Apoptosis induced by cancer chemotherapeutic drugs and its genetic suppression

by

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To my family with love

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AML	acute myeloid leukaemia
araC	cytosine arabinoside or 1-ß-D arabinofuranosylcytosine
araCTP	cytosine arabinoside 5'- triphosphate
bax	*bcl-2* associated **x** gene
bcl-2	B-cell leukaemia/lymphoma gene 2
BHRF1	BadH1 fragment **H** Rightward open reading Frame 1, an EBV gene
BL	Burkitt's lymphoma
Ca^{2+}	ionised form of calcium
CD	cluster of differentiation, as in CD8 on T-cells
C. elegans	***Caenorhabditis elegans*** - a nematode
Chep-BL	Cheptages-BL cell line
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
dCTP	deoxycytidine triphosphate
DHFR	dihydrofolate reductase
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen
EDTA	**ethylenediaminetetraacetic** acid
etop	etoposide
FCS	foetal calf serum
FDCP	Factor Dependent Continuous cell line from the Paterson institute
FDCP-1-B	FDCP-1 sub-clone B
FDCP-1-δ	FDCP-1 sub-clone δ
FdUMP	**fluorodeoxyuridine-5-monophosphate**
FdUrd	5-fluoro-2'-deoxyuridine
FdUTP	**fluorodeoxyuridine-5-triphosphate**
G_1	gap 1, period in the cell cycle prior to S-phase
G_2	gap 2, period in the cell cycle after S-phase and prior to mitosis (M)
G_0	gap 0, period in which cells leave the cell cycle and enter a quiescent state
Gy	Gray; measurement of radioactivity 1Gy = 100 rads
H^3TdR	tritiated thymidine

List of abbeviations

ICAM	Intracellular cell adhesion molecule
IL-2	interleukin-2
IL-3	interleukin-3
IL-6	interleukin-6
Ig	immunoglobulin
kDa	kilodalton
LCL	lymphoblastoid cell line
LFA	lymphocyte function -associated antigen
LMP	latent membrane protein
MCL-1	myeloid cell leukaemia 1
MDR	multidrug resistance
MTX	methotrexate
$NaHCO_3$	sodium hydrogen carbonate
P-170	P-glycoprotein protein product of ***mdr1*** gene
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
RNA	ribonucleic acid
S-phase	period of **DNA** replication in the cell cycle, prior to G_2 and M
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
TMP	thymidine monophosphate
Topo II	Topoisomerase II
TTP	thymidine triphosphate
TS	thymidylate synthase
UV	ultra-violet

Synopsis

Chapter 1

Synopsis

Apoptosis can be distinguished from necrosis, the classical form of cell death, by several morphological and biochemical criteria. Apoptotic cells, but not necrotic cells, show early condensation of chromatin as well as endonuclease activation resulting in cleavage of the nuclear DNA into oligonucleosomal fragments. Both physiological and low level cytotoxic stimuli have been shown to induce apoptosis, which in some cell models can be suppressed by inhibitors of protein and **RNA** synthesis. The concept of the cell being actively involved in its own death, combined with the demonstration that factors which alter the rate of cell death, such as the proto-oncogene *bcl-2,* can directly affect the number of cells within a population, has resulted in the identification of cell death alongside proliferation and differentiation as a means for controlling celi population growth.

The purpose of this study was to determine if *bcl-2* and the Epstein-Barr virus gene BHRF1, which share 25% primary amino acid sequence homology, could suppress apoptosis in response to a variety of anti-cancer treatments. After demonstrating apoptotic cell death on treatment with chemotherapeutic agents in an **IL-3** dependent cell line (FDCP-1) and three different EBV genome-positive Burkitt's lymphoma cell lines, the survival of EBV-BL cell lines expressing either exogenous *bcl-2* or BHRF1 was examined. Suppression of apoptosis in response to treatment with chemotherapeutic drugs or y radiation was clearly shown in EBV-BL cells expressing *bcl-2* or BHRF1 when compared to control transfectants.

This study has further confirmed that BHRF1 is functionally homologous to *bcl-2*, suggesting that BHRF1 may act to prevent apoptosis during EBV infection. Suppression of chemotherapeutic drug induced cell death by either *bcl-2* or BHRF1 also represents a novel form of drug resistance and may form an alternative mechanism by which multidrug resistance may arise during chemotherapy. The identification and investigation of other genes which produce suppression of apoptosis is also important in order to determine the extent of involvement of apoptotic suppression in the transformation to the malignant state and in the acquisition of multidrug resistance. **A** protocol to screen for 'apoptosis-suppressed cells' in the FDCP-1 **E - 3** dependent cell line was developed to identify new genes involved in the pathway(s) of apoptosis.

Aims and Objectives

Chapter 2

Aims and Objectives

The observation that the proto-oncogene bcl-2 was able to act as a molecular suppressor of apoptosis both *in vitro* (Vaux *et al.,* 1988; Nunez *et al.,* 1990) and *in vivo* (McDonnell *et al.,* 1989) was paramount in demonstrating that cell death was fundamentally important in the growth control of cell populations.

The translocation of a gene on chromosome 18q21 to the immunoglobulin heavy chain gene locus on chromosome 14q32 is strongly associated with follicular lymphoma. The gene on chromosome 18 was named B-cell leukaemia/lymphoma gene **2,** representing the proposed proto-oncogene bcl-2 (Tsujimoto *et al.,* 1984). bcl-2 has since been shown to be oncogenic in nude mice injected with 3T3 cells which express an exogenous form of the gene (Reed *et al.,* 1988), but bcl-2 is not able to produce a transformed cell phenotype in the absence of other genetic changes, Work with bcl-2-Ig transgenic mice demonstrated that they had expanded pre-B and B-cell populations which arose as a direct consequence of the ability of bcl-2 to prevent the death of these cells (McDonnell *et al.,* 1989). Initially the mice exhibited no clinical effects, however, after 12 months many of the mice developed lymphoma and in 50% of the mice this was associated with a deregulated c-myc gene (McDonnell and Korsmeyer 1991). This demonstrated that bcl-2, by providing a survival signal for cells which would otherwise have died to maintain a B-cell population of normal size, had increased the chances of oncogenic mutation(s) occurring to produce clonal malignant outgrowth.

One the first genes to be identified with homology to bcl-2 was the Epstein Barr virus gene BHRF1 (Cleary *et al.,* 1986). This gene is expressed early in the EBV lytic cycle (Pearson *et al.,* 1986) and in some tightly latent cell lines after serum starvation, followed by reculture in high serum concentrations (Kocache and Pearson, 1990). EBV is associated with several human cancers, including endemic Burkitt's lymphoma found in areas defined by the African malaria belt. BHRF1 shares 25% primary amino acid sequence homology with bcl-2, mainly at the C-terminus (Cleary *et al.,* 1986).

Aims and Objectives

One of the aims of this study is to analyse further the extent of functional homology between *bcl-2* and BHRF1. Eukaryotic expression constructs are available for these genes in the form of a BHRF1 construct produced by Dr. David Huen (Dept. Cancer Studies, University of Birmingham) and the Tsujimoto *bcl-2* construct, (used by kind permission of Yoshihide Tsujimoto), as described in Tsujimoto (1989). Functional homology will be determined in transfected cells by the ability of BHRF1 to suppress cell death in a comparable manner to *bcl-2.*

Bcl-2 has also been shown to induce stress resistance in response to several stimuli including heat shock and methotrexate (Tsujimoto 1989). Chemotherapeutic drugs such as methotrexate are known to induce apoptosis (e.g. Wyllie *et al.,* 1980; Li and Kaminskas, 1984; Lennon *et al.,* 1990). *A* primary aim of the project is to investigate whether *bcl-2* or BHRF1 are able to suppress chemotherapeutic drug induced cell death. Initially, induction of apoptosis in three human Burkitt's lymphoma cell lines and a murine IL-3 dependent cell line by chemotherapeutic drugs will be confirmed. This will be demonstrated by morphological criteria using both light and electron microscopy techniques and by biochemical analysis to demonstrate fragmentation of the DNA, a classical marker of apoptosis (Wyllie, 1980). Once this has been established, cells transfected with either a *bel-2* or a BHRF1 expressing construct will be used to investigate whether or not expression of these genes can produce a novel form of multidrug resistance by suppressing apoptosis. One documented form of multidrug resistance is produced by the over expression of a human gene ***mdr1,*** which codes for a glycoprotein able to actively pump naturally occurring chemotherapeutic drugs from the cytoplasm of cancer cells (Ling, 1990). The drugs used in the present study include methotrexate, primarily because it does not interact with the MDR1 glycoprotein and therefore is not actively removed from the cell by this mechanism (Mickisch *et al.,* 1991). *A* demonstration of multidrug resistance in the transfected cells, including resistance to methotrexate, would **suggest** a mechanism for multidrug resistance involving *bcl-2* or BHRF1 which is independent of MDR1 expression.

The discovery of BHRF1, albeit a viral gene, which has homology to *bcl-2,* along with the evidence that *bcl-2* is not able to suppress apoptosis in all cell types (Nunez *et al.,* 1990),

implies that other cellular genes may well function to promote cell survival, and has been recently demonstrated by the discovery of three cellular *bcl-2* homologues (reviewed in Williams and Smith, 1993). **Work** with the IL-3 dependent cell line FDCP-1 has shown that some of these cells appear viable after 7 days, as judged by vital dye exclusion, in the absence of **IL-3** and do not appear to enter apoptosis (N. J. McCarthy, B.Sc. thesis 1990). However, these cells do not appear to grow under these conditions, suggesting that they may be unable either to proliferate or to die, behaviour which is comparable to FDCP-1 cells expressing *bcl-2* (Vaux ***et al.***, 1988). **A** screening protocol is to be developed to assess the phenotype of these 'survivor' cells to examine the possibility that some may be live cells blocked from entering apoptosis on **IL-3** withdrawal. The attainment of cells with this phenotype will enable genotyping of the mutant cells to see whether they over expresses *bcl-2* or a new, previously uncharacterised gene involved in the apoptotic pathway(s), possibly a *bcl-2* homologue. **A** longer term possibility would be the production of mutants using insertional mutagenesis to identify such genes.

Introduction

Chapter 3

Introduction

Section 3.1 Apoptosis and Necrosis

3.1.1 What is apoptosis?

Apoptosis was the word coined in a paper by Wyllie, Kerr and Currie in 1972 for a newly described mode of cell death. This does not mean, of course, that cells had evolved a new way of dying, but that the above authors had identified a mode of cell death which was morphologically and biochemically distinct from the classical form of death known as coagulative necrosis. The fundamental difference between apoptosis and necrosis is that apoptosis represents a controllable form of cell death, which has wide ranging implications in many biological fields. However, it was the morphological characteristics which first alerted Wyllie and colleagues to apoptosis and hence it is useful to start an introduction on this subject from a morphological point of view.

3.1.1.1 Cell death by coagulative necrosis

Necrosis is the classically accepted form of cell death and occurs in response to any severe physiological or environmental deviation i.e. change in temperature, lack of oxygen (ischemia) and injury by agents such as chemical toxins etc., (Trump ***et al.,*** 1973; Wyllie ***et al.,*** 1972; Wyllie ***et al.,*** 1980). Such environmental deviations lead to a loss of homeostatic control by the cell and this is thought to occur in two ways:

- Damage to the plasmalemma, such as that produced by complement attack or osmotic fluctuations, which enables an influx of ions, especially calcium, into the cell (Wyllie ***et al.,*** 1980)
- Damage to respiratory apparatus, such as mitochondria, leads to a lack of ATP which causes a failure of ion pumps in the membrane. This again allows a large scale influx of ions into the cell, including calcium ions, which cause further damage to the mitochondria as well as stimulating

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Ca^{2+} dependent proteases and phospholipases (Laiho and Trump 1975; Trump *et al.,* 1973).

Morphologically, cells dying by necrosis show a distinct pattern of cellular breakdown, see figure 3.1**A,** which consists of two stages, one reversible, the other irreversible (Trump *et al.,* 1973). The reversible stage, in which cells may be able to adapt to environmental changes, is characterised by the dilation of the endoplasmic reticulum along with a slight clumping of the nuclear chromatin. Other changes in the cell also include cytoplasmic swelling, whilst the mitochondria appear to be condensed due to the inner membrane shrinking from the outer. The irreversible stage is reached when the cell is unable to adapt to the environmental change. This stage can be identified by the mitochondria undergoing 'high amplitude swelling' which is characterised by the appearance of dense lipid rich aggregates within the inner membrane, which stili continues to shrink from the outer. The cell continues to swell and the chromatin takes on the appearance of flocculent masses which eventually disappear leaving a nuclear ghost (Wyllie *et al.,* 1980). Finally all membranes rupture and the cell undergoes autolysis and denaturation. Areas of tissue affected by the initial toxic insult often involve large numbers of necrotic cells and this can result in exudative inflammation in viable tissue nearby, damaging these also. Cellular debris resulting from necrosis is phagocytosed by monocytes.

3.1.1.2 Cell death by apoptosis

Celi death by apoptosis is found to occur in many different biological situations. Single apoptotic cells are often found in healthy growing tissues such as small gut crypts. Apoptosis also occurs at many stages of embryonic development, for example, during the formation of digits from a solid limb paddle where interdigital cells between the digits are seen to die (Hmchliffe and Ede 1973). It has also been observed in metamorphosis (reabsorption of the tadpole tail), in endocrine induced atrophy of tissues, in celi mediated immunity and in neoplasms (Wyllie *et al.,* 1980). In many of the cases mentioned above, apoptosis occurs as a control or regulatory process in the tissue, often under normal physiological circumstances. Perhaps one of the best examples of this is the deletion of autoreactive cells in the thymus (Smith *et al.,* 1989; Jenkinson *et al.,* 1989). Immature thymocytes fall into three categories,

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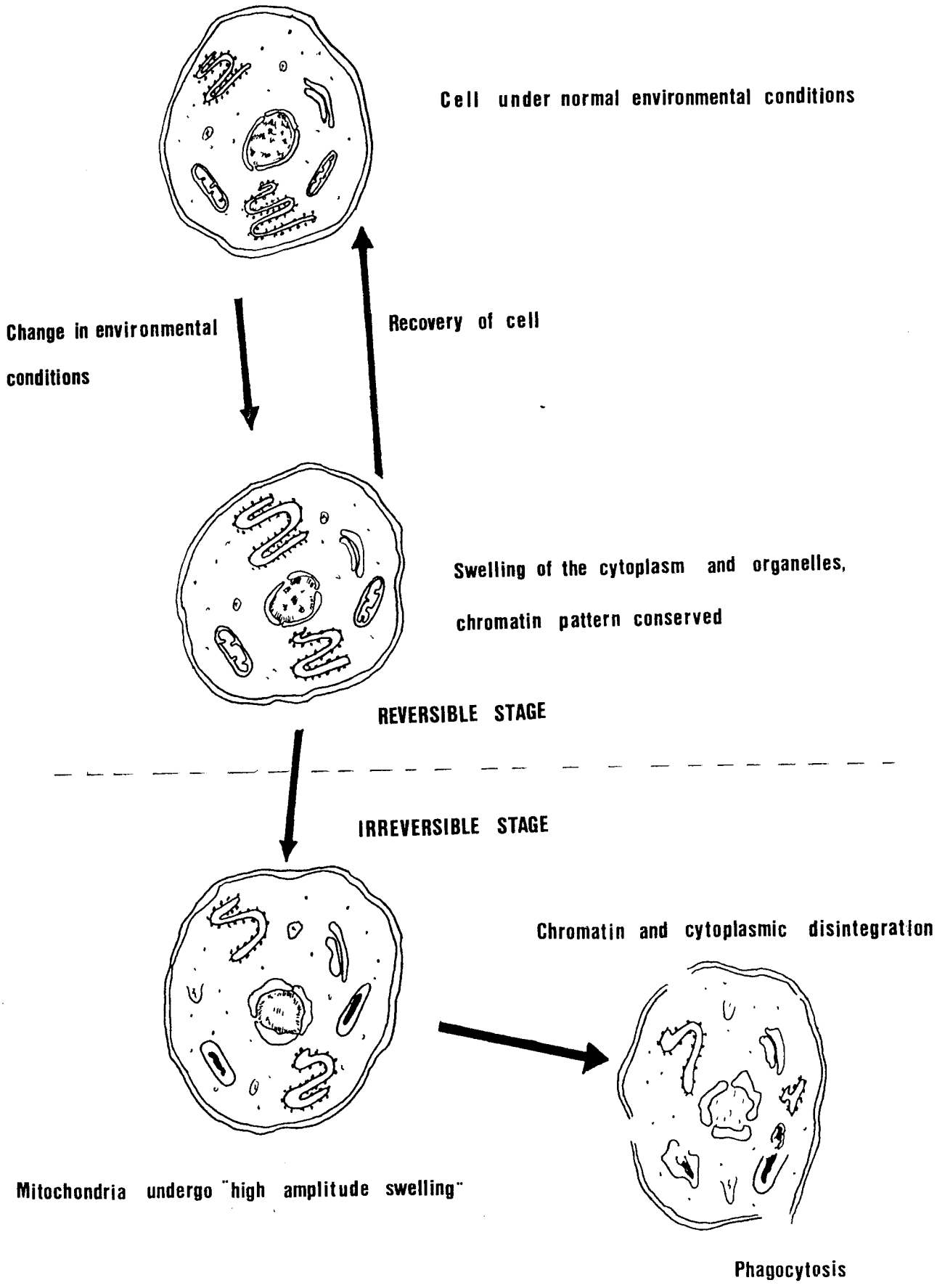
Figure 3.1A The morphology of a cell dying by Necrosis

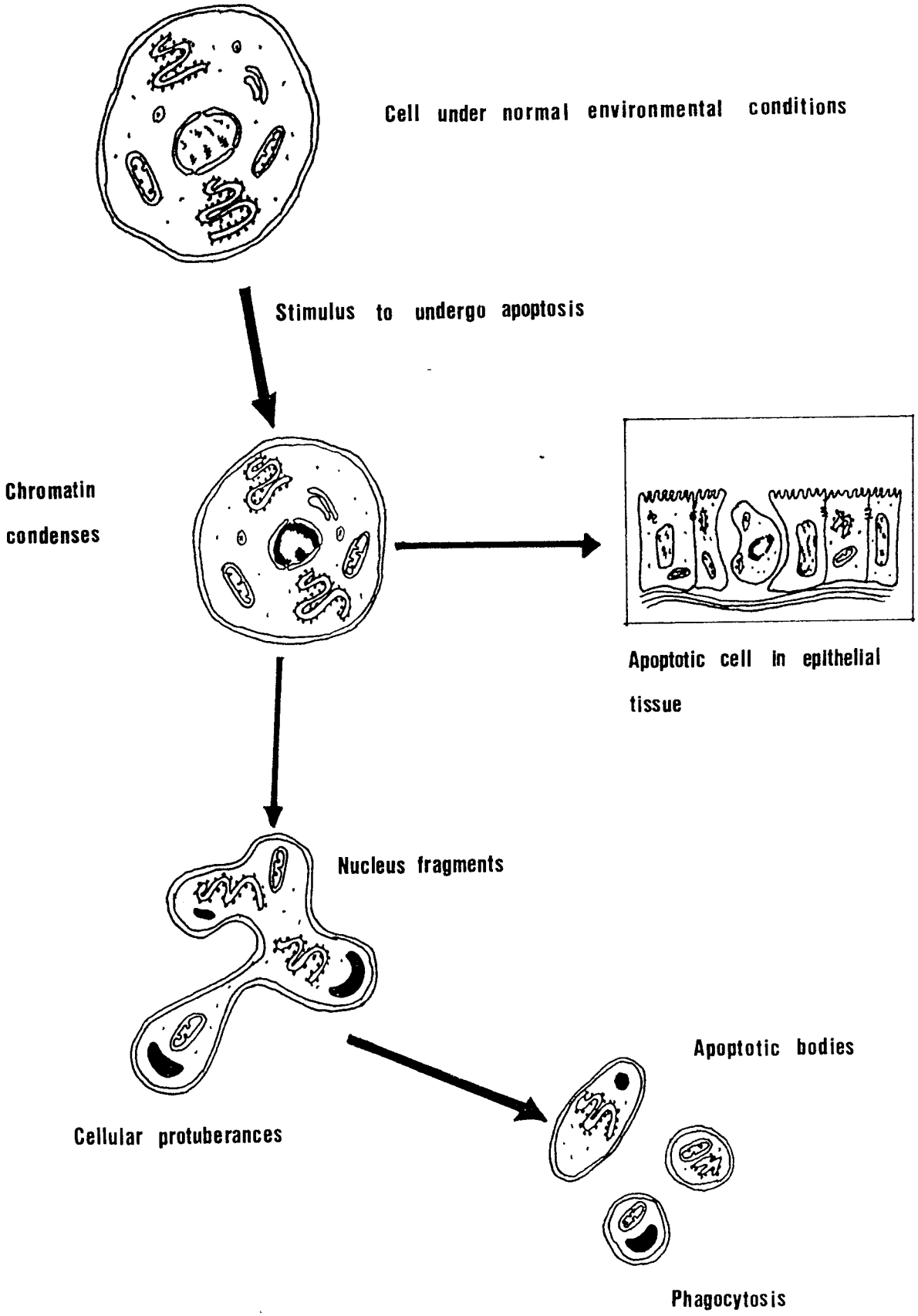


those expressing a useful T cell receptor (TCR), able to recognise **MHC** and antigen, those expressing a useless TCR, unable to recognise self MHC or antigen and those expressing a TCR which recognises self only. The latter two categories are of no use or are dangerous to the body and are therefore signalled to enter apoptosis and die. The former category are useful and able to bind to antigen presented by dendritic cells which results in a signal to proliferate and therefore survive. This in itself is an interesting concept, since most of the control in a tissue was previously thought to occur during the proliferation and differentiation stages of cell development, (see section 3.2.1).

Morphologically, an apoptotic cell is different from a necrotic cell (Wyllie ***et al.,*** 1980; Kerr 1971; Wyllie ***et al.,*** 1972), see figure 3.1B. One of the most prominent and earliest stages of apoptosis involves the nucleus. Chromatin is seen to condense and form aggregates near the nuclear membrane which becomes convoluted, whilst the nucleolus becomes enlarged and appears abnormally granular. In parallel to this, detectable changes in the cytoplasm occur with it becoming condensed and protuberances appearing at the membrane surface. There are two main theories so far which account for the reduction in the volume of the cell cytoplasm. Wyllie's original suggestion was that cells lost intra-cellular fluid selectively by osmosis to combat the efflux of ions such as K+ from the cell (Wyllie 1987).

A second theory and one which is not necessarily independent from the former, is that of microtubule disruption. Early morphological studies suggested that a redistribution of microtubules occurred in apoptosis (Wyllie ***et al.,*** 1980). More recently, Martin and Cotter (1990) followed microtubule patterns in differentiating HL60 cells undergoing apoptosis. Staining of the microtubule network using immunofluorescence revealed a disrupted network when compared to viable cells. Such disruption could be effected by the increase in intracellular calcium observed in apoptosis since microtubules are known to be sensitive to changes in calcium homeostasis (Schliwa, 1976). Conversely, treatment of undifferentiated HL60 cells with microtubule disruptants, such as colchicine and vinblastin, resulted in a loss of viability together with the morphological characteristics, such as chromatin condensation, associated with apoptosis.

Figure 3.1B The morphology of a cell dying by Apoptosis



At the point of cytoplasmic condensation the cell will still appear viable in the presence of vital dyes, therefore indicating no structural failure in the cell membrane. In structured epithelial tissues, cells at this stage will have rounded up and severed all junctions with surrounding viable cells. Progression of the cytoplasmic condensation results in crowding of the organelles, but unlike necrotic cells, the organelles of apoptotic cells are morphologically normal except for the dilation of the endoplasmic reticulum.

Later stages of apoptosis result in fragmentation of the condensed chromatin which forms crescent shaped caps. Migration of the chromatin occurs into the highly convoluted areas of the cell which can bud off, producing apoptotic bodies, some of which contain dark condensed chromatin and others just intact cellular organelles. The apoptotic bodies are normally phagocytosed by the surrounding viable cells or by cells of the mononuclear phagocytic system (see the following subsection). Phagosomes containing apoptotic bodies finally fuse with primay and secondary lysosomes produced by the ingesting cells for degradation.

3.1.2 The recognition and phagocytosis of apoptotic cells

Recognition of apoptotic cells as 'senescent self by macrophages is thought to be mediated partly by the vitronectin receptor, a member of the integrin family of receptors found on macrophage cell surfaces (Savill ***et al.,*** 1990). This receptor is able to recognise a specific amino acid sequence (Arg-Gly-Asp), known as an RGD sequence, which was thought to be expressed on the extracellular matrix of apoptotic cells. However, it would seem that apoptotic cells do not express RGD containing molecules on their cell surface, and instead this sequence may be important in facilitating the recognition of apoptotic cells by a more indirect mechanism. Work by Savill and colleagues (1992) has demonstrated that recognition of apoptotic neutrophils may indeed be via the vitronectin receptor, as well as the CD36 ligand, which are able to bind thrombospondin, a soluble RGD containing protein which may act as a molecular bridge and bind to an anionic site on the apoptotic cell. Interestingly, GM-CSF increases the secretion of thrombospondin by macrophages and hence increases the capacity of macrophages to bind apoptotic cells.

Changes in cell surface charge also appear to be important in apoptotic cell recognition. Changes in pH have been shown to reduce dramatically the binding of apoptotic neutrophils by macrophages (Savill *et al.,* 1989) and reductions in cell surface charge have been documented in apoptotic thymocytes. This may be due in part to the desialylation of surface carbohydrate (Morris *et al.,* 1984) as well as the expression of phosphatidylserine on the plasma membrane (Fadok *et al.,* 1990).

Therefore, recognition of apoptotic cells could be mediated by one or more of the following mechanisms;

- Loss of sialic acid moieties expressed on carbohydrate molecules,
- Recognition through the vitronectin receptor using thrombospondin as a linker protein and
- Recognition of cell surface phosphatidylserine.

Efficient recognition of apoptotic cells is thought to aid the rapid disposal of cell debris which is one of the reasons why apoptosis occurring in healthy tissue is often hard to identify. *In vitro,* apoptotic cells may undergo secondary necrosis which leads to cell swelling and membrane breakdown (Wyllie *et al.,* 1980).

3.1.3 The significance of chromatin degradation in apoptosis

One of the most striking morphological features of apoptosis is the very early condensation of the chromatin. In thymocytes this is concomitant with the activation of the calcium and magnesium dependent endonuclease (Wyllie 1980), and much of the *in vitro* work on the identification of the endonuclease has been carried out on these cells, since they readily undergo apoptosis in the presence of glucocorticoids (Wyllie *et al.,* 1980).

At the point of chromatin condensation, analysis of the DNA by agarose gel electrophoresis reveals the presence of a DNA ladder. The appearance of the DNA is comparable to that of DNA digested with micrococcal nuclease.

The endonuclease is only able to cut linker DNA between nucleosomes, (see diagram **3.2).** DNA within the nucleus is structured, i.e., it undergoes some form of packaging. Some of the proteins involved in the packaging are histones, H1, H2a, H2b, **H3** and H4. These

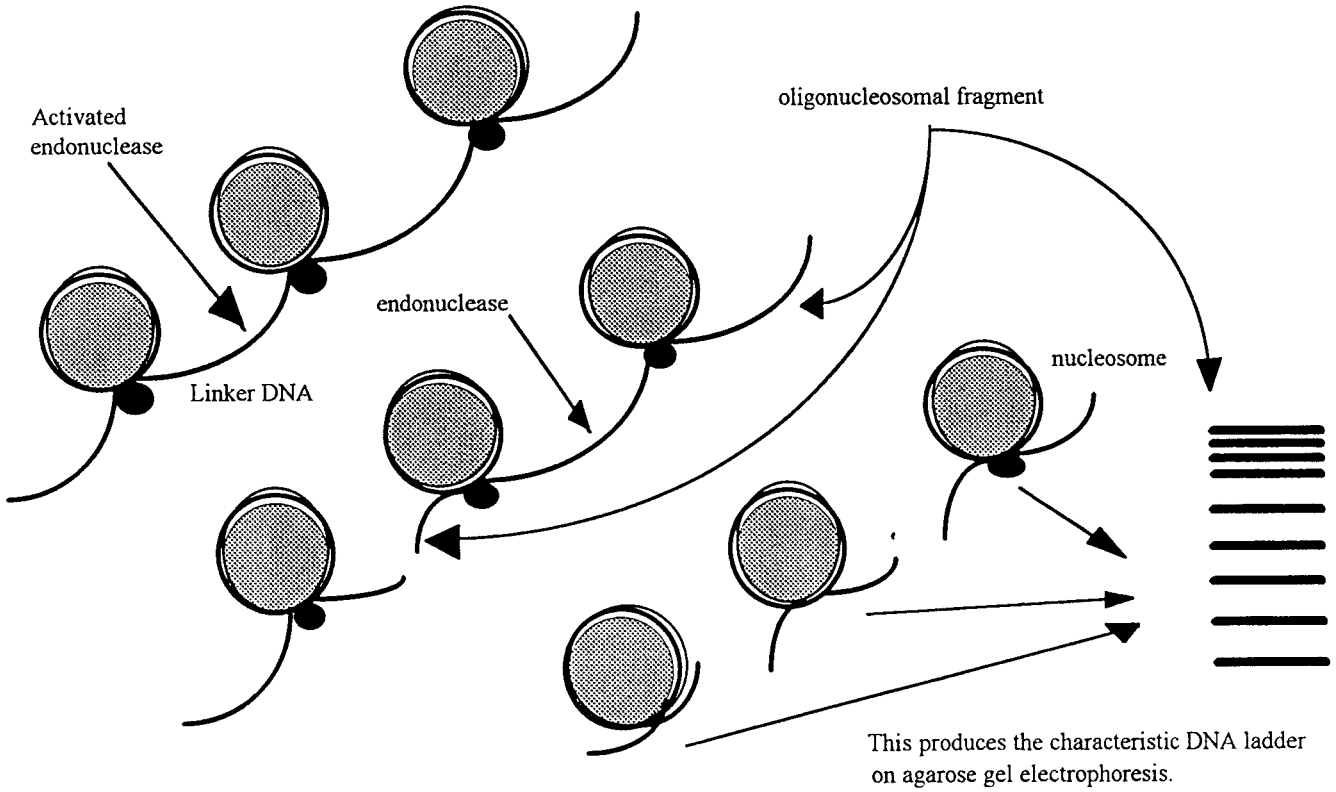


Figure 3.2 The mechanism of endonuclease cleavage of DNA within apoptotic cells

proteins interact with one another and the DNA *to* produce structures known as nucleosomes. Nucleosomes are made up of histone cores around which the DNA is wound. DNA within these complexes is protected from the endonuclease, but linker **DNA** between nucleosomes remains susceptible to cleavage and is digested in the presence of the activated endonuclease. Not all linker **DNA** is cleaved at the same rate due to differing accessibility between active and condensed chromatin and therefore oligonucleosomal fragments are produced resulting in the formation of the characteristic DNA ladder. Recent work by Arends *et al.,* (I 990) has shown that oligonucleosomal fragments of 180-540bp in length are associated primarily with High Mobility Group (HMG) proteins 14 and 17 and are free from the nuclear membrane. Larger fragments however are fixed to the nuclear membrane and are associated with histone H1. This may be explained by the smaller fragments representing active genes more accessible to the endonuclease since actively transcribed genes are believed to associate with HMG proteins. Larger fragments may represent still tightly bound inactive genes to which the endonuclease would have delayed access, hence larger fragments and the presence of histone H1 (Weintraub 1985). Only when a protease is present in conjunction with an activated endonuclease can all the DNA be digested resulting in a smear of DNA being produced on an electrophoresis gel. This result is generally associated with necrotic cell death, where degradation of all cellular organelles is seen. Fragmentation of the **DNA** is thought to be directly responsible for the condensation of the nucleus, since incubation with micrococcal endonuclease results in the same effect (Arends *et al.,* 1990).

Efforts to purify the endonuclease at first proved unsuccessful (Baxter *et al.,* 1989), despite claims that the Ca^{2+}/Mg^{2+} endonuclease is always present in the nuclei of immature thymocytes (Cohen and Duke 1984; Wyllie *et al.,* 1987; Wyllie 1980). However, some candidate endonucleases have now been described, including three Ca^{2+} dependent enzymes present in thymocyte nuclei (Gaido and Cidlowski 1990; Arends *et al.,* 1990; Peitsch *et al.,* 1993). *Ail* three endonucleases appear to be different molecules, with one having been shown to be DNase I, an endonuclease which is found in several tissues other than thymocytes (Peitsch *et al.,* 1993). The presence of three different Ca^{2+} dependent endonucleases implies

that the Ca^{2+} dependent pathway may exhibit some redundancy, but this has not yet been shown and requires fùrther investigation.

Other candidates for the role of the apoptotic endonuclease have been documented in cells other than thymocytes (Barry and Eastman 1993; R. Fernandes personal communication). Both groups have isolated enzymes which are activated by a change in pH levels within the cell, but do not appear to be affected by an increase in the intracellular levels of Ca^{2+}. Barry and Eastman have suggested that the endonuclease found in apoptotic CHO cells after treatment with chemotherapeutic drugs is DNase **II,** activated by a rise in pH within the cytoplasm during apoptosis, the same result has also been shown for **HL60** cells (Barry ***et al.***, 1993). Ca^{2+} dependent endonucleases have also been found in tissues other than thymocytes, such as the endonuclease implicated in rat prostate cells undergoing apoptosis after androgen withdrawal (Kyprianou ***et al.,*** 1988). This suggests that there may be both Ca^{2+} dependent and independent endonucleases present in cells and either can be used depending on the signal received and whether a rise in internal calcium levels occurs. This also implies that there are several different signalling pathways within cells which can effect the same cellular responses, such as endonuclease activation (D. J. McConkey, personal communication).

Section 3.2 The significance of apoptosis

3.2.1 Apoptosis and cell population growth control

In general, control of cell population size was previously thought to be mediated at the cell proliferation and cell differentiation level. With the discovery of apoptosis identifying a pathway of active, controllable cell death, it has now become feasible that control of cell populations can be exerted at the level of cell death, as depicted in figure **3.3.** Many cellular models have reflected this, (see below), along with demonstrating that inhibition of cell death can lead directly to an accumulation of cells within a given population.

Many physiological apoptotic stimuli have been identified, including withdrawal of IL-3 from factor dependent cells (Williams ***et al.,*** 1990), or stimulation of the T-cell receptor in immature thymocytes (Smith ***et al.,*** 1989; McConkey ***et al.,*** 1989d). The response of such cells to these stimuli is in turn dependent on the intracellular state of the cell, (see figure **3.4).**

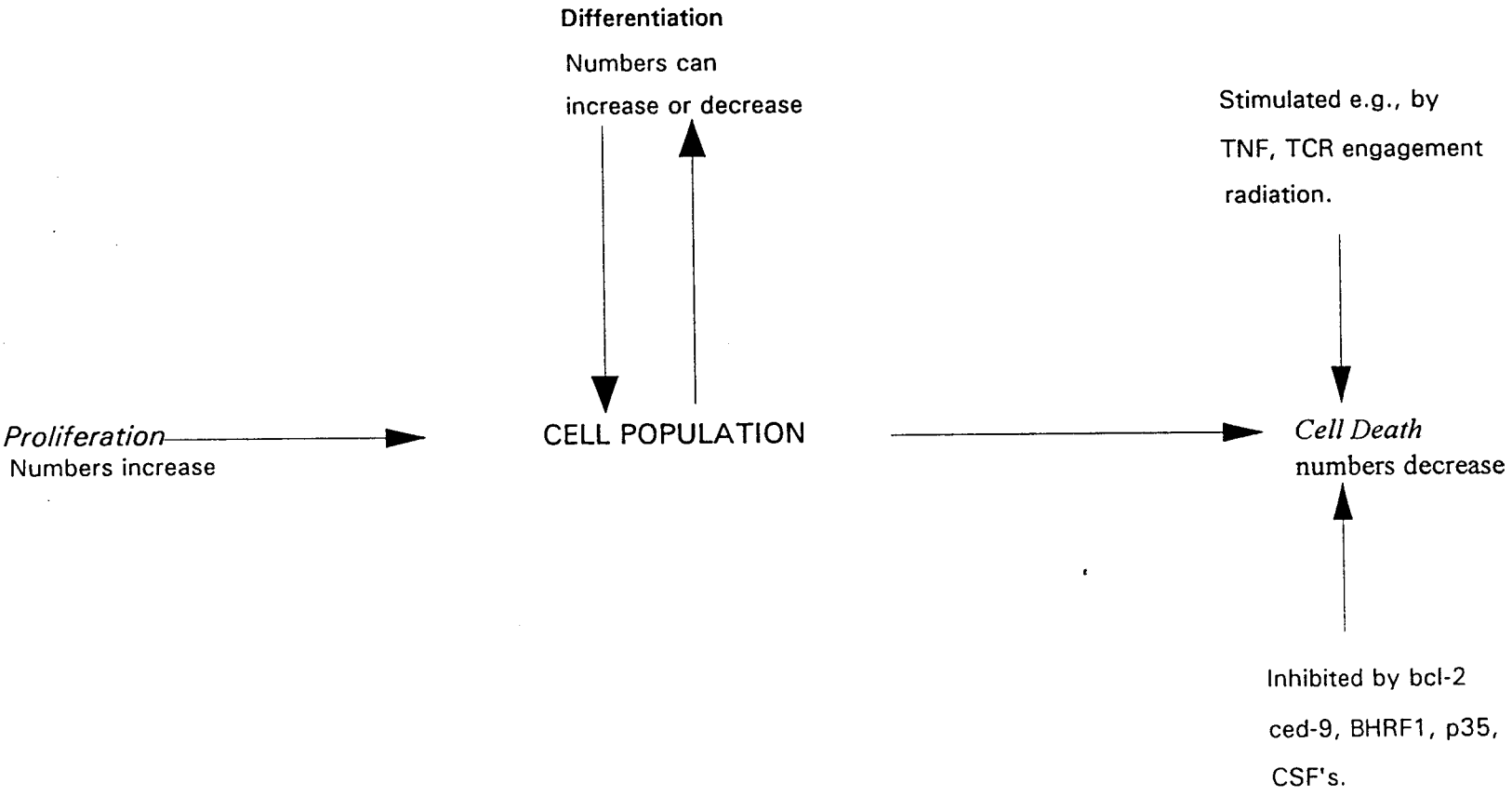


Figure 3.3 The control of cell population numbers can be effected at the proliferation, differentiation and cell death levels. Cell death itself can be regulated by various different stimuli, both positive and negative, which can increase or decrease the numbers of cells survivng in a population, without any change in proliferation and differentiation rates (see text for details).

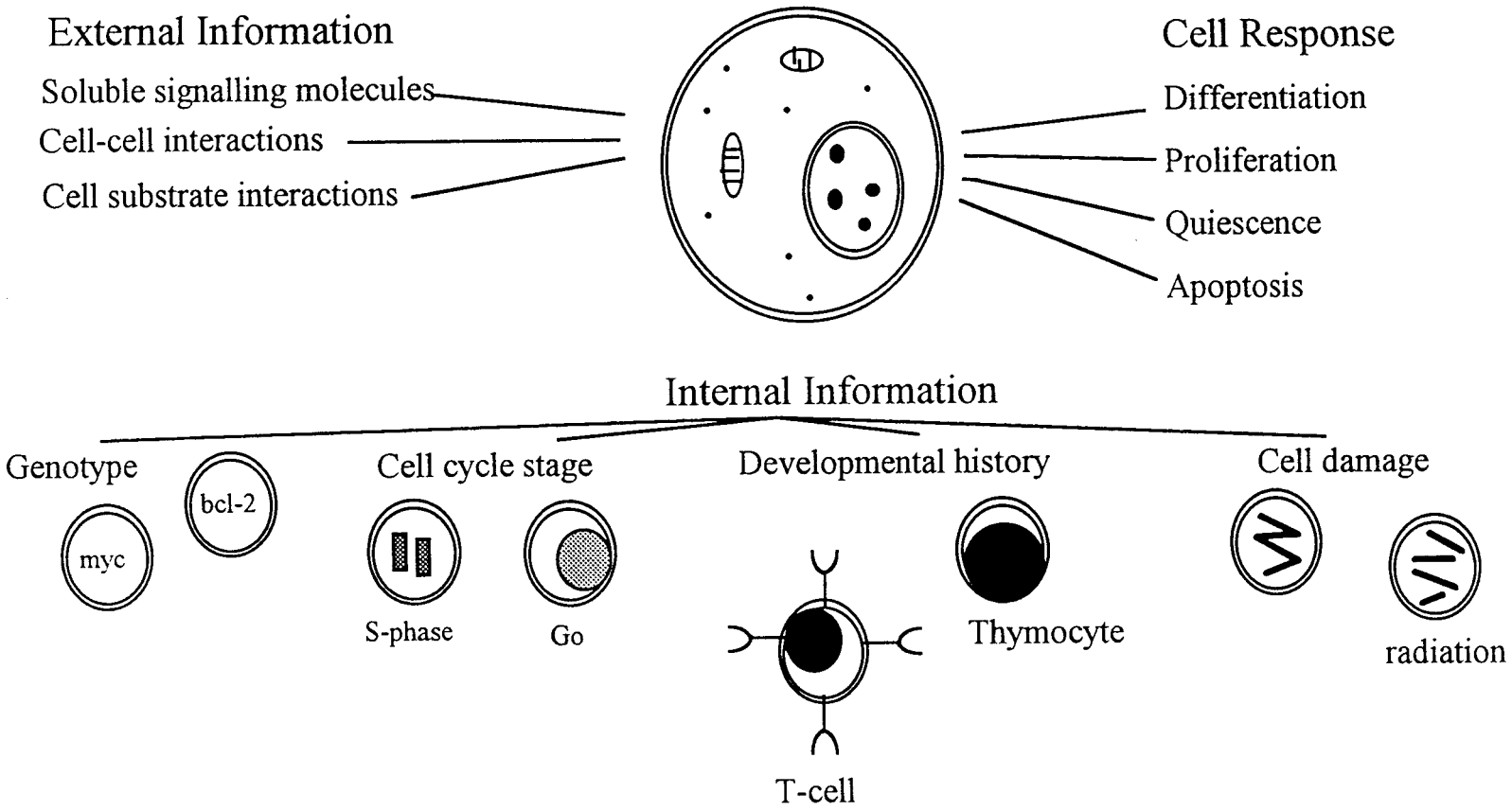


Figure 3.4 External and internal factors governing cell fate

The cellular response to external signalling factors such as hormones and growth factors is dependent on the internal information within the cell. For example, an immature thymocyte which receives an external signal in the form of cross linking of the TCR will enter apoptosis, however if a mature T-cell receives the same stimulus then the cellular response is proliferation.

For example, changes in gene expression can alter the response of a cell to a given stimulus. Deregulated c-myc, for example, causes apoptosis in response to serum withdrawal in fibroblast cells, a signal which in fibroblasts with regulated c-myc results in quiescence and the down regulation of c-myc expression (Evan *et al.,* 1992).

Perhaps one of the best examples for demonstrating cellular growth control carried out at the level of cell death is found in the nematode *Caenorhabditis elegans,* in which the cellular development pathways within this animal have been precisely mapped.

3.2.2 Invertebrate models **of** programmed cell death

In recent years invertebrate models have given an invaluable insight into the genetic controls of cell death. Work in the tobacco hornworm *Manduca sexta* has shown that a host of mRNAs are produced in the final ecdysis before emergence of the adult moth, a process triggered by a fall in the levels of ecdysteroid hormone (Schwartz and Truman 1982, 1983). Regression of intersegmental muscles causes the prominent gene expression to change from myofibrillar proteins to a large number of smaller **mRNAs,** one of which is homologous to TRPM-2 (testosterone repressed prostate message **2),** a gene also expressed during apoptosis within the rat prostate (Wadewitz and Lockshin, 1988; Buttyan *et al.,* 1989; see section 3.4.5). In addition Schwartz and co-workers (1990) demonstrated that this form of cell death requires the activation of several genes including polyubiquitin which could be involved in tagging proteins for degradation.

A definitive plan of gene expression during cell development and death has been mapped in the nematode *Caenorhabditis elegans* (Ellis and Horvitz 1986; Hedgecock *et al.,* 1983; Sulston *et al.,* 1983). The cell lineage development pattern within this animal has been extensively investigated and hence it has been established that exactly 131 cells normally die during the animal's development. Some of the genes involved in the programmed cell deaths have been identified and are shown in figure 3.5. Of particular interest are two genes, programmed cell death gene **3** *(ced* **3)** and *ced 4,* which have been shown to be directly responsible for the death of specific cells during development. Loss of function mutants of *ced 3* or *4* result in an animal where cells which normally die during development do not, but instead take up the same differentiated form as their sister cells (Ellis and Horvitz 1986).

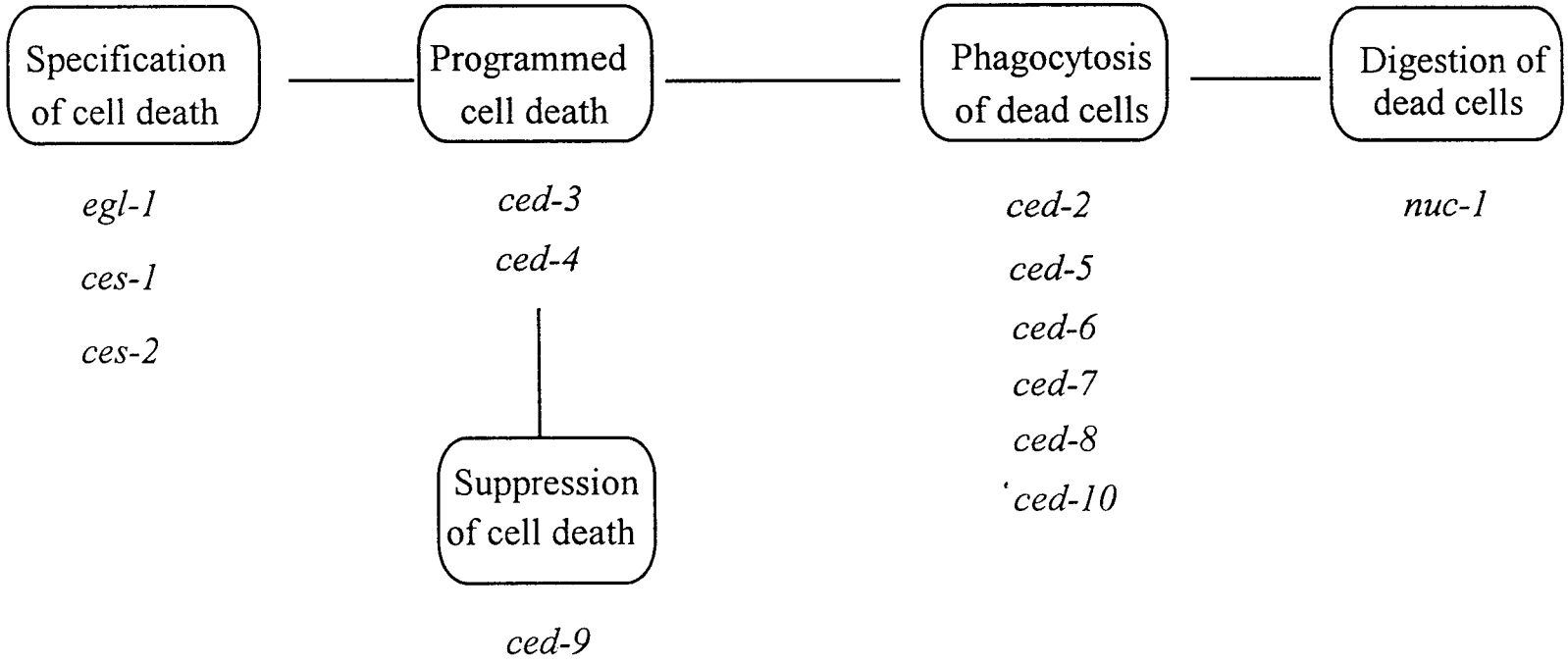


Figure 3.5 Genes involved in the cell death pathway in the nematode *Caenorhabditis elegans.*

Mutations in both *ced 1* and *2* prevent the engulfment of a cell corpse and cause chromatin to remain uncondensed (Hedgecock *et al.*, 1983). Genes *ced 5-8* and *ced 10* are important in the disposal of the cell corpse (Ellis *et al.*, 1991). These genes may facilitate the recognition of cell a corpse by neighbouring cells or may be important in initiating phagocytosis. *nuc 1* is a nuclease involved in DNA degradation in the late stages of cell death and may be present in the engulfing cells and not the dying cells (Sulston 1976; Hedgecock *et al.*, 1983).

Construction of mosaic animals demonstrated that ***ced 3*** and ***4*** gene products acted within the cells which died and not as a killer signal produced by neighbouring or surrounding cells (Yuan and Horvitz 1990).

The genetic control of ***ced 3*** and ***ced 4*** was determined by the discovery of ***ced*** *9* which acts to suppress the expression of ***ced 3*** and ***ced 4,*** thereby blocking cell death. ***Ced*** *9* gain of function mutants illustrated that in the presence of ***ced*** *9* expression no cell death was seen, but in the absence of ***ced*** *9* expression i.e., loss of function mutants, cell death was seen, not only in the **131** cells which normally died, but also in other cells within the nematode (Hengartner ***et al.,*** 1992). This illustrated that ***ced*** *9* was a general genetic suppressor of cell death within these animals, acting on genes in addition to ***ced 3*** and ***ced 4.*** Loss of ***ced*** *9* expression also caused a maternal effect with offspring from ***ced*** *9* mutants being semi sterile and any resulting eggs produced from the offspring failing to develop.

Recent work with ***ced*** 9 loss of function mutants has demonstrated that functional suppression of cell death can be restored in ***ced*** *9* mutants by the transgenic expression of ***bcl-2,*** (Vaux ***et al.,*** 1992), the B cell leukaemidlymphoma gene which in several mammalian cell systems is able to suppress apoptotic cell death (see section 3.5). This neatly identified ***ced*** *9* as a functional homologue of ***bcl-2*** and also demonstrated that the suppression of cell death is an evolutional conserved mechanism for controlling cell population size, implying that genes with similar functions to those identified in **C.** elegans should exist in mammalian cells.

Other genes within the programmed cell death pathway which have been identified are involved in the phagocytosis of dead cells (see figure 3.5). Mutants which lack one or more of these genes do not phagocytose the dead cells, instead the cells are left within the cellular tissues with no apparent deleterious effects (Ellis ***et al.,*** 1991).

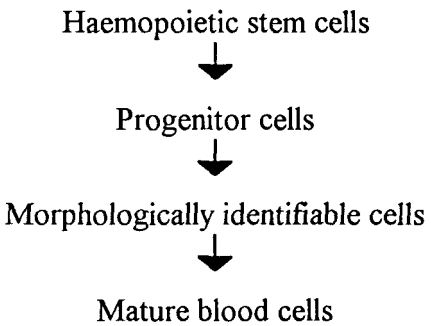
This model has identified specific genes which when activated can either promote or suppress cell death.

3.2.3 Vertebrate models of apoptosis

3.2.3.1 Haemopoiesis

Haemopoiesis is the word used to describe the production and control of all blood cells in the body. The tissue responsible for this is mainly the bone marrow, with additional roles played by the spleen and liver. Production of haemopoietic cells starts early during embryogenesis with stem cells first appearing in the yolk sac of an embryo and then migrating to the liver. Populations of stem cells are transient and those stem cells which seed in the liver and later in the spleen and bone marrow are the only stem cells the organism has. These stem cells must therefore be capable of self renewal and the production of many different blood cell lineages in order to give rise to a mature blood cell population. Haemopoietic stem cells give rise to eight distinct lineages: erythroid, neutrophil-granulocytic, monocytic, eosinophilic, mast cell, megakaryocytic, T-lymphoid and B-lymphoid, as neatly illustrated by the spleen colony forming assay (Metcalf 1988).

The non-lymphoid cell lineages are formed mainly in the bone marrow, with the spleen playing a minor but expandable role in the case of some diseases. For B and T-lymphocytes however, stem cells may give rise to them in the bone marrow, but then T-cell precursors migrate and enter the thymus where they undergo proliferation and differentiation. B-cell precursors stay longer in the bone marrow but eventually migrate to the spleen and lymph nodes where further proliferation and maturation occurs (see Liu ***et al.,*** 1989 and section 3.5.2). Haemopoietic cells show a hierarchy of cell populations which is exhibited in the development in all eight cell lineages:



Introduction

The first and most important cell population is the stem cell. These cells are able to self renew as weil as produce a further differentiated celi population known as progenitor cells. The mechanism by which stem cells are able to reproduce themselves as well as produce progenitor cells is unknown. Evidence suggests that stem cells undergo asymmetrical divisions which give rise to one stem cell and one progenitor cell (Metcalf 1988). Committed progenitor cells produced by the stem cells are further differentiated, being unable to self renew indefinitely or to produce stem cells. Most progenitor cells are unipotential i.e. only able to produce colonies of one celi lineage, although some multipotential progenitor cells have been identified (Metcalf 1988). Progenitor cells give rise to morphologically identifiable cells which then terminally differentiate to produce the mature cell population.

The control of haemopoiesis

Most of the work carried out on this subject has been with cells of the granulocyte/macrophage lineage. The processes described above are thought to be controlled by Colony Stimulating Factors or **CSFs** (Metcalf 1989) and stromal cells, both of which make up the haemopoietic microenvironment (Metcalf 1988; Roberts ***et al.,*** 1987). *In* ***vitro*** haemopoietic stem cells require the presence of stromal cells in order to survive and proliferate (Roberts ***et al.,*** 1987). How the stromal cells are able to support a proliferating stem cell population is not clear, but it is thought that it involves the production and presentation of CSFs by the stromal cells (Spooncer ***et al.,*** 1986; Roberts ***et al.,*** 1987).

There are four main CSFs involved in the granulocyte/macrophage developmental pathway, these are multipotential CSF or multi-CSF also known as interleukin-3 or **IL-3,** granulocyte-macrophage CSF or GM-CSF, granulocyte CSF or G-CSF and macrophage CSF or M-CSF. All CSFs are glycoproteins with Mr between 18,000 and 90,000; a third to a half of which ***is*** made up of carbohydrate. They act by triggering membrane receptors on appropriate cells and each CSF has its own specialised receptor which is not cross reactive, that is, an IL-3 receptor cannot be triggered by the binding of a GM-CSF ligand. **IL-3** is known to promote the growth of both stem and more mature progenitor cells and could therefore be fundamentally important in the control of haemopoiesis.

Introduction

The involvement of CSF in haemopoiesis can be split into two roles, that leading to cell survival and that leading to proliferation. On removal of CSFs from the growth medium most haemopoietic cells will die, as was first noted by Paran and Sachs in 1968. Similarly, a delay in the addition of GM-CSF to granulocyte/macrophage progenitor cells results in a decline in ability to form colonies in soft agar. This occurs due to the cells being unable to survive in the absence of GM-CSF (Metcalf and Merchev, 1982), which has now been shown to be due to the induction of apoptosis in the absence of the required cytokine (e.g., Williams ***et al.,*** 1990). Low concentrations of **IL-3** have been shown simply to keep cells alive and addition of more IL-3 to these cells results in a rise in intracellular pH and stimulation of cellular proliferation. Mature haemopoietic cells also respond to the absence of a specific cytokine, for example, erythrocyte progenitors will undergo apoptosis in the absence of erythropoietin (Koury and Bondurant, 1990a,b). Hence the presence of cytokines is required not only as a proliferative signal, but also as a suppressor of apoptosis. Therefore, control of haemopoietic cell population numbers ***in vivo*** is thought to be primarily at the levei of cell death.

Cells which respond to **CSF** can be said to be 'programmed' in their response (Whetton ***et al.,*** 1988), that is, the actual response of a cell is determined by two points of genetic programming:

- that leading to the display and synthesis of specific receptors and
- that determining whether cells proliferate, enter defined differentiation pathways or exhibit enhanced functional activity on receptor stimulation.

Very recent work by Fairbairn and colleagues (1993) has demonstrated that differentiation of FDCP-mix cells can occur in the absence of cytokine, as long as a survival signal is present. **A** gene which has been demonstrated to suppress apoptosis in the FDCP-1 cell lines is *bcl-2* (Vaux ***et al.,*** 1988) and this acts as a genetic survival signal in the absence of the cytokine. If this gene is expressed in FDCP-mix cells not only do the cells survive in the absence of the cytokine, they also begin to differentiate and produce more mature lineage cells. Differentiation of a single cell also occurred without concomitant proliferation. This suggests that differentiation in some cells is genetically predetermined and the presence of

different cytokines is not required for differentiationto occur per se. Cytokines may simply be required to provide a proliferative signal in order to increase the cell population number.

However, cells can be influenced by the type of CSF present. If a bipotential progenitor cell of the granulocyte/macrophage lineage is incubated with both M-CSF and GM-CSF then an enhanced proliferation and competitive production of cells is seen. This results in both macrophage colonies and granulocyte colonies, the amounts of which depend on the concentrations of M-CSF and GM-CSF present (Metcalf 1989). So, in summary, colony stimulating factors influence haemopoietic cells in four ways:

- in the enhancement of survival, i.e., in the suppression of apoptosis,
- as a proliferative stimulus,
- as an influence in cellular differentiation and
- as an influence in cellular maturation.

3.2.3.2 Induction of apoptosis in the immune system

The study of apoptosis in immature thymocytes has led to morphological and biochemical events within this pathway being widely accepted as definitive landmarks through which a cell has to pass to be classified as apoptotic (Wyllie *et al.*, 1980; Wyllie 1980). However, with the increasing number of cell models studied, it has become apparent that not all apoptotic cells progress in exactly the same manner.

Immature thymocytes and mature T lymphocytes.

Immature thymocytes die by apoptosis on addition of glucocorticoid hormone and have provided the paradigm for apoptosis, defining several characteristics of the process (Wyllie *et al.*, 1972; Wyllie 1980; McConkey *et al.*, 1989a). Addition of a glucocorticoid results in cell lysis after 10 hours with **DNA** fragmentation detectable as early as 1 hour after glucocorticoid addition (Wyllie *et al.*, 1980; McConkey *et al.*, 1989a). By the use of gel electrophoresis, **DNA** can be seen to be cleaved into multiples of approximately 180-200 base pairs i.e., oligonucleosomal sized fragments comparable to chromosomal **DNA** which has been cleaved by micrococcal endonuclease (Wyllie 1980; Arends *et al.*, 1990). In virtually all cases of apoptotic cell death activation of the endonuclease occurs and in thymocytes, it is accepted

that the endonuclease is calcium and magnesium dependent (Cohen and Duke 1984) and probably requires extracellular calcium to remain activated. It is also established that the endonuclease results in fragmentation of the **DNA** into oligonucleosomes by cutting linker regions of **DNA** not protected by the presence of histones.

Glucocorticoid stimulated cell death can be prevented by both inhibitors of **RNA** synthesis, e.g. actinomycin D and by inhibitors of protein synthesis, e.g. cycloheximide (Wyllie *et al.*, 1984; Cohen and Duke 1984).

Binding of the glucocorticoid to its receptor within the thymocyte results in an immediate increase in intracellular calcium (McConkey *et al.*, 1989a). Later stages of apoptosis can be blocked by introducing the Ca^{2+} binding dye quin-2-AM or by placing the cells in Ca^{2+} depleted medium, implying that a calcium signal is required to stimulate cell death (McConkey *et al.*, 1989a). Thymocyte apoptosis can also be induced by incubating the cells in the presence of a calcium ionophore such as ionomycin (e.g., Wyllie ***et al.***, 1984; McConkey 1989b) which presumably mimics the calcium rise associated with glucocorticoid binding.

Much of the pathway through which glucocorticoids stimulate an increase in cytosolic calcium concentration is unknown. However, it is well established that glucocorticoids have specific receptors within the cell which become active on binding the glucocorticoid and are then able to bind DNA. This in turn promotes the transcription of glucocorticoid dependent genes (Luisi *et al.*, 1991; Evans 1988; Yamamoto 1985). It is most unlikely that the expression of the glucocorticoid dependent genes is required for the immediate Ca^{2+} increase, but induced gene product(s) may help to sustain it. McConkey ***et al.***, (1989a) have suggested that such a gene product could be a pore protein involved in the transport of calcium across the nuclear membrane. Blocking of calcium dependent proteins such as calmodulin will also block apoptosis, therefore suggesting that calcium may initiate a signalling cascade involving calmodulin. Other calcium dependent proteins such as proteases and phospholipases, so crucial to necrotic cell death, are not activated in thymocyte apoptosis. Inhibition of these enzymes fails to abrogate apoptotic cell death (McConkey ***et al.***, 1989~).

CEM cells, originally derived from an acute lymphoblastic T cell leukaemia, also undergo apoptosis on exposure to glucocorticoid (Alnemri and Litwack 1989). However, this

study demonstrated that the death of these cells is independent of an intracellular calcium increase. Novobiocin, a topoisomerase II inhibitor, also causes apoptosis in these cells which again is independent of a calcium increase (Alnemri and Litwack 1990). Furthermore, CEM nuclei incubated in the presence of calcium do not undergo DNA fragmentation, suggesting that there is no detectable calcium and magnesium dependent endonuclease in these cells. This is supported by the work of Barry and Eastman (1993) who show that DNase II can become activated in some apoptotic cells due to a drop in pH levels within the cell, rather than an increase in Ca^{2+} concentrations.

Cell death which is independent of protein synthesis has been shown both in **S49** mouse lymphoma cells in response to a glucocorticoid (Vedickis and Bradshaw 1983) and in human leukaemic HL60 cells on treatment with calcium ionophore (Martin ***et al.,*** 1990). Both groups concluded that some cell lines may not require new gene expression in order to activate the endonuclease or produce changes in cellular morphology. This suggests that some cells are continuously primed to enter apoptosis but the required proteins within the cytoplasm are kept in an inactive form until the appropriate apoptotic stimulus is received.

Glucocorticoids and calcium ionophores are not the only means of inducing T-cell apoptosis. Immature $CD4^+$ $CD8^+$ thymocytes expressing the CD3/T-cell receptor (TCR) complex can be signalled to die by apoptosis either by anti-CD3 antibody or by a superantigen complex such as ***Staphylococcus*** enterotoxin B ***(SEB)*** which bind to and signal through the TCR (Smith ***e?al.,*** 1989; McConkey ***et al.,*** 1989d; Jenkinson ***et al.,*** 1989). Apoptotic death of immature double positive thymocytes within thymic cultures is also seen in response to the above stimuli and this has important implications for the production of a tolerant immune system (Kappler ***et al.,*** 1987; Kingston ***et al.,*** 1985). Apoptosis produced by stimulation of the TCR can be mimicked by calcium ionophores (McConkey ***et al.,*** 1989d). Comparable results have been obtained by Ucker ***et al.,*** (1989) in transformed T-cell hybridomas.

Therefore apoptosis in immature thymocytes can be signalled both by antigen binding to the CD3/TCR complex and by binding of glucocorticoid to its intracellular receptor, both resulting in an increase in Cytosolic calcium. Apoptosis can be inhibited by blocking the calcium influx or by inhibiting RNA or protein synthesis. These systems have provided some

of the first information about biochemical signalling pathways leading to apoptosis, but we are still some way from a clearly defined molecular mechanism (see section **3.3).**

Interleukin 2 (IL-2) dependent T-cell clones die by apoptosis on withdrawal of the cytokine (Cohen and Duke 1986). This can be prevented by the presence of phorbol esters (Rodriguez-Tarduchy and Lopez-Rivas 1989), suggesting that PKC activation can block apoptosis in this system. It is not known how removal of a growth factor results in apoptosis, but it has been suggested that such cells are again continuously primed to enter apoptosis, but that the process is actively suppressed by the presence of the appropriate factors (Raff 1992).

Cytotoxic and natural killer cell induced death within the immune system is a very interesting but complex model system. This in part stems from the fact that such cells are known to secrete a wide range of cytotoxic molecules on contact with target cells. Some of these are able to produce necrosis, e.g., Perforin (Duke *et al.,* 1989; Young and Liu 1988), whereas others, such as tumour necrosis factor, can induce both necrosis and apoptosis (Laster *et al.,* 1988). It is interesting to note however that some of these models diverge noticeably from the classical concept of apoptosis. For example, there is evidence for protein synthesis required for cell death that is independent of **DNA** fragmentation (Hirota *et al.,* 1989, reviewed in Golstein *et al.,* 1991). Such models are valuable because they indicate that apoptosis may be modified in certain circumstances to provide cell death more appropriate for the situation e.g., rapid target cell destruction to combat viral infection.

Other ligands expressed on the surface of cells have been found to induce apoptosis on stimulation (Trauth *et al.,* 1989; Yonehara *et al.,* 1989). Trauth and co-workers, by raising monoclonal antibodies to malignant B cells, produced an antibody, anti *APO*-1. This antibody, on binding the APO-1 antigen caused apoptosis. Engagement of the Fas antigen, which is expressed on many cells including myeloid cells, T lymphoblastoid cells and diploid fibroblasts (Yonehara *et al.,* 1989), also leads to cell lysis. Expression of the fas cDNA in normally non-expressing cells can also result in lysis in the presence of anti fas antibodies (Itoh *et al.,* 1991). These two cell ligands have now been shown to be one and the same (Oehm *et al.,* 1992) and to belong to an emerging protein receptor superfamily which includes the tumour necrosis factor receptor, nerve growth factor receptor and CD40.

As a final note to this section, one should also consider radiation-induced cell death. In both lymphocytes and thymocytes this is a valuable model because it provides yet another signal with which cells die, but the rate at which they die can depend on their respective activation states. Both thymocytes and lymphocytes die quickly if they are in the resting state (Yamada and Ohyama 1988; Sellins and Cohen 1987). If the cells are active then they may take much longer to die, with activated lymphocytes this may be a matter of days (Filippovich ***et al.,*** 1988; Lowenthal and Landers 1985). Why this occurs is not clear, but proliferating cells are likely to have different molecules present in the cytoplasm which can suppress apoptosis and this is further implied by the fact that phorbol esters protect lymphocytes from radiation damage (Tomei *et al.,* 1988). However, other reasons have been suggested, Filippovich *et al.,* **(1988)** believing that the rate of apoptosis is dependent on the amount of DNA breaks already present in the cell before radiation exposure.

B-cell models

Work with developing B-cell populations has shown that these cells may also go through positive selection much like thymocytes. **A** proportion of developing centrocytes in follicles of secondary lymphoid organs undergo apoptosis when placed *in vitro* (Liu *et al.,* 1989). However, this can be prevented if the cells are stimulated through their surface immunoglobulin. These cells are thought to represent a population of B cells which have just undergone somatic mutation, and as a result are selected *in **vivo*** on the basis of their affinity for antigen expressed on follicular dendritic cells (MacLennan and Gray 1986). This selection through surface immunoglobulin may prevent cells with high affinity for antigen from undergoing apoptosis as is seen ***in vitro,*** and allow these cells to differentiate into memory B cells. In direct contrast, mature surface **IgM** positive murine B lymphomas die by apoptosis on stimulation of their surface Ig receptors (Hasbold and Klaus 1990). There is evidence that the differing sensitivity of immature and mature thymocytes to apoptotic stimuli, i.e., susceptibility to undergo apoptosis depending on the developmental point of the cell and the stimulus received, may well occur in B cell development, hence allowing for the differing outcomes in response to Ig stimulation.

Further work in this area has shown that a subset of germinal centre B cells, which specifically express the antigen cluster CD 77, undergo apoptosis both *in vivo* and *in vitro* (Mageney *et al.,* 1991). *In vitro,* these cells can be rescued from dying by both interleukin 4 and anti CD40 antibodies. Germinal centre B cells and early myeloid cells can also be rescued by the presence of antibodies to the CD23 receptor and interleukin 1a (Liu *et al.,* 1991a; Mossalayi *et al.,* 1990). In germinal centre B cells this latter signal resulted in differentiation towards the plasmacytoid pathway and may provide an explanation for the role of a subset of follicular dendritic cells known to express CD23. Interestingly these B cells could also be rescued by monoclonal anti CD40 antibodies, but this did not result in differentiation. Liu *et al.,* (1991a) have suggested that this may represent a bifurcation in the development of germinal centre B cells.

Section 3.3 Mechanisms of the apoptotic pathway(s)

3.3.1 Introduction

Extracellular signals can trigger apoptosis through several cytoplasmic pathways, some of which are represented in figure **3.6.** Most of the intracellular signals which induce apoptosis can stimulate other responses (e.g., proliferation or differentiation) in different cell types or in cells under different environmental conditions.

In thymocytes and some other cell systems, induction of apoptosis is associated with an increase in intracellular calcium concentration. This increase can be produced by the production of inositol triphosphate from the hydrolysis of phosphatidylinositol, which causes the release of calcium stored within the endoplasmic reticulum (Berridge 1984). **A** sustained increase in calcium concentration is thought to be maintained by the entry of extracellular calcium through channels in the cytoplasmic membrane.

Cyclic AMP can also act as a second messenger in apoptosis (Bourne *et al.,* 1975; Vedickis and Bradshaw 1983; McConkey *et al.,* 1990c; Lanotte *et al.,* 1991). Cyclic AMP and calcium activated calmodulin pathways are known to interact, e.g. via protein kinase **A,** which is stimulated by cAMP and is thought to act in the endonuclease activation pathway, and can phosphorylate some Ca^{2+} channels and pumps (McConkey *et al.,* 1990c). Protein Kinases C

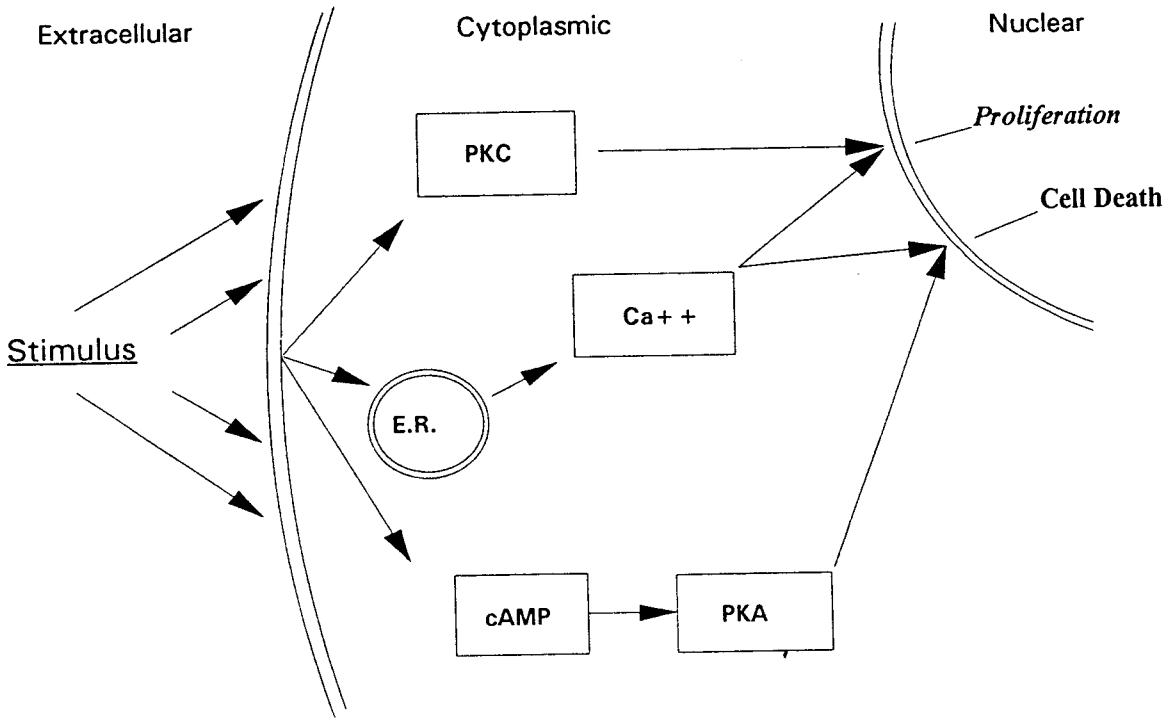


Figure 3.6 Intracellular signalling pathways involved in apoptosis

An extracellular stimulus can trigger several signalling pathways within a cell: (i) The production of PKC, activated by diacylglycerol, one of the cleavage products produced by inositol phospholipid breakdown. This pathway is generally associated with proliferation and in some cell models, PKC has been shown to inhibit apoptosis. (ii) Calcium release plays an important part in the signalling pathway of apoptosis and is again a cellular response to inositol phospholipid breakdown. The presence of a calcium signal with PKC activation has been proposed to result in a 'balanced' signal promoting proliferation, an unbalanced signal such as calcium release may result in cell death. (iii) The direct stimulation of cyclic AMP also causes apoptosis in some cell models. cAMP is known to interact with calcium dependent calmodulin pathways via PKA. Therefore some interaction occurs between PKC and PKA pathways.

and **-A** also interact during cellular signalling (Yoshimasa *et* ***al.,*** 1987) and in apoptosis, stimulation of PKC is able to block DNA fragmentation and cell death stimulated by calcium and cAMP pathways (McConkey *et al.,* 1990c). Therefore, in thymocytes signals within the cell may interact directly i.e., cross talk, via the two protein kinase systems (see figure 3.6).

3.3.2 Cytosolic signalling pathways involved in apoptosis

Thymocyte apoptosis induced by glucocorticoids or Ca^{2+} ionophores can be blocked by phorbol esters (McConkey *et al.,* 1989b). These compounds act by directly stimulating PKC, probably by mimicking diacylglycerol, the physiological activator of PKC (Berridge 1984). This implies that a signal involving PKC activation can block the apoptotic signal. Consistent with this, interleukin 1 (IL-1), which can activate PKC, (Rosoff *et. al.,* 1988), is able to block apoptosis in thymocytes stimulated via the T-cell receptor complex. IL-1 also blocked cell death in thymocytes exposed to glucocorticoids, calcium ionophores and antibodies to the antigen receptor complex (McConkey *et al.,* 1990b). Using the above and other T-cell signalling models, McConkey *et al.,* (1990a) have proposed a mechanism for cell signalling in apoptosis. This is based on the idea of unbalanced cell signalling and follows from the observation that signalling of mature T-cells to proliferate involves the use of two pathways; that given by occupation of the TCR, resulting in a Ca^{2+} / cAMP signal, and that given by an accessory molecule such as IL-1 which involves PKC activation. If however these cells receive a stimulus from the TCR only, then the cells enter a state of non responsiveness, known as clonal anergy, (Jenkins *et al.,* 1987; Jenkins *et al.,* 1988; Quill and Schwartz, 1987) which may be important in the down regulation of the immune response. If an analogous interplay between signals occurs in immature thymocytes then a cell which receives stimulation by both glucocorticoid and IL-1, i.e., both Ca^{2+} /cAMP and PKC pathways, will receive a balanced signal which will not result in cell death. This is also consistent with studies on IL-2 dependent cells where the presence of IL-2 protects the cells from the effects of glucocorticoid (Nieto and Lopez-Rivas, 1989). If only the glucocorticoid or TCR signal is received then an increase in cytosolic calcium, or cAMP activation occurs without the balancing PKC signal and this results in apoptosis (McConkey *et al.,* 1990a). This is a plausible mechanism when considered in T cells, but it should be remembered that immature thymocytes in particular are

a population of cells undergoing a complex process of selection and therefore represent a special case.

Section 3.4 Genes and cell signalling in apoptosis

3.4.1 Introduction

A paper published by Wyllie and colleagues in 1987 illustrated how various deregulated oncogenes could affect the rate of apoptosis within a tumour by determining the apoptotic and mitotic rates in various induced fibroblast tumours in immune compromised animals. In fibroblasts transfected with an **SV40** promoter containing plasmid, only small nodules of fibrosarcomas were produced which corresponded to the cells having a low mitotic rate but a high apoptotic rate. Fibroblasts which over expressed exogenous ***myc*** produced tumours which had both high apoptotic and mitotic rates and produced indolent tumours (see section 3.4.3). Cells over-expressing *ras,* however, produced highly metastatic cancers with a high rate of mitosis and a low rate of apoptosis. These results neatly demonstrated that cancers which showed a low incidence of apoptosis coupled with a high mitotic rate *in vivo,* had a distinct advantage for unrestrained celi population growth. This system also illustrated that even with a relatively high incidence of apoptosis tumours still form, as is seen for the *myc* transformed fibroblasts. **A** reduction in the rate of apoptosis is therefore not the only factor involved in malignancy, but may well represent an early step towards the cancerous state.

3.4.2 Viral gene suppression of apoptosis

The ability of viral genes to mediate cellular transformation has long provided an active area for cancer research. More recently, specific viral genes required for cellular transformation have been identified and genes such as **DNA** tumour virus oncoproteins, adenovirus E1b and human papilloma virus **E6** have been found to bind the tumour suppressor gene p53 (Braithwaite and Jenkins 1989; Gannon and Lane 1987; Sarnow *et al.,* 1982). Hence such genes are able suppress normal p53 activity and G_1 arrest which probably causes the observed increase in proliferation (Momand *et al.,* 1992; Lane and Benchimol 1990; Werness *et al.,* 1990). In addition, recent research has shown that some virai genes may affect cellular

growth either by directly suppressing apoptosis or by upregulating *bcl-2* and indirectly suppressing apoptosis (see section ***3.6),***

3.4.3 *c-myc* and apoptosis

Classically, expression of c-myc during the cell cycle is associated with cell proliferation and, in cases where c-myc is deregulated, cell transformation. c-myc expression is known to occur throughout the cell cycle, and is up-regulated in cells entering G_1 from a G_0 state and down-regulated in cells entering G_0 or terminally differentiating (Cole 1986).

Two recent papers have shown that under certain conditions, continuous expression of ***c-myc*** is associated with apoptosis. Rat-1 fibroblasts, in the presence of low serum, normally enter a quiescent state which corresponds to a decline in the level of c-myc expression (Dean ***et*** *al.,* 1986; Waters ***et*** *al.,* 1991). However, when transfected with a constitutively expressed c-myc gene these cells fail to enter G_0 on removal of serum and instead enter apoptosis (Evan ***et*** *al.,* 1992). Examination of Rat-1 myc clones expressing different levels of the proto-oncogene, revealed that the higher the level of enforced c-myc expression, the faster the resulting entry into apoptosis and the greater the sensitivity to a drop in the concentration of serum. Deregulated c-myc was also shown to induce apoptosis in cells which were growth arrested in other phases of the cell cycle. Similar results have been obtained in an **IL-3-** dependent myeloid cell line continuously expressing c-myc (Askew ***et*** *al.,* 1991). The parental cell line, on removal of **IL-3,** down regulates the expression of both c-myc and ornithine decarboxylase (ODC) and accumulates in G_1 from which cell death follows several hours later. Cells which continuously express c-myc fail to down regulate both ***myc*** and ODC gene expression in response to cytokine withdrawal and do not progress to block in G_1, but instead rapidly enter apoptosis. Similarly in this system, cells were able to enter apoptosis from wherever growth arrest occurred in the cell cycle.

Induction of apoptosis by c-myc requires the same regions within the protein, i.e., the helix-loop-helix domains and the leucine zipper, as are required for Co-transformation and inhibition of differentiation (Freytag *et* ***al.,*** 1990; Stone *et al.,* 1987). Hence, the ability of c-myc to bind **DNA** and other proteins may suggest a role in the initiation of apoptosis in some cases (Evan ***et*** *al.,* 1992). Consistent with this hypothesis is the upregulation of *c-myc*

expression seen in rat ventral prostate cells undergoing apoptosis in response to the removal of androgen (Buttyan ***et*** *al.,* 1989) and the requirement of Myc in activation-induced apoptotic cell death in T-cell hybridomas (Shi ***et*** *al.,* 1992). It also supports the suggestion that apoptosis is in some ways related to cell proliferation since some of the same genes have been implicated in the regulation of both processes (see Ucker 1991 for review).

3.4.4 p53 and its role in apoptosis

Another gene able to promote apoptosis within myeloid cells is the tumour suppressor gene p53 (Yonish-Rouach ***et*** *al.,* 1991). p53 expression normally results in inhibition of proliferation, however, wt p53 expression in M1 cells results in apoptosis. IL-6, however, is able to block the apoptotic response by inducing differentiation possibly involving the ability of **IL-6** to down-regulate *c-myc* (Kimchi ***et al.,*** 1988; Resnitzky and Kimchi 1991).

Acute myeloid leukaemia cells both from patients and ***in vitro*** celi lines have been found to express mutant p53 and this appears to confer a growth advantage (Slingland ***et*** *al.,* 1991). How p53 exerts its effect as a tumour suppressor gene is unclear, but it has been proposed that it may be involved in the cellular response to DNA damage (e.g., Lane 1992). Accumulation of p53 protein within the cell nucleus is often seen after DNA damage producing arrest in G_1 (Maltzman and Czyzyk 1984; Kastan ***et*** *al.,* 1991), which is thought to facilitate repair of the damaged **DNA.** However, if the damage to the genome is too great, apoptosis results. Therefore p53 is proposed to act as a molecular sensor of DNA damage, allowing efficient repair to occur or possibly facilitating the correct genomic environment for cell death to occur (Yonish-Rouach ***et*** *al.,* 1993). Cells which express mutant p53 or have incapacitated p53 due to the presence of viral oncogenes will fail to enter G_1 under the same conditions and continue to proliferate. The resulting failure to deal appropriately with DNA damage leads to genetic instability with an increased risk of progression towards malignancy.

Recent work with p53 null mice has neatly illustrated how p53 can act as a molecular detector of DNA damage. Thymocytes from these mice remained sensitive to apoptotic stimuli, such as glucocorticoids and calcium ionophore, but were insensitive to stimuli such as **y** radiation and double strand breaks induced by the drug etoposide (Clarke ***et*** *al.,* 1993; Lowe ***et al.,*** 1993). This has neatly illustrated that an absence of wild type p53 in cells which

normally express it can lead to a block in one particular apoptotic pathway, i.e., that pathway which is induced directly by damaged to the DNA. Whether or not p53 is involved in apoptotic signalling pathways produced by different signals has yet to be established.

3.4.5 Death genes

Inhibitors of protein synthesis are able to delay apoptosis in many cell systems. This implies that the cell must be active in its own death, i.e., it must synthesise new gene products or continue synthesising particular gene products before apoptosis can occur. Some, but not all of these gene products may be specific to cell death by apoptosis. The genes which may need to be transcribed and their relevant functions are now an intense area of research.

Apoptosis in the rat ventral prostate was one of the first models in which genes expressed during the cell death period were analysed (Buttyan *et al.,* 1988; Sanford *et al.,* 1984). Analysis of known genes revealed expression of c-fos, c-myc, a-tubulin and HSP70 over a **3** day period ***in vivo.*** **This** is a similar progression to that seen in cells stimulated to undergo proliferation (Curran *et al.,* 1985). Another gene identified in rat tissues which was associated with apoptosis was the testosterone repressed prostate message 2 (TRPM-2) gene (Leger *et al.,* 1986; Monpetit *et al.,* 1988; Buttyan *et al.,* 1989). TRPM-2 has been shown to be the rat homologue of sulphated glycoprotein 2 or clusterin (Cheng *et al.,* 1988; Collard and Griswold, 1987), a lectin like molecule secreted by healthy sertoli cells.

Thymocytes have also been widely used to isolate 'death genes'. **mRNA** has been examined from thymocytes undergoing apoptosis after y-irradiation or treatment with glucocorticoids and cAMP analogues (Harrigan *et al.,* 1989; Owens *et al.,* 1991). **RP8** and RP2 were isolated after treatment with y-irradiation and glucocorticoid, and have been shown to have DNA binding motifs and integral membrane properties respectively (Owens *et al.,* 1991).Eleven mRNA clones have also been isolated from glucocorticoid and cAMP analogue treated thymocytes, three of which required the presence of both stimulants in order to be expressed (Harrigan ***et*** *al.,* 1989). Subsequent analysis has revealed that one of the clones encodes a glucocorticoid-induced receptor with homology to the family of G-protein coupled tachykinin receptors (Harrigan *et al.,* 1991). *So* far the role of these isolated genes within the cell death pathway has not been elucidated.

Section 3.5 The importance of *bcl-2* in apoptosis

3.5.1 *bcl-2*, a molecular suppressor of apoptosis

Impairment of apoptosis resulting in predisposition to oncogenesis has been clearly shown in cell systems both *in vitro* and *in vivo* for the proto-oncogene *bcl-2* (Reed *et al.*, 1988; Korsmeyer *et al.*, 1990; Strasser *et al.*, 1991).

Bcl-2's oncogenicity was first implied by its association with follicular lymphoma, which in 90% of human cases involves the t(14;18) translocation. This results in the J_H segment of the immunoglobulin heavy chain gene on 14q32 becoming juxtaposed to a transcriptionally active gene on 18q21 (Cleary and Sklar, 1985), termed the B cell leukaemia/lymphoma gene 2 or *bcl-2* (Tsujimoto *et al.*, 1984). Sequencing revealed that the full Bcl-2 polypeptide is encoded by two exons, the **5'** exon being separated by a 50kb fragment from the second exon which **has** a long 3' untranslated region, Most of the *bcl-2* reading frame is contained within the **5'** exon with the major-breakpoint cluster region (MCR) occurring in the middle of the 3' untranslated region (Cleary and Sklar 1985; Tsujimoto *et al.*, 1985; Bahkshi *et al.*, 1985; Cleary *et al.*, 1986). Why this translocation occurs within such a high percentage of follicular lymphomas is not clear. One possibility is that a region around the *bcl-2* MCR has sequences which are similar to heptamer and nonomer sequences involved in germline **VDJ** rearrangements of the **IgH** gene (Taussig *et al.*, 1989) and this leads to inappropriate rearrangements (Tsujimoto *et al.*, 1988). Another possibility is illegitimate pairing between D_H and J_H ends of chromosome 14 with staggered double strand breaks on chromosome 18 (Bahkshi *et al.*, 1987; Cotter *et al.*, 1990).

Human *bcl-2* gene transcripts generate two primary proteins, Bcl-2a and BcI-2ß of 26kDa and 22kDa respectively, which are generated by alternate splice and polyadenylation site selection (Cleary *et al.*, 1985, Tsujimoto and Croce 1988). Bcl-2a and Bcl-2ß have the first 196 amino acids in common but differ in their C-termini. Early fractionation studies suggested that the Bcl-2 protein was associated with the plasma membrane fraction of the cell and since its DNA sequence revealed no transmembrane or signal peptide domain, Bcl-2 was assigned a location within the inner cytoplasmic membrane (Tsujimoto and Croce 1988).

Several attempts have been made to identify the exact location of Bcl-2 and with the use of monoclonal antibodies raised against human Bcl-2, the protein has been localised to the inner mitochondrial membrane (Hockenbery ***et al.,*** 1990) and other areas within the cytoplasm (Liu ***et al.,*** 1991; Chen-Levy ***et al.,*** 1989, 1990; Hamilton ***et al.,*** 1991).

Of fundamental importance in ***bcl-2s*** meteoric rise to fame was the demonstration that it prolonged the survival of IL-3-dependent cells in the absence of the cytokine (Vaux ***et al.***, 1988). Such cells were subsequently shown to enter apoptosis on withdrawal of the growth factor, indicating clearly that ***bcl-2*** could suppress apoptosis and prolong cell survival in environments in which they would normally die (Williams ***et al.,*** 1990; Rodriguez-Tarduchy ***et al.,*** 1990; Crompton 1991). Suppression of apoptosis by ***bcl-2*** however, is not a universal effect since IL6-dependent cells over expressing ***bcl-2*** are not prevented from entering apoptosis on cytokine removal. Suppression by ***bcl-2*** is therefore associated with particular cell lineages and may be mediated by interactions with in specific cytokine signalling pathways (Nunez ***et al.,*** 1990).

3.5.2 The role of *bcl-2* in B cell selection

Within the immune system Bcl-2 is expressed in thymus, spleen and tonsillar tissue, with staining patterns for both T and B cells within these areas conforming to a similar pattern, (Hockenbery ***et al.,*** 1991). T-cells within the thymus show no staining in the cortex, a region associated with tolerance induction of immature T-cells, but stain positively at the corticomedullary junction and medullar, areas which correspond to mature T-cells (MacDonald ***et al.,*** 1988; Miller ***et al.,*** 1989). **A** similar pattern can be seen in tonsillar tissue containing germinal centres. B cells within the germinal centre dark and light zones show no Bcl-2 expression, but cells at the follicular mantle zone surrounding the germinal centre do express the gene. Both germinal centres and thymic cortex contain large numbers of cells which are often seen to be undergoing the process of apoptosis (McPhee ***et al.,*** 1979; Swartzendruber and Congdon, 1963). Non-expression of Bcl-2 within these areas is thought to correlate with this susceptibility to induction of cell death. BcI-2 expression is also seen in plasma cells and myeloid cells within normal bone marrow (Hamilton ***et al.,*** 1991) as well as in memory B cells (Nunez ***et al.,*** 1991). Mature B and T cells stimulated with mitogens also

produce an increased amount of *bcl-2* **mRNA** (Reed *et al.,* 1987) and in plasma cells, peak antibody secretion correlates with down regulation of *bcl-2* expression (Mushinski *et al.*, 1988).

The ability of B cells to recognise unlimited numbers of foreign antigens is facilitated not only by rearrangement of the light and heavy Ig chain genes, but also by somatic mutation within the variable regions of the Ig chain (Taussig ***et*** *al.,* 1988). This somatic mutation can result in amino acid changes in the complementarity determining regions or hypervariable regions of the Ig receptor which can cause a change in the Ig binding affinity. This 'affinity maturation' occurs after the initial exposure of T-cell dependent B-cells to antigen and on re-challenge, antibodies of higher than average affinity are produced. This process of affinity maturation gives rise to more efficient Ig receptors for recognising a specific type of antigen as well as to receptors of unchanged or lower affinity. It is in the selection of cells displaying higher affinity Ig that *bcl-2* expression is a critical determining factor.

Affinity maturation occurs in the germinal centres, such as those found in tonsillar tissue (MacLennan and Gray, 1986). Germinal centres can be divided anatomically into two areas (Nieuwenhuis and Opstelten 1984), the dark zone which contains proliferating centroblasts and the light zone which contains centrocytes which are generally not proliferating (Searle *et al.,* 1982). Within this region follicular dendritic cells (FDC), are numerous and according to the type of surface protein which they present can be subdivided into two zones within the light zone. The basai zone contains FDC which express I-CAM 1 (CD54) and CD73 (Johnson *et al.,* 1989), and the apical zone contains FDC which express CD23 (Bettler *et al.,* 1989; Johnson *et al.,* 1986). Evidence that selection occurs in these areas has been based on the culture of purified germinal centre B cells which do not express *bcl-2* (see Liu *et al.,* 1989 and section 3.2.3). These cells spontaneously enter apoptosis *in vitro,* but this can be prevented if the cells are stimulated through their surface immunoglobulin i.e., their receptors are cross linked by the use of anti Ig antibodies on the surface of sheep red blood cells. These B cells can also be rescued by anti-CD40 antibody and by soluble CD23 with interleukin-1a (Mangeney *er al.,* 1991, Liu *et al.,* 1991a). In all three cases rescue from apoptosis was associated with induction of *bcl-2* expression.

Expression of bcl-2 appears to be critical in deciding the fate of B-cells within the germinal centre (Liu ***et al.***, 1991b). Centroblasts and centrocytes within the germinal centre do not express bcl-2 whereas cells at the follicular mantle zone surrounding the germinal centre do (Hockenbery ***et al.***, 1991). Cells which fail to interact with antigen in the germinal centre therefore enter apoptosis, but those which contact antigen presented by the follicular dendritic cells are rescued by a specific signal and resume expression of bcl-2 (Liu ***et al.,*** 1991b).

3.5.3 The oncogenicity of *bcl-2*

When first discovered bcl-2 was regarded as only a potential proto-oncogene since there was no direct evidence of its transforming ability. The oncogenicity of bcl-2 was demonstrated by Reed and colleagues in 1988 using constructs producing Bcl-2a and Bcl-2ß which proved to be tumourigenic in 3T3 fibroblasts injected into nude mice. However, unlike ***ras*** and myc, bcl-2 did not have the ability to transform 3T3 cells ***in vitro,*** suggesting secondary somatic changes were required to produce the resulting tumours.

The use of bcl-2 transgenic mice has taken this concept further and has illustrated that artificially induced expression of bcl-2 can provide the first step towards the development of lymphoma.

A bcl-2-immunoglobulin minigene construct, mimicking the t(**14;**18) translocation found in follicular lymphoma, was used to produce bcl-2 transgenic mice (McDonnell ***et al.***, 1989). These bcf-2-Ig transgenic mice displayed tissue specific expression of bcl-2, i.e., bcl-2 expression was mainly restricted to lymphoid tissue including spleen and thymus from about 8 to 13 weeks of age. Examination of the spleens revealed extensive hyperplasia in white pulp zones. Expanded follicular centre areas were evident with an abundance of small follicular centre cells and occasional immunoblasts, macrophages and plasma cells. The mice in general demonstrated an expanded B cell compartment bearing B220, Ia, IgM/IgD and K markers, corresponding to mature B cells when compared to litter mates. The B cells also expressed high levels of AA4.1 showing that the expanded B cell compartment still expressed some immature markers. Bcl-2 did not suppress maturation since plasma cells, immunoblasts and high levels of serum IgD and IgA were present. None of the mice at this stage showed any signs of developing neoplasia and there was no evidence of monoclonal outgrowth. Bcl-2

deregulation within **B** cells therefore resulted in a higher steady state number of B cells and these B cells had a much increased ability to survive in culture in the G_0 phase compared with normal B cells from littermates. This is in some ways analogous to the ability of bcl-2 to prolong myeloid cell survival in **IL-3** deprived conditions (Vaux ***et al.,*** 1988). The high level of surviving G_0 B cells was predicted to increase the chance of a B cell undergoing further genetic change leading to the production of a malignant monoclonal B cell tumour (McDonnell ***et al.,*** 1989).

Further evidence for bcl-2s ability to aid tumour production was demonstrated in another bcl-2 transgenic mouse model. Mice expressing an Eμ-*bcl-2*-22 gene construct (i.e., bcl-2 under the control of the Ig heavy chain enhancer) were crossed with Eμ-*myc* expressing mice (Adams ***et al.,*** 1985) to produce Eμ-*bcl-2/myc* mice (Strasser ***et al.,*** 1990). These mice rapidly developed a disseminated malignant lymphoma, much faster than the Eμ-*myc* littermates. The tumour formed within these mice was unusual since it represented a very primitive haemato-lymphoid stem or progenitor cell. In addition these transgenics again demonstrated excess pre B and B cells, but these cells were not malignant and did not grow well in culture. However, after a number of weeks, spleen and bone marrow cultures did produce a clonai outgrowth of B cells indicating that further genetic alterations were required in these cells to result in malignant clones.

The bcl-2-Ig transgenic mice of McDonnell ***e? al.,*** (1989) went on to develop malignant lymphoma at about 12 months of age (McDonnell and Korsmeyer 1991). This resulted from clonal malignant outgrowth of cells from the original lymphoid hyperplasia. This again indicated that secondary changes occurred to produce the lymphoma and in half of the mice these changes included a deregulated ***myc*** gene.

In summary, bcl-2 is strongly implicated in the development of follicular lymphoma since deregulated bcl-2 expression in transgenic mice produces high numbers of surviving pre B and B cells, an analogous situation to that found in human follicular lymphoma (McDonnell and Korsmeyer 1991). Bcl-2 does this by suppressing signais which would normally result in germinal centre B cell death by apoptosis during clonal selection (Liu ***et al.,*** 1991b). This large

surviving pool increases the chance of cells acquiring random genetic mutations which eventually lead to malignant clonal outgrowth.

It is interesting to note that a number of papers recently published (Hamilton ***et al.***, 1991; Pezzella ***et al.***, 1990) find ***bcl-2*** expression in lymphomas not associated with the t(14;lS) translocation. It may well be that the result of the deregulation of ***bcl-2*** by the IgH enhancer is not the only genetic mishap within lymphoid cells which can result in ***bcl-2*** deregulation.

An interesting offshoot of the effect of artificial induction of ***bcl-2*** expression on B cell development has been found in transgenic Eμ-***bcl-2***-22 mice. Strasser ***et al.***, (1991a) report several perturbations in mature B cell responses. Transgenic mice show prolonged responses to antigen which corresponded to a accumulation of small B cells, immunoglobulin secreting cells and serum immunoglobulin ***in vivo*** and most of the transgenic mice developed autoimmune disease. Mice were shown to be developing and dying of acute renal failure which on examination was due to lymphoid infiltration of the glomeruli. Serum examination revealed the mice contained antibodies against nuclear antigens including histones and double stranded **DNA.** This is often found in patients suffering from autoimmune systemic lupus erythematosus. This was a common finding in the Eμ-***bcl-2*** mice and was therefore not dependent on the point of integration of the transgene. The condition appeared to be due to the failure of the ***bcl-2*** transgenic mice to terminate the immune response in the normal manner, implying that such termination may involve apoptosis under physiological conditions. Strasser ***et al.,*** (1991a) propose that the absence of detectable autoimmunity in ***bcl-2*** transgenics from McDonnell ***et al.***, (1989) may be due to the differences between the mouse stains used to produce the transgenic mice. It remains to be seen whether an accumulation of plasma cells and serum immunoglobulin caused by the deregulation of ***bcl-2*** has any role to play in human autoimmune diseases.

3.5.4 The effect of *bcl-2* expression in developing T cells

Strasser ***et al.***, (1991b) reported the production of ***bcl-2*** transgenics which expressed ***bcl-2*** within the thymocytes and T cells. Sentman ***et al.***, (1991) also constructed transgenic mice which expressed ***bcl-2*** within the thymus by using the lck gene promoter which has been

used successfully to express transgenes within cortical thymocytes (Chaffin *et al.*, 1990). Bcl-2 expression enhances immature thymocyte survival both on culture ***in vitro*** and in the presence of several stimuli which normally result in apoptosis in these cells such as anti-CD3 antibodies (Smith *et al.*, 1989), calcium ionophores (McConkey *et al.*, 1989a), glucocorticoids (Wyllie 1980) and ionising radiation (Sellins and Cohen 1987).

It has been suggested that apoptosis induced by engagement of the CD3/TCR complex by self antigens bound to self MHC molecules maybe involved in the clonal deletion of autoreactive thymocytes, an important element of tolerance induction (Smith *et al.*, 1989). The negative selection of such autoreactive cells is thought to be concomitant with a down-regulation in *bcl-2* expression. Therefore, constant expression of *bcl-2* in the transgenic mice might be expected to inhibit this selection process. However, thymic selection did not appear to be disrupted in the *bcl-2* transgenic mice when the mice were crossed to mice expressing a superantigen which normally causes deletion of a specific set of T cell subsets. Some TCR^{hi} autoreactive thymocytes were detected in one of the mouse models (Strasser *et al.*, 1991b), but this was not reproduced in the *bcl-2* transgenic mice of Sentman *et al.*, (1991). Therefore, the role of *bcl-2* expression in the selection of T cells still needs to be clearly defined. Recent evidence has shown that a cellular homologue of *bcl-2*, ***bcl-x*$_s$** is expressed within thymocytes and is able to inhibit *bcl-2's* ability to suppress cell death (Boise *et al.*, 1993). If the levels of ***bcl-x*$_s$ mRNA** found in thymocytes prove to be representative of the levels of $Bcl\text{-}x_s$ protein expressed, then this may explain why the effects of *bcl-2* expression on T-cell repertoire selection are difficult to detect (Williams and Smith 1993).

Section 3.6 Epstein Barr virus and BHRF1

3.6.1 Introduction

Epstein Barr virus is a typical gammaherpesvirus which infects around 95% of all human populations. The virus is normally acquired asymptomatically in early childhood, but infection in adolescence in 50% of cases leads to infectious mononucleosis. Once acquired the virus persists in the host throughout life due to the persistent (latent) infection of B-lymphocytes (Gratama *et al.*, 1988; Yao *et al.*, 1985). Once the virus is induced into lytic

cycle, virus particles are shed mainly from epithelial tissue and from the oropharynx area (reviewed by Rodgers ***et al.***, 1992).

Epstein Barr virus, or EBV, is associated with several types of human cancers. In Africa in areas which are within the malaria belt, a childhood cancer, Burkitt's lymphoma, (BL) is an endemic disease which is associated with EBV infection (Epstein, 1985). Burkitt's lymphoma has been shown to be either EBV negative or positive and in areas where Burkitt's lymphoma is non-endemic, EBV-negative-BL predominates. In southern China EBV infection is strongly associated with nasopharangeal carcinoma (de The 1982).

Two strains of EBV have been identified, types **A** and **B,** of which B is less efficient than A in the transformation of B-cells (Rowe ***et al.***, 1989; Rickinson ***et al.***, 1987).

3.6.2 Epstein Barr virus infection in B-lymphocytes

EBV binds to B-lymphocytes via a viral glycoprotein receptor, gp350/220 which interacts with CR2, a cell surface receptor for the C3d complement fragment. EBV is able to transform B-lymphocytes of both humans and primates producing continuous latently infected lymphoblastoid cell lines or LCLs which exhibit continuous proliferation ***in vitro*** (Rodgers ***et al.***, 1992). Once EBV is established within a host cell it can undergo one of two life cycles, the lytic or latent cycle. In the latent cycle i.e., non production of virus, cells either express 1 or progress to expressing **8** latent genes. These are the only genes expressed out of the 100 or so genes which are encoded within the EBV genome. Induction of the lytic cycle, i.e., viral production, results in nearly all of the 100 viral genes being expressed.

Primary infection by EBV occurs in the oropharynx where the virus establishes persistent infection of epithelial cells by cycles of infection, virus replication, cell death and virus release followed by infection of new cells (Leibowitz ***et al.***, 1988). Initial infection of B-cells occurs in the oropharynx area by contact of sub-epithelial B-cells, which are largely non-permissive for viral infection, with virus shed from the infected epithelial cells. The ability of EBV to immortalise B-cells has been studied mainly in vitro using lymphoblastoid cell lines which represent the infected form of normal peripheral B-cells. These cells are able to proliferate continuously in vitro and express a restricted pattern of eight EBV latent genes (EBNAs 1, **2,** 3A, 3B, 3C, LP, LMP1 and 2(TP) as well as a defined set of B-cell activation

antigens (e.g., CD23, CD30, CD39 and CD70) (Gregory ***et al.,*** 1987, 1988a,b). The role of each of the eight EBV latent genes in specific B-cell transformation has been analysed by gene transfection assays in various cell lines. Expression of a particular EBV latent gene in B-lymphoma cell lines which do not express EBV (EBV negative Burkitt-type Iymphomas), but are at a similar stage of differentiation as EBV positive Burkitt's lymphoma cells has illustrated which EBV viral genes induce some of the B-cell phenotypic changes occuring in LCLs. For example, EBNAI expression does not result in B-cell growth transformation (Wang ***et al.,*** 1987), but appears to be essential for binding to the latent origin of replication (oriP) and thereby maintaining the EBV genome in an episomal form (Rawlins ***et al.,*** 1985, Yates ***et al.,*** 1985). Both EBNA 2 and LMPl have been shown to induce cellular changes consistent with B-cell activation such as the expression of CD23, CD39 and the reduction in the expression of CALLA and CD10 (Wang ***et*** *al.,* 1987; Wang ***et*** *al.,* 1990a,b), although EBNA 2 is able to upregulate expression of CD23, independently of LMP-1 expression. The clumping fo EBNA 2 transfected cells ***in vitro*** **is** due to CD23 expression (Wang ***et*** *al.,* 1987). EBNA 2 also acts as a transcription factor which can activate the expression of LMP1 (Fahraeus ***et al.,*** 1990; Wang ***et*** *al.,* 1990b), as well as initiating or enhancing the expression of EBNAs1, 3, **4** and 6 (Woisetschiaeger ***et*** *al.,* 1991). This may explain the inability EBNA 2 deficient mutant viruses to initiate the growth transformation of B cells (Hammerschmidt and Sugden 1989). EBNA-2 expression in Rat-1 cells (a fibroblast cell line), although not able to induce growth transformation, can reduce the dependency of these cells for serum growth factors (Dambaugh ***et*** *al.,* 1986). Expression of LW-1 in continuous fibroblast cell lines has transforming effects such as altered morphology, reduced requirement for serum factors and the loss of contact inhibition in monolayer culture (reviewed by Leibowitz ***et*** *al.,* 1988). LMP-1 can also alter the growth of EBV negative Burkitt's lymphoma lines inducing increased intracellular free calcium, cell clumping and increased LFA-1, ICAM-1 and LFA-3 expression (Wang ***et al.,*** 1988; Wang ***et*** *al.,* 1990b). The induction of B-lymphocyte adhesion molecules by LMP-1 results in cell clumping and may enhance cell growth and survival by increasing the number of cells which come into contact with secreted autocrine factors (Liebowitz ***et al.,*** 1988). LMP-1 has also been shown to associate with the cytoskeletal protein vimentin as well as increase its

expression (Birkenbach ***et al.,*** 1989). **A** naturally occurring mutant of LMP-1 which lacks the N-terminus as well as the first four transmembrane domains **(wt** LMP-1 has six membrane spanning domains with both the N and C termini being cytoplasmic) is unable to induce any of the above growth transforming effects and does not associate with the cytoskeleton (Wang et al., 1988), suggesting this loss of vimentin association blocks LMP-1 induced phenotypic changs .

EBNA ***6*** (or EBNA 3c) has also been shown to be involved in B-lymphocyte transformation, inducing expression of CD21 in the EBV negative B-cell line BJAB (Wang ***et al.,*** 1990a). Transfection experiments in Raji BL cell lines, which do not express EBNA 6 due to its partial deletion in the resident EBV virus, has shown that expression of the gene results in increased LMP-1 expression. Increased CD23 expression and expression of the cytoskeletal protein vimentin are also induced, consistent with the increased expression of LMP-1 (Allday ***et al.,*** 1993). These results imply that EBNA 6 co-operates with EBNA 2 in enhancing the expression of LMP1 but whether this interaction occurs at the transcriptional or post-transcriptional level has yet to be established.

Therefore, it appears that several of the latent genes expressed in LCLs are able to induce various increases in specific cellular gene expression associated with B-cell activation. Both EBNA 2 and LMP-1 when expressed separately in fibroblast lines, appear to effect the cells requirement for serum growth factors thereby increasing the cells ability to survive and proliferate in conditions which are growth limiting. Similarly, these viral genes may reduce B-cell requirement for growth factor by increased expression of LFA-I, ICAM-1 and CD23 allowing homotypic B-lymphocyte adhesion and hence either increased autostimulation or closer contact with autocrine secreted growth factors. Superinduction of CD23 expression and the production of its soluble form (Thorely Lawson and Mann 1985) may be very important in maintaining LCL survival since CD23, in combination with IL-la, is a potent survival factor for germinal centre B-celis (Liu ***et al.,*** 1991). LMP-1 induced upregulation of LFA-3, along with ICAM-1 and LFA-1 increases the cytotoxic T-cell response to the infected cells due to the interaction of LFA-3 with CD2 on T-cells (Springer ***et al.,*** 1987). Although not detrimental to LCLs in vitro, up-regulation of these ligands in vivo would lead to the

elimination of the infected B-cell. Hence in both group 1 BL cell lines in vitro and BL tumour cells in vivo LMP-1 is not expressed and therefore neither are LFA-1, 3 and ICAM-1, providing a mechanism by which the EBV infected B-cell can evade T-cell detection (Gregory ***et al.,*** 1988). Recent work has shown that this restricted expression of EBNA 1 is produced **by** the exclusive use of a newly discovered EBV promotor, F (Schaeffer et al., 1991). The expression of the EBNA genes in LCLs appears to be cotrolled by two promotors, the **C** and **W** promotors and switching between these promotors as well as alternative splicing and poly **(A)** site selection, controls the restricted latent gene expression (reviewed in Rodgers et al., 1992). Studies on uncultured peripheral blood lymphocytes have indicated that another form of EBV latency may exist in vivo in which EBNAl is not expressed, only TP1(LMP2A) gene expression is seen, which is consistant with the hypothesis that a minimally active viral epísome exisits in resting B cells and hence evades immune recognition due to the absence of EBNAs 2, 3 and LMP (Qu and Rowe 1992). The role of TP1 in this form of latency is as yet unclear, but it may well represent the normal form of EBV latent infection found in healthy individuals.

Therefore EBV latent gene expression in vivo would normally be predicted to exist in a highly conserved form, but in specific clinical circumstances transformed B-cells, exhibiting a phenotype similar to that of LCLs, occur within seropositive individuals. Primary EBV infection in infants normally occurs without any clinical manefestations. However, infection in later life can lead to a condition known as infectious mononucleosis. 0.5-2% of peripheral blood B-cells are EBNA positive suggesting that fully EBV growth transformed cells are present (Rodgers ***et al.,*** 1992). LCL like cells are also present in seropositive individuals which have undergone organ transplants and hence receive immunosuppressive drugs. These patients can develop B-cell lymphomas which are EBV positive, but on removal of the immunosuppressive drugs and the re-establishment of T-cell responses the lymphomas regress (reviewed by Lenoir and Borakmann 1987). Hence cytotoxic T-cells primed against specific EBV antigens are thought to limit the production of growth transformed B-cells expressing all eight latent genes in healthy EBV carriers.

Therefore, it appears that the differing forms of latent gene expression are produced by the utilisation of different latent promotors and that expression of the latent genes is in part dictated by the likelihood of cytotoxic T-cell interactions i.e., highly restricted latent gene expression in healthy, seropositive individuals, but full latent expression in can be utilised in vitro as seen in LCLs.

The close association of EBV with endemic BL rests on the following well established points:

- Patients with endemic BL have unusually high titres of antibodies to EBV antigens, show a pattern of reactivity which is specific to the disease and undergo changes in antibody levels in association with clinical events.
- All tumour cells of about 98% of properly authenticated cases carry multiple copies of the EBV genome.
- **A** WHO prospective seroepidemiology study found **16** cases of endemic BL in 42,000 Ugandan children over a 2 year period and were able to demonstrate unusually high titres of antibodies to virus capsid antigens in those destined to develop tumours many months or years before clinical manifestations. The **risk** of developing BL for those with high antibody titres was 80x greater than for matched controls.
- The production of Lymphoblastoid cell lines (LCLs) by infection of primary B-cells by EBV.
- EBV is experimentally oncogenic in certain south American sub-human primates.
- Animal herpes viruses that produce natural or experimental malignant tumours provide striking parallels for EBV and endemic BL (Epstein, 1985).

3.6.3 Sensitivity to apoptosis in EBV latently infected B-lymphocytes

Unlike EBV lymphoblastoid cell lines, biopsy isolates of EBV positive Burkitt's lymphoma cell lines only express one latent gene, EBNA 1. However, if these cells are cultured *in vitro* they will eventually express all eight latent genes, EBNAs 1, 2, 3a, 3b, 3c, LP and latent membrane proteins (LMP) 1 and 2 (Gregory *et al.,* 1990). These different forms of latency are referred to as group I or group III cell lines respectively (Gregory *et al.,* 1991) and were first characterised by observing that cultured EBV positive B cell lines either retain the cell surface phenotype of original biopsy cells or show genetic drift and eventually express all **8** latent genes (Rooney *et al.,* 1986; Rowe *et al.,* 1987).

These two groups of cell lines were shown to have differing sensitivities to the induction of apoptosis in response to various stimuli (Gregory *et al.,* 1991). Group I cells readily entered apoptosis in response to serum reduction or the presence of calcium ionophore. However, group III cell lines did not enter apoptosis in response to these stimuli. Group III cell lines have since been shown to express *bcl-2*, mediated by the expression of LMP1, which produces the insensitivity to apoptotic stimuIi (Henderson *et al.,* 1991). How expression of LMP1 induces expression of *bcl-2* has so far not been determined.

The initial sensitivity of group I cell lines to apoptosis may result from the site of tumour cell origin within the germinal centres of lymphoid follicles (Mann *et al.,* 1976; Gregory *et al.,* 1987). B-cells (centroblasts) within this cellular environment undergo somatic hyperrnutation producing immunoglobulin which exhibits either an enhanced binding for antigen, or immunoglobulin with unchanged or lower affinity for antigen. Progeny of the centroblasts, known as centrocytes, leave the cell cycle, re-express surface Ig and pass through the follicular dendritic cell network. Within this environment, if the cells successfùlly bind antigen then they are rescued from apoptosis, cells which do not successfblly bind antigen die. The surviving centrocytes either become memory B cells or antibody secreting plasma cells (see section 3.5). Therefore group I cell lines may act like centrocytes and constantly require survival signals in the absence of *bcl-2* expression.

3.6.4 The Epstein Barr virus gene **BHRF1**

Early data bank searches revealed only one protein with homology to *bcl-2,* the Epstein Barr virus (EBV) gene product BHRF1 (Cleary *et al.,* 1986).

The EBV BamH1 fragment **H** Rightward open reading Frame 1, **BHRF1,** encodes a putative transmembrane protein, (Pfitzner *et al.,* 1987; Pearson *et al.,* 1987) to which *bcl-2* shows sequence homology (Becker *et al.,* 1991; Cleary *et al.,* 1986). BHRF1 expression in EBV cell lines appears to result in the production of a family of **mRNAs** all with the same 3' end but differing in nucleotide chain length (Pfitzner *et al.,* 1987). Exactly what role the BHRF1 protein product plays in EBV transformation is not known. BHRF1 is transiently expressed in some partially permissive latently infected B-lymphocytes (Kocache and Pearson 1990), but is abundantly expressed during the early lytic infection cycle (Hummel *et al.,* 1982a,b; Pearson *et al.,* 1987). The BHRF1 open reading frame consists of 191 codons and the predicted translation product has a potential hydrophobic amino-terminal signal sequence, a putative external domain of approximately 150 amino acids, a 21 amino acid hydrophobic potential transmembrane domain and a carboxy-terminal pentapeptide (Pearson *et al.,* 1987, Pfitzner *et al.,* 1987). The localisation of the protein within the cell has cytoplasmic and perinuclear distribution (Henderson *et al.,* 1993) with some suggestion of membrane association (Kocache and Pearson 1990).

The 25% primary amino acid sequence homology, which occurs over a 150 amino acid region in the carboxy end of the protein, between *bel-2* and BHRF1 suggests that *bel-2* is evolutionarily related to BHRF1 (see figure 3.7) and implies that BHRF1 may exert a similar effect on protection from cell death as *bel-2* has been shown to do (see section **3.5).** This has interesting implications in protection from viral infection in vertebrate cells. **A** paper recently published has identified a gene in Baculovinis, p35, which is able to suppress apoptosis on infection in insect cells (Clem *et al.,* 1991). Prevention of host cell apoptosis by this gene enables the virus to infect and replicate efficiently within the cell. **A** similar role for BHRF1 can be envisaged, especially since the gene is expressed early in the lytic cycle. However, recent results have shown that BHRF1 is not strictly necessary for efficient viral transformation of B-lymphocytes *in vitro,* (Marchini *et al.,* 1990), but its homology to *bcl-2*

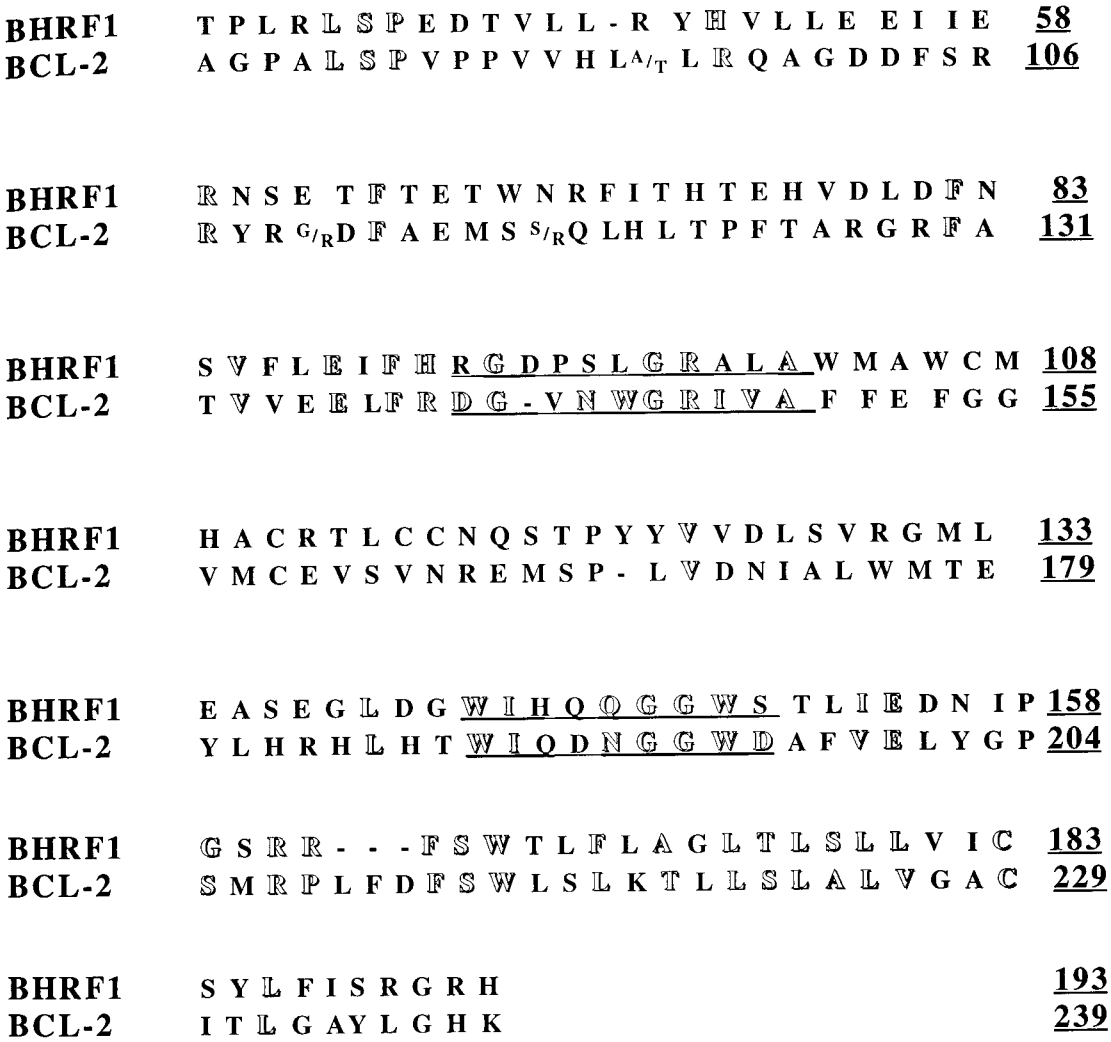


Figure 3.7 Regions of homology between the human *bcl-2* gene and the EBV viral gene BHRF1.

The amino acid sequence for the Epstein Barr virus gene BHRF1 (Pearson et al., 1987) is compared to the BCl-2 sequence (Tsujimoto and Croce 1986; Cleary *et al.*, 1986). Identical or functionally equivilent amino acids are shown in shadow (▒) and underlined letters represent regions of homology which are highly conserved, and therefore probably functionally important, within the *bcl-2* gene familiy (Boise *et al.*, 1993, Oltvai *et al.*, 1993, Williams and Smith 1993). BHRF1 and BCL-2 share 25% primary amino acid sequence homology, with greatest similarity being apparent within the 150 amino acids of the bcl-2 c-terminus(as shown here). No homology is observed between the N-termini of the two proteins (BCL-2 a.a. 1-81; BHRF1 a.a. 1-33)(data not shown).

suggests that a survival signal may be necessary in EBV infected B cells *in vivo* which do not express ***bel-2*** (Henderson ***et al.,*** **1993)**

Section 3.7 Drug resistance in leukaemia/lymphoma

3.7.1 Introduction

Over the past few years improvements in the effective high dose delivery of cytotoxic drugs along with improved primary patient care has meant that remission rates for many cancers have improved dramatically. However, with this success has come the knowledge that some cancers become, or are already to some extent, drug resistant. Lister and Rohaitiner (1984) reported that not only do higher concentrations of drugs increase rate of remission, but that they also increase the rate of relapse free survival. Models for the development of drug resistance are mainly determined by the size of tumour burden at presentation. Goldie and Coldman (1979) proposed that the expectation of cure was related to both the size of the tumour burden at presentation **(N)** and the spontaneous mutation rate towards resistance ***(a).*** The chance of attaining a cure is inversely related to both N and **a** i.e., the higher N and the greater ***a,*** the lower the probability that a cure will occur. Also of great importance is that **a** is related to one drug. In the combination chemotherapy treatments now commonly in use, three or more drugs may be involved and this decreases the overall frequency of resistance, i.e., cells must become resistant to around **3** or more drugs instead of just one, and the chances of all these independent mutations occurring concomitantly will, in theory, be lower.

Resistance to cytotoxic drugs can be placed into two categories; primary and secondary resistance. Many solid tumours are found to be chemoresistant at diagnosis and hence represent a primary resistant tumour. Primary resistance in leukaemia/lymphomais very rare since an initial response to chemotherapy is nearly always seen. Leukaemias which do exhibit primary resistance are those for which the majority of the cell population is resistant at presentation. Secondary resistance occurs when a cell population appears sensitive at diagnosis, but a resistant sub-population emerges as therapy proceeds. On average, mutants with a given resistant phenotype occur at a rate of between 1 in 10^5 and 1 in 10^6 cell divisions.

Tumours will often have around 10^{12} cells at presentation and as a consequence mutation to resistance is likely to occur in a short period of time.

Although several drug resistance mechanisms have been identified, drug resistance is still one of the greatest problems facing the clinical oncologist. A single definitive resistance mechanism has not so far been shown to account for resistance to a plethora of drugs. Indeed it is now thought that drug resistance may be explained by more than one mechanism.

3.7.2 Classification of drug resistance

Throughout evolution cells have acquired the ability to deal with foreign bodies or chemical toxins. It has been suggested that cancer cells may have enhanced efficiency in using some of these detoxification pathways leading to drug resistance. A description of some of these mechanisms is outlined in table 3.1 (Hall and Cattan, 1991).

Although many single drug resistance mechanisms have been identified, i.e., dihydrofolate reductase gene amplification in methotrexate resistant cells, one mechanism identified so far seems to induce multidrug resistance in response to drugs with differing modes of action. Cells which exhibit this form of drug resistance are aptly named multidrug resistant cells or MDR cells.

The MDR phenotype was first characterised in cells displaying reduced permeability, or altered membrane glycoprotein content in the presence of drugs such as colchicine (Ling and Thompson 1973), or the vinca alkaloids (Beck ***et al.,*** 1979). Subsequent studies identified that these cells were able to internally reduce the level of drug within the cytoplasm by actively pumping the drug from the cell (Shen ***et al.,*** 1986; Fojo ***et al.,*** 1985). The protein responsible for this phenomenon was identified as a membrane glycoprotein, (Juliano and Ling 1976), subsequently termed P (for permeability)-glycoprotein.

Genomic analysis revealed that in humans there are two closely related genes, *mdr1* and *mdr2* that encode for highly homologous membrane proteins. Although both genes are referred to as multidrug resistance genes this role has only been shown for ***mdr1.*** The gene codes for a 4.5kb mRNA which produces the 170 kilodalton membrane glycoprotein, known as P-glycoprotein, which acts as an ATP dependent drug efflux pump on contact with cytotoxic drugs from the naturally derived drug compounds i.e., vinca alkaloids, produced

Mechanism	Action	Drugs affected
1. Decreased drug uptake.	Owing to chemical similarities between cytotoxic drugs and naturally occurring metabolites, many drugs are transported across the cell membrane by an active, energy dependent process which works against the concentration gradient. Alterations in the number or structure of these carrier proteins may lead to decreased drug uptake and therefore increased drug resistance.	Methotrexate; a resistant cell line which shows a 93% reduction in methotrexate influx when compared to the parental cell line (Hill *et al.*, 1979). Melphalan; uptake can be decreased by the addition of a variety of amino acids into culture medium which inhibit two carrier systems, a sodium dependent ASC system which is important at low drug concentrations and an L system which is sodium independent and important at high drug concentrations (Begleiter *et al.*, 1979).
2. Increased drug efflux (Multidrug resistance, MDR).	Many sublines of cells in vitro which show increased resistance to anthracyclins have demonstrated cross resistance to the vinca alkaloids and other naturally occurring, functionally unrelated compounds. The MDR phenotype is frequently associated with enhanced drug efflux of the drug across the cell membrane and increased expression of P-glycoprotein.	Vinca alkaloids (vincristine, vinblastin), anthracyclins, (daunorubicin, doxorubicin), epipodophyllotoxins, (teniposide, etoposide), actinomycin D, colchicine.
3. Decreased drug activation.	Several cytotoxic drugs rely on activation after administration before they become effective. Activation may occur in host tissues or in the neoplastic cells themselves. A decrease in the activity of enzymes responsible for this conversion maybe responsible for decreased activation of the drug.	Cytosine arabinoside (ara C) is converted into two phosphate derivatives, 1-β-D- arabinofuranosylcytosine-s-diphosphate (ara-CDP) and 1-β-D-arabinofuranosylcytosine-s-triphosphate (ara-CTP). Characterisation of a resistant cell line has shown resistance was due to the complete absence of one of the enzymes-ara C kinase, which is important for this conversion (Drahovsky and Kieis, 1970).

Table 3.1
Mechanisms of Drug resistance.

Mechanism	Action	Drugs affected.
4.Cellular detoxification.	Glutathione S-tranferases, (GST's), are a group of enzymes which are highly conserved throughout evolution and represent a primitive defence mechanism. They are expressed in the liver and kidney and have the ability to detoxify a wide range of electrophilic, xenobiotic substances by formation of glutathione conjugates. They act as important intracellular binding proteins for potentially harmful products of metabolism, such as bilirubin.	Enhanced GST expression is seen in resistance to adriamycin (Batist *et al.*, 1986, Deffie *et al.*, 1988) and bifuntional alkylating agents (Adam *et al.*, 1985, Gupta *et al.*, 1989).
5.Enhanced compensatory metabolism.	Resistance arises due to changes which effectively limit or reverse the damage caused by the action of a drug on a specific cellular pathway.	Anthracyclins, including adriamycin, probably exert a cytotoxic effect by producing free hydroxy radicals. Highly reactive species produce the peroxidation of proteins and nucleic acids (Powis 1987). An adriamycin resistant human breast cancer cell line shows decreased free radical formation possibly due to an increase in the level of glutathione peroxidase activity resulting conversion of free radicals to inactive forms (Sinha *et al.*, 1987).
6. Biochemical changes in the target of drug action.	This type of resistance may occur as a result of changes in either the level of expression or in the biochemical properties of the target molecule for a given chemotherapeutic agent.	Cells resistant to topoisomerase II effecting drugs e.g., anthracyclins or epipodophyllotoxins, show alterations in both levels of topo.II produced and in the activity of the enzyme.(Davies *et al.*, 1988; Zwelling *et al.*, 1989) Cells showing resistance to methotrexate (MTX) often show an increase in the levels of dihydrofolate reductase (DHFR). This allows the metabolic pathway for tetrahydrofolate formation to occur at the normal rate and restore the normal level of thymidine triphosphate. DHFR increases may occur due to gene amplification or by the formation of double minute chromosomes.

Table 3.1 (cont)

Mechanisms of drug resistance

from the periwinkle plant (e.g., vincristine, vinblastin), anthracyclins, produced by Streptomyces, (e.g., daunorubicin and doxorubicin) and the epipodophyllotoxins produced from plant alcohol extracts, (e.g., etoposide and teniposide). These drugs exhibit differing modes of action, but they can all be effectively removed from MDR cells by the action of P-glycoprotein (Weinstein ***et al.,*** 1990).

Bcl-2 has been shown to mediate "stress resistance" in lymphoblastoid cells by a pathway which is independent of heat shock protein expression (Tsujimoto 1989). On exposure of these cells to stimuli such as heat, ethanol, methotrexate or low serum, a higher rate of survival was seen for the ***bcl-2*** expressing cells. Since stimuli such as methotrexate (Barry ***et al.,*** 1990; Lennon ***et al.,*** 1990) and low serum (Henderson ***et al.,*** 1991; Gregory ***et al.,*** 1991; Wyllie ***et al.,*** 1987) have been shown to induce apoptosis, these effects seem likely to result from specific suppression of apoptotic cell death. This is particularly interesting in the case of methotrexate, a widely used cytotoxic drug, since it implies that deregulated *bcl-2* expression in some cancer cells may contribute to drug resistance (reviewed by Dive and Hickman 1991). It may be possible that ***bcl-2*** or other similar, as yet unidentified genes, could contribute to drug resistance by this mechanism.

3.7.3 Mechanisms of chemotherapeutic drug cytotoxicity

Drugs which are active against a variety of human cancers have differing modes of generating a cytotoxic signal. Since tumours often posses a higher mitotic rate than the normal tissues from which they derive, drugs which affect cells in S-phase exhibit greater cytotoxicity on the tumour cells than the surrounding tissues.

One general mechanism of inhibiting S-phase transition is to limit the supply of nucleic acids required for DNA replication. Many drugs work on this principle but affect different pathways of pyrimidine and purine synthesis. Hydroxyurea, for example, inhibits the enzyme ribonucleoside diphosphate reductase which catalyses the reductive conversion of ribonucleotides to deoxyribonucleotides, a crucial and probable rate limiting step in the synthesis of DNA (Synder 1984). Inhibition of **DNA** synthesis by this mechanism leads to loss dGTP, decrease in dATP and increase in both dTTP and dCTP, although turnover in dTTP is also reduced (Skoog and Nordenskjold 1971). Such loss of deoxyribonucleotides also leads to

an inhibition of **DNA** repair which in turn leads to the formation of **DNA** strand breaks, which initially can be repaired on removal of hydroxyurea (Bacchetti and Whitmore **1969)** and do not cause a loss in cell viability as measured by trypan blue exclusion (Li and Kaminskas **1987).** The early studies of Bacchetti and Whitmore (1969) also illustrate that a second wave of **DNA** strand breaks occurs after removal of the drug and that this leads to cell death. Since hydroxyurea is now known to induce apoptosis (Lemon **et al., 1990),** the second wave of strand breaks are probably due to the activation of the endonuclease. Bacchett and Whitmore also reported a non-S-phase mode of cell killing in which **10-20%** of the cells are killed and suggested that such deaths result from cells just entering S-phase from **G1** and from cells which die through "unbalanced growth" i.e., **DNA** synthesis is reduced but **RNA** and protein synthesis are still occurring.

Induction of cell death by cytosine arabinoside (araC) has also been associated with **DNA** strand breaks and unbalanced growth as discussed above (Fram and Kufe **1982).** In order to be effective, araC must be activated within the celi by conversion to the 5'-monophosphate nucleotide (araCMP), catalysed by deoxycytidine kinase. AraCMP then reacts with other nucleotide kinases to form the diphosphate and triphosphate forms. The triphosphate form araCTP is incorporated into the **DNA** during S-phase and thus causes inhibition of **DNA** synthesis due to a slowing of chain elongation and inhibition of chain termination (Momparler **1968, 1972;** Zahn **et al., 1972).** AraCTP may also inhibit **DNA** polymerase by competing with dCTP for binding to this enzyme (Furth and Cohen **1968).** Other studies have shown that incorporation of araCTP into the **DNA** is associated with a loss of clonogenic survival and suggests that the presence of the arabinose moiety results in alkali-labile lesions that produce single strand breaks (Kufe ***et al.,*** **1980).** It also alters the reactivity of the **3'** terminus due to the conformational and hydrogen bonding differences of the arabinose moiety which is responsible for the decrease in chain elongation (Cozzarelli, **1977).** How the presence of araCTP in the **DNA** triggers cell death is unclear although recent evidence demonstrates that apoptosis is induced as a result treatment with araC and this can be associated with an increase in ***c-jun*** expression (Gunji ***et*** **al., 1991).** Methotrexate is another antimetabolite which is also S-phase specific and induces **DNA** strand breaks in exposed cells

(Li and Kaminskas **1984;** Lonn and Lonn **1986).** Methotrexate inhibits dihydrofolate reductase, an enzyme which catalyses the production of tetrahydrofoiate from dihydrofolate (Werkheiser, **1963).** Tetrahydrofolate is subsequently converted to N^5-N^{10}-methylenetetrahydrofolate which is essential for the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate by the enzyme thymidylate synthase. Therefore methotrexate inhibits both thymidylate and purine nucleotide synthesis (Johns and Bertino **1982)** and accordingly cell death has been postulated to occur due to a lack of thymine and purine (Hryniuk **1972).** Once in the cells methotrexate becomes polyglutamylated and this form of the drug has been found to be a potent inhibitors of other folate-dependent enzymes involved in purine synthesis and folate conversions (Kwok and Tattersall, **1992).** Cells treated with methotrexate show a progressive accumulation of **DNA** single strand breaks with increased exposure time and fewer cells recover on removal from the drug **as** treatment time increases (Li and Kaminskas **1984;** Lonn and Lonn **1986).** Failure of the cells to recover is assumed to be due to an inability to repair increasing numbers of **DNA** strand breaks due to a restriction in supply of deoxythymidine triphosphate and purine nucleotides. This may be true, however, treatment of cells with methotrexate has also been shown to induce apoptosis resulting in the cIeavage of the **DNA** into oligonucleosomal fragments, characteristic of apoptosis (Li and Kaminskas **1987;** Barry et al., **1990).** Precisely which cytotoxic lesion induced by methotrexate actually generates the apoptotic signal has yet to be established, but the inhibition of **DNA** synthesis and the resulting **DNA** damage may be important.

Not all S-phase drugs act primarily by disrupting nucleotide synthesis, other drugs are able to affect enzymes required for **DNA** replication. ***An*** example of the latter are the epipodophyllotoxins which act on topoisomerase II. Mammalian topoisomerase II exists as a homodimer which forms at least two complexes with **DNA** that are thought to exist in rapid equilibrium (reviewed by Liu **1989).** These two complexes are known as the cleavable and non-cleavable complexes and represent the broken and non-broken states of double stranded **DNA** produced by the enzyme. Topoisomerase II and topoisomerase I are required during **DNA** replication and transcription to provide swivel points for the **DNA,** removing the torsional stresses which occur as the **DNA** unwinds from its highly packaged structure to

allow access to enzymes such as DNA polymerase. Topoisomerase II (topo. II) is also required for the segregation of chromatids prior to mitosis since only topo. II can unlink two intertwined DNA circles via its strand passing ability (Yang *et al.,* 1988). Both enzymes induce transient protein bridged DNA breaks on one (topo **I)** or both (topo II) DNA strands. The topoisomerase **II** poisons such as etoposide and teniposide are non-intercalating drugs that stabilise the breakage and re-joining reaction i.e., the cleavable complex and hence induce **DNA** strand breaks (Liu 1989). Such complexes are reversed upon removal of the drug which raises the interesting question of of why the cell dies (Long *et al.,* 1986; Berger et al., 1991). However, recent work has indicated that significant DNA fragmentation often occurs several hours after removal of the drug or on prolonged exposure to the drug (Kaufmann 1989, Walker *et al.,* 1991). This subsequent DNA fragmentation has been shown to be the result of apoptosis-fragmentation of the DNA being produced by the activated endonuclease. It has been proposed that the block of replication forks by topo. II inhibition or cleavable complex formation triggers apoptosis (Jaxel *et al.,* 1988). Exactly how etoposide induced stabilisation of the cleavable complex triggers apoptosis is again unclear. However, results from studies of p53 null mice have indicated that the DNA damage produced by etoposide causes an accumulation of p53 which apeears to neccessary for cell death because in p53 null mice, p53 accumulation cannot occur and the cells do not die (Clarke et al., 1993; Lowe et al., 1993) (see section **3.4.4).** Another possible mechanism for etoposide induced cytotoxicity is by specific genetic alterations resulting from increased sister chromatid exchange (SCE) (Berger *et al.,* 1991). The increased potential for non homologous recombination due to SCE has been postulated to result in genetic loss. The loss of essential genes leads to a loss of a crucial protein(s) which are required for continued cell survival.

The cytotoxic drugs outlined above are most lethal in the S-phase of the cell cycle due to their different mechanisms for inducing blocks in DNA replication and nucleotide synthesis. Although each of the drugs specifically act in different ways and on different cellular targets the damage produced in the cell always appears to signal the induction of apoptosis. The molecular form of the drug induced signals for apoptosis have yet to be fully estblished (Dive and Hickman 1991).

Section 3.8 In summary ...

Apoptosis represents an active, controllable form of cell death involved in the regulation of cell population growth control (Williams ***et*** *al.,* 1992). **A** cell dying by apoptosis goes through characteristic morphological and biochemical changes which can be used to distinguish it from necrosis. Therefore, in response to a given stimulus, the type of cell death occurring can be determined (Wyllie *et al.,* 1980, Wyllie 1980). Several signalling pathways have been identified within apoptotic cells (McConkey *et al.,* 1990a) as have several genes, some of which are more normally associated with proliferation, e.g., c-***myc*** and c-***fos*** (Evan *et al.,* 1992; Buttyan *et al.,* 1989).

The *bcl-2* gene, which **is** abnormally expressed by virtue of the t(14;18) translocation in follicular lymphoma, was the first gene unequivocally identified with the ability to suppress apoptosis (reviewed in McCarthy *et al.,* 1992). Mice transgenic for the *bcl-2* gene have shown how suppression of cell death within the population of B cells increases both the numbers of cells present and the chances that a cell will acquire additional mutagenic changes leading to clonal malignant outgrowth (e.g., McDonnell and Korsmeyer 1991). Several genes have been identified with homology to *bcl-2,* the first of which was the EBV gene BHRF1 (Cleary *et al.,* 1986). Both genes share 25% primary amino acid sequence homology, but functional homology has yet to be determined and **is** one of the aims of this study.

Bcl-2 has also been shown to induce stress resistance in response to stimuli such as methotrexate in cells expressing an exogenous form of the gene (Tsujimoto 1989). This suggests that *bcl-2* may produce resistance to chemotherapeutic drugs.

Finally, deregulation of *bcl-2* is a primary step towards the development of lymphoma, providing the required genetic background for other mutations, such as mutated *myc,* to be viable (Askew *et al.,* 1991). Therefore, the identification of other genes able to suppress apoptosis is important for the further understanding of progressive tumour development.

Chapter 4

Materials and Methods

Section 4.1 General Techniques

4.1.1 Tissue culture and standard aseptic techniques

Severa1 cell lines were used throughout this project in order to characterise different aspects of apoptosis. All tissue culture was carried out in class II cabinets using aseptic techniques. All cell lines were cultured using ready-made **1x** liquid medium (Gibco) supplemented with foetal calf serum (FCS) and 2mM L-glutamine (Flow). Standard and filter top $25cm^2$ or $75cm^2$ culture flasks (Nunc) were used for routine cell culture and all cell lines were incubated at 37°C in a **5%** CO_2 atmosphere.

4.1.2 Determination of cell viability by vital dye exclusion

Cell viability was ascertained by the use of a vital dye, Nigrosin (BDH), used at a final concentration of 0.1% which is non-toxic to the cells. **A** 1% Nigrosin solution was routinely made up in PBS and filter sterilised before use. 90µl of a cell suspension and 10µl of vital dye were mixed in a small eppendorf and 10µl of this was added to a Mod. Fuchs Rosenthal haemocytometer to determine cell viability (Hudson and Hay 1980).

The statistical significance of differences in cell viability was determined using the Student t-test (see appendix **A).**

This method of determining cell viability measures a very late point in apoptotic celi death, i.e., disruption of the cell membrane. Cells which appear viable in a vital dye will not necessarily represent a viable cell which is able to form progeny. Therefore, in experiments where celi viability needed to be stringently assessed, colony formation in soft agar was also used (see section 4.2.3.1).

4.1.3 Analysis of apoptosis

Morphological and biochemical differences exist between cells dying by apoptosis or by necrosis (see section 3.1). These can be used to identify which form of cell death is occurring in a cell population in response to a specific death stimulus.

4.1.3.1 Morphology of apoptotic cells

Cells were assessed for apoptotic morphology by electron microscopy. This method of examining morphology clearly shows evidence of DNA condensation within the nucleus and morphological changes characteristic of apoptosis.

1ml of a cell suspension containing approximately $1x10^6$ cells was pelleted in a 1.5ml eppendorf tube (Sarstedt) using a microfuge at low speed (6500rpm at 4°C) for 5 minutes, after which the supernatant was carefully removed. The eppendorfs were placed on ice and 1ml of ice cold 2.5% glutaraldehyde fixative was carefully added without disturbing the pellet. The cells remained on ice for 2 hours to allow fixative to perfuse through the cell pellet. The pellets were then post-fixed in osmium tetroxide, dehydrated and embedded in epoxy resin. Using an ultramicrotome, 70nm sections were cut from the resulting blocks and stained in uranyl acetate and Reynolds lead citrate. Sections were examined and photographed using a Jeol 1200ex transmission electron microscope. A more detailed summary of the E.M process and the relevant recipes are shown in appendix B.

4.1.3.2 DNA analysis

DNA analysis by agarose gel electrophoresis was carried out as shown in figure 4.1. This method analyses genomic DNA and therefore fragmentation of the DNA is more obvious when the wave of apoptosis includes a substantial proportion of the cell population at the time of analysis. The solutions used in this protocol are shown in table 4.1.

In cases where it was not possible to harvest cells and analyse the DNA on the same day, cells were sampled, pelleted and washed once in ice cold tris buffered saline. The cells were then pelleted in a large eppendorftube, supernatant was removed and the cell pellet was rapidly frozen and stored for up to 7 days at -20°C.

10^6 cells were spun down in a 1.5ml eppendorf using a microcentrifuge at low speed (6500rpm at 4°C) for 5 mins.

The supernatant was removed with a Gilson pipette and the cells were spun again for 15 seconds.

The pellet was resuspended by vortexing in 20µl of buffer 1 containing 0.5% sodium lauryl sarkosinate and 0.5mg/ml proteinase K. The cells were then incubated for 1 hour in a preheated dry block at 50°C.

After 1 hour, any condensation was removed from the lid, (briefly spun in microcentrifuge at high speed), and 10µl of 0.5mg/ml stock RNase A diluted in buffer 1 was added to the eppendorf. This was mixed using a vortex and incubated for a further hour at 50°C.

Gel preparation:
A 2% agarose gel solution (Sigma) was made up in 1x tris phosphate buffer. This was melted in a microwave, set on defrost, and mixed carefully till fully dissolved. Ethidium bromide was added to a final concentration of 0.1µg/ml.
The agarose was poured into a prepared gel tray of the required size, and a gel comb was placed in the gel approx. 3 cm from one end of the gel and this was left to set on a level surface.

Eppendorfs were heated to 70°C in a dry block.

10^6 cells ($3x10^5$ for FDCP-1's) are required for good definition of DNA fragmentation.

The supernatant was removed to prevent any further DNA degradation due to any calcium or magnesium ions present which could have produced further endonuclease activation.

EDTA, which was present in buffer 1, chelates magnesium and calcium ions, thus reducing endonuclease activity further. Sodium lauryl sarkosinate denatures proteins including histones, therefore its presence released the DNA from protein produced structural configuration. Proteinase K degrades proteins including proteases, DNases and histones and prevented any further degradation of the DNA.

The RNase A was boiled to remove any DNase present and was used to remove all RNA from the lysate.

2% agarose was used to give good separation of smaller DNA fragments. Ethidium bromide is an intercalating DNA dye which becomes visible under U.V. light and was used to visualise the DNA once electrophoresis was complete.
The setting of the gel on a level surface gave an even distribution of agarose and therefore even sample separation.

70°C was required to enable melting of the loading buffer.

The condensate from the lid was again spun down, and 10µl of loading buffer containing 1% low gelation temperature agarose was added to the eppendorf. The eppendorf tubes were vortexed, spun briefly at high speed to remove gel from the sides of the tubes and reheated to 70°C.

↓

The samples were loaded into the dry wells of the gel using siliconised Gilson pipette tips, which were cut to give a tip opening just narrower than the well.

↓

The samples were left to set in the wells, then the gel was placed in the electrophoresis apparatus containing the 1% tris phosphate running buffer. This was topped up with buffer so that the wells were fully submerged and any trapped air bubbles were removed from the sample wells.

↓

The gel was run at 10V/cm for 2 hours.

↓

The gel was removed from the apparatus and photograph under U.V. light.

Low gelation temperature agarose was used to enable efficient dry loading of samples into the wells.

↓

Dry loading the samples was used to prevent movement of the high molecular weight DNA.

↓

If the samples were left to stand for too long before placing in buffer then the main gel could have dried out the sample wells. Bubbles were removed to enable the efficient running of the samples.

Figure 4.1 Whole cell lysis and genomic DNA electrophoresis method for detecting DNA fragmentation in apoptotic cells.

Solution	Stock concentration	Final concentration
Buffer 1	10mM EDTA, 50mM Tris HCl at pH 8 containing 0.5% sodium lauryl sarkosinate.	
Proteinase K	20mg/ml stock stored frozen at -20°C in small aliquots.	0.5mg/ml obtained by adding 5µl of stock to 195µl of buffer 1 on ice. Add 20µl to cell pellet.
RNase A	10mg/ml stock stored frozen at -20°C in small aliquots.	0.5mg obtained by adding 10µl of stock to 190µl of buffer 1. Add 10µl to lysate.
Ethidium Bromide	5mg/ml stock stored at room temperature away from strong light.	0.1µg/ml obtained by adding 1µl to a 50ml gel or 2µl to a 100ml gel.
Loading buffer	10mM EDTA at pH 8 which contains 1% low gelation temperature agarose and 40% sucrose.	
Tris phosphate running buffer (10x)	108g Trizma base 15.1ml 85% phosphoric acid 40ml 0.5M EDTA pH8 Make up to 1lt using distilled H_2O, then adjust to pH 8.0-8.2	Dilute 1 in 10 with distilled water to give 1x tris phosphate running buffer.

Table 4.1 Solutions used in whole cell lysis and analysis of genomic DNA

For some cell lines genomic DNA analysis did not prove sensitive enough to detect **DNA** fragmentation, therefore low molecular weight DNA was isolated. Cleavage of the DNA by the endonuclease produces oligonucleosomes representing multiples of 180bp which, on lysing the cells, can be separated from the uncleaved high molecular weight DNA. This method is based upon the published method of Wyllie ***et*** al.,(1982). $5x10^6$ or 10^7 cells were pelleted in a small eppendorf and resuspended in 20µl of cold PBS on ice. Cells were then lysed on ice for 5 minutes in 100µl of lysis buffer containing 5mM Tris-HCI, 5 m M EDTA and 0.5% triton, pH 7.5. The lysate was centrifuged at **18** 000rpm (16000g) for 40 minutes at 4°C to separate the high molecular weight DNA (pellet) from the low molecular weight DNA (supernatant). The resulting supernatant was carefully removed without disturbing the pellet and placed in a labelled **1.5ml** eppendorf on ice. The supernatant was then treated as described for genomic **DNA,** (see figure 4.1 and table 4.1) except that 10x concentrations of proteinase K and RNase **A** were added.

4.1.4 Induction of apoptosis

Four chemotherapeutic drugs were routinely used throughout this project to induce apoptosis; etoposide, an epipodophyllotoxin; methotrexate, cytosine arabinoside (araC) and hydroxyurea, ail anti-metabolites. These drugs were chosen because they all cause cell death via the disruption of different cellular pathways, as outlined in table 4.2.

Ail drugs were made up fresh just before addition to the cells. 4.5mg of methotrexate, 5mg araC and 5mg etoposide were dissolved in 100µl of DMSO (200µl for etoposide), then diluted 1:10 with the appropriate growth medium and filter sterilised using a 0.2µm syringe top filter (Costar). Hydroxyurea was dissolved directly in growth medium and filter sterilised as described above. Serial dilution to the desired 10x concentration was carried out for all drugs and these were kept on ice prior to addition to cell culture. DMSO was used at a final concentration of 0.001% or less as a control in at least three repeat experiments and had no effect on cell viability.

Cytosine arabinoside	Methotrexate	Hydroxyurea	Etoposide
S-phase specific anti metabolite drug. araC acts as a competitive inhibitor of dCTP. araC is metabolised by the cell to produce the effective form of the drug- araCTP, the presence of which causes inhibition of DNA polymerase (Furth and Cohen 1968). Incorporation of araCTP in the DNA can also lead to termination of the polynucleotide chain (Momparler 1972).	Inhibitor of deoxynucleotide synthesis and is therefore S-phase specific. Methotrexate blocks the reduction of dihydrofolate to tetrahydrofolate by inhibiting dihydrofolate reductase (Werkheiser 1963). Tetrahydrofolate acts as a cofactor in methylation of dUMP to dTMP. Therefore in the absence of tetrahydrofolate thymidine becomes depleted, inhibiting DNA synthesis.	S-phase specific drug able to inhibit ribonucleotide reductase, an enzyme which facilitates the conversion of ribonucleotide diphosphate to deoxyribonucleotide diphosphate (dRDP). This limits the supply of dRDP and affects both purine and pyrimidine synthesis (Snyder 1984). May also impair DNA repair synthesis leading to accumulation of DNA strand breaks (Li and Kaminskas 1987).	Etoposide is a member of the epipodophyllotoxin family of drugs and is able to inhibit topoisomerase II. This drug stabilises the transient intermediates of topo II which can be detected in mammalian cells as protein linked DNA single and double strand breaks (Liu 1989).

Table 4.2 Chemotherapeutic drugs and their mechanisms of cellular toxicity.

Section 4.2 FDCP-1 specific methods

4.2.1 FDCP-1 cell lines

4.2.1.1 Introduction

The FDCP-1 cell line, (Factor Dependent Continuous cell line from the Paterson laboratory, Manchester (Dexter ***et al.,*** 1980)), requires the presence of **IL-3** for continued survival and proliferation. This cell line is representative of a haemopoietic, granulocyte/macrophage progenitor cell which is developmentally blocked, i.e., unable to differentiate further to produce mature cell lineages.

FDCP-1 cells were maintained in liquid suspension culture using RPMI 1640 (Gibco) supplemented with 10% FCS (Gibco & Advanced Protein Products), **2mM** L-glutamine and 1% **mIL-3** (see below). Cells were passaged every **2-3** days by dilution to $5x10^4$ cells/ml. Growth of the cells to densities of more than $1x10^6$/ml was carefully avoided since this results in exhaustion of **IL-3** and rapid apoptosis.

Since the FDCP-1 cell line represents a heterogeneous cell population regarding growth and death rates (N. J. McCarthy, unpublished observations) sub-clones of the FDCP-1 cell line were obtained by soft agar cloning and limiting dilution in an attempt to standardise death rates. One of the isolated clones, FDCP-1-B, was used routinely throughout this project since its response to **IL-3** withdrawal resulted in death by apoptosis over a period of 48 hours (O-10% viability), a desirable time course. **A** second clone, FDCP-1-6 was obtained from a culture of FDCP-1 cells which had been deprived of **IL-3** for 7 days, then plated in soft agar with **IL-3** to rescue any surviving cells.

IL-3 was produced in the form of a supernatant obtained from **mIL-3** cells (used by kind permission of Prof. Dr. Melchers, Basel Inst. Immunol.). The **mIL-3** cells are X63Ag8-**653** cells which have been transfected with a plasmid containing the murine **IL-3** gene and therefore secrete large amounts of **IL-3** into their growth medium (Karasuyama and Melchers, 1988). These cells were maintained in RPMI 1640 and **5%** FCS supplemented with 2mM L-glutamine and were passaged every **3** days or once they had reached confluency. Supernatant

from the cells was harvested and screened for FDCP-1 cell growth promotion before use. Batches were stored in 500ml bottles at -20°C and were thawed and filter sterilised before addition to **FDCP**-1 growth medium, or subsequent re-freezing into smaller 10ml aliquots.

4.2.1.2 Removal of cells from IL-3

Removal of cells from IL-3 was carried out by washing the cells twice in RPMI 1640 medium containing 10% FCS and 2mM L-glutamine, but without IL-3. Cells were then cultured in IL-3-deprived medium for periods varying from 24 hours to 7 days. Medium containing low levels of **IL-3,** sufficient to stimulate cell survival, but not proliferation was obtained by adding IL-3 at 0.2% instead of the usual concentration of 1% which is required for cell survival ***and*** proliferation in normal culture conditions.

4.2.2 Protocol for chemotherapeutic drug induced apoptosis

Replicate cultures were set up in a 24 well plate (Nunc) with the desired final concentration of drug being added to the appropriate wells; 0.1μm methotrexate, 0.5μg etoposide, 0.5μg araC and 2mM hydroxyurea (see section 4.1.4). Cells were exposed to the drugs for a maximum time course of 96 hours, with viability analysed at 15, 24, 39, 48 and 72 hours. Morphology was analysed at 24 hours by E.M. and **DNA** integrity was examined at various time points as described in section 4.1.3.

4.2.3 Electroporation

Electroporation was used to introduce a plasmid expressing the Epstein Barr virus gene BHRF1 or a *bcl-2* expressing plasmid into the FDCP-1 cells. BHRF1 was the first of a family of *bcl-2* homologues to be identified and therefore functional homology between the two genes was analysed using the FDCP-1 cell line. The BHRF1 plasmid construct used is shown in figure 4.2. The *bcl-2* construct used was a kind gift from Yoshihide Tsujimoto and is as described in Tsujimoto (1989).

Electroporation of the BHRF1 construct was carried out as described in figure 4.3 and was the method of choice for these cell lines since it is relatively straightforward and because

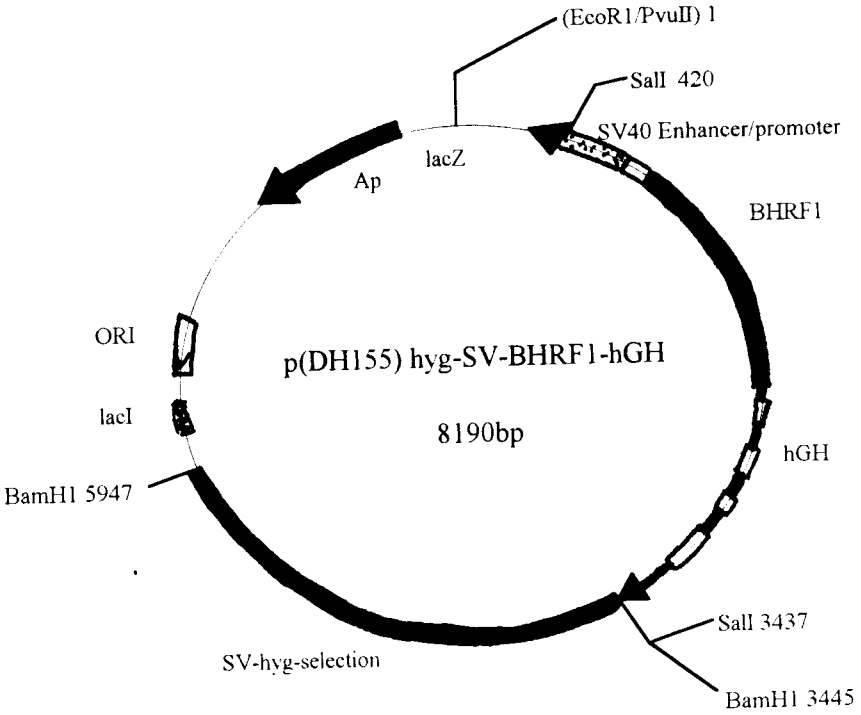


Figure 4.2 Diagrammatic representation of the BHRF1 plasmid construct used in the electroporation of FDCP1 cells

Cell number and viability of FDCP-1 cells was determined and cultures of <90% viability were used. Around 60 million cells were needed per experiment.

↓

Cells were centrifuged at 1600 rpm for 10 minutes and supernatant was removed. Cells were washed twice in ice cold PBS and resuspend in cold PBS at approx. 20 million cells per ml.

↓

0.8ml of this cell suspension was placed in an electroporation cuvette on ice. This was repeated for another two tubes and all 3 were clearly labelled A, B or C.

↓

To cuvette A 10μg (9μl) of HYG-SVBHRF1HGH (plasmid containing BHRF1 gene) was added; to cuvette B 10μg (8μl) of HYG-SV-HGH (control plasmid with no BHRF1 gene) was added; no additions were made to cuvette C (control).

↓

All cuvettes were kept on ice for 15 minutes before electroporation. **Cells were mixed in the cuvette briefly and any water was wiped away from the outside of the cuvette before placing it in electroporation apparatus.**

↓

Electroporation:
For all tubes the apparatus was set to 280 volts, and 960μF. The first cuvette was placed in the holder, with the metal sides facing left and right, and the slide holder was moved into position with the metal sides touching the electrodes. Both charging buttons were pressed and held in until the charge was delivered to the cells (machine beeped when this was done). At this stage the buttons were released and the cuvette removed and placed on ice. Time constant was checked (should be between 17-19) as was actual voltage delivered to the sample to establish that these were of the required levels. This was repeated for the remaining two cuvettes.

↓

After electroporation, cuvettes were left on ice for 15 minutes and then at room temperature for a further 15 minutes.

↓

Cells were resuspended in tissue culture flasks with approx. 17mls of growth medium to give ~ 600,000 cells per ml and were left to recover for 24 hours at 37°C with 5% CO_2.

Figure 4.3 Protocol for electroporation of FDCP-1 cells.

of the clonal nature of the FDCP-1 cell lines, transfectants could be easily selected by cloning in soft agar.

A Bio-Rad Gene-pulser apparatus was used along with a capacitance extender which enables higher voltages up to 0.450kV to be used for the electroporation of eukaryotic cells and was set to 960 microfarads (μF). The FDCP-1 cells were electroporated in phosphate buffered saline (PBS) which has a very low resistance due to high ionic strength. This means that the time constant is much lower in this media, around 17 msecs as opposed to 170 msecs in buffered sucrose. The time constant represents the resistance **x** capacitance, and is the time required for the peak voltage to decay to approximately **37%** of the initial voltage. For the FDCP-1 cells in PBS this was recommended to be between 17 and 19 milliseconds (Muser ***et al.,*** 1989).

Sterile cuvettes (Bio-Rad) were used once per electroporation and care was taken not to touch the two metal sides of the cuvette, thereby allowing maximum contact between the cuvette and the electrodes. Ail appropriate safety procedures were followed during the electroporation protocol.

Approximately 60 million FDCP-1 cells were used per experiment, allowing 20 million per cuvette and cells were of high viability and in log phase growth before treatment. Plasmid constructs were kept frozen in small aliquots at -40°C and thawed a few minutes before addition to the cuvettes.

After electroporation cells were incubated in growth medium for **24** hours at 37°C and 5% CO_2, to allow the cells to recover. Cell viability was then determined by vital dye exclusion (normally around 50%) and live cells were harvested by centrifugation through a nycodenz (Nycomed) gradient by the method shown in figure **4.4.** This enables removal of the dead cells, the presence of which may impair the growth of the remaining live cells, and reduces the number of cells which need to **be** analysed by growth on soft agar. Cells which had incorporated the plasmids were selected in the presence of hygromycin (Sigma) by virtue of the hygromycin resistance gene within the plasmid construct.

Cells electroporated with the *bcl-2* plasmids were treated exactly the same as described for the BHRF1 transfectants in figure **4.3,** except for additions of plasmids and antibiotic

Viability of the cell suspension was determined, using the vital dye nigrosin. Up to 10 million cells were spun at 1600rpm for 10 minutes in a sterile universal.

Cells were resuspended in 0.2ml of growth medium and mixed with 5.2ml of diluted nycodenz in 10-12ml centrifuge tubes (Nunc). This was carefully overlaid with 2ml of growth medium. (Nycodenz was diluted by adding 5.2ml nycodenz to 0.8ml of 'diluent A' (Nycomed) and kept cold and sterile).

Cells were spun for 10 minutes at 1000g (2700rpm) at 10°C.

The centrifuge tube was carefully removed and the interface and medium were harvested, most live cells were found between the nycodenz/medium interface and appeared as a whitish layer. All dead cells were pelleted at the bottom of the centrifuge tube. The live cells were added to 20mls of warmed, gassed growth medium in a universal.

Cells spun for 10 minutes at 1600rpm.

Supernatant was discarded and cells were resuspended in 2ml of growth medium. Another 18mls of medium was added to the cells and they were re-spun for 10 minutes at 1600rpm.

Supernatant was discarded and the pellet was resuspended in 5ml of growth medium. Viability was determined and cells were re-cultured.

Figure 4.4 Protocol for the separation of live and dead cells using a nycodenz gradient.

selection. 10µl of pcΔj-*bcl-2* (bcl-2 expressing plasmid) was added to cuvette **A** and 3.4µl of pcΔj-SV2 (control plasmid) was added to cuvette B. Transfectants were selected for in the presence of the antibiotic geneticin (G418) (Sigma).

For properties of antibiotics see table 4.5 in section 4.3.

4.2.3.1 Soft agar cloning

The ability of FDCP-1 cells to clone on soft agar in the presence of the selection antibiotic hygromycin or geneticin (Sigma) was used to identify which cells had successfiilly incorporated either BHRF1 or bcl-2 plasmids. Plating of the cells was carried out as described in figure 4.5, which represents the double layer method of soft agar cloning. The selection antibiotic was added to the bottom 10ml medium/agar layer at 120% of the required final concentration in order to compensate for the 2mls of agar/mediumtop layer which contained the cells. Final concentrations of between 10 and 20µg hygromycin or 1-2mg geneticin per plate were used. **A** 100x concentration of antibiotic was made up directly in growth medium and filter sterilised before adding the required amount to the cooled (45°C) medium/agarlayer.

Clones which grew in the presence of antibiotic were harvested, grown up in liquid culture and selected again by addition of either 300µg/ml hygromycin or 2mg/mI geneticin to the growth medium. Cells were cultured in the presence of antibiotic for **7** days then in antibiotic free medium for **7** days.

Expression of BHRF1 by the selected clones was assessed by western blotting, to detect protein expression, and by survival of the clones in medium without **IL-3** or low levels of IL-3. The second method was used since the BHRF1 phenotype was predicted to be similar to that of FDCP-1 cells expressing exogenous *bcl-2* i.e., enhanced survival of cells in the absence of IL-3.

Selected clones, both **BHRF1** transfectants and controls as well as bcl-2 transfectants and controls, were removed from IL-3 as described in section 4.2.1.2 and were set up in replicate cultures in the absence of **IL-3.** Viability was determined at 24 hour intervals and compared to that of an untransfected population of FDCP-1 cells.

A 1% Noble agar solution (Difco labs) was made up in triple distilled water (BDH) and sterilised for 20 minutes in a pressure cooker.

↓

The 1% Noble agar was mixed 1:1 with 2x growth medium and incubated at 45°C in a water bath. 2x growth medium = 40% FCS, 20% 10x RPMI, 29% triple distilled water (sterilised), 5% 44g/L $NaHCO_3$, 4% 200mM L-glutamine and 2% mIL-3, adjust to ~ pH 7.

↓

10ml of the agar/medium mix was added to the desired number of 90mm petri dishes (Sterilin) and left to set for 10 minutes. This formed the bottom layer on which the cells were plated. These were then placed in an incubator at 37°C in a 5% CO_2 atmosphere and allowed to equilibrate for 30-60 minutes or overnight in sandwich boxes if required.

↓

The remaining agar / medium mix was kept at 45°C.

↓

The required cell suspension (e.g., $5x10^5$ cells in 1ml in normal growth medium x *n* plates) was placed in a universal and heated briefly to 45°C. The cell suspension was mixed 1:1 with the remaining agar/medium mix.

↓

2ml of this suspension was carefully pipetted onto the previously poured bottom layer and allowed to set.

↓

The agar plates were placed in a clean, dated sandwich box and incubated at 37°C and 5% CO_2 for 2 hours with the lid off the box. Once equilibrated, the lid was replaced and the box was sealed.

↓

After approximately 2 weeks cell colonies of around 40 cells in size should have appeared on the plates. These were counted and harvested if desired. Colonies were removed from the plate by dropping 5µl of medium onto the colony to loosen the cells, then harvesting the colony with a 20µl Gilson pipette, placing it in 1ml of growth medium.

Figure 4.5 Protocol for soft agar cloning

4.2.3.2 Western blotting.

Cells were analysed for BHRF1 expression by the method shown in figure **4.6.** Buffers and solutions used are shown in table **4.3.** The BHRF1 antibody solution was kindly provided by Dr. Sheila Henderson (Dept. Cancer studies, Birmingham).

4.2.3.3 Development of a western blot by the ECL method

Instead of developing the blots using autoradiography, it was decided to use the faster method of electrochemiluminescence(ECL). Washed nitrocellulose membranes were placed in a 1:1 mixture of the two bottled ECL chemicals (Pharmacia) and left at room temperature for exactly one minute. This allows the development of the horse radish Peroxidase stain present on the second antibody which leads to a fluorescent signal in the area where the antibody has attached. The membrane was then quickly removed and carefully rapped in cling film, any air bubbles present were removed. The membrane was subsequently placed in an autorad cartridge and covered with a sheet of light sensitive film. The cartridge was then closed and the film exposed to the membrane for **30** seconds initially, after which the film was removed and placed in developer. This was left for 5 minutes and then transferred to fixative for another 5 minutes. Finally the film was washed in distilled water and hung up to dry. A signal, if present, should mark the film within **30** seconds, if strong, to 30 minutes if very weak, therefore separate pieces of film were exposed for increasing quantities of time to detect the signal.

4.2.4 Selection protocol for apoptosis deficient mutants

4.2.4.1 Introduction

On removal of **FDCP-1** cells from **IL,-3** and subsequent culture in **IL-3** deprived conditions for **7** days, a very low number of cells can appear viable by vital dye assessment **(N. J.** McCarthy, B.Sc. thesis 1990). It was possible that these cells represented spontaneous mutants which were unable to enter apoptosis on removal of **IL-3,** or showed enhanced cell survival, much like a cell expressing exogenous ***bcl-2*** (Vaux ***et al.,*** 1988). Therefore, a protocol was devised to select these cells for further analysis. At the same time the possibility

SDS gel preparation:

Using Bio-Rad apparatus, 1 large and small glass plate (front) were assembled. Spacers were placed between the 2 plates and they were checked to make sure they were level. The bottom edges of plates were smeared with Vaseline and placed firmly in the stand. This was then filled with H_2O and left for 10 minutes to check for leaks.

↓

The resolving gel was mixed, adding the ammonium persulphate last. The gel was pipetted between the glass plates till approx. 3/4 full and then a layer of butan-2-ol was added to allow the gel to set level. Butan-2-ol was then removed and the gel was washed with dH_2O.

↓

The stacking gel was mixed, adding the ammonium persulphate last. This was pipetted on to the running gel until 0.5ml from the top of the front plate and then the comb was added. Air bubbles were removed by running a comb along the top of the plates and then the gel was left to set for 1 hour. The comb was subsequently removed and the gel was washed in running buffer.

↓

10^7 cells were used per sample and placed in a sterile centrifuge tube and spun for 10 minutes at 1000rpm.

↓

The supernatant was carefully removed and the cells were resuspended in 1ml of PBS in a 1.5ml eppendorf tube. Cells were spun for a further 5 minutes at 6500rpm in a microcentrifuge. All PBS was removed and the required amount of gel sonicating buffer was added to the pellet (20µl required per 10^6 cells).

↓

Cells were sonicated on ice and then heated to 100°C for 2 minutes in a dry block.
At this stage cell samples were stored frozen at -20°C for later use if required

↓

20µl of each sample was loaded into separate wells in the 15% SDS gel along with molecular weight markers. The gel was run at 30 volts, with cooling apparatus present, for 4-6 hours or until samples had reached the bottom edge of the gel.

↓

Next, the gel was carefully separated from the glass plates and the stacking layer of the gel was removed. The transfer blot was carefully assembled in the following way:

On the black side of the blot apparatus were placed two pieces of brillo pre-soaked in transfer buffer, on to this was placed one piece of pre-soaked filter paper and bubbles were removed. Next the gel was carefully added onto the filter paper and one corner was trimmed so that the resulting blot could be correctly oriented. Added on to the gel was one piece of pre-soaked nitrocellulose (Hybond-C), cut to the correct size and trimmed on the same corner as the gel. Air bubbles were again removed and another piece of filter paper was placed onto the nitrocellulose (bubbles removed) and then two pieces of pre-soaked brillo were finally added. This blot case was then closed and fastened.

The transfer tank was filled with transfer buffer and the black side of the blot case was placed facing the negative electrode, with the white side facing the positive electrode. The transfer was carried out overnight at 30 volts, 0.12 amps at room temperature.

↓

Gel and nitrocellulose were removed together and placed in dH_2O. The gel was carefully removed, fixed in methanol for 30 minutes and then stained with coomassie blue to check proteins had transferred from gel.

↓

The nitrocellulose membrane was placed in Ponceau S (1% in 3% trichloroacetic acid). This was used to stain up the protein bands to check that the protein had indeed transferred. The stain was removed from the membrane by rinsing in tap water and then it was placed in blotto (5% skimmed milk in 0.02% TBS azide) and agitated overnight at room temperature.

↓

Blotto was then removed and the membrane was washed in PBS tween (1ml Tween 20 in 1lt PBS) for 15-20 minutes. It was subsequently placed in a plastic bag with 3 sides sealed. To this, the first antibody (1:100 dilution of BHRF1 antibody in blotto) was added and agitated in a cold room overnight or for 3 hours at room temperature.

↓

The nitrocellulose was then removed and washed in 0.1% PBS tween for at least 20 minutes. (N.B. antibody solution was saved for re-use).

↓

The nitrocellulose was then placed in a plastic bag with three sides sealed and the second antibody was added, (peroxidase conjugated anti-mouse Ig, 1:5000 dilution in blotto) and the bag sealed. This was agitated for 1 hour at room temperature.

↓

The nitrocellulose was then washed for 1 hour using 3 changes of 0.1% PBS tween.

↓

The nitrocellulose blots were then developed using the ECL technique.

Figure 4.6 SDS gel and western blotting protocol for detection of the BHRF1 protein

Cell sonicating buffer	15% resolving gel for 2 gels	Stacking gel for 2 gels
50mM Tris base 4% sodium dodecylsulphate (SDS) 10% Glycerol 5% β mercaptoethanol 0.01% Bromophenol blue pH 6.8	22.5ml dH_2O 22.5ml resolving buffer (1.5M Tris, 0.4% SDS, 70µl TEMED pH 8.8) 45ml acrylamide (30% acrylamide: 0.8% bisacrylamide made up in fume cupboard) 300µl Ammonium persulphate (added last)	11ml dH_2O 15ml stacking buffer (150µl 20% SDS, 14.8ml 0.25M Tris, 23µl TEMED pH 6.8) 4ml acrylamide 300µl Ammonium persulphate (added last)

SDS gel running buffer	Transfer buffer	Blotto
0.25M Tris base (30.3g/L) 1.92M Glycine (144.2g/L) 1.0% SDS (10g/L) pH 8.3	12g Tris base 57.6g Glycine 3.2L dH_2O 0.8L methanol	5% skimmed milk in 0.02% TBS azide.

Table 4.3 Recipes for SDS gels and running buffers.

of mutating FDCP-1 cells and selecting for a *bcl-2* like survival phenotype was considered and this was incorporated into the selection protocol. The construction of the full protocol will be described in results, but the routine elements are described here.

4.2.4.2 Tritiated thymidine selection

Cells were exposed to varying levels of tritiated thymidine (H^3TdR) in the presence and absence of IL-3. $3x10^5$ cells were added into replicate wells in a **24** well plate which consisted of; controls with IL-3, controls without IL-3, cells with **IL-3** and H^3TdR or cells without IL-3 and with H^3TdR. Cells were selected under these conditions at **37°C** with 5% CO, for three days, after which celi viability was determined by vital dye exclusion and more stringently by the ability of cells to clone in soft agar. Cells were removed from H^3TdR by two washes in growth medium before plating in soft agar at $5x10^5$ cells per plate, or lower, as described previously. Both live and dead cells were plated in soft agar to fully assess viability and therefore the number of viable cells per plate was often considerably lower than the originai number of cells plated. Plates with highIy viable cells were always included as controls to obtain an estimate of maximum cloning efficiency. All plates were gassed, boxed and incubated for 14 days after which colonies of greater than 40 cells were scored.

Sterile H^3TdR, (concentration **lmCi/ml,** 28.8Ci/mA specific activity) (Amersham), was made up to a 1Ox stock concentration in growth medium without IL-3. The appropriate amount of H^3TdR was then added to each well to give the desired 1x concentration, e.g., 30µl of H^3TdR was added to 1.5ml medium to give a stock of 20µCi/ml. 100µl of this was added to 800µl of medium with 10% FCS, with or without **IL-3** and 100µl of cell suspension to give a final H^3TdR concentration of 2µCi/ml.

Section 4.3 Burkitt's lymphoma cell lines, specific materials and methods

4.3.1 Burkitt's lymphoma cell lines

Three different Burkitt's lymphoma (BL) cell lines have been used to examine the effects of over expression of ***bcl-2*** and BHRF1 on apoptosis. Each cell line has differing characteristics which are outlined in table 4.4. Cells were maintained in the presence of RPMI

Group I cell lines	Group III cell lines
Express only one EBV latent gene, EBNA 1 and maintain the phenotype of biopsy cells. These cells are sensitive to apoptosis induced by a variety of stimuli.	Express all eight EBV latent genes, EBNAs 1, 2, 3a, 3b, 3c, LP and LMP 1 and 2. These cells also express *bcl-2* and are generally insensitive to apoptotic stimuli.
Cell lines used	Cell lines used
(i) Cheptages (Chep-BL): Kenyan BL from mandible tumour of 5 year old male, t(8;14) present (Rooney *et al.*, 1986) (ii) Akata (Akata-BL) (Takada and Ono, 1989): Japanese BL t(8;14) present, shows some low level expression of *bcl-2*, but remains sensitive to serum withdrawal.	(i) Raji (Raji-BL) (Rymo *et al.*, 1981): One of two cell lines established from 100 samples of African BL. Has t(8;14) present. It is an unusual cell line in that it has group III features, but does not express *bcl-2* even though LMP 1 is expressed. Virus has deletions in Bam H1 A and Bam H1 E and point mutations in LMP (Hatfull *et al.*, 1988) which may explain why *bcl-2* is not expressed. EBNA 3c is also not expressed.

Table 4.4 Characteristics of Group I and III BL cell lines

Drug and stock concentration	Action
Geneticin, (G418), stock concentration of 80mg/ml made up in growth medium, filter sterilised. Store at 4°C.	Kanamycin like drug which interferes with 80s ribosome function, blocking protein synthesis.
Hygromycin, purchased as **powder**; stock concentration of 10mg/ml **solution**; stock concentration of 10,000 U both made up in growth medium and filter sterilised. Store at 4°C.	Aminoglycosidic antibiotic that inhibits protein synthesis by causing mistranslation from mRNA .

Table 4.5 Stock concentrations and actions of selection antibiotics.

1640 supplemented with 10% FCS (Gibco) and 2mM L-glutamine. Since group 1 cell lines are highly sensitive to changes in serum factor concentrations, each batch of FCS must be screened in order to maintain a high degree of cell viability and growth. Cells were passaged every 2-3 days and were not split below a concentration of 1.3 x 10^5/ml. The Burkitt's lymphoma cell lines were transfected with plasmid constructs expressing either *bcl-2* or BHRF1 (Henderson ***et al.***, 1991, 1993) and were cultured in the presence of selection antibiotics in order to maintain gene expression. Cells were grown either in hygromycin at 300μg/ml (BHRF1 plasmids) or geneticin at 2.5mg/ml *(bcl-2* plasmids) and were cultured in the antibiotics for 7 days and then grown without antibiotics for 4-6 days before re-selecting. All cells were washed free of the drugs 12 to 24 hours before any experiments were carried out. For properties of selection antibiotics see table 4.5.

4.3.2 Induction of apoptosis by chemotherapeutic drugs

BL cell lines were treated with the following drugs; methotrexate, araC and etoposide (Sigma) which were prepared as described in section 4.1.4.

BL cells, with a starting viability of ***> 86%,*** were exposed at $3x10^5$ cells/ml to the desired drug concentration for 14 hours in growth medium at 37°C and 5% CO_2. Treated and untreated controls were washed free of drug by 2 washes in warmed, gassed RPMI, and cells were resuspended in at least 3 replicate wells at $3x10^5$ cells/ml in growth medium. Viability was determined after 48 hours by the exclusion of the vital dye Nigrosin.

Cells were also set up in 1ml replicate cultures with each of the drugs and viability was determined at 24, 48 and 72 hours, by vital dye exclusion, in order to obtain a time course of drug induced death.

4.3.3 Exposure *to* ionising radiation

Pre-washed, replicate cell suspensions of $3x10^5$ celldmi were irradiated on ice in 1.5ml eppendorf tubes and exposed to ***8Gy*** (Chep-BL) or 16Gy (Raji-BL) using a Co^{60} y source. After irradiation cells were pelleted and resuspended in 1ml cultures in fresh, warmed growth medium and incubated in a 24 well plate (Nunc) at 37°C with ***5%*** CO_2. Viability was determined as previously described and at least 3 replicates were counted per treatment.

4.3.4 Fluorescence microscopy

Acridine orange, an intercalating, fluorescent DNA binding dye, was used to identify apoptotic cells with the characteristic condensed nuclear DNA morphology. Differing DNA states within viable and apoptotic cells result in differing access for acridine orange into the DNA. Hence acridine orange gives out a diffuse yellow/green fluorescence when bound to DNA in viable cells and a bright green fluorescence when bound to condensed (and less accessible) fragmented **DNA** in apoptotic cells. RNA in both viable and apoptotic cells emitts a red fluorescence.

Acridine orange was made up to 100μg/ml stock in water and filter sterilised. This was then diluted to give a stock of 25μg/ml. 20μl of this was routinely added to a 20μl cell suspension placed on a microscope slide. Both suspensions were mixed briefly on the slide with a 20μl automatic pipette tip before addition of a coverslip, with care taken to avoid trapping any air bubbles. The resulting cell suspension was then viewed immediately under a Zeiss fluorescence microscope using the x100 oil immersion objective lens. Cells were photographed using 100 **ASA** colour film.

For the purposes of determining the numbers of morphologically normal (viable) and apoptotic cells, equal volumes of a cell suspension and acridine orange were mixed as described above and the numbers of apoptotic vs. live cells were determined by counting all cells visible in a field using the x16 objective lens. At least 8 separate fields were counted per replicate treatment.

4.3.5 Giemsa staining of apoptotic cells

Differences in the morphology of normal and apoptotic cells stained with Giemsa (BDH) relies on the differing states of the DNA within these two cell populations. Apoptotic nuclei, which appear characteristically condensed, stain a deep purple colour, where as viable nuclei stain pink in colour with purple nucleoli often visible. Counter staining is provided by Jenners stain (BDH) which gives the cytoplasm a light purple colour.

10^5 cells in 100μl of PBS were spun onto labelled glass slides in a cytospin centrifuge at 600rpm for **4** minutes. The cells were then fixed in methanol for 10 minutes before staining

in diluted Jenners solution for 5 minutes (Jenners diluted **1:3** with Giemsa buffer). Cytospins were washed free of excess Jenners stain by rinsing in Giemsa buffer and were then stained with diluted Giemsa for 10 minutes (Giemsa diluted **1:10** with Giemsa buffer). Excess Giemsa was removed by washing two to three times in Giemsa buffer followed by one wash with distilled water. Slides were wiped dry, with care taken to avoid the stained cells, and then left for 5-10 minutes to air dry before mounting in DPX.

Cell morphology was photographed under oil with **x100** objective lens using 60 **ASA** artificial light colour film and a glass 82C blue filter which produces a whitish background.

Results

Chapter *5*

Results: IL-3 dependent cells

Section 5.1 Do chemotherapeutic drugs induce apoptosis in FDCP-1 cells?

Several studies examining the mechanisms chemotherapeutic drug toxicity demonstrated that apoptosis was induced as a result of treatment (Wyllie ***et al.,*** 1980). For example, low concentrations of hydroxyurea induce early double strand breaks in cells, probably due to the inhibition of DNA synthesis and repair, but these are repaired on removal from the drug. However, either a prolonged exposure, or higher concentrations of the drug, resulted in subsequent cell death which was associated with the reappearance of double strand breaks and fragmentation of the DNA (Li and Kaminskas 1987). Fragmentation of DNA had also been shown for etoposide and methotrexate (Kaufmann 1989; Li and Kaminskas, 1984). The demonstration of apoptotic morphology on treatment with chemotherapeutic drugs has also been reported in several different cell lines (Lennon ***et al.,*** 1990; Collins ***et al.,*** 1992; Fanidi ***et al.,*** 1992). However, induction of apoptosis by chemotherapeutic agents in the FDCP-1 cell lines was confirmed directly and not assumed.

Previous work using the FDCP-1 cell line had shown conclusively that apoptosis occurred as a result of removal of **IL-3** from the cell culture medium (Williams ***et al.,*** 1990) and that the resulting apoptotic morphology was distinctive, i.e., both fragmentation of the DNA and condensation of the nuclear chromatin was comparable to that observed in thymocytes treated with glucocorticoid (Wyllie ***et al.,*** 1984). Hence, identification of apoptotic morphology in the FDCP-1 cells was predicted to be relatively straight forward.

The demonstration of apoptosis induced by chemotherapeutic drugs in FDCP-1 cells is an important preliminary step before examining the possibility that inhibition of apoptosis, by genes such as *bcl-2,* can have an effect on drug resistance (see section 3.7.2).

FDCP-1 sub-clone B cells (FDCP-1-B) were used for these experiments since they have a more synchronous death rate than the FDCP-1 cell population (see section **5.3).** FDCP-1-B cells were treated with four drugs; methotrexate, hydroxyurea, araC and etoposide,

suitable concentrations of which were ascertained by referring to past papers and using 10 fold higher and lower concentrations as well as the suggested value. Drug concentrations which produced around **80%** loss of viability in **48** hours (see table 5.1) were used in the final experiments. Lower concentrations of drug were opted for in order to mimic the low levels of drug which can be tolerated in patients, compared to higher levels of drug which can be used ***in vitro.***

Cells were constantly exposed to the pre-determined, optimum drug concentration and viability was determined at 15, **24,** 39, **48** and **72** hours. The drop in cell viability produced by the drugs is depicted in figures 5.1A and 5.1B with cell viability being around **20%** or lower after **72** hours. The drop in cell viability observed in the control cell population at 15 hours is often seen in these cells and appears to be due to the relatively low starting cell density ($2x10^5$ cells/ml) compared to the density at which the cells were growing at in culture (6-7 $x10^5$/ml), cell to cell contact being an important factor in maintaining high viability (Dexter ***et al.,* 1980).**

Having established a time course of cell death, cells were examined at **24** hours for apoptotic morphology by electron microscopy. Fragmentation of the DNA was analysed at various different time points.

As mentioned earlier, apoptotic morphology in these cell lines is quite obvious on **IL-3** withdrawal and proved to be almost as clear on treatment with the chemotherapeutic drugs. When compared to untreated cells (see figure **5.2A),** in which nuclei have normal diffuse dark and light staining chromatin, apoptotic cells, which have characteristically condensed, dark staining chromatin, are obvious in cells treated with either 0.1μM methotrexate (B); 0.5μg/ml etoposide (C); 0.5μg/ml araC (D) and **2mM** hydroxyurea **(E).**

Fragmentation of the DNA, another classical marker of apoptosis, was detected in cells treated with hydroxyurea for 15 hours, see figure 5.3 lane ***6.*** For cells treated with methotrexate, araC and etoposide, **DNA** fragmentation was not detected until after 30 hours exposure to the drug and more strongly after **48** hours exposure (see figures **5.4A** and **5.4B** lanes **3,4** & *5,* and lanes **4,** *5* & ***6*** respectively). Fragmentation of the **DNA** caused by hydroxyurea is clearly visible at 15 hours, but decreases in intensity at 30 and **48** hours, probably because **2mM** hydroxyurea induces a more rapid loss of viability in the early stages of

Table 5.1

% viability of FDCP-1 cells treated with various concentrations of cytotoxic drugs for 48 hours

10μM MTX	1μM MTX	0.1μM MTX	0.01μM MTX	20mM HY	2mM HY
5.2 +/- 0.97	13.9 +/- 0.20	13.7 +/- 1.10	68.9 +/- 2.40	3.6 +/- 0.63	16.8 +/- 2.66

4μg/ml etop	1μg/ml etop	0.1μg/ml etop	0.01μg/ml etop	0.5μg/ml araC	0.1μg/ml araC
7.1 +/- 1.50	13.0 +/- 1.67	42.0 +/- 1.50	76.7 +/- 1.27	18.3 +/- 0.90	84.3 +/- 5.90

% viability was determined by vital dye exclusion and is equal to the number of surviving cells expressed as a percentage of the total cell number counted.
(n=3).

Abbreviations:
MTX; methotrexate
HY; hydroxyurea
etop; etoposide

Figure 5.1(A), (B) legend

Displays mean % viabilities +/- standard errors (n=3), determined over a 72 hour period

% viability, as determined by vital dye exclusion, is **expressed as** the **number** of **viable cells divided by the total number** of **cells counted.**

Starting cell density = $2x10^5$/ml

Figure 5.1(A) Viability of FDCP-1-B cells treated with hydroxyurea and methotrexate.

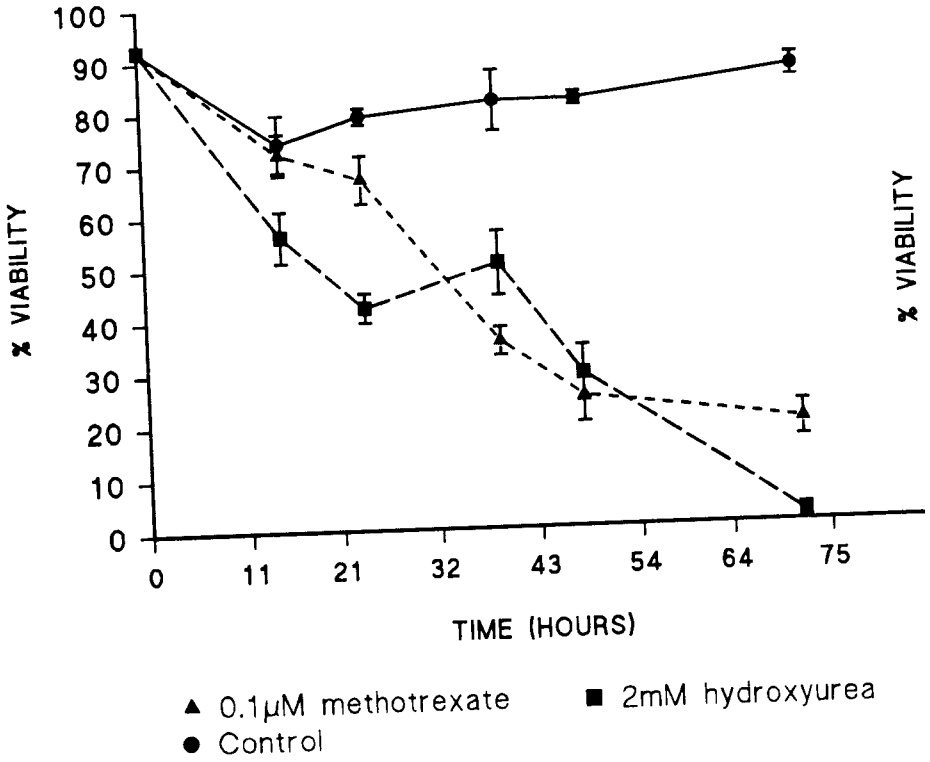


Figure 5.1(B) Viability of FDCP-1-B cells treated with araC and etoposide.

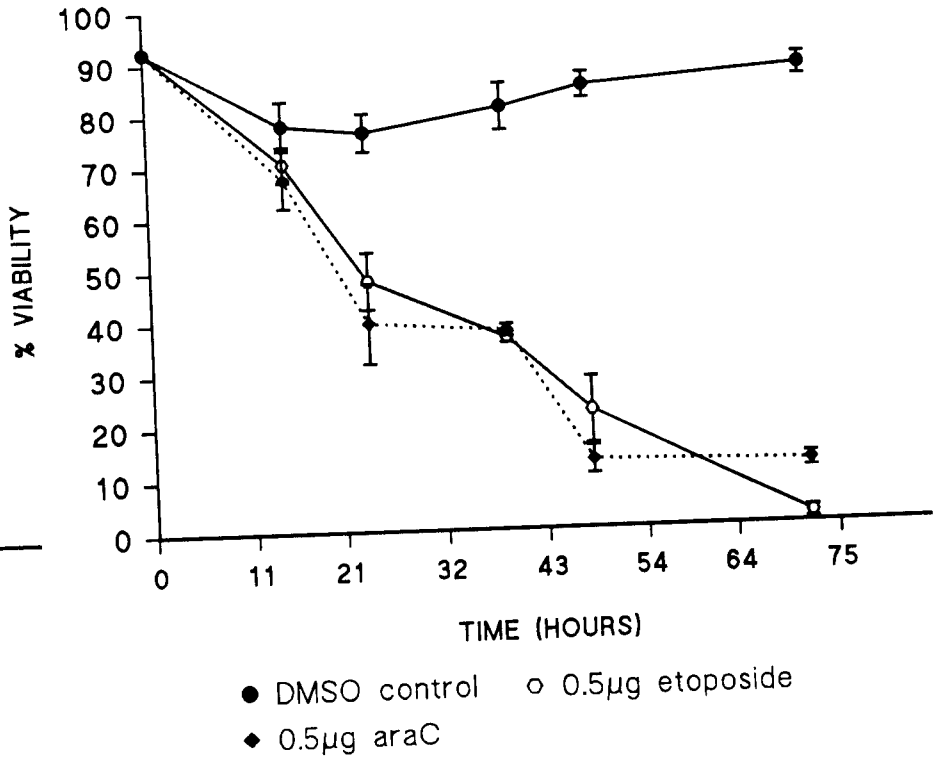


Figure 5.2 Electron micrographs showing induction of apoptosis in FDCP-1-B cells treated with chemotherapeutic drugs

Legend

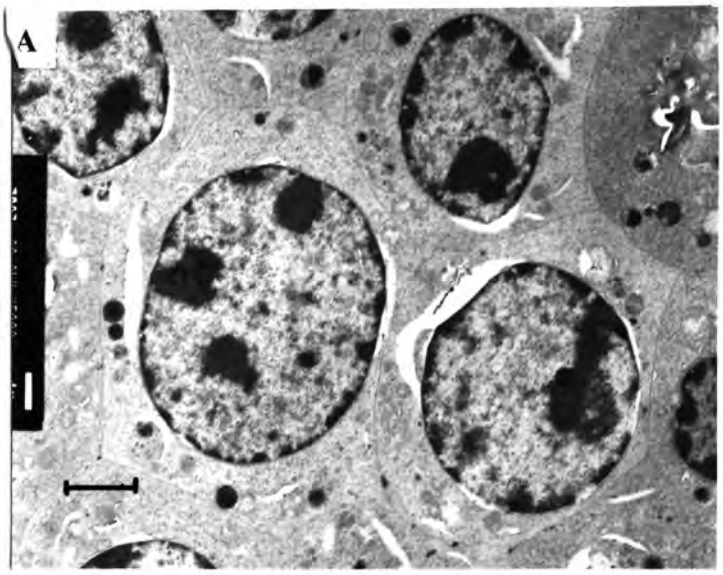
Bar sizes represent 2μM

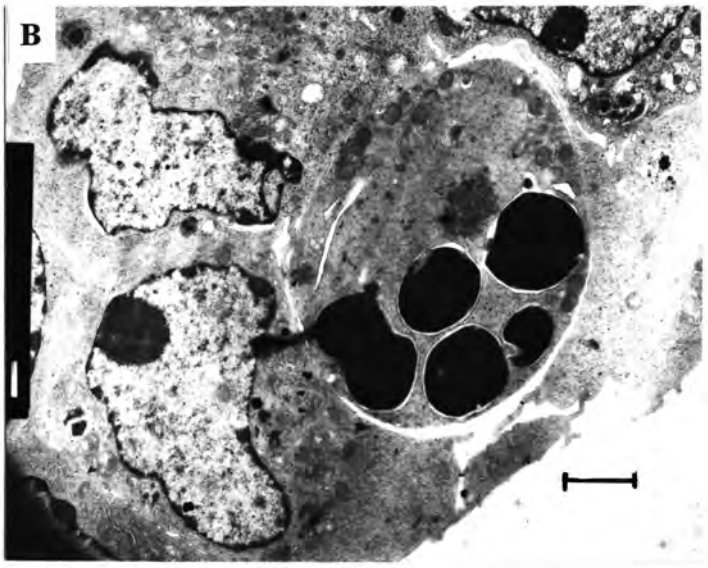
(A) Untreated control cells, note the diffuse appearance of the nuclear chromatin

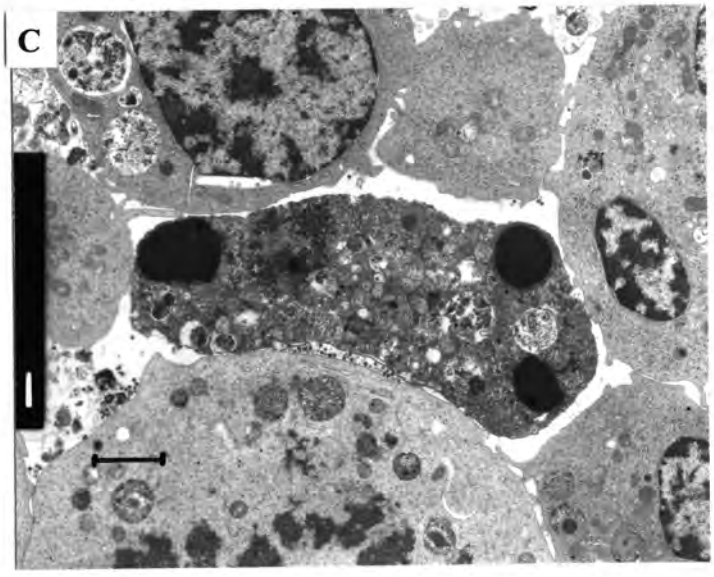
Drug treated cells were exposed to **(B)** 0 1μM methotrexate, **(C)** 0 5μg/ml etoposide.

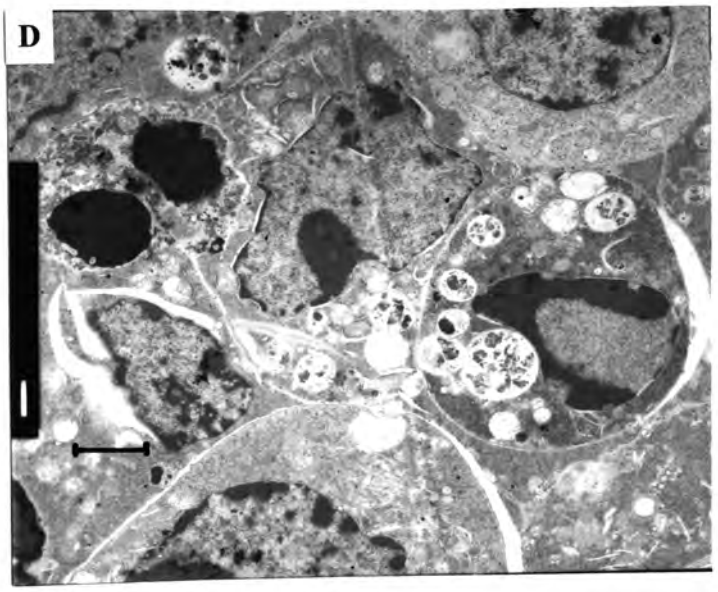
(D) 0 5μg/ml araC and **(E)** 2mM hydroxyurea for 24 hours before anal) sing

Note the highly condensed chromatin identifjing the apoptotic cells


A


B


C


D

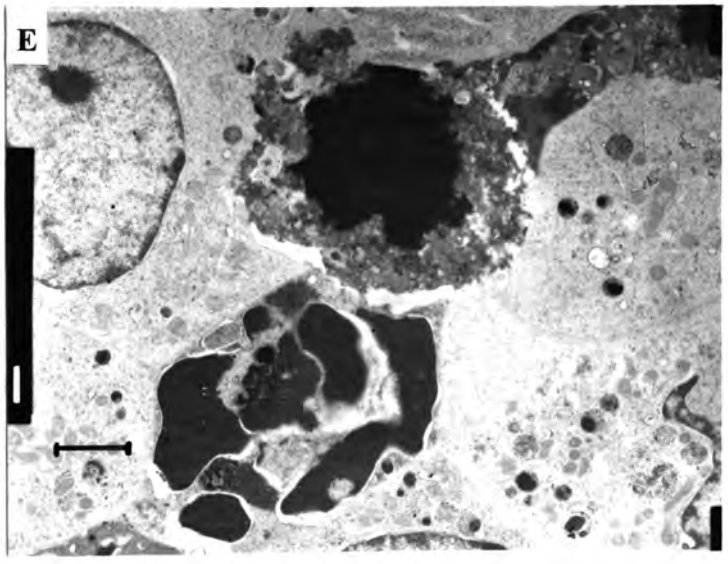

E

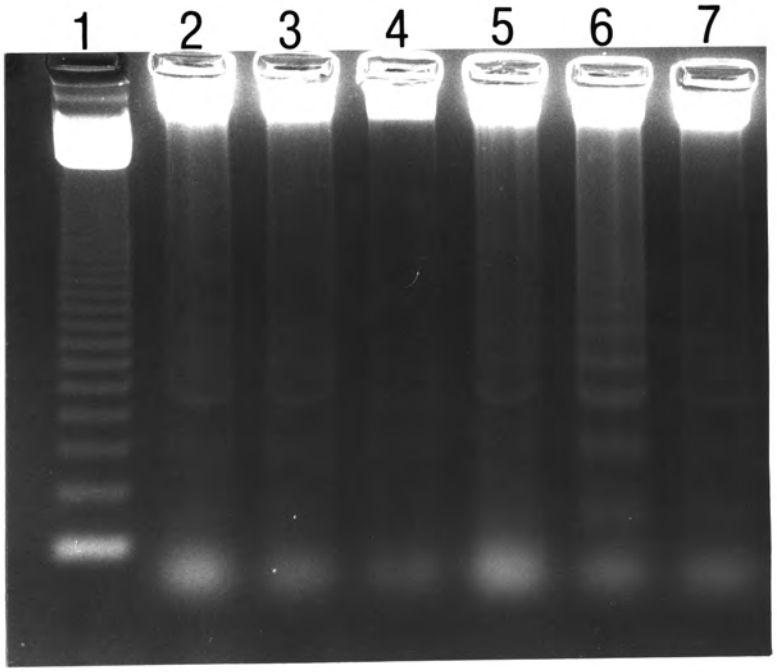
Figure 5.3 Electrophoresis gel showing genomic DNA from FDCP-1-B cells exposed to chemotherapeutic drugs for 15 hours

$3x10^5$ cells were harvested after treatment and analysed as described in materials and methods

Gei lane data

Lane 1	123bp DNA marker
Lane 2	Control untreated cells
Lane 3	Cells treated with 0 5μg/ml araC
Lane 4	Cells treated with 0 1μM methotrexate
Lane 5	0 001% DMSO control
Lane 6	Cells treated with 2mM hydroxyurea
Lane 7	Cells treated with 0 5μg/ml etoposide

*Note the **early** fragmentation of the DNA in response to 2mM hydroxyurea (lane 6) compared to the other drug treated cells (lanes 7.3& 3)*


1
2
3
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5
6
7

Figure 5.4(A) Electrophoresis gel showing genomic DNA from FDCP-1-**B** cells exposed to chemotherapeutic drugs for 30 hours.

3x10^5 cells were harvested after treatment and analysed as described in materials and methods.

Gel lane data:

Lane 1 Control. untreated cells
Lane 2 0.001% DMSO control
Lane 3 Cells treated with 0.5μg/ml etoposide
Lane 4 Cells treated with 0.5μg/ml araC
Lane 5 Cells treated with 0.1μM methotrexate
Lane 6 Cells treated with 2mM hydroxyurea
Lane 7 123bp DNA marker

Note the DNA fragmentation evident in response to etoposide, araC and methotrexate (lanes 3-5 respectively).

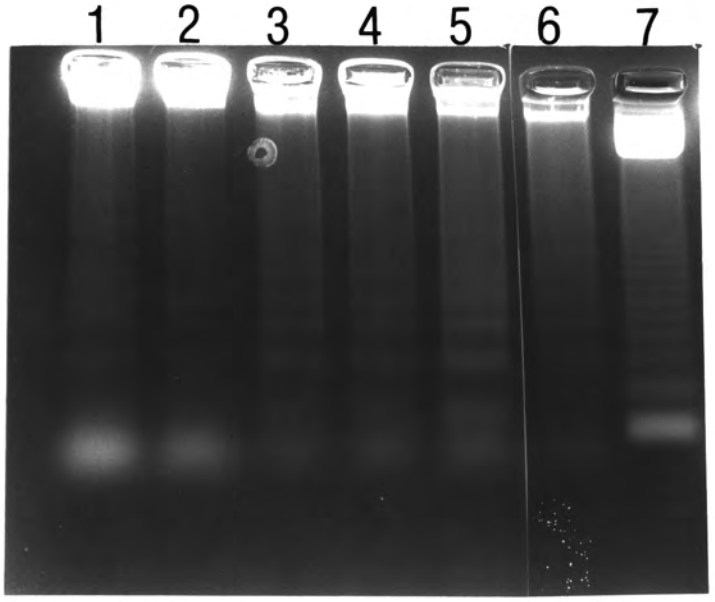
Figure 5.4(B) Electrophoresis gel showing genomic **DNA** from FDCP-1-B cells esposed to chemotherapeutic drugs for **48** hours.

3x10^5 cells were harvested after treatment and analysed as described in materials and methods.

Gel lane data:

Lane 1 123bp DNA marker;
Lane 2 Control untreated cells;
Lane 3 0.001% DMSO control:
Lane 4 Cells treated with 0.5μg/ml etoposide:
Lane 5 Cells treated with 0.1μM methotrexate
Lane 6 Cells treated with 0.5μg/ml araC:
Lane 7 Cells treated with 2mM hydroxyurea.

*Note the less intense **UNA** ladder in cells treated with 2mM hydroxyurea ut **48** hours (lane 7). compared to cells treated with etoposide methotrexate and araC (lanes **4-6).***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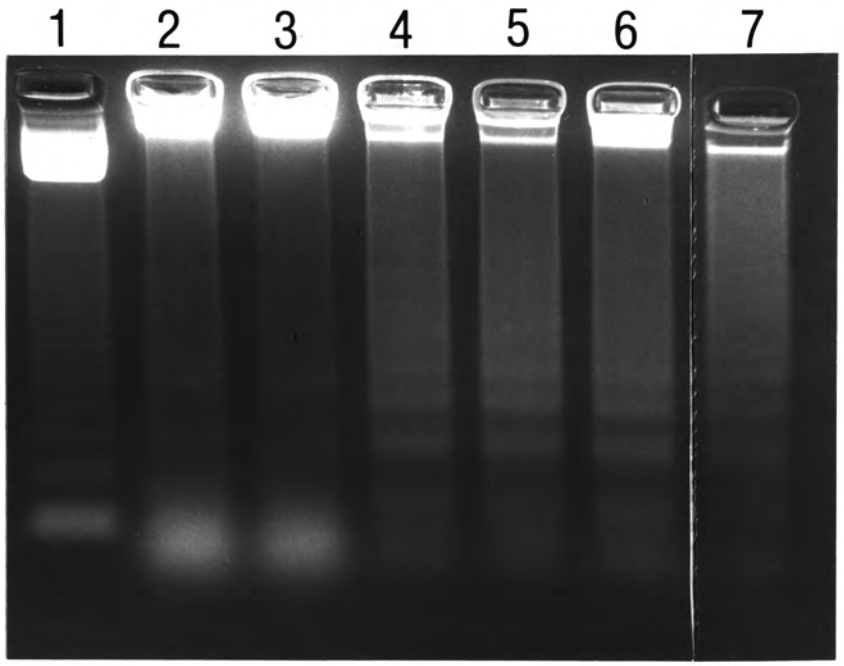
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7

drug exposure when compared to the other drugs (see figure 5.1A and **5.1B)** and may indicate that the level of hydroxyurea used was relatively more potent.

Therefore, in the FDCP-1-B cells both cellular morphology and DNA fragmentation were found to be characteristic of apoptosis, conclusively illustrating that the chemotherapeutic drugs used in this study induced death by apoptosis. These results are in agreement with several papers documenting apoptosis in response to many chemotherapeutic drugs in several different cell lines (Lennon ***et al.,*** 1990; Barry ***et*** *al.,* 1990; Miyashita and Reed 1992; Collins ***et*** *al.,* 1992).

Having established that chemotherapeutic drugs induce apoptosis in the FDCP-1 cells, the ability of *bcl-2* or BHRF1 to prevent apoptosis in these cell lines on treatment with chemotherapeutic drugs was investigated.

Section 5.2 Electroporation of BHRF1 and *bcl-2* into FDCP-1 cells

Both *bcl-2* and its viral homologue BHRF1 were electroporated into FDCP-1 cells to analyse the effect of constant expression of these genes on induction of apoptosis. Since *bcl-2* had been clearly shown to inhibit cell death in the FDCP-1 cell line on **IL-3** withdrawal (Vaux ***et*** *al.,* 1988), analysis of BHRF1 was carried out first.

The EBV gene BHRF1 was one of the first genes to be identified as a homologue of *bcl-2,* exhibiting 25% primary amino acid sequence homology (Clearly ***et*** *al.,* 1986) (see section 3.6.4). In the light of this, albeit limited, homology with *bcl-2,* it was interesting to see if BHRF1 showed some degree of functional homology to *bcl-2* in the FDCP-1 cell lines. Therefore, a plasmid construct containing the BHRF1 open reading frame (designed and produced by Dr. David Huen, Dept Cancer studies, Birmingham) was electroporated into the cells (see section 4.2.3 for details of electroporation protocol and plasmid construct).

After electroporation, cells were plated on soft agar in the presence of the selection antibiotic hygromycin at concentrations of 50μg-10μg per plate. No cell growth was observed at ranges from 50-30μg/plate, but selective clonal cell growth was seen on concentrations between 20 and 10μg/plate (see table 5.2). Colonies representing clones which should have constructs expressing BHRF1 **(A** clones), clones which should have the control plasmid construct (B clones), and "no plasmid" control clones ***(C*** clones) which grew in the presence

Table 5.2

% cloning efficiency of clone-B cell transfectants in the presence of hygromycin

Hygromycin concentration per plate	Number of colonies/plate	Number of live cells plated	% cloning efficiency
20μg	A clones = 4 B clones = 16 C clones = 0	10^5	A = 4×10^{-3} B = 1.6×10^{-2} C = 0
10μg	A clones = 500 B clones = 400 C clones = 8	10^5	A = 0.5 B = 0.4 C = 8×10^{-3}
0μg	A clones = 4135.1 B clones = 7443.2 C clones = 218.0	10^5	A = 4.1 B = 7.4 C = 0.2
17.5μg	A clones = 1558.6 B clones = 2385.1 C clones = 520.0	10^5	A = 1.6 B = 2.4 C = 0.5
0μg	A clones = 4480.4 B clones = 7824.0	10^5	A = 4.5 B = 7.8

The concentrations of hygromycin used above resulted in the selective growth of A and B clones containing plasmid constructs compared to untransfected controls (C). The following clones were harvested from these plates; 20μg plates = A clones A_2 A_3, B clones B_3, B_4; 17.5μg plates = A_β B_β.

of hygromycin were harvested from the plates and grown up in liquid culture. These clones were then re-plated in the presence of hygromycin at the concentration from which they were originally isolated. For example, clone A_2 was isolated from a plate containing 20μg hygromycin and was re-cloned on several plates of the same hygromycin concentration. After two subsequent rounds of re-cloning or maintenance in the presence of 300μg/ml hygromycin in liquid culture, **A** and B clones, isolated from either 20μg/ml or 17.5μg/ml hygromycin plates, (since none or relatively few control cells (C) were able to clone in these concentrations of hygromycin), were analysed for BHRF1 expression by two methods:

(i) The ability of the cells to survive in the absence of IL-3, i.e., identification of a *'bcl-2* like' phenotype.

(ii) Western blot analysis to determine if the BHRF1 protein was expressed.

5.2.1 Analysis by removal from IL-3

If BHRF1 had the ability to function as a molecular suppressor of apoptosis, much like ***bcl-2*** in these cells (Vaux ***et al.,*** 1988), then it is was probable that the cells expressing BHRF1 would show enhanced survival, but no proliferation in the absence of IL-3. Therefore, six of the clones isolated at selective hygromycin concentrations were removed from E-3 and viability was determined by vital dye exclusion, after a **48** hour period. All the **A** clones tested, which in theory may have contained the BHRF1 expressing plasmid, had the same rate of cell death as the B clones, i.e., cells having the control plasmid, and both cell populations **(A** and B) were less viable than a control population of normal FDCP-1 cells, as shown in table 5.3. This suggested that either BHRF1 did not produce the same effect on cell death as *bcl-2,* despite its homology, or the suppressive effect was not as efficient as *bcl-2* and was not evident in the complete absence of IL-3, or finally, although cells were selected and grown in the presence of hygromycin, the cells did not express BHRF1. Expression of the BHRF1 protein within the cells would be clarified by western blotting, so evidence for a less efficient suppressive effect produced by BHRF1 was investigated by analysing cell viability in low levels of **IL,-3.** Previous experiments with FDCP-1 cells illustrated that levels of IL-3 around 0.4% to 0.2% gave enhanced cell survival at **24** hours, but produced a loss in viability at **48**

Table 5.3

% viability +/- S.E. of BHRF1 transfected FDCP-1 cells and controls after 48 hours without IL-3

Cell clone	FDCP1 control	Clone Aβ	Clone Bβ	Clone A_2	Clone A_3	Clone B_3	Clone B_4
with IL3	89.5 +/- 0.23	70.0+/- 2.54	52.9 +/- 2.42	91.3 +/- 1.69	85.7 +/- 1.32	84.8 +/- 2.30	62.2 +/- 3.23
without IL3	4.0 +/- 2.08	3.6 +/- 0.23	0.00	2.3 +/- 0.30	7.2 +/- 0.46	5.1 +/- 1.50	2.1 +/- 1.04

A clones represent FDCP-1 cells transfected with the BHRF1 construct
B clones represent FDCP-1 cells transfected with the control construct

% viability was determined by vital dye exclusion and is equal to the number of surviving cells expressed as a percentage of the total cell number counted.

N.B., loss of viability observed in clones Aβ, Bβ, and B4 incubated with IL-3 is due to a slight overgrowth of the cells and an exhaustion of the IL-3 present.

hours, as shown in figure 5.5. Therefore, all six clones were once again analysed, this time in levels of **IL-3** at 0.2%. The results show that no significant inhibition of cell death was shown in the **A** clones compared to the B clones or the control population of untransfected FDCP-1 cells (see table 5.4).

In the light of the above results, it appears that either BHRF1 does not function like *bcl-2* in these cell lines, or BHRF1 is not being efficiently expressed in these cells. Therefore the clones were analysed by western blotting to determine expression levels of the BHRF1 protein.

5.2.2 Western blotting analysis to detect BHRF1 expression

Western blotting was carried out as described in materials and methods, section 4.2.3. The results obtained illustrated that none of the **A** clones isolated, i.e., those electroporated with the BHRF1 expressing construct, expressed BHRF1 protein at detectable levels, therefore explaining why no inhibition of cell death was seen in these clones on removal of **IL-**3.

Further analysis of these clones would have involved Southern blotting to check that transfection of the plasmid **by** electroporation had resulted in the integration of the BHRF1 gene into the cell's genome, although selection of the cells in hygromycin should have indicated this. However, since time was of the essence and a slightly different BHRF1 expressing plasmid had been transfected into EBV positive Burkitt's lymphoma cell lines by Dr. **S**heila Henderson (Dept. Cancer studies, Birmingham), analysis of the functional homology of BHRF1 to ***bcl-2*** and whether or not both genes were able to suppress chemotherapeutic drug induced apoptosis was transferred to these cell lines (see chapter 6).

Possible reasons why BHRF1 was not expressed in the FDCP-1 cell lines are discussed in chapter 7.

5.2.3 Electroporation of *bcl-2* expressing plasmids

Bcl-2 expressing constructs and controls were electroporated into FDCP-1 cells and these cells were cloned and selected in the presence of the antibiotic geneticin (G418). Electroporation, cell selection in antibiotic and subsequent analysis of selected clones was

Figure 5.5 legend

Displays mean *%*viabilities +/- standard errors (n=3)

% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

Starting cell density = $3x10^5$/ml

1% IL-3 is the concentration which is required to induce both proliferation and survival of **FDCP-1** cells and is used at this level in standard culture conditions. Concentrations of IL-3 used at 0.4% and 0.2% produce limited survival of the cells for a short period of time, delaying the onset of apoptosis when compared to cells in *0%* IL-3.

Figure 5.5 % viability of FDCP-1 cells cultured in decreasing concentrations of IL-3

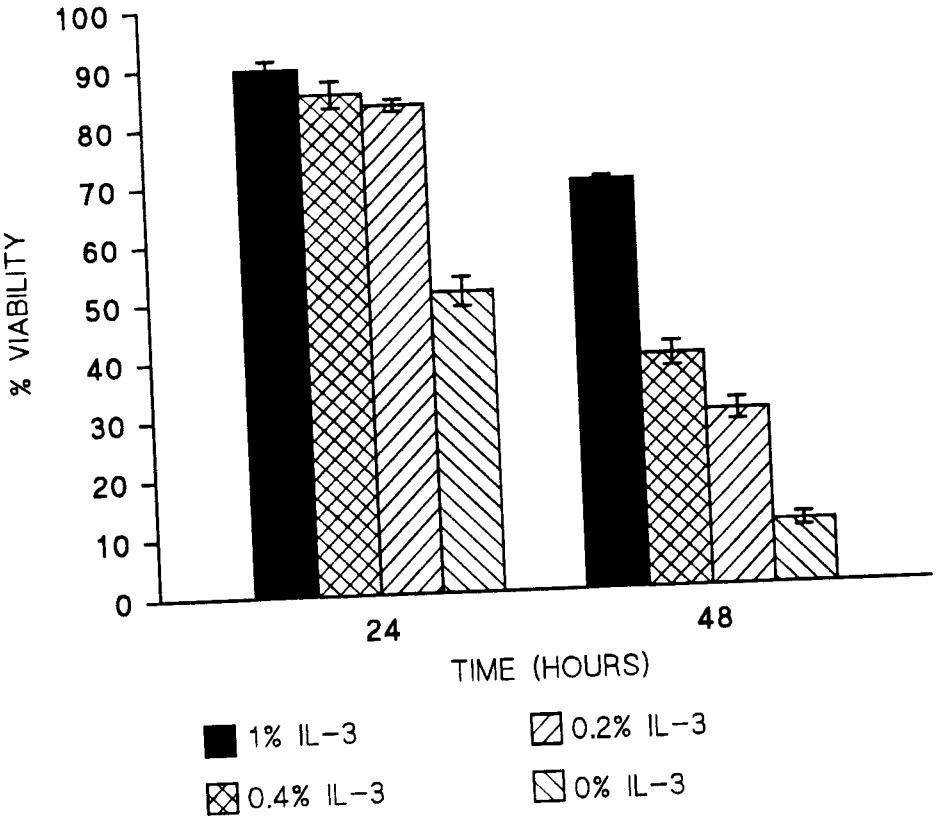


Table 5.4

% viability +/- S.E. of BHRF1 transfectants and controls after 48 hours exposure to 0.2% IL-3

Cell clone	FDCP-1 control	Clone Aβ	Clone A2	Clone A3	Clone Bβ	Clone B3	Clone B4
0.2% IL-3 experiment 1	30.6 +/- 4.04	35.2 +/- 1.04	31.0 +/- 3.70	32.2 +/- 1.67	12.7 +/- 1.39	37.9 +/- 0.80	31.6 +/- 3.23
0.2% IL-3 experiment 2	47.9 +/- 1.80	43.7 +/- 1.20	38.7 +/- 0.40	63.0 +/- 4.40			

A clones represent FDCP-1 cells transfected with the BHRF1 construct
B clones represent FDCP-1 cells transfected with the control construct

% viability was determined by vital dye exclusion and is equal to the number of surviving cells expressed as a percentage of the total cell number counted.

N.B., The difference in viability observed between the FDCP-1 control and clone A_3 in experiment 2 is not statistically significant ($P > 0.05$) ($n=4$)

Table 5.5

% viability +/- S.E. of *bcl-2* transfectants and controls after 48 hours without IL-3

Cell clone	FDCP-1 cells without IL-3	FDCP-1 bcl-2 - X_1 without IL-3	FDCP1-cΔj - Y_1 without IL-3
% viability	30.5 +/- 1.21	10.4 +/- 1.27	1.9 +/- 0.98

% viability was determined as described above from 3 replicate counts, which were representative of repeat experiments.

N.B., Although there is a significant difference between the bcl-2 expressing clone X_1 and the control transfectant Y_1, ($P<0.01$, $n=4$), the survival of the clones is less than the control population of FDCP-1 cells and this would not be a predicted out come in these cell lines when expressing exogenous bcl-2

carried out as described for BHRF1 transfected cells. Although expression of ***bcl-2*** was known to produce enhanced survival of FDCP-1 cells on **IL-3** withdrawal (Vaux ***et al.,*** **1988) this** was not observed in the clones which had been selected for plasmid possession in the presence of **G418** (see table 5.5). Therefore, it appeared that *bcl-2* was not expressed using the Tsujimoto construct in these cells.

Expression of *bcl-2* in the FDCP-1 cell lines by Vaux *et al., (1988)* was produced using a retroviral construct and may have produced much better integration of *bcl-2* into the host cell genome and hence stable *bcl-2* expression. The Tsujimoto construct, used in the present study, is based on an EBV plasmid construct which in cells infected with EBV allows the plasmid to be maintained and transcribed in an episomal form, without integration into host cell **DNA.** Therefore, expression of the gene of interest can occur directly from the plasmid and so does not have the added complication of stable integration of the transfected gene in a suitable area of the genome as occurs with normal plasmid constructs. Hence, expression of the gene is more likely to occur using an EBV construct within an EBV infected cell line (Tsujimoto **1989).** These points will be discussed further in chapter 7.

Section 5.3 Development of a protocol for isolating apoptotic mutants of FDCP-1 cells

Withdrawal of **IL-3** from FDCP-1 cells results in a very low cell viability (–5% on average) by 72 hours as determined by vital dye exclusion. However, a very small, but identifiable set of cells do not become dye permeable, even when **E-3** has been absent for 7 days. These cells may simply be cells which have died, but not lost membrane integrity. However, the cells do not appear to have progressed through apoptosis since they maintain a viable cell appearance, there being no marked change in the cytoplasm and no ruffling of the cell membrane. This suggested the possibility that these cells were viable and unable to die on **IL-3** withdrawal, either because they were factor-independent, or because they were blocked from entering apoptosis. Since FDCP-1 cells are easily cloned in soft agar at low cell concentrations, i.e., <1000 cells per plate, it was plausible that these cells, if actually alive, could be reclaimed from culture after 7 days without **IL-3** by cloning on soft agar medium containing **IL-3.** Any surviving clones could then be harvested from the plates and the

phenotype of these cells could be identified. The clones isolated from these plates were predicted to be of two phenotypes:

(i) Cells which are **IL-3** independent, i.e., are able to secrete their own **IL-3** or short circuit the need for **IL-3** stimulation and hence survive and proliferate in the absence **of** the cytokine (Cook ***et al.,*** 1985; Cleveland ***et al.,*** 1989). These were expected to appear more frequently since FDCP-1 cells are thought to develop **IL-3** independent growth during normal prolonged tissue culture (Askew ***et al.,*** 1991).

(ii) Cells which have a mutation in the apoptotic pathway and cannot enter apoptosis on **IL-3** withdrawal and hence are unable to either grow or die in the absence of **IL-3,** but maintain a G_0 survival state, much like FDCP-1s expressing transfected *bcl-2.*

Three populations of FDCP-1 cells were used throughout the development of the protocol, the FDCP-1s themselves and two subclones, FDCP-1-B and FDCP-1-δ. FDCP-1 cells show asynchronous levels of death on **IL-3** withdrawal (N.J. McCarthy, B.Sc. thesis 1990) and over a prolonged period of time in culture can develop factor independent cells (Elaine Spooncer, personal communication; Askew ***et al.,*** 1991). FDCP-1-**B** cells (isolated by limiting dilution cloning of normal FDCP-1 cells) were used because they have a more rapid rate of cell death on **IL-3** removal when compared to FDCP-1s (O-15% after **48** hours compared to **O-30%** for FDCP-1) and were thought to be less likely to develop factor independent cells. FDCP-1-δ was isolated from an FDCP-1 cell culture which had been deprived of **IL-3** for 7 days and subsequently cloned on soft agar containing **IL-3.** Although it seemed plausible that this clone may have a longer survival period in the absence of **IL-3** than normal FDCP-1 cells, FDCP-1-δ showed no enhanced survival when analysed over a period of **72** hours (see table 5.9).

The number of cells able to survive in the absence of **IL-3** for 7 days was determined for each of the FDCP-1 cell populations by soft agar cloning. Although the FDCP-1 cells clone efficiently in agar, if increasingly greater numbers of live cells are plated, then this eventually causes a loss in colony formation efficiency due to a lack of **IL-3** (see figure **5.6).**

Figure 5.6 legend

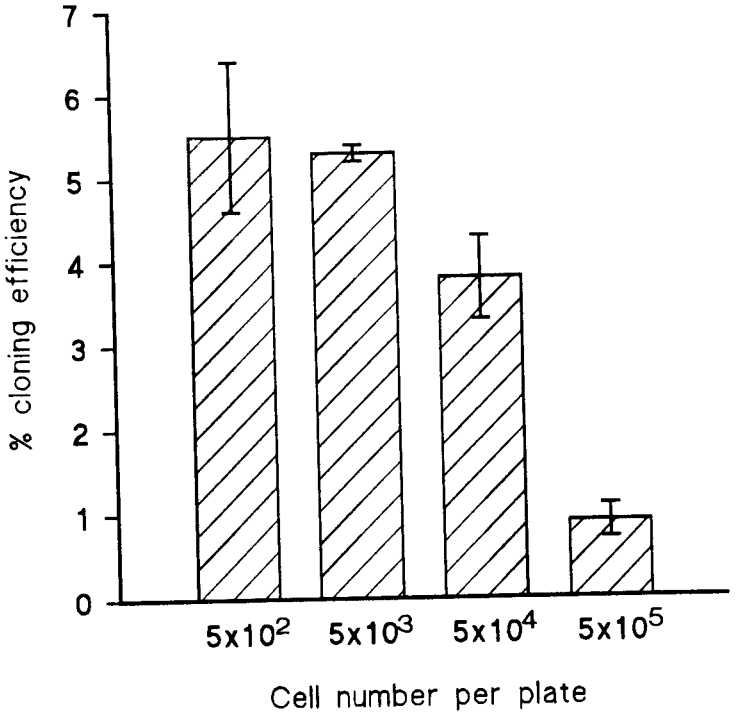
Displays mean Y_0 cloning efficiency +/- standard errors (n=3)

%cloning efficiency is expressed as the number of colonies with > 40 cells present on the plate divided by the number original number of cells present per plate (**5x**10^5 per plate).

N.R. Note the decreasing cloning efficiency seen as the concentration of live cells per plate increases.

Figure 5.6 % cloning efficiency of increasing concentrations of viable FDCP-1 cells

(n=3)



Therefore, cells were never plated above $5x10^5$ cell per plate. After incubation without **IL-3** for 7 days numbers of dead and live cells were ascertained by vital dye exclusion and then cells were either plated directly on to agar plates i.e., both live and dead cells together, or the cells were purified, using a nycodenz gradient in order to separate dead cells from any live cells. The second method was used in case a large number of dead cells present on the plate inhibited efficient colony formation. In order to check that a low level of live cells could be efficientlyisolated from a large number of dead cells, **1000** live cells were added to $5x10^6$ dead cells prior to separation of viable cells by centrifugation through a nycodenz gradient and plating in soft agar. Separation of the added live cells from the dead cells and recovery of the added live cells from the agar plates was found to be efficient, as demonstrated in table 5.6. Therefore, any live cells present after 7 days without **IL-3** should be isolated from the large numbers of dead cells by gradient purification. However, some cells could show a reduction in cell size after 7 days without **IL-3** (as is seen in ***bcl-2*** transfected cells on prolonged **IL-3** withdrawal), producing **a** change in buoyant density, causing some live cells to be lost during gradient separation. Therefore, dead and live cells were also plated out without prior purification. **A** drop in cloning efficiency may arise due to the presence of the dead cells, so to investigate this 1000 live cells were added to plates containing increasing numbers of dead cells. The results, shown in figure **5.7,** illustrate that the presence of dead cells does interfere slightly with live cell cloning efficiency, the average cloning in the presence of dead cells being **2-3%,** as compared to 7 +/- **1.9%** efficiency normally. However, a consistent reduction in plating efficiency was seen whether live cells were plated with 500 dead cells as a background or $5x10^5$ dead cells and therefore a useful number of viable clones can still be isolated using this method, even when a large number of dead cells are present.

The numbers of cells able to survive 7 days without **IL-3** and subsequently clone are shown in table 5.7 and do not appear to be very high, i.e., there doesn't appear to be a large number of spontaneous survival mutants from which it will be difficult to identify the less frequent apoptotic mutants. In order to determine which clones were apoptotic mutants as opposed to **IL-3** independent cells, the differences between the cell cycle states of the two populations in the absence of **IL-3** was exploited.

Table 5.6

% cloning efficiency of 1000 live cells separated from $5x10^6$ dead cells			
	FDCP-1 cells	**FDCP-1 clone-B cells**	**FDCP-1 δ-clone cells**
$5x10^6$ dead cells + 1000 live cells per plate	131.7 +/- 13.4%	62.7 +/- 26.29%	114.84%
Number of replicates	(n=7)	(n=6)	(n=2)

N.B., cloning efficiency is expressed as a % of replicate control plates representing 1000 live cells purified on a nycodenz gradient in the absence of dead cells. In two cases, FDCP-1 and FDCP-1-δ, colony formation in the presence of $5x10^6$ dead cells was higher than that of 1000 live cells in the absence of dead cells and since the latter was used as 100% efficiency this gives colony formation as >100%.

Figure 5.7 legend

Displays mean % cloning efficiency +/- standard errors (n=3)

% cloning efficiency is expressed as the number of colonies with > 40 cells present per plate, divided by the number of live cells originally plated i.e., 1000

Control plate (1000 live cells with no background of dead cells) cloning efficiency = 7 +/- **1.9%**

Figure 5.7 % cloning efficiency of 1000 live cells plated on increasing concentrations of dead cells.

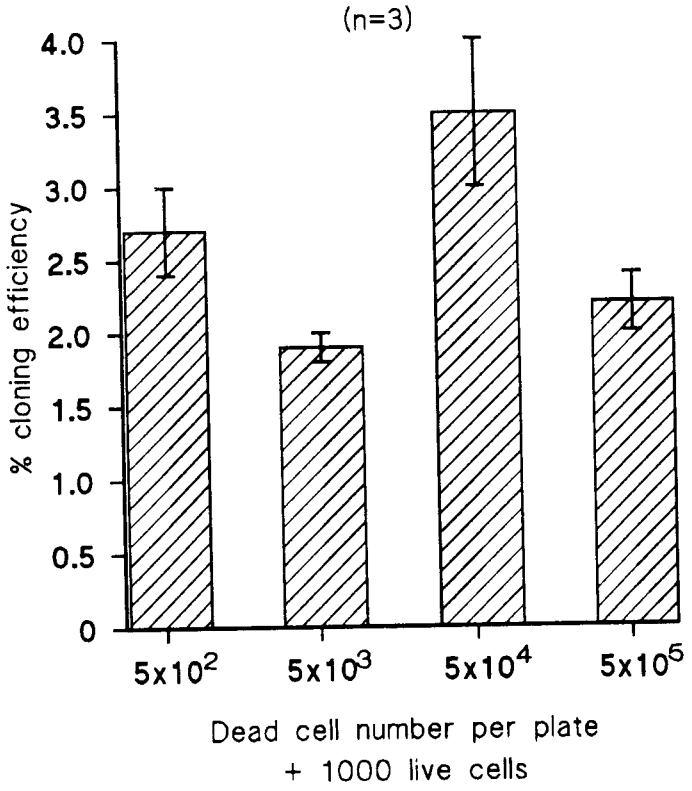


Table 5.7

Rate of cell survival after 7 days without IL-3 as determined by soft agar cloning

	FDCP-1 cells	FDCP-1 clone-B cells	FDCP-1 δ-clone cells
$5x10^5$ dead cells per plate	6.4×10^{-6}	4×10^{-6}	2×10^{-5}
$5x10^6$ nycodenzed dead cells per plate	6.4×10^{-6}	2.6×10^{-6}	1.3×10^{-5}

Table 5.8

Rate of cell survival after full selection protocol

FDCP-1-B cells	FDCP-1 -δ cells
4×10^{-7}	1.93×10^{-5}

The rate of cell survival was determined by dividing the number of isolated colonies by the total number of cells analysed i.e., 10 survivors from 2 populations subjected to nycodenz purification and subsequent plating is divided by 10^7 cells, the number of cells originally incubated in the absence of IL-3 for 7 days. The fact that on average cloning efficiency for 1000 cells per plate is ~5% is also accounted for in the calculation, so that the number is multiplied by 20 to get the survival rate for the population as a whole.

Results: IL-3 dependent cells

The fundamental difference between the two isolated cell types is that one will proliferate and hence be in cell cycle in the absence of IL-3 (IL,-3 independent cells) and one will not (apoptotic mutants, predicted to maintain a G_0 state). Since it was the latter cell population that were of interest in this study, the former population of cells were eliminated by selection in an S-phase specific drug. This was carried out by using a standard tritiated thymidine (H^3TdR) suicide protocol based on the selection of mutant yeast (Thompson *et al.*, 1970) (see section **4.2.4.2).**

In order to obtain a high level of cell kill by H^3TdR, normal FDCP-1 cells, growing in IL-3, were incubated for 72 hours with differing levels of H^3TdR. A 72 hour incubation period with H^3TdR should ensure that all cells progress through the celi cycle to and reach S-phase where death due to the presence of H^3TdR should occur. Viability, after treatment was determined initially by vital dye exclusion and more stringently by colony formation in soft agar. Results are shown in figure **5.8** and illustrate that H^3TdR at $2\mu Ci/ml$ produced a cell kill of **80%,** only leaving 20% of surviving cells to be re-selected, therefore, $2\mu Ci/ml$ was used in the final protocol.

5.3.1 Implementation and testing of the full protocol

After all the preliminary experiments described above, the full selection protocol was constructed and used as shown below:

The required number of cells (between 10^7 and $2x10^7$) were removed from culture and washed free of IL-3 by two washes in RPMI supplemented with 10% FCS and 2mM L-glutamine.

↓

After the second wash and pelleting, cells were resuspended in **5ml** of growth medium without IL-3 and viability and cell number were determined.

↓

After readjustment to $2x10^6$ cells per **mi, 5ml** of the cell suspension was added to 15 **mls** of warmed, pregassed growth medium without **IL-3** in a $75cm^2$ tissue culture flask. The cells were then incubated for **4** days at 37°C with 5% CO_2.

↓

Figure 5.8 (A) legend

FDCP-1 cells were treated with H^3TdR in the presence of IL-3 for 72 hours, before removing from the H^3TdR and cloning in soft agar containing IL-3. % colony formation is expressed **as** the number of colonies per plate, divided by the number of colonies counted per plate from a control, untreated **FDCP-1** cell population

All plates were plated with a starting cell density of $5x10^5$ per plate.

Figure 5.8(B) legend

The % viability of the **FDCP-**1 cell population after 72 hours treatment with H^3TdR in the presence of IL-3 was determined by vital dye exclusion prior to plating on soft agar (as described in figure 5.8(A)).

This graph represents the viabilities of the cells which were subsequently plated giving the results shown in figure **5.8(A).** % viability is expressed as number of viable cells divided by the total number of cells counted.

N.B. 2μCi/ml H^3TdR induced ~80% cell kill of cells proliferafing in the presence ofII>-3.This concentration was chosen-for use in the final protocol to eliminate IL-3 independent cells able to proliferate in the absence qf IL-3.

Figure 5.8(A) % colony formation, as determined by soft agar cloning, for FDCP-1 cells treated with increasing concentrations of tritiated thymidine. (n=3)

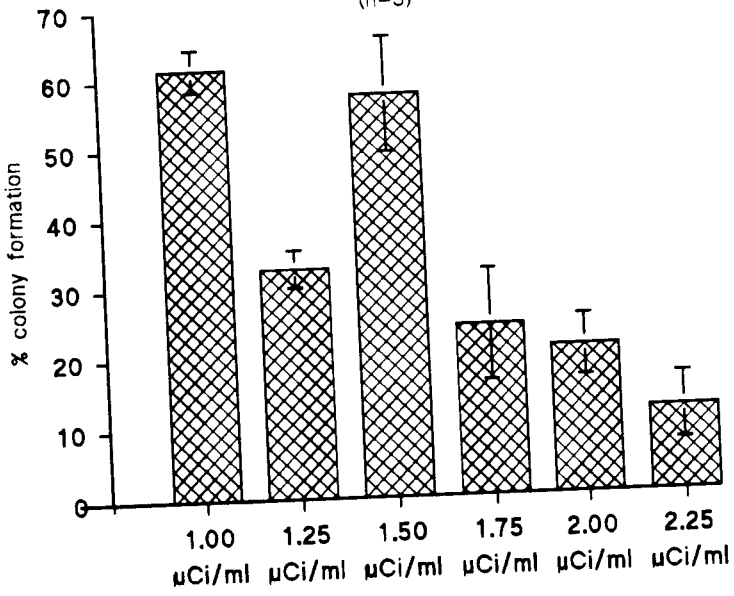
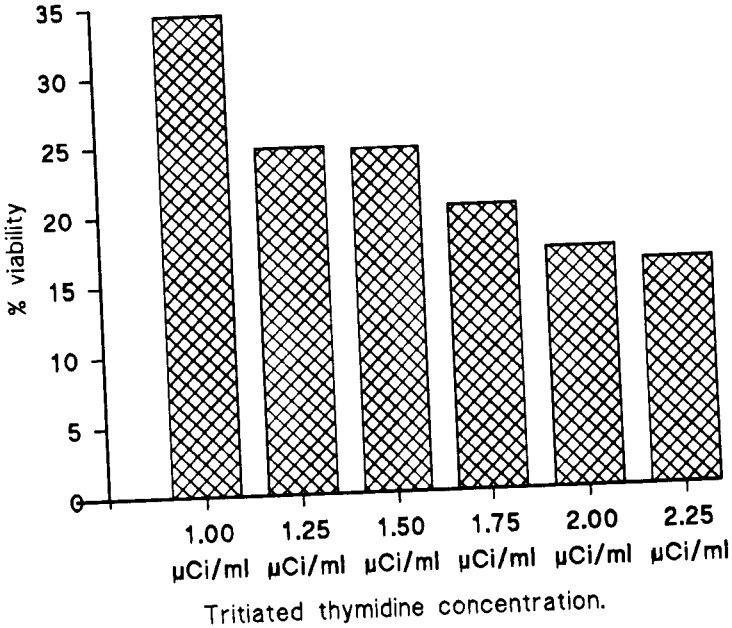


Figure 5.8(B) % Viability, as determined by vital dye exclusion, for FDCP-1 cells treated with increasing concentrations of tritiated thymidine.



Results: IL-3 dependent cells

After 4 days cell viability was determined by vital dye exclusion (on average <1% viability) and cells were purified on nycodenz gradient according to the normal protocol (see materials and methods).

↓

After purification, cells harvested from the medium/nycodenz interface were resuspended in 200µl of growth medium without IL-3 and cell numbers, both live and some dead, were determined (normally between $2x10^5$- $5x10^5$ cells). Cells were then added to 1ml cultures containing 2µCi/ml H^3TdR, again with no IL-3, and were incubated under these conditions for a further 3 days, bringing the total number of days without IL-3 to 7.

↓

Cell number and viability were determined, initially by vital dye exclusion and then by plating in soft agar containing IL-3. Cells which reached this stage normally came to less than $5x10^5$, so only one plate was generally needed. The plates, including live cell control plates to monitor cloning efficiency, were gassed and boxed. Numbers of colonies with greater than 40 cells were scored after 14 days.

Table 5.8 shows the number of FDCP-1-B cells and FDCP-1-δ cells which grew after selection in the above protocol. The results are representative of 5 replicate experiments with the total number of cells having been screened equalling $70x10^6$ for each cell type. The levels of cells surviving the selection protocol may be too low to obtain spontaneous mutants which exhibit a block in the apoptotic pathway, i.e., these rates represent between one and ten colonies formed per seventy million cells. Although some of these colonies were harvested from the plates, they very rarely grew well once placed in liquid culture, suggesting that the clones were non-viable.

To increase the number of survival mutants, the possibility of mutating the FDCP-1 cells using insertional mutagenesis was considered and subsequently carried out in collaboration with Dr. Farzin Farzaneh (Rayne Inst., London). This could increase the numbers of mutants by 10 to 20 fold. Although FDCP-1-B cells have now been successfully mutated using this method, they were not available in time for analysis in the protocol.

Further developments and refinements of this protocol will be discussed in chapter 7

5.3.2 Analysis of clones obtained from plates after 7 days without IL-3

Clones which were isolated after 7 days without IL-3 were analysed to see if they were either factor independent or candidate apoptosis mutants. The clones were removed from **IL-3** and left for 72 hours before determining viability and were compared to a normal population of FDCP-1-δ and FDCP-1-B cells. Clones which were **IL-3** independent will grow by definition in the absence of IL-3 and therefore an increase in cell number would be expected over the 72 hour period. If a clone was specifically blocked from entering apoptosis, then a higher viability compared to normal FDCP-1 cells, but no increase in cell number, would be expected during the period of IL-3 withdrawal. No enhanced survival of the isolated clones was seen compared to normal FDCP-1-B and **-δ** cells, see table 5.9. FDCP-1 and FDCP-mix cells which express ***bcl-2*** generally lose viability slowly over a period of 2-3 weeks, with viability being around 50-70% after 8 days (Vaux ***et al.,*** 1988; Fairbairn et al., 1993), a result which was never observed in these clones on IL-3 withdrawal. This suggests that the cells which are able to survive without **IL-3** for 7 days, as judged initially by vital dye exclusion, do not necessarily represent cells which have either become factor independent or cannot enter apoptosis, but may represent one of the heterogeneous cell phenotypes found within the FDCP-1 cell population. Sub-clones of FDCP-1 cells which show enhanced survival have been obtained previously by limiting dilution cloning without removing the IL-3 (N.J. McCarthy, B.Sc. thesis 1990). These FDCP-1 sub-clones exhibited enhanced survival over 72 hours when compared to parental FDCP-1 cells, but this effect was lost over a period of **3-4** weeks in normal culture conditions (see chapter 7 for further discussion).

Table 5.9

% viability +/- S.E. of 'survivor clones' removed from IL-3 for 72 hours

Cell clone	δ-Clone	Clone B	Survivor 1	Survivor 2	Survivor 3	Survivor 4	Survivor 5
experiment 1	5.1 +/- 0.75	8.9 +/- 1.80	26.8 +/- 3.33	19.5 +/- 1.86	17.4 +/- 2.72	10.3 +/- 1.35	28.2 +/- 2.69
experiment 2		14.3 +/- 2.81	10.7 +/- 0.42	14.0 +/- 2.66	11.9 +/- 0.10	12.4 +/- 1.91	24.1 +/- 0.58

% viability was determined by vital dye exclusion and is equal to the number of surviving cells expressed as a percentage of the total cell number counted.

N.B., Survivor clones represent cells which were cloned on soft agar after FDCP-1-B cells had been cultured in the absence of IL-3 for 7 days. Although higher rates of survival are evident for the survivors in experiment 1 compared to FDCP-1-B and FDCP-1-δ, and for survivor 5 in experiment 2, these were not of the levels one would expect for either factor independent cells (which should proliferate in the absence of IL-3) or for cells with a 'bcl-2 like' phenotype.

Chapter 6

Results: Burkitt's lymphoma cells

Section 6.1 Introduction

Analysis of the effects of ***bcl-2*** and BHRF1 on drug induced apoptosis were investigated using several EBV genome positive BL cell lines which had been transfected with plasmid expressing either BHRF1 or ***bcl-2*** (Henderson ***et al.,*** 1991, 1993; Tsujimoto, 1989).

EBV positive Burkitt's lymphoma **is** primarily a childhood lymphoma endemic in areas defined by the African malaria belt. The lymphoma is highly aggressive, but very sensitive to treatment with chemotherapeutic drugs (Calvalli 1991), possibly due to its origin in the germinal centre and/or the presence of a deregulated c-myc gene produced as a result of the t(8;14) translocation. Several cell lines were established from biopsies, primarily from African children, and have proved very useful as ***in vitro*** models of EBV-BL (Epstein 1985).

Three BL-cell lines have been used as models for drug induced apoptosis in this study, two of which, Cheptages (Rooney ***et al.,*** 1986) and Akata (Takada and Ono 1989), are representative of group **I** cell lines, which are sensitive to apoptosis, and Raji-BL cells (Rymo ***et al.,*** 198*1)* which exhibit some of the group III cell line characteristics, but retain group I like sensitivity to apoptosis. Group I cell lines express only one EBV latent gene, EBNA 1, where as the group III cell lines express all eight EBV latent genes, EBNAs 1, **2,** 3a, 3b, 3c, LP and ***LMP*** 1 and ***2*** (Gregoy ***et aí.,*** 1990). Group III cell lines are thought to be resistant to apoptotic stimuli due to the up-regulation in expression of host cell ***bcl-2*** (Henderson ***et al.***, 1991). However, Raji-BL cell lines do not express ***bcl-2,*** possibly due to point mutations within the LMP 1 gene (Hatfull ***et aí.,*** 1988), which has been proposed to facilitate the up-regulation of host cell ***bcl-2*** in EBV-BL cell lines (Henderson ***et al.,*** 1991; **S.** Henderson, personal communication) and therefore remain sensitive to apoptosis.

BL cell lines latently infected with EBV have circular forms of the EBV genome present i.e., episomes. EBNA 1 is the only gene from the EBV genome which needs to be expressed during latency in order to maintain the episomal state by binding to and activating

the latency origin of replication. This can result in the expression of genes from the episome without prior integration within the host cell **DNA** (Rodgers ***et al.,*** 1992). The construct used for the **BHRF1** expressing plasmid in these cells **was** based on the pHebo vector which can be episomally maintained in the presence of **EBNA** 1 (Henderson ***et al.,*** 1993). Therefore, because the plasmid does not need to integrate within a suitably active expressed region of the host cell genome in order to express the gene of interest, the chance of attaining cells expressing the gene of interest is much increased. The ***bcl-2*** construct used in these cells (Henderson ***et al.,*** 1991) **is** also an EBV based vector (Tsujimoto 1989) and hence the same arguments apply regarding episomal gene expression.

Cells transfected with either BHRF1 or ***bcl-2*** show no significant increase in cell cycle time when compared to control transfectants, as shown in table 6.1.

Although preliminary work illustrated that chemotherapeutic drugs induced apoptosis in the **FDCP-**1 cells (see section **5.** 1), this had not been established for the EBV-BL cell lines used in the present study. Therefore, the cell lines were exposed to three chemotherapeutic drugs; methotrexate, etoposide and araC.

Two different protocols for exposing the cells to the drugs were used; cells were either exposed to the drug for a limited period of **14** hours after which the cells were washed and incubated in normal growth medium for a further **48** hours before determining viability, or cells were constantly incubated in the presence of the drug at slightly lower concentrations than those used for the **14** hour exposure period.

Section 6.2 Induction of apoptosis by chemotherapeutic drugs and y radiation

To investigate whether the BL cell lines underwent apoptosis on treatment with cytotoxic drugs, morphological analysis was carried out by electron microscopy for all three celi lines, by acridine orange fluorescence in Akata-BL and Raji-BL cells and also by Giemsa staining in the Raji-BL cells alone.

Morphological changes characteristic of cell death by apoptosis were seen in treated cells and are shown unequivocally in the electron micrographs.

Table 6.1

Cell cycle times of BL cells transfected with either BHRF1 or *bcl-2* compared to controls

	Chep-BL		Raji-BL		Akata-BL	
	cΔj control transfectant	**bcl-2 transfectant**	**hebo control transfectant**	**BHRF1 transfectant**	**hebo control transfectant**	**BHRF1 transfectant**
Cell cycle time (Hours)	31.6 +/- 7.4	29.0 +/- 6.7	24.7 +/- 3.9	22.6 +/- 3.2	25.3 +/- 3.5	23.1 +/- 1.9
Significance	p<0.1 N.S		p<0.1 N.S		p<0.1 N.S	

N.B., There was no significant difference in cell cycle times between expressors and non expressors.

Results: Burkitt's lymphoma cells

Untreated control Raji-BL cells **(A)** and drug treated cells are shown in figure 6.1 with the condensed chromatin, a characteristic marker of apoptotic cells, being clearly visible in cells treated with 10μM methotrexate **(B),** 1μg/ml etoposide (C) and 1μg/ml araC (D). The induction of apoptosis in response to ionising radiation which has been documented for several cell lines (Sellins and Cohen, 1987; Yamada and Ohyama **1988;** Collins ***et al.,*** 1992), was confirmed for these Burkitt's cell lines. Raji-BL cells were exposed to 16Gy of y radiation and morphology was examined 24 hours after treatment. Figure 6.1(E) illustrates the apoptotic morphology evident at this time point.

Similar apoptotic morphology was observed in drug-treated Akata-BL cells (figure 6.2B) in response to 1μg/ml etoposide and in Chep-BL cell lines in response to 5μM methotrexate **(D),** 1μg/ml etoposide **(E)** and 1μg/ml araC (F). Cells treated with araC often showed additional degradative changes indicating cell damage which could either be independent of the process of apoptosis, or could represent early onset of secondary necrosis (to be discussed further in chapter 7).

Fluorescence microscopy can also be very useful when identifying apoptotic morphology, as well as providing an alternative method for counting live vs. apoptotic cells. Both Raji-BL and Akata-BL cells were examined for apoptotic morphology by staining with the fluorescent dye acridine orange. The different appearance of apoptotic and morphologically normal cells is clearly shown in figure ***6.3.*** Control untreated Raji-BL cells **(A)** show the bright diffuse orange staining of morphologically normal cells with intact **DNA** when compared to Raji-BL cell lines treated with the chemotherapeutic drugs (B, C, D) or y radiation (I) in which the condensed, fragmented green fluorescing **DNA** is evident as a marker for apoptotic morphology. Akata-BL cell lines also demonstrate the differing morphology in response to either 100μM methotrexate (F) or 1μg/ml etoposide **(H).**

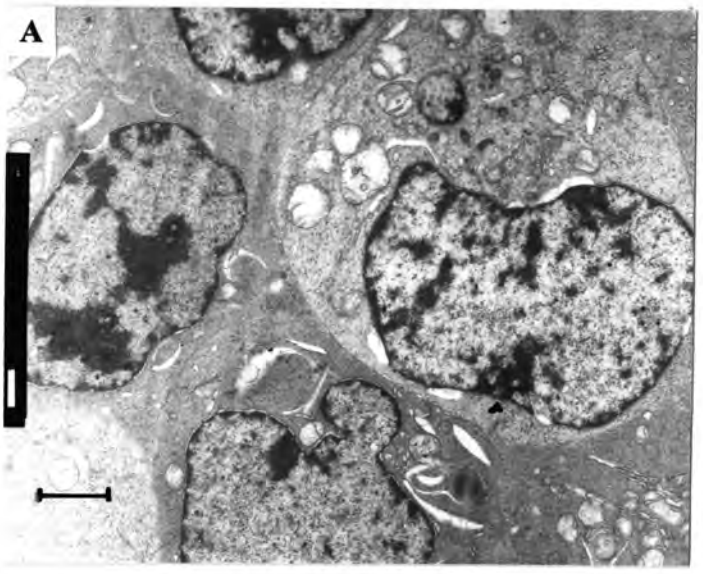
For fùrther illustration of apoptosis in response to chemotherapeutic drugs in these cell lines a third method of cell staining was used. Raji-BL cells were stained with Giemsa (see figure 6.4) which stains normal nuclei a faint pink with purple nucleoli and apoptotic nuclei dark purple, which appear as condensed and fragmented masses within the apoptotic cells.

Figure 6.1 Electron micrographs showing induction of apoptosis in Raji-BL cells treated with chemotherapeutic drugs and y radiation

Legend

Bar sizes represent 2pm

(A) Untreated control cells, note the regular appearance of the cytoplasm and normal nuclear chromatin state Drug treated cells were exposed to **(B)** 10μM methotrexate, **(C)** 1μg/ml etoposide and **(D)** 1μg/ml araC, for 14 hours and subsequently grown at 37°C free of drug for 24 **hours** before morphological analysis Apoptotic morphology **is** clearly shown in all drug treated cells **by** the condensation and margination of the chromatin around the nuclear membrane **(E)** Cells treated with **16Gy** of y radiation again showing the margination of chromatin at the nuclear membrane, indicative of apoptosis, 24 hours post irradiation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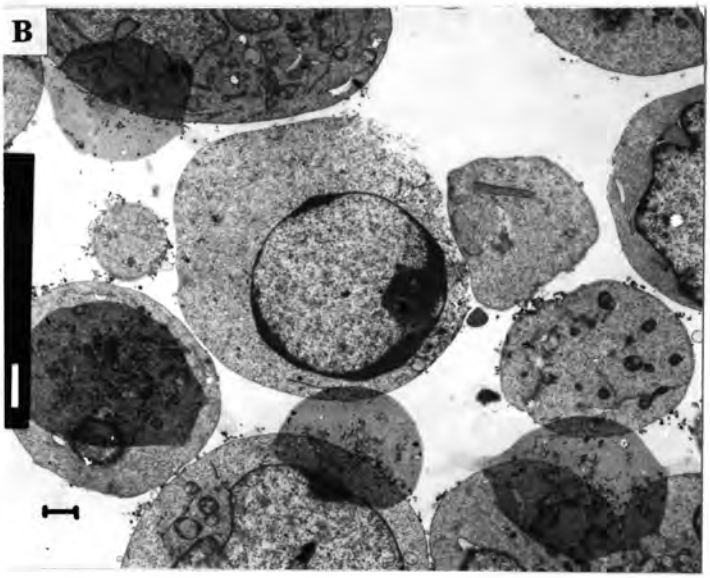
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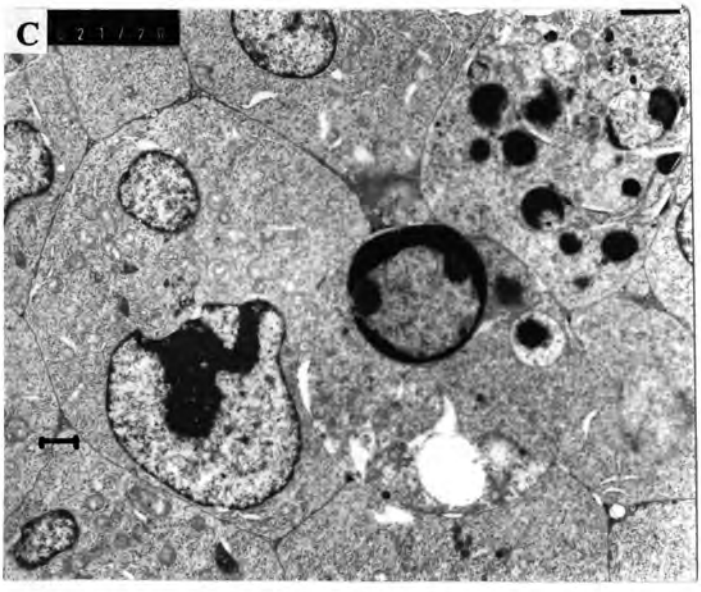
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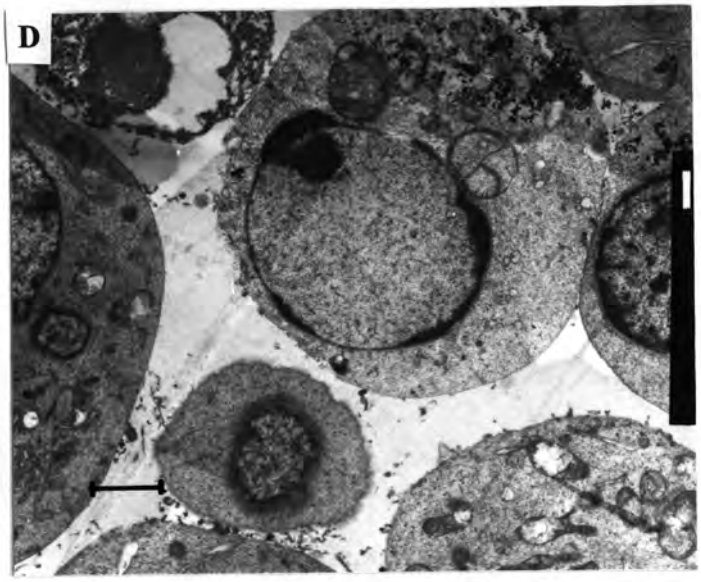
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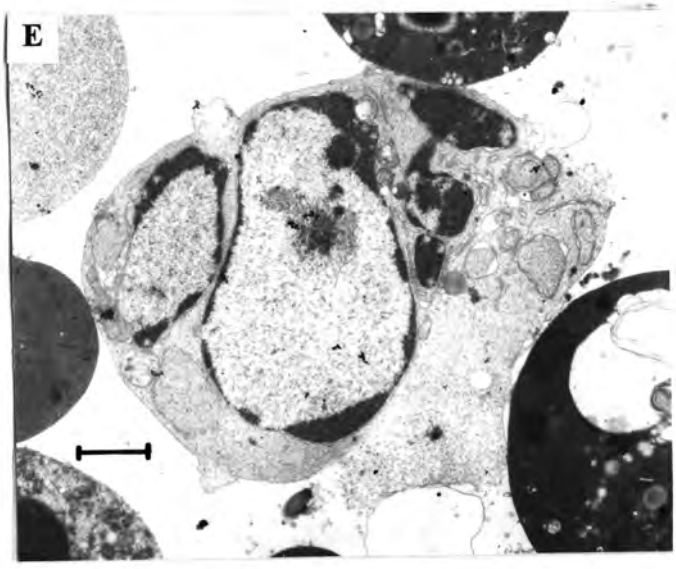

E

Figure *6.2* Electron micrographs showing induction of apoptosis in Akata and Chep-BL cells treated with cytotoxic drugs and y radiation.

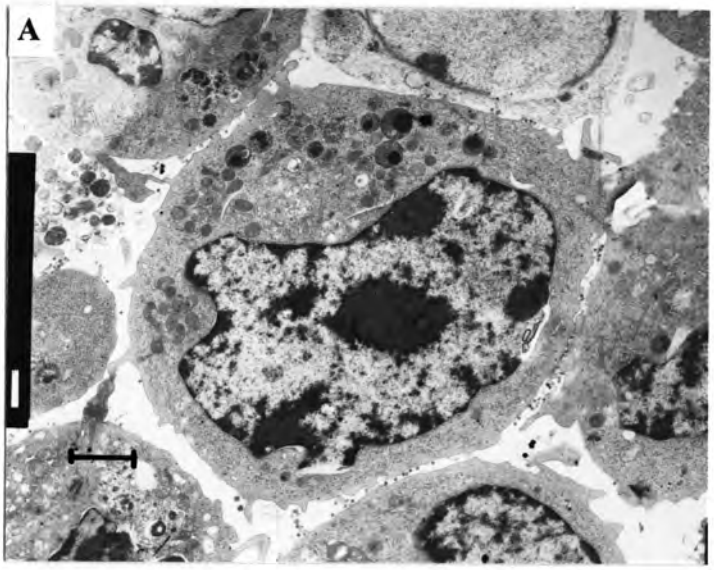
Legend

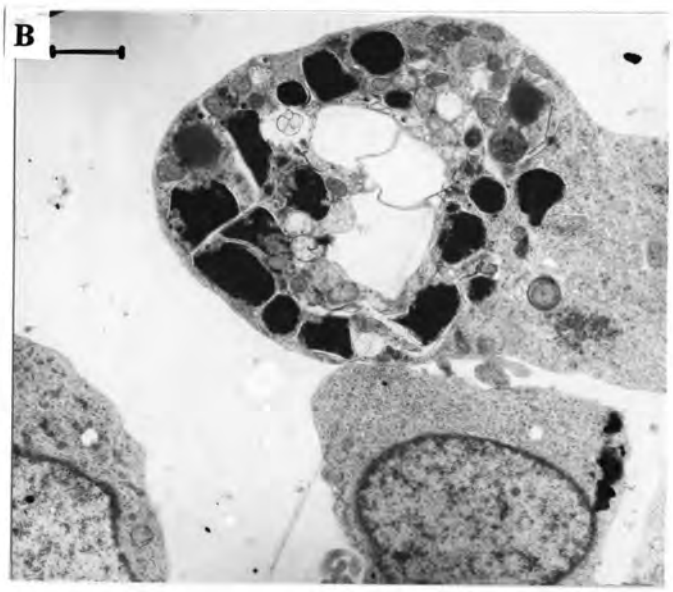
Bar sizes represent 2μm.

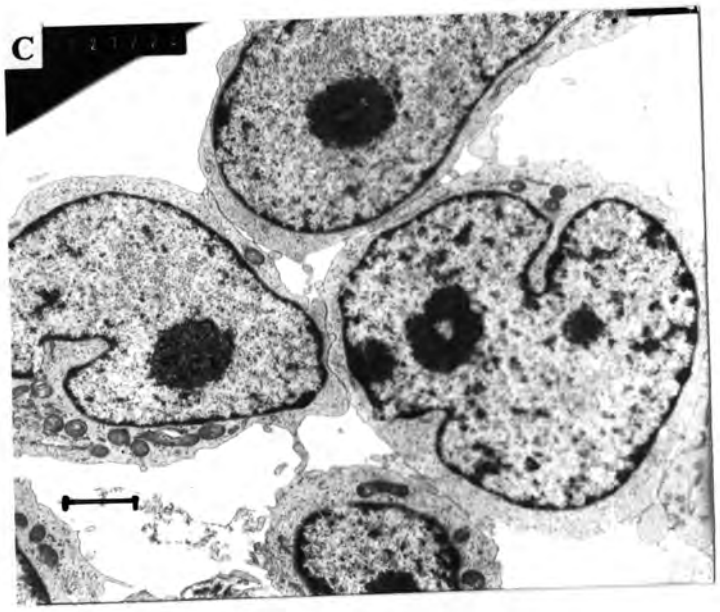
(A) Untreated Akata-BL control cells, note the regular appearance of the cytoplasm and normal nuclear chromatin state. Drug treated Akata-BL cells were exposed to **(B)** 2.5μg/ml etoposide, for **14** hours and subsequently harvested for morphological analysis. Note the highly condensed and fragmented nuclear chromatin.

Untreated Chep-BL control cells are shown in **(C),** again, note the regular appearance of the cells compared to cells treated with **(D)** 5μM methotrexate, **(E)** 1μg/ml etoposide and (**F**) 1μg/ml araC.

Cells were harvested directly after 14 hours exposure to drugs, without the subsequent growth in drug free medium since Chep-BL can often show **a** considerable drop in viability at this point and any later analysis may result in apoptotic morphology being overlooked. Despite this. cells treated with araC showed additional degradative changes, normally associated with necrosis. This suggests that death induced by this drug in this cell line is rapid. which may have resulted in the onset Of secondary necrosis by the time the cells were analysed.


A


B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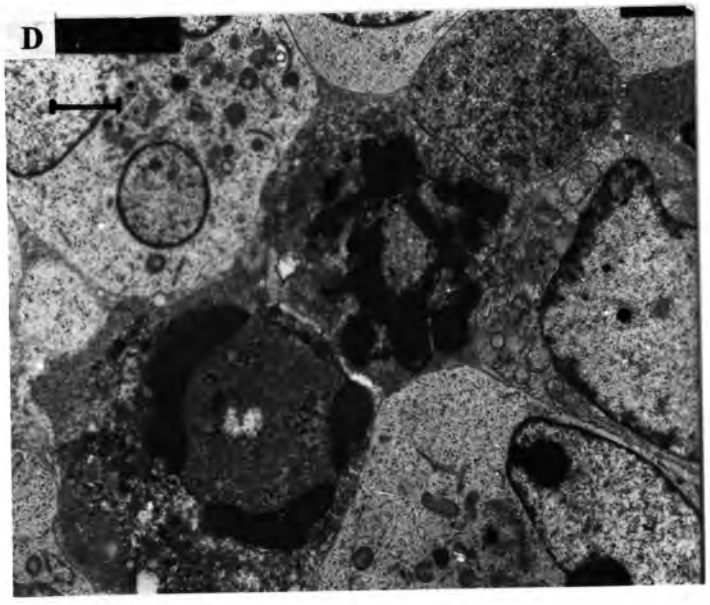
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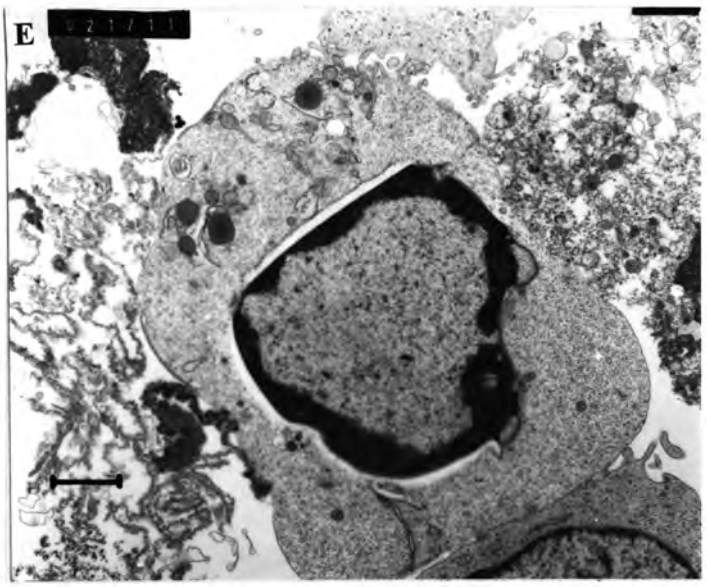

E

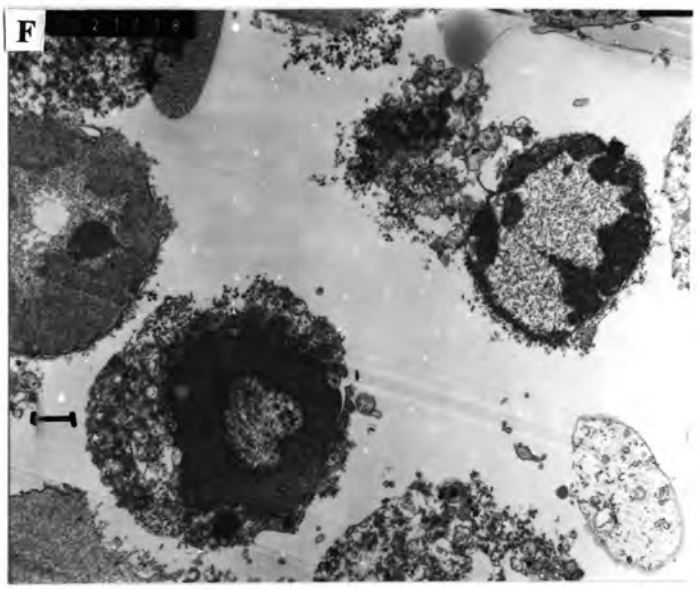

F

Figure 6.3 Apoptotic morphology in drug treated Raji-BL and Akata-BL cells stained with the fluorescent dye acridine orange.

Legend

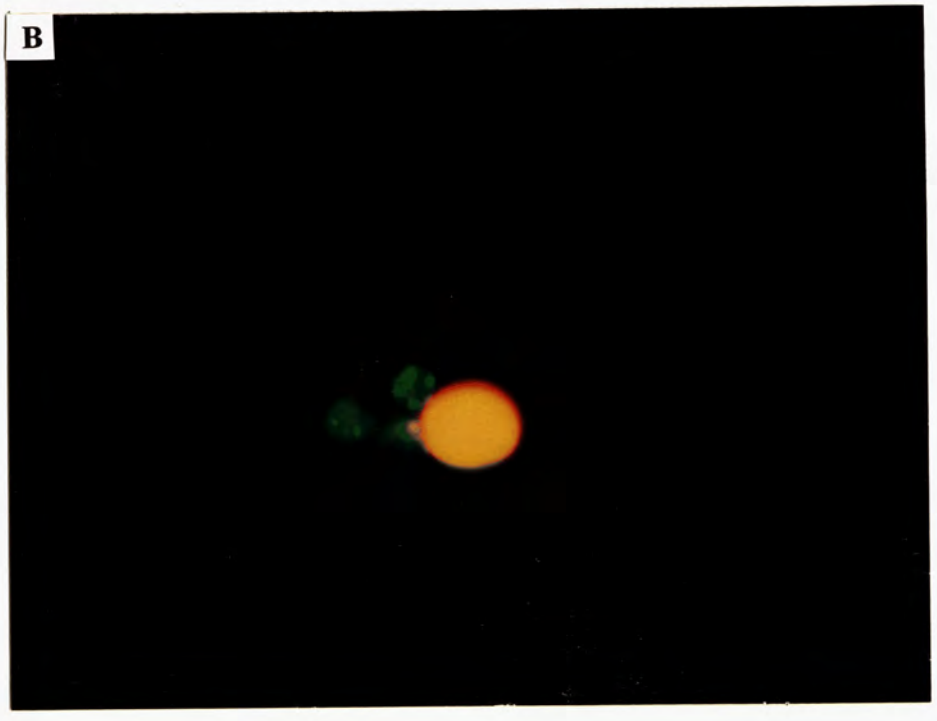
Acridine orange **is** an intercalating DNA dye which produces yeliow/green fluorescence in double stranded DNA and red fluorescence in single stranded DNA or RNA. Therefore viable cells have bright diffuse staining DNA and apoptotic cells have featureless condensed DNA which give off a brighter green fluorescence.

Untreated control Raji-BL cells are shown in **(A)** demonstrating the bright yellow colour associated with viable cells with intact DNA In contrast, cells treated with **(B)** 10μM methotrexate, **(C)** 1μg/ml etoposide and **(D)** 1μg/ml araC appear both condensed as a whole and nuclear chromatic stains green and **is** fragmented within the cell Similar morphologies are show for Akata-BL cells **with** control cells shown in **(E)** and cells treated with 1μg/ml etoposide shown in **(F)**

Cells were treated with drugs for 14 hours and the subsequently grown in drug free conditions for **24** hours before harvesting for morphological analysis

Magnification = x100 oil immersion objective lens


A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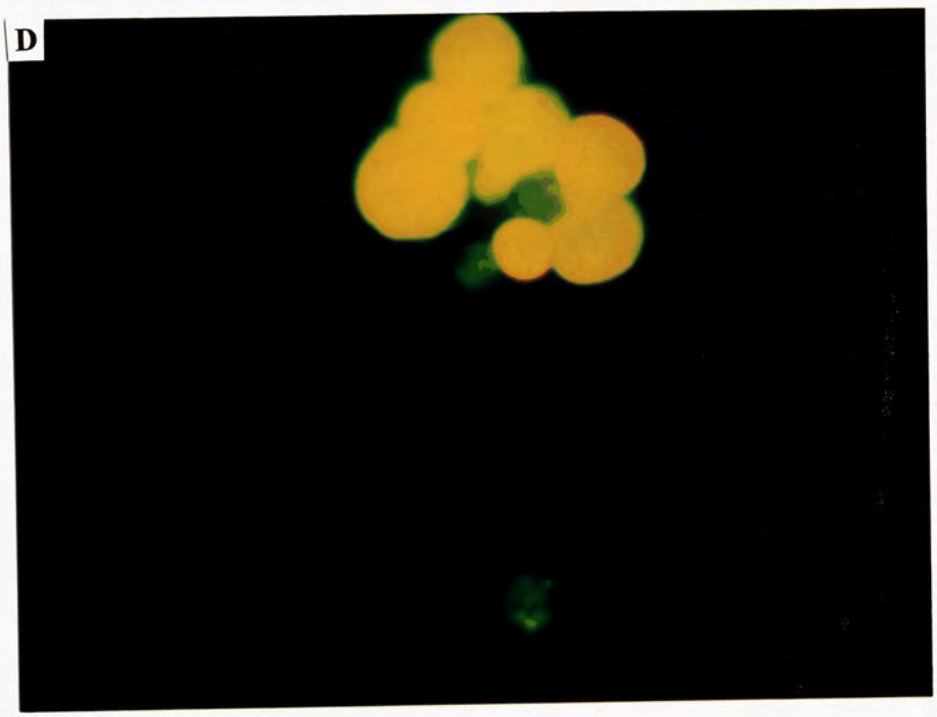
C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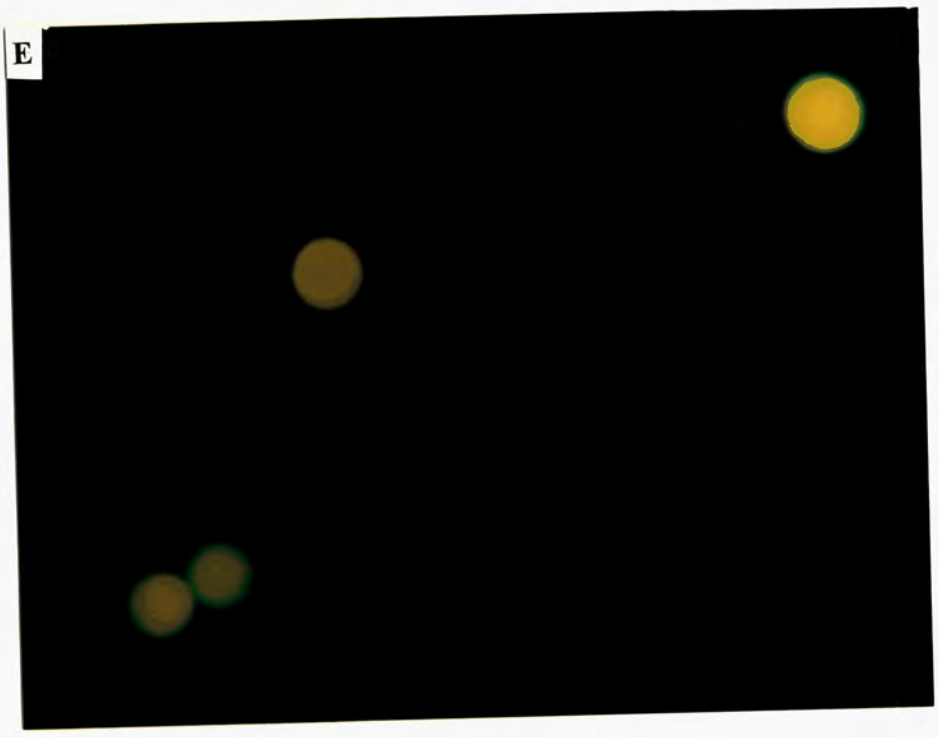
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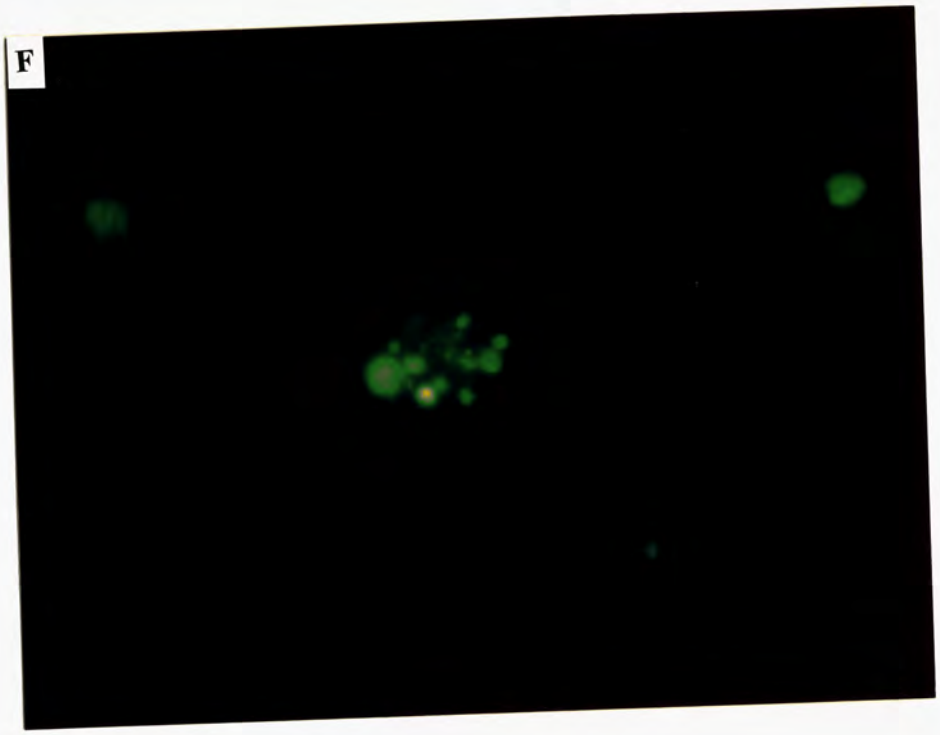

F

Figure 6.3 legend (cont.) These photographs were enlarged *to* illustrate clearly the morphology of single apoptotic cells. Control Raji-BL cells were enlarged ***(G)*** to compare against Akata-BL cells treated with 100μM methotrexate **(H)** and Raji-BL cells treated with 16Gy y-radiation **(I).**

Magnification = originally x100 oil immersion objective lens

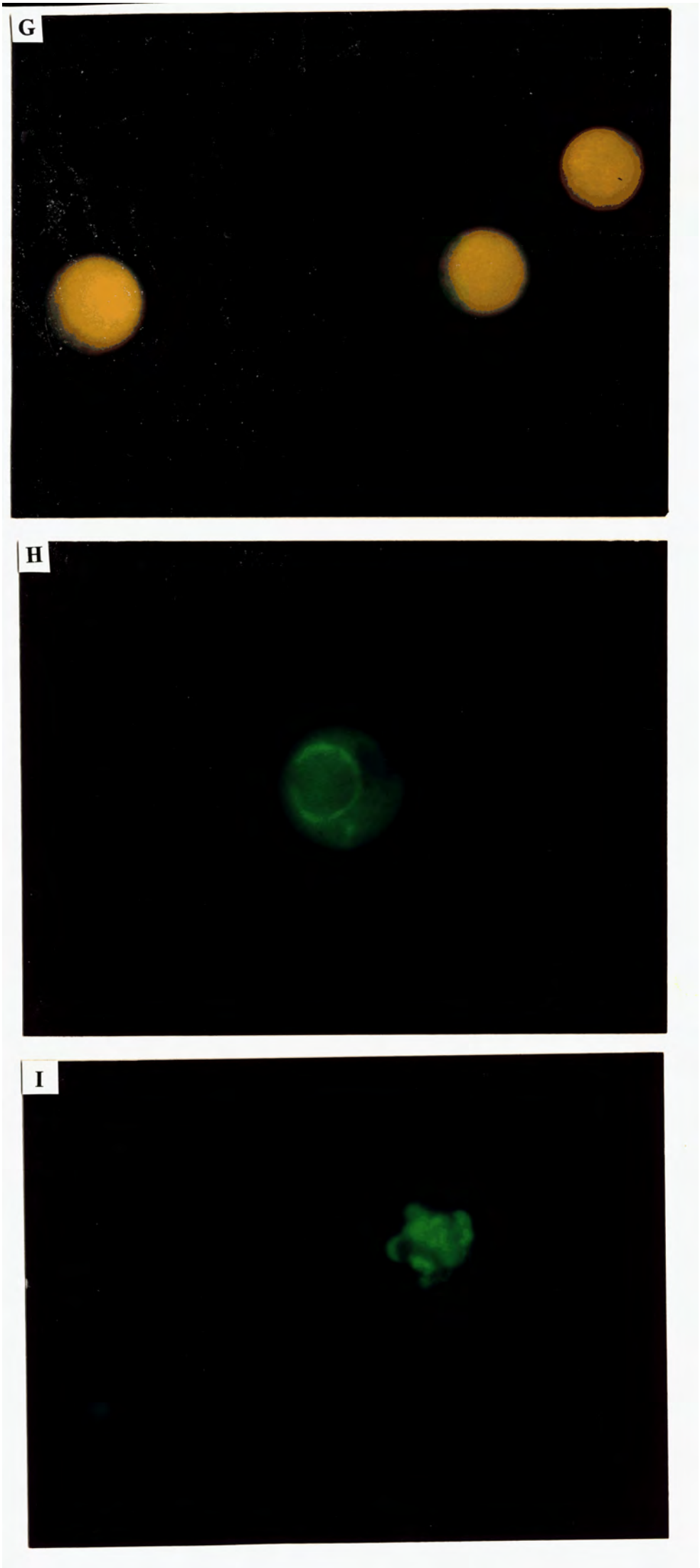
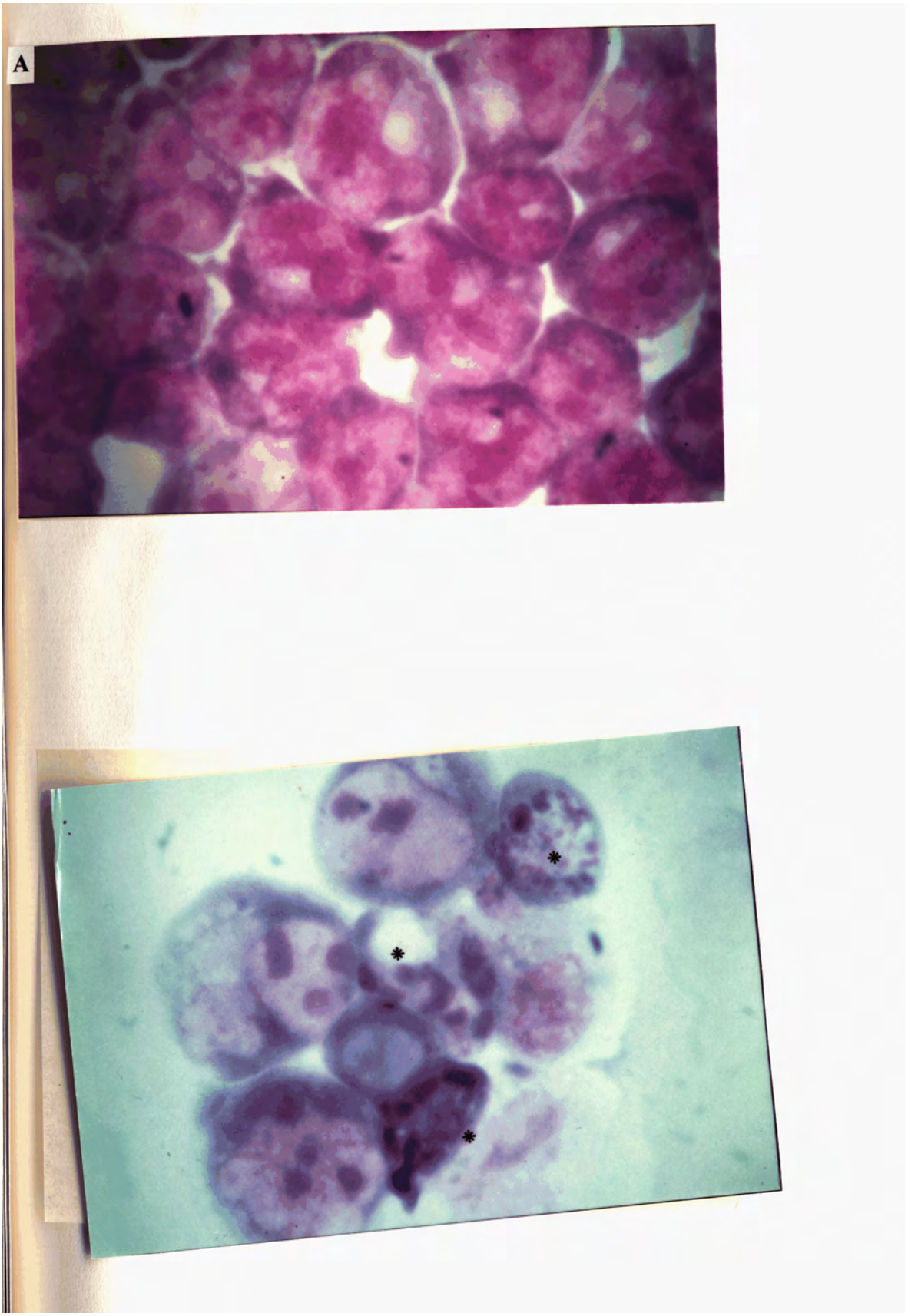

G
H
I

Figure 6.4 Raji-BL cells stained with Geimsa to illustrate apoptotic cells after treatment with chemotherapeutic drugs.

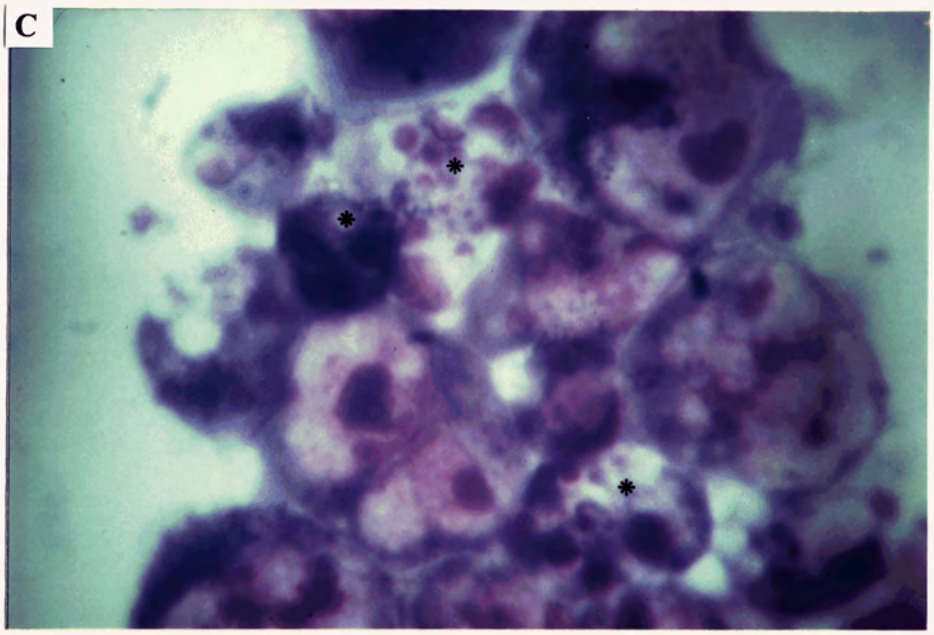
Legend

(A) Untreated control cells, note the pink appearance of the nucleus with purple nucleoli present. Cells treated with **(B)** 10μM methotrexate. **(C)** 1μg/ml etoposide and **(D)** 1μg/ml araC for 14 hours with 24 subsequent drug free growth before harvesting. Control cells are also shown **(E)** in comparison to cells analysed 24 hours after treatment with 16Gy of y-radiation **(F).** Note the dark pink/purple condensed chromatin evident in the apoptotic cells (indicated by *stars).*

Magnification = *x100* oil immersion objective lens.


A

C



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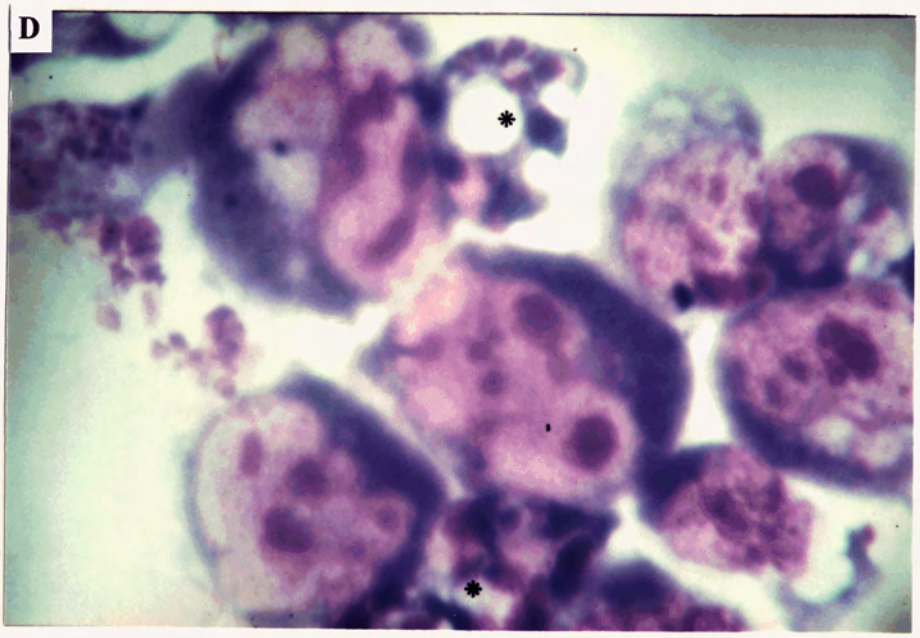
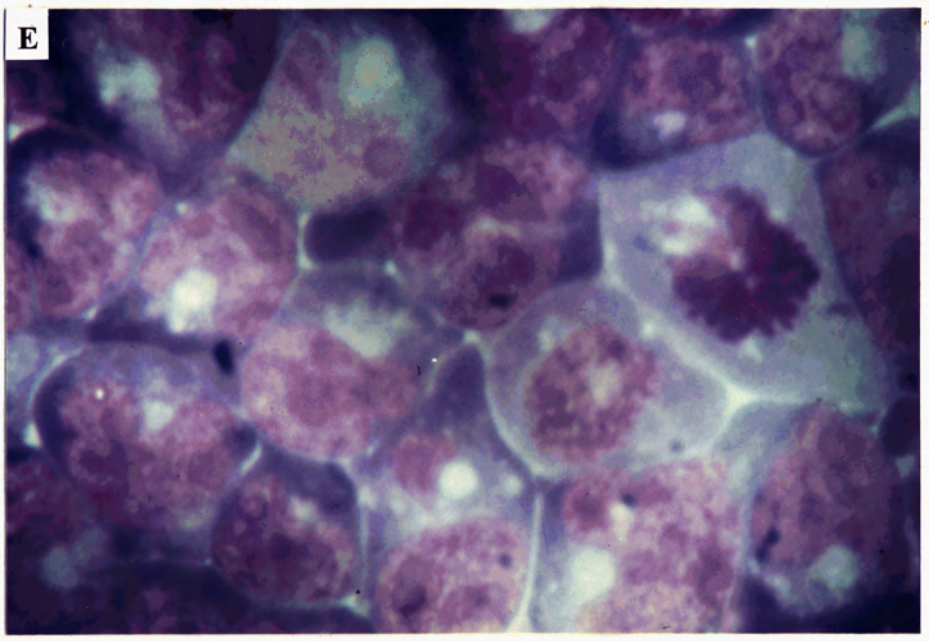
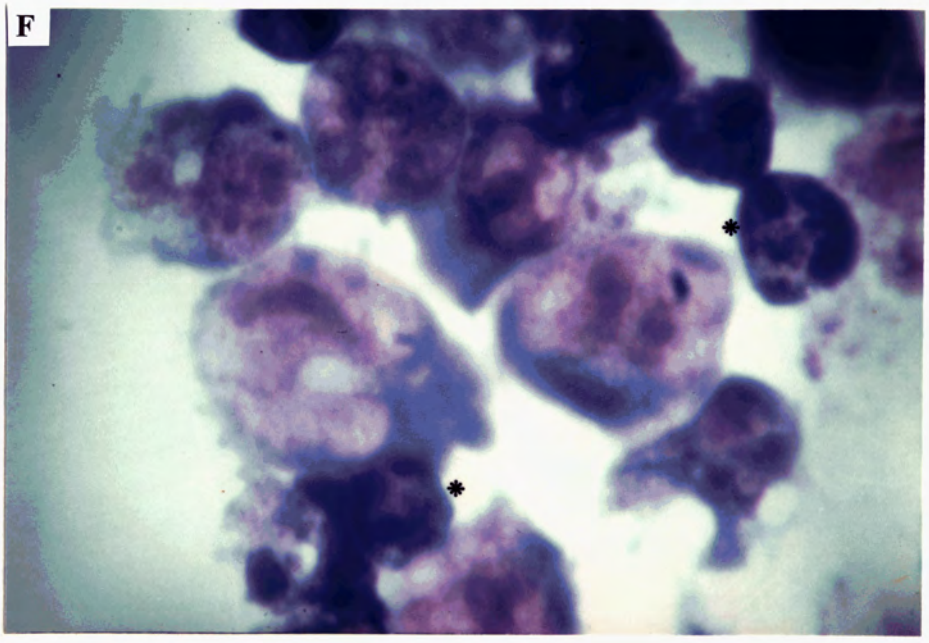



E


F

Results: Burkitt's lymphoma cells

Apoptotic nuclei (marked by *) are evident in Raji-BL cell lines which have been treated with chemotherapeutic drugs (figure 6.4 B, C and D) and with y radiation (F).

Therefore, all three BL cell lines undergo apoptosis in response to chemotherapeutic drugs and in Chep-BL and Raji-BL cell lines, after treatment with y radiation, as determined by morphological analysis.

A further characteristic of many apoptotic cells is the activation of a calcium dependent endonuclease resulting in fragmentation of the DNA into multiples of 180bp (Wyllie 1980; Cohen and Duke 1984). Figure 6.5 shows an electrophoresis gel of low molecular weight DNA from Raji-BL cells after treatment with etoposide, methotrexate or araC, (lanes 3-5) with fragmentation being clearly visible. Electrophoresis of genomic **DNA** at 12 hours post y radiation also demonstrates **DNA** fragmentation in Raji-BL cells (see figure 6.6 lane 3). However, in Chep-BL and Akata-BL cell lines, fragmentation was not detectable regardless of whether genomic or low molecular weight DNA was analysed. This is most likely due to a high level of cells entering apoptosis asynchronously or cells undergoing DNA cleavage which does not produce the characteristic low molecular weight oligonucleosomal fragments, but produces larger, higher molecular weight fragments which are not obvious on agarose gels (Cohen ***et al.,*** 1992; Brown ***et al.,*** 1992; Tomei ***et al.,*** 1993) (see chapter **7** for further discussion).

A further classical point of apoptosis is the suppression of cell death by inhibitors of RNA and protein synthesis (Cohen and Duke 1984; Wyllie ***et al.,*** 1984; McConkey ***et al.,*** 1989a). Although this has since been shown to be dependent on cell type (Vedickis and Bradshaw 1983; Martin ***et al.,*** 1990) it was interesting to determine if new protein or RNA synthesis was required in drug induced apoptosis in these cell lines. Raji-BL cells were incubated with the drugs for 14 hours with or without 0.8μM cycloheximide. Cells were then washed free of both cycloheximide and drugs and were incubated for **a** further 48 hours before determining cell viability. Figure 6.7 clearly illustrates significant inhibition of cell death by cycloheximide in the presence of either 10μM methotrexate ($p<0.01$) or 1μg/ml etoposide ($P<0.001$). A significant inhibition of death was not evident in response to 1μg/ml araC. This implies that both methotrexate and etoposide require active protein synthesis to induce death,

Figure 6.5 Electrophoresis gel showing low molecular weight **DNA** from drug treated and untreated Raji-BL control transfectant cells.

$5x10^6$ cells were exposed to cytotoxic drugs for 14 hours and then washed free of the drug and cultured in normal growth medium for a further 12 hours before analysis (see materials and **methods).**

Gel lane data:

Lane 1 123bp **DNA** marker;
Lane 2 Untreated control;
Lane 3 Cells treated with 1μg/ml etoposide;
Lane 4 Cells treated with 10μM methotrexate;
Lane **5** Cells treated with 1μg/ml araC.

Note the DNA fragmentation evident in cells treated with the chemotherapeutic drugs (lanes 3-5).

Figure 6.6 Electrophoresis gel showing genomic **DNA** from Raji-BL cells exposed to 16Gy of y radiation.

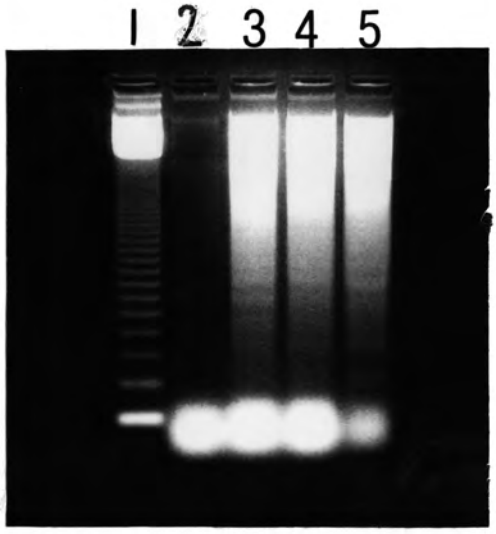
10^6 cells were harvested 12 hours after treatment and analysed as described in materials and methods.

Molecular sizes in kilobase (kb) pairs are indicated

Gel lane data

Lane 1 Control transfectants, untreated.
Lane 2 Control transfectants mock-treated on ice,
Lane 3 Control transfectants exposed to 16Gy y radiation,
Lane 4 BHRF1 transfectants untreated,
Lane **5** BHRF1 transfectants mock-treated on ice,
Lane **6** BHRF1 transfectants exposed to 16Gy y radiation

Note the suppression of fragmentation in the BHRF1 transfectants when compared to the transfected controls in response to y radiation (lanes 6 and 3 respectively).


1 2 3 4 5

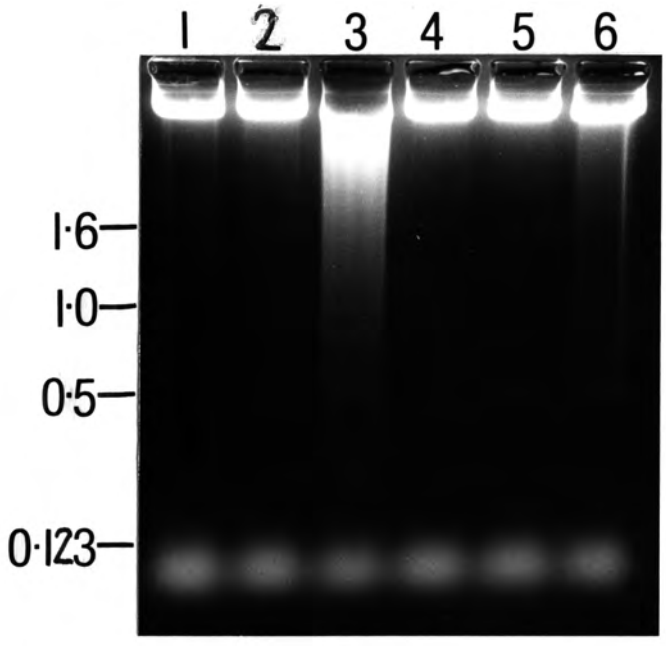

1 2 3 4 5 6
1·6
1·0
0·5
0·123

Figure 6.7 legend

Viability was determined by vital dye exclusion after 14 hours drug exposure with and without cycloheximide and 48 hours drug free and cycloheximide free growth.

Significant suppression ($p<0.01$) is evident for cells treated with cycloheximide and methotrexate (hatched bar) compared to cells treated with methotrexate alone (open bar). A significant difference ($p<0.001$) is also evident between cells treated with cycloheximide and etoposide (hatched bar) compared to cells treated with etoposide alone (open bar). There was no significant difference evident between cells treated with araC with or without cycloheximide.

Means and S.E. are representative of 3 replicate experiments.

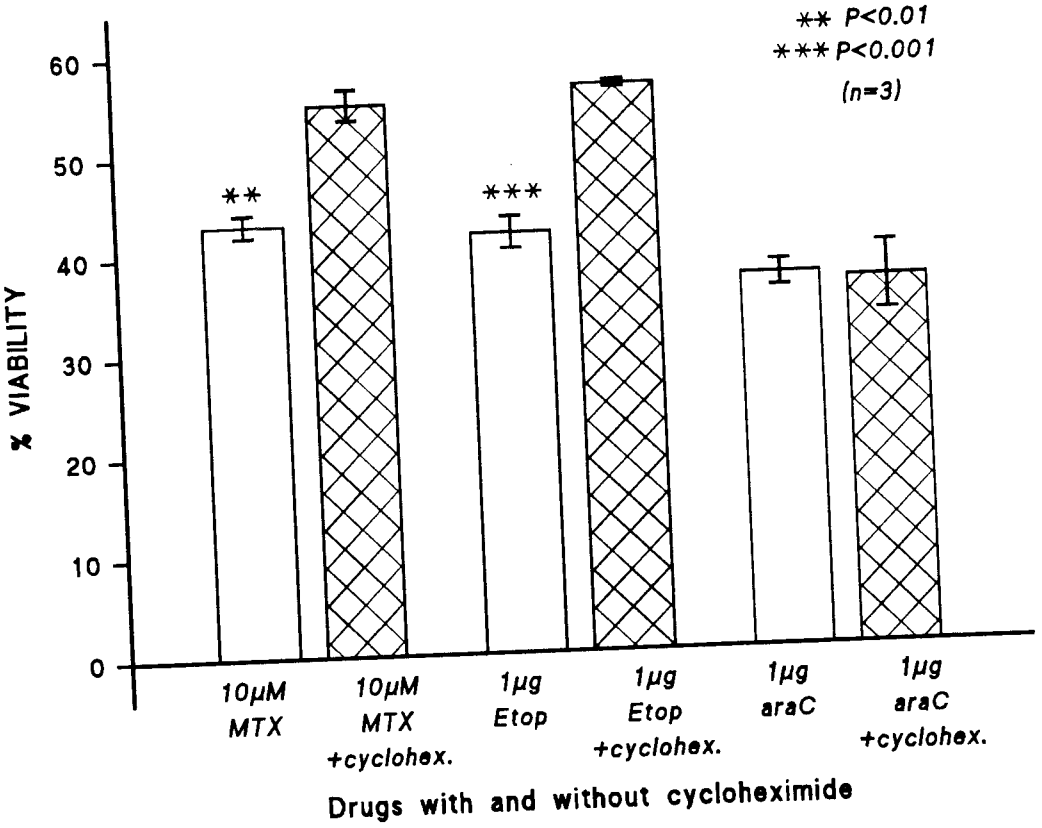
% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

Controls

Raji-BL control transfectants	**% viability at 48 hours +-/ S.E.**
Untreated	91.6 +/- 1.7
0.8μM cycloheximide	89.7 +/- 2.7

Figure 6.7

Viability of Raji-BL hebo control cells after 14 hours exposure to methotrexate, etoposide, araC, with and without 0.8μM cycloheximide.



however, this does not appear to be evident in araC induced death. Inhibition of protein synthesis by cycloheximide may have induced a cell cycle delay which would have caused a drop in toxicity of the S-phase drugs resulting in the suppressive effect. However, etoposide **is** not exclusively S-phase specific and araC, which is an S-phase specific drug, showed no inhibition of death in the presence of cycloheximide, ***so*** the suppression of cell death by cycloheximide appears not to be due to a delay in cell cycle.

Overall, several aspects of drug induced cell death in the BL-cell lines are characteristic of apoptosis, especially in the Raji-BL cells where fragmentation of the DNA is evident and apoptotic morphology is quite clear in response to all stimuli investigated in this study. However, araC does appear to produce morphology in Chep-BL cells which **is** not typical of apoptosis and **is** not affected by the presence of cycloheximide, suggesting new protein synthesis is not required for apoptosis induced by this drug. Therefore, in these cell lines araC may operate to induce apoptosis via different pathways when compared to etoposide and methotrexate.

Section 6.3 Expression of transfected *bcl-2* affords protection against chemotherapeutic drugs and y radiation

Having established that treatment of the BL cell lines with several cytotoxic agents resulted in apoptosis, Chep-BL-bcl-2 transfectants were analysed for protection from cell death in response to these stimuli. Figure 6.8A illustrates the viability of Chep-BL-bcl-2 transfectants and controls after exposure to ***8Gy*** of y radiation. High levels of suppression of apoptosis are seen for Chep-BL-bcl-2 transfectants when compared to controls at **24** and **48** hours post treatment ($p<0.001$).

Bcl-2 is also able to suppress apoptosis in response to cytotoxic drugs as shown in figure 6.8B. Significant inhibition of cell death ($p<0.001$) is seen for Chep-BL-bcl-2 expressing transfectants compared to controls treated with either 1μg/ml etoposide or 5μM methotrexate. Chep-BL-bcl-2 expressors show a smaller inhibition of cell death in response to treatment with araC and the observations are statistically significant at the 10%, but not at the **5%** level.

Figure 6.8(A) legend

Significant suppression of apoptosis is evident for *bcl-2* transfectants (closed bar) when compared to control transfectants (open bar) at 24 and 48 hours ($p<0.001$).

Means and S.E. are shown for 3 replicates from a typical example of 3 separate experiments.

% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

Untreated controls	**Time 0 viability**	**% viability 24 hours +/- S.E.**	**% viability 48 hours +/- S.E.**
Chep-BL control transfectant	90.7 %	81.9 +/- 1.9	84.9 +/- 0.6
Chep-BL *bcl-2* transfectant	95.0 %	96.4 +/- 0.5	95.0 +/- 0.4

Figure 6.8(A)

Viability of Chep–BL cells expressing bcl–2 and controls after exposure to 8Gy of γ radiation

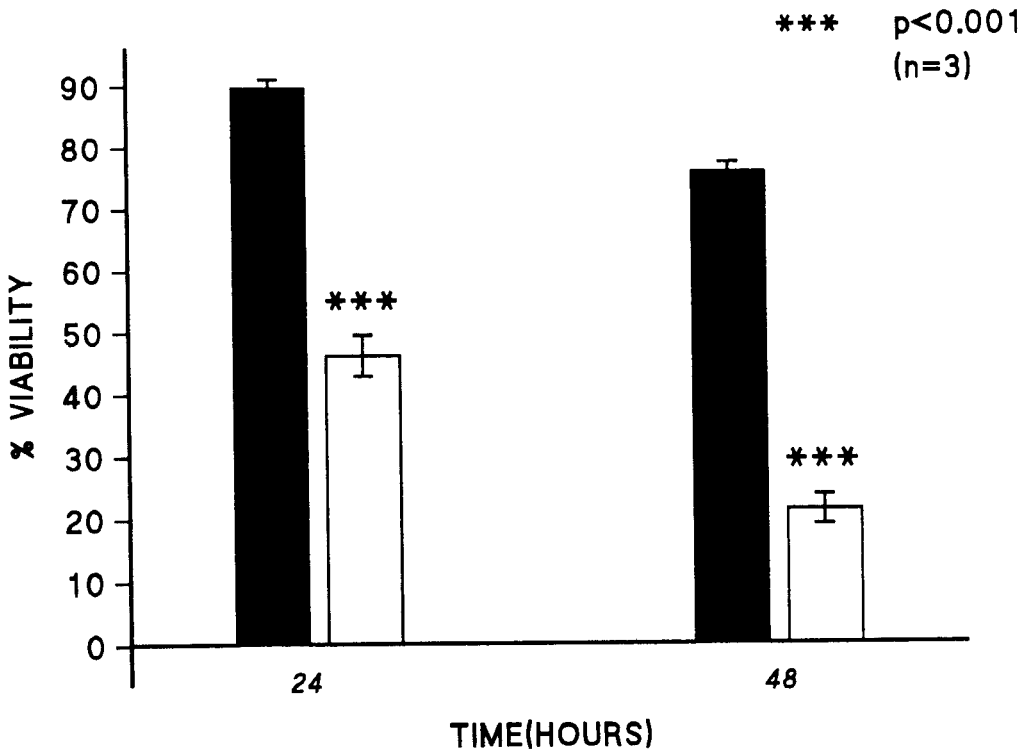


Figure 6.8(B) legend

Significant suppression of cell death is seen in Chep-BL *bcl-2* transfectants (hatched bar) when compared to control transfectants (open bar) in response to 5μM methotrexate ($p<0.001$) and 1μg/ml etoposide ($p<0.001$).

A difference between BHRF1 expressing cells and controls is evident in response to 1μg/ml araC, but this is only significant at the 10% level ($p<0.1$).

Means and S.E. of 3 or more replicate experiments are shown.

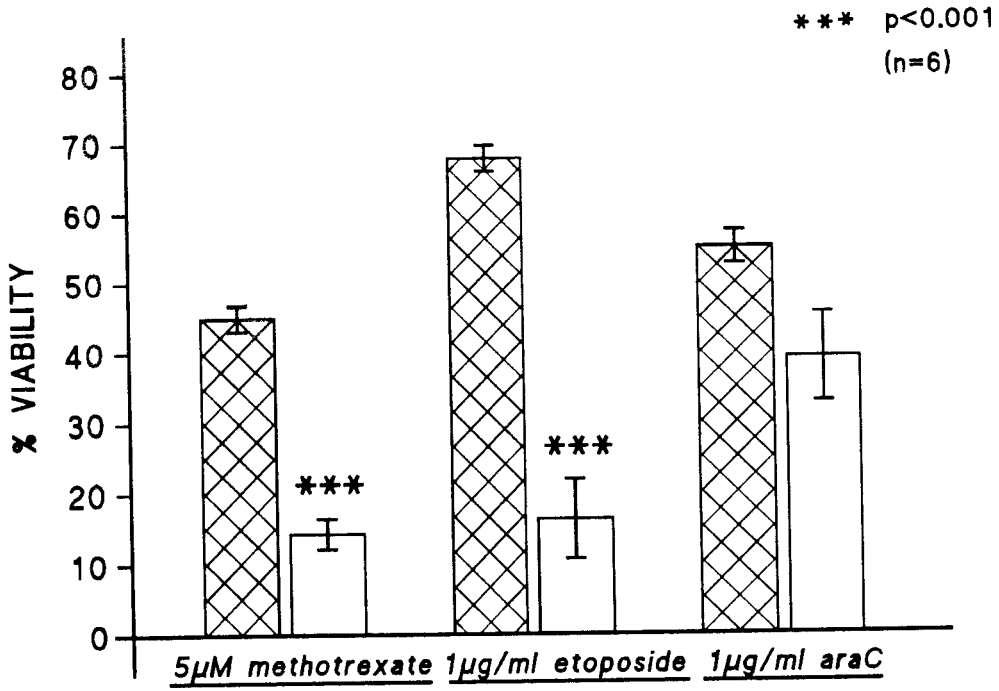
% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

	Time 0 % viability +/- S.E.	**% viability untreated control 48 hours +/- S.E.**	**% viability of DMSO control at 48 hours +/- S.E.**
Chep-BL control transfectant	86.1 +/- 2.3	85.1 +/- 3.2	79.3 +/- 3.5
Chep-BL *bcl-2* transfectant	94.8 +/- 0.9	96.3 +/- 0.7	95.1 +/- 0.6

N.B. Chep-BL control transfectants cultured with and without cycloheximide for 14 hours, show no significant difference between the viabilities as determined at 48 hours post treatment ($p>0.1$).

Figure 6.8(B)

Viability of Chep–BL cells expressing bcl–2 and controls after 14 hours exposure to methotrexate, etoposide and araC and 48 hours subsequent growth



Fluorescence microscopy of cells stained with acridine orange allows identification of cells with apoptotic morphology before cell lysis (see figure **6.3),** potentially representing a more accurate method for establishing numbers of apoptotic vs. viable cells. When apoptosis was monitored by this technique, bcl-2 expression was found to inhibit apoptosis induced by either methotrexate or etoposide, but the inhibition of apoptosis induced by araC was once again found to be relatively small and not highly statistically significant, see figure **6.9A.**

Chep-BL-bcl-2 transfectants and controls were also incubated in the presence of chemotherapeutic drugs for up to 72 hours to determine the rate of cell death. Results of the time course are shown in figure 6.10 and again, although significant inhibition of cell death is seen for bcl-2 expressing transfectants when compared to controls in response to 0.1μM methotrexate **(A)** ($p<0.02$) and 0.5μg/ml etoposide (B) ($p<0.001$) at 72 hours, there is no inhibition of death in cells treated with 0.5μg/ml araC (C) by 72 hours. This latter result may identify a pathway of drug induced cell death for which bcl-2 is not a potent inhibitor or the result may reflect the necrotic morphology sometimes observed in these cells in response to araC. These points will be discussed further in chapter 7.

In order to verify that the inhibition of apoptosis induced by cytotoxic drugs was a reproducible effect produced by the expression of bcl-2 and not a characteristic caused by an unknown mutation in any one clone, two further Chep-BL-bcZ-2 expressing clones were also treated with etoposide. Suppression of apoptosis was again demonstrated and is shown in table 6.2.

These results extend the range of apoptosis-inducing stimuli **for** which bcl-2 provides a protective effect in Chep-BL cells, protection against apoptosis induced by serum withdrawal or calcium ionophore having been previously documented for these cell lines (Henderson ***et al.,*** 1991). The direct demonstration of inhibition of apoptosis induction also confirms that, as found in other cell types, bcl-2 expression increases cell culture viability after treatment with cytotoxic drugs by inhibiting apoptosis rather than stimulating proliferation.

Section 6.4 The EBV gene BHRF1 can also suppress apoptosis

The EBV gene BHRF1 was the first gene shown to have any significant homology to bcl-2 (Cleary ***et al., 1986),*** suggesting the possibility that it too might possess the unusual and

Figure 6.9(A) legend

Transfectants were exposed to chemotherapeutic drugs for 14 hours then washed free of the drugs and grown in drug free medium for 48 hours prior to staining in acridine orange.

Significant suppression of apoptotic morphology is shown for *bcl-2* transfectants (hatched bar) when compared to control transfectants (open bar) in response to 5μM methotrexate ($p<0.05$) and 1μg/ml etoposide ($p<0.02$). Although *bcl-2* expression appears to confer a small protective effect against treatment with 1μg/ml araC, this difference is not statistically significant. Means and S.E. of 3 replicate experiments are shown.

Apoptotic morphology was determined by acridine orange staining, the differences between apoptotic and morphologically normal cells were clearly evident as shown in figure 6.3.

	Time 0 % viability	**% viability 48 hours +/- S.E.**
Chep-BL control transfectant	89.2 +/- 5.3	83.5 +/- 5.1
Chep-BL bcl-2 transfectant	86.7 +/- 3.3	93.1 +/- 1.2

Figure 6.9(A)

Percentage of morphologically normal (non-apoptotic) Chep-BL transfectants expressing bcl-2 and controls after treatment with cytotoxic drugs as determined by acridine orange staining

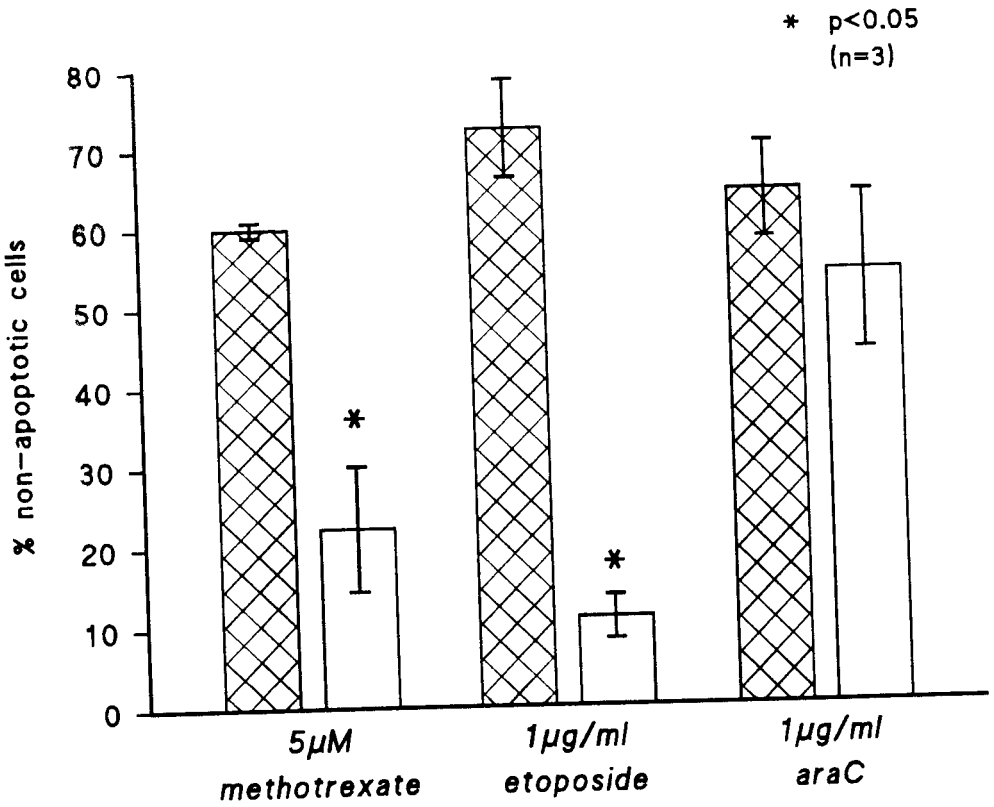


Figure 6.9(B) legend

Transfectants were exposed to chemotherapeutic drugs for 14 hours the washed free of the drugs and grown in drug free medium for 48 hours prior to staining in acridine orange.

Significant suppression of cells with apoptotic morphology is seen for BHRF1 transfectants (hatched bar) when compared to control transfectants (open bar) in response to 10μM methotrexate ($p<0.01$), 1μg/ml etoposide ($p<0.01$) and 1μg/ml araC ($p<0.05$).

Means and S.E. of 3 replicate experiments are shown.

Apoptotic morphology was determined by acridine orange staining, the differences between apoptotic and morphologically normal cells were clearly evident as shown in figure 6.3.

	Time 0 % viability	**% viability 48 hours +/- S.E.**
Raji-BL control transfectant	95.3 +/- 0.8	96.7 +/- 0.8
Raji-BL BHRF1 transfectant	97.1 +/- 0.2	99.2 +/- 0.3

Figure 6.9(B)

Percentage of morphologically normal (non-apoptotic) Raji-BL transfectants expressing BHRF1 and controls after treatment with cytotoxic drugs as determined by acridine orange staining

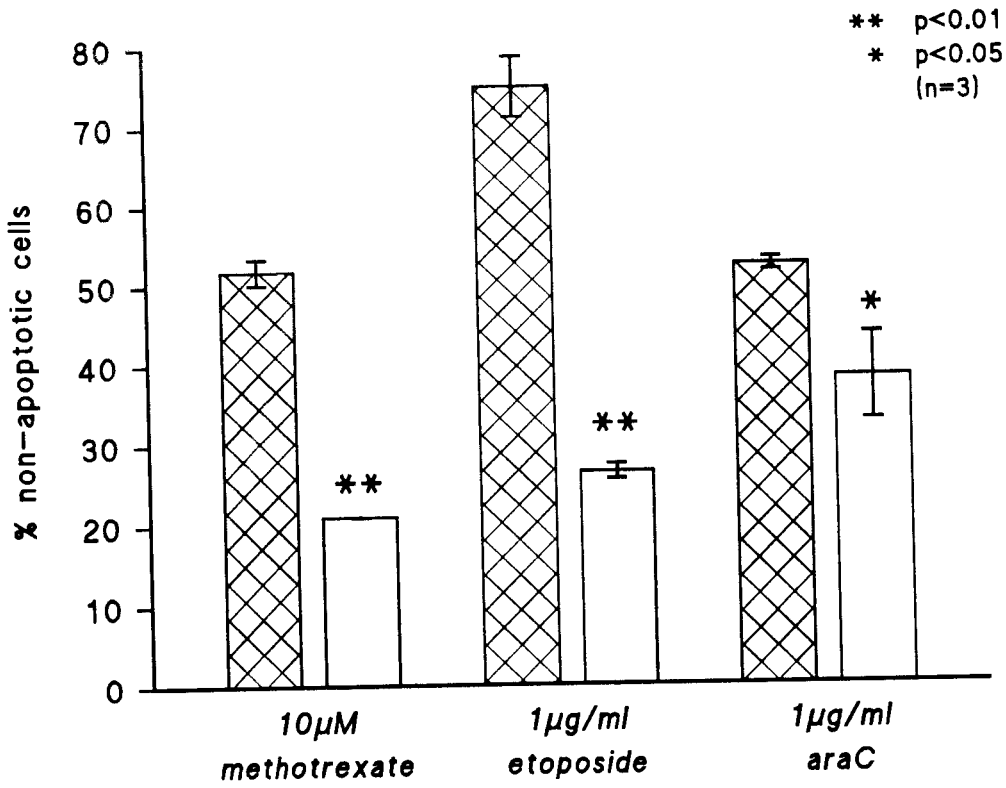


Figure 6.10 legend

Significant suppression of apoptosis is seen for *bcl-2* transfectants. ··◆··· relative to controls --▲-- in response to 1μM methotrexate **(A),** 1μg/ml etoposide **(B).** Cells treated with 0.5μg/ml araC **(C)** do not show significant suppression of cell death. Both *bcl-2* transfectants ■- and controls —●— remain highly viable in the absence of drugs.

Means and **S.E.** are representative of **3** replicate counts. % viability **as** determined **by** vital **dye** exclusion, is expressed as number of viable cells divided by the total number of cells counted.

Figure 6.10 Viability of Chep-BL transfectants expressing *bcl-2* and controls on exposure to methotrexate, etoposide, araC

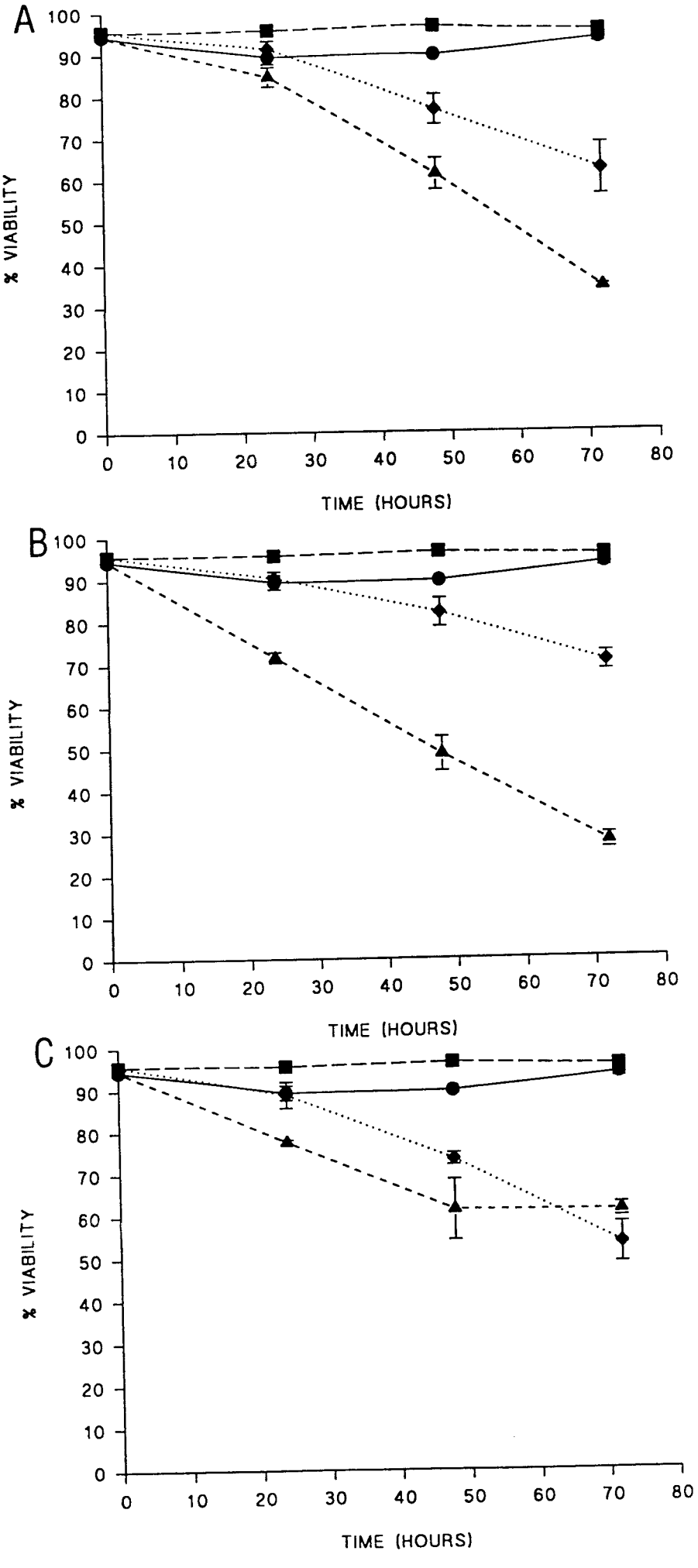


Table 6.2

% viabilities of Chep-BL-*bcl-2* transfectant clones exposed to 1μg/ml etoposide (n=3)

	% Viability +/- S.E.	
Cell clone	**Time 0**	**48 Hours 1μg/ml etoposide**
Chep-cΔj-control transfectant	88.2	6.3+/-3.34
Chep-bcl-2 clone 2c	93.8	46.9+/-6.05
Chep-bcl-2 clone 3	95.7	62.9+/-3.50

% viability was determined by vital dye exclusion and represents the number of live cells counted expressed as a % of the total number of cells counted.

potentially highly significant property of suppressing apoptosis (Williams, 199**i**). Raji-BL cells transfected with BHRF1 or relevant plasmid controls were exposed to 16Gy of y radiation and their viabilities were determined at 24 and 48 hours post treatment. Inhibition of apoptosis is seen for BHRF1-expressing transfectants showing significant inhibition ($p<0.02$) of cell death when compared to controls (see figure 6.11A). This suppression of cell death can also be seen by monitoring **DNA** fragmentation. Figure 6.6 shows a qualitative delay in fragmentation in BHRF1 transfectants compared to controls analysed at 12 hours post irradiation (lanes 6 & **3** respectively).

On treatment with cytotoxic drugs, BHRF1 expressing Raji-BL cells show significant suppression ($p<0.001$) of apoptosis in response to ali three drugs tested, as illustrated in figure 6.11B. Again, greatest suppression is seen in response to etoposide, followed by methotrexate, then araC. These results were also verified by directly counting apoptotic cells stained with acridine orange (see figure 6.9B).

A time course of drug induced cell death was also established in these cells, see figure 6.12. Suppression of cell death is again evident in BHRF1 transfectants when compared to controls in response to methotrexate ($p<0.001$), etoposide ($p<0.001$) and araC **($p<0.05$)** at 72 hours.

To ensure that the effect was not confined to Raji-BL cells, nor indeed the result of an undefined mutation within the Raji-BL transfectants, BHRF1-transfected Akata clones were also studied using etoposide and methotrexate (neither araC or y-radiation could be used with Akata clones since the parent Akata cells are insensitive to high levels of araC (>100μg/ml) and y-radiation (30Gy) see table 6.3). Figure 6.13 shows significant suppression of apoptosis in response to 100μM methotrexate ($p<0.01$) and 1μg/ml etoposide ($p<0.05$), and further indicates that BHRF1 is able to prevent apoptosis in different genetic backgrounds.

Section *6.5* In summary...

The BL cell lines used in this study have been shown to enter apoptosis in response to several chemotherapeutic drugs and for two of the lines, Chep-BL and Raji-BL, in response to y-radiation. Expression of exogenous ***bcl-2*** is able to significantly suppress cell death produced by the above stimuli. This suppression is noticeably smaller for araC, possibly indicating that

Figure 6.11(A) legend

Significant suppression of apoptosis is seen for BHRF1 transfectants (closed bar) when compared to control transfectants (open bar) at 24 and 48 hours after treatment ($p<0.02$).

Means and S.E. are shown for 3 replicates from a typical example of 3 separate experiments.

% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

Untreated controls	**% viability 24 hours +/- S.E.**	**% viability 48 hours +/- S.E.**
Raji-BL control transfectant	88.8 +/- 0.1	92.8 +/- 0.4
Raji-BL BHRF1 transfectant	96.5 +/- 0.9	95.9 +/- 0.7

Figure 6.11(B) legend

Significant suppression of apoptosis is seen in BHRF1 transfectants (hatched bar) compared to control transfectants (open bar) in response to 10μM methotrexate, 1μg/ml etoposide and 1μg/ml araC ($p<0.001$ for all 3 drugs).

Means and S.E. are shown for 3 or more replicate experiments.

% viability, as determined by vital dye exclusion, is expressed as number of viable cells divided by the total number of cells counted.

	Time 0 % viability +/- S.E.	**% viability untreated control 48 hours +/- S.E.**	**% viability of DMSO control at 48 hours +/- S.E.**
Raji-BL control transfectant	95.5 +/- 0.7	95.3 +/- 0.3	95.2 +/- 0.6
Raji-BL BHRF1 transfectant	97.3 +/- 0.9	96.6 +/- 0.7	97.1 +/- 0.3

Figure 6.11(A)

Viability of Raji-BL cells expressing BHRF1 and controls after exposure to 16Gy of γ radiation

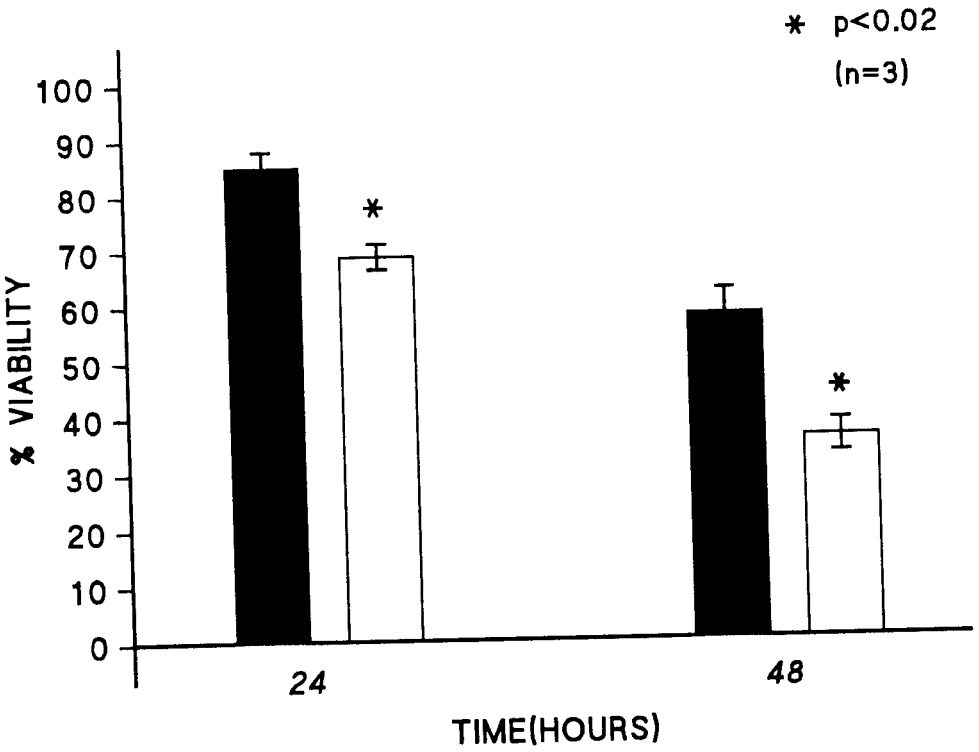


Figure 6.11(B)

Viability to Raji–BL cells expressing BHRF1 and controls after 14 hours exposure to methotrexate, etoposide and araC and 48 hours subsequent growth

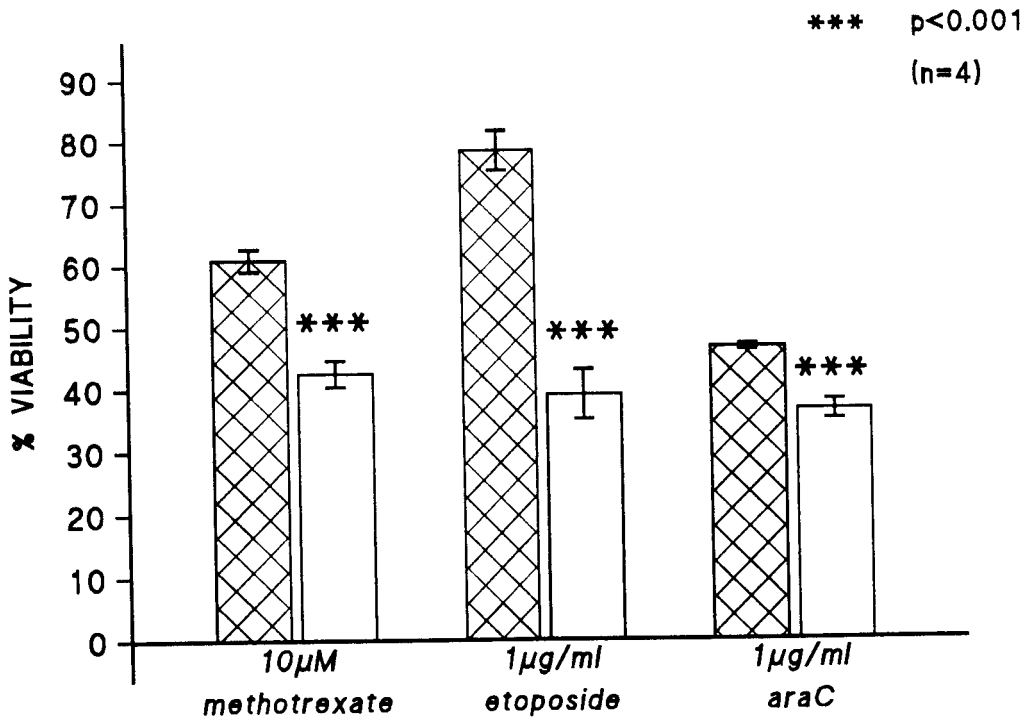
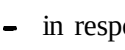
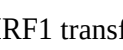


Figure 6.12 legend

Significant suppression of apoptosis is seen for **BHRF 1**transfectants  relative to controls  in response to 1μM methotrexate **(A),** 1μg/ml etoposide **(B)** and 0.5μg/ml araC **(C).** Both BHRF1 transfectants  and controls+ remain highly viable in the absence of drugs.

Means and **S.E.are** representative of *3* replicate counts.

% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

Figure 6.12 Viability of Raji-BL transfectants expressing BHRF1 and controls on exposure to methotrexate, etoposide, araC

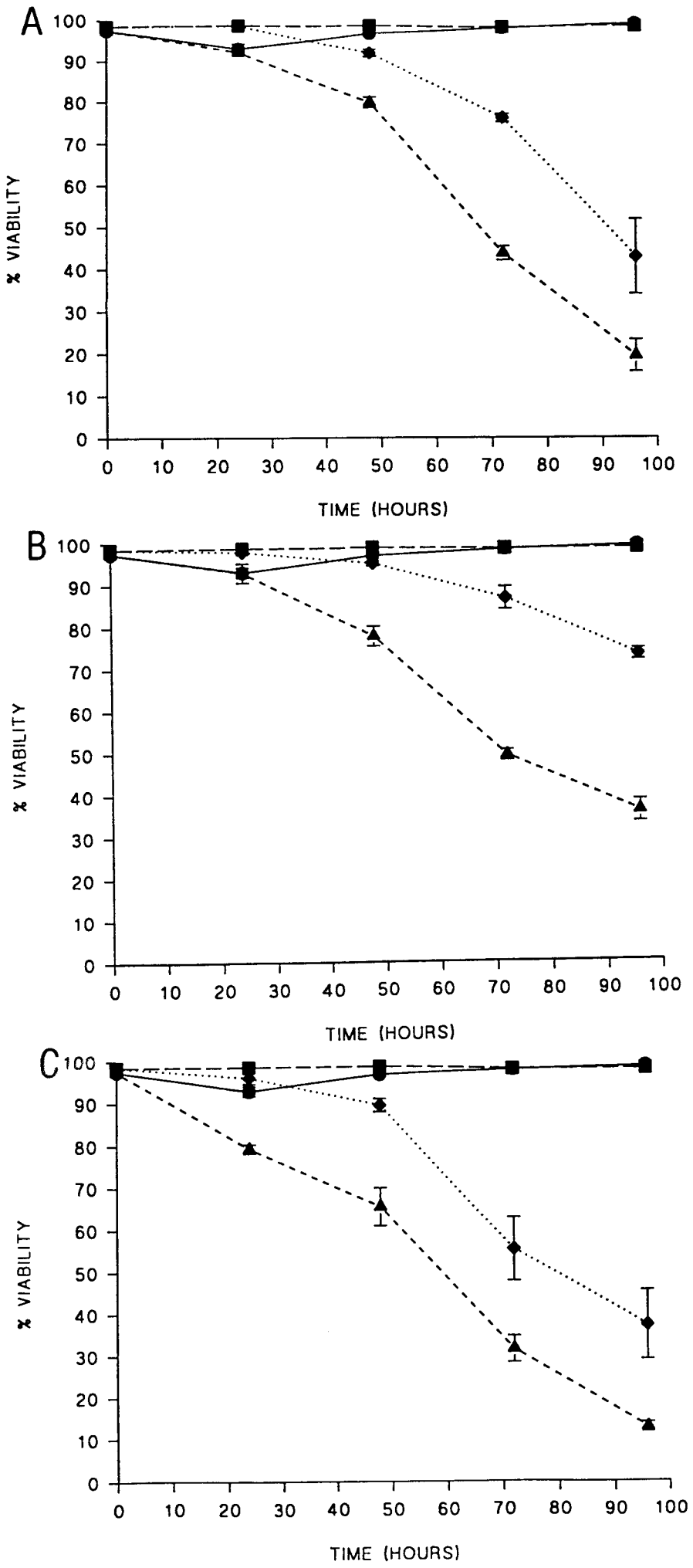


Table 6.3

Levels of Akata-BL parental cell resistance to γ radiation and araC

	% viability +/- S.E. at 48 hours		
Cell clone	**50μg/ml araC**	**100μg/ml araC**	**30Gy γ-radiation**
Akata control transfectant	84.7 +/- 1.9	92.1 +/- 0.5	68.0 +/- 3.3
Akata BHRF1 transfectant	83.9 +/- 2.0	88.7 +/- 0.9	75.8 +/- 2.2

(n=3)

Figure 6.13 legend

Significant suppression of apoptosis is shown for BHRF1 transfectants (hatched bar) when compared to control transfectants (open bar) in response to 100μM methotrexate ($p<0.05$) and 1μg/ml etoposide ($p<0.01$).

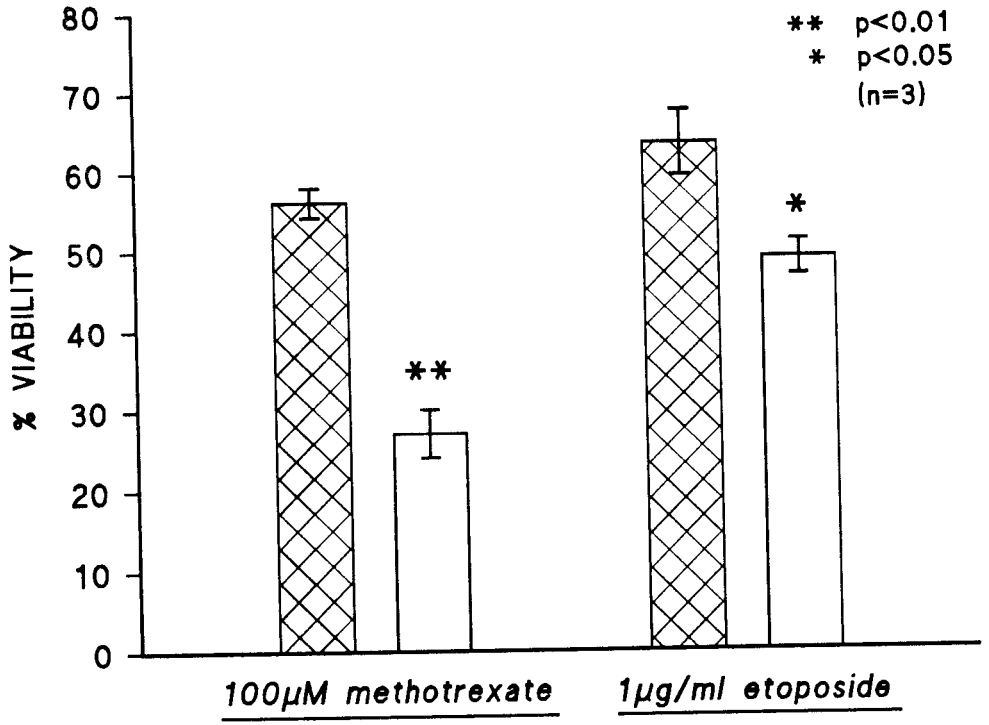
Means and S.E of 3 replicate experiments are shown.

% viability, as determined by vital dye exclusion, is expressed as the number of viable cells divided by the total number of cells counted.

	Time 0 % viability +/- S.E.	**% viability untreated control 48 hours +/- S.E.**	**% viability of DMSO control at 48 hours +/- S.E.**
Akata-BL control transfectant	88.4 +/- 1.7	87.9 +/- 2.6	93.5 +/- 1.3
Akata-BL BHRF1 transfectant	95.6 +/- 0.1	94.4 +/- 0.5	92.8 +/- 0.8

Figure 6.13

Viability of Akata-BL cells expressing BHRF1 and controls after 14 hours exposure to methotrexate and etoposide and 48 hours subsequent growth



araC acts in part through a *bcl-2* independent pathway. BHRF1 expression in two BL cell lines, Raji and Akata also affords protection against drug induced cell death and therefore is functionallyhomologous to *bcl-2* in these cell lines. Since BHRF1 is a viral gene, this result has interesting implications for both efficient viral production i.e., delaying the onset of cell death in order to maximise virus production and the association of viruses such as EBV with human cancers. Both *bcl-2* and BHRF1 induce a novel form of drug resistance in these cell lines by their ability to inhibit, or delay the onset of cell death. Therefore, inhibition of apoptosis by specific genes could represent an alternative mechanism with which to explain multidrug resistance in some cancer cell lines.

Discussion

Chapter 7

Discussion

Section 7.1 Introduction

Perhaps one of the most important features of apoptosis is that this form of cell death is active and can be regulated by both internal and external factors (reviewed in Williams ***et al.,*** 1992). Many cells rely on growth factors to provide a survival stimulus, withdrawal of which results in cell death (Williams ***et al.,*** 1990; Nunez ***et al.,*** 1990; Rodriguez-Tarduchy ***et al.,*** 1990). This suggests that cell populations which overgrow should eventually encounter an environment where growth factors are limiting for some of the cells, which results in apoptosis, thereby maintaining the original population size. However, certain genes, such as ***bcl-2,*** can block apoptosis, resulting in a survival signal which keeps the additional numbers of cells alive. This appears to be an early step in tumour progression, as demonstrated in ***bcl-2*** transgenic mice which have an expanded B-cell population, the increased numbers of which heighten the chances of other oncogenic mutations occurring, resulting in clonal malignant outgrowth (McDonnell ***et al.,*** 1989: McDonnell and Korsmeyer 1991). Therefore, genes which actively suppress apoptosis can provide an environment which is suitable for the acquisition of further malignant mutations (Askew ***et*** *al.,* 1991).

The first gene **to** be identified with homology to ***bcl-2*** was the Epstein Barr virus gene BHRF1, the two genes sharing 25% primary amino acid sequence homology. This suggested that BHRFl may possess the ability to suppress apoptosis much like ***bcl-2,*** and for this reason, the effect of both ***bcl-2*** and BHRF1 on chemotherapeutic drug induced cell death was investigated. The anti-cancer drugs and y-irradiation were chosen as a death stimulus primarily because of a paper published by Tsujimoto (1989) which suggested that ***bcl-2*** was able to suppress chemotherapeutic drug induced and radiation induced cell death. This suppressive effect could represent a novel form of multidrug resistance, if shown to occur in response to a variety of chemotherapeutic agents.

Discussion

In this study, induction of apoptotic cell death by anti-cancer treatments has been demonstrated in an **IL-3** dependent cell line as well as three EBV positive Burkitt's lymphoma cell lines. The morphological examination of the cells was carried out using both light and electron microscopy and evidence for the characteristic endonuclease activation resulting in fragmentation of the **DNA** was shown by agarose gel electrophoresis. Expression of either ***bcl-2*** or BHRFl in the EBV-BL lines produced suppression of apoptosis on treatment with several chemotherapeutic agents and y-radiation, as determined by vital dye exclusion. These results have broadened the stimuli for which BHRFl acts as an apoptotic suppressor, resistance to serum withdrawal and treatment with calcium ionophore having been previously shown (Henderson ***et al.,*** 1993). The results further illustrate that the suppression of apoptosis produced by either ***bcl-2*** or BHRF1 can produce a novel form of drug resistance. The ability of BHRF1 to suppress apoptosis also has implications for EBV infection and virus production in human cells.

Although ***bcl-2*** was the first gene identified with the ability to suppress apoptosis, it was not thought to be the only gene able to produce this effect for several reasons, including the fact that ***bcl-2*** is unable to prevent cell death in response to all apoptotic signals so far investigated. Indeed, three new cellular genes which have homology to ***bcl-2*** have recently been identified (reviewed in Williams and Smith 1993). The identification of genes with homology to ***bcl-2,*** or of other genes which are able to suppress apoptosis, is important since these genes may represent early genetic mutations essential for the successful progression of a cell along the malignant pathway. In this study a protocol has been designed to identify **E-3** dependent cells which are blocked from entering apoptosis so that new genes which act within the apoptotic pathway can be identified.

Section 7.2 Transfection of FDCP-1 cells with *bcl-2* or BHRF1

The EBV gene BHRFl was one of the first genes to be identified with homology to ***bcl-2,*** and now forms part of an extending ***bcl-2*** family (Williams and Smith 1993), which includes ***bcl-x*** and *bax* (Boise ***et al.,*** 1993; Oltvai ***et al.,*** 1993). BHRFl is expressed primarily in the lytic cycle of EBV infection with some expression in the latent cycle after cells have been recultured after serum starvation (Kocache and Pearson 1990). BHRF1's homology to

bcl-2 was thought to reflect the fact that it was expressed in the lytic cycle and may protect against cell death during viral infection, a hypothesis which was supported by the discovery of a baculovirus gene product, p35, which suppressed insect cell apoptosis during infection (Clem ***et al.,*** 1991). This suggested that some viral genomes may have evolved genes which suppress cell death during infection and that induction of cell death by the infected host cell may represent a primitive cell defence mechanism. Several other viral genes have now been identified which suppress cell death during infection (Nielan ***et al.,*** 1993; White ***et al.,*** 1992), indicating a widespread viral mechanism of celi death suppression potentially aimed at prolonging viral replication.

Since a role for BHRFl in the EBV lytic cycle had been suggested, it was very important to determine whether or not BHRFl was functionally homologous to ***bcl-2.*** **A** BHRFl expressing construct was produced by Dr. David Huen (Dept of Cancer Studies, Birmingham) which could be expressed in several cell lines including the factor dependent FDCP-1 cell lines. **A** ***bcl-2*** construct (a gift from Y. Tsujimoto) was used to compare the effects of FDCP-1 cells transfected with ***bcl-2*** with the effect of BHRFl transfected cells, enabling a direct comparison of the functional homology. The FDCP-1 cell lines were initially used as hosts for the ***bcl-2*** and BHRF1 constructs for several reasons, the primary one being that ***bcl-2*** had already been shown to suppress apoptosis on withdrawal of IL-3 in these cell lines (Vaux ***et al.,*** 1988). Therefore, if BHRFl was functionally homologous to ***bcl-2,*** the phenotypic effect should be the same on withdrawal of **IL-3** and easily identifiable.

Although one of the objectives of this study was to examine the potential functional homology between BHRF1 and ***bcl-2,*** the primary objective was to determine whether or not suppression of apoptosis by ***bcl-2*** or BHRFl, was able to induce drug resistance in response to anticancer treatments. ***As*** mentioned above, the main impetus for this study came from a paper by Tsujimoto (1989) which described stress resistance induced by ***bcl-2*** in response to a variety of treatments including methotrexate, suggesting that ***bcl-2*** suppressed chemotherapeutic drug induced cell death. Drugs such as methotrexate, araC, etoposide and hydroxyurea have been shown in this study to induce apoptosis in the FDCP-1 cell lines (see

section 5.1), so any delay in the onset of cell death caused by *bcl-2* or BHRF1 in this cell line was likely to be due to the suppression of apoptosis.

The initial investigation to examine the possibility of functional homology between *bcl-2* and BHRF1 expressed in the FDCP-1 cells, was by induction of apoptosis on removal of IL-3 (Williams *et al.*, 1990). The phenotypic effect of exogenous *bcl-2* expression in FDCP-1 cells on removal from the cytokine is a survival in G_0 of the cell cycle (Vaux *et al.*, 1988; Fairbairn *et al.*, 1993) and for cells expressing BHRF1 this was also the predicted phenotype.

Cells transfected with either BHRF1 or *bcl-2* expressing constructs were removed from IL-3 for 2-3 days and then viability was compared to control transfectants and untransfected FDCP-1 cells. Approximately *60* million cells were electroporated in the presence of either the *bcl-2* plasmid construct or the BHRF1 construct and no cells were produced which expressed the genes at a level sufficient to suppress apoptosis in the absence of IL-3 (see section **5.2).** There are several reasons why this may have occurred.

As mentioned above, *bcl-2* has been expressed in these cell lines before (Vaux *et al.*, **1988),** however, the transfection of the *bcl-2* construct was not by electroporation into the FDCP-1 cells, but involved using a retroviral construct. The use of viral transfection in general ensures that the gene of interest is effectively integrated within the genome of the host cell and this results in the stable expression of a copy or copies of the gene. There is also a high efficiency of gene transfer into the target cell mainly due to the exploitation of the retrovirus life cycle. Retroviral RNA encoding the gene of interest is turned into DNA by the viral gene reverse transcriptase. The resulting viral DNA circularises and integrates into the host cell genome, aided by a viral DNA site-specific recombinase (Varmus 1988). Interestingly, much of the work in FDCP-1 cells which has involved expression of other genes such as *myc* (Cleveland *et al.*, 1990) and raf (Rapp *et al.*, 1990) has also been produced by using retroviral constructs. It is probable that to attain efficient expression of a gene within FDCP-1 cells retroviral expressors are most efficient.

Transfection of plasmid constructs by electroporation can result in a low yield of cells expressing the transfected gene (~0.1% Frohman and Martin 1989), but this is often off-set by the simplicity of electroporation in comparison to retroviral infection. Retroviral infection of

cells involves the use of a defective viral construct, containing the gene of interest, which **is** unable to efficiently replicate itself due to the absence of virus capsid antigens. The virus can only be expressed in a virus packaging cell line and viral infection of the cells of interest is attained by coculture with the lethally irradiated packaging cell line which expresses the virus (Leslie Fairbairn, personal communication). Cells are selected in antibiotic after virus infection since cells which have incorporated the plasmid will be resistant to the antibiotic by virtue of a resistance gene present in the construct. The transfected cell line must be checked to make sure that the virus construct has not mutated and is being expressed from the cells. This possibility, along with the influence of the viral genes in the construct, increases the risk that the gene could be efficiently expressed in other organisms. Since **I** had not previously worked with retroviral constructs together with the fact that electroporation is a safer method, retroviral transfection was considered, but decided against.

A second point to be made concerning the ***bcl-2*** construct used in the FDCP-1 cells is that the construct is based on an EBV plasmid. Cells infected with EBV can express the virus in an episomal form. EBV genes can be expressed from the episome as long as the infected cell expresses EBNA 1. Expression of this gene is necessary to maintain the episomal state and to act as an enhancer for the latency origin of replication. EBV infected cells, which express EBNA 1, electroporated with constructs based on EBV plasmids are therefore able to maintain the plasmid in the episomal form and express genes from it without requiring successful integration into the host cell DNA. Non-EBV plasmid constructs which are electroporated into cells lose this advantage in that once in the cell they must integrate within a actively expressed area of the host cell genome. This introduces another reduction in the chances of attaining an expressible integrated version of the gene. Since the FDCP-1 cells do not express EBNA 1, the episomal form the plasmid cannot be maintained and so to gain expression of the ***bcl-2*** gene successful integration and expression of the gene must occur within the host cell genome. Therefore, this reduces the probability of obtaining clones which efficiently express the gene.

The **BHRF1** construct used in the FDCP-1 cells, engineered by Dr. David Huen, was not based on an EBV construct, and therefore relied on normal host cell integration after

electroporation. Although not successfully expressed in the FDCP-1 **cell** lines, a similar construct was expressed in EBV positive BL cell lines *(S.* Henderson Ph.D. thesis 1992) and expressed a truncated version of the BHRF1 protein which suppressed apoptosis on culturing the cells in low serum concentrations. The reason for expression of the truncated protein was not clear, however a second recombinant plasmid based on the pHebo plasmid, which can be episomally maintained in EBV infected cells, was engineered with the human growth hormone gene, present in the first construct, replaced by an intron from the ß-globin gene. This construct, when successfully expressed in the same EBV-BL cells resulted in the full length expression of the BHRFl protein (Henderson ***et al.,*** 1993). It is unclear whether or not the truncated expression of BHRFl produced inefficient expression of BHRFl in the FDCP-1 cells. Since BHRF1 and ***bcl-2*** were successfullyexpressed in the EBV-BL cell lines, analysis of the functional homology between the two genes and the ability of the genes to suppress chemotherapeutic drug induced apoptosis was transferred to these cell lines. As a final point to this section, Dr Graham Cowling (personal communication) has also reported that the FDCP cell lines as a whole are difficult to transfect successfully.

Section 7.3 Protocol for the selection of apoptotic mutants

Removal of FDCP-1 cells from IL-3 for **7** days can often result in some cells which appear viable as determined by vital dye exclusion **(N. J.** McCarthy, B.Sc. Thesis 1990). When such cultures are re-cloned on soft agar containing IL-3, surviving clones can be obtained as shown in section 5.3. However, these clones were not representative of the cell types which were predicted to exist i.e., either IL-3 independent cells - cells which secrete their own IL-3 or have another mechanism producing auto stimulation (Cook ***et al.,*** 1985; Askew ***et al.,*** 1991), or apoptotic mutants - cells which are unable to enter apoptosis and remain in a G_0 state for a prolonged period of time, much like a ***bcl-2*** expressing phenotype in FDCP-1 cells. Although several survivor clones, once isolated from the plates, were grown up in liquid culture, none of these showed any enhanced survival in the absence of IL-3 (see FDCP-1-δ-clone and survivor FDCP-1-B-clones, section 5.3.2, table 5.9). **As** mentioned briefly in results, FDCP-1 sub-clones showing some enhanced survival on **IL-3** withdrawal were previously isolated simply by cloning the cells using limiting dilution in the presence of IL-3 **(N.** J.

McCarthy, **B.**Sc. thesis). Interestingly, these sub-clones lost their enhanced survival capacity after **3-4** weeks in normal culture conditions, suggesting that the growth of cells in conditions which are not optimal, such as the growth of a single cell devoid of other cell to cell contacts, causes epigenetic effects leading to a temporary change in cell phenotype. This could also explain the small enhancement of survival seen in the FDCP-1-B survivor clones on the first analysis, which was not repeated on re-analysis several weeks later (see table 5.9). The clones isolated in the present study which survive **IL-3** deprivation also appear to represent cells within the FDCP-1 population which can adapt temporarily to lack of growth factor and may reflect the fact that FDCP-1 cells already have a high rate of self-renewal. Although these cells are not fully transformed i.e., do not produce tumours when injected into mice, they may be sufficiently transformed to enable adaptation in growth limiting environments. One such adaptation maybe the survival, but not proliferation, of cells produced by other growth factors present in the FCS, which is not removed in **IL-3** deprived cultures. Such a phenotype could be lost on re-culturing in **IL-3.** This hypothesis could be tested by culturing cells in the absence of **IL-3** and FCS, or low level FCS to see if any clones survive after 7 days. This is an important consideration for the full screening protocol, since the results presented in this study show a small proportion of cells survive the protocol without any block in the apoptotic pathway, while appearing to have the desired phenotype. However, if these cells do exist due to the above reasons, then they can also be screened out after using the selection protocol. After a period of culture in the presence of **IL-3,** the removal of **IL-3** from these cells should result in cell death within **72** hours, as has been seen for the survival clones analysed so far, and any real apoptotic mutants should lose viability at a much slower rate.

Although the characterisation of **IL-3** independent cells was not important in the development of the screening protocol, the isolation and determination of the numbers of cells with this phenotype was required, especially since such cells had been previously described to occur in this cell line by several groups (Cleveland ***et al.,*** 1989; Askew ***et al.,*** 1991). The **IL-3** dependent cells were predicted to be the most frequent within the FDCP-1 population, large numbers of which would have made the identification of apoptotic mutants difficult, since they were expected to occur at a lower frequency. High numbers of **IL-3** independent cells would

also have caused problems when mutagenising the cells because the numbers of **IL-3** independent cells would have increased significantly. However, the frequency of clones able to survive for 7 days in the absence of **IL-3** was very low, around **5** per million in FDCP-1 and FDCP-1-B cells and **15** per million for FDCP-1-δ. Analysis of some these clones, as described above, did not reveal any **IL-3** independent cells. Therefore, these results indicate that the levels of spontaneous **IL,-3** independent mutants were of a sufficiently low frequency i.e., below 15 per million, to allow the identification of apoptotic mutants.

The absence of cells with the **IL-3** independent phenotype from the experiments carried out in this study is probably due to the fact that the cells were not placed repeatedly in **IL-3** deprived conditions, spontaneous mutants having been produced by continually re-challenging the cells to **IL-3** deprived conditions (Cleveland ***et al.,*** 1989). This suggests that **IL-3** independent cells will only be isolated from the protocol once cells have been repeatedly removed from **IL-3.** However, the repeated selection of cells in H^3TdR should minimise this.

A small number of clones, **2** from 70 million FDCP-1-B cells and 10 from 70 million FDCP-1-6 cells were present on soft agar plates after subjecting the cells to the full selection protocol, including H^3TdR selection (suggesting that the isolated clones were not in cell cycle when in the absence of **IL-3** and therefore not **IL-3** independent). However, H^3TdR selection only kills 80% of cycling FDCP-1 cells in the presence of **IL-3,** therefore 20% of cells are likely to survive this selection protocol suggesting that selection is useful, but not absolute. The few clones which were harvested from the plates did not grow for a prolonged period in normal liquid culture conditions, suggesting that the cells which survived this protocol were not stable mutants and proved to be non-viable when cultured further, Overall, these results demonstrate that the level of apoptotic mutants occurring spontaneously in FDCP-1 cells is likely to be too low to efficiently isolate and characterise new genes involved within the apoptotic pathway, but isolation of such mutants produced by mutagenesis should be possible using this protocol.

An alternative method for isolating apoptotic mutants of FDCP-1 cells was to actively mutate them, increasing the numbers of mutants present. Cells could be treated with N-methyl-**N-nitro-N-nitrosoguanine** (MNNG) and the cells subsequently run through the selection

protocol as described in section **5.3.2.** However, although useful mutants would undoubtedly be produced by this method, it would be very difficult to identify which gene(s) have been mutated. For example, if an FDCP-1 apoptotic mutant was produced by **MNNG** mutation, the gene involved would have to be identified, either by screening for a variety of possible genes, such as ***bcl-2,*** or if the gene was unknown, by comparing **DNA** libraries from normal cells and mutant cells deprived of IL-3. In addition, many mutations, such as point mutations will not be detected using this method. However, if the cells were mutagenised by the process of insertional mutagenesis, then the gene(s) affected would be marked (Farzaneh and Gaken 1991; Stocking ***et al.,*** 1988, 1993). Insertional mutagenesis works on the principle of placing the cell lines of interest in contact with a virus, produced by a virus packaging cell line. The virus is normally a defective virus, no longer able to fully replicate due to the inability to produce virus capsid antigens (as described for retroviral infection of ***bcl-2*** into FDCP-1 cell lines, section 7.2). Defective expression of the viral envelope is also useful because its presence results in a low number of integrations per cell by preventing re-infection of the cell with additional copies of the virus. Once a virus enters the cell, it copies its **RNA** to **DNA** by the use of reverse transcriptase and integrates in the host cell **DNA** (Varmus 1988) and this happens for several virus genomes per cell. The mutagenic effect occurs when the virus infects a cell and integrates within a gene of interest, either activating it or disrupting it, to produce the desired mutant phenotype. With this system, however, the mutated gene **is** easy to identify because the gene will be marked by the presence of viral enhancer elements found in the long terminal repeat sequences of the viral DNA, which can be identified by southern blotting. Therefore the genetic area in which the mutation has occurred can be identified, proving especially useful if the mutated gene(s) is unknown. If the mutation required to produce the desired phenotype is a recessive somatic mutation i.e., the mutant phenotype is a loss of function mutant, then two mutation events must occur in order to mutate or knockout both copies of the gene, unless the gene exhibits functional haploidy. This mutant phenotype therefore occurs less often because of the two mutation events required. Gain of function mutants are more frequently the result of insertional mutagenesis because only one copy of the gene needs to be deregulated to produce the phenotypic effect (Farzaneh and Gaken 1991).

Based on the reasons discussed above, insertional mutagenesis was the method used to produce mutant FDCP-1s and was carried out in collaboration with Dr. Farzin Farzaneh (Rayne Inst. London). At first, the mutation of the FDCP-1s was problematic since they were difficult to infect to a high level with the ecotropic virus for which they have receptors. This problem of low level infection was solved and mutant clones of the FDCP-1-B cells have been produced. However, the initial delay in infection meant that the cells were not mutated in time for me to analyse the mutant clones using the devised protocol.

One would predict that some apoptotic mutants would be present after mutagenesis and that not all of the mutants would have deregulated ***bcl-2*** genes- the most obvious possibility to produce the desired phenotype. In the light of the recent papers on the ***bcl-2*** family (Boise ***et al.,*** 1993; Oltvai ***et*** *al.,* 1993; Kozopas ***et al.,*** 1993) it seems likely that a mutant phenotype could be produced by mutating ***bcl-x*** or possibly MCL-1, a ***bcl-2*** homologue found in myeloid cells for which functional homology with ***bcl-2*** has yet to be established. The discovery of these ***bcl-2*** homologues also indicates that other molecular suppressors of apoptosis are likely to exist, some probably without homology to ***bcl-2.*** The identification of these genes may be very important in highlighting those which are involved in the early mutagenic events of oncogenesis.

Section 7.4 Bcl-2 and BHRF1 are functionally homologous and able to suppress apoptosis induced by anticancer drugs and irradiation

Since both BHRFl and ***bcl-2*** had also been transfected into EBV positive Burkitt's lymphoma B-cells, using **EBV** based constructs, the examination of functional homology between ***bcl-2*** and BHRFl was carried out using these celi lines.

When first recorded by Dennis Burkitt (1958) the endemic nature African Burkitt's lymphoma the cancer was a puzzle. However, Epstein suggested that a virus may well be associated with the disease and once a BL cell line was established ***in vitro,*** electron microscopy revealed the presence of numerous virus particles within the cells. The virus was identified as a gammaherpesvirus and named Epstein-Barr virus after Epstein himself and his colleague Yvonne Barr (Epstein 1985). Although not the only factor involved in BL, EBV is able to transform B-cells ***in vitro*** producing lymphoblastoid cell lines (LCLs) which

characteristically express eight EBV latent genes; EBNAs 1, 2, 3a, 3b, 3c, LP, **LMP** 1 and 2, suggesting that certain viral genes may contribute to the cancer. Indeed, **LMP** 1 has been shown to be tumorigenic in nude mice (Wang ***et al.,*** 1985). In addition to EBVs ability to express its own genes within the host, it can also upregulate certain host cell genes producing a highly transformed phenotype e.g., LCLs express high levels of the B-cell activation antigens CD23, CD30, CD39 and CD70 (Thorley-Lawson and Mann, 1985) as well as the cellular adhesion molecules LFA-1, ICAM-1 and LFA-3 (Gregory ***et al.,*** 1988). Interactions between LFA-1 and ICAM-1 are thought to result in the characteristic cell clumping seen during the tissue culture of LCLs. Fresh biopsy cells, however, when first cultured ***in vitro,*** only express one EBV latent gene, EBNA 1, and two tumour markers (CD10 and CD77). The cells also lack detectable expression of a wide range of B cell activation antigens e.g., (CD21, 23, 30, 39, 70, BB1 and G28.10) and adhesion molecules ICAM-1 and LFA-3, but show some expression of LFA-I. These cells are known as group I BL cell lines (Gregory ***et al.,*** 1990). If such cells are subsequently passaged ***in vitro,*** then drifting of the phenotype is seen producing cell lines much like LCLs. These cell lines are known as Group III cell lines and express all eight latent genes as well as activation antigens and cell adhesion molecules as described for LCLs. Some cells show an apparent intermediate phenotype, expressing both CD10 and CD77 as well as some **of** the B-cell activation adhesion/antigen molecules, but further passages result in attainment of the group III phenotype (Gregory ***et al.,*** 1990). As well as differing in the expression of both EBV latent genes and B-cell activation antigens/adhesion molecules, group I and III cell lines differ in their sensitivity to apoptotic stimuli, the former being highly sensitive and the latter very insensitive (Gregory ***et al.,*** 1991). These differences were primarily thought to be due to the expression LMP 1 in group III cells, which is able to upregulate host cell ***bcl-2*** expression and suppress apoptosis (Henderson ***et al.,*** 1991). However, it would appear that LMP 1 may not be able to cause the upregulation in ***bcl-2*** expression since this effect is not seen in other cell lines in which expression of LMP1 has been analysed (B. Sugden, personal communication).

Since group III cell lines already express ***bcl-2,*** these in general were not used to express either ***bcl-2*** or BHRF1. However, one group III cell line, Raji-BL, does not express

bcl-2. The reason for this is unclear, but the Raji EBV genome has mutations found in both the EBNA 3c gene and the LMP 1 gene, possibly affecting EBV's ability to unpregulate host cell *bcl-2* efficiently **(S.** Henderson, personal communication). Therefore, Raji-BL remains sensitive to apoptosis and was used to express the BHRF1 construct. The other two cell lines used were Akata- and Chep-BL both of which are stable group I cell lines and are sensitive to apoptotic stimuli. Interestingly, Akata-BL does express low levels of *bcl-2* and although this makes the cell slightly more resistant to certain apoptotic stimuli, it does not prevent the cells from dying when placed in low serum concentrations (Henderson ***et al.,*** 1993).

The BL cell lines used in this study had been shown to enter apoptosis in low level serum and in response to calcium ionophore (Gregory ***et al.,*** 1991; Henderson ***et al.,*** 1991, 1993), but, had not been incubated with chemotherapeutic drugs. Apoptosis was clearly demonstrated to occur in response to methotrexate, etoposide and araC as well as y-radiation (see section ***6.2).*** Suppression of cell death in response to methotrexate and etoposide was clearly shown in Chep-BL cells expressing *bcl-2* and Raji and Akata-BL cells expressing BHRF1. Suppression of apoptosis in response to y-radiation was also evident in Chep-BL cells expressing *bcl-2* and Raji-BL cells expressing BHRF1 when compared to controls. Akata-BL cells proved to be resistant to treatment with y-radiation **(up** to 30Gy) and therefore could not be used to examine the resistance produced by the expression of **BHRF1,** Methotrexate is also relatively well tolerated, being at a higher concentration, **100μM,** in Akata-BL cells, whereas it is only used at **10μM** in Raji-BL cells to induce a comparable –60% loss in viability, **48** hours post drug treatment. Akata-BL cells also show an inherent resistance to high concentrations of araC, no real loss of viability being evident after treatment with **100μg/ml**. Therefore, in response to certain anticancer treatments, Akata-BL show greater resistance when compared to Chep and Raji-BL. Why Akata are resistant to these stimuli is not clear, but suppression of apoptosis in EBV cells may be mediated by as yet unidentified mechanisms which are independent of *bcl-2* expression (Milner ***et al.,*** 1992). **A** direct comparison of group I Mutu-BL lines with group III Mutu-BL lines demonstrated that the levels of *bcl-2* expressed in group III cells did not account fully for the resistance seen to apoptotic stimuli such as serum withdrawal, anti Ig antibodies or ionomycin. **A** comparable level of resistance in group I cells

transfected with ***bcl-2*** plasmids is only seen when ***bcl-2*** is over expressed five fold above the levels of expression found in group III cells. Therefore, group III cells exhibit a degree of apoptotic suppression which is ***bcl-2*** independent and may be attributable to EBV's ability to upregulate other host cell genes which are as yet unidentified. Prolonged passage of group I cells (around p240-250 compared to p40-50) can also produce resistance to anti Ig antibodies without a concomitant upregulation of ***bcl-2.*** Exposure of group I Mutu-BL to IFN ***a*** can also suppress apoptosis in response to calcium ionophore, anti-Ig antibodies and serum depravation without any upregulation in ***bcl-2*** expression being apparent. Whether or not the ***bcl-2*** independent suppression mechanism could be due to upregulation of a ***bcl-2*** homologue such as ***bcl-x***$_L$ remains to be seen.

Chep-BL cells on the other hand have a greater sensitivity to the presence of chemotherapeutic drugs and y-radiation. For example, substantial cell death was evident after treatment with ***8Gy*** of y-radiation compared to 16Gy needed to induce a comparable amount of cell death in Raji-BL cells. The effect of ***bcl-2*** expression on araC induced cell death in Chep-BL cells was interesting in that the cells showed around a 60% loss in viability post drug treatment, and although suppression of cell death was evident at 48 hours post treatment, the difference was not statistically significant at the ***5%*** level. The reason for this is unclear, but it does not seem to be the result of higher levels of toxicity produced by araC, since the overall loss in membrane integrity at 48 hours is less for araC than for either methotrexate or etoposide. The morphological appearance of the cells after 14 hours incubation with araC is not classically apoptotic, however, it may be that the cells had already progressed through apoptosis by 14 hours and had started to undergo secondary necrosis. In the light of the above results it would seem unlikely that araC induces necrosis in the Chep-BL cell lines, so this does not explain why ***bcl-2*** is unable to significantly inhibit araC induced cell death in this cell line. **A** protection from araC induced cell death by Bcl-2 has been shown in S.49, WEHI 7.2 and 697 pre-B-leukaemia cell lines (Miyashita and Reed 1992, 1993). However, some cell lines transfected with ***bcl-2*** can show no resistance to a specific drug, whereas another cell line transfected with ***bcl-2*** **is** resistant to the drug. For example, in this present study and **in** the work of Miyashita and Reed (1992), resistance to methotrexate in a variety of ***bcl-2***

transfected cell lines has been shown. However, resistance to methotrexate is not seen in small cell lung cancer cell lines expressing exogenous ***bcl-2,*** but resistance to other drugs such as adriamycin is (Ohmori ***et al.,*** **1993).** Such conflicting results could possibly reflect differing cellular responses to chemotherapeutic drugs which occur due to the transformed nature of the cell lines themselves.

Evidence of DNA fragmentation in response to treatment with both cytotoxic drugs and y-radiation proved to be elusive, but was demonstrated in the Raji-BL cells (see chapter 6, figures 6.5 and ***6.6).*** Fragmentation of the DNA in Akata and Chep-BL was not detected either by analysis of genomic DNA or by separation of low molecular weight DNA by centrifugation using as many as **107** cells. Endonuclease activation producing oligonucleosomal DNA fragments in Chep-BL cells during incubation in low serum concentrations has been shown by agarose gel electrophoresis (Henderson ***et al.,*** **1991),** but only after sampling 10^8 cells and harvesting the low molecular weight DNA by Centrifugation. Whether the absence of a detectable DNA ladder is due to asynchronous apoptosis or relatively little fragmentation to low molecular weight DNA is not clear. For some cell lines which morphologically go through apoptosis, DNA fragmentation is not always evident (Cohen ***et al.,*** **1992),** possibly due to limited cleavage of the DNA into high molecular weight fragments (Brown ***et al.,*** **1992;** Tomei ***et al.,*** **1993).** This implies that not all cells which die by apoptosis necessarily have to fragment their DNA to the extent documented in thymocytes on treatment with glucocorticoid. Destruction of the DNA so that it becomes unreadable by RNA polymerase is hypothetically the most important outcome of endonuclease activation and may explain why not all cells fully fragment their DNA, but do cut the DNA into higher molecular fragments, which are not visible on agarose gels. The other possibility is that the BL cell lines enter apoptosis very asynchronously and therefore to detect any fragmentation a large number of cells need to be sampled and low molecular weight DNA separated from high molecular weight DNA by centrifugation. **This** was the method by which fragmentation produced by cytotoxic drugs in Raji-BL cells was detected, but did not solve the problems of detecting fragmentation in either Akata or Chep-BL cells.

Discussion

The results presented in chapter ***6*** clearly demonstrate that the BL cell lines Raji, Chep and Akata undergo death by apoptosis in response to chemotherapeutic drugs and y-radiation, which can be suppressed by the expression of transfected ***bcl-2*** or BHRF1. These results also demonstrate that ***bcl-2*** and BHRF1, as well as being able to inhibit apoptosis induced by serum withdrawal and calcium ionophore (Henderson ***et al.,*** 1993), can also suppress apoptosis induced by anti-cancer treatments, thus extending the boundaries of their functional homology.

Although suppression of apoptosis **is** evident **48** hours post drug or y-radiation treatment, whether or not the BHRF1 or ***bcl-2*** can keep the surviving cells alive indefinitely is not clear. ***An*** attempt to address this point was made by analysing colony formation in soft agar after treatment of the cells with low levels of y-radiation (1Gy). Although control Raji-BL transfectants would plate in soft agar with an efficiency of ***~5%,*** this was very rarely repeatable and was partly dependent on the cells having been cultured in the absence of the selection antibiotic hygromycin for at least 72 hours. Neither Akata or Chep-BL cells would form colonies on soft agar. Hence the actual survival state of these cells after anti-cancer treatment is still undetermined. Similar results have been documented for the Mutu-BL cell lines (Fisher ***et al.,*** 1993). Colony formation in 697 cells (pre-B-cell leukaemia line) transfected with ***bcl-2*** was assessed after treatment with dexamethasone, methotrexate and vincristine (Miyashita and Reed, 1993). ***bcl-2*** transfected cells treated with dexamethasone retained the ability to clone (53%) when compared to untreated controls, however, colony formation was significantly reduced for 697-Bcl-2 cells treated with 10μM methotrexate ***(6%)*** and completely abolished for 697-Bcl-2 cells treated with vincristine. Differences in the toxicity of the drugs was not thought to be responsible for this result. All ***bcl-2*** transfected cells were resistant to the above drugs as determined by vital dye exclusion, indicating that this measure of cell viability does not reflect colony forming ability. Although the results of Miyashita and Reed are limited in their relevance to all anti-cancer drugs acting on many different cell lines, they suggest that ***bcl-2*** cannot block apoptosis in response to all chemotherapeutic drugs to the degree of maintaining colony forming ability. Therefore

suppression of apoptosis may prove to be only one factor involved in multidrug resistance (see below).

Section 7.5 BHRF1 and *bcl-2* suppress chemotherapeutic drug induced apoptosis-implications for multidrug resistance

The question of whether or not *bcl-2* or BHRF1 induce increased cell survival or simply delay cell death is important when addressing the ability of such genes to produce drug resistance. Several recent papers have documented suppression of chemotherapeutic drug induced cell death produced by the deregulated expression of *bcl-2* (Fanidi *et al.,* 1993; Miyashita and Reed 1992, 1993; Collins ***et al.,*** 1992; Walton *et al.,* 1993; Fisher *et al.,* 1993), many of whom have suggested that this represents an alternative form of multidrug resistance. The basis for this argument was that the documented mechanisms of drug resistance (as outlined in section 3.7) could not realistically be induced simultaneously by simply upregulating the expression of one gene, i.e., ***bcl-2.*** For example, in the present study, three drugs were used, methotrexate, araC and etoposide, all of which have different documented classical resistance mechanisms, i.e., either target enzyme increases (dihydrofolate reductase in methotrexate resistance, increases in amount of topoisomerase II and in its activity in etoposide resistance (Zwelling *et al.,* 1989) or decrease in drug activation (araC is converted into araCDP and araCTP in order to be incorporated into the DNA, absence of an enzyme araC-kinase inhibits this producing resistance (Drahovskyand Keis, 1970). It would be highly surprising, although not impossible, for the inappropriate expression of *bcl-2,* or indeed BHRF1, to result in all these effects. There is, however, a more realistic possibility, i.e., that ***bcl-2*** has an effect on the multidrug resistance gene ***mdr1.*** This human gene codes for a 170kDa protein product which is an energy dependent transport protein. In some human cancers, such as liver and kidney, this protein, known as p170, is highly over expressed resulting in the active removal of drugs such as the vinca alkaloids, epipodophyllotoxins and anthracyclins from the cytoplasm. Because of this rapid removal, drugs never reach toxic levels within the cell and so the cells are effectively drug resistant. p170 is only active on naturally occurring cytotoxic drugs such as those listed above, but since many of these drugs

are used in chemotherapy regimes against many human cancers, this form of multidrug resistance can produce very real problems for clinical treatment.

Although many cell lines have been produced ***in vitro*** with **MDR1** over expression by continuous growth in the presence of MDR1 associated drugs, over expression of MDR1 in clinical specimens has only recently been characterised (Goldstein ***et*** al., 1988). Cancers which show high levels of MDR1 expression are commonly those of kidney, adrenal gland, colon and liver, presumably because non-cancerous cells from the corresponding normal tissues have been shown to express the MDR1 protein. In general, these cancers are resistant to chemotherapy (Fojo ***et*** al., 1987). The physiological role of MDR 1 is not clear, but it may act as a detoxification system, hence its high level expression in the liver and kidney (Weinstein ***et*** al., 1990).

Not all drug resistant tumours however, whether exhibiting primary (intrinsic) or secondary (acquired) drug resistance, can be explained by the MDR1 mechanism. MDR1 expressing cells have mainly been studied ***in vitro,*** with drug resistant cell lines being produced by exposing cells to progressively higher concentrations of an MDR1 drug. This can result in cell lines which exhibit resistance which is 100 times greater than any documented ***in vivo.*** Therefore, although ***in vitro*** studies have identified the mechanism behind MDR1 resistance, ***in vivo*** studies have proved somewhat inconclusive as to which cancers are drug resistant due to MDR1 over expression. As mentioned above MDR1 expression in cancers of the liver, kidney, adrenal glands and the colon is well documented, but its relevance to leukaemia/lymphoma drug resistance is less clear.

p170 expression is found in leukaemias such as acute myeloid leukaemia ***(AML),*** chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia **(CML)** in blast crisis, but its expression does not always correlate with the type of drug resistance which is observed.

In CML for example, cells can acquire resistance to hydroxyurea when the disease enters the stage of blast crisis. In cells at this stage the MDR 1 gene is expressed, but only at levels which are comparable to cells which remain drug sensitive (Weide ***et*** al., 1990). It may be that the drug resistance found in blast crisis is due in part to the constant activation of ***abl***

produced by the t(9;22) translocation, resulting in the ***bcrlabf*** fusion protein found in almost all cases of CML (de-Klein et al., 1982). Expression of a temperature sensitive mutant of v-abl in myeloid cells has shown that expression of the gene produces very high levels of drug resistance to busulfan (C. Dive, personal communication) and hydroxyurea (R. Chapman, personal communication). This implies that a constant activity of abl is able to suppress apoptosis, a factor which was unknown when the MDR analysis was carried out by Weide and colleagues in 1990. Although MDR1 expression may produce resistance to certain drugs in CML cells it does not seem to be responsible for the drug resistance which is observed during blast crisis. The resistance produced by v-abl to chemotherapeutic drugs now suggests that the bcrlabl fusion gene could be involved in drug resistance in CML.

MDR1 expression in patients with CLL shows no defined pattern which correlates with either stages of the disease or acquisition of drug resistance due to previous treatment with MDR1 associated drugs, indeed some patients have high levels of MDR1 expression at diagnosis (Shustik e? al., 1991). The facts which lead Shustik and colleagues to draw the above conclusions were that no gain of MDR1 expression was seen during chemotherapy for patients who were MDR1 negative at the start of treatment, but MDR1 expression was lost by some patients who had MDR1 positive CLL cells at the start of treatment. Expression of MDR1 was lost either during treatment with chlorambucil,a drug not affected by the presence of MDR1, or spontaneously in the absence of treatment.

The work described above indicates that multidrug resistance may not be explained solely by the over expression of MDR1, indeed it is now thought that drug cross resistance is most likely to be multi-factorial. This implies that there are other unknown mechanisms involved in drug resistance which could be explained in part by the ability of genes to suppress apoptosis in response to chemotherapy.

This present study has shown that the expression of either *bcI-2* or BHRFl in EBV-BL cell lines will cause drug resistance to methotrexate, etoposide and araC, but whether this is independent of known resistance mechanisms described above, cannot be fully addressed. However, since etoposide is the only drug affected by MDR1 expression used in this study and

resistance to methotrexate, araC and y radiation is also seen, it is probable that *bcl-2* and BHRF1 cause a novel form of multidrug resistance.

A paper which addresses the role of ***bcl-2*** in drug resistance directly has recently been published in which ***bcl-2*** produces drug resistance in response to, amongst others, 5-fluorodeoxyuridine (Fisher ***et al.,* 1993).** This drug inhibits the thymidylate synthase (TS) enzyme, which is important in the methylation of dUMP to TMP, causing a reduction in TTP pools. Inhibition of TS also produces **DNA** strand breaks due to the mis-incorporation of dUTP into the **DNA** and the incorporation of FdUTP into the **DNA.** Since the levels of TS are easy to identify and the pharmacological mechanisms of FdUrd toxicity are established, the effect of ***bcl-2*** expression on these parameters can be analysed. ***bcl-2*** was expressed in EBV Mutu-BL group I cell lines using the Tsujimoto plasmid construct (Milner ***et al.,* 1992;** Tsujimoto, **1989).** Resistance to FdUrd was shown in the ***bcl-2*** transfectants with the pharmacological pathways remaining unaltered in the ***bcl-2*** transfectants when compared to controls (Fisher ***et al.,* 1993).** This demonstrated that ***bcl-2,*** by suppressing apoptosis in response to chemotherapeutic drugs, produces a form of multidrug resistance which is independent of some of the classical mechanisms of drug resistance. Although these results further support the hypothesis that ***bcl-2*** does induce a novel form of drug resistance, multidrug resistance mechanisms such as **MDR1** have yet to be fully investigated in relation to ***bcl-2*** expression.

Whether ***bcl-2*** induced resistance enables cells to survive and continue to proliferate on removal of the drug, or whether it simply delays the onset of death has yet to be fully established. **As** mentioned in section **7.4, *bcl-2*** is able to completely prevent apoptosis in a significant proportion of **697** pre-B leukaemia cells of which **53%** are able to clone in semi-solid medium after treatment with dexamethasone. However, ***bcl-2*** is unable to fully prevent apoptosis in these cells after treatment with vincristine since no colony formation is seen (Miyashita and Reed **1993).** Therefore, ***bcl-2*** may not be able to completely protect cells from apoptosis in response to all chemotherapeutic drugs, but much more research has yet to be carried out on this subject.

Section 7.6 BHRF1 is a molecular suppressor of apoptosis-implications for EBV virology

A role for genes which are able to suppress apoptosis during virai host cell infection in order to increase viral production through delaying host cell death has become apparent over the past few years. Clem and colleagues (1991) identified a baculovirus gene product, p35, which is able to suppress apoptosis in infected insect cells. **A** second apoptosis suppressing gene, iap, has also been found in this virus (Crook ***et*** al., 1993).

Transformation of primary rodent cells by adenovirus is facilitated by both E1a and E1b protein products (Branton ***et*** al., 1985). Although E1a is capable of producing cellular immortalisation, Co-expression of E1b is required for high efficiency transformation. The E1b gene encodes two distinct proteins of 19kDa and 55kDa, either of which is able to enhance the transforming ability of E1a. Transformation of cells by E1a alone **is** inefficient due to the cell's inability to by-pass a phase of cell death which occurs after focus formation. This cell death bears the hallmarks of apoptosis and can be prevented by the expression of the 19kDa E1b protein (White ***et*** al 1991, White ***et*** al 1992, Hashimoto ***et*** al., 1991). Two separate reports have shown that the cell death induced both by ***TNFa*** (White ***et*** al., 1992) and also by anti-Fas antibodies (Hashimoto ***et*** al., 1991) can be prevented by expression of the E1b 19kDa protein product. Both anti-Fas antibodies and *TNFa* have been shown to produce apoptosis in a number of cell lines (Duvall and Wyllie 1986; Laster ***et*** al 1988; Trauth ***et*** al 1989; Kyprianou ***et*** al., 1991; Itoh ***et*** al., 1991; Yonehara ***et*** al., 1990), implying that the adenovirus E1b 19kDa protein product acts as a suppressor of apoptosis. Two other oncogenes, v-abl and HER2/ERBB2, have also been shown to suppress ***TNFa*** cytotoxicity and so may suppress apoptosis (Suen ***et*** al., 1990, Hudzaik ***et*** al., 1988). Both genes have tyrosine kinase activity which, in the case of c-abl, has been shown to be synergistic with c-myc in producing factor-independent cells (see Cleveland ***et*** al., 1988 and section 7.7).

In the case of p35 and E1b 19kDa proteins, the viral proteins appear to prevent apoptosis in order to obtain prolonged replication of the virus without killing the host cell. However, in the case of E1b it appears that the gene is also able to prevent apoptosis induced by external stimuli such as *TNFa.*

Although the above argument can be used to define a role for BHRF1 in EBV infection, especially since BHRFl is expressed almost exclusively in the lytic cycle, work by Marchini and colleagues (1991) has shown that BHRFl is not essential for effective infection and virus production ***in vitro.*** However, they could not rule out the possibility that BHRFl may be expressed in group I cell lines (BL tumour cells) ***in vivo*** which express EBNA 1, but none of the other latent genes and therefore presumably do not have the ability to upregulate the expression of host cell *bcl-2*. EBV latent infection resulting in the group I phenotype is thought to promote an escape from host T cell immune surveillance since the cells express only one antigen instead of all eight (Gregory ***et al.,*** 1988). If a gene is required to prolong EBV infected host cell survival ***in vivo*** and *bcl-2* cannot be upregulated, BHRF1 may be able to fulfil this function (Marchini ***et al.,*** 1991; Henderson ***et al.,*** 1993). Additionally, the BHRFl promoter is not far from the promoter which drives EBNA 1 expression suggesting that both could be upregulated by a common element. The effect of BHRF1 on EBV replication in *bcl-2* null cells needs to be investigated to establish a clear role for BHRFl in EBV infection.

The existence of viral genes which are able to suppress both cell death during viral infection and apoptosis in response to stimuli such as *TNFa* has implications in the identification of the molecular pathways by which some viral genes are able to transform cells.

Section 7.7 Relevance of c-myc

Three recent papers have illustrated that c-myc appears to be involved in the nuclear decision of a cell to enter apoptosis (Askew ***et al.,*** 1991; Evan ***et al.,*** 1992; Shi ***et al.,*** 1992). Deregulated expression of c-myc induces apoptosis in conditions where growth factors are limiting and the cells would normally enter a G_0 state by down regulating c-myc expression (Askew ***et al.,*** 1991; Evan ***et al.,*** 1992). Induction of apoptosis by c-myc under certain conditions appears to contradict its role as a Co-transforming gene. The decision of a cell expressing c-myc to proliferate or die may depend heavily on the signals the cell is receiving both from its environment and its genome (see Williams ***et al.,*** 1992). The ability of c-myc to induce apoptosis in these cells could act as a safety mechanism against deregulation of c-myc expression leading to malignancy (Askew ***et al.,*** 1991). **A** cell which constantly expressed c-myc would gain the ability to continuously proliferate in normal cellular conditions but would

require secondary changes to grow independently of appropriate survival signals from the surrounding environment. Continuous expression of c-myc in the absence of other changes will trigger apoptosis if proliferation is no longer stimulated by the surrounding environment. Evidence for this hypothesis has been demonstrated in the Eμ-*myc* transgenic mouse model in which B-cells from these mice have been shown to die *in* vitro more rapidly than B-cells from normal littermates when removed from a cell feeder layer (Langdon ***et*** al., 1988). In Eμ-*bcl*-2/myc mice however, bcl-2 provides a survival signal which blocks the apoptotic pathway and presumably provides c-myc with a genomic environment amenable to continuous cell proliferation. Deregulation of c-myc, therefore, would only lead to tumourigenesis if other genetic changes prevent the induction of apoptosis. The presence of a deregulated c-myc gene in BL cells may be one of the reasons why EBV has two survival mechanisms, upregulation of host cell bcl-2 and expression of BHRFl, by which it can suppress the death of the cell, especially when growth factors are limiting.

Other transformation studies in cell lines using different combinations of ***myc*** and other oncogenes also support the hypothesis of mutually compatible oncogenic mutations. Preneoplastic bursal stem cell populations transformed with the myelocytomatosis virus, which contains v-myc, show a greater sensitivity to apoptosis induced by y-radiation when compared to the normal embryonic B cell population (Neiman ***et*** al., 1991). If the preneoplastic cells are then infected with a virus containing the v-rel oncogene, a neoplastic lymphoid population results which is insensitive to apoptosis induced by either y-radiation or dexamethasone. This result is paralleled in neoplastic populations derived from v-myc and v-rel transformed bursal lymphocytes. Therefore, transformation by v-rel and progression from preneoplastic to neoplastic stages in v-myc induced lymphoma is accompanied by the suppression of apoptosis.

The proto-oncogenes abl and c-raf have also been shown to synergise with c-myc in transformation studies. In genetically normal myeloid cells c-Raf is phosphorylated in the presence of mitogenic stimuli and is therefore thought to be involved in the signal transduction pathway of such effectors (Rapp ***et*** al., 1988). Over expression of c-raf has been shown to produce IL-3 independent cells after several challenges in IL-3 deprived conditions and this effect is greatly enhanced in the presence of virally expressed myc. The IL-3 independent

clones obtained did not exhibit autocrine production of IL-3, suggesting that an alteration in the growth factor signalling pathway may have occurred, by-passing the need for IL-3 receptor stimulation. Interestingly, abl and other tyrosine kinase oncogenes have also been shown to result in factor independent cell growth in the myeloid cell line FDCP-1 (Cook ***et*** al., 1985; Cleveland ***et*** al., 1988). Expression of exogenous c-myc in this cell line results in partial or full abrogation of growth factor dependence (Rapp ***et*** al., 1985, Dean ***et*** al., 1987). Factor independent clones could also be subsequently derived from the partially factor independent clones, suggesting that secondary genetic changes other than c-myc activation were required for factor independence. It is not surprising that abrogation of IL-3 dependence by abl and other tyrosine kinase oncogenes is associated with the constitutive expression of c-myc since in the FDCP-1 cell line c-myc expression is normally regulated by the presence of IL-3. Recently, expression of a temperature sensitive v-abl gene has been shown to produce suppression of apoptosis at the permissive temperature (32°C) on IL-3 withdrawal in a myeloid cell line, but the cells do not proliferate in the absence of the cytokine (Evans ***et*** al., 1993), again suggesting that additional mutations are required to produce factor independent cells. The involvement of abl in IL-3 independence has been investigated further by examining raf-1 expression in FDDP-2 cells, a subclone of the FDCP-1 cell line which express a temperature sensitive v-abl gene construct (Cleveland ***et*** al., 1989). At the non-permissive temperature (39°C) v-abl has low activity and the cells are IL-3 dependent. Addition of IL-3 results in c-raf-1 phosphorylation and therefore activation. At the permissive temperature (32°C) v-Ab1 is active and as a result the cells are IL-3 independent and show constant activation of c-ruf-1, a state which is not altered by the addition of IL-3 (Rapp ***et*** al., 1990). Both c-abl and c-raf therefore seem to lie on the same signal transduction pathway resulting in similar synergistic effects with c-myc in producing factor independence. If such oncogenes are able to mimic the relevant intracellular signals normally provided by IL-3 then this would provide the conditions alluded to earlier enabling c-myc to produce constant proliferation without the possibility of a proliferative block leading to apoptosis.

A high level of c-myc expression has also been correlated with a more rapid response to DNA damage in the presence of teniposide and etoposide, two cytotoxic drugs which

inhibit topoisomerase activity (Bertrand ***et*** al., 1991). Such cytotoxic drugs can result in two types of DNA damage; (i) primary damage, which is reversible and represents a stage where double stranded breaks occur within the DNA on exposure to the drug and are subsequently repaired after drug removal; (ii) secondary DNA damage which is irreversible and results from an attempt to repair the primary DNA damage and resume normal cell growth. Secondary damage is characterised by fragmentation of the DNA into oligonucleosome sized fragments, representing the onset of apoptosis. Cell lines, such as the promyelocytic HL60 line, which have a high c-myc expression level, responded in a comparable manner to other human tumour cell lines to primary DNA damage induced by the drugs. However, the HL60 cells and other high level c-myc expressing tumour cells had a more rapid onset of secondary DNA damage which often resulted in more rapid cell death. Etoposide has been shown to induce DNA fragmentation and apoptosis in other cell lines (Kaufmann 1989; Fanidi ***et*** al., 1992; Miyashita and Reed 1992) suggesting that a high level of c-myc expression may promote cell death by apoptosis in response to chemotherapeutic drugs.

Burkitt's lymphoma cell lines express deregulated c-myc by virtue of the t(8;14) translocation associated with both EBV positive and negative BL (Dalla-Favera ***et*** al., 1982). Burkitt's lymphoma is very sensitive to treatment with either chemotherapeutic drugs or radiation, and the possibility exists that this **is** due to deregulated c-myc. The sensitivity of these cells could also be a reflection of the site of tumour origin, i.e., centrocytes, present in the germinal centre. These cells have undergone a process of somatic hypermutation and now the require a positive signal to prevent apoptosis. Cells in this situation may be ready to apoptose, with all the required proteins being present, therefore withdrawal of growth factor or presence of a stressful environment will result in rapid cell death. These hypotheses have not been fully investigated in Burkitt's cells as yet, but the fact that biopsy cells drift towards the group III phenotype and up regulate bcl-2 suggests that a continual survival signal is advantageous for these cells ***in vitro.***

In addition, inhibition of c-Myc synthesis within T-cell hybridomas by antisense oligonucleotides results in the suppression of activation-induced cell death (Shi ***et*** al., 1992). Under certain conditions both immature T-cells (Smith ***et*** al., 1989) and some T-cell

hybridomas (Ucker *et al.,* 1989; Odaka *et al.,* 1990) undergo apoptosis in response to **CD3/T-**cell receptor activation. This stimulus in both mature and immature T cells is associated with a cascade of gene expression including the early induction of c-myc (Altman *et al.,* 1990), therefore illustrating that in this cell system c-myc expression is an essential component for activation-induced cell death to occur.

Therefore, under specific conditions and in specific cells, the level of c-myc expression can be important in deciding whether a cell lives or dies, but it is unlikely to be an essential gene for all documented occurrences of apoptosis.

Section 7.8 Significance of p53 expression during apoptosis

Expression of wild type p53 in a myeloid cell line M1, which normally does not express the protein, results in apoptosis (Yonish-Rouach *et al.,* 1991). The reasons behind this cellular response were, and still are to a degree, unknown. Wild type p53 is known to act as a tumour suppressor gene, producing a block in G_1 when expressed in transformed cell lines which lack **wt** p53 expression, whereas cells expressing active **wt** p53 are more refractory to this effect (Michalovitz *et al.,* 1990; Chen *et al.,* 1990). The mechanism by which **wt** p53 produces this cell cycle arrest is unknown. It would appear that M1 cells are not able to block in G_1 when expressing exogenous **wt** p53, but they do show preferential induction of apoptosis in G_1 rather than in S phase (Yonish-Rouach *et al.,* 1993). Why this occurs is not clear, but it may be due to the internal genetic environment of the M1 ceils (Yonish-Rouach *et al.,* 1993) where there is a conflict between proliferation signals and growth arrest. Initially it was suggested that this may be because the cells produce high levels of c-Myc, which can lead to apoptosis in the absence of the cytokine, without the cells blocking in G_1 (Evan *et al.,* 1992, Askew *et al.,* 1991). However, wt p53 expression produces repression of c-myc **mRNA,** therefore this gene is not highly expressed when M1 cells enter apoptosis and does not explain the induction of apoptosis by **wt** p53 in this cell line.

An upregulation in the expression of p53 has been documented after treatment of cells with agents which cause **DNA** damage, allowing cells to block in G_1, presumably to facilitate effective **DNA** repair prior to re-entering the cell cycle (Kastan *et al.,* 1991). It has been suggested in the light of Yonish-Rouach's work that p53 signals **DNA** damage effecting a cell

cycle block and if the **DNA** damage incurred by the cell is too great then the cell undergoes apoptosis (Lane 1992). The role of p53 as a sensor of **DNA** damage was neatly illustrated by examining apoptosis in thymocytes from p53-null mice (Clarke ***et al.,*** 1993; Lowe ***et al.,*** 1993). p53 nullizygous thymocytes treated with glucocorticoid or anti-CD3 antibodies still undergo death by apoptosis as normal, but when treated with agents which damage **DNA** such as etoposide or radiation, the thymocytes are resistant. Therefore, in the absence of p53 the cell is not signalled to leave the cell cycle and repair the **DNA** damage, nor to die. This effectively means that a cell can progress through the cell cycle with significant levels of damage, the replication or repair of which ***is*** likely to lead to mutation.

p53 expression may also be important in the induction of apoptosis in response to growth factor withdrawal (Yonish-Rouach ***et al.,*** 1991; Lotem and Sachs 1993). Exposure of M1 cells to **IL-6** leads to an inhibition of apoptosis induced by **wt** p53 expression . **IL-6** does not produce this effect by inhibiting p53 expression, and appears to act downstream of p53. **IL-6** induces differentiation in these cells under these conditions and the cells become cytokine dependent. **This** cytokine dependence is irreversible and independent of wt p53 expression by this stage. Therefore expression **of wt** p53 in these cells under the above conditions re-asserted growth inhibitory 'circuits' within the cells implying that normal growth control had been at least partially restored within these cells.

The possible involvement of p53 in apoptosis induced by cytokine withdrawal suggests that p53 expression or repression may have important implications for the ability of tumour cells to evade death. Most human tumours show mutation of the p53 gene affecting p53 expression (Levine ***et al.,*** 1991), but it appears that this is not a primary requirement for cancers to develop. However, p53 may be more involved in cells acquiring a more aggressive phenotype possibly leading to metastic growth. The point at which p53 mutation occurs in tumour development may be dependent on the preceding oncogenic activation events. Murine prostate cells, for example, which express exogenous ***ras*** produce hyperplasia within the prostate only after mutation of the p53 gene. If the same cells are transfected with c-myc as well as ***ras*** then malignant cells are produced in the presence of wild type p53 (Lu ***et al*** 1992). The latter cells are also unaffected by the expression of exogenous wild type p53 illustrating

that ***ras*** and ***myc*** are together able to promote tumour growth without the need to block p53 expression. This again illustrates ways in which successful tumour cells gain mutations which enable the highest chance of continuous cell growth and survival, independent of the external and internal cellular environment. This has also been reflected in lymphomas arising in **Ep-*myclbcl-2*** transgenic mice which surprisingly did not show high levels of p53 mutation (T. J. McDonnell, personal communication). The explanation for this is likely to be ***bcl-2's*** ability to suppress p53 induced apoptosis in response to **DNA** damage, even though **wt** p53 is induced normally. This has suggested that p53 mutations maintain a similar genetic survival advantage to deregulated ***bcl-2,*** i.e., as mentioned in the previous section, ***bcl-2*** provides the survival signal which complements deregulated c-myc allowing constant proliferation. In the absence of expression of a complementary oncogene like ***bcl-2,*** cells which express deregulated c-myc must mutate another gene which gives a survival advantage. In some cases such a step could involve mutating p53 which may block cell death on growth factor withdrawal (Lotem and Sachs 1993)

Expression of **wt** p53 in Burkitt's lymphoma cell lines has been shown to induce apoptosis (Yonish-Roauch, personal communication). The cells used in the present study all show different p53 expression patterns, Chep-BL express **wt** p53, Raji-BL express mutant p53 and Akata-BL express no p53 (Lawrence Young, personal communication; Duthu ***et al.,*** 1992). Wt p53 expression in Chep-BL may help to produce the extreme sensitivity of these cells to changes in serum concentration when compared to Raji and Akata-BL. Raji-BL express mutated p53, due to mis-sense mutations in both alleles. Mutant p53 in this cell line may provide some protection against cell death since Raji-BL lack ***bcl-2*** expression, which is normally seen in group III cell lines. However, mutations in p53 do not result in resistance to apoptosis in Raji-BL cells. Akata-BL are relatively resistant to some apoptotic stimuli when compared to Chep-BL. In addition, Akata-BL unusually express low levels of ***bcl-2*** (Henderson ***et al.,*** 1993), but, very high levels of ***bcl-2*** expression are required to induce the **same** degree of resistance in group **I** cell lines as is found in group III cells (Mïlner ***et al.,*** 1992). Therefore, the low level expression of ***bcl-2*** in Akata is unlikely to increase the resistance of this cell line significantly. The non-expression of p53 may explain why Akata-BL

show resistance to induction of apoptosis in response to certain stimuli such as y-radiation. However, when considering the results obtained in p53 null thymocytes, Akata-BL cells should also be insensitive to other stimuli which damage the DNA, such as the presence of etoposide. Akata-BL cells remain sensitive to etoposide in the absence of p53 and this is difficult to reconcile with the hypothesis put forward to explain the role of p53 in the induction of apoptosis after DNA damage.

Future research into the interaction of p53 effector pathways with specific oncogene products such as Bcl-2 and C-Myc should provide further insight into the molecular mechanisms by which apoptosis is induced or suppressed. Of particular interest will be the role of p53 in the blocking of cells in G_1 after induction of **DNA** damage by chemotherapy, and the pathways by which some cells may escape such a pause in the cell cycle and as a result progress further towards metastic growth or possibly drug resistance.

Section 7.9 The growing *bcl-2* family

The existence of other cellular genes with functional homology to *bcl-2* has long been assumed. Initial studies illustrated that *bcl-2* was able to prolong cell survival in B cells from *bcl-2* transgenic mice and also in mature memory B cells (McDonnell ***et al.,*** **1989;** McDonnell and Korsmeyer **1991;** Nunez ***et al.,*** **1991).** The ability of *bcl-2* to suppress apoptosis in cytokine dependent cells **is** also well documented (Vaux ***et al.,*** **1988;** Williams ***et al,,*** **1990;** Rodriguez-Tarduchy ***et al.,*** **1990),** but apoptosis is not prevented by *bcl-2* when expressed in cells dependent on **IL-6** (Nunez ***et al.,*** **1990)** and is not expressed in all human cell lineages (Hockenbery ***et al.,*** **1990).** It would be unlikely that one single gene would control cell population growth by being able to prevent apoptosis. Such an important part of the development of multicellular organisms would surely be controlled by many genes acting in different tissues and in response to different external stimuli.

Painstaking investigations carried out in invertebrates have shown how a hierarchy of genes are able to control cellular development. The existence of death regulating genes was shown clearly by studying cell lineage development in the nematode C. *elegans* (Ellis and Horvitz **1986).** Two genes, ***ced* 3** and ***ced 4*** were found to be necessary for cell death to occur and acted in a cell autonomous manner (Yuan and Horvitz **1990).** These genes were found to

be regulated by *ced 9,* gain of function mutants of which exhibited no cell death during lineage development (Hengartner *et al.,* 1992). This suggested that *ced 9* was comparable to *bcl-2* in function as an active suppressor of cell death and this was confirmed by the ability of *bcl-2* to substitute for *ced 9* to prevent cell death in C. *elegans* (Vaux *et al.,* 1992). Again the functional homology between *ced 9* and *bcl-2* suggested that this type of gene had an important role in multi-cellular organisms and that, for more complex animals, several genes would be expected to fulfil this role.

The discovery of BHRF1 and its low level homology to *bcl-2,* along with other viral genes which suppress cell death during infection, has suggested that these genes are related through evolution. Indeed, another viral gene LMW5-HL from African swine fever virus also shows limited sequence homology to *bcl-2* (Neilan *et al.,* 1993).

One of the first mammalian genes to be identified with homology to *bcl-2* was MCL-1 (myeloid cell leukaemia-1). MCL-1 was identified primarily as an early response gene involved in the differentiation of M1 cells and has homology to *bcl-2* by virtue of similar carboxyl terminus sequences (Kozopas *et al.,* 1993). The 139 amino acid carboxyl terminal of MCL-1 shares 35% primary amino acid sequence homology with the corresponding region of *bcl-2.* Two amino acid stretches within these portions exhibited striking similarities. Both MCL-1 and BHRFl share greater homology with *bcl-2* than with each other, possibly suggesting a diverging pattern of evolution. Functional homology to *bcl-2* has so far not been established for MCL-1, however this might be expected in the light of work by Fairbairn and colleagues (1993) who have demonstrated that *bcl-2* expression can facilitate differentiation in the absence of IL-3 in early myeloid progenitor cells, without the need for proliferation in some cases.

Very recently, two genes have been identified which exhibit around **40%** homology to *bcl-2* (Oltvai *et al.,* 1993; Boise *et al.,* 1993). *bax* (Bcl-2 associated **x** gene **)** was isolated from **IL-3** dependent **cells** by immunoprecipitation of *bcl-2,* the two proteins again sharing homology in the c-terminus region. *bax* has several alternative **mRNA** forms **a,** ß, and y **,** of which **a** appears to be the dominant form. Expression of a *bax a* containing plasmid in an **IL-3** dependent cell line did not result in suppression of cell death, indeed high levels of Bax **a**

increased the rate of cell death on cytokine removal, but had no effect on cell viability in the presence of IL-3. If ***bcl-2*** and *bax* ***a*** are Co-expressed in the same cell then homo- and hetero-dimers of these molecules are found. Bax preferentially exists in the form of homodimers, but high expression of Bcl-2 identified Bax/Bcl-2 heterodimers and Bcl-2 monomers. The presence of Bax ***a*** can considerably limit the suppressive effect of Bcl-2, and therefore Oltvai and colleagues have suggested that Bax may act to regulate Bcl-2 through the ratio of Bcl-2 monomers to Bcl-2/Bax heterodimers and Bax/Bax homodimers.

bcl-x was identified by low stringency hybridisation with cDNA from the chicken Bursa of Fabricius, one of the early sites of haemopoietic development within this animal (Boise ***et al.***, 1993). The chicken ***bcl-x*** gene, which is expressed from a different genetic locus from chicken ***bcl-2,*** was used to identifya bcl-x-like gene in humans. Alternate **mRNA** splicing produces two forms of Bcl-x in human cells, $Bcl\text{-}x_L$ and $Bcl\text{-}x_s$. ***bcl-x,*** contains an open reading frame of 233 amino acids with similar domains to ***bcl-2. bcl-x,*** encodes a 170 amino acid protein, its truncated length being due to the absence of 63 amino acids which correspond to the region of highest homology to ***bcl-2.*** These two different transcripts result from differential usage of two 5' splice sites within the first coding exon.

Expression of either $Bcl\text{-}x_L$ or Bcl-x, in IL-3 dependent cells revealed that $Bcl\text{-}x_L$ acts as a functional suppressor of apoptosis on IL-3 withdrawal, much like ***bcl-2,*** but that bcl-x, did not suppress cell death. Co-expression experiments using $Bcl\text{-}2\alpha$ expressed with either $Bcl\text{-}x_L$ or Bcl-x, has revealed that expression of $Bcl\text{-}x_L$ with Bcl-2 does not produce a synergistic survival effect on removal **of** IL-3. Expression of $Bcl\text{-}x_s$ with Bcl-2 demonstrated that Bcl-x, was able to block the effect of Bcl-2, resulting in cell death on IL-3 withdrawal. The ability of Bcl-x, to function in this manner is perhaps not surprising when the two main regions of amino acid homology between ***bcl-2*** and $bcl\text{-}x_L$ are absent from ***bcl-x,.*** This also suggests that the functional activity of ***bcl-2*** is dependent on these two regions. The different roles of $Bcl\text{-}x_L$ and Bcl-x, are reflected in the tissues in which their mRNA is expressed. Immature, double positive thymocytes express high levels of ***bcl-x,*** **mRNA,** a reflection of a thymocytes readiness to enter apoptosis unless rescued. This may also explain why ***bcl-2*** transgenic mice expressing ***bcl-2*** in thymocytes, still undergo successful negative selection

(Sentman ***et al.,*** 1991; Strasser ***et al.,*** 1991). Conversely, high levels of *bcl-x*$_L$ **mRNA** are expressed in mature neural structures and may contribute to the longevity of these post mitotic cells.

The emerging ***bcl-2*** gene family now consists of BHRF1, LMW5-HL, MCL-1, ***bax, bcl-x*** and also ***ced*** 9 (from C. ***elegans)*** which exhibits functional homology (Vaux ***et al.,*** 1992) as well as some low level amino acid sequence similarities (M. Hengartner, personal communication). The existence of these genes illustrates that the regulation of death is understandably under various levels of control, possibly comparable to the regulation of Myc by Max and Mad (Blackwood and Eisenman 1991; Littlewood ***et al.,*** 1992; Ayer ***et al.,*** 1993). The highly homologous regions within the ***bcl-2*** gene family are almost all focused on two regions, **4** and **5**, and this has identified these areas as highly important to the function of Bcl-**2.** This may aid the search in identifyinghow the ***bcl-2*** protein functions within the cell and how this is associated with its membrane form. The emerging ***bcl-2*** family has also shown that some of the genes, such as ***bax*** and ***bcl-x,*** have a relatively high degree of homology, but that other genes, such as BHRFl have low level homology, but all are involved in the regulation of cell death. Whether the genes which suppress cell death are related through evolution has yet to be fully investigated, but the ability of ***bcl-2*** to substitute for ***ced*** 9 in *C. **elegans,*** coupled with the varying degrees of homology between all the genes, suggests that this is likely.

Section 7.10 In conclusion...

When first observed, apoptosis represented a new and morphologically distinct classification of cell death. **As** research into the phenomenon increased, apoptosis was shown to be an active, controllable form of cell death, akin to cell suicide, the cell having to transcribe, or have transcribed, specific genes in order to die. Comparable models from invertebrates, such as *C. **elegans,*** implied that cell death could be used to limit the number of cells which make up a given cell population. With the discovery of the gene ***bcl-2,*** which was able to suppress this active cell death in mammalian cells, control of cell population size at the cell death level was recognised in higher order animals, including mammals. ***bcl-2*** transgenic mice demonstrated that ***bcl-2*** could inhibit cell death ***in vivo*** increasing the cell population number and thereby heightening the chances of mutations occurring, resulting in clonal tumour

outgrowth. bcl-2 now appears to be one of the early sites of advantageous mutations which complement the deregulation of genes such as c-myc, giving the autonomous growth advantage which tumour cells exhibit. **A** similar role may also be attributed to c-abl since this gene is also able to prevent cell death on cytokine withdrawal (Evans ***et*** aí., 1993) and is actively expressed, by virtue of the bcrlabl fusion protein, in myeloid leukaemia cells.

After the initial published identification and long term study of apoptosis in thymocytes, diversification of analysis into other cell lines revealed that not all cells conform exactly to one morphological or biochemical pattern of apoptosis. For example, CEM cells treated with novobiocin undergo **DNA** cleavage due to the activation of a Ca^{2+} independent endonuclease (Alnemri and Litwack 1990).

Cellular genes with classically defined roles in the cell cycle, such as p53, c-myc and c-***fos,*** have also been shown to be involved in some pathways of apoptosis. p53, for example, appears primarily to signal the growth arrest of cells in the presence of **DNA** damage which, if irreparable, leads to apoptosis. In general, p53 may be a gene which is primarily required to trigger the expression of genes involved in specific pathways of apoptosis, i.e., after **DNA** damage or possibly during cytokine withdrawal. Therefore, research at present time has associated p53 with specific apoptotic stimuli suggesting the existence of multiple apoptotic pathways. This is supported by the observation that bcl-2 dependent and independent forms of cell death occur (Cuende ***et al.,*** 1993; Milner ***et al.,*** 1992) suggesting that there are apoptotic pathways which ***bcl-2*** is able to inhibit and other pathways on which it does not act. In addition, the identification of several endonucleases, some of which are Ca^{2+} dependent and others pH dependent, also suggests that different cytoplasmic pathways can activate the endonuclease without the need for a definitive Ca^{2+} signal (D. J. McConkey, personal communication). Conversely, the two different mechanisms of endonuclease activation may be present to accommodate activation signals produced by a number of different cytoplasmic pathways.

The expression of either ***bcl-2*** or BHRFl in the three BL cell lines used in this study has shown that genes which suppress apoptosis can produce a novel form of drug resistance. The varying genetic backgrounds of the three BL cell lines produced very useful and

sometimes thought provoking results. Chep-BL cell lines are unquestionably sensitive to apoptotic stimuli, a factor which may be aided by the presence of **wt** p53, or possibly other genes such as ***bcl-x,*** which have not yet been investigated. The expression of transfected ***bcl-2*** in Chep-BL lines produced suppression of apoptosis in response to methotrexate, etoposide and y-radiation, but had a smaller, not statistically significant effect on cell death in response to araC. This result is consistent with the morphological appearance of the cells which are not classically apoptotic after 14 hours exposure to araC. It has not been possible to address whether earlier time points i.e., **<14** hours drug exposure, would have revealed apoptotic morphology. However, since the cells do not show a rapid reduction in membrane integrity after treatment with araC, especially when compared to methotrexate and etoposide, this implies that cell death is not by rapid lysis as would be expected in necrosis. araC itself may induce death by a number of pathways, which appear to be independent of protein synthesis, and therefore may produce morphology which is not distinctively apoptotic. The inability of ***bcl-2*** to suppress araC induced cell death in Chep-BL cells could also be attributable to the cell line itself Other cells, such as T-cell lymphomas and lung cancer cell lines, which have been transfected with ***bcl-2,*** produce contradictory results when addressing for which drugs Bcl-2 can produce resistance (Myashita and Reed 1992; Ohmori ***et al.,*** 1993).

Perhaps the most fundamental point behind the discovery of apoptosis is that it represents an active, controllable, physiological form of cell death which can be produced by a number of cellular pathways, resulting in the commitment to death and the marking of the celi for phagocytosis either by a passing macrophage or a surrounding viable cell. Whether or not cells **fully** cleave their **DNA** into oligonucleosomal fragments in multiples of ~180bp, or show a reduction in cytoplasmic volume may not be of crucial significance to the cell, but the ability to die when required is fundamentally important for cell population growth control. Cancer research as a whole in the last 10 years has shown that tumour cells bearing mutations which facilitate the survival of the cells irrespective of their surrounding environment have been positively selected for. It would now appear that these mutations provide the required backgrounds to allow genes, which produce constant proliferation, to be tolerated in growth limiting environments.

Viruses also appear to have gained survival advantages in expressing genes such as BHRFl, p35 and LMW5-HL which prevent apoptosis of the host cell during infection (Levine ***et al.,*** 1993). The work presented in this thesis and the work of others has shown that such viral genes are also able to suppress apoptosis in response to chemotherapeutic drugs and ***TNFa.*** Whether the expression of such genes is important to cancer development and drug resistance ***in vivo*** has yet to be closely investigated.

BHRF1 is not essential for the production of LCLs ***in vitro*** by EBV, but the expression of the gene could be required for the survival of EBV cells ***in vivo,*** especially since ***bcl-2*** is not thought to be expressed by EBV-BL biopsy cells. **An** additional possibility exists in that EBV-BL cells ***in vivo*** may express a different ***bcl-2*** homologue which provides an adequate survival background to tolerate the presence of deregulated c-myc, present in BL cells.

These possibilities need to be examined in order to determine whether the viral apoptosis suppressing genes can contribute towards cancer formation and acquired drug resistance and if so, how this can be clinically circumvented.

Appendices

Appendix A

Error handling and statistics

Section A.1 Calculation of mean % viability

The calculation shown below illustrates the method used to calculate the mean % viability and standard error from the raw replicate cell counts.

This example is taken from figure 6.11(A), Raji-BL BHRF1 transfectants treated with 16Gy of γ-radiation, viability at 48 hours;

$$\% \text{ viability} = \frac{\text{Number of live cells (bold)}}{\text{Total number of cells counted (bold + normal)}}$$

Replicate 1		%	*Replicate 2*		%	*Replicate 3*		%
22	17	56.4	**19**	18	51.4	**21**	13	61.8
22	14	61.1	**26**	25	51.0	**23**	22	51.1
30	9	76.9	**16**	18	47.1	**20**	13	64.5
	Mean	64.8		*Mean*	49.8		*Mean*	59.1

The n replicate means ($x_i : i = 1....n$) are then averaged to give the true statistical mean ($\bar{x}$) and the standard error of the mean ($\sigma_{n-1}/\sqrt{n}$) where

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

the variance

$$\sigma_{n-1}^2 = \frac{\sum x_i^2 - \bar{x}\sum x_i}{n-1}$$

and the standard deviation $\sigma_{n-1}/\sqrt{\text{variance}}$. From this calculation the computer finds the mean $\bar{x} = 57.9$ and the standard deviation $\sigma_{n-1} = 7.586$. The standard error $= \sigma_{n-1}/\sqrt{3} = 4.380$ and so finally we can state that the mean % viability with its standard error:

$$\underline{57.9 \pm 4.4\% \ (n = 3)}$$

Section A.2 Significance testing

Significance tests between different culture conditions were carried out using the student *t*-test. When comparing two sample values x_1 (from n_1 replicates) whose standard deviation is SD_1 and x_2 (from n_2 replicates) with standard deviation SD_2, their standard deviations must be pooled using

$$\text{pooled S.D. } (S) = \sqrt{\frac{(SD_1)^2(n_1-1)+(SD_2)^2(n_2-1)}{n_1+n_2-2}}$$

From this we can calculate the standard error difference

$$\text{S.E.}_{diff} = \sqrt{\frac{S^2}{n_1}+\frac{S^2}{n_2}}$$

which is then used to calculate t from

$$t = \frac{|x_1 - x_2|}{\text{S.E.}_{diff}}$$

Finally, the value P (the probability for the null hypothesis - no difference between populations) is obtained from the standard t-test tables of probability using t with the (n_1+n_2-2) degrees of freedom.

For example, let us compare % viabilities of Raji-BL BHRF1 transfectants (57.91 ± 7.58) with control transfectants (35.74 ± 5.09) 48 hours after treatment with 16Gy of γ-radiation as shown in figure 6.11(A). In both cases the standard deviations are shown and there were three replicates ($n_1 = n_2 = 3$).

Using the above equations, the pooled standard deviation S = 6.45, $\text{S.E.}_{diff} = 5.27$ and so $t = 4.21$. From the tables we find

$$\underline{0.01 < P < 0.02}$$

indicating a significant difference between the two viabilities since $P < 0.05$.

Appendix B

Electron microscopy methods

Section B.1 Routine preparation of samples

Cell samples for examination by electron microscopy were prepared as described below by members of the E.M. unit in the department of Biochemistry, Birmingham university.

Step 1	**Preparation of cells**	
	Pellet cells in a small eppendorf and remove supernatant.	
Step 2	**Chemical fixation**	
	a) Primary fixative - 2.5% Glutaraldehyde ~ 2 hours on ice.	
	b) Secondary fixative - 1% Osmium tetroxide ~ 1 hour	
Step 3	**Dehydration**	
	70% ethanol	15 mins
	90% ethanol	15 mins
	100% ethanol	15 mins
	100% ethanol	15 mins
	100% ethanol	15 mins
	Propylene oxide	15 mins
	Propylene oxide	15 mins
	Propylene oxide/ resin	45 mins
Step 4	**Embedding**	
	Embed in epoxy resin under vacuum in plastic moulds 20mins	
Step 5	**Polymerisation**	
	At atmospheric pressure for 24 hours at 60°C	
Step 6	**Trimming**	
	Trim specimen block to the area of interest	
Step 7	**Ultra thin sectioning**	
	Cut 70nm sections using an ultramicrotome and collect sections on to electron microscope grids	
Step 8	**Staining**	
	Stain sections with Uranyl acetate and Reynolds lead citrate	
Step 9	**Examine sections using the transmission electron microscope.**	

Section B.2 Mammalian Fixatives for E.M.

The following solutions were made up to use in the preparation of the cell pellet for E.M. examination. Osmium tetroxide was made up and used by members of the E.M. unit only.

Phosphate buffer

Solution A 2.7g Potassium dihydrogen phosphate made up to 100ml with distilled water.

Solution B 0.8g Sodium hydroxide made up to 100ml with distilled water.

Solution C 50ml of solution A and 39ml of solution B to give 0.2M pH 7.2 (check pH).

2.5% Glutaraldehyde Fixative

25ml solution C

10ml 25% Glutaraldehyde (stored at 4°C) (BDH)

1.71g Sucrose (BDH)

1% Osmium tetroxide

2.5ml Solution C

0.1g Osmium tetroxide

0.855g Sucrose

7.5ml Distilled water

(*Use only in a fume cupboard with gloves*)

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