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GROWTH-, DIFFERENTIATION-, AND CELL-DEATH-ASSOCIATED EXPRESSION OF THE *BCL-2* PROTO-ONCOGENE IN HUMAN MYELOID LEUKAEMIA CELLS

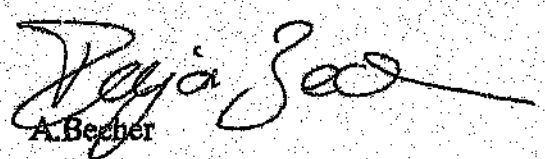
Anja Becher

A Dissertation Submitted to the Faculty of Science
University of the Witwatersrand, Johannesburg
for the Degree of Masters of Science

Johannesburg 1995

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



A.Becher

10 Day of February, 1995.

DEDICATION

This dissertation is dedicated to my parents, who have, with their love and encouragement, helped me to realise many of my dreams.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Nerina Savage, my supervisor, for her guidance and support throughout this project. Your dedication to science has been an inspiration to me.

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UNPUBLISHED CONFERENCE PROCEEDINGS

The role of cAMP in the production of IL-1ß in a human monocytic cell line

(presented in form of a poster during the 12th Annual Congress of Biochemistry ' ' Stellenbosch in 1994)

LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
bcl-2	*bcl-2* gene and *bcl-2* mRNA product
BCL-3	*bcl-2* gene protein product
BSA	Bovine Serum Albumin
C	Catalytic
Ca	Calcium
cAMP	cyclic Adenosine Monophosphate
cdc	Cell Division Cycle
cDNA	copy DNA
CLL	Chronic Lymphocytic Leukaemia
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
CTP	Cytidine Triphosphate
d	deoxy
DAB	3,3'-diaminobenzidine tetrachloride
dbcAMP	N6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
DEPC	diethyl pyrocarbonate
DNA	Deoxyribonucleic Acid

EDTA	Ethylenediamine-N,N,N',N'-tetra-acetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
FITC	Fluoro-isothiocyanate
G_0	Quiescent phase
G_1	Gap phase 1
G_2	Gap phase 2
GDP	Guanosine 5'-biphospate
GTP	Guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
I	Interphase
IL	Interleukin
M	Mitosis
MHC	Major Histocomparibility Complex
MOPS	3-(N-morpholino)propanesulphonic acid
MPF	Maturation Promoting Factor
NB	Neuroblastoma
NF-1	Neurofibromatosis-1
NRE	Negative Regulatory Element
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction

PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol-12-myristate-13-acetate
PNRE	p53-dependent Negative Regulatory Element
R	Regulatory
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
S	Synthesis
SDS	Sodium Dodecyl Sulphate
TBS	TRIS-buffered Saline
TCR	T Cell Receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
TTP	Thymidine Triphosphate
UV	Ultraviolet

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ABSTRACT

This study evaluates the expression of the mRNA and protein products of the *bcl-2* proto-oncogene in the human myelocytic THP-1 and HL-60 cell lines. The *bcl-2* proto-oncogene codes for a protein termed BCL-2 that is able to inhibit some pathways of programmed cell death. Both cell lines are capable of undergoing programmed cell death, as is shown by the presence of some apoptosing cells in cell morphology studies after 24 hour stimulations with the protein synthesis inhibitor puromycin (1 ng/ml). Some HL-60 cells, but no THP-1 cells, undergo apoptosis upon 24 hour co-stimulation with the phorbol ester PMA (16 nM) and the PKA activator dbcAMP (50 μM). Stimulating with PMA or dbcAMP alone does not induce detectable levels of apoptosis in THP-1 and HL-60 cells.

BCL-2 expression is inversely related to differentiation in myeloid cells, and mature monocytes express no BCL-2. Cell cycle analyses and [^{3}H]thymidine uptake studies show that PMA (16 nM) decreases proliferation both in THP-1 cells and the less differentiated HL-60 cells, while dbcAMP (50 μM) decreases proliferation markedly in THP-1 cells only. Cell adhesion and cell morphology studies show that PMA, but not dbcAMP, induces differentiation in both cell lines.

Stimulating cells with PMA for 24 hours induces the expression of IL-1ß mRNA and protein in THP-1 cells, but not in the less differentiated HL-60 cells,

indicating that IL-1ß expression is related to differentiation in these cell lines.

The expression of bcl-2 mRNA and BCL-2 protein is analysed and compared in both cell lines, both under undifferentiated conditions and after induction of differentiation. *bcl-2* mRNA expression is evaluated using reverse transciption and polymerase chain reaction amplification methods, and is shown to decrease upon PMA-stimulation in both cell lines. *bcl-2* mRNA expression is shown to decrease to less than 5 % of control values in THP-1 cells (n=2) and to between 10 % and 20 % of control values in HL-60 cells (n=3) after 24 hours of PMA stimulation. *bcl-2* mRNA expression after dbcAMP stimulation may decrease or may remain unchanged in both cell lines when compared to unstimulated cells.

Immunodetection of BCL-2 protein expression using Western blotting and slotblotting methods is described quantitatively in HL-60 cells, but only qualitatively in THP-1 cells where low levels of BCL-2 expression made quantitation technically difficult. BCL-2 expression is shown to decrease to 78 % of control values by 24 hours after PMA stimulation in HL-60 cells. The BCL-2 protein expression is between 5 X and 15 X lower in THP-1 cells than in HL-60 cells. The inverse relationship of myeloid cell differentiation to *bcl-2* mRNA and BCL-2 protein expression is confirmed by these results. Stimulation with dbcAMP does not cause changes in BCL-2 protein expression in THP-1 cells.

Immunocytochemical detection of BCL-2 protein in whole cells is described. The protein is shown to be located mainly in the cytoplasm. Stronger positive staining is observed in HL-60 cells than in THP-1 cells, thus confirming the results obtained from protein slotblots.

This study confirms the inverse relationship of myeloid cell differentiation to *bcl-2* mRNA expression and to BCL-2 protein expression. Monocytes and tissue macrophages have a high turnover rate compared to their haematopoietic stem cells, and are consistently replaced by newly differentiated progeny cells. High BCL-2 expression in the long-lived stem cells will help protect them from cell death. Decreases in BCL-2 expression during differentiation will lead to increased susceptibility to programmed cell death, and will allow for the high turnover rate observed in monocytes and macrophages. Tissue homeostasis is maintained by this balance between cell proliferation and cell death.

SECTION 1

INTRODUCTION

For the human body to function optimally, its constituent parts have to be subordinate to the system as a whole. Cells are, therefore, not autonomous units, but interact with, and are controlled by, their environment. Hormones and other regulatory factors act upon the cell, ultimately affecting enzyme and protein activation and inactivation, gene transcription and translation.

When the cell obtains a certain degree of autonomy from its environment, it may become a threat to the rest of the system. Such a cell would be termed a cancer or tumour cell.

Cancer cells have developed some independence and are, thus, affected less by their surrounding milieu and its regulatory network. They will follow their own internal programme and will, usually, remain in a proliferating, less differentiated state.

1.1. MULTISTEP CARCINOGENESIS

The conversion of a normal cell to a malignant cancer is thought to be a multi-step process that cannot be attributed solely to the mutation of a single gene. Rather, there must be a series of changes in the properties of the collection of cells that make up the developing tumour. In humans, measurements of age-dependent tumour incidence suggest that five or six independent rate limiting steps have to occur for a tumour to develop (Peto, 1977).

Experimental models, such as the chemical carcinogenesis of mouse skin, imply at least three distinct stages in tumourigenesis: initiation, promotion, and progression (Farber and Cameron, 1980). Initiation is considered to involve mutational events and is, therefore, irreversible. Promotion is a reversible, epigenetic event involving non-mutational alterations in gene expression. In mice, initiation can be achieved through a single painting of a carcinogen onto the skin. The mouse skin treated in such a way will remain outwardly normal in the absence of further intervention. However, if a second agent (promoter) is subsequently applied, malignant papillomas appear in the areas previously exposed to the initiating agent. Certain phorbol esters are able to act as tumour promoters. During tumour progression, distinct changes

occur that convert cells into promoter-independent, autonomously growing cells. The different stages of tumour development represent barriers which ensure that successful completion of the tumourigenic process is a rarely achieved event (Weinberg, 1989).

The overexpression or change in function of certain regulatory genes may initiate cells to become cancerous. Most of these genes code for transcription factors or molecules involved in the signal transduction pathway. Genes that cause or promote cancer when mutated or inappropriately expressed are known as proto-oncogenes. Genes whose loss of function promotes or causes cancer are known as tumour suppressor genes.

In the haematopoietic system, cells are derived from a single class of progenitors known as pluripotent stem cells. Stem cells have the ability to renew themselves,as well as to produce progeny destined to differentiate. These progeny subsequently become committed to a single haematopoietic lineage with a concomitant decrease in their ability to replicate. Leukaemias are clonal cancers, and will usually arise from uncommitted or partially committed haematopoietic stem cells. In accordance with multistep tumourigenesis, at least two mutational events are needed for leukaemias to arise. One of these events will allow the uncoupling of proliferation and differentiation and will thus lead to uncontrolled cell accumulation. The other will cause an extension of the permissive environment by decreasing the cell's

dependence on external growth factors. Both oncogenes and tumour suppressor genes may be involved in these mutational events.(For reviews on the development of normal and neoplastic haematopoietic tissue, refer to Rothstein, 1993, and Sullivan, 1993, respectively).

1.2. PROTO-ONCOGENES AND TUMOUR SUPPRESSOR GENES

Proto-oncogenes are normal cellular genes that are involved in the control of cell growth, proliferation, differentiation, and death. If mutated or inappropriately expressed, however, they may become oncogenic and contribute to tumourigenesis. According to the oncogene paradigm (Weinberg, 1989), a tumour cell clone develops via a series of oncogene activations, which then collaborate to achieve the fully malignant phenotype.

Proto-oncogenes were first discovered as the cellular counterparts of the cell-transforming genes of oncogenic retroviruses (Martin, 1970). These viruses have an unusual replicative cycle: the RNA of their genome is reverse transcribed into DNA, which is subsequently integrated into the chromosomal DNA of the infected host cell. This integrated DNA then serves as a template for the production of viral progeny RNA. During viral RNA production, host cell DNA may be transcribed along with the viral DNA. Once these host

DNA sequences are removed from the stringent controls of the host cell, they may readily undergo independent mutations, yet remain within the viral genome. During viral evolution, mutations arose that gave some viruses an advantage when infecting their hosts. As proto-oncogenes are involved in the control of cell growth, proliferation, differentiation, and death, the introduction of a mutated proto-oncogene into the host cell may contribute to virus-stimulated transformation of the cell. Retroviruses have become an important tool for discovering cellular proto-oncogenes whose existence would otherwise be less apparent.

Most cellular proto-oncogenes become activated oncogenes by somatic mutations which usually cause minor changes in gene structure.

Tumour suppressor genes are able to suppress or inhibit the proliferation of cells. Evidence for their existence was first obtained from experiments involving the fusion of malignant cells with their normal counterparts, which would often result in the suppression of the malignancy in the hybrid cell (see Harris, 1988, for review). The concept developed that some cancerous cells contained a mutant form of a tumour suppressor gene, which failed to function properly. This function could be restored when cell fusion delivered a single dominant wild-type allele of the gene to the formerly cancerous cell.

Thus, a single mutational event is sufficient to activate a proto-oncogene

and allow its protein product to contribute to abnormal cellular growth. By contrast, tumour suppressor genes require two independent mutational events to contribute to abnormal growth. Cancerous mutations in tumour suppressor genes are loss-of-function mutations that act recessively to the wild-type. Mutations in proto-oncogenes are usually gain-of-function mutations and are, therefore, of a dominant nature (for reviews, see Weinberg, 1989, and Levine, 1993).

Both proto-oncogenes and tumour suppressor genes play positive and negative regulatory roles in cell proliferation, cell differentiation, and growth arrest.

1.3. CELL PROLIFERATION

Dividing eukaryotic cells exhibit a well-defined cell cycle, which consists of transitions from one regulatory state to another (Murray *et al.* 1989). Broadly, two phases can be identified: the mitotic phase (M), and the interphase (I). The interphase can be divided further into two gap phases (G_1 and G_2), which are separated by a DNA synthesis phase (S). Cells may withdraw from the cell cycle and enter a quiescent G_0 state in response to reduced serum growth factor concentrations or increased cell density.

Cycling may be arrested at two stages within the cell cycle, either in G_1, or in G_2. One of the G_1 checkpoints is controlled by the p53 tumour suppressor protein. p53 protein concentrations are increased within the cell upon DNA damage. This has an anti-proliferative effect, arresting the cell cycle at G_1, and allowing for DNA repair prior to completion of the cell cycle (Yin *et al*, 1992).

One central and rate-limiting function in the transition from G_2 to M is performed by a protein kinase called the maturation- (or M phase-) promoting factor (MPF). MPF consists of two components: the $p34^{cdc2}$ (cell division cycle 2) serine/threonine protein kinase catalytic subunit, and a B cyclin protein. The biochemical role of cyclin B remains unclear, though potential functions include specification of substrates or subcellular localization of MPF. Cyclin B accumulates during interphase, forms a complex with $p34^{cdc2}$, and thereby activates the MPF kinase activity. During mitosis, cyclin B is degraded, and the MPF kinase activity is switched off. Thus, the system is reset to its interphase state during mitosis (Murray *et al*, 1989 and 1991, Norbury and Nurse, 1992).

The cell cycle is exceedingly complex, with mutation studies on the yeast *Saccharomyces cerevisiae* showing that at least 32 different genes are involved in the cell division cycle (Hartwell, 1978). It is plausible that at least as many, not all as yet identified, play a role in the human cell division cycle.

1.4. CELL DIFFERENTIATION

While cell proliferation refers specifically to the replication process, differentiation is the process by which cells acquire and develop the structures and macromolecules necessary for specialized function. Proliferation and differentiation may occur simultaneously or independently (Rothstein, 1993), and similar proto-oncogenes are expressed during both processes (Zelenka, 1990).

Since only a small fraction of the total genes present in a differentiated cell are expressed at any given time, negative regulatory mechanisms that prevent inappropriate gene expression are very important. These regulatory mechanisms are thought to control the differentiated state actively at the molecular level, thus making cell differentiation reversible. Gene expression, therefore, depends on the composition of regulatory proteins within the cell (Blau, 1992).

Signals from the cell's external and internal environments are transduced to the nucleus, where they influence cell proliferation, differentiation, quiescence, and death (figure 1.1).

1.5. SIGNAL TRANSDUCTION

Cells are able to regulate gene expression in response to changes in the environment during proliferation and differentiation. These alterations in gene expression are often induced by growth factors and other signals external to the cell.

The first step in signal transduction is the interaction of the agonist with its receptor, which may be located in the cell membrane, in the cytoplasm, or in the nucleus. Binding produces conformational and, thus, functional changes in the receptor. This initiates a signal that may be transmitted further by cytoplasmic mediators, or second messengers, and usually ends with a key molecule that regulates transcription. Changes in gene expression can occur in the absence of protein synthesis, indicating that they are often brought about by post-translational modifications of transcription factors (Jackson, 1992).

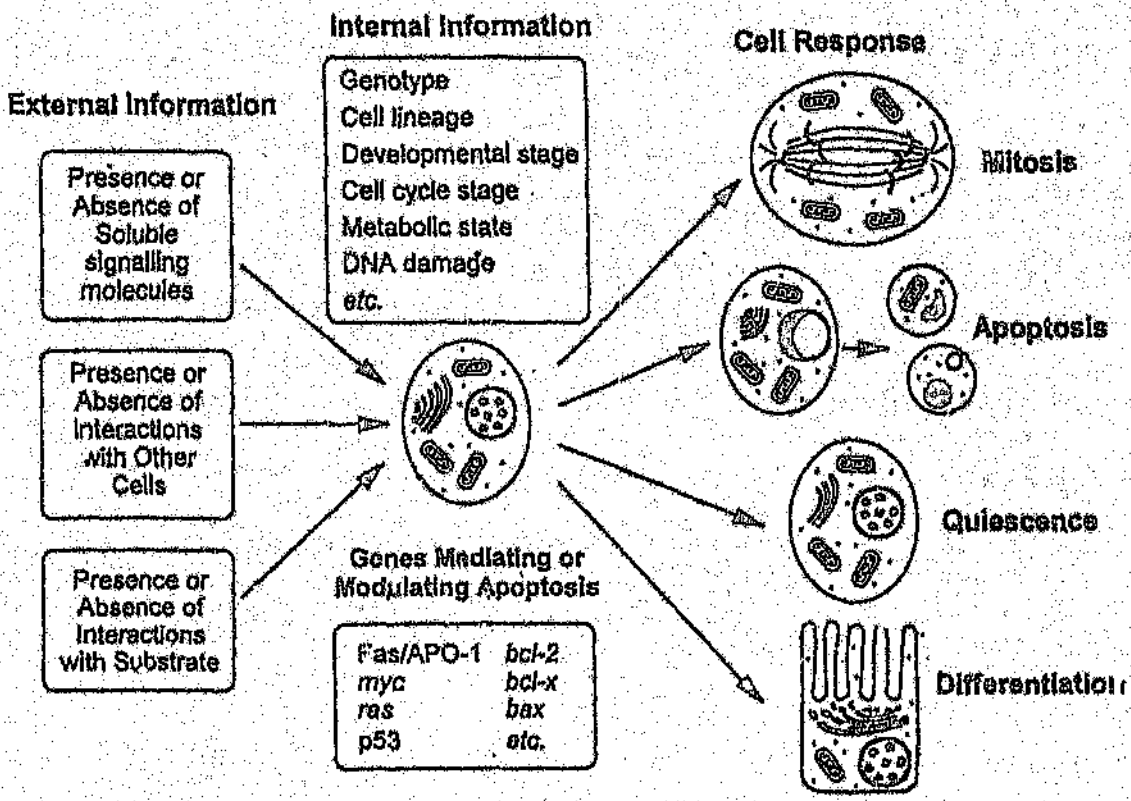


Figure 1.1

Cellular responses to signals from the external and internal environment

Signals from the cell's external and internal environment influence cell proliferation ("mitosis"), differentiation, quiescence, and cell death ("apoptosis"). The gene products that mediate cell death are discussed in sections 1.17 and 1.19. (Reproduced from Williams and Smith, 1993.)

The most frequent reaction used to modify the activity of transcription factors is a change in their state of phosphorylation (Jackson, 1992). Often, phosphorylation changes the DNA-binding capacity of a transcription factor. The transcription factor AP-1, for example, will bind to DNA after inhibitory phosphate moieties have been removed subsequent to the activation of protein kinase C (PKC). The serum response factor, on the other hand, is a transcription factor that is positively regulated by phosphorylation, and

increases the transcription of responsive genes in its phosphorylated state (Jackson, 1992).

Phosphates are incorporated into enzymes by protein kinases using (adenosine triphosphate) ATP, and can be removed by protein phosphatases. The most common occurrence involves the phosphorylation or dephosphorylation of hydroxyl groups of serine, threonine, and tyrosine, although tyrosine phosphorylation accounts for less than one percent of all protein phosphorylations.

Two prominent serine/threonine-specific kinases, both activated by second messengers, play central roles in signal transduction: the calcium/phospholipid-activated protein kinase C (PKC), and the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA).

PROTEIN KINASE C (PKC)

One key component of the second messenger system is the PKC family of enzymes. PKC plays an important role in the pathways leading to cell proliferation, differentiation, and death (Knox *et al*, 1993), and is activated by diacylglycerols and arachidonic acid *in vivo*. These compounds, in turn, are generated by hydrolysis of phospholipids in the plasma membrane during initiation of signal transduction. PKC may play a role in tumour promotion, in that it also constitutes a receptor for mitogenic phorbol esters such as 12-O-

tetradecanoylphorbol-13-acetate (TPA) and phorbol-12-myristate-13-acetate (PMA) (Blumberg *et al*,1984; Angel *et al*, 1987). Among the genes whose transcription is induced or increased by phorbol esters are the cellular proto-oncogenes *c-fos*, *c-myc*, and *c-sis* (Angel et al, 1987).

The PKC family consists of at least nine isoenzymes that can be divided into two main groups: the calcium-dependent or conventional PKCs, and the calcium-independent or novel PKCs (Hug and Sarre, 1993). These isoenzymes exhibit subtly different kinetic properties and substrate specificities (Farago and Nishizuka, 1990), and are expressed in a tissue-specific manner. Specific patterns of PKC isoenzyme expression have been linked to certain developmental stages (Hug and Sarre, 1993), and may be involved in guaranteeing an ordered sequence of proliferation and differentiation events. Subcellular localization patterns of the PKC isoenzymes are also associated with changes in proliferation and differentiation, and with the regulation of cell death (Knox *et al*, 1993).

Activation of PKC by phorbol esters leads to changes in the cellular signalling pathways, including stimulation of MAP kinase activity (Adams and Parker, 1991) and phosphorylation of components of the transcriptional and translational machinery (Boyle *et al*, 1991, Morley *et al*, 1991).

PKC is usually translocated from the cytosol to the plasma membrane

upon activation. This is often followed by cleavage of the enzyme into its regulatory and catalytic domains (Pontremoli *et al*, 1990), and its eventual degradation.

In most cell lines investigated, treatment with the phorbol ester PMA will cause this translocation of PKC from the cytosol to the plasma membrane, and, after prolonged PMA treatment, the eventual depletion of PKC in the cell. PMA sensitivity, however, seems to vary from one PKC isoenzyme to the next, and in different tissues (Nishikawa *et al*, 1990).

When the human myeloid HL-60 cell line is exposed to the phorbol ester TPA, differentiation toward a monocyte/macrophage phenotype takes place (Vandenbark *et al*, 1984). This effect of TPA is concentration-dependent. Higher concentrations (10 nM and above) will inhibit proliferation and cause differentiation, while lower concentrations (0.1-1 nM) will inhibit differentiation and promote proliferation (Trayner and Clemens,1992). It is likely that the lower concentration more closely resembles the *in vivo* situation during the stimulation of PKC by diacylglycerols, where major down-regulation of PKC does not occur (Clemens *et al*,1992). Diacylglycerols, unlike TPA, are metabolized by the cell and, therefore, do not lead to prolonged activation of PKC (Pongracz *et al*, 1994). Thus, the *in vivo* response to PKC activation is likely to be proliferation rather than differentiation.

High concentrations of TPA, on the other hand, rapidly down-regulate at least some species of PKC (Zylber-Katz and Glazer, 1985; Kraft *et al*, 1987). Where the long-term differentiation effects of exposure to high concentrations of phorbol ester are studied, the question therefore arises of whether the observed responses are due to the initial activation of PKC or due to its subsequent down-regulation (Brooks *et al*, 1991), and whether these effects can be compared to those caused by the physiological activation of PKC.

It is not clear whether all observed effects of phorbol esters can be achieved by the activation of PKC alone (Kreutter *et al*, 1985), and it has been suggested that some cellular responses to phorbol esters occur independently of this enzyme (Murphy *et al*, 1991).

PROTEIN KINASE A (PKA)

PKA is another important member of the cellular signal transduction pathway, and is activated by cyclic AMP (cAMP). The PKA enzyme is a tetramer that is made up of two regulatory subunits (R) and two catalytic subunits (C). Each R subunit contains two binding sites for cAMP. Upon cAMP binding, the enzyme dissociates into an R dimer and two activated catalytic subunits:

$$R_2C_2 + 4\ cAMP \rightarrow R_2cAMP_4 + 2\ C$$

Two types of PKA have been identified, type I and type II. The two types

differ in their R subunits which have molecular weights of 49 000 and 55 000 for types I and II, respectively. Type I has a higher affinity than type II for cAMP, and, while both types can undergo phosphorylation, only type II can undergo autophosphorylation. The proportions of the two kinases vary markedly from one tissue to another. In the rat, for example, adipose tissue consists almost exclusively of type II, while heart tissue consists to more than eighty percent of type I. It is possible that the different proportions reflect differences in the sensitivity of tissues to changes in cAMP concentrations (for review on mechanisms of action of cAMP refer to Martin, 1988). The two kinase types may perform different functions, with the type I kinase being primarily involved in cell proliferation and the type II kinase in cell differentiation (Cho-Chung, 1990).

Activation of PKA causes the phosphorylation of the cAMP response element binding protein (CREB). This increases CREB activity, thereby stimulating the transcription of specific genes containing the cAMP response (or regulatory) element (CRE). CRE has the properties of a transcriptional enhancer in that it regulates transcription from a promoter in an orientation- and position-independent fashion.

cAMP-regulated genes seem to share two common characteristics: They are expressed in hormone- and regulatory factor-responsive tissues, and their rate of transcription is rapidly altered in response to changing cAMP levels

(Roesler *et al*, 1988). cAMP concentrations within the cell increase upon activation of adenylate cyclase, the activity of which is, in turn, controlled by the activation of a G-protein linked cell membrane receptor. Changes in cAMP concentrations are linked to changes in cell metabolism (Martin *et al*, 1990). Enzymes that are regulated by cAMP-dependent phosphorylation include phosphorylase kinase, glycogen synthetase, pyruvate kinase, and cholesterol esterase (Martin, 1988).

The function of many, if not all, proto-oncogene and tumour-suppressor gene products can be fitted into one or another step of the cell proliferation or cell differentiation signalling pathway.

1.6. CELLULAR PROTO-ONCOGENES AND TUMOUR SUPPRESSOR GENES IN SIGNAL TRANSDUCTION

Some proto-oncogene products (oncoproteins) mimic growth factors. The *int-2* gene, for example, codes for a protein with significant similarity to the fibroblast growth factor family (Dickson *et al*, 1987), while the *v-sis* oncoprotein is nearly identical to the ß-chain of the platelet-derived growth factor (Doolittle *et al*, 1983).

Other oncogenes encode homologs of cell surface receptors. *c-erbB*, for

example, encodes a homolog of the receptor for epidermal growth factor (Yamamoto *et al*, 1983). Oncoproteins can also be found further downstream in the cell signalling pathway, functioning as tyrosine kinases (*c-src*), serine and threonine kinases (*c-raf*), GTPases (the *ras* family), and transcriptional regulators (*c-fos*, *c-jun*)(see Studzinski, 1989, for review).

Like oncoproteins, tumour suppressor proteins are involved in parts of the mitogenic signal transduction pathway. The neurofibromatosis-1 (NF-1) gene product, for example, is homologous to the GTPase activating proteins (GAPs) that regulate the *ras* proto-oncogene product. The ras-GTP form of the protein activates adenylate cyclase, thereby sending a signal for cell proliferation or differentiation. NF-1 is thought to produce the inactive *ras*-GDP form of this protein, thus decreasing the signal production (Martin *et al*, 1990, and Xu *et al*, 1990).

Tumour suppressor gene products may also act directly as transcriptional regulators. The retinoblastoma susceptibility (Rb) gene product, for example, binds to a cellular transcription factor called E2F during G_0 or G_1 in the cell cycle, thus preventing this factor from causing transcription of a set of genes presumably required for entry into the S-phase of the cell cycle (Chellappan *et al*, 1991). The Rb protein activity seems to be regulated by phosphorylation, with no or poor phosphorylation during G_0/G_1, and high levels of phosphorylation through S-, G_2- and M-phase. A phosphatase returns the Rb

protein to an unphosphorylated state in late mitosis (Buchkovich *et al*, 1989, and Ludlow *et al*, 1990).

The cell and its environment actively control not only cell proliferation and differentiation, but also some forms of cell death.

1.7. PROGRAMMED CELL DEATH

Cell death may occur via one of two broad mechanisms, referred to, respectively, as necrosis and programmed cell death (PCD).

Necrosis refers to an uncontrolled and rapid form of cell death, typically affecting contiguous patches or areas of tissues, and is characterized by swelling of cytoplasmic organelles, deregulation of osmotic pressure and, finally, cell lysis. Intracellular contents are released into the circulating fluids and provoke an inflammatory response (for review, see Vaux, 1993).

PCD, in contrast, is a controlled death process, where the dying cells are actively involved in their own demise. These cells are observed as isolated dying cells that have lost contact with their neighbours and shrunk (Wyllie *et al*, 1980). The term "apoptosis" is used to define the morphological and physiological changes caused by PCD. Apoptotic features include the shrinking

of affected cells, the condensation of chromatin to the nuclear membrane, and the development of cytoplasmic membrane protuberances. Cytoplasmic organelles are left intact throughout most of the apoptotic process (Eastman, 1993). Apoptotic changes occur rapidly, and eventually lead to the disintegration of the cell into a series of membrane-bound, condensed apoptotic bodies (Wyllie, 1993). While internucleosomal fragmentation of DNA is often a late event in PCD, it is not always detected (Fesus, 1993). This may be due to its actual absence, or due to limited digestion that is not resolved into detectable fragments upon electrophoresis (Eastman, 1993). The endonuclease responsible for DNA fragmentation is dependent on calcium and magnesium for its action, and is functionally and antigenically identical to DNase 1 (Fesus, 1993).

The apoptotic bodies ensure that there is no inflammatory infiltrate during PCD. The dying cells are phagocytosed by neighbouring cells, usually as soon as the process of PCD has begun and before the cell has died. Because an inflammatory reaction is not elicited, PCD is a good method for removing unwanted cells in an otherwise healthy tissue (for review, see Cohen, 1991, and Vaux, 1993).

PCD death was initially defined as an active process due to the need for protein synthesis. Inhibitors of macromolecular synthesis may block cell death in some cases, indicating that the cell is actively involved in its own demise

and produces the macromolecules needed for the process. However, many cell types are not protected from death by protein synthesis inhibitors. In these cases the cell death machinery presumably already exists. The protein synthesis inhibitors may themselves trigger cell death in some cells. In these cells the death machinery is probably present and actively suppressed by macromolecules. Once these macromolecules are no longer synthesized, the cells will undergo PCD (for review, see Vaux, 1993).

As with proliferation and differentiation, PCD is a gene-directed process that can be induced or suppressed by extracellular and intracellular signals (figure 1.2). The Fas/Apo antigen, for example, is a cell surface protein that has the ability to transduce apoptotic signals to the cell (Itoh *et al*, 1993). Like with deregulation of proliferation and differentiation, deregulation of PCD can play a role in the development of cancer. The regulation of cell numbers requires a balance between cell proliferation and cell death, with neoplasia being caused either by an increase in proliferation, or a decrease in death.

Interestingly, PCD may also represent a defence mechanism against cancer. The apoptotic threshold may vary in different cell types, and cancer cells generally seem to have a lower threshold than their healthy counterparts. Many dominantly acting oncoproteins have the ability both to transform cells and to sensitize them for PCD (Fisher, 1994). PCD may, thus, be a cellular defence against deregulated growth, and this defence may potentially be

disrupted in those tumour cells that do not respond to antineoplastic therapies.

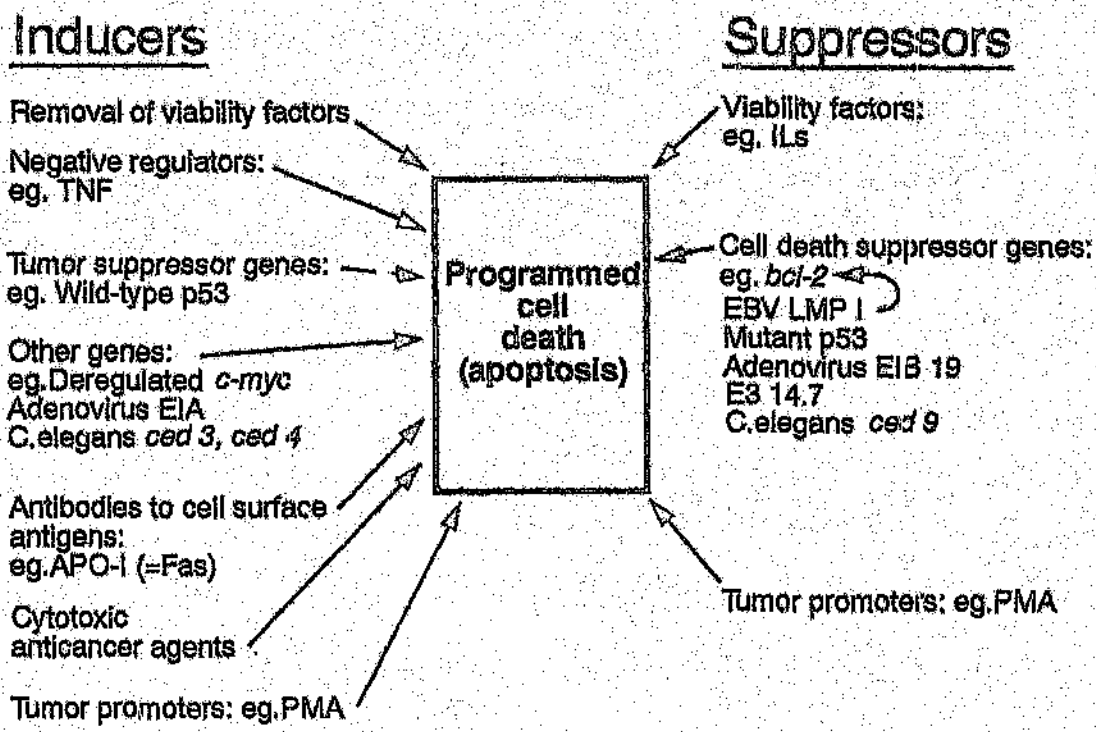


Figure 1.2.

Inducers and suppressors of programmed cell death

Programmed cell death is a gene-directed process that can be induced or suppressed by changes in the concentrations of regulatory gene products (discussed further later in the text). (Adapted from Sachs and Lotem, 1993.)

Recently, a proto-oncogene, called *bcl-2*, was discovered, that can inhibit programmed cell death in some systems under certain conditions.

1.3. THE DISCOVERY OF BCL-2

The *bcl-2* transcriptional unit was discovered solely by virtue of its localization at the site of frequent chromosomal translocations in lymphocytic malignancies. Specific chromosomal translocations are recurrently found in distinct types of malignancies (Yunis, 1983), and molecular cloning of these chromosomal breakpoints has aided in the discovery of novel proto-oncogenes.

Fukuhara *et al* (1979) described a specific chromosomal translocation occurring in at least four of their test-group of thirteen patients (ie. approximately 30 %) with poorly differentiated lymphocytic malignant non-Hodgkin's lymphoma. This translocation involved the donation of material from chromosome 18 to the end of chromosome 14, and seemed to give the cells a proliferative advantage.

Yunis *et al* (1982) studied lymph node biopsy specimens from non-Hodgkin's lymphoma patients using a high-resolution cytogenic technique for chromosomal analysis. A translocation between chromosomes 18 and 14 was found in 16 of 19 cases (ie. approximately 85 %) of follicular-cell lymphoma.

The chromosomal fragment donated from chromosome 18 was found to be specific, showing a constant breakpoint (band q21) in all 16 cases investigated.

The term B-cell lymphoma/leukaemia 2 (*bcl-2*) was proposed for the DNA fragment from chromosome 18 by Tsujimoto *et al* (1984). The findings of this group suggested that the *bcl-2* gene may be consistently involved in B-cell malignancies with the t(14;18) translocation. *bcl-2* is moved into juxtaposition with the immunoglobulin heavy chain region upon translocation, thereby falling under the influences of a transcriptional enhancer located between the heavy joining chain (J_H) gene and the switch region of the human heavy μ chain gene.

bcl-2 and hybrid *bcl-2*/immunoglobulin cDNAs were cloned by Cleary *et al* (1986), and by Tsujimoto *et al* (1986). The *bcl-2* gene was shown to be composed of three exons, the first of which is untranslated. Exons II and III together encode two closely similar proteins, the *bcl-2α* 26 kd protein and the *bcl-2β* 21 kd protein, which differ only in their carboxyl terminal portions. Both proteins were shown to share homology with the predicted Epstein-Barr virus (EBV) protein BHRF-1 synthesized in the early phase of virus infection.

However, even though *bcl-2* had been accepted as a candidate proto-oncogene, no specific biological functions of the *bcl-2* protein product (termed BCL-2) had been described. This changed when Vaux *et al* (1988) published

their finding that BCL-2 expression promoted the survival of pre-B cell lines deprived of their growth factor interleukin-3 (IL-3). Cells infected with the *bcl-2* expression vector persisted in a G_0 state after IL-3 withdrawal, rather than proliferating. It was argued that BCL-2 provided a distinct survival signal to the cells, perhaps contributing to neoplasia by allowing a clone to persist until other oncogenes, that promote proliferation, become activated

Pezzella *et al* (1990) used monoclonal and polyclonal antibodies directed against BCL-2 to show that it was not only expressed in the neoplastic germinal centres of most follicular lymphomas, but also in normal lymphoid cells, of both the B- and T-cell types, and in a substantial number of lymphoproliferative disorders, such as hairy cell leukaemia and Ki-1 lymphoma, in which the 14;18 translocation is not found. These findings indicated that the t(14;18) translocation was not necessary for the expression of BCL-2, and that immunohistological demonstration of this molecule was, therefore, not specific for the t(14;18) translocation.

Thus, *bcl-2* was first discovered in certain B-cell malignancies, where its protein product seemed to prolong cell survival. Once the protein was shown to be present in normal healthy lymphoid cells, however, it was no longer thought of solely as a by-product of certain neoplasias. The distinct function(s) and mechanism(s) of action of BCL-2 remained elusive. Some clues arose when its cell- and tissue-distribution were studied.

1.9. BCL-2 TISSUE DISTRIBUTION

Hockenbery *et al* (1991) used monoclonal antibodies directed against BCL-2 to study its tissue distribution, and found that expression of the protein was topographically restricted to cell lineages and tissues that are characterized by apoptotic cell turnover. These included immature B- and T-cells, glandular epithelium, the basal layer of the epidermis, and neurons. *bcl-2* mRNA and BCL-2 protein expression has since been observed in exocrine glands, basal keratinocytes, cells at the base of colon crypts, and in embryonic kidney and cartilage, where cells are undergoing morphological transition from undifferentiated stem cells to committed precursor cells (Lu *et al*, 1993).

Further studies have revealed that *bcl-2* mRNA and BCL-2 protein expression is linked to specific developmental stages of certain cell and tissue types.

1.10. BCL-2 EXPRESSION IN THE BRAIN

PCD is a wide-spread mechanism for actively removing superfluous cells and cells that have served their purpose (Williams, 1991), and plays an important role in the developing nervous system (Martin *et al*, 1988). Early on in the

development of the embryo, many more neurons are produced than are ultimately required by the nervous system. Surplus neurons eventually die off as part of the process that models the brain and its nerves into their final form (Barinaga, 1993). Some neurons undergo PCD because they arose in the wrong place or are no longer needed. Most, however, are killed off as part of a "size-matching" process, whereby a large excess of nerve cells are initially produced, and then compete for survival until the right number remains. This system is a safety device to ensure that enough neurons are made, as genes do not code for the exact number of neurons that are to be produced.

Recent research has indicated that levels of BCL-2 expression may play a role in neuronal death and survival. When embryonic neurons are cultured shortly after innervating their synaptic targets, they die unless the culture medium is supplemented with an appropriate neurotrophic factor. Allsopp *et al* (1993) have shown that PCD of neurotrophic factor-deprived embryonic chicken neurons can be prevented by introduction of a *bcl-2* expression vector into the cells. Three types of cranial neurons were used in the study, and, while cell death could be prevented by BCL-2 expression in cutaneous and proprioceptive sensory neurons, it could not be prevented in ciliary neurons. These findings suggest that BCL-2 may function in preventing PCD in certain kinds of neurons, and that at least two intracellular mechanisms of PCD associated with neurotrophic factor deprivation exist, one that can be prevented by BCL-2 and one that is unaffected by this molecule.

Introduction of a *bcl-2* expression vector also inhibited PCD in a conditionally immortalized central neural cell line, CSM14.1 (Zhong *et al*, 1993). This protection from cell death included inhibition of neuronal death due to membrane peroxidation, calcium ionophores, and serum, glucose and growth factor withdrawal.

While the two above-mentioned studies involved the introduction of a *bcl-2* expression vector into neural cells, other studies were performed that measured the constitutive expression of *bcl-2* mRNA and BCL-2 protein in neural tissue.

bcl-2 mRNA expression was found to be high in the foetal murine brain, and seemed to be developmentally regulated (Abe-Dohmae *et al*, 1993). *bcl-2α* and *bcl-2β* mRNA levels were highest on embryonic day 15, being about two and three times higher than those of adult, respectively. This time period corresponds well with the peak of neurogenesis in the foetal brain, suggesting that *bcl-2* may be involved in events occurring during brain development. After day 15, levels decreased gradually, reaching adult levels by two to three weeks postnatally.

An association between BCL-2 protein levels and cell morphology was also found in neuroblastoma (NB) cell lines (Tanaka *et al*, 1993). Neuroblastoma is the most common extracranial solid tumour of childhood

(Seeger *et al*, 1982). NB cell lines can be classified according to their predominant morphology. Thus, cells that originated in the paraspinal sympathetic ganglia or the adrenal medulla and that express genes associated with neuronal differentiation are referred to as neuroblastic- (or "N"-), type cells. Non-neuronal cells that grow tightly adherent to their substratum and are related to Schwann, glial, and melanocytic cells are known as "S"- (substratum-) type cells. Cells displaying intermediate morphological characteristics are termed "I"-type cells. The S-type cell lines studied uniformly contained little or no BCL-2 protein, while the N-types contained relatively high levels. When the N-type cell line SH-SY5Y was induced to differentiate upon TPA stimulation, a five- to ten-fold elevation in BCL-2 levels was measured within one day. Conversely, when the I-type BE(2)-C clone was induced to differentiate towards an S-phenotype using 5'-bromodeoxyuridine. This resulted in an approximately 50 % decrease in BCL-2 levels within three days.

Thus, BCL-2 expression is high in the adult brain and seems to increase with increasing neural differentiation. BCL-2 seems to help protect neurons from cell death and may, therefore, be linked to their longevity. Developing neurons will compete against one another for growth factors, and death of those neurons that "lost the battle" may be coordinated by decreasing levels of BCL-2 expression. Fully differentiated neurons that have integrated into the adult neural network will, therefore, display higher levels of BCL-2 expression

and will, thus, be protected from cell death.

In the developed brain, neurons that die are not replaced. It is, therefore, important that unnecessary death is inhibited in order to ensure adequate brain functioning. High levels of BCL-2 expression in the differentiated and integrated neurons may have been selected for during evolution to protect neurons from certain forms of cell death.

During murine foetal brain development, BCL-2 expression peaks at a time when cell generation in the cerebral cortex and hypothalamus is at its highest (Abé-Dohmae *et al*, 1993). This, again, suggests a role for BCL-2 protein product in protecting neurons from cell death.

Newly emerging evidence seems to link BCL-2 expression to hormone levels in hormone-responsive tissues, such as the endometrium and the prostate.

1.11. BCL-2 EXPRESSION IN THE ENDOMETRIUM AND PROSTATE

Hormonal regulation of BCL-2 protein expression is displayed in the uterine endometrium (Gompel *et al*, 1994, Otsuki *et al*, 1994) and in the prostate (MacDonnell *et al*, 1992; Colombel *et al*, 1993).

ENDOMETRIUM

The endometrium undergoes a rapid process of proliferation, differentiation, and cell death under the influence of ovarian oestrogens (mainly oestradiol) and progestins (mainly progesterone) during the female sexual cycle ("menstrual" cycle). This cycle operates through various phases. The endometrium will first go through a proliferative period up to the stage of ovulation under the influence of high levels of oestrogens and low levels of progestins. During this phase of the cycle, BCL-2 expression in the glandular epithelium is up-regulated, reaching maximum levels by the end of the proliferative stage.

During the latter, secretory, half of the cycle, oestrogens and progestins are released in large quantities by the corpus luteum. High levels of progestins cause marked swelling and secretory development of the endometrium, thereby producing appropriate conditions for implantation of a fertilized ovum. BCL-2 protein disappears rapidly in the glandular epithelium of the endometrium as soon as secretory characteristics appear.

Approximately two days before the end of the cycle, the level of ovarian oestrogens and progestins decreases sharply, and menstruation follows. No BCL-2 expression can be detected in the glandular epithelium of endometrial samples during menstruation.

These studies strongly suggest hormonal regulation of BCL-2 protein expression. Up-regulation of BCL-2 synthesis by oestrogens and down-regulation by progestins could play a role in physiological homeostasis of the uterine endometrium.

Further studies might show that BCL-2 expression also displays cyclic variations due to hormone regulation in female breast tissue.

PROSTATE

In normal prostate glands of the adult, BCL-2 expression is restricted to the basal cells, with the more differentiated epithelial cells staining negative for BCL-2 (Colombel *et al*, 1993). Interestingly, the basal cells are thought to represent the stem cell layer in the adult prostate (Colombel *et al*, 1993). Furthermore, BCL-2 is expressed during epithelial development in the foetal prostate. Thus, BCL-2 expression in the prostate seems to be linked to the state of differentiation of the cell.

Basal cells that express BCL-2 are resistant to androgen-induced apoptosis (Rouleau *et al*, 1990). Interestingly, : ndrogen ablation in castrated rats will cause an increased BCL-2 expression, thus indicating an inverse relationship between androgen concentration and BCL-2 expression in the prostate epithelium (McDonnell *et al*,1992).

BCL-2 expression in haematopoietic cells has been widely studied and seems to be differentiation-dependent.

1.12. BCL-2 EXPRESSION IN LYMPHOCYTES

Lymphocytes are haematopoietic cells that develop in the primary lymphoid organs: T cells in the thymus and B cells in the foetal liver and bone marrow. These cells then migrate to secondary lymphoid tissue where they respond to antigen.

B CELLS

There are two main stages during B cell development when the cell risks undergoing PCD (Cohen, 1991):

First, the majority of pre-B cells that undergo D (diversity segment gene)-

to-J (joining segment gene) rearrangements of the immunoglobulin heavy- and light-chain loci, fail to rearrange their genes in such a way as to produce a complete transcript. Cells with such nonsense rearrangements are probably programmed to die.

Second, in pre-B cells that have successfully rearranged their heavy- and light-chain loci, functional surface immunoglobulins (sIg) will be produced. During the window period in which sIgM, but not sIgD, are produced, an encounter with a specific antigen will cause deletion of that pre-B cell. As the antigens most likely to be present in the bone marrow are self molecules, this is one way of deleting autoreactive B cell clones.

Most splenic B cells display a rapid turnover, with 80 to 90 % dying within 18 to 24 hours. Only those B cells exposed to antigenic stimulation are rescued from death, and then leave the spleen to recirculate to the lymph nodes. This rapid turnover of splenic B cells allows a fresh set of receptors to be available constantly, while antigen-stimulated cells survive to become long-lived memory cells.

Mature, healthy B cells do not express *bcl-2* mRNA (Schena *et al*, 1992) or BCL-2 protein (Hockenbery *et al*, 1991). BCL-2 expression does, however, occur in precursor B cells and returns in memory B cells (Hockenbery et al, 1991). This BCL-2 expression pattern may contribute to cell survival patterns.

Experiments were performed on transgenic mice that expressed the *bcl-2* gene controlled by an immunoglobulin regulatory sequence (Strasser et al, 1991a). These mice had a great excess of non-neoplastic pre-B cells, B lymphocytes, plasma cells, and serum immunoglobulins. They also displayed a heightened and prolonged antibody-forming cell response to immunization. These findings are attributable to increased longevity of the B-lineage cells, and are consistent with the notion that constitutive BCL-2 expression interferes with mechanisms of normal B cell development and turnover. Within the first year of life, 60 % of the mice developed some form of autoimmunity, which was probably linked to the increased longevity of autoreactive pre-B cells.

Schena *et al* (1992) have shown that B-chronic lymphocytic leukaemia (B-CLL) cells isolated from eleven patients expressed high levels of BCL-2, which may confer a survival advantage and favour their relentless accumulation. This expression could be down-regulated transiently when cells were exposed to the differentiation agent TPA. *bcl-2* mRNA expression decreased transiently to 35 % of control values by three hours after exposure to TPA, but increased to fourfold the control value by 12 hours post-induction. These opposite effects could be due to the differences in the short-term and long-term effects of phorbol ester stimulation. Thus, the initial activation of PKC leads to a decrease in BCL-2 expression, while the long-term down-regulation leads to

differentiation and BCL-2 up-regulation. The eventual up-regulation in response to differentiation may be a malignant aberration. However, it may also indicate that the cells investigated were affiliated to long-term memory B cells. Further differentiation toward the memory B cell morphology and physiology would, thus, be accompanied by the increase in BCL-2 expression that is also observed when healthy B cells differentiate toward memory B cells (Hockenbery *et al*,1991).

In contrast, proliferation-inducing agents caused a sustained decrease in *bcl-2* mRNA and BCL-2 protein concentrations in B-CLL cells. This corresponds with the decrease in BCL-2 concentrations observed during terminal differentiation of B cells (Hockenbery *et al*, 1991, Schena *et al*, 1992).

T CELLS

T (thymus-derived) lymphocytes play central roles in the growth regulation of other haematopoietic cell types via secretion of growth and differentiation factors, and in immune responses via their T cell receptor (TCR) for antig... The TCR recognises cell-associated fragments of foreign antigens, which are displayed in association with a class of immunoglobulin-like molecules known as the major histocompatibility complex (MHC) of an antigen-presenting cell. For many T cells, another critical set of molecules is required to mediate

recognition of the antigen/MHC complexes, namely the CD4 or CD8 glycoproteins of the immunoglobulin gene superfamily. The majority of mature T cells expresses either CD4 or CD8, and their cross-linking with the TCR complex strongly enhances T cell activation. Free, native antigen is generally invisible to T cells (for review, see Rothenberg, 1992).

PCD plays an important role in the deletion of autoreactive T cell precursors during intrathymal development. Precursor $CD4^-CD8^-$ cells and mature $CD4^+CD8^-/CD4^-CD8^+$ (single positive) thymocytes express relatively abundant mRNA for *bcl-2*, whereas $CD4^+CD8^+$ (double positive) thymocytes express low levels of BCL-2 (McConkey *et al*, 1994).

Transgenic mice have been generated that overexpress BCL-2 in immature T cells in the thymal cortex. These cells normally express only low levels of BCL-2, and its overexpression places a possible antidote to PCD proximal to positive and negative selection of pre-T cells in the thymus (Sentman *et al*, 1991). Even though the total number of thymocytes remained constant, T cell maturation was altered in that the subset of cells representing the transition from double positive to single positive cells was markedly increased. It has been proposed that this population represents an intermediate stage of thymocyte development following positive selection (Guidos *et al*, 1990; Shortman *et al*, 1991). BCL-2 could, however, not rescue self-reactive T cells from negative selection, indicating that BCL-2-dependent and BCL-2-

independent death pathways may exist in the thymus (Korsmeyer, 1992a).

The inability to benefit from BCL-2 expression seems to be confined to very early T cells, with immature T cells acquiring responsiveness to BCL-2 as soon as they first start displaying the TCR antigen-receptor complex at the cell surface (Strasser *et al*, 1994). These data support the concept that BCL-2 expression can promote the survival of immature and mature T cells, but also that other, BCL-2 independent, cell death pathways contribute to the control of lymphocyte numbers.

Evidence that BCL-2 does not interfere with all pathways of cell death was also obtained from BCL-2 transgenic mice T lymphocytes treated with apoptosis-inducing γ-radiation, glucocorticoids, ionomycin, and PMA (Strasser *et al*, 1991b). All transgenic thymocytes, peripheral T cells, and activated T cells resisted killing by these agents. The transgenic cells were not, however, resistant to killing by complement or cytotoxic T cells.

The average lifespan of primed memory T cells is between 18 and 31 months in humans (Akbar *et al*, 1993), even though the memory response to an antigenic encounter may persist for decades. Thus, continuous restimulation and proliferation of responsive clones is required to maintain T cell memory. Surprisingly, primed peripheral T cells commonly down-regulate BCL-2 expression (Akbar *et al*, 1993). This could be a mechanism for controlling T

numbers by removing unwanted T cells after an infection or other immune stimulus. However, primed cells are more responsive to the cytokine interleukin-2 (IL-2), and will upregulate BCL-2 expression in its presence (Akbar *et al*, 1993). Antigenic persistence would ensure the ongoing availability of IL-2, thereby keeping at least some activated T cells in cycle while sustaining high BCL-2 levels and, thus, cell stability. IL-2-dependent BCL-2 expression was also demonstrated in a cytotoxic T cell line (Deng and Podack, 1993), where expression of endogenous BCL-2 was down-regulated within eight hours of IL-2 withdrawal and was not detected after three days. Addition of IL-2 induced BCL-2 expression within eight hours and led to full recovery of BCL-2 expression within 24 hours.

1.13. BCL-2 EXPRESSION IN MYELOID CELLS

Myelopoiesis starts with a pluripotent haematopoietic stem cell. The first progenitor cell arising from this stem cell is the colony forming unit which can give rise to granulocytes (neutrophils, basophils, or eosinophils), erythrocytes, monocytes (which represent a replacement pool for tissue-fixed macrophages), and megakaryocytes under the influence of colony stimulating factors and several interleukins including IL-1, IL-2, IL-3, IL-5, and IL-11 (Rothstein, 1993).

BCL-2 levels in normal myeloid cells are inversely related to maturation (Delia *et al*, 1992). Thus, myeloblasts and promyelocytes are largely BCL-2 positive whereas metamyelocytes and polymorphonuclear cells are mostly BCL-2 negative and monocytes totally negative. Furthermore, induction of leukaemic HL-60 cells toward the monocyte and granulocyte pathways was accompanied by a marked decrease in *bcl-2* mRNA and protein concentrations. Whether a link between differentiation and decreased BCL-2 expression in myeloid cells is a necessary occurrence or is just coincidental, remains to be established. It has been shown (Park *et al*, 1994), however, that, at least in HL-60 cells, a vector mediated increase in BCL-2 expression still allows for retinoic acid induced cell differentiation to occur. Thus, at least in this case, a decrease in BCL-2 expression is not necessarily linked to differentiation.

BCL-2 expression has been linked to increased resistance to certain stresses such as heat shock and ethanol. Interestingly, high expression of BCL-2 is also associated with a low complete remission rate after intensive chemotherapy in acute myeloid leukaemia patients (Campos *et al*, 1993), thus implicating BCL-2 in resistance to chemotherapy.

Even though studies on cell and tissue distribution patterns of *bcl-2* mRNA and protein expression have helped to elucidate the physiological function of BCL-2, its biochemical function and mode of action have remained

speculative. Research on *the bcl-2* gene and BCL-2 protein structures is, however, aiding in the search for answers.

1.14. BCL-2 GENE AND PROTEIN STRUCTURE

The *bcl-2* gene consists of three exons, with the second and third exon separated by an extremely large, 225 kb intron. Two promoters are responsible for the initiation of transcription: P1, and P2 (Seto *et al*, 1988).

The predominant P1 promoter is GC-rich and TATA-less, displaying multiple start sites and seven consensus binding sites for the Sp1 transcription factor. This promoter structure is usually associated with constitutively expressed genes (Dynan and Tjian, 1983), and seems somewhat unusual for a gene like *bcl-2*.

The P2 promoter is located approximately 1.3 kb downstream from P1, and includes both a TATA- and a CCAAT-box. It also displays two initiation sites, and an octamer motif identical to that found in the simian virus 40 (SV40) enhancer. This promoter initiated only a small fraction of *bcl-2* mRNA in the cell lines studied by Seto *et al* (1988).

Due to an alternative splicing event, the *bcl-2* gene can code for two protein products, BCL-2α and BCL-2ß, the first consisting of 239 amino acids and the second of 205 amino acids (Tsujimoto *et al*, 1986). The p26 and p22 proteins are identical in their first 195 amino acids, but differ in their carboxyl ends. The carboxyl end of the p26-BCL-2α protein contains a stretch of 17 hydrophobic amino acids that have been shown to constitute a transmembrane domain.

The hydrophobic region is followed by two positively charged amino acids, histidine and lysine, that probably serve to anchor the BCL-2 protein in the membrane into which it is inserted (Chen-Levy, 1990). Quantitatively, p26-BCL-2α is far more important, in that p22-BCL-2ß is rarely observed in cells (Chen-Levy and Cleary, 1989).

Tanaka and his group (1993) have demonstrated that BCL-2 needs its hydrophobic carboxyl end in order to function properly within the cell. Experiments were performed using the interleukin-3 (IL-3)-dependent haematopoietic cell line 32D. In contrast to p26-BCL-2α, p22-BCL-2ß did not prolong cell survival after IL-3 withdrawal when expressed in 32D cells. However, when the transmembrane domain of the interleukin-2 receptor p55 subunit was fused to p22-BCL-2ß, this chimeric protein was able to extend cell survival. It, therefore, seems likely that it is the lack of a transmembrane domain that renders p22-BCL-2ß defective in extending cell survival.

bcl-2 gene transcriptional regulation is an important determinant of steady state *bcl-2* mRNA levels. The gene contains a 1.3 kb negative regulatory element (NRE) in its 5'-untranslated region that inhibits transcription initiated at its P1 promoter (Young and Korsmeyer, 1993). The 5'-region of *bcl-2* is unusual in that it is extremely long and is highly conserved between the mouse and human genes throughout its length (Negrini *et al*, 1987).

NRE activity is mediated by multiple, functionally redundant elements. This functional redundancy is also observed in other regulatory elements, such as silencers (Baniahmad *et al*, 1990) and enhancers (Herr and Clarke, 1986). Although the NRE can inhibit transcription in either orientation, the activity of its subfragments seems to be orientation-dependent. The NRE functions in a position-dependent manner. It does not exhibit activity when placed in the 3'-region of the *bcl-2* gene and, thus, does not meet the definition of a silencer.

The 5'-untranslated region of the *bcl-2* gene contains a second regulatory element, called the p53-dependent negative regulatory element (or PNRE) (Miyashita *et al*, 1994). This 195-base pair segment mediates the repression of *bcl-2* gene expression via the tumour suppressor gene product p53 in an orientation- and position-independent manner. Whether the observed decrease in BCL-2 protein expression occurs via the p53 protein directly or through another transcriptional regulator whose production is regulated by p53

remains to be determined.

Even though the integral membrane domain of BCL-2α product has been identified, the location of the this protein within the cell remains controversial.

1.15. SUBCELLULAR LOCATION OF THE BCL-2 PROTEIN

Although BCL-2 is membrane-associated, its subcellular location is controversial. Based on subcellular fractionation and immunofluorescence experiments, Chen-Levy *et al* (1989) reported that the protein resides mostly in perinuclear membranes, with about half being associated with the nucleus and the rest with other intracellular membranes. Using similar methods, Hockenbery *et al* (1990) reported that the protein is found primarily in the inner mitochondrial membrane. Since then, various studies have found BCL-2 associated to varying degrees with the mitochondria and other non-nuclear compartments (Liu *et al*, 1991; Hamilton et al, 1991), nuclear envelope and outer mitochondrial membrane (Monaghan *et al*, 1992, Nakai *et al*, 1993), and the endoplasmic reticulum, nuclear envelope and mitochondria (Jacobson *et al*, 1993).

In an effort to clarify the confusion surrounding the intracellular location

of BCL-2, Krajewski *et al* (1993) undertook a multidisciplinary approach to localize the protein in a B cell lymphoma cell line. A combination of methods was used, including subcellular fractionation analysis, immune microscopy studies entailing laser-scanning and electron microscopy, and six different antibodies directed against BCL-2. The protein was found to reside primarily in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane.

The immuno-electron microscopy data suggested a non-uniform distribution pattern of BCL-2. This may be caused by BCL-2 being targeted to specialized regions in the membrane. Since BCL-2 seems to lack organelle-specific targeting sequences, it may non-specifically enter large protein complexes in certain intracellular membranes and be held there through protein-protein interactions. Possible candidates for such membrane proteins would be those associated with the nuclear pore complex and the junctional complexes in mitochondria, both of which are involved in protein transport across membranes (Pfanner and Neupert, 1990; Newmeyer *et al*, 1994). Thus, though highly speculative, the possibility exists that BCL-2 might execute its effect on PCD by playing a role in the regulation of certain membrane transport systems.

More confusing data has, however, emerged with respect to BCL-2 location and function in the form of two separate sets of experiments. One set

of studies ... cells lacking a functional mitochondrial respiratory chain (Jacobson *et al*, 1993), and cell-free extracts with and without mitochondria (Newmeyer *et al*, 1994). The other study used cells lacking a nucleus (Jacobson *et al*, 1994a).

In an experiment by Jacobson *et al* (1993), a human fibroblast cell line was depleted of mitochondrial DNA, thereby removing the genetic coding for thirteen polypeptide components of the mitochondrial electron transport chain and making the cells unable to carry out oxidative phosphorylation. These cells could, however, still be induced to undergo PCD by either serum deprivation or staurosporine treatment. Furthermore, transfection of the cells with a *bcl-2* expression vector led to protection from cell death under apoptosis-inducing conditions. These findings seem to exclude the possibility that either PCD or the protective effect of BCL-2 involves the mitochondrial respiratory chain, at least in the cells studied. Mitochondria are, however, required for PCD to occur, at least in the cell-free Xenopus egg extract systems studied by Newmeyer *et al* (1994). These systems could only undergo PCD in the presence of a mitochondrial fraction, and this PCD could be inhibited in the presence of BCL-2. Thus, BCL-2 probably depends at least partially on the mitochondrial system, but not on a functional respiratory chain, for its inhibitory effect on PCD.

In a subsequent study, Jacobson *et al* (1994a) showed that cells of the

human fibroblast GM701 cell line, the rat oligodendrocyte precursor cell line CG-4, and their corresponding anucleate cytoplasts could all undergo PCD after staurosporine treatment or growth-factor withdrawal. This process could be inhibited by the introduction of a *bcl-2* expression vector into the cells. Enucleated cells retained their mitochondrial function for approximately 24 hours after plating. Addition of staurosporine induced death in over 80 % of GM701 cells and cytoplasts by 12 hours post-induction, whereas virtually no whole cells and less than 20 % of cytoplasts had died by 12 hours in the absence of staurosporine. These results imply that BCL-2 can effectively protect at least some cells from PCD both in the presence and absence of a nucleus.

Thus, in summary, BCL-2 has been localized to the outer mitochondrial membrane, endoplasmic reticulum, and nuclear membrane. The protein cannot function as an inhibitor of PCD in the absence of its integral membrane domain and, therefore, seems to need membrane insertion to be active. Cells lacking nuclei and cells lacking a functional mitochondrial respiratory chain can still undergo PCD and are protected from death by BCL-2. Intact mitochondria are, however, a requirement for PCD, at least in some systems.

Lipid membranes are sites in which reactive oxygen intermediates engage in potentially damaging lipid peroxidation chain reactions (Wyllie, 1994). Part

of the function of BCL-2 could, thus, perhaps be to protect cells from death by inhibiting lipid peroxidation.

I.16. THE ROLE OF BCL-2 IN THE ANTIOXIDANT PATHWAY

Recent studies (Hockenbery *et al*, 1993) indicate a role for BCL-2 in an antioxidant pathway, where it acts after the generation of superoxide and peroxides to prevent cellular damage including lipid peroxidation, and DNA and protein damage. Oxygen radicals are produced as a by-product of functional electron transport chains in the mitochondria, the nuclear envelope, and the endoplasmic reticulum. These are also the three sites where BCL-2 has been primarily localized. Thus, BCL-2 may itself act as a scavenger of oxygen radicals, or it may associate with a protein that acts in such a manner. Oxygen radicals may play a role as signalling molecules that regulate the cell's redox state and affect the activity of responsive transcription factors (eg. AP-1) (Hockenbery *et al*, 1993). Oxygen radicals also trigger the release of mitochondrial Ca^{2+}, an event that is often, but not always, observed during PCD. Increased BCL-2 concentrations have been shown to decrease the release of Ca^{2+} from the endoplasmic reticulum, and the protein may thus function in part by affecting intracellular Ca^{2+} concentrations (Lam *et al*, 1994).

A putative role for BCL-2 in the antioxidant pathway is supported by studies done on *bcl-2* gene-ablated mice (Veis *et al*, 1993). These mice developed two potentially redox related pathologies. Firstly, they developed polycystic kidneys that were reminiscent of those kidney abnormalities caused by metabolic toxins acting on redox enzymes. Furthermore, these gene-ablated mice had hypopigmented hair, and melanogenesis is thought to be regulated through peroxide and its enzymatic detoxification (Halaban and Moellmann, 1990).

Interestingly, these gene-ablated mice underwent apparently normal prenatal development and were born at roughly the expected frequency. This was probably due to the existence of other, BCL-2-related, molecules that provide a redundancy in death repressor activity during mouse embryogenesis. Massive cell death was, however, observed in the spleen and thymus at various ages (Veis *et al*, 1993).

Some further clues as to the action of BCL-2 have been provided by cell death studies in the nematode *Caenorhabditis elegans*.

1.17. CELL DEATH IN THE NEMATODE CAENORHABDITIS ELEGANS

In the nematode *C.elegans*, 131 of the 1090 somatic cells generated during its development undergo programmed cell death (Sulston *et al*, 1983). Fourteen genes have been identified that can be placed into a genetic pathway of apoptosis (Ellis *et al*, 1991), three of which are involved in the regulation and execution of all 131 developmental cell deaths occurring in *C.elegans*. Two of these three genes, namely *ced-3* and *ced-4*, need to be expressed in cells scheduled to undergo apoptosis (Ellis and Horvitz, 1986). The third gene, *ced-9*, negatively regulates apoptosis, in that mutations that cause *ced-9* gain-of-function will prevent the deaths of those 131 cells programmed to die, while inactivation of *ced-9* will cause cells that normally live to undergo apoptosis (Hengartner *et al*, 1992).

The *ced-9* protein shows significant sequence homology (23 % homology over the entire length of the protein) and structural similarity to BCL-2 (Hengartner and Horvitz, 1994b). Furthermore, overexpression of human BCL-2 prevents the normal cell deaths that usually occur during *C.elegans* development, and substitution of BCL-2 for *ced-9* can prevent the deaths of cells that should normally survive (Hengartner and Horvitz, 1994).

The *C.elegans ced-9 gene* shares a promoter with the *cyt-1* gene, which encodes a cytochrome b_{560} homologue. This allows the concerted regulation

of the two genes and indicates that they may be linked to a common biological function. Thus, *cyt-1* may perhaps play a role in PCD in *C.elegans*. Interestingly, human cytochrome b_{560} is part of the mitochondrial electron transport chain, and BCL-2 has been suggested to play a role in decreasing the damaging effects of the oxygen radicals that are produced during mitochondrial respiration (Hockenbery *et al*, 1993). Thus, the two genes may also be functionally linked in humans.

The amino acid sequence of the CED-3 protein is similar (28 % overall homology) to the mammalian interleukin-1ß (IL-1ß) converting enzyme (ICE) (Yuan *et al*, 1993). This cysteine protease cleaves the inactive 33 kd or 31 kd precursor of IL-1ß between Asp^{116} and Ala^{117}, releasing the 153 carboxy-terminal amino acids that constitute the active cytokine (Cerretti, *et al*, 1992; Thornberry *et al*, 1992). The similarity between the *ced-3* protein and ICE suggests that ICE may control PCD during vertebrate development, just as *ced-3* controls cell death during C.elegans development. Recent studies by Miura *et al* (1993) have shown that overexpression of ICE causes Rat-1 fibroblasts to undergo PCD, and that this death can be inhibited by BCL-2. ICE activity can also be inhibited by crmA, a cowpox virus gene product that inhibits inflammation (Ray *et al*, 1992) and that can prevent vertebrate neuronal death induced by deprivation of nerve growth factor (Gagliardini *et al*, 1994).

Interestingly, an *Ice/ced-3*-related gene that encodes both positive and negative regulators of programmed cell death, has been discovered (Wang *et al*, 1994). This gene, termed *Ich-1*, codes for mRNA that produces two distinct products upon alternative splicing. The two mRNA species code for two types of protein: ICH-1_L and ICH-1_S. Surprisingly, while ICH-1_L overexpression induces programmed cell death, overexpression of ICH-1_S results in the suppression of cell death.

In contrast to *ced-3* and *ced-9*, attempts in cloning a *ced-4* homologue in vertebrates have been unsuccessful so far. However, two regions have been identified within the 549 amino acid sequence of the *ced-4* protein that are similar to the calcium-binding domain of calmodulin known as the EF hand, suggesting that *ced-4* activity may be regulated by calcium in *C.elegans*.

Recently, three homologs of BCL-2, namely MCL-1, BCL-x and BAX, have been discovered in vertebrates. Thus, a clear family of BCL-2-like proteins is emerging.

1.13. BCL-2-LIKE GENES IN VERTEBRATES

MCL-1

The *mcl-1* gene, which codes for a protein with sequence similarity to BCL-2, was isolated from a human myeloid leukaemia cell line upon phorbol ester-induced differentiation along the monocyte/macrophage pathway (Kozopas *et al*, 1993). This increase in MCL-1 expression in response to monocyte differentiation is unexpected in that BCL-2 expression decreases in response to monocyte differentiation. The similarity between the two proteins is in their hydrophobic carboxyl portion, suggesting that MCL-1 may also be membrane-associated. The amino-terminal portion of MCL-1 differs from that of BCL-2 in that it contains two PEST sequences (rich in proline, glutamic acid, serine, and threonine) and four pairs of arginines. Pest sequences and pairs of arginines are present in a variety of oncoproteins and other proteins that undergo rapid turnover and are usually expressed primarily in the early stages of differentiation (Kozopas *et al*, 1993). One might speculate that MCL-1 protein influences cell viability in the early stages of induction of differentiation, and that its mechanism of action may be comparable to that of BCL-2.

BCL-X

bcl-x is a *bcl-2*-related gene that is highly expressed in the lymphoid and central nervous system. Two distinct *bcl-x* mRNA species, resulting from the

alternative use of two separate 5' splice sites located within the first coding exon of the *bcl-x* gene, have been isolated (Boise *et al*, 1993).

The longer mRNA, *bcl-x*$_L$, encodes a protein that appears similar in size and predicted structure to BCL-2. When transfected into an IL-3-dependent cell line, BCL-X$_L$ prevented PCD following growth factor withdrawal to an even greater extent than did BCL-2 overexpression. After eight days in growth factor depleted culture, essentially no loss of cell viability had occurred in the BCL-X$_L$ transfected cells, whereas approximately 50 % of cells overexpressing BCL-2 did not survive the same time period. Cotransfection of BCL-2 and BCL-X$_L$ did not improve cell survival beyond that of transfection with BCL-X$_L$ alone.

The shorter *bcl-x*$_S$ mRNA contains a deletion of 63 amino acids from the *bcl-x*$_L$ open reading frame that constitute the region of highest amino acid identity between the BCL-X$_L$ and BCL-2 proteins. When BCL-X$_S$ was transfected into the IL-3-dependent cell line, no protection from cell death followed the withdrawal of growth factor. Furthermore, the cells died with kinetics indistinguishable from control cells upon IL-3 deprivation. Surprisingly, cotransfection of BCL-2 with BCL-X$_S$ resulted in cells that were sensitive to growth factor withdrawal, undergoing PCD upon IL-3 deprivation. The approximate average survival of BCL-2 transfected cells four days after IL-3 removal was 80 %, while that of clones coexpressing BCL-2 and BCL-X$_S$

was 10 %. Thus, BCL-X_S seems to play a dominant role to BCL-2 in the regulation of apoptosis. (See table 1.1.)

One way in which BCL-X_S could act to inhibit BCL-2 function is by forming an inactive heterodimer protein complex with BCL-2. No evidence for this interaction has, however, been obtained. BCL-X_S could, perhaps, bind to and enhance the function of downstream mediators of apoptosis, while BCL-X_L would bind to and prevent the action of such mediators. The effect of BCL-X_S would have to compete with and override that of the BCL-2 protein, perhaps by a higher affinity interaction with the putative downstream regulators of PCD. The expression of BCL-X_S protein could account for those situations when BCL-2 expression does not overcome cell death, such as during the negative selection of T lymphocytes.

Intercellular Proteins Involved in Programmed Cell Death			
C.elegans	Mammalian	Role in Cell Death	Function
CED-9	Bcl - 2	prevents	Opposes Bax
?	Bax	promotes	Opposes Bcl-2
?	Bcl-x (long)	prevents	Opposes Bcl-x (short)
?	Bcl-x (short)	promotes	Opposes Bcl-x (long)
CED-3	ICE	promotes	Protease
?	?	promotes	?

Table 1.1.

Intracellular proteins involved in programmed cell death

Proteins that are expressed during the induction and suppression of PCD interact with one another, and have been evolutionary conserved from the nematode *C.elegans* to mammals. (From Barinaga 1994.)

Murine BCL-X_L is highly expressed in the brain. It is also highly expressed in the bone marrow and thymus, but is down-regulated in the spleen and lymph nodes. High expression levels have been observed during embryogenesis, where it may be able to substitute for BCL-2. This could account for the apparently normal development of bcl-2 gene-ablated mice (Nunez, 1994).

BAX

BAX has extensive amino acid homology with BCL-2, and can homodimerize with itself or heterodimerize with BCL-2 *in vivo* (Oltvai *et al*, 1993). While BAX prefers homodimerization, overexpression of BCL-2 will compete for this homodimerization and produce BAX/BCL-2 heterodimers. The ratio of these two proteins seems to determine a cell's susceptibility to death following an apoptotic stimulus.

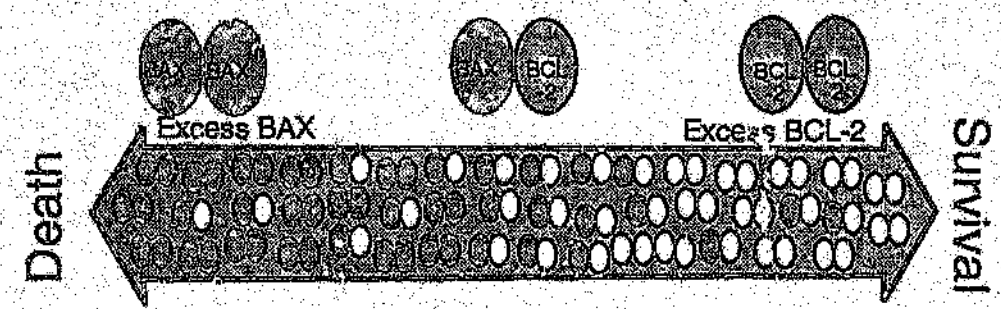


Figure 1.3.

Cell death and survival in relation to the BCL-2/BAX ratio

The ratio of BCL-2 proteins to BAX proteins, which bind either to themselves or to each other, influences cell death and survival. (Adapted from Barinaga, 1994.)

When BCL-2 is in excess, cells are protected. When, however, BAX predominates and forms homodimers, cells are susceptible to PCD (figure 1.3). Whether BAX codes for a death effector protein that is neutralized by

BCL-2, or BCL-2 codes for a death repressor molecule that is neutralized by BAX, remains to be determined. The response of cells to a death signal thus seems to depend on a preset ratio of BAX to BCL-2 molecules.

The most highly conserved domains amongst BCL-2 and BAX, called BCL-2 homology 1 and 2 (BH1 and BH2), are separated by about thirty amino acids, and are critical for BCL-2 function (Yin *et al*, 1994). BCL-2 proteins that contain specific amino acid substitutions within these two regions were still able to homodimerize, but were unable to dimerize with BAX. Interestingly, BCL-2 proteins that demonstrated a diminished interaction with BAX, also displayed decreased repression of PCD cell death. Thus, BCL-2 seems to have to bind to BAX in order to exert its death-repressor activity (see figure 1.4).

BAX mRNA is expressed in a variety of tissues, including lymphoid tissue, lung, stomach, kidney, thymus, lymph node, bone marrow, spleen, pancreas, brain, and heart and smooth muscle tissue. As with BCL-2, two forms of BAX exist: a membrane-bound BAXα, and a cytosolic BAXß. Cytosolic BAXß may provide an additional level of regulation by dimerizing with membrane-bound BCL-2 or BAXα (Oltvai *et al*, 1993).

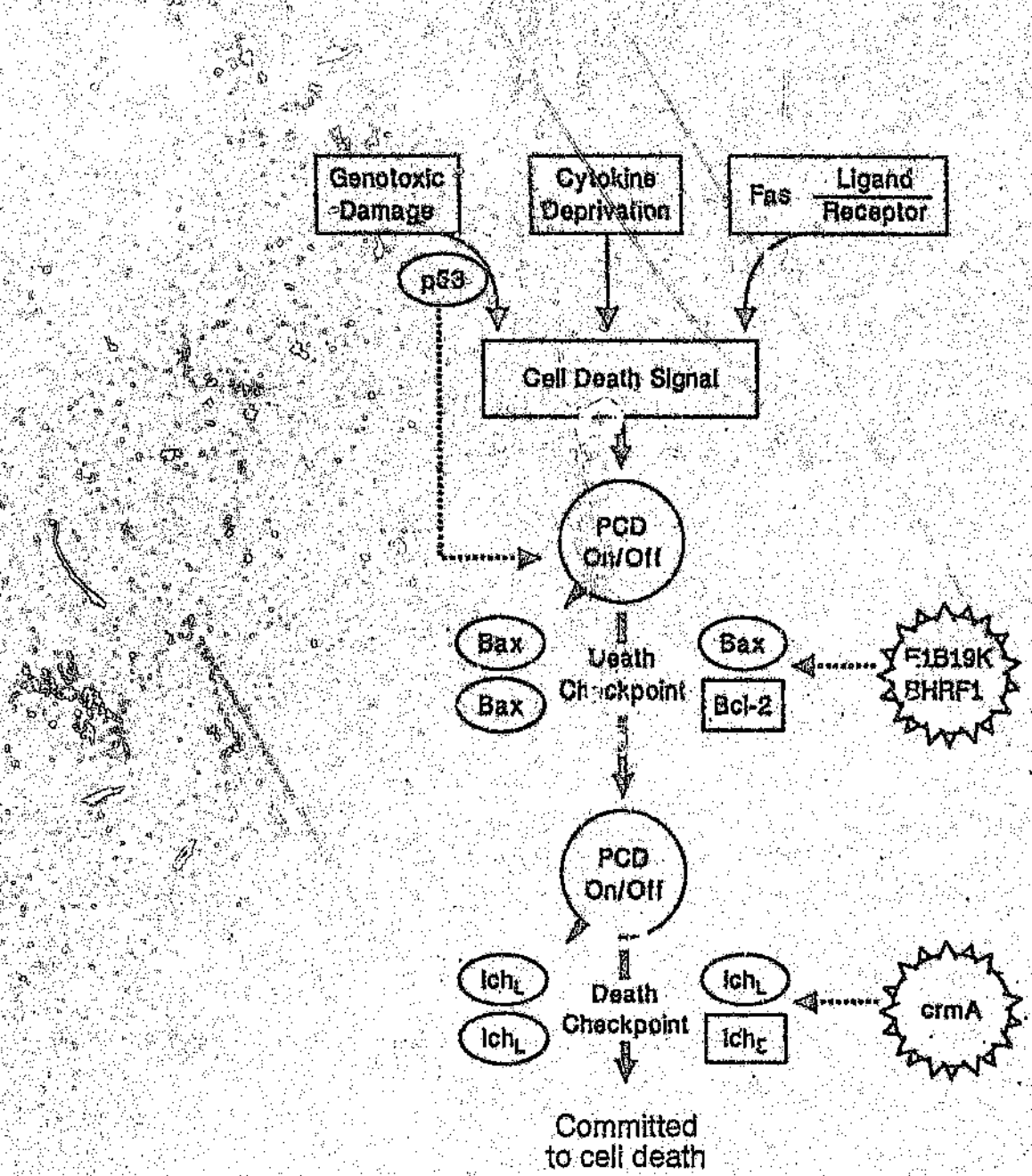


Figure 1.4.

Model of cell death checkpoints analogous to rheostat switches

The BCL-2/BAX ratio and the ICH-1$_S$/ICH-1$_L$ ratio (discussed in section 1.16) are influenced by signals from the cell's external and internal environments. These ratios, in turn, influence the induction or suppression of PCD. (Adapted from Oltvai and Korsmeyer, 1994.)

1.19. BCL-2-LIKE GENES IN VIRUSES

The adenovirus E1B 19 kD protein protects against a cell death programme induced in the host cell by viral infection. BCL-2 can functionally substitute for the E1B 19 kD protein, and both proteins promote cell survival through interaction with a common set of cellular proteins (Boyd *et al*, 1994).

The Epstein-Barr virus BHRF1 protein resembles BCL-2 in its subcellular localization and its capacity to enhance B-cell survival. Thus, BHRF1 and BCL-2 are functionally homologous (Henderson *et al*, 1993).

Apart from interacting with its own family of related proteins, BCL-2 also interacts with the *ras* and *myc* oncoproteins, and with the p53 tumour suppressor protein.

1.20. INTERACTION OF BCL-2 WITH RAS, MYC, AND P53

RAS

Members of the *ras* family of genes code for the GTPase proteins that are involved in the regulation of cell proliferation and differentiation (Spaargaren *et al*, 1994). The non-transforming human RAS-related protein, R-RAS, has

been shown to bind to BCL-2 (Fernandez-Sarabia and Bischoff, 1993). Thus, R-RAS could be a component of a signal transduction pathway that mediates signals involved in the induction of PCD. Overexpression of BCL-2 could alter the ability of R-RAS to transmit its signal, thereby interrupting the transduction of an PCD-inducing message.

MYC

MYC belongs to a group of cellular messengers that are coded for by "immediate early response" genes which are expressed early on in the mitogenic event and do not require de novo protein synthesis for activation. Both *myc* mRNA and MYC protein are expressed throughout the cell cycle in proliferating cells, and MYC has been implicated in the control of normal cell proliferation in numerous studies (for an extensive review, see Marcu *et al*, 1992). MYC has also been implicated in the negative control of cell differentiation and growth arrest, in that down-regulation of MYC expression accompanies both of these processes, and sustained MYC expression can block them (Waters *et al*, 1991). Interestingly, while deregulated expression of MYC may lead to an increase in cell proliferation, it has also been implicated in the induction of PCD in the presence of a block to cell proliferation (Evan *et al*, 1992). It has been suggested (Bissonnette *et al*, 1992) that MYC induces PCD in the absence of a cell survival signal such as growth factor binding, and cell proliferation in presence of a survival signal. PCD induced by MYC can be inhibited by BCL-2 expression (Bissonnette *et al*, 1992; Fanidi *et al*, 1992),

and BCL-2 may, thus, substitute for such a survival signal. Mitogenic rates of cells expressing MYC are similar irrespective of BCL-2 expression, suggesting that BCL-2 does not inhibit all functions of MYC, but specifically blocks its induction of PCD (Fanidi *et al*, 1992). This cooperation provides a mechanism for the oncogenic synergy between these two oncoproteins.

p53

p53 is a DNA-binding protein that functions as a transcriptional regulator (Unger *et al*, 1992). It has anti-proliferative properties, arresting the cell cycle at G_1, and may induce PCD and sensitivity to chemotherapeutic drugs and radiation (Clarke *et al*, 1994). These results suggest that p53 serves as an important checkpoint in the cell cycle before entering S phase. Interestingly, p53 is able to down-regulate BCL-2 expression in some cases (Miyashita *et al*, 1994), which may be one mechanism by which it modulates its effect on PCD. Overexpression of BCL-2, on the other hand, is able to block p53-induced PCD (Ryan *et al*, 1994) (see table 1.2 for summary of effects of c-MYC, p53, and BCL-2 on PCD).

Deregulated Expression of	Apoptosis		
	Induction	Enhancement	Suppression
Wild-type p53	+	-	-
c-myc	-	+	-
Mutant p53	-	-	+
bcl-2	-	-	+

Table 1.2.

Effects of c-MYC, p53, and BCL-2 on PCD

The oncogene products c-MYC and BCL-2 and the wild-type and mutated forms of the proto-oncogene product p53 influence PCD. (From Sachs and Lotem, 1993.)

Although p53 is an important mediator of PCD, p53-independent mechanisms of PCD do exist (Clarke et al, 1993; Strasser et al, 1994). Thus, PCD has been observed to occur in HL-60 cells, even though they lack p53 expression (Cotter and McCarthy, 1994).

Cells that coexpress BCL-2 and MYC, completely overcome the growth-inhibitory and apoptotic effects of p53 (Ryan et al, 1994). It is probable that, while BCL-2 plays a role in protecting cells from p53-induced PCD, MYC overcomes p53-induced cell cycle arrest .

1.2. THE CURRENT STUDY IN PERSPECTIVE

The growth-, differentiation-, and cell-death-associated expression of *bcl-2* mRNA and BCL-2 protein was studied in two myelocytic leukaemia cell lines.

Leukaemias arise from malignant transformations of haematopoietic cells. These cells proliferate primarily in the bone marrow and lymphoid tissues, where they interfere with normal haematopoiesis and immunity. Malignant cells will ultimately move into the peripheral blood system, and will infiltrate other tissues. Tumourigenesis is a multi-step process, and leukaemias arise following malignant transformation of a single haematopoietic progenitor. This is followed by cellular replication and clonal expansion (for a review refer to Champlin and Golde, 1991).

The two cell lines used in the current study were the monocytic leukaemic THP-1 cell line (Tsuchiya *et al*, 1980), and the promyelocytic leukaemic HL-60 cell line (Gallagher *et al*, 1979). The use of cell lines in this study had these

advantages: Firstly, the cell lines provided homogenous cultures of a single cell type and the lack of contamination from other cell types allowed for easier interpretation of the results obtained. Thus, any changes observed could be attributed to the cell line under observation. Secondly, the cell cultures allowed for large quantities of one cell type to be harvested at any one time. The number of cells that can be obtained from a patient or a healthy subject at any one time is, of course, more limited.

Approximately eighty percent of myelocytic leukaemias are positive for BCL-2, although this protein is not expressed in mature monocytes (Delia *et al*, 1992). BCL-2-positive cells exhibit prolonged survival upon growth factor withdrawal and an increased resistance to stress (Campos *et al*, 1993). High expression of BCL-2 in myelocytic leukaemias is also associated with a poor response to chemotherapy and short patient survival times (Campos *et al*, 1993). Thus, BCL-2 seems to play a role in the development and/or maintenance of the leukaemic state. It, therefore, seemed worthwhile to study the expression of BCL-2 in leukaemic cells.

THP-1 cells and HL-60 cells were exposed to the PKC-activating phorbol ester PMA, and to the PKA-activating cAMP analog dibutyryl-cAMP (dbcAMP). The effects of PMA and dbcAMP on proliferation, differentiation, and cell death were studied in relation to *bcl-2* mRNA and BCL-2 protein expression.

The THP-1 and HL-60 cell lines both belong to the myelocytic haematopoietic lineage yet vary in their state of differentiation. The expression of BCL-2 has been shown to be differentiation-linked, and to be present in HL-60 cells but absent in normal monocytes (Delia *et al*, 1992). It was thought useful to assay THP-1 cells for the presence or absence of *bcl-2* mRNA and BCL-2 protein, and to compare this expression to that found in HL-60 cells in conditions of cell differentiation and death.

The THP-1 cell line is thought to resemble normal monocytes closely (Auwerx, 1991). HL-60 cells, on the other hand, aberrantly express both c-MYC and p53. These two proteins interact with BCL-2 and play a role in programmed cell death.

HL-60 cells contain a genomic amplification of *c-myc*, and therefore express unusually high levels of the c-MYC protein (Collins, 1987). Deregulated c-MYC expression has been linked both to uncontrolled cell proliferation, and to programmed cell death. Increased c-MYC expression, in particular, may be a potent inducer of programmed cell death (Evan *et al*, 1992). c-MYC expression decreases upon differentiation.

For these reasons it was decided to examine the effect that high c-MYC concentrations may have on apoptosis-induction, differentiation-induction, and BCL-2 expression in HL-60 cells, and to compare the results to those obtained

in THP-1 cells, which do not aberrantly express c-MYC.

HL-60 cells do not express p53 (Collins, 1987). Haematopoietic cells from p53 gene-ablated mice are more resistant to some forms of programmed cell death (Lotem and Sachs, 1993), although p53-independent cell death pathways also exist (Strasser *et al*, 1994). Apoptosis-induction and BCL-2 expression studies were also considered in HL-60 cells because of their lack of p53. Results too were compared to those obtained in THP-1 cells.

BCL-2 protein was examined by Western blotting and slot blotting followed by immunodetection and also by immunocytochemical analysis of whole cells. *bcl-2* mRNA was analysed by reverse transcription and polymerase chain reaction amplification (PCR).

In summary, the effects of proliferation, differentiation, and cell death, especially in relation to *bcl-2* mRNA and BCL-2 protein expression, were studied and compared in the THP-1 and HL-60 cell lines.

SECTION 2

MATERIALS AND METHODS

2.1. CELLS

2.1.1. CELL CULTURE

THP-1 cells and HL-60 cells (ATTC) were cultured at 37°C in sealed 1 litre flasks (Nunclon, Denmark) in RPMI (Highveld Biological, South Africa) medium containing HEPES (0,05 M), sodium bicarbonate (1,85 g/L), penicillin G (100 000 U/L)(Sigma, USA), streptomycin (80 000 U/L)(Sigma, USA), and 10 % (v/v) heat-inactivated foetal calf serum (Delta Bioproducts, South Africa). The medium was sterilized by filtration through a 0.22 μm filter (Millipore). Cells were passaged at maximum proliferation (approximately every four to five days), at which stage cells were removed and used for experimentation.

2.1.2. STIMULATIONS

Cells were centrifuged for 10 minutes at 200 g in a Beckman model TJ-6 centrifuge (USA). The resultant pellet was washed in sterile saline to remove last traces of medium and cell debris, and was resuspended in fresh RPMI medium containing 10 % foetal calf serum. The concentration of viable cells was estimated by means of the trypan blue exclusion method. One volume of

cells was added to three volumes of dye, and the number of intact cells that excluded the dye were counted in the 25 centre fields of a counting chamber (Weber Scientific International, England). As the centre fields of the chamber hold 0,1 μl, the resultant cell number was multiplied by the dilution factor and 10^4 to obtain the number of cells / ml. Cell viability exceeding 95 % was considered satisfactory.

Cells were resuspended to an approximate final concentration of 50 000 cells / ml for thymidine incorporation analysis, $0.5x10^6$ cells / ml for DNA profile analysis, and between $1x10^6$ and $6x10^6$ cells / ml for RNA and protein analysis.

THP-1 and HL-60 cells were stimulated with Phorbol-12-Myristate 13-Acetate (PMA) (Sigma USA, Lot 61H0038)(16 nM) and/or N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP)(Sigma USA, Lot 112H7805)(50 μM), or puromycin (0.5 ng/ml and 1 ng/ml), and incubated at 37°C for between 3 hours and 48 hours in a humidified incubator with 5 % CO_2. Incubations were performed in 55 mm diameter petri dishes, 24-well plates, or 96-well plates (Nunclon, Denmark).

2.2. CELL MORPHOLOGY

After stimulation, adherent cells were gently removed from the base of the petri dishes using a disposable cell scraper (Costar, USA). Cells were resuspended, and aliquots removed. Cytospins of cells were performed by centrifuging the cells onto glass slides. The cells were then stained with Giemsa.

2.3. CD33 SURFACE ANTIGEN EXPRESSION

CD33 is a surface antigen of unknown function that is found on myeloid precursor cells (Horejsi, 1991). CD33 antibodies were used on THP-1 and HL-60 cells. The two cell lines were then monitored for differences in CD33 expression, both before and after stimulation.

Cells (approximately $6x10^6$ cells per experiment) were washed in saline and resuspended in wash solution (0.2 % bovine serum albumin [BSA] in phosphate buffered saline [PBS], pH 7.3). Monoclonal murine anti-human CD33 antibody linked to the fluorescent fluoro-isothiocyanate (FITC) molecule (Coulter CD33:FITC [MY9] #6602985) (10x dilution) was added to the samples. Control samples of cells were exposed to a non-specific FITC-labelled antibody (anti-mouse) to correct for background fluorescence. After a 30 minute incubation on ice, cells were pelleted (200 g, 5 minutes), washed

in wash solution, and fixed with BSA (1.5 % w/v in PBS, pH 7.4) and formalin (1.5 % v/v in PBS, pH 7.4). The fluorescence was read on an EPICS Profile flow cytometer. The amount of fluorescence was assumed to be proportional to the number of CD33 antigens per cell. The sample flow rate was adjusted to between 10 and 15 μl/minute, and the sheath pressure to 7.5 psi. Between 5 000 and 10 000 cells were analysed.

2.4. DNA PROFILE ANALYSIS USING FLOW CYTOMETRY

Flow cytometry was used to enumerate the subpopulation of cells present in specific phases of the cell cycle. During flow cytometry, cells in free suspension are passed through a liquid pipeline in single file, and then move through a laser beam. This causes the laser light to scatter. Forward scatter measures the size of the cell or particle, while side scatter measures its complexity (Glencross, 1994).

After stimulation, THP-1 and HL-60 cells were pelleted at 200 g, resuspended in a solution containing propidium iodide (50 μg/ml), Triton-X (1 mg/ml) and sodium citrate (1 mg/ml). Cells were analysed on an EPICS-PROFILE FLOW CYTOMETER. Propidium iodide is a fluorescent mutagen that intercalates DNA. Thus, the amount of fluorescence emitted by a cell will

be related to the amount of DNA within that cell and, therefore, to its DNA ploidy. Non-mitotic diploid (2n) cells will generate roughly half as much fluorescent light as mitotic tetraploid (4n) cells. When DNA is synthesized in the S-phase of the cell cycle, and the cell, thus, moves from a diploid to a tetraploid state, an intermediate amount of fluorescence will be generated.

From the amount of fluorescent light generated during flow cytometry, the proportion of cells residing in the G_0/G_1-, S-, or G_2/M-phases of the cell cycle could be estimated. A histogram was produced, which displayed the cell number on the Y-axis and the amount of fluorescence on the X-axis (Figure 2.1). Two peaks appeared, one representing cells in the G_0/G_1 phase, and the other representing cells in the G_2/M phase of the cell cycle. The width of each peak corresponded to a narrow range of fluorescent intensities. The width of the first peak was used to indicate the range of fluorescence representative of the G_0/G_1 phase. When these values were doubled to represent the G_2/M phase, they corresponded well with the second peak on the histogram. Cells represented by the area between the two peaks were assumed to be residing in the S-phase of the cell cycle.

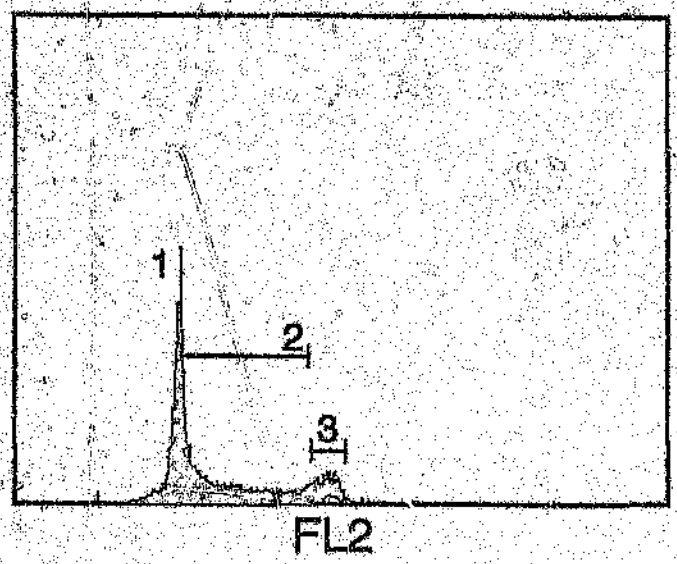


Figure 2.1.

Flow cytometrical histogram showing cell cycle distribution patterns

Flow cytometry was used to estimate the proportion of cells residing in G_0/G_1 (diploid, ie. 2n) (first peak on the histogram), G_2/M (tetraploid, ie. 4n)(second peak on the histogram), and S (between 2n and 4n)(area between the two peaks).(Refer to section 2.4 for more detailed discussion.)

2.5. THYMIDINE INCORPORATION

The incorporation of radioactive thymidine was used as an indication of the amount of DNA synthesis and thus, cell division, that was occurring in response to PMA and/or dbcAMP. THP-1 cells were exposed to PMA and/or dbcAMP for 4 days. On day three, the cells were pulsed with radioactively

labelled [^{3}H]thymidine (Amersham International, England). On day four, the cells were lysed, and the DNA was harvested onto 3M Whatman filter paper. Circles of filter paper representing cells from individual experiments were emersed in aquagel scintillation cocktail, and the radioactivity of these fluid samples was subsequently measured using a Packard Tri-carb liquid scintillation counter.

2.6. RNA ISOLATION AND INTEGRITY ANALYSIS

2.6.1. RNA ISOLATION

Care was taken to keep RNase contamination to a minimum during handling of cells and cell extracts. To ensure minimal RNase contamination, all RNA solutions were made up using diethyl pyrocarbonate (DEPC) (Sigma, USA) H_2O (0.2% DEPC v/v). (Mili Q H_2O) was incubated with DEPC overnight to allow for ribonuclease inactivation, after which it was autoclaved to inactivate the DEPC which would otherwise cause RNA carboxymethylation).

RNA was extracted using the method described by Chomczynski and Sacchi (1987). After stimulation, cells were transferred from the petri dishes to sterile 5 ml tubes. Adherent cells were gently removed from the base of the dishes using a disposable cell scraper (Costar, USA). Cells were pelleted by

centrifuging for ten minutes at 750 g. The resultant pellet was resuspended in a denaturing solution (solution D)(0.5 ml) containing guanidine thiocyanate (4 M), sodium citrate (25 mM), sarcosyl (0.5% w/v), and ß-mercaptoethanol (0.36 ml / 50 ml). A guanidine isothiocyanate stock solution without mercaptoethanol was prepared that could be stored for up to 3 months at room temperature. After addition of ß-mercaptoethanol the solution had to be used within one month.

Suspensions were transferred to Eppendorff tubes and sequentially mixed with sodium acetate (50µl)(2 M, pH 4), cold water-saturated phenol (0.5 ml) and chloroform-isoamylalcohol (150 µl) (49:1 v/v). (The chloroform denatures proteins and facilitates separation of the aqueous and organic phases, while the isoamylalcohol reduces foaming). The tubes were inverted several times and shaken vigorously for 10 seconds to facilitate mixing, and were then incubated on ice for 20 minutes. This was followed by a centrifugation at 10 000 g for 15 minutes at 4°C in a Heraeus biofuge. The RNA was subsequently found in the top aqueous phase and the DNA and denatured proteins into the interphase and the phenol phase. The RNA phase was placed in a sterile Eppendorff tube, mixed with two volumes of isopropanol, and allowed to precipitate at -20°C overnight.

After its precipitation, the RNA was pelleted by centrifuging for 15 minutes at 10 000 g and 4°C. A second precipitation was performed with 0.3

ml solution D and 1 ml isopropanol at -20°C for 2 hours.

The RNA was repelleted by centrifuging for 15 minutes at 13 000 g and 4°C. The resultant pellet was resuspended in 75 % ethanol (200 µl) and incubated at 4°C for 20 minutes. The pellet resulting from a subsequent centrifugation for 15 minutes at 13 000 g and 4°C was air dried and dissolved at 65°C in either SDS (0.5 %)(for Northern blotting) or Baxter water (for reverse transcriptase [RT] reactions and polymerase chain reaction [PCR] amplifications). RNA was stored at -20°C for up to 1 month.

2.6.2. QUANTITATIVE DETERMINATION OF RNA

A pure preparation of RNA has an $A_{260/280}$ value of 2.0. A high degree of sample purity is required for Northern blotting, RT-PCR and for accurate quantitation of RNA. The presence of proteins, DNA or phenol may interfere with the accurate quantitation of RNA present in the sample.

The RNA concentrations in the respective samples was estimated using the following formula:

$$[RNA]\ (\mu g / ml) = 40 \times A_{260} \times \text{dilution factor}$$

Approximately 10 µg of each RNA sample was used for Northern blotting, while 0.5 µg of each RNA sample was needed for reverse transcriptase

reactions and subsequent polymerase chain reaction amplifications (RT-PCR).

2.6.3. DENATURING AGAROSE GEL ELECTROPHORESIS OF TOTAL CELLULAR RNA

RNA was separated according to size via agarose gel electrophoresis. For this procedure, a 1 % (w/v) agarose (Sigma) gel was prepared and buffered with MOPS (3-[N-morpholino]propanesulphonic acid) buffer (0.02 M MOPS, 0.005 M sodium acetate, 0.001 M EDTA). The mixture was heated to allow for melting of the agarose. Addition of formaldehyde (37 %)(54 µl/ml) to the gel ensured denaturation of the RNA. The gel was cast in a horizontal chamber, with a comb being placed vertically into the chamber to allow for the formation of sample loading wells in the gel. The gel was allowed to set before samples were loaded.

Samples (10 µg) were resuspended in sample buffer containing deionized formamide (480 µl/ml)(its high ionic strength promotes heteroduplex formation), 10 x MOPS buffer (107 µl/ml), formaldehyde (37 %),(173 µl/ml), DEPC H_2O (180 µl/ml), glycerol (53 µl/ml)(to increase the density of the sample buffer which allows for efficient loading of the sample into the wells of the gel), and bromophenol blue (6.7 µl/ml)(a low molecular mass dye that allows for easy estimation of electrophoretic speed and efficiency). 1 x MOPS buffer was used as electrophoresis buffer, and samples were electrophoresed at 60 V for two to three hours.

2.6.4. ESTIMATION OF RNA INTEGRITY

The eukaryotic ribosomes of mammals contain approximately 60 % RNA and 40 % protein. The ribosomal RNA is made up of 28 S + 18 S + 5.8 S + 5 S subunits, which become easily visible under ultraviolet illumination if ethidium bromide (0.5 μg/ml)(a mutagen and intercalator of folded ribosomal RNA regions) is added to the agarose gel and to the electrophoresis buffer. Thus, the total RNA integrity in the electrophoresed sample may be estimated from the integrity of the ribosomal RNA species.

2.7. NORTHERN BLOT ANALYSIS OF IL-1β RNA

RNA was transblotted onto nylon (Hybond, Amersham, England) and was detected using a radioactively labelled probe.

2.7.1. NORTHERN BLOTTING OF RNA

RNA samples (approximately 10 μg total RNA per sample) were electrophoresed in the absence of ethidium bromide. After electrophoresis, gels were washed for 15 minutes in 10 x SSC buffer containing sodium chloride (1.5 M) and sodium citrate (0.15 M)(to restore the pH for transfer). Truncating the gel above the wells allowed for easier handling. Washed gels were placed on parafilm-covered plates (to reduce RNase contamination) with

the RNA side (ie. the side that was at the bottom of the gel during electrophoresis) facing up. A piece of nylon membrane (Hybond-N, Amersham) was cut to size (approximately 2 mm larger than the gel on each side) and placed on the gel. This was followed by two rectangles of 3M Whatman filter paper. A closed system was established by taping the membrane, filter paper, and gel down onto the glass plate with wide parafilm strips along all sides. Tissue paper was placed on top of this system to aid the capillary action, and was held down with a weighted glass plate. After 14 to 18 hours the transblot was considered complete, and the nylon membranes were washed and equilibrated in 2 x SSC buffer for 15 minutes. The membranes were then air dried , wrapped in cling wrap, and exposed to UV-light at 312 nm to ensure RNA cross-linking to the nylon.

2.7.2. PREHYBRIDIZATION OF RNA TRANSBLOTS

Prehybridization refers to the non-specific binding of DNA to areas of the blot not occupied by RNA, thus making these sites less accessible to the specific radioactively labelled probe used during later hybridization procedures. The prehybridization solution used contained single stranded herring sperm DNA at a final concentration of 0.8 mg/ml. (Stock herring sperm DNA was prepared at a concentration of 10 mg / ml, aspirated 20 times through an 18 gauge needle and sonicated at 16-20 Hz.)

The DNA was denatured by boiling at 96°C for 5 minutes, and was

subsequently stored on ice to prevent reannealing. DNA was added to a prehybridization solution containing 5 x SSPE buffer (3 M sodium chloride, 0.2 M sodium phosphate, and 0.02 M ethylenedinitrilo-tetraacetic acid (EDTA))(pH 7.4), 49 % (v/v) deionized formamide, Denhardts solution (5.4 % (v/v) of a filtered 100 x solution containing ficoll (20 g/l), polyvinylpyrollidine (20 g/l), and bovine serum albumin (20 g/l)), and 0.5 x sodium dodecyl sulphate (SDS).

Blots were prehybridized for at least 6 hours in plastic pockets or boxes with approximately 12.5 ml of solution per blot. Prehybridization was carried out at 42°C in a shaking waterbath.

2.7.3. RADIOACTIVE RANDOM PRIMER LABELLING OF cDNA PROBES

Random primer labelling makes use of the Klenow enzyme. This unnatural enzyme results from the large C-terminal fragment of DNA polymerase 1 after its proteolysis by subtilisin or trypsin, and contains the polymerase and 3'-exonuclease activities. The 5'-exonuclease activity is absent in this enzyme fragment, thus making it useful for synthesis of DNA in vitro when one wishes to avoid DNA degradation.

The IL-1β cDNA clone was grown up in bacterial plasmids and excised by specific restriction endonucleases.

The IL-1β probe (1075 bases)(a gift from Professor C.A.Dinarello) was isolated using PstI (from Provincia stuartii; cleavage site at CTGCA↓G) and SstI (from Streptomyces stanford; cleavage site differs from recognition site) restriction endonucleases.

A Promega labelling kit was used for the random primer labelling reaction. System components were thawed on ice, while the Klenow enzyme was kept at -20°C prior to use and returned immediately after use. The DNA template (25 ng per labelling) was denatured by heating in an Eppendorff tube at 96°C for 2 minutes and was immediately chilled on ice to prevent reannealing. The reaction was assembled in a separate tube by addition of a dNTP mixture (2 μl) containing 20 μM each of dATP, dGTP and dTTP to 5 x labelling buffer (10 μl).

To this was added the denatured DNA template , acetylated (nuclease free) bovine serum albumin (BSA)(2 μl at 10 mg/ml), [α-^{32}P]dCTP (50 μCi, Amersham,Ayelsbury, England), and the Klenow enzyme (5 units). The reaction was gently mixed and incubated at room temperature for 60 minutes, after which the enzyme was inactivated by heating the reaction tube at 96°C for 2 minutes. The tube was stored on ice after heating to prevent annealing of template and probe.

2.7.4. HYBRIDIZATION OF RNA TRANSBLOTS

The prehybridization solution was replaced by a hybridization solution

containing the labelled probe. The hybridization solution was prepared using 5 x SSPE, formamide (50 % (v/v)), Denhardts (5.3 % (v/v) of a 100 x solution), herring sperm DNA (0.4 mg %), and polyethylene glycol (10 % (v/v) of a 40 % solution). Hybridization was performed overnight at 42°C in a shaking waterbath.

2.7.5. STRINGENCY WASHES

Blots were washed twice with 2 x SSPE and SDS (0.1 %) at 42°C for 15 minutes. A third wash was performed with 1 x SSPE and SDS (0.1 %) at 42°C for 30 minutes, after which blots were left at room temperature for 15 minutes in 0.1 x SSPE and SDS (0.1 %). Washed blots were wrapped in cling wrap and exposed to X-ray film for between 24 hours and 14 days.

2.7.6. SCANNING PROCEDURES

Autoradiograms were scanned using a densitometer (Biomed Instruments). A curve was obtained, and the relative absorbance units were calculated by integration of the area under this curve.

2.8. RIA ANALYSIS OF IL-1ß PROTEIN

IL-1ß protein concentrations were measured in cell supernatants using the IL-1ß [^{125}I] assay system (Amersham, England). THP-1 cell supernatants were diluted 1:5 and HL-60 cell supernatants 1:4 prior to analysis.

The system is based on the competition between the unlabelled IL-1ß in the assay sample and a fixed quantity of ^{125}I-labelled human recombinant IL-1ß for a limited number of binding sites on an IL-1ß-specific antibody. The amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive sample IL-1ß.

Antiserum (25 µl) was added to the diluted supernatants (25 µl), and the reactions were incubated at room temperature for 4 hours. Non-specific binding was measured using only buffer (50 µl), while the zero standard was prepared by adding buffer (25 µl) to antiserum (25 µl). A standard curve was prepared from samples containing standard levels of IL-1ß ranging from 0.0775 to 10 fmol/tube.

Subsequent to the first incubation, [^{125}I]-IL-1ß (25 µl) was added to the samples. A second incubation followed at 4°C for 16 to 24 hours.

After the second incubation period, secondary antibody reagent (62 µl)

was added, and samples were left to stand at room temperature for 10 minutes. The antibody was bound to magnetizable polymer particles, which allowed for the magnetic separation of the antibody-bound fraction from the supernatant fraction after centrifugation.

Tubes were sealed with parafilm and analysed for radioactivity using a Gamma-scintillation counter. Analysis was performed for 1 minute/sample.

2.9. PCR ANALYSIS OF BCL-2 RNA

RNA was reverse-transcribed to produce copy DNA (cDNA). The polymerase chain reaction (PCR) technique was subsequently used as a semi-quantitative technique for *bcl-2* RNA analysis. Two sets of primers were used in combination: one set specific for ß-actin cDNA, and one for *bcl-2* cDNA.

2.9.1. REVERSE TRANSCRIPTION (RT) OF TOTAL RNA

Reverse transcriptase was initially isolated from retroviruses which use this enzyme to make a DNA copy of their RNA genome for insertion into the host cell genome. Reverse transcriptase uses viral RNA as a template for synthesizing a complementary DNA strand. A specific type of transfer RNA serves as a primer for the enzyme.

Here, RNA was reverse-transcribed into cDNA using the following method: Initially, 0.5 μg of total RNA (in 1 μl) was incubated at 60°C for 5 minutes with synthetic oligodeoxythymidine primers (oligo-dTs) (1 μl of a 500μg/ml solution). These oligo-dTs bind to the poly-A tails of the RNA molecules, thereby offering an initiation site for the reverse transcriptase enzyme.

The final RT reaction mixture (20 μl) contained an RNAse inhibitor (20 units) (Amersham, England), deoxyribonucleotides (dNTPs) (1 mM each of dATP, dGTP, dTTP, and dCTP), reverse transcription buffer (50 mM Tris hydrochloric acid pH 8.3, 50 mM potassium chloride, 10 mM magnesium chloride, 10 mM DTT, 0.5 mM spermidine) (Promega, USA) (Promega), and the reverse transcriptase enzyme (5 units) (Promega). The reaction was incubated at 37 °C for one hour, thereby providing time for the reverse transcriptase enzyme to synthesize cDNA from the RNA templates using the dNTPs provided.

The reverse transcriptase was inactivated subsequently by heating the reaction to 70 °C for 5 minutes and then placing it on ice for 5 minutes.

2.9.2. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF SPECIFIC BCL-2 AND ß-ACTIN cDNA SEQUENCES

The PCR technique, which was first described in 1985 (Saiki *et al, 1985*), allows for the exponential generation of short pieces of DNA. The DNA strands are limited in length by two synthetically produced oligonucleotide primers that bind to specific complementary sequences on the DNA. The two primers bind to the plus and the minus strand of the DNA, respectively, with the 3'-OH ends pointing toward one another. A thermostable DNA polymerase is used to catalyze the 5' to 3' primer extensions under the direction of the template DNA. The amplified DNA between and including the two primers is known as the target sequence (Bloch,1991; Carman, 1991).

The PCR reaction consists of three steps, each having its own optimal temperature. The primer-template annealing temperature depends on the melting temperatures of the primers, and will usually lie somewhere between 45°C and 60°C. Enzyme-catalyzed primer extension is usually performed at or near 72°C, while strand separation of the double stranded DNA product to single stranded templates occurs at between 90°C and 96°C (Bloch, 1991; Carman, 1991).

Here, a set of primers was designed that was specific for *bcl-2* DNA and cDNA. Because the primers spanned an intron, PCR amplification of intron-less cDNA and of genomic intron-containing DNA would yield different-sized

products. Thus, the possible presence of contaminating genomic DNA would not interfere with the results obtained.

The *bcl-2* 5' primer bound within exon 1, and was homologous to nucleotide residues 1865 to 1880 of the human *bcl-2α* cDNA, and residues 553 to 568 of the human *bcl-2ß* cDNA (GenBank). The *bcl-2* 3' primer bound within exon 2, and was complementary to residues 2046 to 2059 of the human *bcl-2α* cDNA, and residues 734 to 747 of the human *bcl-2ß* cDNA (GenBank). The PCR product obtained would thus be 195 base pairs in length and would not distinguish between the α- and ß-forms of the *bcl-2* cDNA. The sequences of the two primers were as follows:

5' primer: 5'...AGCTCTTCAGGGACGG...3' (16 nucleotides)
3' primer: 3'...ACGGAAACACCTTG...5' (14 nucleotides)

The PCR reaction efficiency may vary from one reaction to the next, and an internal control is needed to correct for this. Here, ß-actin cDNA amplification was used as an internal amplification control. ß-actin was assumed to be constitutively expressed (Delfau et al, 1990; Stark et al, 1993).

Two primers were obtained (courtesy of R.M.D.Lastrucci). The 5' actin primer bound across exons 2 and 3, and was homologous to residues 393 to 411, while the 3' actin primer bound within exon 3, and was complementary

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to residues 792 to 807. The PCR product would, therefore, be 415 base pairs long (refer to Ponte *et al*, 1984, for DNA sequence of the ß-actin gene). The sequences of the two actin primers were as follows:

5' primer: 5'...AAGATGACCCAGATCATGT...3' (19 nucleotides)

3' primer: 3'...CCGTTACTCGCCAAGGC...5' (17 nucleotides)

The polymerase chain reaction mixture contained the RT cDNA product (10 μl, ie. half of the cDNA produced from 0.5 μg of total RNA), ß-actin primers (0.15 μM each of the 5' and 3' primers), *bcl-2* primers (1 μM each of the 5' and 3' primers), reaction buffer (10 mM Tris-hydrochloric acid buffer, pH 8.8, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1 % Triton X-100), and thermostable DNA polymerase (1 unit) (Dynazyme).

The reaction was temperature-regulated on a thermocycler (Techne PHC2, UK). First, the reaction mixture was heated at 96°C for 5 minutes to allow for denaturation of the cDNA. Next, the reaction was cycled through three different temperature stages 30 times. These stages included a denaturation and DNA strand separation step at 93°C for 30 seconds, followed by a primer annealing step at 45°C for 30 seconds and a primer extension step at 72°C for 60 seconds. Finally, 5 minutes at 72°C ensured that all primer extensions could be completed.

2.9.3. GEL ELECTROPHORESIS OF PCR PRODUCTS

PCR products were electrophoresed on a 2 % agarose gel. The gel was made up using TAE buffer (1 mM Tris, 0.5 mM sodium acetate, 25 μM EDTA final concentrations), and ethidium bromide (0.1 μl / 1 ml of gel) for UV-visualization of the DNA. TAE was used as the electrophoresis buffer, and ethidium bromide was again added for DNA UV-visualization. Samples were loaded in TAE buffer containing 50 % (v/v) glycerol and 0.001 % (w/v) bromophenol blue. Gels were electrophoresed at 4°C and 60 V.

2.9.4. ANALYSIS OF PCR PRODUCTS

Gels were photographed and scanned. Bands corresponding with the *bcl-2* and ß-actin cDNA PCR products were scanned using a densitometer. The scanning programme used was the Zeinah Laser and Camera Floppy Programme from Biomed Instruments (USA). A curve was obtained, and the area under this curve was integrated to yield relative absorbance units. Results obtained for *bcl-2* were adjusted according to the internal ß-actin control.

2.10. BCL-2 PROTEIN ANALYSIS

2.10.1. ISOLATION OF TOTAL PROTEIN FROM CELLS

THP-1 and HL-60 cells were spun down, washed, and frozen at -70°C after

stimulation.

For protein isolation, cell pellets were thawed and suspended in an appropriate amount (usually between 0.3 ml and 0.5 ml) of lysing buffer containing Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol)(50 mM,pH 8.4), EDTA (ethylenediaminetetraacetic acid)(5 mM), EGTA (ethylenebis(oxyethylenenitrilo tetraacetic acid)(5 mM), and Triton X-100 (0.1 %). The protein inhibitor phenylmethylsulfonyl fluoride (PMSF)(2 % (v/v) of a 100 mM stock stored at -20°C in isopropanol) was added to the lysing buffer just prior to use. Cells were incubated in lysing buffer at 4°C for 30 minutes.

After incubation, the suspensions were homogenized in a small glass homogenizer using approximately 50 strokes, and the cell debris pelleted by centrifuging at 4°C and 10 000 g for 10 minutes. Supernatants containing the proteins were removed and stored at -20°C.

2.10.2. DETERMINATION OF PROTEIN CONCENTRATIONS

Protein concentration was determined according to the method of Bradford (1976).

A standard curve was prepared using five duplicate serial dilutions of a bovine serum albumin solution (50 mg %). Samples were diluted appropriately, and 1 ml of Bradford reagent was added to each tube of sample

and standard. Bradford reagent contained Coomassie brilliant blue G250 (100 mg/l), ethanol (50ml of 95 % (v/v) /l), and phosphoric acid (100 ml of 85 % (v/v)/l). The reagent was filtered using a number 1 Whatman filter, and was stored at 4^0C.

Tubes were incubated at room temperature with the Bradford reagent for 10 minutes, and the absorbance at 595 nm was determined within one hour. A standard concentration curve was plotted using the Beckman Quant II soft-pac module (quadratic), and the concentration of protein in each sample was estimated from this curve.

2.10.3. PROTEIN GEL ELECTROPHORESIS

Protein samples were electrophoresed on a denaturing SDS/polyacrylamide gel system under discontinuous conditions. By using a stacking gel and a resolving gel of different gel densities, sharp, highly concentrated zones of material are produced.

Proteins were stacked in a 4 % polyacrylamide gel at pH 6.8, and were resolved in a 15 % polyacrylamide gel at pH 8.8. In the stacking gel, larger pore sizes allow the molecules to move freely but allow for the proteins to stack within the electrical field depending on their molecular size and charge. The resolving gel with its smaller pore sizes separates molecules according to their molecular size or their charge-to-mass ratio.

A 30 % acrylamide stock solution was prepared using acrylamide (29.1 % w/v) and N,N'-methylene bis-acrylamide (0.9 % w/v), and was stored at 4°C for up to two weeks.

The resolving gel was prepared using the acylamide stock solution (50 % (v/v)), TRIS buffer (0.375 M)(pH 8.8), sodium dodecyl sulphate (SDS)(0.1 % (w/v)), N,N,N',N'-tetramethylethylenediamine (TEMED)(0.33 µl/ml), and ammonium persulphate (0.033 % (w/v)).

The stacking gel was prepared using the acrylamide stock solution (13.3 % (w/v)), TRIS buffer (0.125 M)
(pH 6.8), SDS (0.1 % (w/v)), TEMED (0.33 µl/ml), and ammonium persulphate (0.033 % (w/v)).

Gels were degassed prior to addition of TEMED and ammonium persulphate, as the reaction producing free radicals from ammonium persulphate, which is catalyzed by TEMED, is inhibited by free oxygen. (Free radicals catalyze the polymerization of acrylamide with the cross-linking agent N,N'-methylene bis-acrylamide).

The running gel was poured into the gel casting unit, overlayered with 50 - 100 µl of distilled water or isopropanol (to inhibit oxidation), and allowed to set. The stacking gel was then added, and a well-forming comb was placed

into the top of the gel before the gel was allowed to set. After the gel had set, the comb was gently eased out of the gel, while the wells were rinsed with distilled water.

Protein samples were prepared by diluting to 100 µl with TRIS buffer (50 mM)(pH 7.4), and adding 100 µl of sample buffer (containing TRIS buffer (0.125 M)(pH 6.8), SDS (4 % (w/v)), ß-mercaptoethanol (10 % (v/v)), glycerol (20 % (v/v)), and bromophenol blue (0.1 mg/ml)). Samples were heated at 96°C for three minutes, stored on ice, and centrifuged for two minutes before loading. The rainbow molecular mass marker (5 µl) was heated at 96°C with sample buffer (5 µl) for one minute.

The unit was attached to a water bath at 10°C, and samples were loaded into the wells. Samples were allowed to stack at 10 mA/gel and 500 V, after which resolution was achieved at 15 mA/gel and 500 V. The tank electrode buffer contained TRIS buffer (0.025 M)(pH 8.3), glycine (0.192 M), and SDS (0.1 % (w/v)).

2.10.4. WESTERN BLOTTING

Proteins were transblotted onto nitrocellulose (Hybond C) at 200 mA for three hours using a transblotting chamber and a transblotting buffer containing TRIS (25 mM), glycine (1.12 % w/v), methanol (20 % v/v), and SDS (0.1 %).

For transblotting, the protein gel plus nitrocellulose membrane were sandwiched between Whatman no.3 filter paper and thin sponges, and inserted into a transblotting cassette (system components were equilibrated in transblot buffer prior to system assembly). Air bubbles were avoided so that a uniform electrical field was assured during transblotting. The transblotting chamber was filled with transblotting buffer, and the cassette was placed into the chamber with the protein gel facing the anode and the nitrocellulose facing the cathode.

2.10.5. STAINING OF NITROCELLULOSE TRANSBLOT

Transblots were stained to evaluate the integrity of proteins after the isolation and processing methods used. The stain was prepared by adding india ink to TRIS-buffered saline (TRIS (20 mM), sodium chloride (500 mM))(pH 7.5) containing the detergent poly(oxyethylene)$_n$-sorbitan-monolaurate (Tween 20)(0.05 % (v/v)).

Blots were stained at room temperature for one hour. Protein immunodetection cannot be performed on these stained blots.

2.10.6. PROTEIN IMMUNODETECTION

After the proteins were transblotted onto nitrocellulose, open binding sites on the membrane were blocked by incubating in bovine serum albumin (3 % (w/v) in TRIS-buffered saline) overnight at 4°C. The following procedures

were then performed at room temperature.

Membranes were washed in TRIS-buffered saline (TBS) for 15 minutes to remove unbound bovine serum albumin, and were subsequently exposed to the primary antibody (DAKO-bcl-2, a monoclonal mouse anti-human bcl-2 antibody) for 4 hours. The antibody was diluted 1:100 with 5 ml antibody buffer (bovine serum albumin (1 % (w/v)) in TRIS-buffered saline containing Tween 20 (0.05 % (v/v))(TTBS), and incubation took place in a rotating chamber.

Membranes were subsequently washed in TTBS (40 ml) for 15 minutes to remove any excess primary antibody, and were then exposed to the secondary antibody (biotinylated anti-mouse immunoglobulin, Amersham)(diluted 1:200 in 5 ml TTBS) for one hour in a rotating chamber.

After washing them in TTBS (40 ml) for 15 minutes, the membranes were incubated with streptavidin-biotinylated horseradish peroxidase complex (Amersham)(diluted 1:200 in 5 ml TTBS) for one hour in a rotating incubator.

To stain for peroxidase activity, membranes were placed in freshly prepared colour development solution containing 4 chloro-1-naphthol (0.05 % (w/v)), ice-cold ethanol (16.7 % (v/v) of 97 % ethanol), TBS (83.3 % (v/v)), and hydrogen peroxide (0.6 µl/ml). Membranes were gently shaken in the

solution until bands appeared. The reaction was stopped by rinsing the blot in tap water.

The membranes were scanned using a densitometer. A curve was obtain ', and the relative absorbance units were calculated by integrating the area under this curve.

2.10.7. SLOT BLOTTING OF PROTEIN SAMPLES

Once the specificity of the antibody was determined, slot blotting of protein samples (using a Hoefer pr600 SlotBlot, USA) provided a faster and easier method of protein analysis than the conventional gel electrophoresis and transblotting of samples.

The slot blotting system (SlotBlot) was assembled with the filterpaper and nitrocellulose membrane having been equilibrated in TBS buffer previously. The SlotBlot was attached to a vacuum pump and a liquid trap. Vacuum was applied for a few seconds and then switched off before the samples were loaded. Samples were diluted to 100 µl with TBS buffer, and each sample was pipetted against the bottom side wall of one of the wells in the SlotBlot's top block. Total cellular HL-60 protein (15 µg) and total cellular THP-1 protein (90 µg) was loaded. The vacuum was turned back on and the samples were allowed to be pulled through onto the nitrocellulose membrane. Each slot was subsequently rinsed twice with 1 ml of TBS buffer.

The nitrocellulose membrane blot was used for protein immunodetection as described in section 2.10 *b*.

2.11. IMMUNOCYTOCHEMICAL DETECTION OF THE BCL-2 PROTEIN IN WHOLE CELLS

2.11.1. PRETREATMENT OF SLIDES

Slides were pretreated before they were used to mount THP-1 and HL-60 cells. Slides were cleaned by immersion in liquid detergent (1 % v/v) for 30 minutes, and subsequently washed in tap water for 30 minutes and rinsed in distilled water twice for 3 minutes. A final ethanol (95 % v/v) rinse was performed twice for 5 minutes, after which slides were allowed to air-dry. Slides were then treated with 3-(triethoxysilyl)-propylamine (2 % v/v in acetone) for 5 minutes, rinsed in acetone for 1 minute twice and in distilled water for 1 minute twice, and were dried overnight at 37°C. Treated slides could then be stored at room temperature.

2.11.2. FIXING OF CELLS

Cell suspensions were pipetted onto the slide and smeared across the surface. Once the slides were partially dry, they were fixed for 5 to 10 minutes by immersion in a solution of double-filtered, saturated aqueous solution of picric

acid containing paraformaldehyde (20 g / 150 ml). Slides were subsequently rinsed in phosphate-buffered saline until the yellow colour of the picric acid solution had faded.

2.11.3. IMMUNODETECTION OF BCL-2

Slides were fully immersed in citrate buffer (0.01 M, pH 6.0), and were microwaved for 5 minutes. This step was repeated. The slides were allowed to cool in the citrate buffer for 20 minutes, and were subsequently rinsed in phosphate buffered saline (PBS).

Incubation of slides in quenching solution (3 parts hydrogen peroxide to one part methanol) for 30 minutes ensured that endogenous peroxidases would be blocked. Slides were rinsed three times in PBS, and were then incubated with goat serum (5 % v/v in PBS) for 20 minutes to block non-specific antigen sites. The Bcl-2 antibody (Dako) (100 x dilution, 50 µl per slide) was applied to drained slides, which were then incubated at room temperature for one hour. Following three rinses in PBS, the slides were incubated with the secondary biotinylated anti-mouse antibody (Dako) (200 x dilution) for 30 minutes at room temperature. Slides were subsequently rinsed as before, and incubated with streptavidin-biotinylated horseradish peroxidase (Dako) (200 x dilution) for 30 minutes at room temperature. PBS-rinsed slides were then placed in a solution containing 3,3'-diaminobenzidine tetrachloride (DAB) (0.5 mg / 0.5 ml PBS + 0.5 ml distilled water) and

hydrogen peroxide (one drop / 50 ml) for 5 minutes. This produces brown granular staining in positive cells. Slides were rinsed with tap water for 10 minutes, and DAB was neutralized using a bleach solution (5 % v/v).

Slides were counterstained with Meyer's haematoxylin solution containing haematoxylin (2 g/l), ammonium alum (50 g/l), sodium iodate (0.2 g/l), citric acid (4 g/l), and chloral hydrate (50 g/l). This produces a blue background staining. Cells were subsequently dehydrated by washing in increasing concentrations of ethanol. Mounting of slides was performed using xylene and entellan.

Control slides for non-specific secondary antiboby binding were prepared without addition of the primary antibody. Slides were subsequently treated the same way as sample slides.

SECTION 3

RESULTS

3.1. CELL MORPHOLOGY

HL-60 and THP-1 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), PMA plus dbcAMP (16 nM and 50 μM, respectively), and puromycin (1 μg/ml and 0.5 μg/ml).

Approximately 50 % of unstimulated and dbcAMP-stimulated THP-1 cells adhered to the petri-dishes (n=2). Stimulations with PMA and PMA plus dbcAMP increased cell adherence to approximately 95 % (n=2). No detectable adherence was present in unstimulated and dbcAMP-stimulated HL-60 cells. A marked increase in adherence was, however, noted upon PMA-stimulation, and upon stimulation with PMA plus dbcAMP (data not presented).

Cytospins of stimulated cells and unstimulated control cells were subsequently performed by centrifuging the cells onto glass slides (n=1). The cells were then stained, and representative fields were photographed (see section 2.2 for methodology).

Unstimulated THP-1 cells, though roughly polygonal in shape, appeared somewhat indented and irregular (figure 3.1). The cell nuclei, in contrast, were almost sperical and contained well-defined nucleoli. Separation of the nucleus into discrete masses of chromatin, which is a highly typical feature of apoptosis (Martin *et al*, 1994), was not observed on this slide. The THP-1 cells contained large vacuoles in their cytoplasm. No mitotic cells were observed.

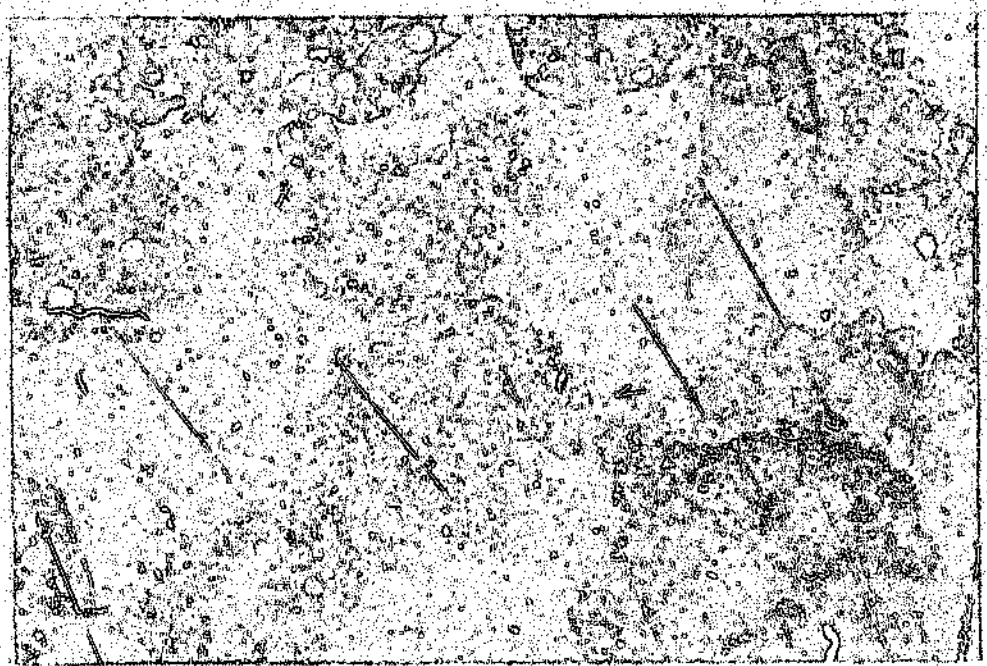


Figure 3.1.

Morphological features of unstimulated THP-1 cells

Unstimulated THP-1 cells were centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was subsequently taken through a light microscope at 1 500 X magnification.

PMA-stimulated THP-1 cells seemed more spherical than their unstimulated counterparts (figure 3.2), and portrayed eccentrically placed ovoid or kidney-shaped nuclei. Large vacuoles were clearly visible in the cytoplasm. No apoptotic nuclear fragmentation was observed in PMA-stimulated THP-1 cells.

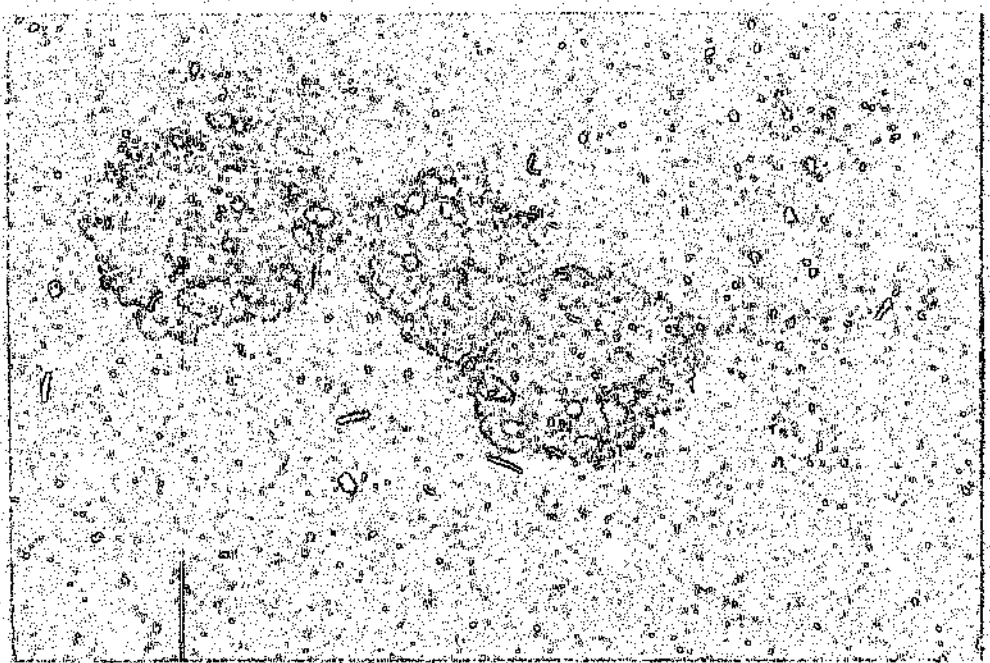


Figure 3.2.

Morphological features of PMA-stimulated THP-1 cells

THP-1 cells exposed to PMA (16 nM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

THP-1 cells stimulated with dbcAMP displayed distinctly heteromorphous nuclei and a few very large, elongated vacuoles (figure 3.3). Cells were roughly spherical in shape, and no apoptotic nuclear fragmentation was visible.

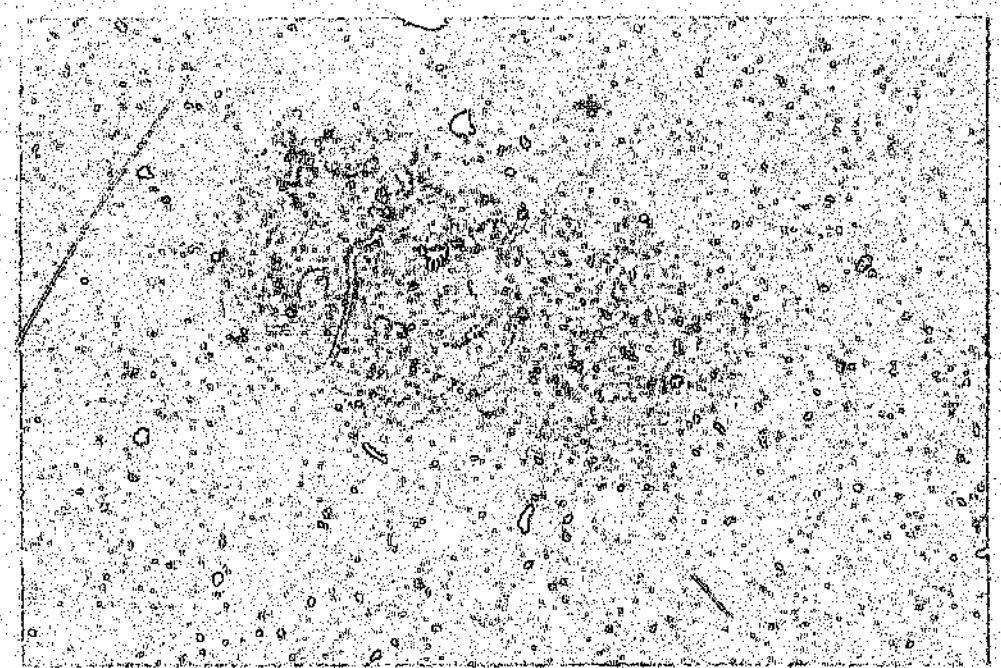


Figure 3.3.

Morphological features of dbcAMP-stimulated THP-1 cells

THP-1 cells were exposed to dbcAMP (50 μM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

THP-1 cells co-stimulated with PMA and dbcAMP combined the morphological features observed in stimulations with PMA or dbcAMP alone (figure 3.4). Most cells were roughly spherical in shape, and contained ovoid, kidney-shaped, or convoluted nuclei. Large, elongated vacuoles were present in the cytoplasm. Again, no apoptotic nuclear fragmentation was observed.

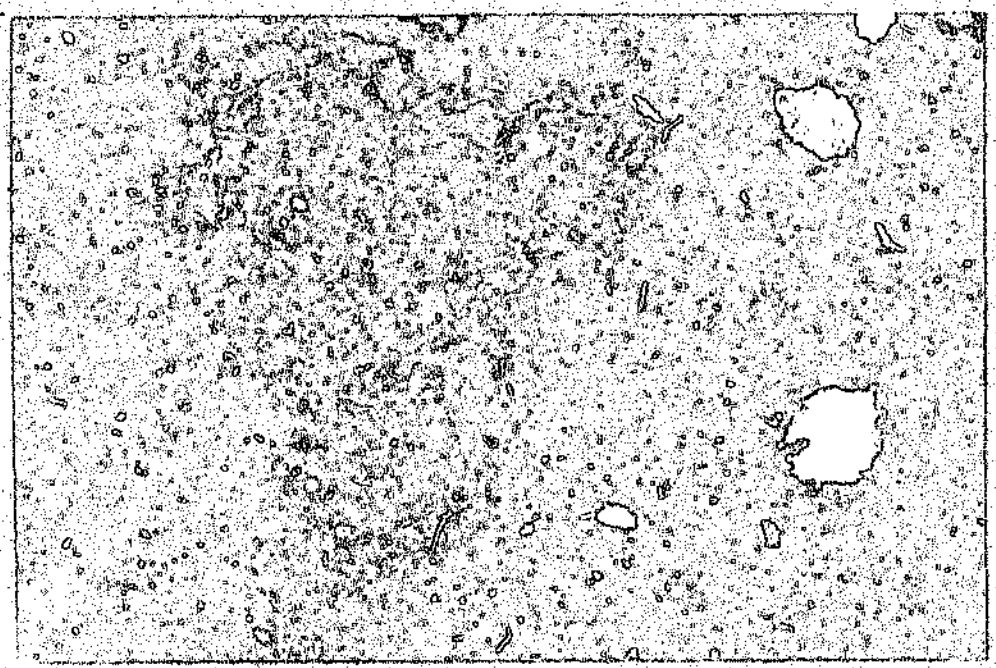


Figure 3.4.

Morphological features of THP-1 cells co-stimulated with PMA and dbcAMP

THP-1 cells were exposed to PMA (16 nM) in conjunction with dbcAMP (50 uM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

Apoptotic features were, however, clearly visible in some of the THP-1 cells stimulated with puromycin (1 μg/ml) (figure 3.5). While most of the cells were regular in shape, with roughly spherical nuclei and polygonal outer cytoplasmic boundaries, a few unusual cells were observed (see cells indicated by arr ws in figure 3.5). These cells were condensed, and their nuclei had fragmented into the discrete compact masses typical of apoptosis (Martin *et al*, 1994). No apoptotic features were observed in THP-1 cells that were stimulated with a lower dose of puromycin (0.5 μg/ml) (results not shown).

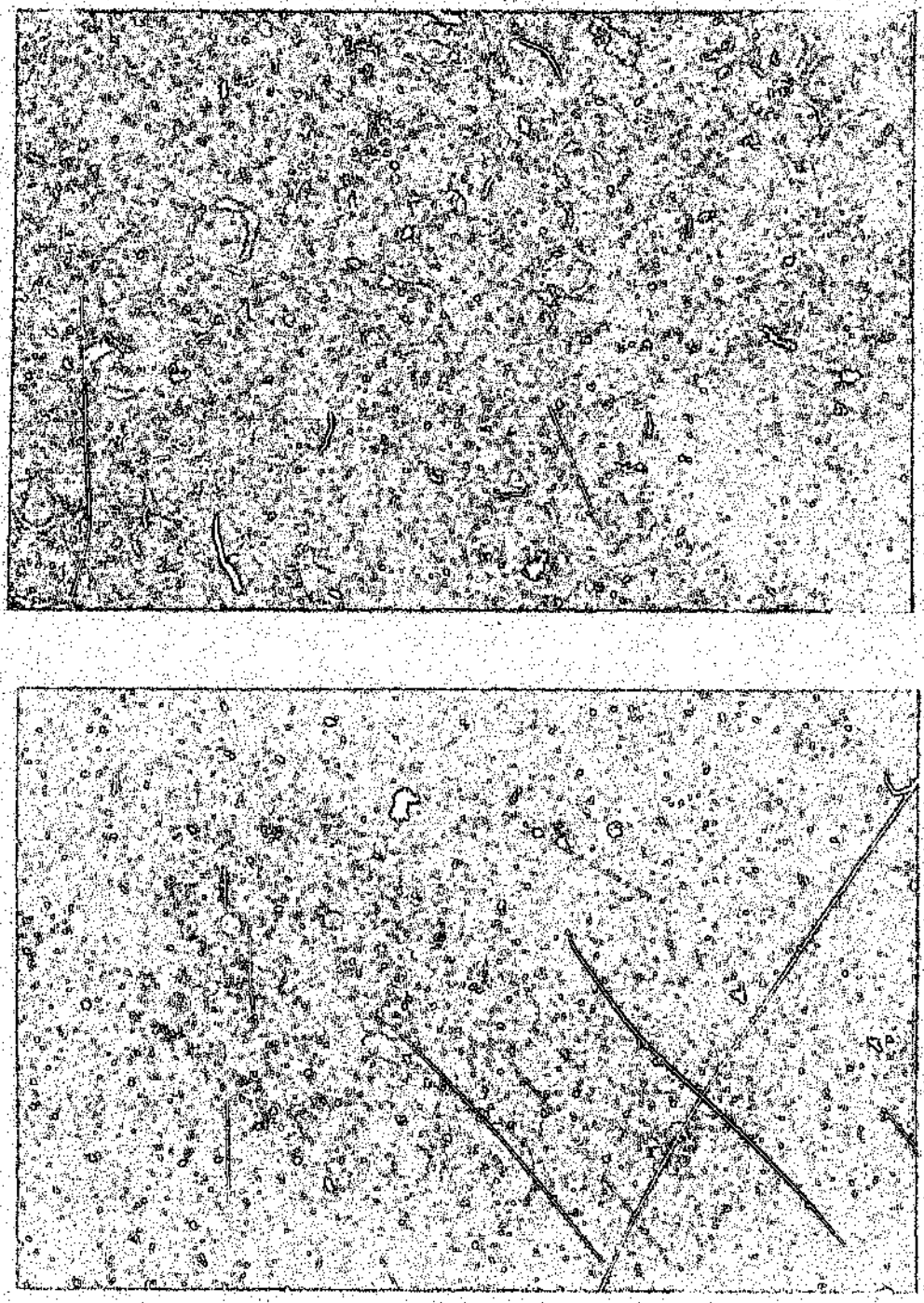


Figure 3.5.

Morphological features of puromycin-stimulated THP-1 cells

THP-1 cells were exposed to puromycin (1 μg/ml) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. Photomicrographs were taken through a light microscope at 600 X magnification (top) and 1 500 X magnification (bottom).

Unstimulated HL-60 cells were roughly sperical in shape, and contained large round or convoluted nuclei with visible nucleoli (figure 3.6). Small vacoules were present in the cell cytoplasm. As with unstimulated THP-1 cells, no mitotic or apoptotic features were observed in these HL-60 cells.

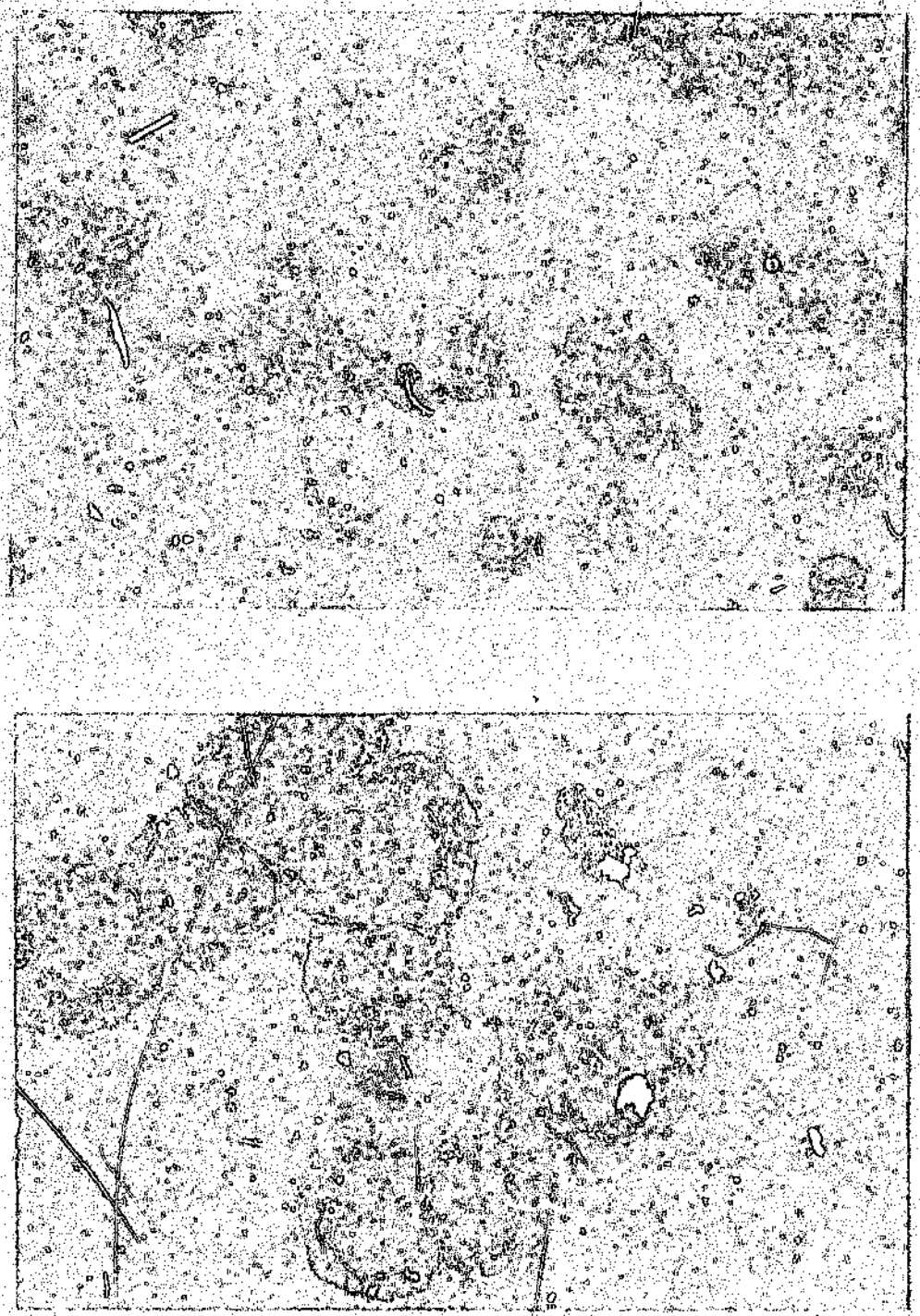


Figure 3.6.

Morphological features of unstimulated HL-60 cells

Unstimulated HL-60 cells were centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was subsequently taken through a light microscope at 600 X magnification (top) and 1 500 X magnification (bottom).

PMA-stimulated HL-60 cells appeared more ovoid than their unstimulated counterparts, and had more elongated nuclei (figure 3.7). Small vacuoles were present in the cytoplasm. Again, no distinct apoptotic features were observed on the slide.

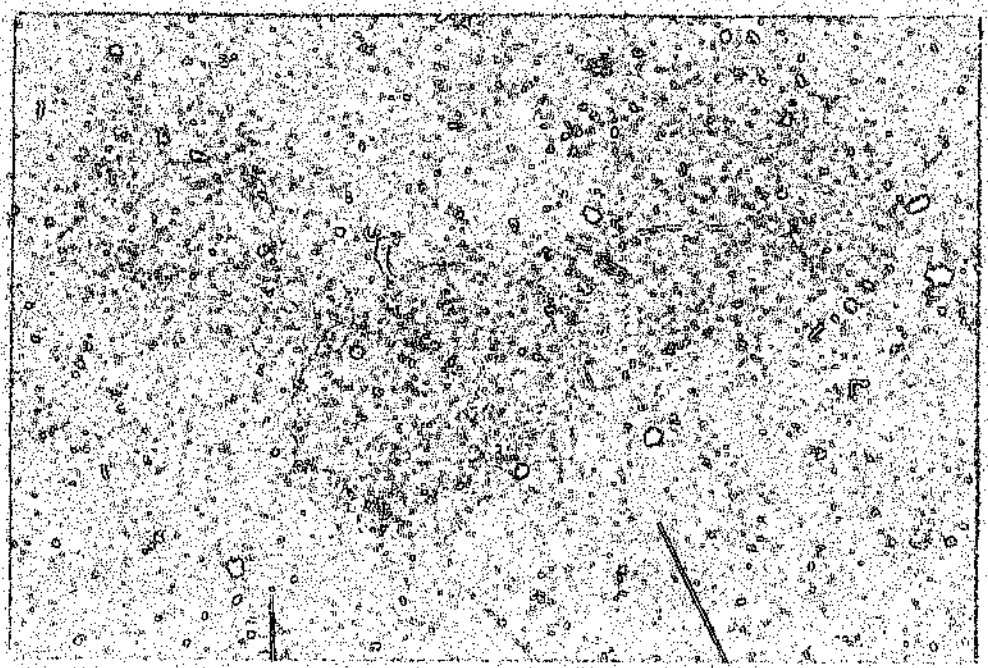


Figure 3.7.

Morphological features of PMA-stimulated HL-60 cells

HL-60 cells exposed to PMA (16 nM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

dbcAMP-stimulated HL-60 cells resembled their unstimulated counterparts, displaying roughly spherical cell shapes and nuclei (figure 3.8). Nucleoli were clearly visible, and small vacuoles were present in the cytoplasm. No apoptotic nuclear fragmentation was observed.

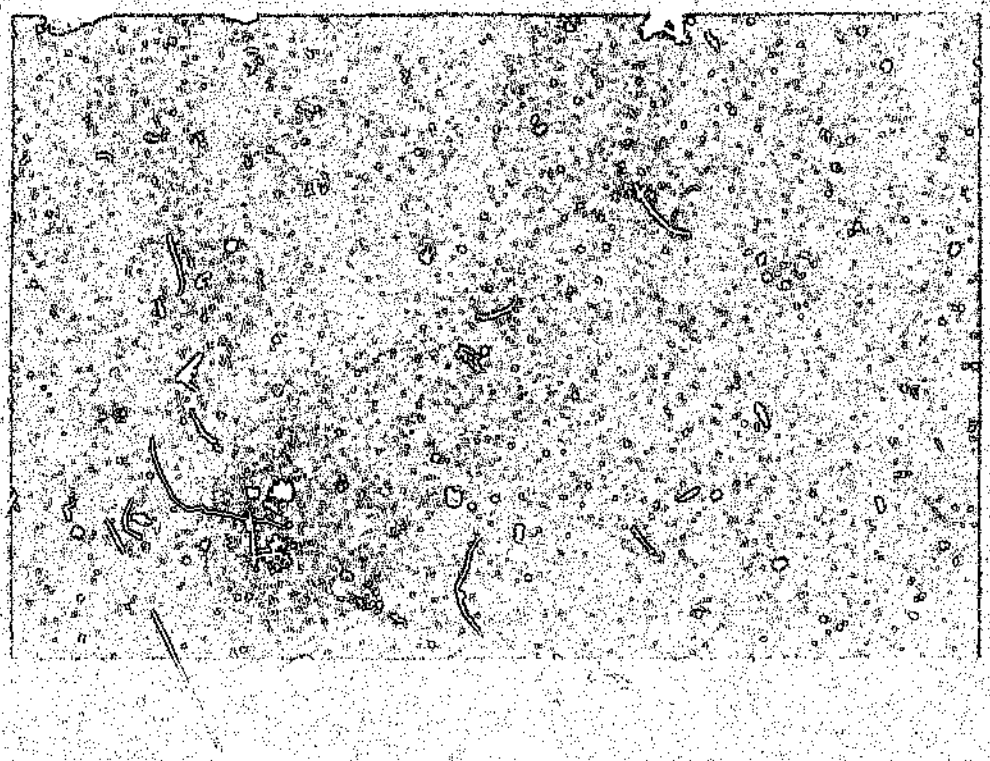


Figure 3.8.

Morphological features of dbcAMP-stimulated HL-60 cells

HL-60 cells were exposed to dbcAMP (50 μM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

Interestingly, some of the HL-60 cells co-stimulated with PMA and dbcAMP portrayed the morpholigical features characteristic of apoptosis (figure 3.9). Most of the cells appeared similar to PMA-stimulated cells, with ovoid cell-shapes, small cytoplasmic vacuoles, and elongated nuclei. However, a few cells on the slide displayed condensed and fragmented apoptotic nuclei (see, for example, the cell indicated by an arrow in figure 3.9).

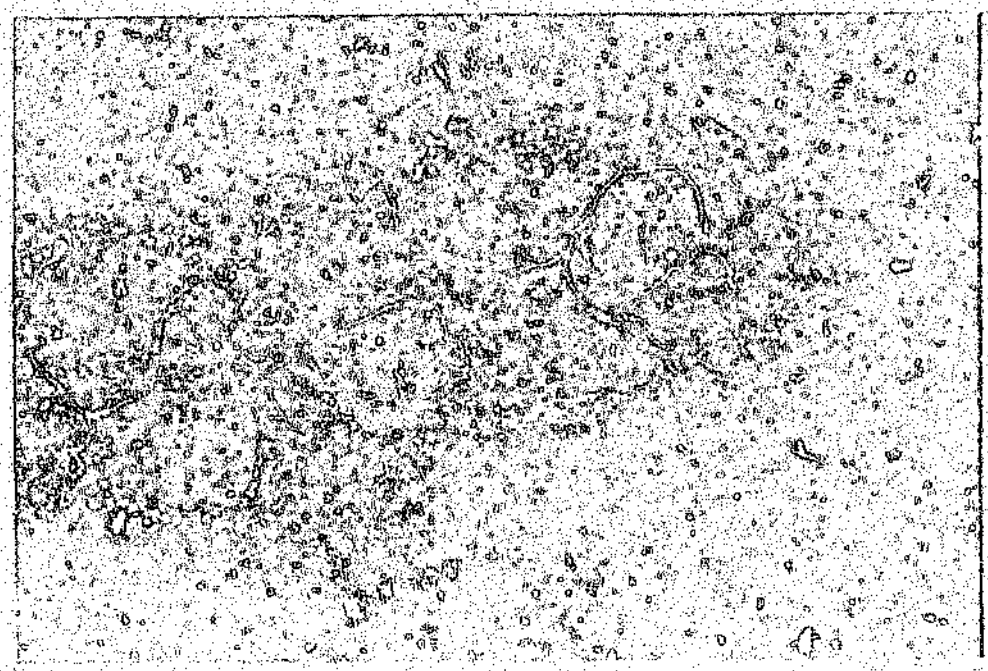


Figure 3.9.

Morphological features of HL-60 cells co-stimulated with PMA and dbcAMP

HL-60 cells were exposed to PMA (16 nM) in conjunction with dbcAMP (50 μM) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. A photomicrograph was taken through a light microscope at 1 500 X magnification.

Apoptotic features were observed in quite a few HL-60 cells stimulated with puromycin (1 μg/ml) (see cells indicated by arrows in figure 3.10). These cells appeared condensed, and contained discrete, compacted nuclear fragments. Those cells that did not display these apoptotic features, resembled unstimulated control

HL-60 cells, but were more vacuolated. Stimulation with the lower dose of puromycin (0.5 μg/ml) also led to apoptotic features in some HL-60 cells (results not shown). Interestingly, this is the same dose that did not lead to observable apoptotic features in THP-1 cells.

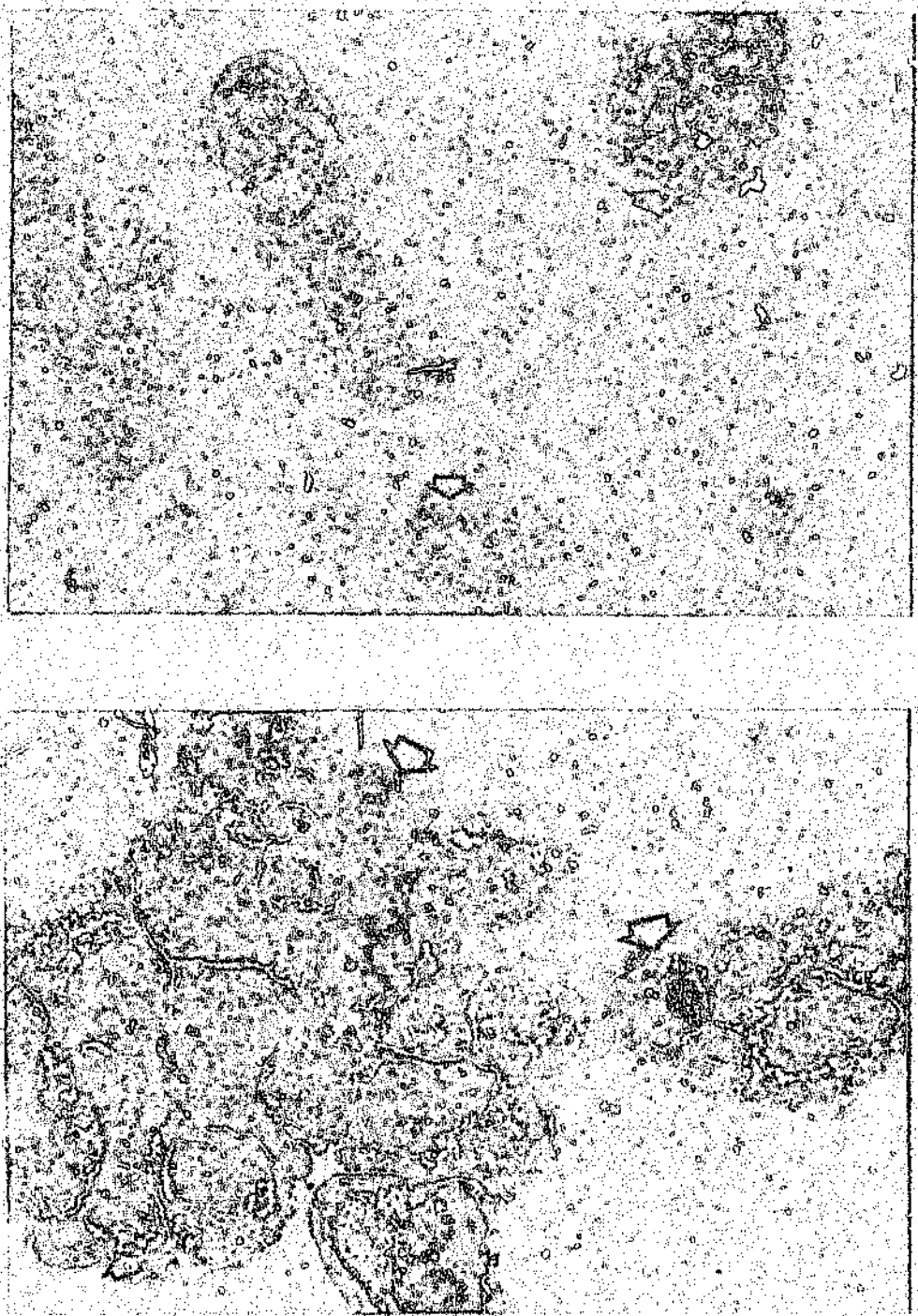


Figure 3.10.

Morphological features of puromycin-stimulated

HL-60 cells

HL-60 cells were exposed to puromycin (1 µg/ml) for 24 hours, and subsequently centrifuged onto a glass slide and stained with Giemsa. Photomicrographs were taken through a light microscope at 600 X magnification (top) and 1 500 X magnification (bottom).

3.2. CD33 SURFACE ANTIGEN EXPRESSION

THP-1 and HL-60 cells were stimulated with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively) for 24 hours. Following this stimulation, cells were incubated with a fluorescently labelled monoclonal antibody directed against the human myelocytic CD33 cell surface receptor (n=1)(for methodology, see section 2.3).

Stimulated and unstimulated THP-1 and HL-60 cells displayed similar levels of CD33 expression. Slight decreases were, however, observed in THP-1 cells stimulated with PMA (alone and in combination with dbcAMP), when compared to unstimulated and dbcAMP-stimulated cells (figure 3.11.). A similar trend was observed in HL-60 cells (figure 3.12.). Unexpectedly, unstimulated THP-1 cells displayed higher levels of CD33 expression than unstimulated HL-60 cells (figure 3.13.). Of course, repeat experiments are needed to confirm the results obtained.

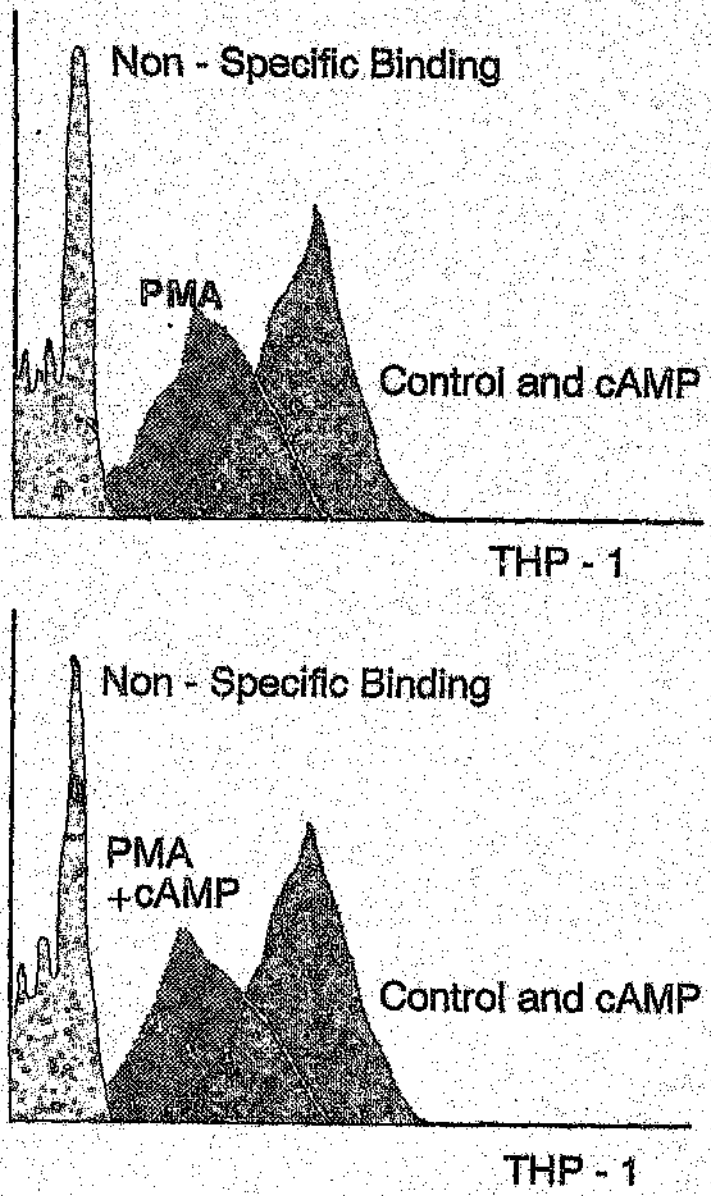
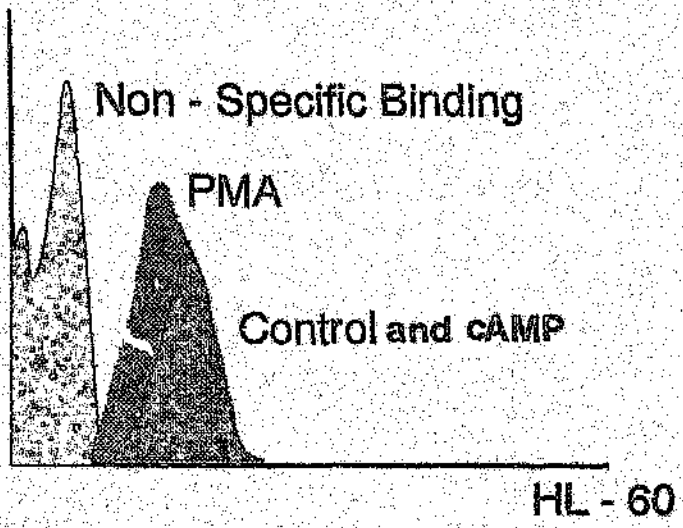


Figure 3.11.

CD33 expression in THP-1 cells

THP-1 cells were stimulated with PMA (16 nM) (top), dbcAMP (50 μM) (top and bottom), and PMA plus dbcAMP (bottom). Cells were exposed to fluorescently labelled anti-human CD33 antibody prior to flow cytometrical analysis. Control cells were exposed to fluorecently labelled anti-mouse antibody to monitor for non-specific binding (n=1).



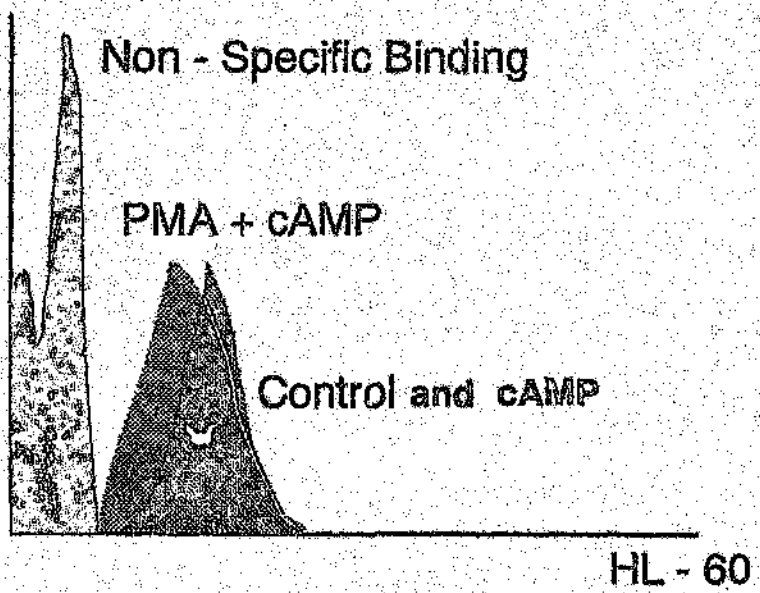


Figure 3.12.

CD33 expression in HL-60 cells

HL-60 cells were stimulated with PMA (16 nM) (top), dbcAMP (50 μM) (top and bottom), and PMA plus dbcAMP (bottom). Cells were exposed to fluorescently labelled anti-human CD33 antibody prior to flow cytometrical analysis. Control cells were exposed to fluorecently labelled anti-mouse antibody to monitor for non-specific binding (n=1).

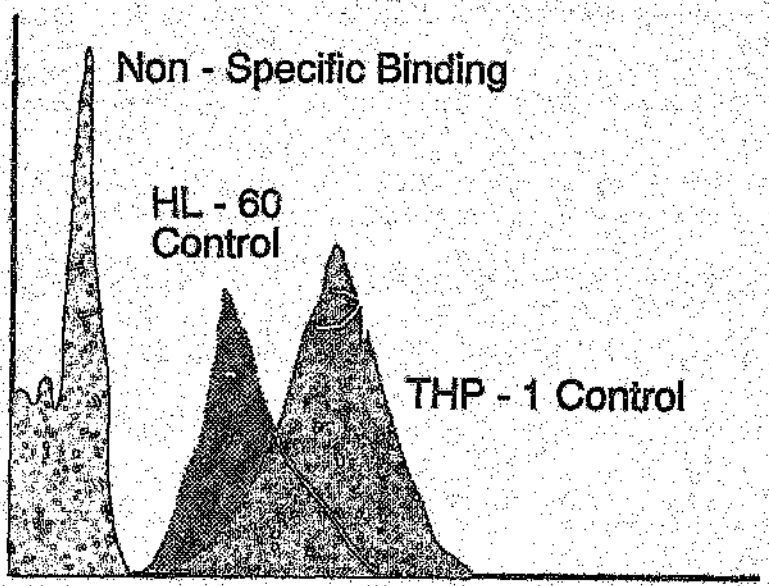


Figure 3.13.

CD33 expression in unstimulated THP-1 and HL-60 cells

Unstimulated THP-1 and HL-60 cells were exposed to fluorescently labelled anti-human CD33 antibody prior to flow cytometrical analysis. Control cells were exposed to fluorecently labelled anti-mouse antibody to monitor for non-specific binding (n=1).

3.3. DNA PROFILE ANALYSIS

Cell cycle distribution patterns were analysed in THP-1 and HL-60 cells using flow cytometry. Cells were stimulated with PMA (16nM)-, dbcAMP (50 μM)-, and PMA plus dbcAMP (16 nM and 50 μM, respectively)(n=2). (See section 2.4 for methods.)

In THP-1 cells, PMA stimulation caused an increase in the proportion of cells residing both in the G_0/G_1 and the G_2/M-phase of the cell cycle (figures 3.14 and 3.16). An average increase of 25 % (the actual values were 30 % and 20 %) was observed for cells residing in the G_0/G_1 phase of the cell cycle, and of 72.5 % (61 % and 83 %) for cells residing in the G_2/M phase as compared to unstimulated control cells. This was accompanied by a 48.5 % (45 % and 52 %) decrease in those cells residing in the S phase (figure 3.15).

dbcAMP stimulation, on the other hand, resulted in cell cycle distribution patterns that more closely resembled those of unstimulated control cells. Stimulation only caused an average increases of 4 % (0 % and 8 %) and 8.5 % (0 % and 17 %) in the proportion of cells residing in the G0/G1 phase and the G_2/M phase of the cell cycle, respectively, when compared to unstimulated control cells (figures 3.14 and 3.16). This was accompanied by a decrease of 5.5 % (5 % and 6 %) in cells residing in the S phase (figure 4.15).

Stimulation with PMA in conjunction with dbcAMP caused cell cycle distribution patterns similar to those observed for PMA stimulation alone. Thus, increases of 23 % (16 % and 30 %) and 81 % (62 % and 100 %) were observed for cells residing in the G_0/G_1 and G_2/M phases of the cell cycle, respectively (figures 3.14 and 3.16). This was accompanied by a 47.5 % (45 % and 50 %) decrease of cells residing in the S phase of the cell cycle (figure 3.15).

Patterns in HL-60 cells showed similar trends to those in THP-1 cells. PMA stimulation caused an increase of 40 % (32 % and 48 %) in cells residing in the G_0/G_1 phase of the cell cycle (figure 3.17), while the proportion of cells residing in the G_2/M phase was increased by 103 % (94 % and 112 %) when compared to unstimulated control cells (figure 3.19). Cell numbers in the S phase were decreased by 75.5 % (72 % and 78 %) (figure 3.18).

dbcAMP stimulation caused little change when compared to control distribution levels (figures 3.17, 3.18, and 3.19), with only a 7 % (3 % and 10 %) increase in cells residing in G_0/G_1, and a 11.5 % (0 % and 23 %) decrease in cells residing in G_2/M. S phase distribution levels did not change, on average, when compared to control levels.

Co-stimulation with PMA and dbcAMP resulted in cell cycle distribution patterns similar to those observed with PMA stimulation alone. Increases of

38 % (33 % and 43 %) and 77 % (54 % and 100 %) were observed for cells residing in the G_0/G_1 phase and G_2/M-phase of the cell cycle, respectively (figures 3.17 and 3.19). A decrease of 64 % (47 % and 81 %) occurred in cells residing in the S phase of the cell cycle (figure 3.18).

(Results for each experiment are given as averages of the two values obtained, with the actual values obtained appearing in brackets).

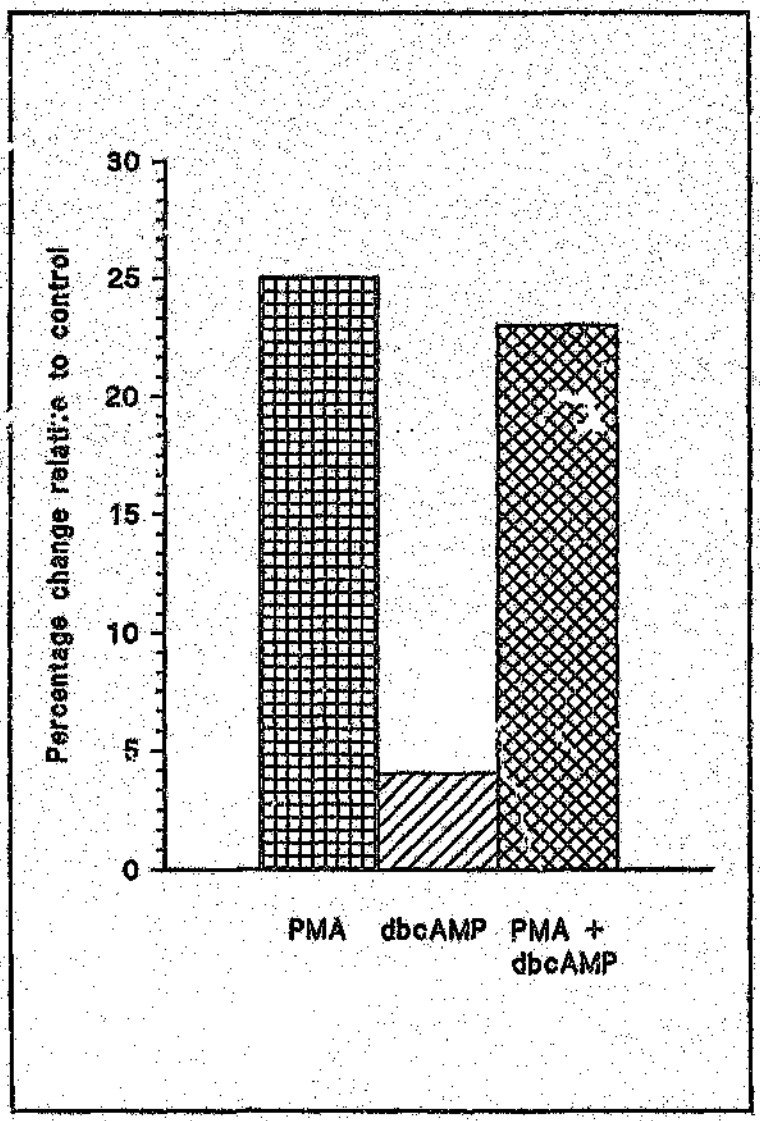


Figure 3.14.

THP-1 cell cycle distribution pattern: Changes in G_0/G_1

THP-1 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results for G_0/G_1 are given as percentage differences between these standards and the stimulated cells (n=2).

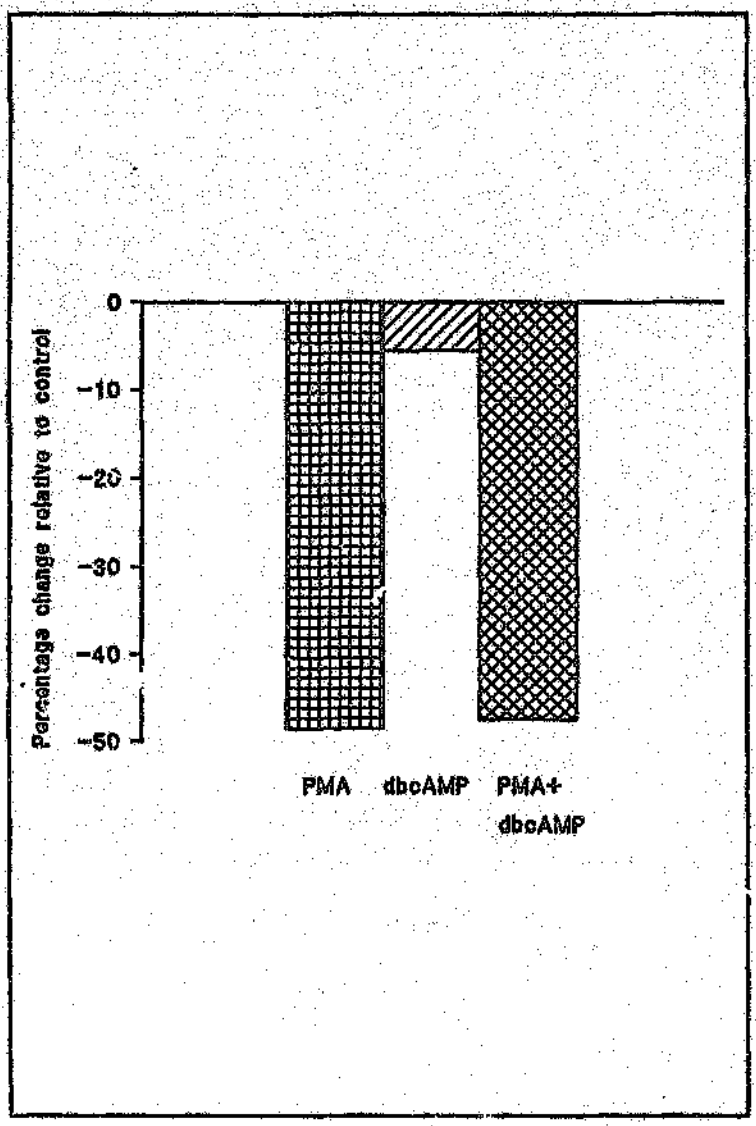


Figure 3.15.

THP-1 cell cycle distribution pattern: Changes in S

THP-1 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results in S are given as percentage differences between these standards and the stimulated cells (n=3).

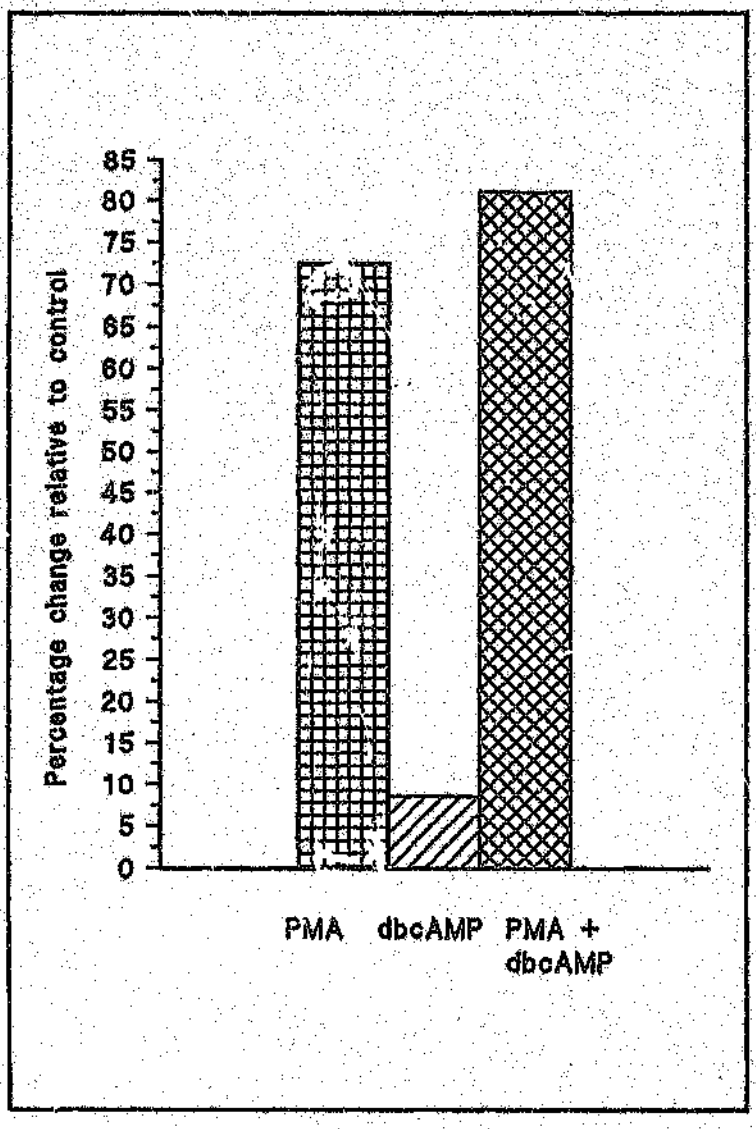


Figure 3.16.

THP-1 cell cycle distribution pattern: Changes in G_2/M

THP-1 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results for G_2/M are given as percentage differences between these standards and the stimulated cells (n=2).

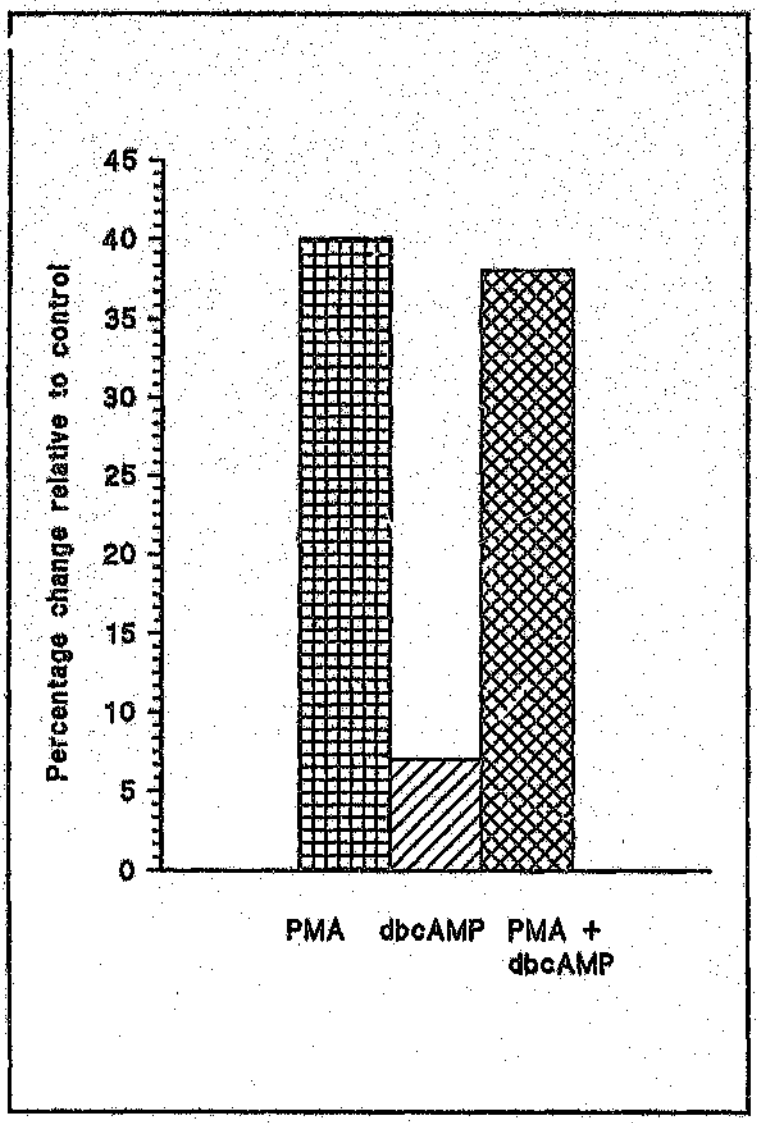


Figure 3.17.

HL-60 cell cycle distribution pattern: Changes in G_0/G_1

HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results for G_1/G_2 are given as percentage differences between these standards and the stimulated cells (n=2).

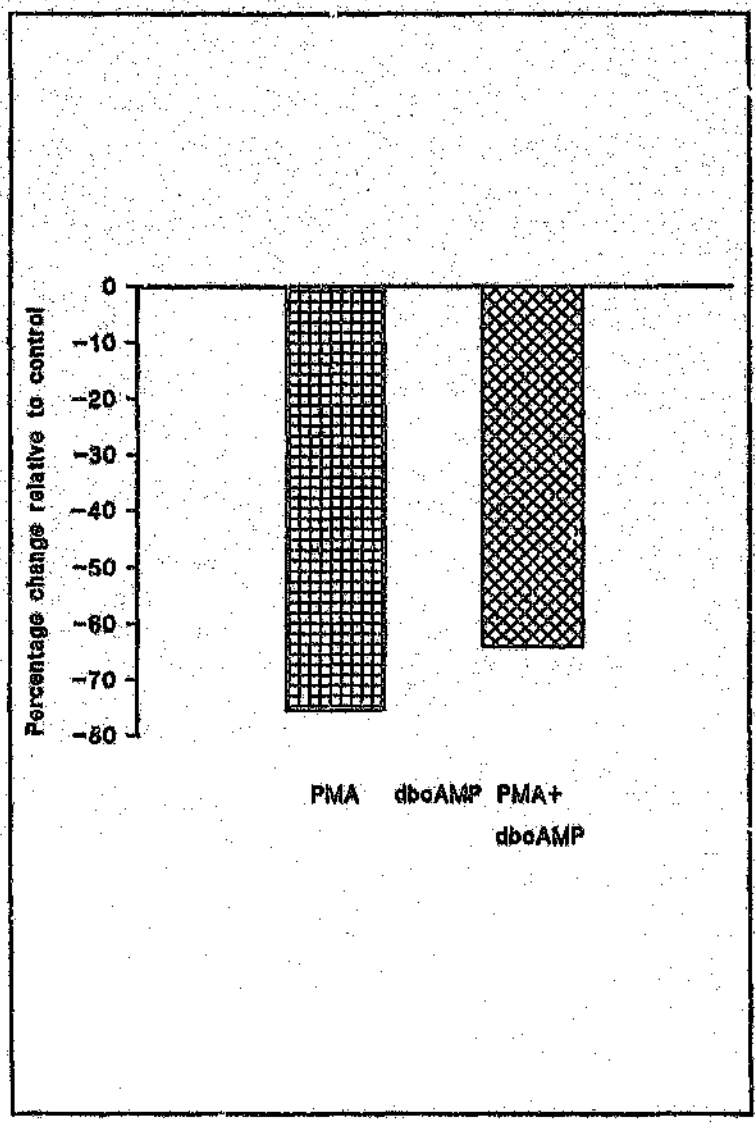


Figure 3.18.

HL-60 cell cycle distribution pattern: Changes in S

HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results for S are given as percentage diff ' uces between these standards and the stimulated cells (n=2).

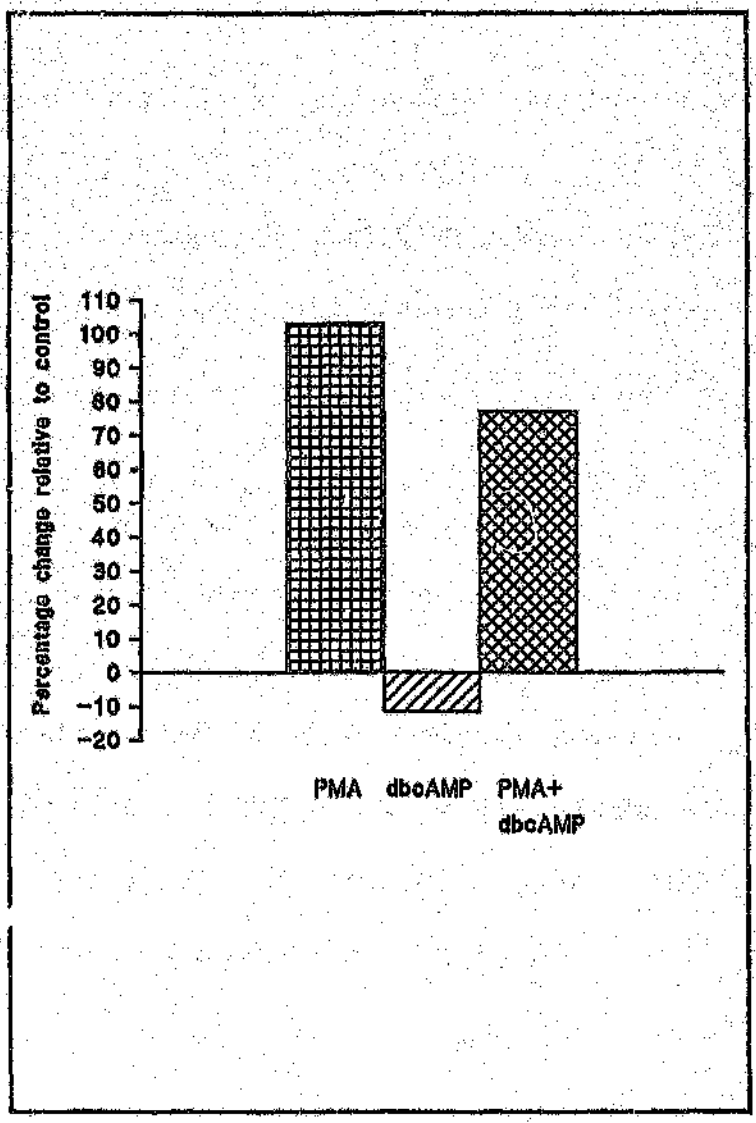


Figure 3.19.

HL-60 cell cycle distribution pattern: Changes in G_2/M

HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cells were subsequently permeabilized using Triton X, and exposed to the DNA intercalator propidium iodide. Analyses of cell cycle distribution patterns were performed using flow cytometry. Unstimulated control cells were used as standards, and the results for G_2/M are given as percentage differences between these standards and the stimulated cells (n=2).

3.4. THYMIDINE INCORPORATION

THP-1 cells were exposed to PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively) for 4 days. Incorporation of radioactively labelled thymidine ([^{3}H]thymidine) over the last 24 hours of stimulation was measured in counts per minute (cpm)(n=2). (For methodology, refer to section 2.5.)

Exposure to PMA resulted in the loss of cell proliferation by day four (80 cpm, compared to 50380 cpm for unstimulated cells). This cytostasis was observed both with PMA stimulation alone, and with PMA stimulation in conjunction with dbcAMP stimulation (48 cpm). Stimulation with dbcAMP alone, on the other hand, did not halt cell proliferation, but caused it to decrease by approximately fifty percent by day four (25220 cpm), when compared to unstimulated control cells (figure 3.20).

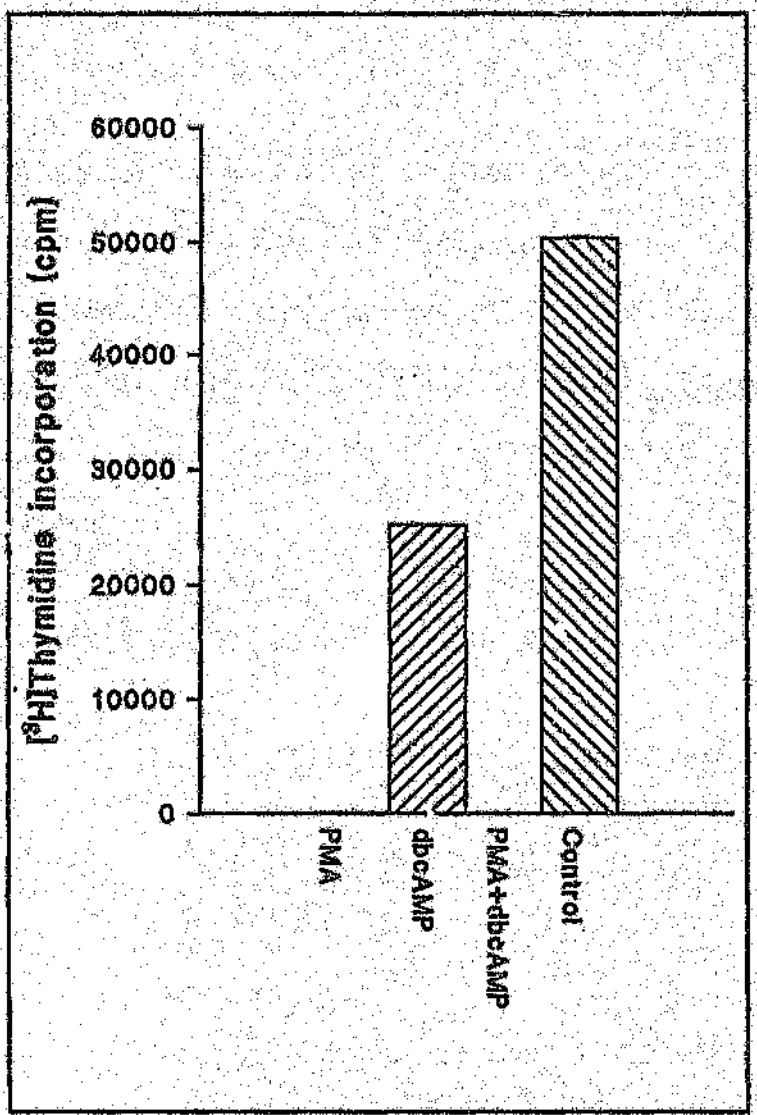


Figure 3.20.

THP-1 cell proliferation as estimated by

[^{3}H]thymidine incorporation

THP-1 cells were exposed to PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP for four days. [3H]thymidine was added during the last 24 hours, after which radioactive incorporation was measured (n=2).

3.5. RNA INTEGRITY ANALYSIS

RNA was isolated from stimulated and unstimulated THP-1 and HL-60 cells using the method described by Chomczynski and Sacchi (1987) (refer to section 2.6.1). RNA was subsequently electrophoresed on agarose gels under denaturing conditions in the presence of ethidium bromide, and was found to be intact (see figure 3.21 for an example of an integrity gel of THP-1 RNA). The three visible bands represent the 28S, 18S, and 5S bands of ribosomal RNA, respectively, and serve as markers of RNA integrity.

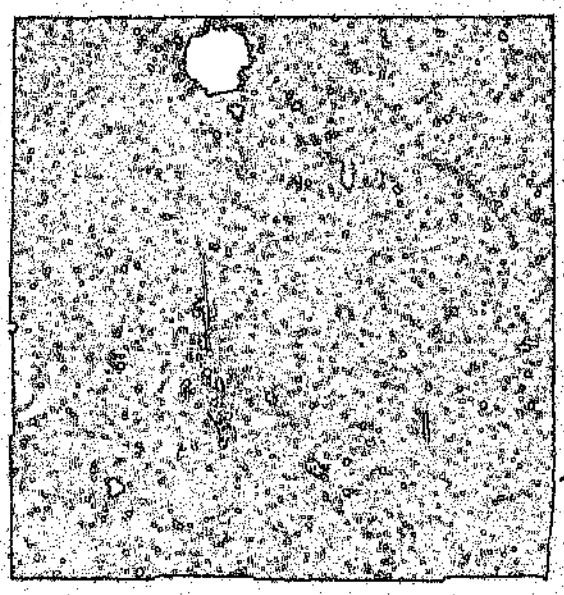


Figure 3.21

RNA integrity gel

Isolated THP-1 RNA was electrophoresed on a 1 % agarose gel in the presence of ethidium bromide. The three bands represent the 28S, 18S and 5S ribosomal RNA subunits, respectively. (Direction of electrophoresis is from top to bottom).

3.6. NORTHERN BLOT ANALYSIS OF IL-1ß mRNA EXPRESSION

IL-1ß expression is linked to increased differentiation of THP-1 (Cochran *et al*, 1989; Gaffney *et al*, 1989; Siljander *et al*, 1993) and HL-60 cells (Sun-sang and Walters, 1991).

THP-1 and HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively). RNA was isolated from these samples and checked for integrity (section 3.5) prior to Northern blotting analysis (refer to section 2.7 for methods).

Neither unstimulated nor stimulated HL-60 cells produced detectable levels of IL-1ß mRNA. THP-1 cells did, however, produce IL-1ß mRNA when stimulated with PMA alone and in combination with dbcAMP (see figure 3.22 for an example of an autoradiograph). This experiment was performed thrice, and similar results were obtained.

The blots were subsequently stripped of the IL-1ß probe and were reprobed for ß-actin mRNA. ß-actin is considered to be constitutively expressed in cells (Delfau *et al*, 1990; Stark *et al*, 1993) and may thus be used as an internal standard to correct for unequal RNA loading. Unfortunately, the signals obtained were consistently very low, even for those samples that showed high IL-1ß mRNA expression (results not shown). This might have been due to interference with or loss of RNA during stripping of the blots.

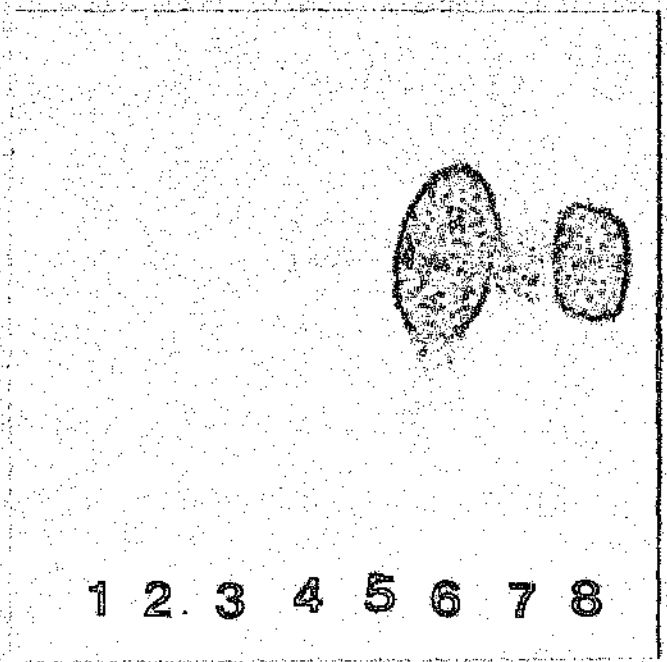


Figure 3.22.

IL-1ß mRNA expression

RNA was isolated, transblotted after electrophoresis, and incubated with a ^{32}P-labelled IL-1ß probe. Blots were subsequently exposed to X-ray film for 1-2 weeks, and autoradiographs were obtained. The representative autoradiograph pictured here shows IL-1ß mRNA expression in HL-60 cells (lanes 1-4) and THP-1 cells (lanes 5-8) that were stimulated for 24 hours with PMA (16 nM) (lanes 2 and 6), dbcAMP (50 µM) (lanes 3 and 7) and PMA plus dbcAMP (lanes 4 and 8).

3.7. RIA ANALYSIS OF IL-1ß PROTEIN EXPRESSION

THP-1 and HL-60 cells were incubated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively). Cell supernatants were subsequently assayed for secreted IL-1ß protein (n=3) (refer to section 2.8 for methodology).

No IL-1ß protein was detected in those supernatants that were obtained from unstimulated and stimulated HL-60 cells. Unstimulated and dbcAMP-stimulated THP-1 cells also did not secrete detectable amounts of IL-1ß protein. PMA-stimulated and PMA plus dbcAMP-stimulated THP-1 cells did, however, secrete IL-1ß protein at detectable amounts (29.2 ± 2.12 fmol/10^6 cells and 31.7 ± 1.54 fmol/10^6 cells, respectively; n=3) (figure 3.23).

(Results given as mean ± standard error of the mean.)

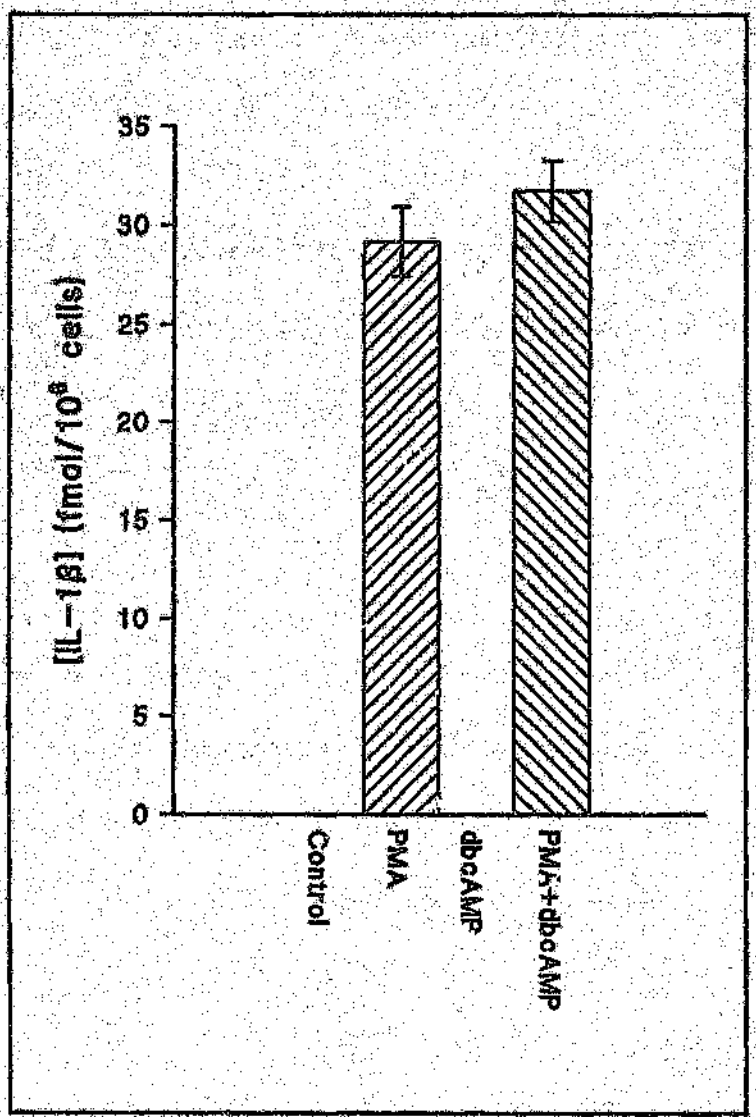


Figure 3.23

IL-1B PROTEIN EXPRESSION

THP-1 and HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP. Cell supernatants were subsequently isolated and assayed for IL-1B protein secretion using RIA.

3.8. RT-PCR ANALYSIS OF *bCL-2* mRNA EXPRESSION

The PCR technique was used to semi-quantitate *bcl-2* mRNA in stimulated and unstimulated THP-1 and HL-60 cells.

THP-1 and HL-60 cells were stimulated with PMA (16nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively). RNA was isolated and reverse transcribed into cDNA. This cDNA then served as a template for specific primer-directed PCR amplification. Two sets of primers were added: one specific for a section of the *bcl-2* cDNA, and the other for a section of the ß-actin cDNA (refer to section 2.9 for methodology). Experiments were performed thrice, and the PCR products were electrophoresed on 2 % agarose gels in the presence of ethidium bromide (figure 3.24). The bands were of the predicted sizes (195 bp and 415 bp for *bcl-2* and ß-actin, respectively), and were visualized and photographed under UV light. The photographs were subsequently scanned using a densitometer scanner. Tube-to-tube differences in PCR efficiency were corrected for by assuming that ß-actin mRNA expression remained constant within one type of cell line (results are depicted in tables 3.1 and 3.2).

bcl-2 mRNA was detected in all samples tested (figure 3.24).

In THP-1 cells (figure 3.24, first four lanes in each photograph, and table 3.1), PMA-stimulation caused a decrease in *bcl-2* mRNA expression. While

bcl-2 mRNA decreased to 80 % of the control value in one of the experiments, it decreased to less than 5 % of the control value in the other. The third experiment could not be evaluated, as no ß-actin or *bcl-2* mRNA was detected. dcAMP stimulation caused no changes in *bcl-2* mRNA expression in two of the studies, while it caused a decrease to 50 % of control values in the third study. Co-stimulation with PMA and dbcAMP caused decreases to 50 % of the control value in one experiment and to less than 5 % of the control value in the other two samples.

In HL-60 cells (figure 3.24, last four lanes in each photograph, and table 3.2), PMA-stimulation caused a decrease in *bcl-2* mRNA expression to between 10 % and 20 % of control values. dbcAMP-stimulation, while decreasing bcl-2 mRNA values to 50 % and 70 %, respectively, in two of the studies performed, did not change the expression relative to control values in the remaining study. Co-stimulation with PMA and dbcAMP caused decreases to between 5 % and 55 % of the control values.

bcl-2 mRNA expression relative to ß-actin expression in unstimulated HL-60 cells was between 25 % and 45 % higher than in unstimulated THP-1 cells. These values are, however, highly speculative, as they are based on the unsubstantiated assumption that ß-actin mRNA expression is similar in the two cell lines, and that the efficiency of the RT-PCR is not affected by the source of the mRNA.

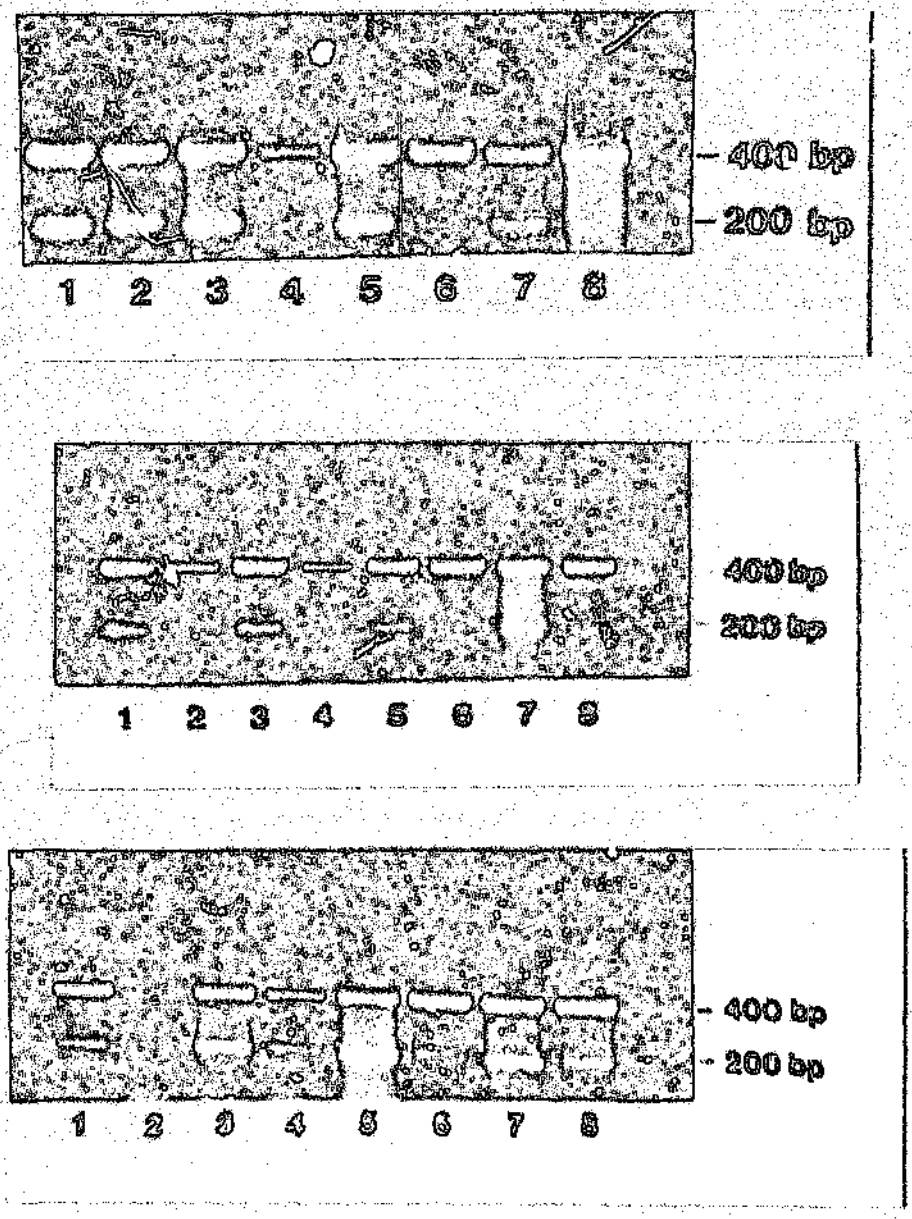


Figure 3.24.

***bcl-2* mRNA expression analysis using RT-PCR**

RNA was extracted from stimulated (24 hours) and unstimulated THP-1 and HL-60 cells. RT-PCR was subsequently performed. ß-actin mRNA was assumed to be expressed constitutively, and was thus used as as an internal standard to correct for sample-to-sample differences in PCR efficiency. PCR products were 195 bp and 415 bp in length for *bcl-2* and ß-actin, respectively, and were resolved on 2 % agarose gels in the presence of ethidium bromide together with a molecular mass marker. Bands were visualized under UV light. RT-PCR was performed on RNA from THP-1 cells (lanes 1-4) and HL-60 cells (lanes 5-8) stimulated with PMA (16 nM) (lanes 2 and 6), dbcAMP (50 µM) (lanes 3 and 7), and PMA plus dbcAMP (lanes 4 and 8). RT-PCR was also performed on RNA from unstimulated control cells (lanes 1 and 5).

CONTROL	PMA	dbcAMP	PMA + dbcAMP
100 %	80 %	50 %	>5 %
100 %	>5 %	100 %	>5 %
100 %	- (*)	100 %	50 %

Table 3.1.

Densitometer scanning results of bcl-2 mRNA expression

in THP-1 cells

Photographs obtained from gel electrophoresis of THP-1 cell RT-PCR products (see figure 3.24) were scanned using a densitometer scanner. Results are depicted for each experiment relative to the control values (corrected relative to *ß*-actin mRNA).
* indicates, that no results were obtained either for actin or for *bcl-2*

CONTROL	PMA	dbcAMP	PMA + dbcAMP
100 %	10 %	50 %	5 %
100 %	20 %	100 %	55 %
100 %	20 %	70 %	50 %

Table 3.2.

Densitometer scanning results of bcl-2 mRNA expression

in HL-60 cells

Photographs obtained from gel electrophoresis of HL-60 cell RT-PCR products (see figure 3.24) were scanned using a densitometer scanner. Results are depicted for each experiment relative to the control values (corrected relative to ß-actin mRNA).

3.9. WESTERN BLOT ANALYSIS OF BCL-2 PROTEIN EXPRESSION

THP-1 and HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively). Cellular proteins were extracted and were quantitated according to the method of Bradford (1976) (refer to section 2.10.2). Proteins were subequently electrophoresed on polyacrylamide gels together with a molecular mass marker, after which Western blotting and protein immunodetection were performed (see sections 2.10.3 to 2.10.6 for methodologies).

Western blots were initially stained with India ink. This stained the protein bands and enabled an estimation of protein integrity and adequate protein transfer (figure 3.25).

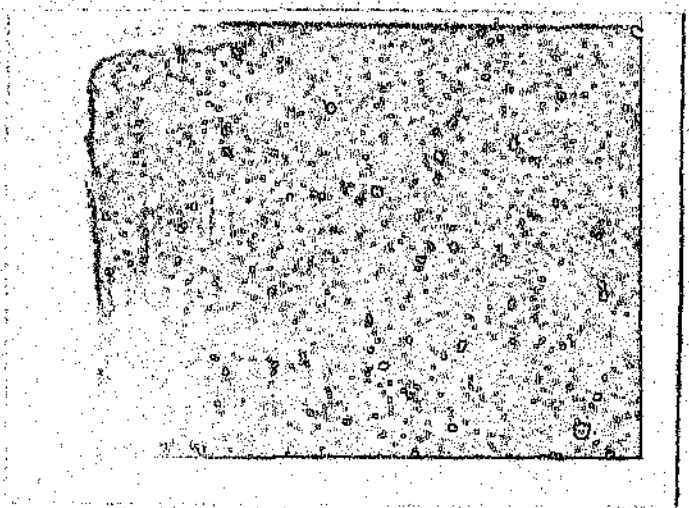


Figure 3.25

India ink-stained Western blot

A Western blot was stained with India ink to assess protein integrity and confirm adequate protein transfer (non-equal amounts of protein loaded).

BCL-2 antibody binding was found to be specific upon immunodetection, yielding one discrete band of approximately 26 kD per protein sample with Western blot analysis both for THP-1 cells (figure 3.26) and HL-60 cells (results not shown).

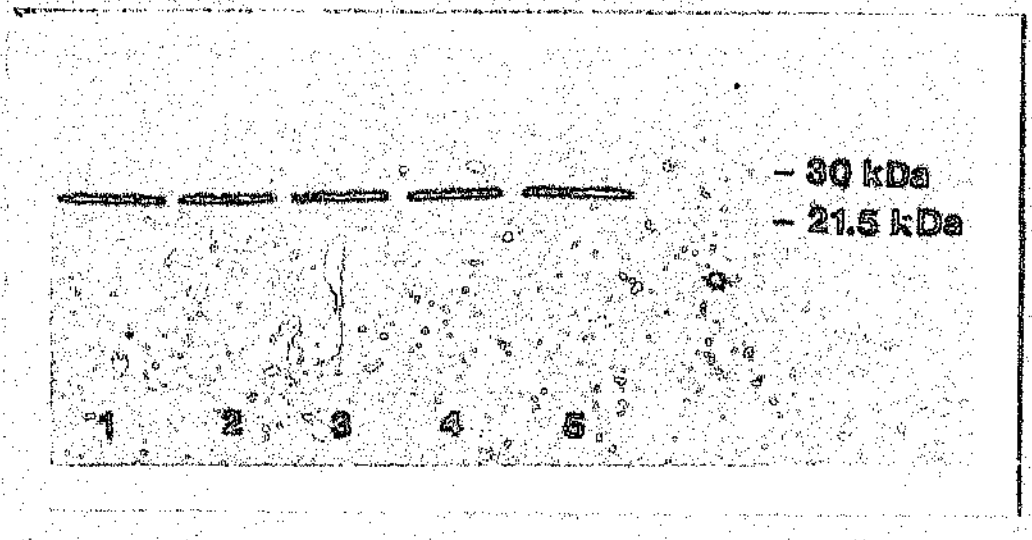


Figure 3.26.

BCL-2 antibody specificity in THP-1 cells

Protein samples from THP-1 cells were electrophoresed on a denaturing SDS/polyacrylamide gel system under discontinuous conditions using a 4 % stacking gel and a 15 % resolving gel. 12µ µg of totel protein per sample were loaded and run together with a molecular mass marker. Proteins were subsequently transblotted onto a nitrocellulose membrane and detected using an anti-BCL-2 monoclonal antibody (DAKO) in conjunction with the biotin-streptavidin system.

3.10. SLOTBLOT ANALYSIS OF BCL-2 PROTEIN EXPRESSION

Slot blotting of protein samples was performed once the specificity of the antibody had been determined. This is a simpler and more direct method of protein transblotting, where samples are applied to wells of a slot blotting system, and are absorbed onto a nitrocellulose membrane using vacuum suction (refer to section 2.10.7). Thus, the protein electrophoresis step is eliminated. This decreases sample handling, and may thus decrease the possibility of protein loss or degradation.

Serial dilutions were performed on protein samples isolated from 7 and HL-60 cells (figure 3.27). Antibody binding was linear between 0 μg and 20 μg of total protein for HL-60 cells (as determined by densitometry scanning; the whole area of the band was scanned), and 15 μg of protein per sample were thus used in subsequent experiments.

BCL-2 protein was detected in unstimulated and stimulated HL-60 and THP-1 cells (figure 3.28). Expression levels were, however, approximately 5 to 15 times lower in THP-1 cells than in HL-60 cells (figure 3.27 and 3.28).

The low band intensities for samples from THP-1 cells led to difficulties in optical density scanning procedures, thus making qualitative assessments unreliable (see figure 3.28, lanes 18-21).

In HL-60 cells, exposure to PMA for 24 hours led to a reproducible decrease of about 20 % in BCL-2 expression (78.33 ± 1.5 % of control value) (n=3) (figure 3.29). dbcAMP-stimulation resulted in BCL-2 protein amounts similar to those in unstimulated control cells (99.0 ± 3.5 % of control value) (n=3) (figure 3.29). Co-stimulation with PMA and dbcAMP led to amounts of BCL-2 expression that were lower than control amounts but higher than those after PMA-stimulation (92.5 % average, actual values were 90.38 % and 97.62 % of control) (n=2) (figure 3.29).

(Results for n=3 are presented as mean ± standard error of the mean.)



Figure 3.27.

BCL-2 protein detection in serial dilutions

of THP-1 and HL-60 protein samples

Serial dilutions of proteins isolated from unstimulated THP-1 cells (top) and HL-60 cells (bottom) were performed. Suspensions containing 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg, 35 µg, and 40 µg of protein (from left to right) were slot blotted and analysed.

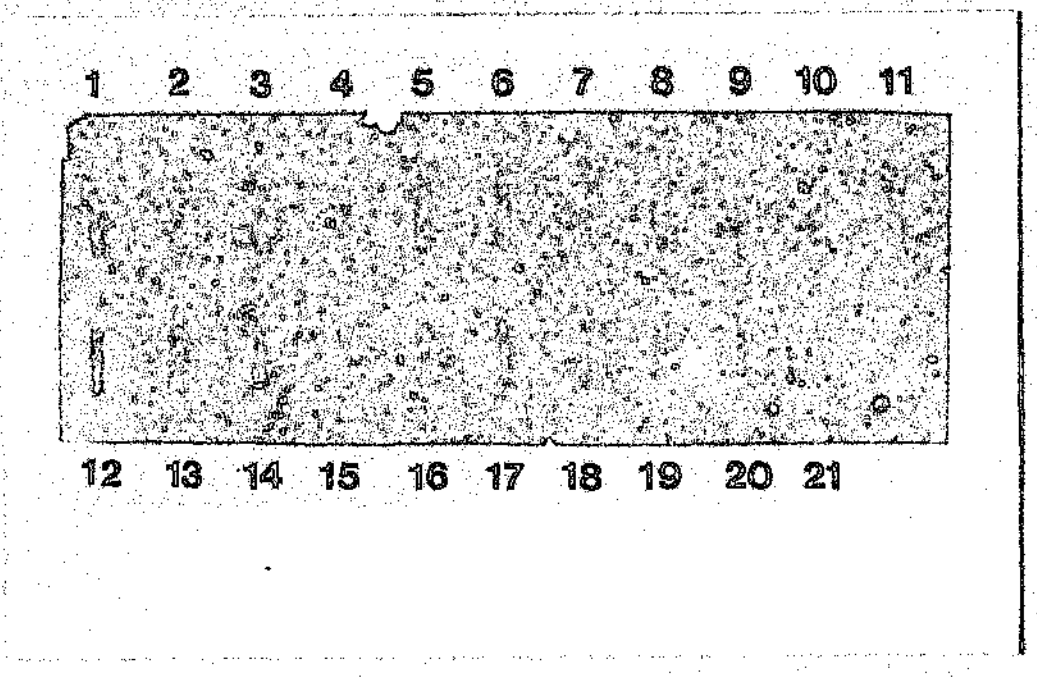


Figure 3.23.

BCL-2 protein detection in unstimulated and stimulated

THP-1 and HL-60 cells

THP-1 and HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM) and PMA plus dbcAMP (16 nM and 50 μM, respectively). Protein was isolated, slot blotted, and immunostained using a monoclonal anti-BCL-2 antibody in conjunction with the biotin-streptavidin system. A representative blot is shown. Protein from three separate sets of experiments were slot blotted here (represented by lanes
1-11 and 12-17 for two separate HL-60 cell experiments, and lanes 18-21 for a THP-1 cell experiment). Lanes 1-3, 12-13, and 18 represent samples from unstimulated cells, lanes 4, 5, 15, and 19 from PMA-stimulated cells, lanes 8, 9, 16, and 20 from dbcAMP-stimulated cells, and lanes 10, 11, 17, and 21 from PMA + dbcAMP-stimulated cells (lanes 6 and 7 represent samples from cells stimulated with a higher concentration of PMA (32 nM).

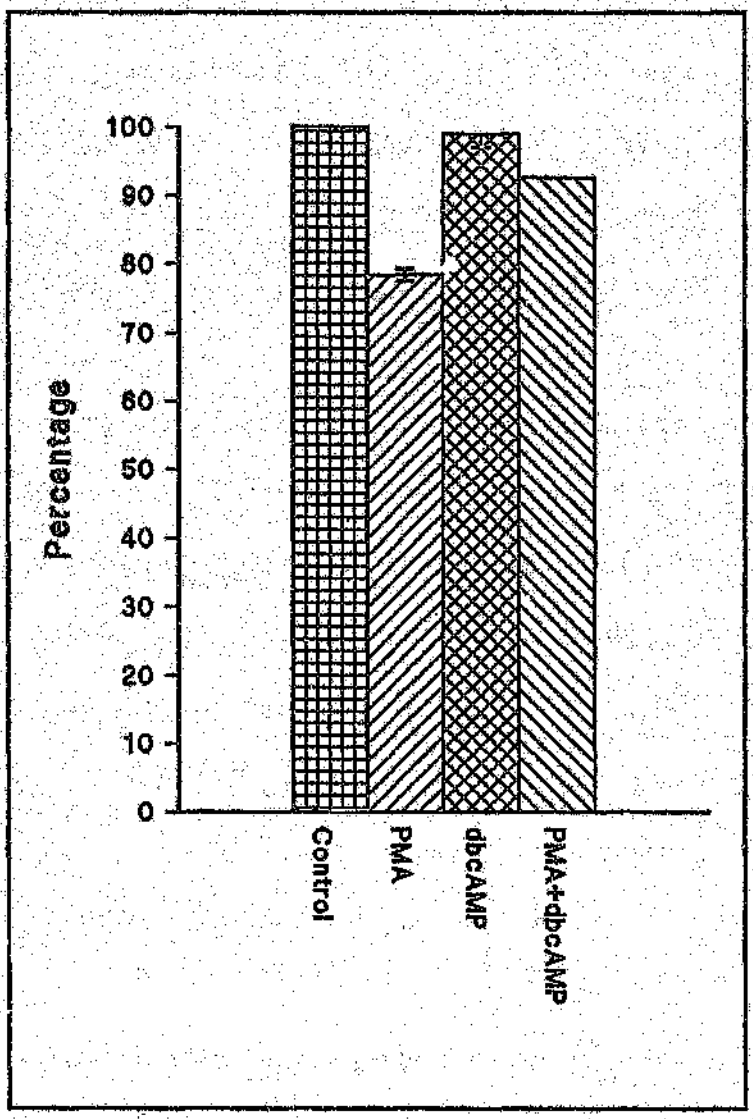


Figure 3.29.

Bar graph depicting changes in BCL-2 expression in unstimulated and stimulated HL-60 cells

HL-60 cells were stimulated for 24 hours with PMA (16 nM), dbcAMP (50 μM), and PMA plus dbcAMP (16 nM and 50 μM, respectively). Protein was isolated from stimulated cells and from unstimulated control cells, and was slot blotted, immunostained and densitometry- scanned. Experiments were repeated thrice, and results are depicted here in the form of a bar graph.

3.11. CELLULAR LOCALIZATION OF BCL-2

THP-1 and HL-60 cells were stimulated with PMA (16 nM) for 24 hours. BCL-2 protein immunocytochemical analyses were subsequently performed on slide-mounted unstimulated and PMA-stimulated cells (refer to section 2.11 for methods).

Positive staining for BCL-2 was found in unstimulated and stimulated THP-1 cells (figures 3.31 and 3.32, respectively) and HL-60 cells (figures 3.34 and 3.35, respectively). However, while positive staining was prominent in cell smears from HL-60 cells, it was, in general, more faint and obscure in THP-1 cell smears. Negative controls were performed without addition of the primary anti-BCL-2 antibody. No staining was observed in these THP-1 and HL-60 cell smears (figures 3.30 and 3.33, respectively), thus showing that secondary antibody-binding was specific for the primary anti-BCL-2 antibody.

Staining was observed primarily in the cytoplasm of unstimulated and PMA-stimulated THP-1 and HL-60 cells. Some staining was seen over the nuclei (see, for example, figure 3.31). It was, however, not clear whether this was true nuclear staining or staining of the cytoplasm that lay over the nucleus. No differences in the pattern of staining were detected between unstimulated and stimulated cells, and between THP-1 and HL-60 cells. Cytoplasmic staining was granular rather than uniform, suggesting some form of compartmentalization or organelle-association of BCL-2.

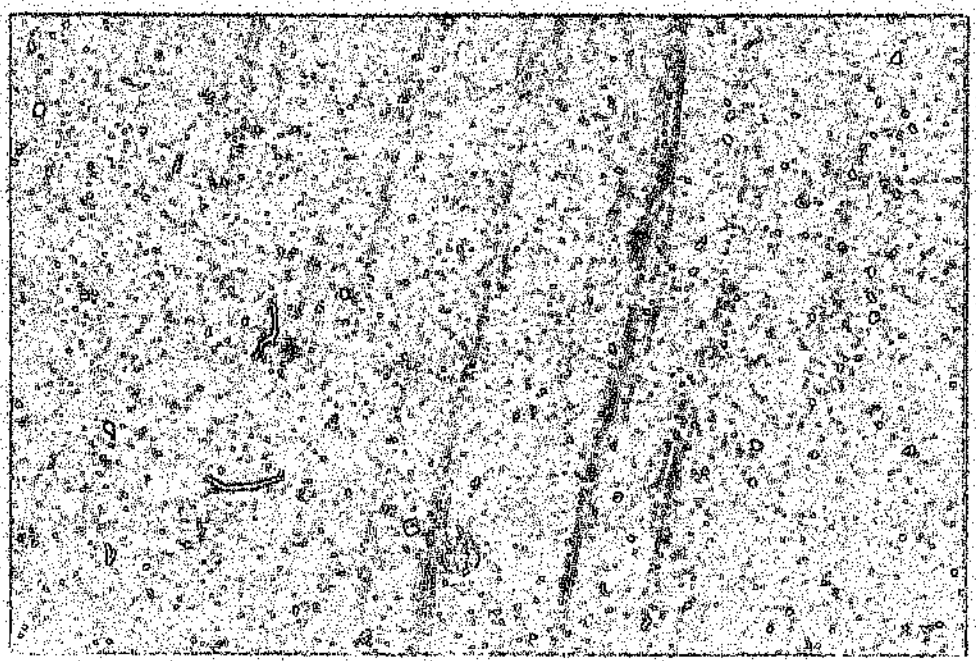


Figure 3.30.

Negative control for BCL-2 antibody staining

in THP-1 cells

Unstimulated THP-1 cells were fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). The primary anti-BCL-2 antibody was, however, not added. Slides were photographed at 600 X magnification. No staining was observed.

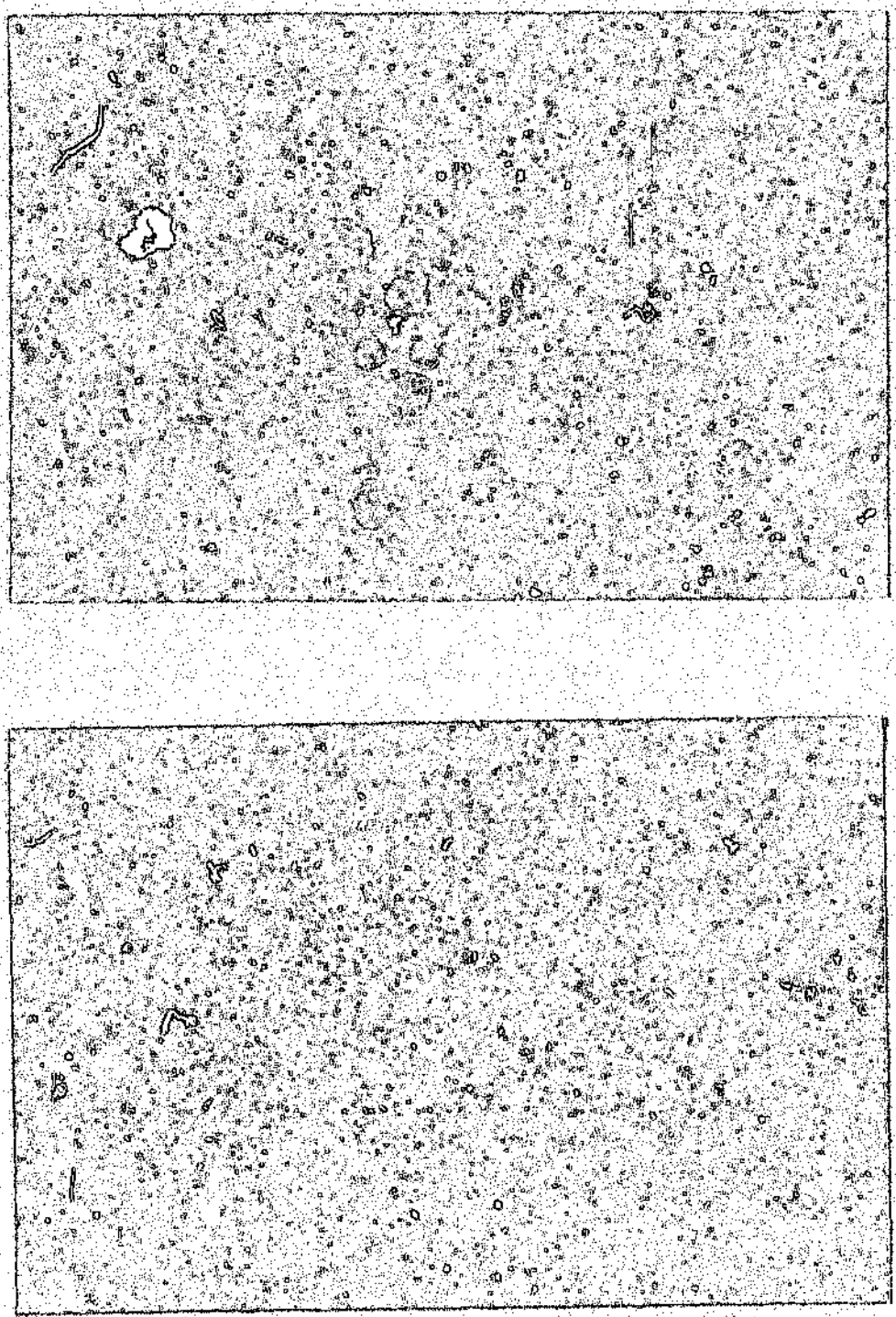


Figure 3.31.

BCL-2 antibody staining in unstimulated THP-1 cells

Unstimulated THP-1 cells were fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). Slides were subsequently photographed at 500 X magnification (top) and 1 500 X magnification (bottom).

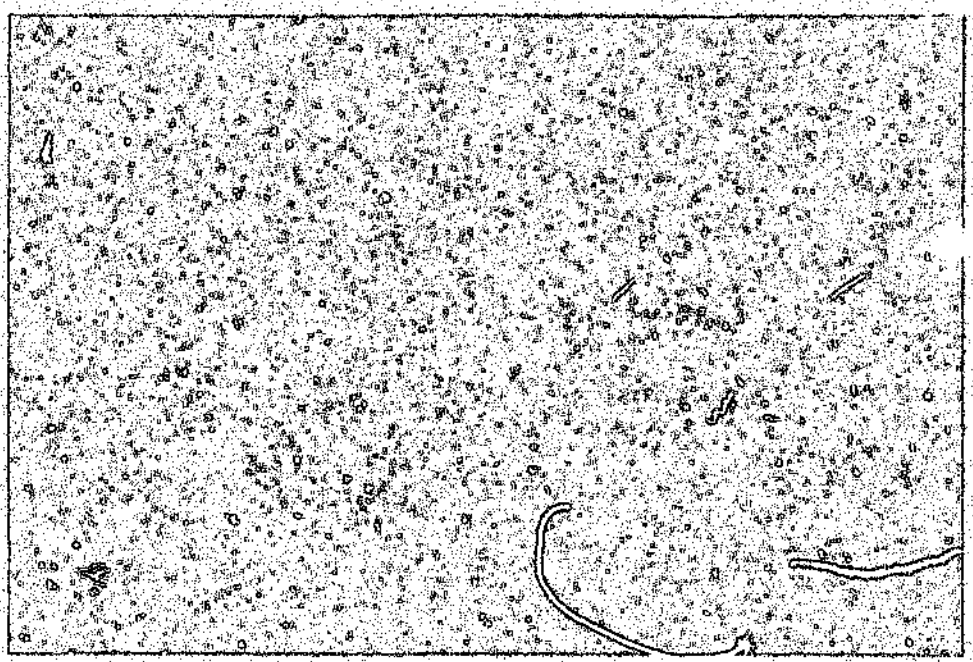


Figure 3.32.

BCL-2 antibody staining in PMA-stimulated THP-1 cells

THP-1 cells were stimulated for 24 hours with PMA (16 nM). They were subsequently fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). Slides were photographed at 1 500 X magnification.

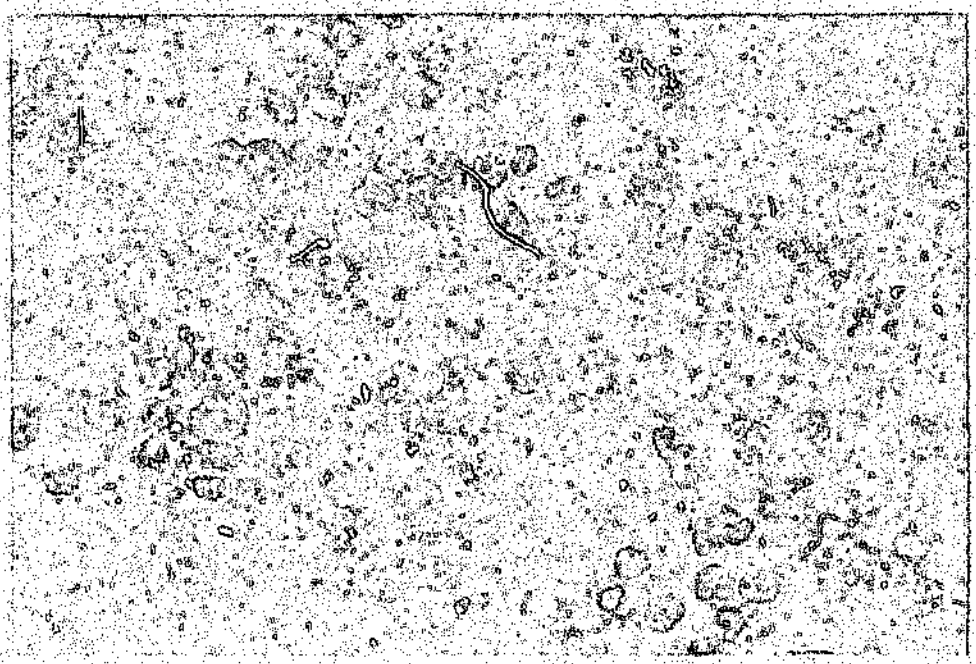


Figure 3.33.

Negative control for BCL-2 antibody staining

in HL-60 cells

Unstimulated HL-60 cells were fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). The primary anti-BCL-2 antibody was, however, not added. Slides were photographed at 600 X magnification. No staining was observed.

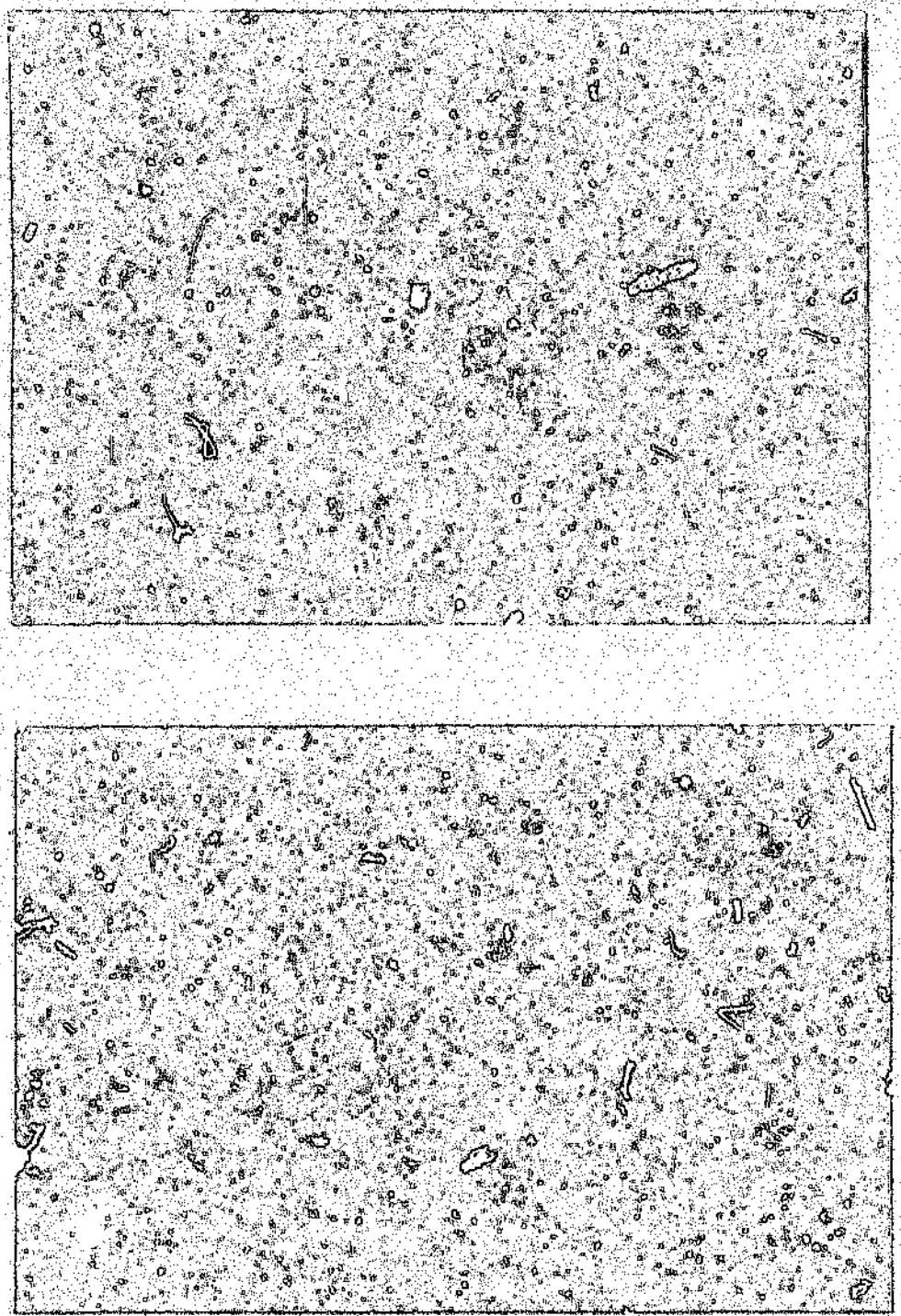


Figure 3.34.

BCL-2 antibody staining in unstimulated HL-60 cells

Unstimulated HL-60 cells were fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). Slides were subsequently photographed at 600 X magnification (top) and 1 500 X magnification (bottom).

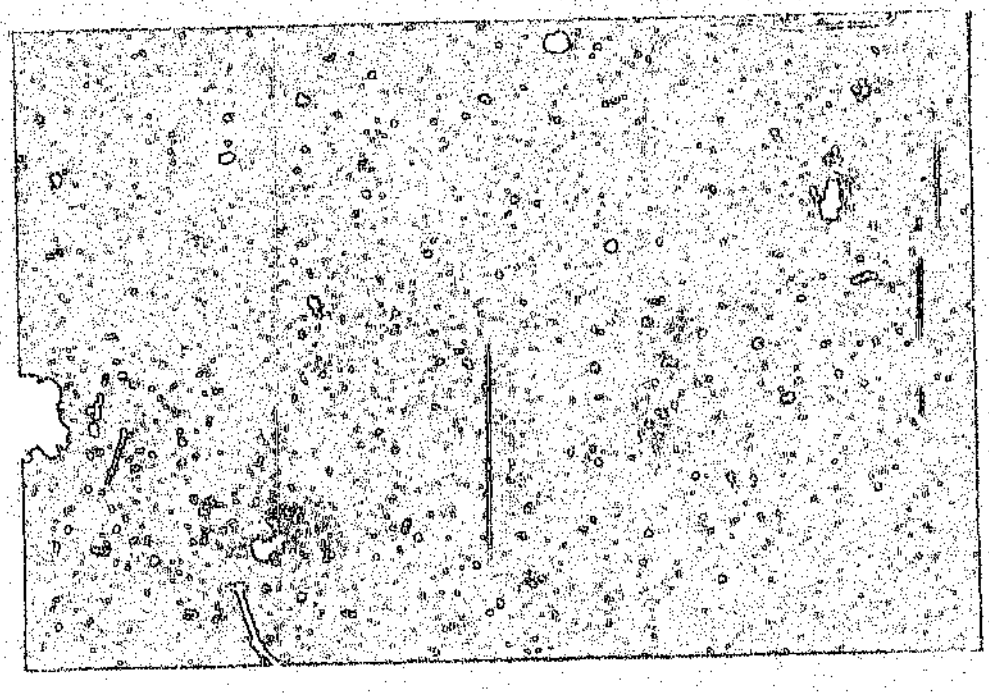


Figure 3.35.

BCL-2 antibody staining in PMA-stimulated HL-60 cells

HL-60 cells were stimulated for 24 hours with PMA (16 nM). They were subsequently fixed onto glass slides, and underwent immunocytochemical BCL-2 detection procedures (refer to section 2.11 for methods). Slides were photographed at 1 000 X magnification.

SECTION 4

DISCUSSION

The effects of PMA- and dbcAMP-stimulation on proliferation, differentiation, and cell death were studied in relation to *bcl-2* mRNA and BCL-2 protein expression in two human myeloid leukaemia cell lines. The human myeloid THP-1 and HL-60 cell lines were both derived from leukaemia patients. However, while the THP-1 cells were cultured from a patient with acute monocytic leukaemia (Tsuchiya *et al*, 1980), HL-60 cells were derived from a patient with acute promyelocytic leukaemia (Gallagher *et al*, 1979). HL-60 cells are, therefore, less differentiated than THP-1 cells (Koeffler, 1983; Auwerx, 1991).

Both cell types represent precursor cells of mature monocytes. Haematopoietic cells are derived from pluripotent stem cells. These stem cells produce progeny that differentiate and become committed to a single haematopoietic lineage. The more differentiated THP-1 cells are committed to the monocyte/macrophage lineage (Auwerx, 1991), while the less differentiated HL-60 cells may differentiate toward the monocyte/macrophage or the granulocyte lineage (Collins, 1987).

Differentiation of haematopoietic cells is linked to a decrease in their rate of proliferation (Rothstein, 1993). BCL-2 protein is expressed in myeloid precursor cells, but not in mature monocytes (Delia *et al*, 1992). Furthermore, BCL-2 expression is inversely related to differentiation in normal myeloid cells (Delia *et al*, 1992). While detectable amounts of BCL-2 are present in HL-60 cells (Delia *et al*, 1992; Campos *et al*, 1993), expression decreases when these cells are induced to differentiate. Mature monocytes do not express BCL-2 (Delia *et al*, 1992). It would, therefore, be expected that HL-60 cells express higher amounts of BCL-2 than do THP-1 cells. Furthermore, BCL-2 expression should decrease in both cell lines upon induction of differentiation.

High amounts of BCL-2 protein may protect cells from PCD. Mature monocytes and tissue macrophages have a high turnover rate , surviving for a few days, and then being replaced by newly differentiated precursor cells (Rothstein, 1993). A decrease in BCL-2 expression upon differentiation may allow for this increased susceptibility to cell death, thus keeping cell numbers relatively constant. Indeed, high expression of BCL-2 has been linked to the development of certain leukaemias (Delia *et al*, 1992).

BCL-2 is a member of a group of proteins that interact to regulate PCD. BCL-2 interacts with the R-RAS GTPase (Fernandez-Sarabia and Bischoff, 1993). GTPases are located at the cytoplasmic membrane, where they translocate signals from membrane receptors to adenylate kinases. R-RAS

thus represents an upstream component of a signal transduction pathway. BCL-2 may exert its effect by binding to R-RAS and inhibiting signal transduction. However, different signalling pathways have been linked with PCD, and BCL-2 is capable of inhibiting more than one of them. It is, thus, probable that BCL-2 acts primarily where these signalling pathways converge, which would be downstream of R-RAS.

BCL-2 also interacts with a homologous protein, called BAX (Zoltan *et al*, 1993; Yin *et al*, 1994). BCL-2 and BAX may form homodimers, or they may dimerize with each other. BCL-2 homodimers are unable to protect cells from death, and it is probable that BCL-2 inhibits PCD by dimerizing with BAX. BAX homodimers promote cell death, and BCL-2 may inhibit this process by decreasing the concentration of unbound BAX available for homodimerization. BCL-2 concentrations decrease when myeloid cells differentiate. It is not yet clear whether BAX levels decrease concurrently. Differentiated myeloid cells are more susceptible to PCD than their less differentiated counterparts (Rothstein, 1993). A decrease in BCL-2 concentration upon differentiation, that is not parallelled by a decrease in BAX, would favour the formation of BAX homodimers and, thus, promote PCD.

BCL-2 interacts with p53 and inhibits p53-induced PCD. p53 is a transcriptional regulator that can cause growth arrest and apoptosis (Clarke

et al, 1993; Lowe *et al*, 1993). p53 expression increases during DNA damage and leads to cell cycle arrest. If the damage is not repaired by the cell, PCD occurs. Interestingly, the *bcl-2* gene contains a negative response element for p53 (Miyashita *et al*, 1994). Overexpression of p53 during DNA damage might, therefore, lead to a concurrent decrease in BCL-2. This decreased BCL-2 expression would, in turn, increase the probability of cell death. BCL-2 can inhibit p53-induced programmed cell death (Ryan *et al*, 1994), indicating that it acts downstream of p53. p53 expression is cell cycle-related, and does not change notably upon differentiation.

BCL-2 may cooperate with c-MYC in tumourigenesis. Deregulated c-MYC expression can lead either to uncontrolled proliferation or to PCD (Evan *et al*, 1992). In the presence of BCL-2, c-MYC-induced PCD is inhibited, and proliferation will occur. Thus, BCL-2 suppresses the cell death signal of deregulated c-MYC expression without affecting c-MYC's ability to promote cell proliferation (Bissonnette *et al*, 1992; Fanidi *et al*, 1992). These two proteins also synergize to overcome the effects of high p53 expression: while BCL-2 inhibits the PCD induced by p53, c-MYC overcomes p53-induced cell cycle arrest. c-MYC is usually down-regulated upon differentiation.

Two BCL-2-related proteins, BCL-X_L and BCL-X_S, also play a role within the cell death pathways (Boise *et al*, 1993). While the action of BCL-X_L is similar to that of BCL-2, BCL-X_S induces programmed cell death. BCL-X_S is

capable of overriding the inhibitory effect of BCL-2 on cell death.

PCD is an important defence mechanism used by the cell against viral infection, DNA damage, and tumourigenesis (Fisher, 1994). Some redundancy of death promoter and inhibitor proteins may ensure that a series of genomic mutations is needed before this defence system becomes ineffective.

HL-60 cells are unusual in that they aberrantly express both c-MYC and p53 (Collins, 1987). The *c-myc* gene is amplified 15- to 30-fold in HL-60 cells which leads to high amounts of steady state *c-myc* mRNA. p53 expression, on the other hand, is absent in HL-60 cells due to an extensive deletion of its gene locus (Collins, 1987).

This increase in expression of a protein which cooperates with BCL-2, together with the lack of expression of a protein that antagonises BCL-2 makes the HL-60 cell line an interesting system in which to study cell death and differentiation.

Gene expression in the more differentiated THP-1 cell line is thought to follow more closely the gene expression patterns of normal monocytes (Auwerx, 1991). THP-1 cells, therefore, provide a good comparative system, both for HL-60 cells and for normal monocytes, which do not express BCL-2, but have relatively normal expression of c-MYC and p53.

4.1. CELL MORPHOLOGY

4.1.a) THP-1 cells

THP-1 cells have been previously described as roughly polygonal in shape with a 12-15 μm diameter, and displaying irregular or slightly indented nuclei with well-defined nucleoli, and cytoplasmic vacuoles (Tsuchiya *et al*, 1980). The THP-1 cells used in this study fitted this description in general. They were, however, larger in size (about 20 μm in diameter), and had more irregular cell shapes. These differences are probably due to the different techniques used. The cytospins used in this study are more likely to flatten out cells than are the cell smears used by Tsuchiya *et al* (1980). When comparing the irregular THP-1 cell morphologies subsequent to cytospins (for example, figure 3.1) to the almost round cell shapes obtained from cell smears (for example, figure 3.30), this becomes apparent. Furthermore, cell diameters calculated from these cell smears were smaller (15 μm to 20 μm) than those calculated for cytospins.

Upon stimulation with PMA, the THP-1 cell nuclei became more irregular in shape. This change of shape of the THP-1 cell nucleus upon stimulation with PMA has also been observed by other researchers (Tsuchiya *et al*,1980), and probably accompanies the differentiation of THP-1 cells to a macrophage-like state (Tsuchiya *et al*, 1982; Auwerx, 1991).

Exposure to PMA also caused an increase in adherence of THP-1 cells. While approximately 50 % of unstimulated or dbcAMP-stimulated THP-1 cells adhered to plastic petri dishes, more than 95 % of cells were adherent 24 hours after addition of PMA or PMA plus dbcAMP. Although an increase in adherence alone should not serve as a criterion of differentiation (Harris and Ralph, 1985), it is likely to signify such in combination with the observed changes in cell morphology. Moreover, stimulation with PMA led to cytostasis (see section 3.4), an event usually observed upon THP-1 cell differentiation (Auwerx, 1991). Thus, exposure to PMA for 24 hours initiated differentiation to a macrophage-like state in THP-1 cells.

Stimulation with dbcAMP also caused more irregular shapes of cell nuclei in THP-1 cells. This change was, however, not accompanied by an increase in cell adherence (data not presented). Moreover, while cell proliferation did decrease by approximately 50 % (see section 3.4), this did not resemble the cytostasis observed upon PMA stimulation and is, thus, unlikely to signify terminal cell differentiation. Furthermore, while PMA-stimulated THP-1 cells portrayed the indented, horseshoe-shaped nuclei typical of macrophages, the nuclei of dbcAMP-stimulated cells were far more random and eccentric in shape. The change in nuclear morphology upon dbcAMP-stimulation may be caused by a change in nuclear activity or function that is not related, or only partially related, to cellular differentiation. This putative change in activity may also be observed in the cytoplasm of dbcAMP-stimulated cells, where

vacuoles are larger and more elongated than in unstimulated or PMA-stimulated cells. Thus, while exposure to dbcAMP caused changes in THP-1 cell morphology, these changes are unlikely to be linked to terminal cellular differentiation. More likely, dbcAMP caused a decrease in proliferation independently of promoting differentiation.

Stimulation with puromycin led to apoptosis-like features in some THP-1 cells. Apoptosis was observed in very few cells only. This rare occurrence of apoptosis was confirmed upon DNA electrophoresis, where no chromosomal fragmentation was detected on agarose gels (data not presented). Thus, inhibition of protein synthesis could cause the apoptotic morphology of PCD in some THP-1 cells. Most cells, however, seemed resistant to the effects of puromycin, at least at the concentrations and exposure-times used in this study.

The role that protein synthesis plays in the initiation of PCD is controversial. Thus, while inhibition of protein synthesis may lead to apoptosis in some cell types, it fails to do so in others, and may even protect some cells from it (Vaux, 1993). This apparent paradox could be explained by a model in which all cells have the potential to undergo PCD. However, while some cells already have the macromolecular components of the cell death machinery present in the cell in a suppressed state, others still need to synthesize them. It may be assumed that the components of a cell death

pathway are already in place and are actively suppressed in those cells where protein synthesis inhibition leads to death. In such cell types, continuous synthesis of death-inhibitory proteins is required for the cells to survive. In contrast, components of a cell death pathway may be absent in other types of cells. These cells are then unable to initiate PCD once the production of cell death proteins is inhibited.

The interaction of BCL-2 and BAX fits into this model. BCL-2 heterodimerises with BAX, and the ratio of the two proteins to one another determines survival or death following a cell death stimulus (Oltvai *et al*, 1993). When BCL-2 is present in excess, BCL-2 homodimerization increases and cell death decreases. Similarly, when BAX prevails, both BAX homodimerization and the occurrence of cell death increase (Wyllie, 1994). If a cell was in the process of synthesizing BAX, inhibition of protein synthesis could protect from the potentially lethal decrease in the BCL-2/BAX ratio. Conversely, if a cell was in the process of synthesizing BCL-2, inhibition of protein synthesis would cause this potentially lethal decrease in the BCL-2/BAX ratio. This model does, obviously, depend on the half lives of BCL-2 and BAX.

There are, of course, other proteins which could disturb the stoichiometry of cell survival and cell death. Some of these have been identified and include BCL-X_S and BCL-X_L, c-MYC, and p53. When protein synthesis is inhibited

in cells that are expressing these proteins, the critical stoichiometry that favours either cell survival or death might change. Thus, inhibiting protein synthesis may cause a cascade of events, with the eventual outcome depending on the proteins that are present in the cell and those that the cell was in the process of synthesizing at the time of synthesis-inhibition.

No apoptotic morphology was observed in unstimulated THP-1 cells, nor in those stimulated with PMA, dbcAMP, or PMA plus dbcAMP. Similarly, no apoptosis was observed in cells stimulated with a low dose of puromycin (0.5 μg/ml). In THP-1 cells exposed to higher doses of puromycin (1 μg/ml) morphological features of PCD became apparent. Thus, these cells probably contain components that actively suppress cell death. The depletion of these components after exposure to puromycin lead to visible signs of PCD in some cells. Cell death was, however, only observed in a few cells. This might have been due to the relatively short exposure time to puromycin. Davidoff and Mendelow (1993) have found that apoptosis in the less differentiated HL-60 cells is only initiated after 24 hours of exposure to puromycin (0.9 μM). The low level of apoptosis observed could also be due to the low concentration of puromycin used, as cell death in U937 cells is readily observed after 24 hours in the presence of a ten-fold higher concentration of puromycin (10 μg/ml) (Kochi and Collier, 1993). It should, however, be noted that some toxins cause cell death by apoptosis at lower, pharmacologically more relevant doses, and cell death via necrosis at higher concentrations (Eastman, 1993).

In summary, THP-1 cells are capable of undergoing apoptosis and therefore, probably, PCD. This event was, however, not observed in unstimulated THP-1 cells, nor in THP-1 cells stimulated with PMA, dbcAMP, or PMA plus dbcAMP. It could, of course, be that long-term differentiation would eventually lead to an increase in PCD. Interestingly, activators of PKC (such as PMA) have been reported both to protect from and to induce PCD, depending on the system into which they are introduced (Eastman, 1993). In THP-1 cells, apoptosis was not observed in response to PMA stimulation, indicating that PMA may induce differentiation rather than cell death in these cells. Of course, terminal differentiation of haematopoietic cells does lead to an increased susceptibility to PCD (Rothstein, 1993). PMA-stimulation may, therefore, have initiated differentiation in these cells without inducing terminal differentiation, at least not at 24 hours.

These cell morphology studies were performed once only, and would have to be repeated to confirm the results obtained.

4.1.b) HL-60 cells

HL-60 cells have been shown to be almost spherical in shape, with cytoplasmic vacuolation, large round nuclei and distinct nucleoli (Martin *et al*, 1994). The HL-60 cells used in this study fitted this description well. As with the THP-1 cells, the observed HL-60 cell diameter of approximately 20 μm was probably

exaggerated by the cytospin technique.

Upon PMA stimulation, HL-60 cell nuclei became more ovoid, indicating differentiation to a macrophage-like state (Collins, 1987). This differentiation was accompanied by a marked increase in cell adherence (data not presented). Furthermore, PMA-stimulation led to a decrease in proliferation, as indicated by a decrease in the number of cells entering the S-phase of the cell cycle (see section 3.3). Induction of differentiation of HL-60 cells to a macrophage-like state by phorbol esters has been previously described to be accompanied by an increase in adherence to plastic and a decrease in proliferation (Collins, 1987). No apoptotic cell morphologies were observed upon PMA stimulation. Similar results were obtained by Pongracz *et al* (1994), who have shown that PMA has little effect on apoptosis in HL-60 cells.

Stimulation of HL-60 cells with dbcAMP did not cause noticeable changes in cell morphology when compared to unstimulated control cells. Thus, any effect that exposure to dbcAMP might have had on these cells did not visibly influence their morphology, and did, therefore, probably not include induction of differentiation. dbcAMP also failed to induce HL-60 cell adherence to plastic (data not presented) or a change in the number of cells entering the S-phase of the cell cycle (see section 3.3). Thus, dbcAMP did not cause noticeable changes in cell morphology, proliferation, or differentiation in HL-60 cells.

In contrast to THP-1 cells, some HL-60 cells stimulated with a combination of PMA and dbcAMP displayed apoptotic characteristics. It seems as though the potential effect of PMA as an inducer of PCD (Eastman, 1993) was triggered or enhanced by the presence of dbcAMP. Interestingly, it has been shown recently that a cAMP analogue can induce PCD death in a rat leukaemia cell line (Duprez *et al*, 1993). Thus, the combination of two potential activators of PCD seemed able to induce apoptotic morphology in some HL-60 cells, but not in THP-1 cells.

HL-60 cells were also more susceptible than THP-1 cells to apoptosis and therefore, probably, to PCD induced by puromycin. While only the higher concentration of puromycin (1 μg/ml) caused apoptotic cell morphology in some THP-1 cells, both the lower (0.5 μg/ml) and higher concentrations were capable of inducing similar morphologies in a subpopulation of HL-60 cells.

These studies on cell morphology would have to be repeated to confirm the results obtained. However, if HL-60 cells do have an increased susceptibility to PCD, the question arises as to what causes this increase. HL-60 cells express unusually high levels of c-MYC (Collins, 1987), and an increased expression of c-MYC has been implicated as a potent inducer of PCD (Evan *et al*, 1992). Thus, the high level of c-MYC in HL-60 cells may make these cells more sensitive toward potential cell death-inducing agents.

On the other hand, HL-60 cells do not express p53 (Collins, 1987), and haematopoietic cells from mice deficient in p53 are more resistant to some forms of PCD (Lotem and Sachs, 1993). It could, thus, be assumed that HL-60 cells have a decreased susceptibility to cell death due to a lack in p53 protein. However, p53-independent pathways of PCD do exist. Cell death caused by PMA or DNA damaging agents has been shown to occur in the absence of functional p53, at least in proliferating lymphoid cells (Strasser *et al*, 1994). Apoptosis in HL-60 cells has been observed by Cotter and McCarthy (1994) in response to terminal differentiation. BCL-2 levels decreased steadily during induction of differentiation in those studies. However, cells only became more susceptible to apoptosis once terminal differentiation had been reached. Thus, susceptibility to p53-independent forms of PCD may be increased in HL-60 cells by high c-MYC expression.

In summary, HL-60 cells are able to undergo apoptosis and therefore, probably, PCD. Apoptosis was observed both in response to puromycin-stimulation and to PMA plus dbcAMP-stimulation. No apoptosis was observed in unstimulated cells, or in cells stimulated with PMA or dbcAMP alone. Thus, the cell death-inducing properties of PMA only became apparent once co-stimulation with dbcAMP occurred. This may point toward a synergistic effect of PMA and dbcAMP on PCD in HL-60 cells. Interestingly, evidence does exist of that increased intracellular levels of cAMP may sensitize cells to PCD (Kroemer and Martinez-A., 1994). HL-60 cells were more susceptible to

apoptosis than THP-1 cells. This might be caused by an increased c-MYC expression. It may, however, also be due to the increased susceptibility of certain cancer cells to apoptosis (Fisher, 1994).

4.2. CD33 SURFACE ANTIGEN EXPRESSION

CD33 is a cell surface antigen of unknown function (Horejsi, 1991), and is expressed on myeloid progenitor cells including myeloblasts, promyelocytes and myelocytes, promonocytes and monocytes (Simmons and Seed, 1988). It is absent from granulocytes and from pluripotent stem cells, and can thus be used as a differentiation marker to discriminate between different progenitor cells of the haematopoietic system (Simmons and Seed, 1988).

CD33 expression decreased in THP-1 cells upon stimulation with PMA, and PMA plus dbcAMP. dbcAMP-stimulation alone did not cause a similar decrease in CD33 expression. Furthermore, the decreases caused by PMA alone, and those caused by PMA plus dbcAMP were almost identical. Thus, the observed changes are most probably effected by PMA alone.

In HL-60 cells stimulated with PMA, and PMA plus dbcAMP, a very slight decrease in CD33 expression was observed. Again, no decrease was observed

upon dbcAMP stimulation alone, and the observed changes are probably due only to PMA. This corroborates results obtained showing that PMA affects differentiation.

CD33 antigen expression decreased upon PMA-stimulation and, therefore, upon differentiation, both in THP-1 cells and in HL-60 cells. It could, thus, be expected that the less differentiated HL-60 cells should express a higher level of CD33 than their more differentiated THP-1 cell counterparts. This was, however, not observed. Levels of CD33 expression seemed virtually identical in the two cell lines, with HL-60 cells, if anything, even showing a slight increase in expression when compared to THP-1 cells. This somewhat surprising result may be caused by or linked to one of the many genetic abnormalities found in HL-60 cells (Collins, 1987). It may, of course, also indicate that THP-1 cell dedifferentiation had occurred in culture. This is, however, unlikely, as unstimulated THP-1 cells were more adherent than unstimulated HL-60 cells (50 % versus 0 %, respectively). THP-1 cells also produced IL-1ß upon PMA-stimulation, whereas HL-60 cells did not. IL-1ß expression is linked to differentiation in myelocytic cells (Cochran *et al*, 1989). Moreover, HL-60 cells expressed higher levels of BCL-2 than did THP-1 cells, and BCL-2 expression is inversely related to myeloid cell differentiation (Delia *et al*, 1992).

The differences in CD33 expression were very slight, and increased

incubation times subsequent to addition of differentiation-inducing agents may be needed for more complete differentiation to occur and for more obvious changes to be observed. PMA may also be ineffective in inducing complete differentiation at the concentration used.

4.3. DNA PROFILE ANALYSIS

Exposure to PMA caused a decrease in the proportion of cells residing in the S-phase and an increase in the proportion of cells residing in the G_0/G_1- and the G_2/M-phases of the cell cycle both in THP-1 and HL-60 cells. When cell proliferation is inhibited, cells accumulate at one of at least two cell cycle checkpoints in either the G_1 or the G_2 phase of the cell cycle (Strasser et al, 1994), thereby causing a decrease in the proportion of cells residing in the S-phase. The observed results thus indicate that PMA inhibited proliferation both in THP-1 and in HL-60 cells.

While stimulation with PMA plus dbcAMP caused cell cycle distribution patterns similar to those observed for PMA-stimulation alone, dbcAMP-stimulation by itself caused little change when compared to distribution levels in unstimulated cells. This was observed both in THP-1 and HL-60 cells, although a slight decrease in the proportion of cells residing in the S-phase

observed in THP-1 cells. Thus, while dbcAMP caused a slight decrease in proliferation in THP-1 cells, it had little effect on HL-60 cells.

When cells are induced to PCD, they will accumulate in G_1 or in G_2 (Strasser *et al*, 1994). Interestingly, one of the checkpoints in G_1 is thought to be regulated by p53, and cells lacking this protein seem to accumulate mainly in G_2 upon induction of cell death (Livingstone *et al*, 1992). However, in cells expressing BCL-2, a significant G_0/G_1 population is also present (Strasser *et al*, 1994). Thus, the G_0/G_1 population in arrested HL-60 cells, that is observed despite the absence of p53, may be due to high BCL-2 expression. Of course, PMA-induced differentiation differs from induction of PCD in that it causes a significant amount of growth arrest. This will lead to cell accumulation in the resting G_0-phase of the cell cycle quite independently of the p53 G_1 checkpoint. The increased G_0/G_1 population may thus reflect these "resting" cells. Terminal differentiation in haematopoietic cells is, however, linked to cell death (Rothstein, 1993), and the observed decrease in cells residing in the S phase of the cell cycle during differentiation may have a similar effect to the decrease observed during PCD.

Cells displaying characteristic features of PCD can be quantified flow cytometrically as a "subdiploid" population due to apoptotic chromatin condensation and DNA cleavage (Nicoletti *et al*, 1991). No such subpopulation was evident in DNA cell cycle analysis studies, indicated that no significant amount of apoptosis or programmed cell death was occurring in unstimulated

or stimulated THP-1 and HL-60 cells. This corroborates with the morphological data and the DNA electrophoresis data, where few cells were observed to have undergone apoptosis.

In summary, cell cycle distribution patterns confirmed that PMA-stimulation caused decreased proliferation both in THP-1 and in HL-60 cells, whereas dbcAMP had a weaker effect on inhibition of proliferation in THP-1 cells and no effect on proliferation in HL-60 cells. They also showed that no significant amount of apoptotic cell morphologies characteristic of PCD were present in the cells studied. This tied in well with the morphological data obtained.

4.4. THYMIDINE INCORPORATION

Exposure to PMA led to cytostasis by day four, while stimulation with dbcAMP led to a decrease in proliferation, but not to cytostasis. This confirms the results obtained from cell morphological studies and DNA profile analyses. Together with cell adherence studies, these results show that PMA caused a decrease in proliferation and an increase in cell differentiation in THP-1 cells.

dbcAMP caused a decrease in proliferation in THP-1 cells, as indicated by DNA profile analyses and thymidine incorporation studies. This decrease in proliferation did not, however, seem to be linked to cellular differentiation events, as illustrated by the failure of dbcAMP to cause an increase in cell adherence, and by cell morphology studies.

Although no thymidine incorporation studies were performed on HL-60 cells, the cell morphology and adherence studies, together with DNA profile analyses show that PMA also causes a decrease in proliferation and an increase in differentiation in HL-60 cells. dbcAMP did not seem to cause a noticeable decrease in proliferation in HL-60 cells, as indicated by DNA profile studies. Neither did it induce cell differentiation, as illustrated by cell morphological studies, and by its failure to cause cell adherence.

PMA-stimulation at the concentration used (16 nM) thus seemed effective in inducing at least partial differentiation in THP-1 and HL-60 cells.

4.5. IL-1ß mRNA AND PROTEIN EXPRESSION

Unstimulated THP-1 cells did not produce detectable levels of IL-1ß mRNA or secreted IL-1ß protein. This correlates well with previous studies using

THP-1 cells (Cochran and Finch-Arietta, 1989) and blood monocytes (Schindler *et al*, 1990), and is thought to be related to the developmental stage of the cells (Cochran and Finch-Arietta, 1989).

In contrast, THP-1 cells stimulated with PMA did produce IL-1ß mRNA and secreted IL-1ß protein. This induction of IL-1ß expression in response to PMA has also been observed by other researchers (Fenton *et al*, 1988; Turner *et al*, 1988; Gaffney *et al*, 1989). Some researchers, however, argue that PKC is involved in the secretion of IL-1ß only, and not in its production (Bakouche *et al*, 1992), while others have found that PKC activation via PMA also causes an increase in IL-1ß mRNA stability (Siljander *et al*, 1993). It should, of course, be noted that PMA elicits some cellular responses independently of PKC (Murphy *et al*, 1991), and that the induction of IL-1ß mRNA and protein synthesis may be partially or totally independent of PKC's activation by PMA. It has been suggested (Cochran *et al*, 1989) that the production of IL-1ß mRNA and protein is related to the developmental stage of the THP-1 cell, with IL-1ß only being detectable once macrophage-like differentiation has occurred. Interestingly, activation of PKC alone is thought to be insufficient in causing macrophage-like differentiation events (Kreutter *et al*, 1985), and the differentiation caused by PMA may thus be, at least partially, independent of PKC. Some compounds that bind to and activate PKC have even been found to inhibit differentiation (Kraft *et al*, 1986). In these studies, however, it is important to note that, at least in some cases, short- and long-term

phorbol ester treatment have opposite effects on cell proliferation (Moud *et al*, 1991). This is probably due to the initial activation of PKC, which is followed by its down-regulation upon prolonged stimulation (Brooks *et al*, 1991). Different concentrations of phorbol ester can also have opposing effects, with lower concentrations inducing a proliferation response and higher concentrations a differentiation response (Clemens *et al*, 1992). Interestingly, phorbol ester-induced differentiation can be inhibited in THP-1 cells upon treatment with the PKC-inhibitor staurosporine (Barendsen *et al*, 1990).

In summary, PMA induces IL-1ß production in THP-1 cells. Whether this induction is in response to PKC activation alone or to its subsequent down-regulation, or whether PMA affects IL-1ß production by a PKC-independent pathway, is not clear at present.

Interestingly, production of IL-1ß has been described previously to occur mainly in the late G_1-phase of the cell cycle (Matsushima, 1986), and stimulation with PMA, and with PMA plus dbcAMP, has been found here to increase the proportion of THP-1 cells residing in the G_0/G_1-phase by approximately 40 %.

Exposure of THP-1 cells to dbcAMP did not cause the production of IL-1ß mRNA or protein. This is in accordance with previous studies (Hurme, 1990; Serkkola *et al*, 1992) which shows that dbcAMP-stimulation does not

induce IL-1ß expression in THP-1 cells, and may even inhibit it. Murine macrophages are capable of expressing IL-1ß mRNA upon dbcAMP-stimulation (Ohmori *et al*, 1990). However, between 100 and 500 μM dbcAMP were needed to elicit this response, which exceeds the concentrations of dbcAMP (50 μM) used in the present study.

Surprisingly, the reported augmentation by dbcAMP of the PMA-induced production of IL-1ß mRNA and protein (Hurme, 1990; Sun-sang and Walters, 1991; Serkkola *et al*, 1993) was not observed in THP-1 cells, at least not at the protein level. The reported effect is more profound at the mRNA level than at the protein level. However, the approximately 10 % increase observed in the expression of the secreted protein in the current study does not compare well to the four-fold increase described by Hurme (1990). Whether this is due to the higher concentration of dbcAMP used (100 μM, compared to 50 μM in this study), or whether PMA was not efficient in priming cells for the dbcAMP response, is not clear. Of course, some phenotypic drift might have occurred in the cultured THP-1 cells that made them resistant to dbcAMP augmentation of PMA-induced IL-1ß expression.

Unstimulated HL-60 cells did not produce detectable levels of IL-1ß mRNA or protein. This corresponds with observations described elsewhere (Sun-sang and Walters, 1991). However, while low expression of IL-1ß has previously been detected in HL-60 cells in response to PMA (Sun-sang and

Walters, 1991), this response was not observed in the current study. It is probable that the low levels of IL-1ß production described for HL-60 cells were insufficient for detection. Similarly, no IL-1ß was detected in cells co-stimulated with PMA and dbcAMP. Production of IL-1ß has been linked to the state of differentiation of monocyte-like cells (Cochran *et al*, 1989), and the less differentiated HL-60 cells are, therefore, likely to have a decreased capability of producing IL-1ß when compared to their more differentiated THP-1 counterparts. This may also explain why no detectable levels of IL-1ß expression were present in HL-60 cells and confirms their less differentiated state.

4.6. *bcl-2* mRNA AND BCL-2 PROTEIN EXPRESSION

4.6.a) THP-1 cells

bcl-2 mRNA and BCL-2 protein were detected in unstimulated and stimulated THP-1 cells. However, the protein expression was consistently too low to yield representative results from densitometer scanning. Protein bands could be detected by densitometer scanning of the Western blots and slotblots. However, a lack of band intensity resulted in high background/band ratios and, thus, decreased the resolution obtained. Representative and reproducible results were, thus, not obtained for BCL-2 protein expression in THP-1 cells.

No reports on the presence or absence of BCL-2 expression in THP-1 cells exist in the general scientific literature to date. It has, however, been previously shown that BCL-2 expression is inversely related to maturation in normal myeloid cells (Delia *et al*, 1992). Thus, while promyelocytes are largely BCL-2 positive, monocytes are totally negative (Delia *et al*, 1992). THP-1 cells are thought to resemble normal monocytes closely, but are slightly less differentiated (Auwerx, 1991). Unlike mature monocytes, THP-1 cells did express BCL-2 protein. This expression was, however, very low, with 90 μg to 120 μg of total cellular protein yielding only faint bands upon immunodetection. Furthermore, BCL-2 protein expression was between 5 and 15 times lower in THP-1 cells than in HL-60 cells. The low concentration of BCL-2 protein is consistent with the hypothesis that BCL-2 expression is inversely related to maturation in myeloid cells (Delia *et al*, 1992). Thus, THP-1 cells have lower concentrations of BCL-2 than HL-60 cells, but higher concentrations than their more differentiated monocyte counterparts. Low levels of BCL-2 expression in these cells is, therefore, consistent with the notion that expression decreases as myeloid differentiation increases.

bcl-2 mRNA was expressed in THP-1 cells. Expression of *bcl-2* mRNA was decreased in PMA-stimulated cells with respect to control values. PMA induces differentiation in THP-1 cells (Auwerx, 1991). It thus seems that

PMA-induced differentiation of THP-1 cells is linked to a decrease in *bcl-2* mRNA expression. Whether the two events of increased differentiation and decreased *bcl-2* mRNA expression are necessarily linked is not clear. However, differentiation and BCL-2 protein expression have been uncoupled in HL-60 cells (Park *et al*, 1994). Thus, differentiation and *bcl-2* expression may be regulated by two distinct pathways, both of which may be activated by changes in PKC activity. Uncoupling of these two pathways would then be possible by inhibiting one of the pathways subsequent to PKC activation.

Varying results were obtained for dbcAMP-stimulated THP-1 cells, with two experiments yielding no change in *bcl-2* mRNA levels and one experiment yielding a decrease of approximately 50 % upon stimulation. Increased intracellular cAMP levels have been linked to an increased susceptibility to cell death (Kroemer and Martinez-A., 1994). This increased susceptibility may be mediated by a decrease in BCL-2 expression, which would be reflected at the mRNA level. A critical threshold level of cAMP may have to be reached before a signal is given that decreases *bcl-2* expression, thus causing a decrease in only one of the samples tested. Higher concentrations of dbcAMP may be needed to ensure that threshold levels are always reached and to yield more consistent results.

dbcAMP caused some cytostasis in THP-1 cells, without affecting their state of differentiation. Cellular differentiation is usually linked to a decrease

in cell proliferation. It is, thus, possible that the observed inverse relationship between differentiation and *bcl-2* expression (Delia *et al*, 1992) is due both to the actual differentiation events and to a decrease in the rate of proliferation. *bcl-2* mRNA expression may become inhibited once cell proliferation has decreased beyond a certain threshold level.

PMA plus dbcAMP-stimulated THP-1 cells showed a consistent decrease in *bcl-2* mRNA expression. This correlates with the notion that PMA stimulation does lead to a decrease in *bcl-2* expression, and dbcAMP may potentially decrease *bcl-2* expression.

4.6.b) HL-60 cells

BCL-2 protein expression was between 5 X and 15 X higher in HL-60 cells than in THP-1 cells. This is consistent with the finding that BCL-2 expression is higher in less differentiated myeloid cell types than in their more differentiated counterparts (Delia, 1992). It also correlates with the observed down-regulation of BCL-2 by p53 (Miyashita et al, 1994), which will obviously not occur in the p53-lacking HL-60 cell line. Furthermore, mice deficient in p53 exhibit increases in BCL-2 protein levels (Miyashita et al, 1994), which is also consistent with the increased amounts observed in HL-60 cells.

The observed difference in BCL-2 protein expression between THP-1 and HL-60 cells did not seem to be reflected at the mRNA level for unstimulated

cells. *bcl-2* mRNA expression in HL-60 cells was found to be only between 25 % and 45 % higher than in THP-1 cells. These results have to be treated with caution, as they are based on the unsubstantiated assumption that RT-PCR results obtained from two different cell lines are comparable. However, posttranscriptional regulation of *bcl-2* has been described in lymphoblasts, where mitogen-stimulation will cause a loss of BCL-2 protein expression with a concurrent increase in bcl-2 mRNA levels (Akagi *et al*, 1994). It is, therefore, possible that post-transcriptional regulation is, at least in part, responsible for the observed differences in BCl-2 protein expression in the two cell lines studied.

PMA-stimulation caused a decrease in *bcl-2* mRNA and BCL-2 protein expression in HL-60 cells. This correlates with previous reports of decreases in *bcl-2* mRNA and protein levels upon induction of differentiation in HL-60 cells (Delia *et al*, 1992), and with results obtained for THP-1 cells (section 4.6.a). Surprisingly, decreases were more profound at the mRNA level (where expression was found to be between 10 % and 20 % of control levels) than at the protein level (where expression was approximately 80 % of control levels). This, again, suggests, that BCL-2 expression is, at least partially, regulated at the post-transcriptional level.

BCL-2 protein expression was still high after a 24 incubation with PMA.

Mature monocytes do not express BCL-2 (Delia et al, 1992), and the high expression in HL-60 cells after PMA stimulation may reflect incomplete differentiation. Higher concentrations of PMA or longer incubation times may be needed to induce terminal differentiation and increased BCL-2 down-regulation in HL-60 cells.

Similar trends to those in HL-60 cells stimulated with PMA alone were observed in HL-60 cells that were co-stimulated with PMA and dbcAMP. Both *bcl-2* mRNA and BCL-2 protein concentrations were decreased upon stimulation with PMA + dbcAMP. mRNA levels decreased to between 5 % and 55 % of control levels, while protein levels decreased only to approximately 92 % of control levels.

It is, of course, the BCL-2 protein rather than the *bcl-2* mRNA that protects cells from PCD. If decreases in mRNA levels are not reflected to the same extent at the protein level, this might indicate the presence of a post-transcriptional "buffering" mechanism. Regulatory macromolecules may exist that increase the *bcl-2* mRNA stability or decrease the BCL-2 protein breakdown in response to decreased *bcl-2* gene transcription. This may be a protective mechanism against transient PCD signals.

Decreases in BCL-2 levels upon PMA-stimulation should make cells more susceptible to apoptosis and PCD. This was, however, not observed in cell

morphological studies. Thus, other proteins that protect from cell death, such as BCL-X_L, may have substituted for BCL-2. Alternatively, the observed decrease in BCL-2 protein levels may not have been sufficient in causing a marked increase in susceptibility apoptosis and PCD, at least not within the first 24 hours post-stimulation.

No overall change in BCL-2 protein expression was observed in response to dbcAMP stimulation in HL-60 cells. At the mRNA level, varying results were obtained upon dbcAMP-stimulation, with two experiments yielding decreased *bcl-2* mRNA expression (50 % and 70 % of control levels, respectively), and one experiment yielding no change in expression. Again, as with THP-1 cells, this might have been due to an antiproliferative effect of dbcAMP, which may potentially decrease *bcl-2* transcription once a certain threshold level of cAMP is reached. It is, of course, also possible that *bcl-2* gene transcription is influenced by PKA-activation in some other way independently of the observed decrease in cell proliferation. Increased cAMP levels have been linked to increased susceptibility to PCD (Park *et al*, 1994). This effect may operate via decreased *bcl-2* mRNA and BCL-2 protein levels.

4.7. CELLULAR LOCALIZATION OF BCL-2

BCL-2 was present in the cytoplasm of THP-1 cells and HL-60 cells. The distribution of the protein was localized rather than uniform, suggesting some form of compartmentalization or organelle-association of BCL-2. This correlates with previous studies which have found BCL-2 in the outer mitochondrial membrane (Monoghan *et al*, 1992; Krajewski *et al*, 1993; Nakai *et al*, 1993) and the endoplasmic reticulum (Jacobson *et al*, 1993; Krajewski *et al*, 1993). Previous studies have also localized BCL-2 to the nuclear envelope (Chen-Levy *et al*, 1989; Jacobson *et al*, 1993; Krajewski *et al*, 1993). Staining was also observed over the nuclear areas in THP-1 and HL-60 cells. Whether this was true nuclear staining or a result of staining of the cytoplasm that overlay the nucleus, was not clear.

Staining was more pronounced in HL-60 cells than in THP-1 cells. HL-60 cells showed more intense staining at the single cell level than did THP-1 cells. Furthermore, more HL-60 cells than THP-1 cells stained positive for BCL-2. This corroborated results obtained for BCL-2 protein expression in the two cell lines, where expression was found to be 5 to 15 times higher in HL-60 cells than in THP-1 cells.

SECTION 5

CONCLUSION AND FUTURE PROSPECTS

Studies were performed on THP-1 cells and the less differentiated HL-60 cells. These cell lines were chosen because of their common myeloid haematopoietic cell lineage together with their varying levels of differentiation. HL-60 cells, in particular, were also chosen for their unusually high expression of c-MYC and their lack of expression of p53.

Both cell lines were assayed for *bcl-2* mRNA and BCL-2 protein, and for changes in these upon PMA and dbcAMP stimulation. These studies were thought to be interesting with respect to HL-60 cells, where the effect of c-MYC on BCL-2 expression would be increased, and the effect of p53 on BCL-2 expression would be absent. The studies were also considered useful in THP-1 cells, both as a comparison to normal monocytes, and as a comparison to HL-60 cells, with THP-1 cells being intermediate in differentiation between HL-60 cells and monocytes.

Unstimulated THP-1 and HL-60 cells showed high levels of proliferation. Once PMA-stimulation had occurred, cell proliferation decreased and differentiation increased. dbcAMP-stimulation caused some decrease in proliferation levels, especially in THP-1 cells, but no observable increases in

differentiation.

PMA-induced differentiation was confirmed by cell adhesion experiments, cell morphology, and cell proliferation studies. dbcAMP stimulation caused a decrease in proliferation without observable differentiation. Incubation periods were limited 24 hours in most experiments, and it would be useful to study the effects of longer incubation times. The changes observed after PMA-stimulation and after dbcAMP-stimulation may become more pronounced after 4 to 5 days.

Few apoptotic cells were detected for both cell lines upon stimulation with puromycin. Increased incubation periods and increased concentrations of puromycin should be used in future studies, as these might increase the amount of cell death occuring. *bcl-2* mRNA and BCL-2 protein expression should also be analysed with respect to puromycin treatment in THP-1 cells and HL-60 cells.

Both cell lines were assayed for *bcl-2* mRNA and BCL-2 protein, and expression was found to be inversely related to differentiation. This conclusion was reached from two sets of results. Firstly, THP-1 cells expressed lower levels of *bcl-2* mRNA and BCL-2 protein than HL-60 cells. Secondly, bcl-2 mRNA levels decreased in both cell lines upon PMA-induced differentiation. BCL-2 protein levels decreased in HL-60 cells upon PMA stimulation.

Changes in *bcl-2* mRNA were more profound than changes in BCL-2 protein upon stimulation with PMA, dbcAMP, and PMA plus dbcAMP. This indicates that post-transcriptional mechanism(s) of BCL-2 expression regulation may exist. This could, perhaps, occur by increased RNA stability or an increased protein half-life. Both hypotheses should be tested in future studies.

P53 is a negative regulator of BCL-2, and the absence of p53 may account for the high levels of BCL-2 expression in HL-60 cells. One way in which p53 may negatively regulate BCL-2 is via the PNRE at the transcriptional level (Young and Korsmeyer, 1993). However, *bcl-2* mRNA concentrations in HL-60 cells seemed to be only between 25 % and 45 % lower than those in THP-1 cells, while, at the protein level, expression was 5 to 15 times higher in HL-60 cells than in THP-2 cells. THP-1 cells expressed very low concentrations of BCL-2 protein, even though their *bcl-2* mRNA concentrations were more comparable to those in HL-60 cells. This implies that post-transcriptional methods of BCL-2 regulation exist, at least in THP-1 and HL-60 cells.

bcl-2 mRNA and BCL-2 protein concentrations decreased in HL-60 cells upon PMA stimulation. *bcl-2* mRNA levels similarly decreased in THP-1 cells upon PMA stimulation. Exposure to PMA also induced a decrease in proliferation and an increase in differentiation in these cells. This decrease in *bcl-2* mRNA and BCL-2 protein expression may, thus, be caused by increased

differentiation. However, differentiation and BCL-2 protein expression have been uncoupled in other studies (Park *et al*, 1994). Thus, two pathways may exist, one causing differentiation and one regulating BCL-2 expression. Both are induced by the activation of PKC (or its subsequent down-regulation) by PMA. However, other signals may cause dissociation of the two pathways by having an effect downstream of PKC on one of the pathways but not on the other.

Decreases in *bcl-2* mRNA expression were more profound than decreases in BCL-2 protein expression in HL-60 cells in response to PMA. The rate of transcription may have been decreased in response to a signal, and this decrease was not carried through to the protein level to the same extent. Perhaps the BCL-2 protein half life is longer than the *bcl-2* mRNA half life. Some buffering system may exist in response to changes in *bcl-2* mRNA concentrations that results in relatively stable protein concentrations. Cofactors, ions, or macromolecules may exist that can temporarily stabilize BCL-2 protein in response to decreased *bcl-2* mRNA concentrations. Longer incubation times should be used to determine whether the BCL-2 protein concentration does eventually drop and reflect *bcl-2* mRNA concentrations more accurately. *bcl-2* mRNA and BCL-2 protein half lives should also be measured.

HL-60 cel were more susceptible to puromycin-induced programmed cell death than were THP-1 cells. This could be due to the high level of c-MYC expression in HL-60 cells, which is a potent inducer of programmed cell death (Evan *et al*, 1992; Davidoff and Mendelow, 1993). Furthermore, PMA plus dbcAMP-stimulation caused some cell death in HL-60 cells, but not in THP-1 cells. PMA has been shown previously to be able both to protect from cell death and to induce it (Eastman, 1993). The potentially death-inducing effect of PMA seems to be activated or increased by dbcAMP in HL-60 cells. Increased intracellular levels of cAMP have been shown previously to sensitize cells to PCD (Park *et al*, 1994).

Lack of p53 expression did not protect HL-60 cells from programmed cell death, indicating that p53-independent cell death pathways do exist in myeloid cells (Strasser *et al*, 1994).

In conclusion, the link between differentiation and decreased *bcl-2* mRNA and BCL-2 protein levels in myeloid cells was confirmed. Decreasing levels of *bcl-2* mRNA and BCL-2 protein seemed to be linked also to decreased proliferation without concurrent differentiation, as was observed upon dbcAMP-stimulation. Interestingly, the changes in expression due to decreased proliferation and increased differentiation were more pronounced at the mRNA level than at the protein level.

SECTION 6

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