between 47-127 IL-10 receptor sites per cell, whose binding specificity of IL-10 was reflected in a dissociation constant estimated as ranging from 168-426 x 10^{-12} mol/L. Binding of IL-10 to its receptor on B-CLL cells results in a pattern of phosphorylation of signal transducer and activators of transcription 1 (STAT1) and STAT3 proteins that is identical to that seen in normal T-cells and monocytes and similar to IFN-α (STAT1 & 3) and IFN-γ (STAT1) receptor activation in B-CLL. Interestingly, at concentrations above 10 ng/ml, IL-10 consistently and significantly inhibited, in a dose-dependent manner, TPA-induced proliferation of B-CLL cells as measured by ^{3}H-TdR incorporation (cells from 5 patients cultured for 7 days). IL-10 had the opposite effect on the differentiation of B-CLL cells from 10 patients. Incubation of B-CLL cells in the presence of IL-10 and TPA was shown to significantly enhance the production of IgM compared to those cells incubated with TPA alone. However, in contrast to the study by Fluckinger, which suggested that IL-10 induces apoptosis in B-CLL cells associated with a reduction in cellular Bcl-2 levels, no increase in the rate of apoptosis was detected in those cells cultured with the cytokine. When cells from 7 B-CLL cases were cultured in 25 ng/ml of IL-10 for 24 hours there was no increase in apoptosis detected compared with cells cultured in medium alone. Similar results were obtained when culture was extended in 3 cases from 24 to 72 hours. There was no statistically significant increase in the rate of apoptosis in 6 cell samples cultured with IL-10, but there appeared to be a trend towards improved survival and in individual cases cell survival was clearly higher. Furthermore, IL-10 induced an increased rate of cell survival comparable to that of IFN-γ, when used at 25 ng/ml to counteract the effect of HC at a concentration of 5 x 10^{-4} mol/L.

As one might expect in a study showing no statistically significant improvement or loss of cell viability, no change in Bcl-2 levels was induced by IL-10. The authors suggest that the contrast between their study and that of Fluckinger is explained by the different methods of cell selection and cryopreservation, the latter being reported to induce a substantial reduction in IL-10 binding specificity compared to freshly isolated cells (Jurlander, 1997).

Furthermore, a third study suggests that IL-10 increases B-CLL cell viability *in vitro*. In an investigation using cells from 4 B-CLL patients, only 3 of which were $CD5^+$, blood was obtained and PBMC were isolated by Ficoll/Hypaque density gradient centrifugation. No further purification was undertaken on the basis that in all four cases the leukaemic cells accounted for >90% of the blood lymphocyte population. In comparison to *in vitro* culture of PBMC from B-CLL patients in medium alone, and the culture of PBMC cells from normal individuals with IL-10, which showed no improvement in survival, culture of PBMC from B-CLL patients with IL-10 at 1.0-50 ng/ml for between 10-21 days offered statistically significant increased survival, suggesting that IL-10 offers protection from apoptosis. In culture, the PBMC from B-CLL patients were found to spontaneously produce IL-10 using ELISA, in comparison to the sera from normal individuals in which IL-10 was undetectable. The authors suggest that this is evidence of the autocrine involvement of IL-10 in extending the life span of B-CLL cells *in vitro*, but given the absence of any attempt to further purify the PBMC there can be no certainty that IL-10 is being produced by B-CLL cells. Bcl-2 expression was examined by flow cytometry and Western blotting without detecting any difference in its expression in PBMC cultured with and without IL-10 (Kitabayashi, 1995).

Doubts about IL-10's ability to increase the rate of *in vitro* apoptosis observed in B-CLL cells are also raised by a study attempting to determine the effect of BM stromal cells on the survival of B-CLL cells. In a study of cells from 9 B-CLL patients, where apoptosis was shown to be increased from ~22% to 42±5% when cells were cultured in BM stromal cell conditioned medium, the use of an anti-IL-10 antibody failed to prevent the increase, suggesting that IL-10 was not instrumental in mediating it (Lagneaux, 1998).

Given the small sample size of some of these studies, the limited degree of cell purification in one study, the conflicting evidence of the effect of IL-10 on cell viability and particularly its effect on Bcl-2 levels, it is difficult to draw any clear conclusions from the results, but on balance IL-10 appears not to reduce the viability of B-CLL cells *in vitro*.

However, although no attempt was made to relate IL-10 to the expression of Bcl-2 family proteins, a further series of studies of the effect of IL-10 on B-CLL cells, throws these inconsistencies into even sharper contrast.

A study of the B-CLL cells from 35 patients which sought to relate the expression of IL-10 mRNA to disease progression, came to the conclusion that the expression of IL-10 mRNA was inversely related to disease progression in an autocrine fashion. Transcripts of the gene were detected by RT-PCR in 11/20 patients with non-progressive disease, but in only 2/15 with progressive disease. Disease progression being defined by a progression in the previous 3 months of disease-related anaemia (haemoglobin < 100 g/l), thrombocytopenia (platelets < 100 x 10^9/l), enlargement of spleen/liver/lymph nodes ≥ 25%, doubling of blood lymphocyte counts, and/or the appearance of constitutional symptoms (Sjöberg, 1996). Although the authors suggest that "effective autocrine loops may be generated by intracellular cycling of cytokines", no attempt was made to demonstrate production of IL-10 by these cells, to determine the serum levels of IL-10 in these patients, or to show the *in vitro* effect of IL-10 on these neoplastic cells, and the results are called into question by two other studies. In contrast a study of 123 serum samples from 97 B-CLL patients showed no correlation between disease progression (which the authors failed to define) and IL-10 serum levels. However, a correlation was noted between IL-10 serum levels and the Rai stage, with higher levels detected in the sera of patients with Rai stage 3-4, compared to levels in Rai stage 0-2 (Egle, 1996). Notwithstanding the failure of this study to detect a statistical correlation between disease progression and IL-10 sera levels, at face value the detection of higher levels of IL-10 in the cells from patients with B-CLL Rai stage 3-4 rests very uneasily with the lower levels of IL-10 mRNA found in patients with progressive disease reported by Sjöberg *et. al.* Furthermore, another study comparing the serum IL-10 levels from 55 HIV-negative B-CLL patients with those of 48 healthy donors, was unable detect any statistically significant difference in the B-CLL group when analysed by Binet stage or between the two groups (Knauf, 1995).

9.5 IFN-α

Studies investigating the effect of IFN-α on Bcl-2 levels in, and the rate of *in vitro* apoptosis of B-CLL cells, have also produced inconsistent results. Despite having been used at one time as a treatment for B-CLL, IFN-α has also been shown to reduce the level of apoptosis in B-CLL cells and to preserve the *ex vivo* levels of Bcl-2.

Apoptosis was observed in 9/16 samples of B-CLL cells after 30 hours of *in vitro* culture, but this was reduced from a mean of 36.8% apoptotic cells to 16.8% when the cells were cultured in medium containing 10^3 U/ml IFN-α. Additionally, culture of the cells with IFN-α protected the leukaemic cells from hydrocortisone-induced apoptosis. Bcl-2 levels were found to be preserved in cells from patients resistant to apoptosis for up to 30 days of culture in medium alone, but levels of Bcl-2 were rapidly reduced in cells sensitive to apoptosis. The loss of Bcl-2 was inhibited by culture with IFN-α (Panayiotidis, 1994).

These results were confirmed by a study of 12 B-CLL patients, none of whom showed any evidence of a t(14;18) translocation, which indicated that IFN-α increases cellular levels of Bcl-2 *in vitro* and *in vivo* and protects cells from apoptosis *in vitro*. Cells from 10 patients with different stages of disease were incubated in medium alone and with IFN-α for 18 hours, before being analysed for Bcl-2 using indirect immunofluorescence and flow cytometry. Cells incubated in medium alone were 79.7±8.4% positive for Bcl-2 with a mean cell fluorescence of 24.8±6.2. Cells incubated with 500 U/ml of IFN-α showed significant increases in Bcl-2 as indicated by 94.6±3.2% being Bcl-2-positive with a mean cell fluorescence of 49.6±5.8. The IFN-α-induced increase in Bcl-2 was accompanied by reduced rates of apoptosis, including that induced by incubation with 10^{-5} M of HC and exposure to 1500 rads of γ-radiation before incubation. The rates of a DNA fragmentation after 18 hours, as quantified by a modified diphenylamine reaction in which the DNA in the supernatant was calculated as a % of the total DNA in the supernatant and pellet, was 35.6±10.3% in medium alone, 63.9±12.6% in medium plus HC and 80.0±2.9% when pre-γ -irradiated. These rates were reduced respectively to 6.6±5.8%, 10.8±4.5% and 5.4± 1.6%, when IFN-α was introduced to the culture conditions. However, the use of IFN-α to treat B-CLL suggests that any relationship between IFN-α, Bcl-2 levels and sensitivity to apoptosis is not a simple one. Anomalously, and in contrast to the reduction in white blood cell counts that are detected when the disease is treated with IFN-α, 8 B-CLL patients receiving 3 megaunits of IFN-α subcutaneously 3 times weekly for 3-6 weeks, showed an increase in Bcl-2 levels in cells isolated after treatment compared to those detected before administration of IFN-α, (Jewell, 1994).

Although IFN-α was found to reduce the B-CLL cell burden when used to treat one patient (3×10^6 3 times weekly), it was also found to protect B-CLL cells from *in vitro* apoptosis in 6/8 patients. 16% of cells cultured for 96 hours with the 2b isoform of IFN-α exhibited signs of apoptosis measured by propidium iodide labelling in flow cytometric analysis and DNA fragmentation assays, compared to to 70% in control cultures.

The results were not dependent on type and the 2a isoform of IFN-α produced similar results. However, in disagreement with the two other studies quoted, no link was detected between the IFN-α-induced lower rates of apoptosis and maintained or increased levels of Bcl-2. Indeed lower basal levels of Bcl-2 were detected after 24, 48 and 72 hours of incubation with IFN-α (Chaouchi, 1994).

9.6 bFGF

Basic fibroblast growth factor (BFGF) also protects cells from B-CLL cell lines from fludarabine-induced apoptosis in a manner that appears to involve the upregulation of *bcl-2* mRNA and Bcl-2. Cells from the EBV-transformed cell line 183CLL and cells from the EBV-negative WSU-CLL were incubated with 100 μM fludarabine alone or fludarabine and bFGF at 100 ng/ml. Incubation with fludarabine alone resulted in a reduction in cell viability as measured by trypan blue exclusion compared to control cultures. Addition of bFGF to the culture conditions resulted in both a delay in the onset of, and reduction in the percentage of cells undergoing apoptosis. Protection from apoptosis appears to be associated with upregulation of *bcl-2* mRNA expression and the 183CLL cell line, which expresses low amounts of steady-state *bcl-2* mRNA, showed a significant upregulation of the 8.5 kb transcript after stimulation with 10 ng/ml bFGF. A significant accumulation of the transcript was detected after 2 hours, with maximal response at 24 hours. No difference in the half-life of *bcl-2* mRNA from bFGF-stimulated and unstimulated 183CLL cells was detected. Similarly, increased expression of Bcl-2 was detected in 183CLL cells, WSU-CLL cells and cells from three B-CLL patients purified by Ficoll-Hypaque density gradient centrifugation only, after exposure to bFGF. Densitometric analysis of protein bands indicated that bFGF induced a 1.5- to 2.3-fold increase in Bcl-2 levels (König, 1997b).

9.7 *p53*.

Significant evidence exists to suggest that the Bcl-2:Bax ratio can be modulated either directly or indirectly by the *p53* gene product, but the evidence for its involvement in the pathogenesis of B-CLL is unfortunately both limited and contradictory. For example, evidence in support of *p53*'s influence on Bcl-2:Bax ratios comes from studies using M1 myeloid leukaemia cells and mice. Induction of a temperature-sensitive form of the tumour suppressor *p53* gene in transfected M1 cells, a murine myeloblastic leukaemia cell line that contains no P53 protein, reduces *bcl-2* mRNA to less than 10% of normal levels after 19 hours.

In contrast the level of *bax* mRNA is rapidly increased after 4 hours. However, the actual impact of induction of this gene may be underestimated, as the semi-quantitative RT-PCR method used to measure *bcl-2* mRNA levels and the Northern blotting analysis of *bax* mRNA take no account of the many cells with low Bcl-2:Bax ratios that may have undergone apoptosis before analysis. Measurement of the levels of Bcl-2 and Bax in *p53*-deficient mice homozygous for *p53* gene disruptions (-/-) indicate that the loss of *p53* appears insufficient to cause elevations of Bcl-2 and reductions in Bax in all tissues *in vivo*, but it appears to exert an influence in several tissues including lymph nodes, spleen, epithelial cells of the prostate, several neuronal cell populations, and thymocytes. For example, in the thymus Bcl-2 and Bax expression was largely confined to medullary cells with little staining of cortical thymocytes observed in either *p53*-deficient or normal mice. However, in the *p53*-deficient mice, Bcl-2 immunostaining of medullary thymocytes was significantly stronger whilst that of Bax was weaker. The absence of any effect on Bcl-2 levels in certain murine tissues such as liver, heart, and skeletal indicates that *p53* is not the only determinant of Bcl-2 and Bax levels in certain tissues, but such limitations do not negate the findings of this study (Miyashita, 1994).

Further evidence in support of the *p53* gene's ability to influence the Bcl-2:Bax ratio comes from an investigation using M1 myeloid leukaemia cells. These were stably transfected with a plasmid to establish an M1p53 cell line which expresses wild-type (wt) *p53* under the control of a temperature-sensitive transgene, the effect of which was compared to untransfected M1 myeloid leukaemia cells exposed to TGFβ1. Activation of the wt *p53* function at the permissive temperature of 32.5°c resulted in the apoptotic death of the entire population of 0.15×10^6 within 24 hours. Treatment of M1 cells with 10 ng/ml of TGFβ1 also resulted in apoptotic death, but the process was much slower than with induction of apoptosis via the temperature-sensitive *p53*, and was incomplete after 5 days. Bcl-2 expression was rapidly downregulated in M1 cells following induction of apoptosis with both the temperature-sensitive *p53* and TGFβ1. However, the rapid 20-fold increase in the level of *bax* mRNA seen in the presence of the protein synthesis inhibitor cycloheximide, and the 10-fold increase in the level of Bax, seen in response to *p53* activation, is not detected with TGFβ1. Ectopic expression of Bcl-2, using recombinant plasmids constructed and packaged as infectious amphotrophic retroviruses, blocks apoptosis in M1 cells induced by TGFβ1, but only delays apoptosis induced by temperature-activated *p53*. This would appear to suggest that Bax is an immediate early response target gene for P53 and assuming that the mechanism functions similarly in B-CLL cells, adds weight to the view that their fate is determined by the balance between inducible Bcl-2 and Bax (Selvakumaran, 1994).

Although no attempt was made to measure the relative levels of Bcl-2 and Bax, support for the theory that *p53* induces apoptosis through manipulation of the relative Bcl-2 and Bax levels comes from a study indicating that p53-induced apoptosis can be overcome by overproduction of Bcl-2. About 90% of cells from a V-myc-induced T-cell lymphoma cell line died apoptotically within 3 days of the induction of a transfected and temperature-sensitive wt *p53* gene. However, in two double transfectant clones containing also a retrovirally-driven *bcl-2* construct, Bcl-2 was expressed at high levels and apoptosis was almost completely inhibited. A further clone expressing Bcl-2 at lower levels partially protected the T-cell lymphoma cell line from *p53*-induced apoptosis (Wang, 1993). This response is certainly compatible with *p53* causing apoptosis through its effect on Bcl-2 and Bax, but unfortunately the evidence for its involvement in B-CLL in this manner is both scanty and contradictory.

Circumstantial evidence in support of *p53*'s involvement in the control of apoptosis in B-CLL comes from a study involving 30 B-CLL patients. 15 of these, who were defined as having non-progressive disease and 15 having progressive disease, showed a clear correlation between progressive B-CLL and aberrant expression of p53 protein using both enzyme-linked immunosorbent assay (ELISA) and FACS. In contrast, the leukaemic cells from 15/15 non-progressive patients, normal resting B-cells and activated B-cells, were all found to be negative for p53 protein using the PAb260 monoclonal antibody. However, 9/15 samples from progressive B-CLL patients were found to be positive for this antibody which recognises a conformational alteration which is normally characteristic of mutant p53 protein. The levels of *p53* mRNA were similar in both groups, so it remains possible that the aberrant expression of PAb260-recognised P53 protein arises from post-transcriptional modifications that induce functional and conformational changes. However, this does not entirely negate the fact that an apparently abnormal form of P53 was expressed in the B-CLL cells from patients with progressive disease (Aguilar-Santileses, 1994).

The influence of the *p53* gene in B-CLL is contradicted by a further two studies which failed to find a connection between Bcl-2:Bax ratios and *p53* mutations, or between levels of *p53* mRNA detected and *bax* mRNA levels, *bcl-2:bax* mRNA ratios, or disease progression. When the neoplastic cells from 21 B-CLL patients were studied to determine if there was any connection between the mutant form of P53 and Bcl-2/Bax levels and sensitivity to 9-amino-20(s)-campothecin- or fludarabine-induced apoptosis, none could be detected. The 7/21 patients with a detected *p53* mutation displayed highly variable levels of Bcl-2/Bax, as did the 14/21 with wt P53.

A relationship between high Bcl-2:Bax ratios and resistance to 9-amino-20(s)-campothecin- or fludarabine-induced apoptosis was detected in 7/8 samples, but the resistance to induced apoptosis was unrelated to P53 status (Thomas, 1996) Similarly, when the levels of *p53* mRNA in B-CLL cells from 38 patients were analysed by competitive PCR, there was no obvious relationship between the absolute *p53* mRNA values and *bax* mRNA levels, *bcl-2:bax* mRNA ratios, or between leukaemic cells from patients with progressive and non-progressive disease (Aguilar-Santileses, 1996).

Some explanation for these contradictory results may come from a study which found that the leukaemic cells from 15 B-CLL patients expressed significant amounts of *bcl-2* mRNA and Bcl-2 on isolation, but after incubation with theophylline, chlorambucil and particularly theophylline and chlorambucil in combination, this was reduced and accompanied by significant increases in apoptosis. However, theophylline and chlorambucil have opposite effects on P53 expression. Incubation with theophylline reduced expression of P53 by a mean of 68%, in contrast to incubation with chlorambucil and to a lesser extent theophylline and chlorambucil, which increased levels of P53 by a mean of 45% and 20% respectively. Although no attempt was made to relate P53 levels to the Bcl-2:Bax ratio, the theophylline-induced reduced expression of P53 and Bcl-2, combined with the chlorambucil-induced increased level of P53 and reduced level of Bcl-2, suggests that there may be P53-dependent and P53-independent pathways to influence the Bcl-2:Bax ratio (Mentz, 1996). Such a phenomenon would offer a possible explanation for the contradictory results from the other studies referred to in previous paragraphs.

The evidence available is too contradictory to enable any meaningful conclusions to be formed, but given that *p53* is reported involved in over 50% of all neoplasias, the matter is clearly of such potential importance in the pathogenesis of B-CLL as to warrant more definitive and exhaustive investigation.

9.8 BM stromal cells

Culturing cells from B-CLL patients with human BM stromal cells reduces the rate of apoptosis *in vitro* in a manner that correlates with maintenance of Bcl-2 levels. When B-CLL cells were cultured in medium alone they were 22±3% and 21±6% apoptotic after 48 hours in two separate tests, but when cultured directly on normal BM stromal cells apoptosis was reduced to 8±2% and Bcl-2 expression was preserved.

In contrast when a microporous membrane was used to separate the B-CLL and stromal cells, the rate of apoptosis increased from ~22% to 38±7%. The rate of apoptosis was similarly increased to 42±5% in B-CLL cells cultured in stromal cell conditioned medium. These results suggest that culture on BM stromal cells inhibits apoptosis, but that BM stromal cells release a soluble factor which promotes apoptosis. Use of antibodies subsequently excluded the possibility of this soluble factor being IL-6, IL-10 or TGF-β. Use of antibodies similarly showed that adhesion of neoplastic B-CLL cells to stromal cells is partially mediated by the β1 and β2 integrins acting simultaneously, in particular CD18/CD11a and CD49d, and that the lower survival rate of umbilical cord blood B-cells cultured on BM stromal cells could be explained by a lower rate of adhesion. Similarly the β1/ β2 integrin ligands on stromal cells, ICAM-1 (CD54) and VCAM-1 (CD106), are simultaneously involved, but the fact that the complete blocking of adhesion of B-CLL cells to stromal cells could not be achieved by targeting these CDs with antibodies, suggests that other mechanisms are involved in the adhesion process (Lagneaux, 1998). A study described in detail para 9.12.2 suggests that IL-7 may mediate apoptosis reduction caused by ligation of the β2 integrin.

Given the propensity of B-CLL to invade the BM it is particularly interesting that BM stromal cells may play an important role in the survival and accumulation of neoplastic B-CLL cells. Although no attempt was made to relate survival to Bcl-2 levels, the ability of BM stromal cells to promote cell survival is further supported by a study of cells from 10 patients. In 10 B-CLL cell cultures using medium alone, only a mean of 14.7% of the cells remained viable after 10 days of culture and all cells were dead after 30 days. In comparison, a mean of 47% of the B-CLL cells cultured on BM stromal cells obtained from normal BM donors were alive after 10 days of culture and after 30 days of culture between 12-65% of the cells from 7/10 cultures remained viable. Adherence to BM stromal cells again appeared critical for the anti-apoptotic effect and separation of the B-CLL cells from the BM stromal cells with a 0.45 μM culture filter resulted in the anti-apoptotic effect being lost. Culture on BM stromal cells offers similar protection from HC-induced apoptosis. In medium plus HC, a mean of only 2.4% of the cells were alive after 10 days of culture and all were dead after 30 days. In comparison a mean of 20.5% and 5.8% respectively were alive when cultured directly on BM stromal cells plus HC (Panayiotidis, 1996).

9.9 CD95/APO-1/Fas and CD40.

Although ligation of CD95 appears to induce apoptosis by a Bcl-2-independent route in lymphoid cells, the effect of Bcl-2 family members on CD95-induced apoptosis in B-CLL cells remains very unclear with studies showing widely differing results. However, the connection between Bcl-2 family members and CD95-induced apoptosis is of particular interest, as a means of inducing apoptosis in Bcl-2-expressing B-CLL cells that is independent of Bcl-2, is at least as interesting as one that is not.

A series of studies using lymphoid cells from mice and investigating CD95-induced apoptosis via the FADD/caspase-8 apoptotic pathway, suggests that Bcl-2 does not inhibit CD95-induced apoptosis. In CD95-induced apoptosis in B-lymphoid cell lines, thymocytes, and activated T-cells, Bcl-2 expression is not downregulated, Bax and Bcl-x_S are not upregulated, and Bcl-2 offers very little protection from apoptosis. In contrast expression of the cowpox virus caspase-inhibiting cytokine response modifier A (crmA), blocks apoptosis induced by CD95 ligation, but not that induced by γ-irradiation, corticosteroids or serum deprivation, which are blocked by Bcl-2. Furthermore, transgenically expressed Bcl-2 markedly augments the survival and abnormal accumulation of lymphocytes in CD95-deficient lpr mice (Green, 1998; Newton, 1998; Smith, 1996; Strasser, 1995; Thornberry, 1998). These studies suggest that CD95 and Bcl-2 are involved in regulating different apoptotic pathways, but on the basis of the evidence available it remains unclear what connection if any there is between CD95-induced apoptosis and Bcl-2 levels in B-CLL.

A study involving a total of 21 B-CLL patients suggests that Bcl-2 can inhibit CD95-induced apoptosis. When analysed, the cells from 10 patients were found to be weakly positive for CD95 using a specific IgM MoAb, with a mean of 15.6% of the cells (range 5-41%) expressing the antigen. CD95 expression was not changed during spontaneous or HC-induced apoptosis, and culture with medium alone, IL-4, IL-10, or PMA did not modulate its expression. However, culture of $CD95^{+}$ B-CLL cells with IFN-α upregulated CD95 expression in 15/19 samples and CD95 mRNA expression in 5/7. Similarly, culture with IFN-γ increased CD95 expression in 21/21 and CD95 mRNA expression in 7/7. Culture with an anti-CD95 MoAb failed to induce apoptosis in B-CLL cells in which expression of CD95 had been increased by culture with IFN-γ, in contrast to its effect on $CD95^{+}$ Jurkat cells and four $CD95^{+}$ lymphoblastoid cell lines, where the anti-CD95 MoAb induced apoptosis. This may be due to higher levels of Bcl-2 expressed in the cells.

The cells from 4 B-CLL patients analysed for Bcl-2 expression using anti-Bcl-2 MoAb, FITC-conjugated rabbit anti-mouse Ig, and FACS, were found to be 90-96% positive for Bcl-2 (Panayiotidis, 1995).

A further study has linked downregulation of *bcl-2* expression very tenuously with CD95-induced apoptosis in B-CLL cells. The neoplastic cells from a total of 9 B-CLL patients were analysed for *bcl-2* levels and the extent of apoptosis, in relation to exposure to SAC, IL-2, and anti-CD95. The study purports to show that treatment of B-CLL cells with SAC and IL-2 results in an increased expression of surface CD95 and a greater sensitivity to CD95-induced apoptosis through a downregulation in the expression of *bcl-2*. However, it has to be noted that of the total of 9 patients, the cells from only 2 were analysed for CD95 expression before and after activation with SAC, IL-2 or a combination of the two, and only one of the second pair had been included in the first analysis. Although 5 patients showed downregulation of *bcl-2* mRNA in response to activation with SAC and IL-2, and in 3 patients activation with SAC and IL-2 led to an increase in CD95-induced apoptosis, the cells from none of the patients were subjected to all of the analyses. Furthermore, only the cells from 1 patient were subjected to 3/4 analyses. Treatment with SAC plus IL-2 bears very little relationship to the conditions prevailing naturally *in vivo* and given the very small sample sizes, the study offers very little other than what may be an insight into the idiosyncrasies of the cells of a single case of B-CLL. The value of this insight is emphasised very strongly by the cells from a further B-CLL patient, which had a high constitutive expression of CD95 before activation and a CD95 epitope density similar to the other tested cells after activation with SAC and IL-2. After incubation with SAC, IL-2, and anti-CD95, the cells reacted in a directly opposite manner by high ^{3}H-TdR incorporation and low apoptosis (Mapara, 1993).

Very limited support for the results obtained in murine lymphoid cells comes from a study attempting to determine the relative effects of CD40 and CD95 on apoptosis in B-CLL cells. Analysis of the leukaemic cells from B-CLL patients indicated that an average of 85.5% of the cells from 10/10 patients cells were positive for CD40 with a range of 54-97%. Similarly all 10 samples expressed Bcl-2 with a mean of 86.8% of the cells being positive (range 42-100%). Fewer of the cells and patients were positive for CD95. Only 4/10 samples had >10% of the cells CD95^{+}, with a mean of 9.4% overall. However, incubation of the cells from 10/10 patients with NIH3T3 cells stably expressing the human CD40 ligand (CD40L) increased the CD95^{+} cells very significantly to a mean of 74.0% (range 18-95%) and also increased the mean fluorescence intensity from 0.58 arbitrary units to 3.27.

Incubation with anti-CD95 MoAb did not increase the rate of apoptosis in unactivated B-CLL cells and CD40 triggering did not significantly inhibit apoptosis. However, ligation of CD95 significantly increased the rate of apoptosis in 2/10 cells in which CD95 had been upregulated by ligation of CD40. No differences in Bcl-2 levels in B-CLL cells were detected before and after *in vitro* culture and no correlation between Bcl-2 levels and sensitivity to CD95-induced apoptosis was detected (Wang, 1997).

However, a subsequent study contrasts sharply with the findings of the investigation by Wang *et. al.* Exogenous CD40 ligand (CD40L) rescues B-CLL cells from spontaneous, soluble CD95 ligand- and fludarabine-induced *in vitro* apoptosis in a dose- dependent manner at concentrations between 0.10-1.0 ng/ml. Incubation of B-CLL cells for 24 hours with medium alone resulted in 21.42±4.6% becoming apoptotic and incubation with medium plus CD95 ligand resulted in 46.2±8.6% becoming apoptotic. Addition of CD40L at 1 ng/ml to the culture conditions reduced the percentage of apoptotic cells to 5.3±1.4% and 21.0±6.1% respectively, suggesting that CD40 antagonises almost exactly the effect of CD95. Similarly CD40L rescued B-CLL cells cultured with fludarabine from *in vitro* apoptosis. Interestingly the sera of B-CLL patients appears to contain higher levels of active soluble CD40L than the sera from normal healthy donors. The sera from 51 B-CLL patients was found to contain a mean value of 0.80 ng/ml of CD40L in comparison to that from 55 healthy donors, where the mean level was 0.29 ng/ml. Incubation of B-CLL with varying concentrations of autologous plasma showed that those with the highest concentrations of CD40L had the highest percentage of surviving cells after 24 hours, a phenomenon that could be reversed by anti-CD40L antibody at 25 µg/ml. Although it had no apparent effect on the expression of *bcl-*x_S or *bcl-2* mRNA, incubation of B-CLL cells with CD40L upregulated *bcl-*x_L mRNA and CD95 receptor expression in 6 samples within 3-6 hours (Younes, 1998). Whilst confirming the effect of CD40 ligation on CD95 expression, this study raises questions about the effect of CD40 ligation on apoptosis in B-CLL, and on the role that Bcl-x_L might play in countering CD95-induced apoptosis in B-CLL.

Overall the results of these attempts to determine what role CD40 and particularly CD95 might play in the pathogenesis of B-CLL have produced results that are too inconsistent for conclusions to be drawn from them. However, the advantage presented by a means of inducing Bcl-2-independent apoptosis in B-CLL is of such potential therapeutic interest as to warrant further clarification of how CD40 and CD95 function and oppose each other.

9.10 CD6

An explanation for the frequently inconsistent and contradictory results from attempts to link external stimulation to the rate of apoptosis and Bcl-2 levels, comes from a study investigating the role of IgM and CD6 in B-CLL. CD6 (activated leukocyte cell adhesion molecule) expression on B-CLL cells from 26 patients was found to be higher than that expressed on normal tonsillar B-cells, but not associated with disease progression. Levels were very variable, with cells from 22/26 patients positive for CD6 and the cells from 20/26 >75% $CD6^+$. In the 3 cases assessed for CD6 expression using indirect immuno-fluorescence with the anti-CD6 MoAb IOR-T1, CD6 expression increased after 3 days *in vitro* incubation with SAC + IL-2, or TPA. However, B-CLL cells reacted variably to stimulation with various combinations of SAC, IL-2, TPA, or IOR-T1, which had no consistent proliferative effect. *In vitro*, cells from 10 patients showed a significant increase in the number with apoptotic nuclei observed with uv microscopy, from a mean of 13.2% to 39.7% when cultured with anti-IgM MoAb. The degree of apoptosis in these circumstances correlates with cell surface IgM expression, but the percentage of apoptotic cells was reduced in 8/10 populations co-cultured with anti-IgM and the anti-CD6 MoAb IOR-T1. The induction of apoptosis by anti-IgM in B-CLL cells and the protection offered against anti-IgM-induced apoptosis by IOR-T1 appears to be mediated by changes in *bcl-2* and bax_α mRNA levels. In 5 cultures anti-IgM was found to reduce the *bcl-2*/bax_α mRNA ratio by a reduction in *bcl-2* mRNA and particularly by an upregulation of bax_α mRNA. Simultaneous culture with IgM and anti-CD6 MoAb IOR-T1 in 4 cases resulted in a significant reduction in bax_α mRNA without any change in *bcl-2* mRNA, raising the *bcl-2*/bax_α mRNA ratio in a manner that correlated with IOR-T1-mediated protection of anti-IgM-treated cells. Changes at the mRNA level appear to translate into similar changes at the protein level and in B-CLL cells from 3 patients co-cultured with anti-IgM and IOR-T1, the unchanged *bcl-2* mRNA and reduced bax_α mRNA levels were reflected in unchanged Bcl-2 and reduced Bax_α levels. In two further cell cultures Bax_α protein levels were higher after culture with anti-IgM than after culture with medium alone. In the circumstances it appears that it may be the Bcl-2/Bax ratio that is modulated through CD6 in B-CLL cells, where appropriately CD6 expression appears to be higher than on normal tonsillar B-cells (Osorio, 1997).

9.11 CDw32

When BM- and peripheral blood-derived B-CLL cells from 17 previously untreated patients were analysed for survival and apoptosis in relation to Bcl-2 expression with a number of soluble and membrane-bound factors, the most efficient stimulus to *in vitro* survival was co-cultivation with CDw32-transfected murine fibroblasts where direct contact between the fibroblasts and leukaemic cells was permitted. In comparison to control cultures there was a reduction in the number of cells undergoing apoptosis of 90% as assessed by trypan blue exclusion. The prolongation of survival by CDw32-transfected murine fibroblasts was accompanied by a reduction in apoptosis which was associated with the partial prevention of the loss of Bcl-2. The association between CDw32, the reduced rate of apoptosis, and preservation of Bcl-2 levels is given added weight by a further co-cultivation using a murine fibroblast cell line C58 which was not transfected with CDw32 and which failed to prevent the loss of Bcl-2 or to offer any similar protection from apoptosis (Buske, 1997).

9.12 Others

Other studies have implicated other cytokines in the control of apoptosis in B-CLL cells, although in many cases the sample numbers are small and function has not been related to the Bcl-2 family. A study using the leukaemic cells from 6 patients suggested that as well as IL-4 which is discussed in earlier paragraphs, the cytokines IFN-γ, IL-2, IL-6, IL-13 and TNF-α were also capable of suppressing spontaneous *in vitro* apoptosis. In comparison to cells cultured in medium alone, culture with IL-4 and IFN-γ increased *in vitro* viability by 15-40% in respectively 6/6 and 5/6 cell populations. Culture with IL-2 and IL-6 increased the viability of the same 4/6 cell populations by 15-30%, where the ability of IL-2 to suppress apoptosis in these cells generally correlated with surface expression of the low-affinity IL-2 receptor, CD25. The effect of TNF-α was to increase cell viability in 4/6 populations by between 5-20% and IL-13 significantly increased viability in 1/6 populations by 29.0%. When Bcl-2 levels were measured in 4 of 6 cell populations using flow cytometry at various intervals between 4-7 days, all of these cytokines appeared to sustain rather than upregulate Bcl-2 expression (Tangye, 1997). Although Bcl-2 levels were not assessed, the suggestion that IL-13 protects B-CLL cells from spontaneous apoptosis, is supported by a further study using cells from 12 patients. IL-13 is effective at reducing the rate of apoptosis in culture at 100-200 ng/ml, with the number of B-CLL cells with apoptotic nuclei reduced from 68±24% to 32±12% after 4 days culture at the higher concentration, in comparison to culture in medium alone (Chaouchi, 1996)

IL-7 has also been tenuously connected to the prevention of apoptosis in B-CLL cells, but without any attempt to relate its effect to Bcl-2 family members. Apoptosis was inhibited when B-CLL cells were cultured *in vitro* on a monolayer of EA.hy926 human umbilical cord endothelial hybrid cells, with the coincident kinetics of IL-7 mRNA down- regulation and the reduced rate of apoptosis, suggesting that prevention of apoptosis is linked to the retention of IL-7 mRNA. In contrast, the rate of apoptosis was higher and IL-7 mRNA was downregulated in B-CLL cells cultured on a monolayer of A549/8 carcinoma cells, the fusion partner used to generate the EA.hy926 hybrid cells, and those cultured in the absence of other cells. As appeared also to be the case with BM stromal cells (para 9.8.1), the effect of monoclonal antibodies directed against the β2-integrin, CD11b/CD18, on B-CLL cells, suggests that it is involved in mediating the interaction between B-CLL and EA.hy926 hybrid cells (Long, 1995). However, the anti-apoptotic effect of IL-7 is called into question to some extent by a series of *in vitro* experiments involving the B-CLL cells from 10 patients. This suggested that IL-1 and IL-7, and in contrast to the results obtained by Tangye *et. al.*, IL-2 and IL-6, failed to inhibit apoptosis as assessed by morphology and DNA fragmentation. If this study is correct, then IL-7 must improve *in vitro* B-CLL cell viability in an intracellular autocrine fashion (Buschle, 1993).

IFN-γ also appears to extend the life span of B-CLL cells in an autocrine fashion that shows parallels with the effects of other cytokines, but unfortunately no attempt was made to determine if this involves Bcl-2. *In vitro* culture of B-CLL cells from 10 patients in medium alone resulted in the recovery of 56±6.1% viable cells after 5 days, with 5-20% of the cells showing morphologic evidence of apoptosis, and DNA fragmentation into multiples of 180 bp after 16 hours. In contrast, culture with 100 U/ml of IFN-γ increased viability to 94±3.6% after 5 days without any evidence of cell proliferation. Concentrations of IFN-γ as low as 25 U/ml were effective, with maximum inhibition of apoptosis achieved at IFN-γ concentrations between 100-1000 U/ml. The sera of 7/10 B-CLL patients contained IFN-γ at concentrations between 60-200 pg/ml, in contrast to the sera from 10 healthy donors where IFN-γ concentrations were undetectable (<20 pg/ml). Production of IFN-γ mRNA was found to be at high levels in the cells from 3 B-CLL patients activated with TPA and calcium ionophore and, in the 2 cases tested, 74% and 82% of the leukaemic cells were found to synthesise IFN-γ transcripts, suggesting that IFN-γ may play a part in increasing the life span of B-CLL cells in an autocrine fashion (Buschle, 1993).

10 Conclusions.

The conclusions that can be drawn from the involvement of the Bcl-2 family in the pathogenesis of B-CLL particularly include, that the life span of B-CLL cells appears to be extended *in vitro* by higher ratios of Bcl-2 to Bax rather than absolute levels of Bcl-2, that involvement extends beyond Bcl-2 and Bax and may involve the expression of multiple family members, that the deregulated expression of Bcl-2 caused by genetic translocation may be greater than initial studies suggested, that Bcl-2 and Bax levels in B-CLL cells may be subject to deregulation and control by a wide range of largely extra-cellular influences, and that B-CLL cells may conform to the general apoptotic paradigm with Bcl-2 family members regulating cell survival through pore-forming/inhibiting activity in intracellular membranes.

Given the large number of studies which have linked expression of members of the Bcl-2 family to the neoplastic process in B-CLL, the link with the Bcl-2 family is now beyond doubt and, if the studies by Gottardi (1996), Kitada (1998) *et. al.* are representative, aberrant and overlapping expression of members of the Bcl-2 family is common in B-CLL. Unfortunately, only limited information is available about the causes and extent of the inappropriate expression of these proteins in B-CLL, the number of patients in which Bcl-2 family members may play a significant role in the neoplastic process, the extent to which expression may vary within the clone and provide the setting for the chemotherapeutic selection of refractory clones, the extent to which expression of more than one pro-survival member of the family may contribute to the neoplasm, and particularly very little to relate the nature of abnormal expression of Bcl-2 family members to the highly variable clinical outcome.

There appears to be no simple correlation between Bcl-2 levels and the clinical progression or stage of the disease. Likewise, attempts to show an inverse relationship between the sensitivity of B-CLL cells to *in vitro*-induced apoptosis and absolute levels of Bcl-2, and by inference extended life span *in vivo,* have produced conflicting results in a large number of studies. However, the absence of any clear relationship between Bcl-2 levels and disease progression, and the contradictory findings of studies trying to link the *in vitro* survival times of B-CLL cells and Bcl-2 levels, are explained by the co-expression of pro-apoptotic family members such as Bax. Higher Bcl-2:Bax ratios correlate with progressive disease, lack of response to therapy, and resistance to the *in vitro* apoptosis induced by a wide range of cytotoxic agents.

In contrast lower Bcl-2:Bax ratios correlated with non-progressive disease, lack of previous treatment, and increased sensitivity to the induction of apoptosis using cytotoxic agents *in vitro*.

The relationship between higher Bcl-2:Bax ratios and progressive disease, lack of response to therapy, and resistance to the induction of *in vitro* apoptosis, is very much in keeping with clinical observations of an often relentless accumulation of neoplastic B-CLL cells accompanied by an acquired tolerance of cytotoxic drugs. In particular the effect of higher Bcl-2:Bax ratios in B-CLL appears to conform to the Ollvai, Korsmeyer and Milliman paradigm, allowing speculation that the interaction of the pro- and anti-apoptotic members of the Bcl-2 family in B-CLL cells corresponds to a common cellular mechanism. The wider evidence confirms the role of the BH3 domain as the pro-apoptotic domain, with BH1-4 apparently essential for anti-apoptotic function, although fundamental questions about the way in which the BH3-only pro-apoptotic members function in comparison to the BH1-3 pro-apoptotic members, remain unanswered. A wide range of experiments suggest that homo- and heterogenous interactions between family members are essential to apoptotic function. Unfortunately the experimental conditions frequently bear too little resemblance to conditions prevailing naturally in the cell for meaningful conclusions to be drawn from them and the mechanics of apoptosis remain essentially unclear. It is difficult to infer anything significant from experimental conditions that involve, for example, the removal of the hydrophobic TM domain from family members when it is thought to play an essential role in locating these proteins to intracellular membranes and when some studies have suggested it to be important to Bcl-2's anti-apoptotic function. The fact that these proteins apparently continue to fulfil their proposed function without a TM domain does not necessarily indicate that the TM domain is unnecessary to function. They may be targeting a protein with which they interact before it has reached its normal site and interference with function in such an abnormal manner says very little about normal function. Tagging of family members such as Bcl-2, Bcl-x_L and Bax with haemagglutin and FLAG epitopes is also common in experimental procedures, as are plate binding assays. It remains possible that these modifications, as well as the removal of the TM domain, may induce their observed effects via changes in the protein's conformation or by steric hindrance, and again may say very little about the function of the family members in a more natural setting.

What is especially intriguing and remains largely unexplored and unexplained, is the apparently pore-forming structure of various members of the family, particularly Bcl-2 and Bcl-x_L, and their location in mitochondrial membranes.

Unless this is an occasion where form does not follow function, nothing has been discovered to exclude the possibility that their putative pore-forming structure plays an essential role in the mitochondrial control of apoptosis.

Whilst the conditions in vesicles and planar lipid bilayers may bear very little similarity to the conditions prevailing in the mitochondrial membranes at the contact sites between the inner and outer membranes, where Bcl-2-family proteins are known to insert, the ability of Bcl-2, Bcl-x_L, and Bax to form conductance channels in synthetic membranes offers the opportunity of speculating about the manner in which the Bcl-2 family may adjust the apoptotic threshold of the cell. In this context it is worth remembering that the ability of Bcl-2, Bcl-x_L, and Bax to form conductance channels in synthetic membranes appears to be linked to acidic lipid membranes, pH and voltage. Furthermore, the channels they form are of different and varying conductance states, they display a degree of ion-selectivity, α -helices 5 and 6 in Bcl-2 (and by inference their equivalents in the other family membranes that possess them) are particularly important to pore forming function, there is a suggestion of conductance being linked to the oligomerisation of these proteins, and Bcl-2 appears to be capable of inhibiting the channel-forming activity of Bax in lipid vesicles.

The stoichiometric relationship between Bcl-2 and the PBR, combined with the fact that Bcl-2 and Bax are both capable of forming open channels of large conductances up to ~2 nS, raises questions about how full a role the Bcl-2 family may play in the formation of the PT pore. It is certainly possible, even likely, that the Bcl-2 family may regulate the opening of the PT pore, and hence apoptosis, by allowing the passage of ions into or out of mitochondria, but to what extent may proteins belonging to the Bcl-2 family be involved in the formation of the pore?

Single-channel measurements of excised mitochondrial patches and reconstituted purified ADP/ATP carrier (AAC), have suggested that when converted into a large unselective channel, the AAC is a key component of the PT pore. This channel is low cation-selective, is pH sensitive, closing completely at pH 5.2, and its conductance has multiple sublevels varying from 300-600 pS. This channel shows remarkable similarities to those formed in synthetic membranes by Bcl-2, Bcl-x_L, and Bax, all of which appear to be capable of forming conductance channels of this magnitude in planar lipid bilayers, posing the question of whether they are involved directly in the formation of the PT pore?

Clearly that question cannot be answered on the basis of present knowledge, but it should make a potentially fruitful area of research and if the relationship of Bcl-2 family members to the PT pore is not that of participants, it will be interesting to note the physical relationship between Bcl-2 family-formed pores and the PT pore.

Regardless of whether Bcl-2 family members are actually involved in the PT pore's structure, it will be also be interesting to determine if the manner in which pro- and anti-apoptotic members of the Bcl-2 family interact *in vitro,* is a reflection of the nature of interactions when they are located in intracellular membranes. The range of experiments to try and assess how Bcl-2, Bcl-x_L, and Bax function when embedded in synthetic membranes, although valuable, has been very limited. In particular, although experiments have shown that Bcl-2, Bcl-x_L, and Bax form conductance channels in synthetic membranes, this is not evidence that these proteins form channels in isolation of each other *in vivo*. The ability of Bcl-2 to inhibit carboxyfluorescein release through Bax conductance channels in liposomes may be a more accurate reflection of the functioning of these apoptosis-associated proteins in more natural circumstances. If that should prove to be the case it will be interesting to see if the fate of the cell is determined by the cellular stoichiometry of Bcl-2 family members, with individual conductance channels formed by both pro- and anti-apoptotic family members and either the open/closed conformation of the pores, or the nature and direction of ions allowed to pass through them, determined by the balance between them.

Attempts to determine the reasons for the apparently unregulated expression of members of the Bcl-2 family appear to have concentrated almost exclusively on Bcl-2 itself, and particularly on the possibility that expression of the protein is deregulated as a result of a genetic translation or extracellular stimulation. A number of studies using Southern blotting, including studies involving cohorts of 100 and 170 B-CLL patients, have found little or no evidence to suggest that the t(14;18) translocation of the Bcl-2 gene, or the less common translocation to the Igκ or Igλ loci, is common in B-CLL. However, the rates of detection ranging from 0% to 12%, most of which range from 0-3%, are contradicted by the apparently more sensitive techniques of pulsed gel electrophoresis and fluorescence in situ hybridisation. Although these studies involve much smaller cohorts, they have shown a very high incidence of translocation, 100% in one case, and have been shown to be highly effective in detecting the t(14;18) translocation in follicular lymphoma. These studies appear to deserve a degree of credence that is disproportionate to the number of patients involved.

Although larger studies would be necessary to confirm the situation, it is clearly possible that deregulation of Bcl-2 expression in B-CLL as a result of the translocation of the *bcl-2* gene is more common than currently envisaged. However, it is clear that cellular Bcl-2 levels are modulated throughout, for example, B-lymphopoiesis, as it becomes expedient to either make the developing cell more vulnerable to deletion or increase its longevity. In the circumstances, a malfunction of any of the mechanisms that modulate the levels of Bcl-2 family members could just as easily result in inappropriate expression of anti-apoptotic proteins as a translocation. In the absence of any substantial evidence to the contrary, this type of malfunction certainly cannot be excluded as being instrumental in the pathogenesis of B-CLL in at least some patients and it remains possible, if Bcl-2 family member deregulation can be shown to be a ubiquitous feature of B-CLL, that the nature of the deregulation may contribute to the character and aggressiveness of the disease.

The most interesting, and potentially most productive aspect of the role of the Bcl-2 family of proteins in the pathogenesis of B-CLL, is the extent to which their levels may be modulated by largely extracellular influences including IL-4, IL-5, IL-8, IL-10, IFN-α, IFN-γ, bFGF, TGFβ1, BM stromal cells, CDw32, CD95, CD6, CD40, IgM, and the *p53* gene. It is clear that these may play a significant role in the pathogenesis of the disease and as such, present numerous opportunities for further research aimed at understanding the cause of the disease. However, a better understanding of their role may also provide the opportunity to manipulate the mechanisms involved to significant therapeutic advantage.

The fact that lower Bcl-2:Bax ratios correlate with non-progressive disease, lack of previous treatment, and particularly increased sensitivity to the induction of apoptosis using cytotoxic agents *in vitro*, combined with the ability to manipulate levels of Bcl-2 and Bax, and by inference other family members involved in B-CLL, potentially offers opportunities to treat the disease, or to make existing treatments more effective by preceding them with a modulation phase. During this modulation phase the neoplastic cells could be made more vulnerable, e.g. to chemotherapy, by having their ratios of anti-apoptotic to pro-apoptotic family members reduced, an option which might be of particular value in refractory disease. Manipulation of receptor or cytokine levels often has an adverse effect on normal cells, but the wide range of entities which affect the apoptotic threshold in B-CLL cells provides an opportunity to exploit a potential synergy between them, with levels of individual cytokines, or receptors modulated in such a way as not to have undue pathological effects on normal cell populations.

Of the entities that appear to regulate the Bcl-2:Bax ratio in B-CLL cells, IL-4, IL-5, IL-8, CD6, BM stromal cells, CDw32, the *p53* gene and CD95 appear to be of particular value, or of such potential value as to make them worthy of further investigation. The effect of CD95 is potentially very interesting if its ligation were eventually proven to induce an apoptotic pathway unregulated by Bcl-2, as a Bcl-2-independent pathway could prove a very useful adjunct to therapy in any disease associated with the protein. However, a great deal more work is necessary to establish the extent of the influence on molecular entities listed above on the various members of the Bcl-2 family in B-CLL, the extent to which these play an active part in the neoplastic process in the disease, and the mechanisms through which they act.

Bibliography.

Adachi, M. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1989) vol 86 2771-4.

Adachi, M. *et. al.*; Oncogene (1989) vol 4 1073-5.

Adachi, M. *et. al.*; Journal of Experimental Medicine (1990) vol 171 559-64.

Adachi, M. *et. al.*; Blood (1996) vol 88(11) 4118-23.

Adams, J. M. & Cory, S.; Science (1998) vol 281 1322-6.

Aguilar-Santelises, M. ; International Journal of Cancer (1994) vol 58 474-9.

Aguilar-Santelises, M. *et. al.*; International Journal of Cancer (1996) vol 69 114-9.

Akahani, S. *et. al.*; Cancer Research (1997) vol 57 5272-6.

Amiel, A. *et. al.*; Cancer Genetics and Cytogenetics (1994) vol 73 165-8.

Ansell, S. M. *et. al.*; American Journal of Hematology (1999) vol 60(2) 99-104.

Antonsson, B. *et. al.*; Science (1997) vol 277(5324) 370-2.

Arpin, C. *et. al.*; Science (1995) vol 268(5211) 720-2.

Ashkenazi, A. *et. al.*; Science (1998) vol 281(5381) 1305-8.

Bakshi, A. *et. al.*; Cell (1985) vol 41 899-906.

Barondes, S.H. *et. al.*; Cell (1994) vol 76 597-8.

Bartal, A. *et. al.*; Acta Haematologica (1978) vol 60(3) 161-71.

Bastion, Y. *et.al.*; Annals of Oncology (1992) vol 3(2) 171-2.

Berberich, I. *et.al.*; Journal of Immunology (1994) vol 153(10) 4357-66.

Bernardi, P. *et. al.*; Journal of Bioenergetics & Biomembranes (1996) vol 28(2) 131-8.

Bertrand, F. E. *et. al.*; Blood (1997) vol 90(2) 736-44.

Billips, L. G. *et. al.*; Journal of Experimental Medicine (1995) vol 182(4) 973-82.

Boise, L.H. *et. al.*; Cell (1993) vol 74 597-608.

Boldin, M. P. *et. al.*; Cell (1996) vol 86(6) 803-15.

Borner, C. *et. al.*; Journal of Cell Biology (1994) (vol) 126 1059-68.

Bossy-Wetzel, E. *et. al.*; EMBO Journal (1998) vol 17(1) 37-49.

Boyd, J.M. *et. al.*; Oncogene (1995) vol 11 1921-8.

Bredesen, D. E.; Annals of Neurology (1995) vol 38(6) 839-51.

Brincker, H.; Scandinavian Journal of Haematology (1982) vol 29(3) 341-9.

Brittinger, G. *et. al.*; Annals of Hematology (1997) vol 74 291-4.

Brustovetsky, N. & Klingenberg, M.; Biochemistry (1996) vol 35(26) 8483-8.

Buschle, M. *et. al.*; Journal of Experimental medicine (1993) vol 177 213-8.

Buske, C. *et. al.*; Experimental Hematology (1997) vol 25 329-37.

Byrnes, R. K. *et. al.*, American Journal of Clinical Pathology (1995) vol 104(2) 199-203.

Caligaris-Cappio, F. *et. al.*; Blood Cells (1993) vol 19 601-13.

Candelas, S. *et. al.*; Immunity (1997) vol 6(5) 501-8.

Carayon, P. *et. al.*; Blood (1996) vol 87(8) 3170-8

Castedo, M. *et. al.*; Journal of Immunology (1996) vol 157(2) 512-21.

Chaouchi, N. *et. al.*; Clinical Immunology and Immunopathology (1994) vol 73(2) 197-204.

Chaouchi, N. *et. al.*; Blood (1996) vol 87(3) 1022-9.

Chen, G. *et. al.*; Journal of Biological Chemistry (1996) 271(39) 24221-5.

Cheng, E.H-Y. *et. al.*; Nature (1996) 379(6565) 554-6.

Cheng, E.H.-Y. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1997) vol 94 690-4.

Cheng, E.H. *et. al.*; Science (1997) vol 278(5345) 1966-8.

Chittenden, T. *et. al.*; The EMBO Journal (1995) 14(22) 5589-96.

Choe, J. *et. al.*; Journal of Immunology (1996) vol 157(3) 1006-16.

Choi, M. *et. al.*; Immunity (1994) vol 1 179-87.

Choi, S. S. *et. al.*; Oncogene (1995) vol 11 1693-8.

Clark, E. A. *et. al.*; Journal of Immunology (1989) vol 143(12) 3873-80.

Clark, E. A. & Lane, P. J.; Annual Review of Immunology (1991) vol 9 97-127.

Cleary, M.L. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1985) vol 82 7439-43.

Clevenger, C. V. *et. al.*; Molecular Endocrinology (1997) vol 11(5) 608-18.

Cohen, J. J. *et. al.*; Immunology Today (1993) vol 14(3) 26-30.

Collins, R.J. *et. al.*; British Journal of Haematology (1989) vol 71 343-50.

Cory, S *et. al.*; Annual review of Immunology (1995) vol 13 513-43.

Cruz-Reyes, J. & Tata, J. R.; Gene (1995) vol 158 171-9.

Cryns, V. & Yuan, J.; Genes & Developmeny (1998) vol 12(11) 1551-70.

Cuneo, A. *et. al.*; British Journal of Haematology (1996) vol 92(2) 375-81.

Cutrona, G. *et. al.*; European Journal of Immunology (1997) vol 27(1) 234-8.

Cuttner, J.; Cancer Investigation (1992) vol 10(2) 103-9.

Dameshek, W.; Blood (1967) vol XX1X no. 4 pt. II 566-84.

Danescu, M. *et. al.*; Journal of Experimental medicine (1992) vol 176 1319-26.

Das, R. *et. al.*; Oncogene (1996) vol 12 947-51.

Davi, F. *et. al.*; Blood (1997) vol 90(10) 4014-21.

de Jong, D. *et. al.*; Cancer Research (1994) vol 54(1) 256-60.

Deveraux, Q.L. *et. al.*; EMBO Journal (1998) vol 17(8) 2215-23.

Diaz, J-L. *et. al.*; Journal of Biological Chemistry (1997) 272 11350-5.

Diele, L. F. & Ketchum, L. H.; Seminars in Oncology (1998) vol 25(1) 80-97.

Dighiero, G; *Nouvelle Revue Française d'Hématologie* (1988) vol 30 283-8.

Dighiero, G.; Hematology and Cell Therapy (1996) vol 38 S41-S61.

Di Raimondo, F. *et.al.*; Leukemia & Lymphoma (1993) vol 11 63-8.

Dorshkind, K.; Journal of Immunology (1991) vol 146(12) 4204-8.

Douglas, R. S. *et. al.*; Blood (1997) vol 89 (3) 941-7.

D'Sa-Eipper, C. *et. al.*; Cancer Research (1996) vol 56 3879-82.

Dyer, M. J. S. *et. al.*; Blood (1994) vol 83 (12) 3682-8.

Dyer, M. J. S. *et. al.*; British Journal of Haematology (1997) vol 97 669-72.

Efremov, D.G. *et. al.*; Blood (1996) vol 87(9) 3869-76.

Egle, A. *et. al.*; British Journal of Haematology (1996) vol 94 210-2.

Eguchi, Y. *et. al.*; Cancer Research (1997) vol 57(10) 1835-40.

Ehlich, A. *et. al.*; Cell (1993) vol 72(5) 695-704.

Elangovan, B. *et. al.*; The Journal of Biological Chemistry (1997) vol 272 (39) 24494-8.

Evan, G. & Littlewood, T.; Science (1998) vol 281 1317-22.

Evans, D. L. & Mansel, R. E.; Journal of Molecular Evolution (1995) 41 775-83.

Fluckinger, A-C. *et. al.*; Journal of Experimental Medicine (1994) vol 179 91-9.

Forster, I. *et. al.*; European Journal of Immunology (1987) vol 17 521-8.

Francia di Celle, P. *et. al.*; Blood (1996) vol 87 (10) 4382-9.

Frankfurt, O. S. *et. al.*; Leukemia Research (1997) vol 21 (1) 9-16.

Freedman, A. S. *et. al.*; Blood (1989) vol 73(1) 202-8.

Freedman, A. S. & Nadler, L. M.; Chapter 1 of Chronic Lymphocytic Leukemia Ed. by B.D. Cheson, published Marcel Dekker (1993) 297-336.

Fulcher, D.A. & Basten, A.; Immunology and Cell Biology (1997) vol 75 446-55.

Gaidano, G. *et. al.*; American Journal of Pathology (1994) vol 144(6) 1312-9.

Galy, A. *et. al.*; Immunity (1995) vol 3(4) 459-73.

Ghia, P. *et. al.*; European Journal of Immunology (1995) vol 25(11) 3108-14.

Ghia, P. *et. al.*; Journal of Experimental Medicine (1996) vol 184(6) 2217-29.

Ghia, P. *et. al.*; Immunology Today (1998) vol 19(10) 480-5.

Gibson, L. *et. al.*; Oncogene (1996) vol 13 665-75.

Giles, F. J. *et. al.*, Seminars in Oncology (1998) vol 25(1) 117-25.

Gillet, G. *et. al.*; EMBO Journal (1995) vol 14 (7) 1372-1381.

Glimcher, L. H. & Kara, C. J.; Annual Review of Immunology (1992) vol 10 13-49.

Gong, S. *et. al.*; Science (1996) vol 272(5260) 411-4.

Gottardi, D. *et. al.*; British Journal of Haematology (1996) vol 94 612-8.

Grawunder, U. *et. al.*; Immunity (1995) 3(5) 601-8.

Green, D. R. & Reed, J. C.; Science (1998) vol 281 1309-1312.

Hagman, J. *et. al.*; Genes & Development (1993) vol 7(5) 760-73.

Hamblin, T.J. *et. al.*; Journal of Clinical Pathology (1986) vol 39 713-9.

Han, J. *et. al.*; Genes & Development (1995) 10 461-77.

Han, J. *et. al.*; Molecular and Cellular Biology (1996) vol 16 (10) 5857-64.

Han, S. *et. al.*; Journal of Immunology (1995) vol 155 556-67.

Han, S. *et. al.*; Science (1996) vol 274(5295) 2094-7.

Han, T.; Blood Cells (1987) vol 12 481-3.

Hanada, M. *et. al.*; Blood (1993) vol 82 (6) 1820-8.

Hanada, M. *et. al.*; Journal of Biological Chemistry (1995) 270(20) 11962-9.

Harada, K. *et. al.*; Journal of Experimental Medicine (1991) vol 173(2) 409-15.

Hardy, R. R. *et. al.*; Journal of Experimental Medicine (1991) vol 173 1213-25.

Hartley, S. B. *et. al.*; Cell (1993) vol 72 325-35.

Henderson, A. *et. al.*; Annual review of Immunology (1998) vol 16 163-200.

Hengartner, M. O. *et. al.*; Cell (1994) vol 76 665-76.

Hirayama, F. & Ogawa, M.; Blood Cells (1994) vol 20(2-3) 341-7.

Hohfeld, J. & Jentsch, S.; EMBO Journal (1997) vol 16(20) 6209-16.

Hohfeld, J.; Biological Chemistry (1998) vol 379(3) 269-74.

Holder, M. *et. al.*; European Journal of Immunology (1995) vol 25(11) 3160-4.

Hsu, S.Y. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1997) vol 94 12401-6.

Hsu, Y. T. *et. al.*; Journal of Biological Chemistry (1998) vol 273(17) 10777-83.

Hunter, J. J. *et. al.*; Molecular and Cellular Biology (1996) vol 16 (3) 877-83.

Inohara, N. *et. al.*; EMBO Journal (1997) vol 16 (7) 1686-94.

Ishida, T. *et. al.*; Journal of Immunology (1995) vol 155(12) 5527-35.

Jacobson, M. D. *et. al.*; Nature (1993) vol 361(6410) 365-9.

Jacobson, M. D. *et. al.*; Nature (1995) vol 374(6525) 814-6.

Jewell, N. *et. al.*; EMBO Journal (1994) vol 16 (7) 1686-94.

Johnson, S. *et. al.*; Lancet (1996) vol 347(9013) 1432-1438.

Jonsson, V. *et. al.*; Leukemia (1996) vol 10(2) 327-32.

Juliusson, G. & Gahrton, G.; Chapter 4 of Chronic Lymphocytic Leukemia ed. by B.D. Cheson, published by Marcel Dekker Inc. (1993) 83-103.

Jurgensmeier, J. M. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1998) vol 95(9) 4997-5002.

Jurlander, J. *et. al.*; Blood (1997) vol 89 (11) 4146-52.

Kantor, A.B. *et. al.*; Annual Review of Immunology (1993) vol 11 501-38.

Karasuyama, H. *et. al.*; Journal of Experimental Medicine (1993) vol 178(2) 469-78.

Karasuyama, H. *et. al.*; Cell (1994) vol 77(1) 133-43.

Keating, M. J.; In chapter 15 of Chronic Lymphocytic Leukemia Ed. by B.D. Cheson published Marcel Dekker 1993 297-336.

Keating, M. J.; Chapter 26 Leukemia 6th. Ed. published W.B. Saunders & Co (1996) 554-86.

Kelsoe, G.; Advances in Immunology (1995) vol 60 267-88.

Kelsoe, G.; Immunity (1996) vol 4(2) 107-11.

Kelsoe, G.; Seminars in Immunology (1996) vol 8(3) 179-84.

Kenny, J.J. *et. al.*, Oncogene (1997) vol 14 997-1001.

Kerr, J. F. R. *et. al.*; British Journal of Cancer (1972) vol 26 239-57.

Kerr, J. F. R. *et. al.*; Chapter 2 of Apoptosis: The molecular basis of cell death published Cold Spring Harbor Laboratory Press (1991) 5-30.

Khanim, F. *et. al.*; Journal of General Virology (1997) vol 78 2987-99.

Khouri, I. F. *et. al.*; Journal of Clinical Oncology (1994) vol 12(4) 748-58.

Khouri, I. F. *et. al.*; British Journal of Haematology (1997) vol 97(2) 466-73.

Kiefer, M. C. *et. al.*; Nature (1995) vol 374 736-9.

Kincade, P. W. *et. al.*; Annual Review of Immunology (1989) vol 7 111-43.

Kipps, T. J.; Advances in immunology (1989) vol 47 117-85.

Kitabayashi, A. *et. al.*; International Journal of Hematology (1995) vol 62 99-106.

Kitada, S. *et. al.*; Blood (1998) vol 91(9) 3379-89.

Kitamura, D. *et. al.*; Nature (1991) vol 350(6317) 423-6.

Kitamura, D. *et. al.*; Cell (1992) vol 69(5) 823-31.

Knauf, W. U. *et. al.*; Blood (1995) vol 86 (11) 4382-3.

König, A. *et. al.*; Blood (1997) vol 90 (2) 562-70.

König, A. *et. al.*; Leukemia (1997) vol 11 258-265.

Koopman, G. *et. al.*; European Journal of Immunology (1997) vol 27(1) 1-7.

Korsmeyer, S. J.; Trends in Genetics (1995) vol 11(3) 101-5.

Kothakota, S.. *et. al.*; Science (1997) vol 278(5336) 294-8.

Kozopas, K. M. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1993) vol 90 3516-20.

Krajewski, S. *et. al.*; Cancer Research (1993) vol 53(19) 4701-14.

Krajewski, S. *et. al.*; American Journal of Pathology (1994) vol 145 (6) 1323-36.

Kroemer, G. *et. al.*; Immunology Today (1997) vol 18 44-51.

Kuppers, R. *et. al.*; EMBO Journal (1993) vol 12(13) 4955-67.

Lagneaux, L. *et. al.*; Blood (1998) vol 91 (7) 2387-96.

Lagresle, C. *et. al.*; Journal of Immunology (1995) vol 154(11) 5746-56.

Laytragoon-Lewin, N. *et. al.*; International Journal of Cancer (1998) vol 76 909-12.

LeBien, T. W.; Chapter 5 Leukemia 6th. Ed. published W.B. Saunders & Co (1996) 65-82.

Lee, L. C. *et. al.*; The journal of Biological Chemistry (1996) vol 271(38) 23284-8.

Lee, M. Y. *et. al.*; Blood (1993) vol 82(7) 2062-8.

Lenschow, D. J. *et. al.*; Annual Review of Immunology (1996) vol 14 233-58.

Li, P. *et. al.* Cell (1997) vol 91(4) 479-89.

Li, Y. S. *et. al.* Journal of Experimental Medicine (1993) vol 178(3) 951-60.

Li, Y. S. *et. al.* Immunity (1996) vol 5(6) 527-35.

Li, Y. Y. *et. al.*; Journal of Immunology (1996) vol 157(4) 1440-7.

Li, Z. *et. al.* Immunity (1996) vol 5(6) 575-89.

Lin, E. Y. *et. al.*; The Journal of Immunology (1993) vol 151 (4) 1979-88.

Lin, W. C. *et. al.* Science (1993) vol 260(5110) 953-9.

Lin, Y. *et. al.*; Science (1997) vol 276(5312) 596-9.

Lindhout, E. *et. al.*; Clinical & Experimental Immunology (1993) vol 91 (2) 330-6.

Lindhout, E. *et. al.*; Journal of Experimental Medicine (1995) vol 181(6) 1985-95.

Linet, M. S. *et. al.*; Chapter 2 of Chronic Lymphocytic Leukaemia Ed. by Aaron Polliack and Daniel Catovsky, published by Harwood Academic Publishers (1988) 11-32.

Linet M. S. *et. al.*; American Journal of Epidemiology (1989) vol 130(4) 655-64.

Lishner, M. *et. al.*; Cancer Genetics and Cytogenetics (1995) vol 85 118-23.

Liu, Y.-J. *et. al.*; Nature (1989) vol 342 929-31.

Liu, Y.-J. *et. al.*; European Journal of Immunology (1991) vol 21 (8) 1905-10.

Liu, Y. J. *et. al.*; Immunity (1996) vol 4(3) 241-50.

Liu, Y. J. *et. al.*; Immunity (1996) vol 4(6) 603-13.

Liu, Y. J. *et. al.*; Seminars in Immunology (1996) vol 8(3) 169-77.

Liu, X. *et. al.*; Cell (1996) vol 86(1) 147-157.

Loken, M. R. *et. al.*; Blood (1987) vol 70(5) 1316-24.

Long, B. W. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1995) vol 92 1416-20.

Lotz, M. *et. al.*; Journal of Experimental Medicine (1994) vol 179 999-1004.

Ma, A. *et. al.*; The EMBO Journal (1992) vol 11(7) 2727-34.

MacLennan, I.C.; Annual Review of Immunology (1994) vol 12 117-39.

Mainou-Fowler, T. *et. al.*; Blood (1994) vol 84 (7) 2297-304.

Mancini, M. *et. al.*; Journal of Cell Biology (1998) vol 140(6) 1485-95.

Mapara, M. *et. al.*; European Journal of Immunology (1993) vol 23 702-8.

Maraskovsky, E. *et. al.*, International Immunology (1998) vol 10(9) 1367-75.

Marchetti, P. *et. al.*; Journal of Experimental Medicine (1996) vol 184(3) 1155-60.

Martinez-Valdez, H. *et. al.*; Journal of Experimental Medicine (1996) vol 183(3) 971-7.

Matolscy, A. *et. al.*, Blood (1994) vol 83(5) 1363-72.

Matolscy, A. (1) *et. al.*, American Journal of Pathology (1995) vol 147(1) 207-16.

Matolscy, A. (2) *et. al.*, Annals of the New York Academy of Sciences (1995) vol 764 496-503.

Matsumoto, M. *et. al.*, Science (1996) vol 271(5253) 1289-91.

McConkey, D.J. *et. al.*; The Journal of Immunology (1996) vol 156(7) 2624-30.

Meffre, E. *et. al.*; European Journal of Immunology (1996) vol 26(9) 2172-29.

Melchers, F. *et. al.*; Current Opinion in Immunology (1995) 7(2) 214-27.

Mentz, F. *et. al.*; Blood (1996) vol 88 (6) 2172-2182.

Merup, M. *et. al.*; British Journal of Haematology (1996) vol 92 647-52.

Michallet, M. *et. al.*; Annals of Internal Medicine (1996) vol 124(3) 311-5.

Middleton, G. *et. al.*; Development (1996) 122 695-701.

Minegishi, Y. *et. al.*; Journal of Experimental Medicine (1998) vol 187(1) 71-7.

Minn, A. J. *et. al.*; Journal of Biological Chemistry (1996) vol 271(11) 6306-12.

Minn, A. J. *et. al.*; Nature (1997) vol 385(6614) 353-7.

Miyashita, T. *et. al.*; Oncogene (1994) vol 9 1799-805.

Mond, J. J. *et. al.*; Annual Review of Immunology (1995) vol 13 655-92.

Montserrat, E. *et. al.*; Leukemia & Lymphoma (1996) vol 21(5-6) 467-72.

Montserrat, E. *et. al.*; Annals of Oncology (1997) vol 8 (Suppl. 1) S93-S101.

Muchmore, S. W. *et. al.*; Letters To Nature (1996) vol 381 335-31.

Muzio, M. *et. al.*; Cell (1996) vol 85(6) 817-27.

Nashiro, Y. *et. al.*; Oncogene (1999) vol 18(21) 3244-51.

Natkuman, Y. *et. al.*; Journal of Immunology (1994) vol 153(8) 3476-84.

Nava, V.E. *et. al.*; Journal of Virology (1997) vol 71 (5) 4118-4122.

Newton, K. *et. al.*; EMBO Journal (1998) vol 17(3) 706-18.

Nilsson, K; Chapter 2 of Chronic Lymphocytic Leukemia Ed. by B.D. Cheson published Marcel Dekker (1993) 33-62.

O'Connor, L. *et. al.*; EMBO Journal (1998) vol 17(2) 384-95.

Oltvai, Z. N. *et. al.*; Cell (1993) vol 74 609-19.

Oltvai, Z. N. *et. al.*; Cell (1994) vol 79 189-92.

Ohno, T. *et. al.*, Blood (1998) vol 91(5) 1757-61.

Oostendorp, R. A. & Dormer, P; Leukemia & Lymphoma (1997) vol 24 (5-6) 423-35.

Orth, K. *et. al.*; Journal of Biological Chemistry (1996) vol 271(28) 16443-6.

Osorio, L. M. *et. al.*; Blood (1997) vol 89 (8) 2833-41.

Osorio, L. M. *et. al.*; Leukemia & lymphoma (1998) vol 30 247-56.

Ottilie, S. *et. al.*; Journal of Biological Chemistry (1997) vol 272(27) 16955-61.

Panayotidis, P *et. al.*; British Journal of Haematology (1993) vol 85 439-45.

Panayotidis, P *et. al.*; British Journal of Haematology (1994) vol 86 169-73.

Panayotidis, P *et. al.*; Leukemia (1995) vol 9 1227-32.

Panayotidis, P *et. al.*; British Journal of Haematology (1996) vol 92 97-103.

Parker, M. W. & Pattus, F.; Trends in Biological Science (1993) vol 18 391-5.

Pegoraro, L. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1984) vol 81 7166-70.

Pepper, C. *et. al.*; British Journal of Haematology (1996) vol 95 513-7.

Pepper, C. *et. al.*; British Journal of Cancer (1997) vol 76(7) 935-8.

Pepper, C. *et. al.*; Leukemia & Lymphoma (1998) vol 28(3-4) 355-61.

Petrella, T. *et. al.*, Anticancer Research (1997) vol 17(5B) 3907-13.

Pound, J. D. *et.al.*; Blood (1997) vol 89(3) 919-28.

Prak, E. L. *et. al.*; Journal of Experimental Medicine (1995) vol 182(2) 541-8.

Pribyl, J. A. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1996) vol 93(19) 10348-53.

Pritsch, O. *et.al.*; Seminars in Oncology (1998) vol 25(1) 34-41.

Pu, Q.Q. *et. al.*; British Journal of Haematology (1997) vol 98 413-7.

Rabinowe, S. N. *et. al.*; Blood (1993) vol 82(4) 1366-76.

Raghoebier, S. *et. al.*; Blood (1991) vol 77(7) 1560-4.

Rajewsky, K. *et. al.*; Nature (1996) vol 381(6585) 751-8.

Rawlings, D. J. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1995) vol 92(5) 1570-4.

Rechavi, G. *et. al.*; Leukemia (1989) vol 3(1) 57-60.

Reed, J.C. *et. al.*; Mechanisms of Lymphocyte Activation and Immune Regulation VI, Ed. by Gupta & Cohen, Plenum Press, New York (1996) 99-112.

Reed, J. C; Nature (1997) vol 387 773-6.

Revilla, Y. *et. al.*; Virology (1997) vol 228 400-4.

Rheaume, E. *et. al.*; EMBO Journal (1997) 16(21) 6346-54.

Ribera, J. M. *et. al.*; Blood Cells (1987) vol 12 471-9.

Rickert, R. C. *et. al.*; Nature (1995) vol 376(6538) 352-5.

Rinkenberger, J. L. *et. al.*; Immunity (1996) vol 5(4) 377-86.

Robertson, L. E. *et. al.*; Leukemia (1996) vol 10 456-9.

Rolink, A. *et. al.*; Journal of Experimental Medicine (1993) vol 178(4) 1263-70.

Rudel, T & Bokoch, G. M.; Science (1997) vol 276(5318) 1571-4.

Ruzek, M. C. & Mathur, A.; European Journal of Immunology (1995) vol 25(8) 2228-33.

Ryan, D. H. *et. al.*; Blood (1997) vol 89(3) 929-40.

Sanz, E. *et. al.*; Journal of Experimental Medicine (1996) vol 183(6) 2693-8.

Sato, T. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1994) vol 91 9238-42.

Sattler, M. *et. al.*; Science (1997) vol 275 983-6.

Schena, M. *et. al.*; Blood (1992) vol 11 2981-9.

Schendel, S.L. *et. al.*;Proceedings of the National Academy of Science of the USA (1997) vol 94 5113-8.

Schlesinger, P.H. *et. al.*; Proceedings of the National Academy of Science of the USA (1997) vol 94 11357-62.

Scheuerman, R. H. & Racila, E.; Leukemia & Lymphoma (1995) vol 18(5-6) 385-97.

Schulz, J. B. *et. al.*; Journal of Neurochemistry (1997) vol 69(5) 2075-86.

Sedlak, T. W. *et. al.*; Proceedings of the National Academy of Science of the USA (1995) vol 92 7834-8.

Selvakumaran, M. *et. al.*; Oncogene (1994) vol 9 1791-8.

Shibasaki, F. *et. al.*; Nature (1997) vol 386(6626) 728-31.

Shimizu, S. *et. al.*; Nature (1995) vol 374(6525) 811-3.

Silacci, P. *et. al.*; Journal of Experimental Medicine (1994) vol 180(4) 1329-36.

Silverman, G. A. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1990) vol 87 9913-7.

Simonen, M. *et. al.*; European Journal of Biochemistry (1997) vol 249 85-91.

Simonian, P .L. *et. al.*; Journal of Biological Chemistry (1996) vol 271(37) 22764-72.

Simonian, P. L. *et. al.*; Journal of Biological Chemistry (1996) vol 271(50) 32073-77.

Simonian, P.L. *et. al.*; Oncogene (1997) vol 15 1871-5.

Sjöberg, J. *et. al.*; British Journal of Haematology (1996) vol 92(2) 393-400.

Smith, K. G. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1995) vol 92(25) 11628-32.

Smith, K. G. *et. al.*; EMBO Journal (1996) vol 15(19) 5167-76.

Sorensen, J. M. *et. al.*; Journal of Clinical Oncology (1997) vol 15(2) 458-65.

Sprent, J. & Tough, D. F.; Science (1994) vol 265(5177) 1395-400.

Stephan, R. P. *et. al.*; Journal of Immunology (1997) vol 158(4) 1598-609.

Stephan, R. P. *et. al.*; Blood (1998) vol 91(1) 75-88.

Strasser, A. *et. al.*; EMBO Journal (1995) vol 14(24) 6136-47.

Susin S. A. *et. al.*; Journal of Experimental Medicine (1996) vol 184(4) 1331-41.

Susin S. A. *et. al.*; Journal of Experimental Medicine (1997) vol 186(1) 25-37.

Takahashi, A. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1996) vol 93(16) 8395-400.

Takano, Y. S. *et. al.*; Journal of Pathology (1991) vol 163(4) 329-36.

Takayama, S. *et. al.*, Cell (1995) vol 80 279-284.

Tanaka, S. *et. al.*; The Journal of Biological Chemistry (1993) vol 268 (15) 10920-6.

Tangye, S. G. *et. al.*; British Journal of Haematology (1996) vol 92 950-3.

Tangye, S. G. *et. al.*; Immunology and Cell Biology (1997) vol 75 127-35.

Tedder, T. F. *et. al.*; Annual Review of Immunology (1997) vol 15 481-504.

Terada, S. *et. al.*; Cytotechnology (1997) vol 25(1-3) 17-23.

Thomas, A. *et. al.*; Oncogene (1996) vol 12 1055-62.

Thornberry, N. A. & Lazebnik, Y.; Science (1998) vol 281 1312-6.

Torres, R. M. *et. al.*; Science (1996) vol 272(5269) 1804-8.

Tsiagbe, V. K. *et. al.*; Critical Review of Immunology (1996) vol 16 381-421.

Tsujimoto, Y. *et. al.*; Science (1984) vol 226 1097-9.

Tsujimoto, Y. *et. al.*; Science (1985) vol 228 1440-3.

Tsujimoto, Y. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1986) vol 83 (14) 5214-8.

Turner, C. A. Jnr. *et. al.*; Cell (1994) vol 77(2) 297-306.

Vander Heiden, M. G. *et. al.*; Cell (1997) vol 91(5) 559-62.

von Freeden-Jeffry, U. *et. al.*; Journal of Experimental Medicine (1995) vol 181(4) 1519-26.

Walker, P. R. & Sikorska, M; Biochemistry & Cell Biology (1997) vol 75(4); 287-99.

Wang, D. *et. al.*; British Journal of Haematology (1997) vol 97 409-17.

Wang, H-G. *et. al.*; Oncogene (1994) vol 9 2751-6.

Wang, H-G. *et. al.*; Cell (1996) vol 87 629-38.

Wang, H-G. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1996) vol 93 7063-8.

Wang, K. *et. al.*; Genes & Development (1996) vol 10 2859-69.

Wang, Y. *et. al.*, Oncogene (1993) vol 8(12) 3427-31.

Wasserman, R. *et. al.*; Journal of Immunology (1997) vol 158(3) 1133-8.

Wen, L. P. *et. al.*; Journal of Biological Chemistry (1997) vol 272(41) 26056-61.

Witte, P.L. *et. al.*; European Journal of Immunology (1993) vol 23(8) 1809-1817.

Xiang, J. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1996) vol 93(25) 14559-63.

Xue, D. & Horovitz, H. R.; Nature (1997) vol 390(6657) 305-8.

Yamada, T. *et. al.*; Laboratory Investigation (1996) vol 74(2) 384-94.

Yang, E. *et. al.*; Cell (1995) vol 80 285-91.

Yang, R-Y. *et. al.*; Proceedings of the National Academy of Sciences of the USA (1996) vol 93 6737-42.

Yin, X-M. *et. al.*; Letters To Nature (1994) vol 369 321-3.

Younes, A. *et. al.*; British Journal of Haematology (1998) vol 100 135-41.

Zamzami, N. *et. al.*; Journal of Experimental Medicine (1995) vol 181(5) 1661-72.

Zamzami, N. *et. al.*; Journal of Experimental Medicine (1995) vol 182(2) 367-77.

Zamzami, N. *et. al.*; Journal of Experimental Medicine (1996) vol 183(4) 1533-1544.

Zamzami, N. *et. al.*; FEBS Letters (1996) vol 384(1) 53-7.

Zamzami, N. *et. al.*; Journal of Bioenergetics and Biomembranes (1997) vol 29(2) 185-93.

Zamzami, N. *et. al.*; Oncogene (1998) vol 16(17) 2265-82.

Zelentz, A. D. *et. al.*; Blood (1991) vol 78 (6) 1552-60.

Zha, H. *et. al.*; Journal of Biological Chemistry (1996) vol 271(13) 7440-4.

Zha, J. *et. al.*; Cell (1996) vol 87 619-28.

Zha, J. *et. al.*; Journal of Biological Chemistry (1997) vol 272(39) 24101-4.

Zhang, H. *et. al.*; Biochemical and Biophysical Research Communications (1995) vol 208(3) 950-6.

Zhang, M. *et. al.*; Oncology Reports (1998) vol 5 23-30.

Zoratti, M & Szabo, I; Biochimica et Biophysica Acta (1995) vol 1241(2) 139-176.

Zou, H. *et. al.*; Cell (1997) vol 90(3) 405-13.